SOME PHYSIOLOGICAL EFFECTS OF THE INSECTICIDE KEPONE IN THE LABORATORY MOUSE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

James Joseph Huber, B.Sc., M.Sc.

******

The Ohio State University
1963

Approved by

Adviser
Department of Zoology

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. George W. Ware, Department of Zoology, The Ohio State University, for his cooperation and encouragement while serving as my adviser.

Thanks is expressed to the Bureau of Sport Fisheries and Wildlife and to Dr. Eugene H. Dustman, Assistant Chief of the Branch of Wildlife Research for the research grant which helped finance this study.

I am indebted to the Natural Resources Institute and to Dr. Charles A. Dambach, Director, for the research assistantship which allowed me to carry out this project.

To the Department of Zoology, The Ohio State University, which provided the necessary facilities, and to its many staff members for their assistance at various times during this study, I am grateful. In this regard, Dr. Eugene Good and Dr. Tony J. Peterle deserve special appreciation.

Recognition is due Dr. Walter F. Loeb and Dr. Walter Venzke, College of Veterinary Medicine, The Ohio State University, for their technical assistance and advice.

Thanks is due to the Allied Chemical Corporation, Morristown, New Jersey, for their cooperation in supplying the technical grade Kepone used in this study.

To Carol Ann Huber, my wife, appreciation is extended for her encouragement, patience and typing assistance.

CONTENTS

TABLES

INTRODUCTION

Perspective and Objectives

Since World War II, insecticides have become a prominent part of our way of life. Their use has enabled our agricultural production and our public health standard to rise to their present levels. These chemical compounds which kill insects usually are also harmful to other forms of life including man.

The harmful effects of insecticides on forms of life other than insects has been demonstrated many times by entomologists and wildlife specialists such as DeWitt and George (1960) and Rudd and Genelly (1956) among many. These detrimental effects may result from the extreme toxicity of some insecticides such as parathion or from the persistence of compounds with relatively low toxicity, such as DDT. This persistence in the environment is, perhaps, the greatest potential hazard of insecticide application. Such persistence may result in the accumulation of toxic quantities or sufficient amounts to affect the reproductive or other physiological processes of many kinds of animals for many years.

These potential dangers of insecticides were not taken too seriously for many years despite the warnings of

many entomologists and wildlife specialists. In 1949, the American Association of Economic Entomology issued the following resolution:

> Whereas - New chemicals with insecticidal possibilities are appearing rapidly, many of which may be released prematurely,
>
> Be It Recorded - That entomologists, manufacturers, formulators and processors use extreme caution in promoting, releasing and/or recommending new insecticides for general use. This should be done only after careful, thorough experimental work on effectiveness, toxicity to man, animals and plants, and relationship to other insecticides.

In 1962, Rachel Carson's "Silent Spring" brought to the public's attention the potential hazards of insecticides to non-insect forms of life. Although Miss Carson's publication may be criticized for its sensationalism and biased presentation of otherwise accurate scientific information, she did accomplish her objective, that being to alert the public to the potential hazards that the indiscriminate use of pesticides may have on beneficial life forms.

The President's Scientific Advisory Committee Report in 1963 emphasized the potential dangers of insecticidal compounds and stressed the importance of testing such compounds by thorough experimental research for effectiveness, acute, and chronic toxicity to man,

animals and plants, and their long term effects on the environment of man.

Dr. Jerome Weisner, chairman of the President's Science Advisory Committee, while appearing before the Senate Government Operations subcommittee made the following statement concerning pesticides, "Man's uncontrolled use of poisonous chemicals, including pesticides, is potentially a greater hazard then radio-active fallout" (Consumer Reports, 1963).

The reports issued by the American Association of Economic Entomology, the President's Science Advisory Committee and other responsible groups leaves little doubt of the necessity for further toxicological research, especially in reference to the effects of low-level chronic toxicity over an extended period of time. In view of these considerations, the objectives of this study were:

1. To determine the chronic toxicity of Kepone to the laboratory mouse.
2. To determine some of the reproductive and physiological disturbances in the laboratory mouse which occur during the ingestion of sublethal concentrations of Kepone.
3. To determine if any major pathological changes occur in the organs of mice treated for extended periods of time at sublethal dosages.

4. To determine if Kepone is accumulated in mice when fed continuous sublethal dosages.
5. To determine the major organs in which Kepone is accumulated.
6. To determine if Kepone is excreted following the withdrawal of the treated diet.
7. To determine if Kepone passes through the milk and placentae of treated females.
8. To observe any abnormal behavior or syndrome which may be related and used as an indication of chronic Kepone poisoning.

REVIEW OF THE LITERATURE

Since reviews of the mammalian reproductive mechanism and anatomy can be found in recent texts such as Lloyd (1959), Turner (1955), Guyton (1961), Ham and Leeson (1961), Anderson (1961), Prosser and Brown (1961) and Nalbandov (1958), this review will concern itself with the literature pertaining to hormonal and insecticide studies which apply to this study.

Chlorinated Organic Insecticides

Introduction

A chlorinated organic insecticide is any organic compound containing chlorine and carbon and used for the control of some insect. However, beyond this broad similarity, the compounds vary widely in chemical structure and activity. Although much is known about many aspects of pharmacology of some of these materials, the basic mode of action is not known for a single one of them.

It is entirely possible that chlorinated organic insecticides of significantly different chemical structure have different modes of action; it is certain that there are qualitative as well as quantitative differences in their physiological action (Table 1).

Table 1. Acute Oral LD_{50} Values of 15 Chlorinated Organic Insecticides for Adult Male White Rats (Clinical Handbook on Economic Poisons, 1963)

Insecticide	LD_{50} (mg/kg body weight)
Aldrin	39
Chlordane	335
DDA	740
DDE	880
DDT	113
Dieldrin	46
Endrin	18
Heptachlor	100
Isodrin	16
Kelthone	1100
Kepone	95[a]
Lindane	88
Methoxychlor	6000
Mirex	4000[b]
Toxaphene	90

[a]Martin (1961).

[b]Allied Chemical Corporation (1962).

Physiological Action and Pathology

The following summary of physiological action and pathology is taken from the Clinical Handbook on Economic Poisons, 1963. In general, chlorinated organic insecticides affect the central nervous system of mammals, but the exact mechanism of this action either in man or in animals has not been elucidated. Some act as central nervous system stimulants (chlordane), others as depressants (beta isomer of benzene hexachloride).

Some compounds or certain degradation products are stored in fat. Such storage results either from a single large dose or from repeated small doses. The materials stored in the fat appear to be largely inactive since the total amount stored in an experimental animal often may be greater than the lethal dose if given at one time.

The insecticides or their derivatives usually may be demonstrated in milk and urine. The compounds stored in the fat are eliminated at varying rates when further dosage is discontinued.

Laboratory examinations in mammals are usually negative and always nonspecific except that the insecticide or its derivatives may be demonstrated in stomach contents, urine, or tissues, especially fat.

In experimental animals killed by large doses of chlorinated organic insecticides, dilatation of blood vessels and even small hemorrhages secondary to convulsions

may be encountered. Prolonged feeding of sublethal dosages of these compounds produces microscopic changes in the liver and kidneys in some experimental animals. This has not been demonstrated clearly in man in connection with uncomplicated poisoning.

The outstanding changes, following repeated feeding in rodents, are found in the liver. These changes consist of enlargement, centrolobular hypertrophy, margination of cytoplasmic granules, fatty infiltration, and lipospheres. Similar changes may occur separately or in a different order following a wide variety of toxicants. These changes have not been found in the higher animals nor in man. All animals show liver cell necrosis at high levels of dosage, but again the changes are non-specific.

Nervous symptoms such as tremors and convulsions are characteristic of chlorinated organic insecticide poisoning, but these symptoms may be induced by a variety of other economic poisons and by less specific neurologic disease.

Phenobarbitol and a few other barbiturates known to control convulsions of central origin have been used successfully in the control of convulsions induced by some chlorinated organic insecticides; calcium gluconate has been less successful.

Kepone

Kepone, the copyrighted trade name for decachloro-octahydro-1,3,4-metheno-2H-cyclobuta (6d) pentalen-2-one, was developed by the Allied Chemical Corporation in 1958. Formerly called compound GC-1189, it is a chlorinated, polycyclic ketone with a molecular weight of 491 and the empirical formula of $C_{10}Cl_{10}O$.

It is a stable, white to tan solid which sublimes with some decomposition at about 350° C. It is practically insoluble in water (0.4% at 100° C) but it is soluble in a strongly alkaline aqueous solvent. It is readily soluble in acetone, but less soluble in benzene and light petroleum. It readily forms hydrates (10-13%) on exposure to ordinary temperatures and humidities (Martin, 1961).

Kepone is manufactured by the condensation of two moles of hexachlorocyclopentadiene in the presence of sulphur trioxide, and hydrolysis to the ketone. It is registered and sold as a 50% wettable powder, an emulsifiable concentrate, and as a pelleted ant and cockroach bait.

Anhydrous Kepone is known to form additional compounds; but little is known about their formation. The metabolism of Kepone within any animal is unknown (Martin, 1961).

Mortality and Tolerance Studies

The following review is intended to give some idea of the tolerance which certain animals have displayed in past studies to several different chlorinated organic insecticides.

Aldrin

Aldrin is reported to have no effect on rat longevity in tests conducted over two years at 25 ppm dietary (Treon and Cleveland, 1955), At 300 ppm all rats died within two weeks. Borgman (1952) reported tolerance of aldrin by rats up to 75 ppm for three months, and up to 50 ppm for 16 months. Less than 50% of the test rats survived the 100 ppm level in chronic tests of 16 months. In tests by Lehman (1952a), tissue damage could be detected at 25 ppm in chronic tests. There was 100% mortality of 8-10-week-old turkeys in 14 days when fed 25 ppm aldrin (Anderson et al., 1952).

BHC

Lehman (1951) gave the LD_{50} of the gamma isomer of benzene hexachloride (lindane) as 125 mg/kg for rats. On the other hand, the LD_{50} for the beta isomer of BHC was 6000 mg/kg.

Chlordane

In tests by Ambrose et al. (1953) no mortality occurred among rats fed a diet containing 80 ppm for 400 days. Lehman (1952a) reported mortality in chronic tests with rats at 400 ppm, but not at 75 ppm.

DDT

Rats tolerated 400 ppm DDT in their diet for six months without significant mortality (Ortega et al., 1956). At 1000 ppm all rats died within 80 days (Smith and Stohlman, 1944). Laug and Fitzhugh (1946) reported 10 of 12 rats died in two years fed a diet containing 200 ppm. No gross effects appeared in rats below 100 ppm (Lehman, 1952a).

Dieldrin

Dieldrin is the chemical compound to which aldrin is converted in the body of the rat. Rat longevity was not affected over two years by a diet containing 25 ppm (Treon and Cleveland, 1955). Rats also tolerated 75 ppm for six months. Ortega et al. (1957) in similar chronic tests reported a tolerance up to 25 ppm dieldrin by adult rats. Quail are susceptible to dieldrin at 5 ppm (DeWitt, 1956), and Genelly and Rudd (1956) reported male pheasants are killed by a level of 50 ppm.

Endrin

Rats tolerated up to 5 ppm for 80 weeks, but 60% mortality occurred at 25 ppm for females and 60% at 50 ppm for males (Treon et al., 1955). At 106 weeks this sex difference was insignificant.

Kepone

Sherman and Ross (1961) reported 440 ppm fatal to young chicks within 15 days. DeWitt and George (1960), based on preliminary tests against young quail and pheasants, made a comparison of the tentative relative toxicity of Kepone to 12 other pesticides. Phosdrin and lindane were more toxic while BHC, malathion, toxaphene and seven others were less toxic. As shown in Table 1, Kepone is eleventh in order of toxicity among the 15 insecticides included in this table. Little data are available concerning the toxicity of Kepone to mammals.

Methoxychlor

Methoxychlor caused no significant mortality when fed to rats at a concentration of 1600 ppm for two years (Hodge et al., 1950). With 45 days of exposure, 10,000 ppm caused no mortality, but at 30,000 ppm, 80% of the test rats died.

Toxaphene

Toxaphene can be tolerated by rats up to 200 ppm for a year with no gross changes (Ortega et al., 1957). There are, however, intracellular alterations in the liver at such exposures. Lehman (1952a) reported no gross effects at 100 ppm for two years with rats, and considered toxaphene one-half as potent as aldrin in chronic tests with rats and equal to lindane.

Food and Water Consumption and Growth

Aldrin

The depression of growth in young male turkeys on a diet containing 3 ppm is reported by Anderson et al. (1952). At 6.25 ppm both sexes demonstrated growth depression. Borgman (1952), working with rats, stated that treated animals fed 150 ppm aldrin equalled or surpassed the corresponding controls in body weight after 16 months. No significant difference was found in the food intake. Quail and pheasant demonstrated slight depression of growth only during the first few weeks when fed sublethal levels of aldrin (DeWitt, 1956). Treon and Cleveland (1955), using sublethal doses of 2.5 to 25 ppm, reported the growth rate of treated rats was equal to or greater than that of the controls. Ball et al. (1953) reported increased weight with no change in length of animals fed up to 20 ppm. This weight increase correlated

with increased food consumption of 180 to 270% by the treated animals. These rats returned to a normal growth rate within one week after the withdrawal of aldrin from their diet.

BHC

The beta isomer of BHC depressed growth of young rats at 100 ppm (Nelson et al., 1950).

Chlordane

Laying pullets lost weight and appetite in proportion to the chlordane dosage (Rosenberg et al., 1950).

DDT

DeWitt (1956) reports a slight depression of growth in quail during the first few weeks with a sublethal level of 100 ppm. Rats fed up to 25 ppm had growth rates equal to or greater than that of the corresponding controls (Treon, 1955). There was no interference of growth in 21-day-old rats fed up to 50 ppm DDT for 15 to 27 weeks (Nelson et al., 1950). Conley (1951a) reported that DDT ingested by dogs elevated their metabolic rate which was reflected in their increased food and water intake and a decrease in body weight. Burlington and Lindeman (1950) using chicks reported no food and water intake or weight

gain difference between controls and those injected with 2 to 3 grams DDT over a period of 80 days. There was no change in the growth rate of rats ingesting up to 400 ppm DDT for 35 days. At 1000 ppm all rats showed striking weight loss.

Dieldrin

A slight depression of growth occurred only during the first few weeks in quail and pheasants fed a sublethal dose of 1.0 ppm (DeWitt, 1956). Treon and Cleveland (1955), using sublethal doses of 1 to 25 ppm, reported no growth retardation of rats. At 50 to 100 ppm, growth retardation occurred at different periods, but final weights equalled or surpassed those of the controls. Pheasants ate less as the dosage of dieldrin increased from 25 to 50 ppm. Genelly and Rudd (1956) suggested the reduction in numbers of eggs may be due to this factor. No weight gain difference occurred among chicks fed sublethal doses of 20 to 30 ppm (Sherman and Rosenberg, 1953).

Endrin

Quail and pheasants experienced a slight depression of growth during the first few weeks when given a sublethal level of 1.0 ppm endrin (DeWitt, 1956). There was no difference in weight gained among chicks fed a sublethal

level of 1.0 ppm (Sherman and Rosenberg, 1953). Treon et al. (1955), using sublethal levels report that treated animals equalled or surpassed the controls in weight. Rats which survived higher dosages of 50 to 100 ppm also equalled or surpassed the corresponding control animals after 20 and 40 weeks.

Kepone

Chicks fed dietary levels of Kepone ranging from 22 to 220 ppm equalled or surpassed the controls in their growth rate (Sherman and Ross, 1961). At 440 ppm growth retardation was evident although food consumption did not decrease.

Methoxychlor

Food consumption and the growth rate of rats were decreased by 10,000 ppm (Hodge et al., 1950). Growth was inhibited and 80% of the rats died in 45 days at a concentration of 30,000 ppm.

Toxaphene

Genelly and Rudd (1956) stated that pheasants ate less when given toxaphene at a dietary level of 300 ppm.

In summary, growth and feeding rates of rats do not seem to be retarded at sublethal levels of chlorinated organic insecticides, but may equal or even surpass their

corresponding controls. Depression of such rates does not occur until near lethal levels are given in the diet. Birds appear to be much more sensitive than mammals to chlorinated organic insecticides. Because of their greater sensitivity, their growth rates are affected at much lower concentrations.

Major Organ Weights

Aldrin

Livers of male adult rats increased in size in three months on dietary levels of 75 or 150 ppm aldrin, but no enlargement occurred at 5 ppm (Borgman, 1952). Female rats showed no liver enlargement over nine months at 25 ppm, but the liver did increase in size at 45 ppm over the same period of time. In two-year experiments, liver enlargement occurred at dietary levels of 50 or 150 ppm, but not at 5 or 10 ppm. Treon and Cleveland (1955) reported liver enlargement in rats on dietary levels of 2.5, 12.5, and 25 ppm in 18 months.

BHC

Nelson et al. (1950) reported liver enlargement in rats fed prolonged diets of 10 ppm beta - BHC. The alpha and gamma isomers of BHC produced liver enlargement only at levels exceeding 50 ppm.

Chlordane

Nelson et al. (1950) reported liver enlargement of rats fed 10 ppm chlordane.

DDT

Prolonged feeding of 50 or 100 ppm resulted in significant enlargement of rat livers (Nelson et al., 1950). Laug and Fitzhugh (1946) reported liver and kidney enlargement in rats fed 100 to 800 ppm DDT for six months. This enlargement was most notable at the higher doses. Significant increases in liver size were observed in two months when rats were fed dietary levels of 400 ppm DDT, but no increases were noted at 200 ppm (Ortega et al., 1956). Twenty-five ppm DDT increased liver weights in rats significantly when ingested over an 18 month period (Treon and Cleveland, 1955).

Dieldrin

Dietary levels of 2.5, 12.5 and 25 ppm over an 18 month period produced significant liver enlargement in rats (Treon and Cleveland, 1955). The liver/body weight ratios relevant to the levels of dieldrin in the diets of male rats were: 25.0 ppm, 3.29 grams of liver per 100 grams of body weight; 12.5 ppm, 3.13 grams of liver; 2.5 ppm, 2.92 grams. The corresponding value in the control group was 2.55 grams per 100 grams of body weight.

Endrin

The livers of male rats increased in weight when 5 ppm endrin was fed to rats for two years. The liver in the female was unaffected in similar tests. Contrariwise, kidneys in female rats were relatively enlarged while those of the males were not (Treon et al., 1955). The feeding of endrin to rats at 1.0 ppm for a period of two years did not alter any of the visceral weights significantly.

The weights of the livers and kidneys of dogs in relation to total body weight were increased after the dogs had been fed diets containing 8 ppm endrin for six months. Dogs fed 3 ppm endrin for 19 months had enlarged hearts and kidneys, but the livers, spleens and body fat bore normal relationships to the total body weight (Treon et al., 1955).

Kepone

Sherman and Ross (1961) reported no increase in liver weight in chicks fed a diet containing 400 ppm for two weeks. Chick oviducts and vents showed swelling and enlargement, but other viscera appeared normal.

Methoxychlor

Rats weighing 50-55 grams each had retardation in the growth of their testes when fed diets containing 10,000 or 30,000 ppm methoxychlor for 45 days. This did

not occur when rats weighing 70-75 grams were used (Hodge et al., 1950).

Toxaphene

Liver enlargement was noted in rats fed a diet containing 25 ppm toxaphene in chronic tests (Fitzhugh and Nelson, 1951).

Two conclusions may be drawn from the studies just reviewed. First, the weights of the major body organs were not recorded in the majority of the studies. Second, when such weights were recorded, some alteration did occur in some organs, especially in the liver. These alterations were most significant at high sublethal doses of the insecticide. It appears that enlargement and alteration of the liver is one of the major characteristics resulting from the ingestion of sublethal levels of most chlorinated organic insecticides.

Reproduction Data

Aldrin

Reproduction was impaired in rats fed a dietary level exceeding 10 ppm, although no significant mortality occurred in the adults below 50 ppm (Borgman, 1952). Aldrin at a concentration of 12.5 ppm reduced the number of pregnancies in rats, but had no effect on litter size (Treon and Cleveland, 1955). A level of 2.5 ppm had no

effects on rat reproduction. In suckling rats, levels of 12.5 or 25 ppm produced severe mortality. One ppm dietary aldrin during the reproductive period of quail did not reduce egg production, fertility or hatchability, but did cause a high mortality in new-born chicks (DeWitt, 1956). When aldrin was fed for several weeks to quail preceding their reproductive period, egg production was affected with significant mortality of the adults.

BHC

Black et al. (1950) reported no effect on egg production in chickens when technical BHC is included in their diet at a concentration of 84 ppm.

Chlordane

Egg production in chickens showed a slight decrease within 28 days at a dietary concentration of 500 ppm, a steady decrease after seven days at 1500 ppm, a complete inhibition of egg production in seven to 14 days at 2500 ppm, and a complete inhibition of egg production in five days at 5000 ppm (Rosenberg et al., 1950).

DDT

Treon and Cleveland (1955) reported no effect on rat reproduction at 25 ppm. Genelly and Rudd (1956) noted a significant reduction in egg production and hatchability

in pheasants at 400 ppm dietary. In studies by DeWitt (1956), 100 ppm DDT did not reduce egg production, fertility or hatchability in quail. However, there was a high mortality of chicks after birth. At 200 ppm, 87% of the quail chicks died within six weeks after hatching.

Dieldrin

A dietary 2.5 ppm dieldrin reduced the number of pregnancies in rats, but had no effect on litter size (Treon and Cleveland, 1955). A level of 12.5 or 25 ppm in the diet of nursing rats resulted in severe mortality to the sucklings. Kitselman (1953) stated that sublethal doses of dieldrin did not alter the reproductive capacity of dogs, but some pups died after one to three days of nursing while others were born dead or died the same day. He concluded that pups were more tolerant to such chemicals *in utero* than after birth. DeWitt and George (1960) reported a decrease in the hatchability of quail eggs and a reduction in viability of chicks whose parents had been fed 1.0 ppm dieldrin for 16 to 20 weeks.

Kepone

Good *et al.* (1963) reported an inverse relationship between dosage and the reproductive capacity of mice of mixed parentage. With the reproduction of the control mice being equal to 100% in reference to the number of young

produced, 17.5, 25, 30 and 37.5 ppm Kepone reduced the reproductive rate 14.8, 49.8, 93.5 and 92.4%, respectively. The litter size and the number of litters were reduced in treated mice. In tests with BALB/cJaxGnMc pure strain mice, a 10 ppm dietary level reduced the reproductive rate 53.5%. This reduction in reproduction was principally due to the production of fewer litters by the treated mice.

Reproduction of quail was completely inhibited when Kepone was included in the winter and spring diets at the rate of 50 ppm (DeWitt and George, 1960). Pleasant reproduction was almost completely inhibited at both 25 and 50 ppm (0.7 and 0.5 viable chicks per hen as compared with 13.3 for controls). Reproduction of quail was sharply reduced when the diet contained 1, 5, 10, or 25 ppm in both winter and reproductive periods. Many of the chicks were crippled or defective at hatching. Pheasants fed 5 or 10 ppm produced less than 50% of the number of viable chicks produced by the controls. Male pheasants reared on diets containing 50, 100, or 150 ppm Kepone did not develop characteristic coloration; their plumage resembled that of adult females. Histopathological examination of these birds revealed marked abnormalities of testicular tissue and the presence of numbers of malformed sperm.

In conclusion, it appears that reproduction can be reduced in birds and mammals without significant mortality to the adults.

Vaginal Smears

A normal estrous cycle can be determined by vaginal smears as demonstrated by Allen (1922) in mice and by Long and Evans (1922) in rats.

Three methods are in common use for taking vaginal smears (Snell, 1941). (1) The medicine dropper or lavage method. A medicine dropper drawn to a fine point and containing a few drops of water in inserted into the vagina, the water ejected and immediately sucked in again. The water with its contents is then transferred to a slide for examination. (2) The spatula or curette. The vaginal cells are removed by means of curette. The cells are then transferred to a drop of water on a slide. (3) The cotton swab method. Vaginal cells are removed with a fine, moist, cotton swab and then put in some water on a slide.

Wade and Doisy (1935) showed that the cotton swab method can cause irritation to the vagina and disturb the estrous cycle. Rogers and Allen (1937) reported that the curette method can also be disturbing to the estrous cycle in rodents if used frequently. In studies by Emery and Schwabe (1936), the medicine dropper method was shown to be the least irritating method with little disturbance of the estrous cycle.

The estrous cycle of the mouse or rat can be conveniently divided into five stages, namely, proestrus, estrus, metestrus-1, metestrus-2 and diestrus. The first

two stages are anabolic during which active growth is in progress in various parts of the genital tract. They culminate in ovulation and the mating response. The second two are catabolic stages characterized by degenerative changes in the genital tract. The last stage, diestrus, is a period of relative quiescence (Snell, 1941).

Vaginal smears are characterized by three types of cells: leukocytes, cornified cells, and nucleated epithelial cells. Leukocytes are very small, round cells. Cornified cells are very large, flattened, angular in outline, quite regular in size, and lack nuclei. Nucleated epithelial cells are intermediate in size, round to oval, with clear cytoplasm and a central nucleus that takes a strong methylene blue stain.

The characteristic appearance of the five stages of the normal estrous cycle is as follows:

1. Proestrus = E* to EC or ECL
2. Estrus = EC^{+} to C^{++}
3. Metestrus - 1 = LC^{++} (C in clumps)
4. Metestrus - 2 = CL^{+} to L^{++}
5. Diestrus = EL^{+} (varying amounts of mucous)

* E = nucleated epithelial cells, C = cornified cells, L = leukocytes, + indicates many cells, ++ indicates very many cells.

The principle of the vaginal smear method is based on the fact that no part of the mouse genital tract undergoes

more striking histological changes during the estrous cycle than the epithelium of the vagina.

In proestrus the epithelium consists of three layers (Snell, 1941). The outer layer is composed of epithelial cells. Below this is the stratum granulosum which, with the approach of estrus, becomes the stratum corneum. Third is the stratum germinativum, some seven cell layers thick. During proestrus the cells of the outer layer are delaminated into the vagina, producing the characteristic nucleated smear. As estrus approaches, cells from the cornified layer become exposed and are delaminated producing the cornified appearance of the estrus smear. The onset of metestrus-1 is characterized by the peeling off of the rest of the cornified layer in clumps, along with the first signs of leukocyte infiltration of the stratum germinativum. During metestrus-2 there is some delamination of the stratum germinativum layer which by now has become heavily infiltrated with leukocytes. As a result of the delimination of these superficial layers, the vaginal epithelium at diestrus contains only one layer, the stratum germinativum, some three to seven cell layers in thickness. Active growth of the stratum germinativum is noticeable in late diestrus and by early proestrus, a stratum granulosum has formed several cell layers, thus completing the cycle.

In the literature, only two insecticide studies were found which reported the use and results of the vaginal smear method. Thienes et al. (1946) reported that daily doses of 2 mg/kg of nicotine interrupted the estrous cycle of rats which resulted in the abolishment or decrease in the occurrence of estrus. A smaller dose resulted in no interference.

Aldrin at a dietary 20 ppm disturbed the estrous cycle of rats with significant estrus repression (Ball et al., 1953). They began with four groups of 30-day-old rats which were fed a diet containing 0, 5, 10, and 20 ppm aldrin, respectively. Each group contained 25 females and the following observations were made: in the control group, one case of 10-day constant anestrus and no cases of constant estrus occurred; at 5 ppm, 8 cases of 10-day constant estrus and one 10-day constant anestrus; at 10 ppm, 4 cases of 10-day constant estrus and 3 cases of 10-day constant anestrus; at 20 ppm no cases of constant estrus and 8 cases of constant anestrus. Twenty percent of the smears of the control group were estrus. They reported that adaptation of the treated rats began during the 18th week of treatment and that by the 37th week, there was no significant difference between the treated and control vaginal smear readings. In conclusion, they stated that a 5 or 10 ppm dietary level of aldrin increased the occurrence of estrus while a 20 ppm level decreased it.

Histological Studies

The literature contains much information on micropathological changes in animals exposed to chlorinated organic insecticides. A review of some of the more important histological findings are summarized below under specific insecticide subheadings.

Aldrin

In rats, 15 ppm dietary over a 24 month period resulted in centrolobular hepatic cell enlargement with peripheral migration of cytoplasmic granules (Borgman, 1952). Dogs fed sublethal doses of 0.2 to 2.0 mg/kg body weight of aldrin showed some degeneration of ganglionic cells of the cerebral cortex, focal necrosis of the liver, and degeneration of parenchyma of the liver, kidney and brain. Such cells regenerate after the removal of aldrin from the diet (Kitselman, 1953).

It is interesting to note that the tremors produced by feeding frogs aldrin are prevented in the rear leg if the sciatic nerve is cut, while pithing the brain terminates the tremors in the whole animal (Waud, 1952).

BHC

The feeding of <u>beta</u> - BHC at 10 ppm caused hepatic cell enlargement and peripheral migration of granules in cells of rat livers (Fitzhugh and Nelson, 1951). Lindane,

at 50 ppm dietary, resulted in similar liver cell alteration in rats (Ortega, 1958). Conley (1951b) reported liver damage, slight brain hemorrhage, and congested spleen in dogs fed technical grade BHC. Liver enlargement, necrosis, congestion, and fatty degeneration were found in rats fed gamma - BHC (Doisy and Bocklage, 1949).

Chlordane

Choudhury and Robinson (1950), after feeding goats toxic amounts of chlordane, found congestion of the brain, spinal cord, adrenals, and liver. There was nerve cell degeneration, proliferation of the reticulo-endothelial cells of the spleen, acute glomerulonephritis and swelling of the intestinal tract. The liver contained focal necrosis, atrophy and degeneration of hepatic cords, and congestion of the sinusoids.

Fitzhugh and Nelson (1951) report that a dietary level as low as 2.5 ppm chlordane will cause hepatic cell enlargement and peripheral migration of granules in rat liver cells. Ortega (1958) also reports hepatic cell enlargement, RNA granule migration to the cell periphery, lipoprotein inclusions, and changes in sinusoid permeability at 2.5 ppm with rats. Focal necrosis of the liver and degenerative changes in the proximal convoluted tubules of the kidney were noted by Stohlman et al. (1950) in rats fed up to 50 mg/kg per day.

Chlordane caused atrophic ova and reduction in comb size in laying pullets at a dietary level of 2500 ppm (Rosenberg et al., 1950).

DDT

A dietary level of 5 ppm caused cell enlargement and peripheral migration of granules in the cells of rat livers (Fitzhugh and Nelson, 1951). Laug et al. (1950), feeding rats 1 to 50 ppm DDT for 15 to 27 weeks, noted that males showed more hepatic change than females. This change consisted of the characteristic response of rodents to chlorinated organic poisoning, that being hepatic cell enlargement with peripheral migration of the granules especially at centrolobular locations. No necrosis was present.

A dietary level of 1000 ppm for one year resulted in focal necrosis of the rat liver, deposition of fat droplets in the adrenal medulla and cardiac muscle, and nephritis with diffuse degeneration of the convoluted tubules (Lillie et al., 1947). These alterations were not noted at the 200 and 500 ppm. Ortega et al. (1956) reported necrosis of rat liver only at 1000 ppm for 25 days or 5000 ppm for 5 days. Treon and Cleveland (1955) stated that more liver cell alteration was noted in rats fed DDT at a level of 25 ppm than that produced by aldrin or dieldrin at the same dosage.

DDT fed to dogs over a long period of time caused lesions of the central nervous system (Philips and Gilman, 1946). Such lesions may cause symptoms of DDT poisoning to persist after the removal of DDT from the diet. Since in dogs phenobarbitol is able to control convulsions which occur after the ingestion of DDT, the motor cortex, which is the part of the brain acted upon by phenobarbitol, is thought to be very sensitive to DDT. Ventricular fibrillation of the heart sometimes occurs in dogs, but it has never been noted in rodents.

Conley (1951a) reported that dogs fed DDT for a long period of time have adrenal hemorrhage, centro-lobular necrosis of the liver, excitation of the afferent pathways of the central nervous system, and possible physical interference at the lipid surface of nerve cells. This possible interference with nerve cells is suspected because of the vacuolization found around large nerve cells in the spinal cord and the cerebral motor cortex nuclei. Ventricular fibrillation, which can be induced by adrenalin stimulation through the hypothalamus, was also reported.

Burlington and Lindeman (1950) reported testicular retardation in chicks fed 2 to 3 grams DDT over an 80 day period.

Dieldrin

Characteristic hepatic cell enlargement, peripheral migration of granules and liposphere inclusions result when

rats are fed 5 ppm dieldrin (Ortega, 1958). These cell changes were reversible if high dosages were avoided. In dogs, 0.2 to 2.0 mg/kg body weight produced cell changes similar to those produced by the same levels of aldrin (Kitselman, 1953).

Endrin

Centrolobular necrosis and fatty degeneration of the liver occurred in rats fed endrin at levels of 5 or 25 ppm for two years (Treon et al., 1955). There were also diffuse degeneration of the kidney, adrenal, and brain of those which did not survive. These effects were not noted at 1 or 5 ppm in animals that lived.

In a similar study by Zavon (1961), rats fed 5 ppm endrin for two years had normal viscera. At 25 and 50 ppm, the brain, liver, kidney, and adrenal showed varying degrees of degeneration. A 100 ppm dietary level produced degenerative changes in the brain and liver along with necrosis of the uriniferous tubules.

Toxaphene

Fitzhugh and Nelson (1951) reported characteristic liver cell enlargement and alteration when toxaphene is fed to rats at a 25 ppm concentration. Ortega (1958) noted similar liver alteration when rats ingested toxaphene.

Such changes may eventually lead to central necrosis of the liver.

In conclusion, chlorinated organic insecticides at certain dietary levels can result in the alteration of certain body organs when ingested over an extended period of time. Hepatic cell hypertrophy and peripheral migration of cytoplasmic granules are some of the first pathological signs noted by some workers of chlorinated organic insecticide poisoning in rodents. These liver changes can eventually lead to a necrosis of the liver if ingestion of the toxic material is continued. Most of these cell alterations are reversible if excessive doses are avoided. At higher levels, the kidney commonly shows degenerative changes in the uriniferous tubules. Continued ingestion eventually results in degenerative changes in the brain. These changes are most notable in animals that die after long exposure to the toxic compound.

Serological Studies

In studies on chicks by Burlington and Lindeman (1952), DDT caused no alteration in number of red blood cells nor of hemoglobin levels. The blood sugar, plasma protein, calcium, phosphorous, fibrinogen and prothrombin were all within normal levels. The tremors which these chicks exhibited were not due to hypocalcemia.

The blood sugar level was normal before and after the convulsions exhibited by rats given large doses of gamma - BHC (Doisy and Bocklage, 1949). In studies by Waud (1952) with aldrin and cats, the blood sugar rose from 100 to 240 milligrams per 100 milliliters blood (mg/100 ml) before the onset of tremors and to 355 mg/100 ml after their onset. Adrenalectomy prevented this rise in blood sugar in cats. In dogs, the blood pressure declined in relation to the dose of aldrin with no other detectable blood changes.

Prolonged feeding of DDT reduced the number of cells and decreased the hemoglobin in rats (McNamara et al., 1946). Weikel (1958) reported that the absorption of sublethal doses of endrin had no effect on the hematopoietic activity of blood-forming organs such as the liver, spleen, and bone marrow in rats. Endrin does not inhibit the phosphate exchange rate in whole blood as demonstrated with dieldrin, aldrin, and DDT.

Crevier et al. (1954) found that carbon tetrachloride and chloroform increased the level of serum esterase by as much as 150% in 58 days when rats were given dietary levels of 100 ppm. Serum esterase is formed in the liver of the rat. Heptachlor and BHC had little effect on the esterase level. They also found that aldrin increased the esterase level in hypophysectomized or thyroidectomized rats, but not in normal rats. A sharp

rise in serum esterase was found prior to death in all rats.

Borgman (1952) found no differences in the hemoglobin content, the number of erythrocytes or leucocytes, and the types of white blood cells in the peripheral blood of rats fed 150 ppm aldrin for 16 months.

Hormones and Reproduction

In the following review, only the highpoints of the estrous cycle and the interrelationships of the various female reproductive hormones are given.

The Estrous Cycle

Sexually mature female mice, maintained in the laboratory separated from males, repeat the estrous cycle throughout the year at intervals of approximately five days. As described previously in detail under the heading, Vaginal Smears, it is possible to determine the stage of the estrous cycle by an examination of smears from the vagina. Diestrus, proestrus, estrus, metestrus-1, and metestrus-2 represent the five phases of the normal estrous cycle.

During diestrus the ovaries contain large corpora lutea and small vesicular follicles. Since the estrous cycles of mice are completed rapidly, the corpora lutea from the previous estrous cycle are also present. The

uterus is small and anemic with the lumen small and slitlike. The endometrial glands are collapsed and atrophic (Snell, 1941). Proestrus is characterized by the rapid swelling of the vesicular follicles. The uterus distends greatly with a marked water content increase and a pronounced vascular engorgement of the entire organ. Distension and hyperemia of the uterus reaches its maximum peak at estrus. Ovulation occurs approximately ten hours after the onset of estrus usually between 10 P.M. and 4 A.M. The period of "heat" lasts for about thirteen to fourteen hours. At metestrus-1 the ovary contains many newly formed corpora lutea and only small vesicular follicles. The uterus decreases in vascularity and distension.

Ovulation in Mammals

The process of ovulation is still not clearly understood. The latest concept is that ovulation is a phenomenon initiated in the gonad through the action of the gonadotrophic hormones of hypophysial origin. As outlined by Turner (1955), the following pertinent points concerning ovulation have been established in the past few years:

1. Though oogenesis and follicular formation can proceed to a certain stage in the absence of pituitary gonadotrophins, hypophysectomy prevents preovulatory swelling and rupture of the Graafian follicle.

2. Hypophysectomized female rats parabiotically joined with castrate partners develop a condition of constant vaginal estrus, but they do not ovulate, mate or form corpora lutea. While under the prolonged influence of follicle-stimulating hormone (FSH) and small amounts of luteinizing hormone (LH), the ovaries develop large follicles which do not ovulate, but eventually degenerate. Tremendously large follicles may be produced by the administration of extracts containing FSH and traces of LH to hypophysectomized mammals, but, despite the large size of the follicles, ovulation does not occur. These observations prove that ovulation does not occur automatically when the follicles reach mature size, and indicate that the process cannot be explained on the basis of increased liquor folliculi secretion and a consequent elevation of pressure within the antrum.

3. The constantly estrous rats, produced by parabiosis, ovulate, mate, and form corpora lutea when given an injection of purified LH. These experiments indicate that ovulation can be induced by the administration of a proper mixture of FSH and LH. A correct ratio between the amounts of FSH and LH seems to be absolutely essential for the full expression of the ovarian follicle. Though ovulation in the rabbit is conditioned normally by cervical stimulation, it may be induced purely by the injection of FSH and LH.

4. Ovulation is not dependent upon the nerve supply to the ovaries. Normal ovulation may occur in ovarian grafts and in excised follicles deprived of all nervous connections, provided the proper gonadotrophic hormones have been permitted to act.

5. The release of ovulatory gonadotrophins from the pituitary seems to be regulated by means of neuro-humoral agents from the hypothalamus. Rabbits may continue to copulate after detaching the pituitary gland from the base of the brain, but ovulation does not occur in these animals. Under such conditions, ovulation can be induced by the administration of FSH and LH. In normal rabbits, ovulation may be induced by electrical stimulation of certain areas of the brain, but not after the pituitary is separated from the brain by section of the infundibular stalk.

In relation to human ovulation, two recent studies are of interest. A study by Brown (1959) indicates estrogen may be very important in the normal ovulatory menstrual cycle of humans. In normal ovulatory cycles, at least one and usually two peaks of estrogen excretion were observed. One peak was highly characteristic and occurred at about mid-cycle just prior to ovulation. During anovulatory menstrual cycles, the estrogen level remained more or less constant.

Simpson et al. (1956) employing monkeys, reported mid-cycle peaks of FSH and LH just prior to ovulation in which LH appeared to increase relatively more than FSH.

The Hormonic Regulation of the Estrous Cycle

The regulation of the reproductive cycle is not completely understood and the present explanation is only a hypothesis which best fits the evidence accumulated thus far.

Beginning with the relatively quiescent ovary of the diestrus phase, the follicles at this time are immature and appear to secrete only small amounts of estrogen. When the level of estrogen in the blood is low, the pituitary secretes increasing amounts of FSH and some LH. As the follicles grow under the synergistic influence of FSH and LH, they release more estrogen. The increasing output of estrogen during proestrus depresses the release of FSH and encourages the secretion of luteotrophin (LTH). LTH activates the secretion of progesterone from the luteal-like tissue of the follicles, which in conjunction with estrogen, brings about the change from diestrus to proestrus to estrus.

Sexual receptivity begins during the transition from proestrus to estrus and is conditioned by the synergistic action of estrogen and progesterone. The average duration of the receptive period in mice is 13-14

hours. Ovulation occurs near the end of estrus, approximately ten hours after the beginning of sexual receptivity, at a time when FSH and LH are present in proper proportions. The discharge of LH from the pituitary seems to be inhibited by high levels of progesterone in the blood. The corpora lutea secrete progesterone for only a short time unless pregnancy supervenes.

A few of the effects of estrogen and progesterone on the vagina and uterus should be noted at this time. After oophorectomy of the adult mouse the vaginal epithelium becomes thin and mitotically inactive and only the diestrus type smear is obtainable. Administration of estrogen will cause marked growth of the vaginal wall with resulting proestrus and estrus type smears. As the effect of the estrogen wears off, the vagina passes through metestrous and then remains in diestrus again unless more estrogen is administered. By continuing the estrogen injections a constant vaginal estrus is obtained in the castrate mouse.

The uterus of the oophorectomized mouse responds in a similar manner to estrogen injections. After oophorectomy the uteri atrophy in size and the endometrial glands involute. Mitoses, distension, and an increase in water content follow the injection of estrogen. The endometrial glands undergo hyperplasia. According to Nelson (1939), one effect on the uterus after prolonged

estrogen treatment is the production of cystic hyperplasia of the endometrial glands. It should be noted that in normal cycles, estrogen and progesterone are needed for the complete differentiation of the uterus. Progesterone is required for implantation of the fertilized egg.

Pituitary and Ovarian Interrelationships

In a study by Fevold (1941), the administration of purified FSH to hypophysectomized rats increases ovarian weight by causing the differentiation and growth of numerous follicles. If the FSH is freed sufficiently from LH, it does not produce any luteinization. In the rat highly purified FSH produces follicular growth, but, since the uterus and vagina remain infantile, it is believed that such enlarged follicles do not liberate effective amounts of estrogen. However, when minute amounts of LH are added to the FSH, the ovary secretes enough estrogen to bring the sex accessories into an estrus state. Thus, it appears that FSH in combination with LH is essential for the secretion of estrogen, the level of LH being subminimal for ovulation and luteinization.

The LH, administered alone, produces little effect upon ovarian follicles. Corpora lutea are not formed unless the follicles have been matured to an appropriate degree by FSH.

The simultaneous administration of FSH and LH to hypophysectomized rats produces a much greater increase in ovarian weight than when comparable amounts are administered separately. In other words, there appears to be a synergistic interaction between the two hormones.

Various experiments have demonstrated that estrogen decreases the secretion of FSH and that castration increases the output of FSH (Meyer et al., 1946). Furthermore, there is evidence that moderate amounts of estrogen may increase the release of LH. Thus the two ovarian hormones may exert opposite effects upon the secretion of gonadotrophins by the pituitary gland.

When a hypophysectomized female rat is united parabiotically with an oophorectomized female, the ovaries of the hypophysectomized partner are stimulated by the gonadotrophic hormones secreted by the intact pituitary of the castrate partner. After oophorectomy the pituitary becomes predominantly a follicle-stimulating mechanism. As a result of the FSH from the oophorectomized female, many large follicles appear in the hypophysectomized parabiont. These follicles neither ovulate nor luteinize, but become cystic and eventually degenerate. Under the influence of constant estrogen secretion by the cystic ovaries, the uterus becomes inflated with a watery secretion, and the vaginal smears indicate that the hypophysectomized

animal is maintained for long periods in an estrus state (Witschi and Levine, 1934).

If LH is administered to such a female paraboint, the follicles ovulate and luteinize. This indicates that the pituitary of the oophorectomized female provides little LH.

The Nervous System and Reproduction

The changes in the ovary which culminate in ovulation have been known to be controlled by hormones of the pituitary as reported in the classical work of Smith and Engle (1927). In recent years evidence has accumulated which reveals that the release of the pituitary hormones necessary for ovulation are stimulated via the central nervous system.

Certain species, including the rabbit and cat, require the neural stimulus accompanying coitus to induce ovulation. These "reflex" ovulators are a small minority group when compared with the number of species that ovulate cyclically or seasonally in a so-called "spontaneous" manner.

Many workers including Harris (1959) reported that weak localized stimulation to certain parts of the hypothalamus induced ovulation in rabbits. Sawyer (1959) blocked ovulation in rabbits with or without inducing

ovarian atrophy by placing small bilateral electrolytic lesions in the basal tuberal nuclei of the hypothalamus.

In spontaneously ovulating animals, anterior hypothalamic lesions block the ovulatory cycle in a state of constant estrus, as first reported in the guinea pig by Dey (1943) and confirmed in the rat by Hillarp (1949) and others. Harris (1959) and Greep and Barnett (1951) induced ovarian atrophy and permanent anestrus by destruction of the pituitary stalk in the rat.

The act of ovulation forms a link in the chain of events essential for the survival of the species. These are (1) the ripening of a follicle, (2) the rupture of a follicle and discharge of an ovum, and (3) the synchronization of the behavioral response of the organism, so that at the time of ovulation the female is receptive to the male.

These three processes, by the work of many individuals in the past 20 years, have been related to the hypothalamus. The first two are regulated by the gonadotrophic secretion of the pituitary gland which is under the control of the hypothalamus. The latter, the estrus behavior of the female, appears to be evoked by a sufficient concentration of ovarian hormones in the blood acting on some neural mechanism in the posterior hypothalamus.

Donovan and Harris (1956) working with ferrets showed that lesions placed in the anterior hypothalamus accelerated estrus while lesions placed elsewhere produced an anestrous state. They believed that estrus producing lesions act by destroying some nervous mechanism which normally inhibits the release of FSH.

Dey (1943) noted the presence of permanent estrus, associated with ovaries containing ripe follicles but no corpora lutea, in guinea pigs which had lesions placed in their anterior hypothalamus. Barrnett and Mayer (1954), Greer (1953), and Hillarp (1949) have made similar observations on the rat.

It can be stated, then, that anterior hypothalmus lesions may convert either the anestrous female ferret or the regularly cyclic female rat into a state of constant estrus. It should be noted that in the case of the ferret, the animal is changed from a state of reproductive quiescence to one of apparently full activity, whereas in the rat the conversion is from one type of active state to another. At the present time the case of the ferret may be regarded as a release effect on FSH secretion, and in the rat as a blockading action on the rhythmic release of LH.

In general, the process of ovulation seems to require for its initiation a sudden increase in the secretion of LH against a background of FSH secretion. Markee *et al.* (1946) found that stimulation of the rabbit's

pituitary did not result in ovulation, whereas stimulation of the hypothalamus did result in ovulation. The use of the remote-control method by Harris (1959) confirmed Markee's findings. The evidence indicated that the hypothalamus, in some manner, stimulated the release of LH from the pituitary.

The method of stimulation from the hypothalamus to the anterior pituitary is believed to be by way of the hypophysial portal system. These vessels drain blood from the median eminence of the hypothalamus to anterior pituitary (Worthington, 1955). Any permanent blockade of this portal system results in an atrophic condition of the reproductive system (Harris, 1959). Apparently some hormonal substances liberated by nerve endings in the median eminence, is transported by these vessels to the anterior pituitary to activate or inhibit the cells of the gland.

The experiment of Nikitovitch-Winer and Everett (1957) confirmed the above results. These workers hypophysectomized female rats and transplanted the pars distalis to the kidney capsule. No estrous cycles occurred in these animals. The grafts were then removed from the kidney to the median eminence. These animals with transplants under the median eminence, which is vascularized by the hypophysial portal vessels, regained normal

reproductive function as shown by cyclic estrous changes, pregnancy, and normal ovaries, uteri and vaginas.

The above examples have demonstrated that the relationship between the central nervous and endocrine systems is one of reciprocity. Just as the nervous system maintains and regulates endocrine function, so do the hormones react back on the nervous system to modify its activity. For example, the ovarian hormones exert actions on the central nervous system, which cause the female to be receptive at the time of ovulation. Estrogenic substances implanted in the posterior hypothalamus can evoke the mating behavior in the spayed cat (Harris, 1959).

Bioassay

Reliable chemical methods for the quantitative determination of pituitary gonadotrophins are not available, and assays must be conducted biologically. Since there are many bioassay methods, only the two employed in this study will be reviewed. A review of other methods can be found in Gray and Bacharach (1961).

LH Assay

According to Greep *et al.* (1941) the enlargement of the ventral lobe of the prostate in hypophysectomized immature male rats is specific for LH activity and is not affected by the presence of FSH. The addition of purified

FSH to purified LH injections did not enhance the enlargement of the ventral lobe of the prostate in such animals.

These findings have been confirmed by many workers including McArthur (1952), Loraine and Brown (1956), and Apostolakis (1959). Lorain and Brown (1956) found that the index of precision of this method was less than 0.2 which is highly acceptable in the clinical field today.

FSH Assay

Steelman and Pohley (1953) described tests with intact rats which confirmed the observations of Bates and Schooley (1942) that human chorionic gonadotrophin will augment the action of FSH on the weight of the ovary. This test is convenient, precise and sensitive and has been used with success for estimations of FSH activity in urine and in pituitary tissue by various workers including Neal et al. (1954), Fletcher and Brown (1957), and Steelman and Segaloff (1959). The specificity and precision of this test is very satisfactory. Simpson et al. (1956) made parallel assays on the same pituitary extracts by the antral test and the "augmentation" test with similar results. According to Simpson the precision of the antral test is below 0.15, a figure with which the augmentation test is comparable.

Insecticide Residues

Chlorinated organic insecticides and often times their metabolites can be stored in the various parts of

the animal body. The recent application of gas-liquid chromatography to the detection of insecticide residues promises to increase the sensitivity and accuracy of residue measurements. A summary of some chlorinated insecticide residue studies is given in the following paragraphs.

Aldrin

Male rats fed aldrin for 12 weeks showed greatest accumulation in the fat followed by the kidney, liver and brain. There was much less aldrin stored in the kidney and more in the liver of the female as compared with the male (Kunze and Laug, 1953). Treon and Cleveland (1955) demonstrated that the withdrawal of aldrin from the diet of rats after seven to 18 months on 25 ppm did not result in release of the insecticide from adipose tissue rapid enough to induce poisoning.

Borgman (1952) reported the following ppm accumulations for female rats after nine months ingestion:

dietary (ppm)	fat	liver	kidney	brain	muscle
5	20	1.5	1.0	2.0	2.0
15	35	4.0	1.0	1.0	3.0
25	48	2.5	1.5	--	2.0
45	80	3.5	2.1	2.6	1.5

Little residue was found after aldrin was removed from the diet for nine weeks. Ban *et al.* (1956) revealed that

aldrin is converted to dieldrin and stored as such in the body of rats. Apparently, the aldrin residues reported in older studies are actually dieldrin.

Chlordane

In a study by Ambrose et al. (1953), male rats fed 320 ppm accumulated 43, 41, and 81 ppm in the fat in 5, 148, and 407 days, respectively. Twice as much chlordane was found in the females. The withdrawal of chlordane from the diet resulted in a prompt drop in storage with little residue found after 30 days.

DDT

DDT, the most intensively studied insecticide, has been found to accumulate in body tissues at dietary concentrations as low as 1.0 ppm (Laug et al., 1950). In one week, measurable amounts can be found in the fat of rats, with the females showing the greater accumulation. As the dose is increased, more is stored in body tissues, but in an inverse relationship due to incomplete gut absorption at very high levels.

When DDT is fed in concentrations below 440 ppm to rats, maximum storage values are reached in 54 days, after which they remain constant (Woodard and Ofner, 1946). At higher concentrations, residues continue to increase. Females contained 150 and 300 ppm DDT in their fat after

54 days ingestion of 12.5 and 25 ppm, respectively. The metabolism of DDT appears to take place in the liver since it disappears so quickly from this organ.

Ortega et al. (1956) reported that rats store DDT in proportion to the dose concentration. At dietary levels up to 200 ppm, storage values reached an equilibrium within six months. DDT is gradually lost after its removal from the diet of rats. After 14 months, measurable amounts of DDT could still be found in the rat tissues.

Kunze et al. (1949) found a progressive rise in stroage of DDT up to maximum values in 23 weeks. At 1 to 10 ppm, maximum accumulation is reached sooner. After the removal of DDT from their diet, 50% remained after one month and 25% after three months. Following two years ingestion of DDT by rats the following values were obtained:

DDT in Diet (ppm)	DDT in Fat (ppm)
10	119
100	103
200	313
400	1091
800	4220

Withdrawal of food from animals on a very high dosage of DDT for two years caused characteristic tremors. Presumably, such animals were metabolizing body fat containing large amounts of DDT.

In a study by Durham et al. (1956), male rats injected with diethylstilbestrol, an estrogenic compound, while given a diet containing DDT, stored more DDT and had larger livers than the corresponding controls. Female rats, under similar circumstances, had larger livers and less storage of DDT than corresponding female controls which were not injected with testosterone propionate, a compound with androgenic activity.

Dieldrin

Rats fed dieldrin for 12 weeks showed an accumulation pattern similar to that for aldrin (Kunze and Laug, 1953).

Endrin

Rats fed endrin for 12 weeks gave a similar accumulation pattern as reported above for aldrin and dieldrin (Kunze and Laug, 1953). Endrin disappears gradually from the tissues of animals after removed from their diet. Dieldrin and DDT behave in a similar manner, but the evidence indicates that these compounds, especially DDT, accumulate in the fatty tissues to a greater extent and that, conversely, they tend to disappear less rapidly than does endrin under the same circumstances (Treon et al., 1955).

Heptachlor

At a low dose such as 30 ppm, only the metabolite of heptachlor, heptachlor epoxide, is found in rat tissues. At higher doses, both heptachlor and the epoxide form are found in rat tissues. When rats were fed a 30 ppm dietary for two weeks, 165 ppm heptachlor epoxide were found in the fat (Davidow and Radomski, 1952).

Toxaphene

Lehman (1952b) found no trace of toxaphene in rat tissues after two years ingestion at a concentration of 100 ppm. At 400 ppm, 180 ppm toxaphene were found in the body fat.

There seems to be no generalization which can be made concerning the accumulation of chlorinated organic insecticides except that some do accumulate. DDT, a relatively mild toxicant to mammals, is stored readily even at low dietary levels. Toxaphene, another very mild toxicant, is not stored except at very high doses.

The accumulation of an insecticide in the body of a mammal seems to depend mostly on the stability of the chemical compound and the enzymatic-excretion potentialities of that mammal in relation to the compound. It has been stated that detoxification involves the polarizing and the decreasing of the fat solubility of the toxicant so that

the kidney or other possible excretion pathways do not continue to reabsorb it passively. Possibly this involves the oxidation or conjugation of the toxicant within the microsomes and the RNA components of the liver cells which are very permeable to fat soluble substances.

METHODS AND MATERIALS

Rearing Facilities and Maintenance

In the initial part of this study from January to December of 1962, the laboratory animals were housed in a wooden structure located on the campus of The Ohio State University, Columbus, Ohio. The temperature was controlled by means of fans, windows and a gas stove. During the cooler months, the temperatures ranged from 65 to 75° F, while in the warmer months it ranged from 70 to 90° F. Temperatures were recorded by means of a hygrothermograph. The humidity was not controlled.

Unless otherwise specified, white mice of the BALB/cJaxGnMc strain, produced by sibling matings for more than 80 generations, were utilized in this study. The animals were confined in galvanized steel (6 x 12 x 5 inches) or plastic (7 x 12 x 5 inches) cages and provided with food and water. Not more than four animals were maintained in a single cage except when a young litter was present. The cages and cedar shavings litter were cleaned once a week and the water changed twice a week. Purina Mouse Chow, obtained from the Ralston Checkerboard Square Company, St. Louis, Missouri, was used throughout the study.

In December of 1962, the mouse colony was moved to the rearing room in annex of the Botany and Zoology Building of The Ohio State University. This rearing room was air-conditioned, with constant temperature controls which maintained the temperature between 70-74° F and a relative humidity of 20-40%.

The animals were confined in stainless steel cages identical in design to the previously mentioned metal cages. Care procedures remained the same.

During the course of this study there were no outbreaks of disease and less than 5% mortality of control animals.

Mortality Studies

In order to approximate the chronic toxicity of Kepone to the laboratory mouse, groups of mice were fed various dietary levels of Kepone. Tests were carried out with individuals and with groups of two to four adult and juvenile mice of both sexes. Diets impregnated with 0, 10, 30, 40, 50, 60, 70, 80 and 100 ppm Kepone were evaluated.

The treated and control mice were confined in metal cages, the number ranging from one to four per cage. In one exception, 16 male mice were confined in a large cage and given a diet containing 100 ppm. A minimum of 12 animals were fed at each dietary level. All mice were observed each day for tremor or other signs of poisoning.

Food pellets were ground by means of a burr-mill to pieces approximately one-fourth inch diameter to give better distribution of the toxicant. Each concentration of Kepone was dissolved in 25 ml acetone which was applied by pipette to one kilogram of chow in a slowly rotating, one gallon pickle jar. The chow was rotated for 20 minutes at 20 rpm to insure thorough distribution of the toxicant. The acetone was allowed to evaporate from the open containers at least 24 hours before the chow was offered to the mice. Food was freshly treated every two or three weeks. Desired concentrations of Kepone were prepared from a 93.6% pure technical grade material.

Food and Water Consumption

Food consumption was measured by weighing the feed containers and their contents. The difference between two successive weighings represented the amount of food consumed. The amount of waste was insignificant, because the food particles were too large to pass through the mesh of the food containers. Water consumption was computed in a similar manner by successive weighings of the water bottles to the nearest tenth of a gram.

Measurements were also made of the entire food consumption of the mouse colony. As the amount of food purchased and treated was known as well as the number of

mice present, an estimate was made of individual consumption from these data.

In the initial screening test, the food consumption of 10 adult males was measured daily for 10 days. Eight adults were placed in two metal cages, four per cage. Single adult males were placed in two additional cages. Measurements were made on single and groups of animals because it was observed that single animals tend to consume more food than when they have one or more companions. One-half of these animals were fed a diet containing 40 ppm Kepone while the remaining five animals served as the controls. All animals were five to six-months-old, weighing 25-30 grams each.

In another screening test involving adult males weighing 30-35 grams each, two males in individual cages and two males in a single cage had their water and food consumption measured for two weeks. They were then fed 100 ppm Kepone and their food and water intake measured daily until all had died.

The food and water consumption of 20 adult virgin females weighing 22-27 grams each, fed a dietary level of 40 ppm Kepone, were compared with 20 control females for 31 and 48 days, respectively. There were four females per metal cage.

Juveniles 21-24-days-old weighing 8-13 grams each were placed on various dietary levels of Kepone and their

food and water consumption measured for two months or until death. The dosage levels tested were 0, 40, 60, 70, 80 and 100 ppm. Two females and two males were fed at each concentration. One of each sex was housed separately and the other two paired in a single cage. Due to the short life span of juveniles on the treated levels of 70, 80 and 100 ppm, these concentrations were repeated giving a total sample of four females and four males. At the 70, 80, and 100 ppm levels, food and water consumption were measured daily. At the remaining levels measurements were made twice a week.

Growth Measurements

Juveniles of both sexes, 21 to 24-days-old weighing 8 to 13 grams each, were placed on various dietary levels of Kepone to learn of any effect on growth. These data were collected by weighing the animals twice a week during the course of their mortality and food and water consumption tests. Mice on dietary levels of 70, 80 and 100 ppm were weighed daily. All weighings were performed on a Harvard trip balance or on a dispensing balance to 0.1 gram.

Most of the animals used during the course of this study were weighed prior to any treatment, after any tests, and before their dissection for examination. Although such data do not represent growth rates, they do provide information on the variation of normal body weights.

Major Organ Weights

During specific tests or at their termination, treated and control mice were sacrificed and examined. Individual organs were carefully dissected out immediately after the animal was killed, and weighed. Therefore, there was little chance for dehydration of the organs before they were weighed. The following weights were recorded: total body, liver, kidneys, heart, spleen, testes, ovaries, uterus, adrenals, brain and pituitary. Total body and liver weights were determined by a trip balance or a dispensing type balance to the nearest tenth of a gram. The remaining organs were weighed on a single-pan analytical balance to one-tenth of a milligram. The majority of the organs were placed into Bouin's fixative for 24 hours after they were weighed, and then into 70% alcohol so that histological sections could be made if desired.

To determine when exceptionally enlarged livers develop and if the fat content of such livers increases, two adult males fed 40 ppm and two controls were sacrificed at each of the following intervals: 7, 10, 14, 21, 28, 42, 56 and 120 days. During the entire project the liver weights of most of the sacrificed animals were recorded along with their history of treatment. This provided information concerning liver enlargement over a wide range of treatments, age groups and sexes.

The fat content of the livers was determined by the following procedure:

1. After killing the animal, its liver was immediately dissected out and weighed to the nearest tenth of a gram.
2. The liver was then macerated with a porcelain mortar and pestle in a medium of anhydrous sodium sulfate. The amount of sodium sulfate was equal to four times the weight of the liver.
3. The macerated liver was extracted with diethyl ether for four hours using a Soxhlet extraction apparatus.
4. The solvent was evaporated from the fat extract and the remaining extract was weighed to the nearest tenth of a gram.

Reproduction Data

In the beginning of this study four groups of adult mice, each group composed of eight pairs, were fed 10, 30 and 37.5 ppm Kepone, respectively, for one month prior to mating. These mice were of mixed parentage and will be designated as group A in the remaining pages.

These treatment levels were selected because of previous work by Good *et al.* (1963) in which reproduction

was severely reduced at the 30 and 37.5 concentrations without severe mortality.

After both sexes had been fed Kepone for one month at the indicated concentrations, the pairs were mated, one male and one female per plastic cage. Males were caged continuously with the females until pregnancy, after which the males were removed until the young were born and weaned. If pregnancy did not occur within three weeks, the original male was replaced.

The females were checked daily for pregnancies and litters. After the birth of a litter the following information was recorded: mother, father, date of litter, number of young plus observations concerning maternal care, presence of a nest, and tremors. Reproduction data were collected from group A for 100 days from January until May, 1962. In May all pairs were separated in preparation for future tests.

In June, 1962, matings were made between treated males and control females and control males and treated females. The treated females and males were from group A and the control females and males from the first offspring of the controlfemales of group A. All treated animals had been fed 40 ppm for a minimum period of two months.

Ten control males were put in individual plastic cages and each mated with two females, one treated and one control. The treated females were marked by toe clippings.

Ten treated males were mated in a similar manner. The males were separated from the females after a two-week mating period. During the mating period all animals were fed a 40 ppm treated diet. After the mating period, previously untreated mice were returned to a control diet and the previously treated mice were maintained on a treated diet.

In August, 1962, 28 pairs of four and one-half-month-old mice of the pure strain BALB/cJaxGnMc were mated to confirm the earlier data collected from group A. These mice will be designated as group B in the remaining pages. Fourteen of the 28 pairs had been fed 40 ppm Kepone for two and one-half months prior to mating. The remaining 14 pairs served as the controls. The procedure followed was that described in relation to group A. Reproduction data were collected from group B for 100 days from August until November, 1962, when all pairs were separated.

Ten control males and 10 control females, all offspring of group B, were mated with 10 treated females and 10 treated males, respectively, from group B. Single pairs were mated in individual plastic cages in November, 1962. The 40 ppm diet was fed to all animals during the four week mating period.

The following experiment was designed to learn if reproduction would resume when treated animals were permanently removed from a treated diet. In December,

1962, the 10 treated males and the 10 treated females from group B were placed on an untreated diet and mated with 10 control females and 10 control males, respectively, also from group B. Each of the control females had borne two previous litters and each of the control males had fertilized females in earlier matings. None of the treated females had borne a litter in previous matings, but some of the treated males had fertilized control females in cross mating tests. Reproduction data were collected for 100 days from December, 1962, until April, 1963. Each pair was placed in an individual cage and the procedure was that described for groups A and B.

Vaginal Smears

As discussed in detail in the literature review, the vaginal smear technique permitted one to find out, in a simple, reliable manner, whether the female estrous cycle was occurring in a normal manner.

The medicine dropper method was employed since it appeared to be the least irritating method to the females. A medicine dropper drawn to a fine point was inserted into the vagina and one or two drops of saline solution were quickly forced out and then sucked back into the medicine dropper. This solution was then placed on a glass slide, a drop of methylene blue added with a cover slide, and then microscopically examined. The methylene blue aided in

differentiating the cell types since the nuclei of the epithelial cells absorbed it intensely. The physiological saline solution prevented the swelling and bursting of the leukocytes.

Vaginal smears were taken daily for three weeks from two groups, each group composed of 20 two-month-old virgin females. These two groups had estrous cycles comparable in length, regularity and occurrence. One group was then fed 40 ppm Kepone. Three weeks and eighteen weeks later, daily vaginal smears were taken of both groups for three and four weeks, respectively.

Vaginal smears were also taken from the breeder groups, A and B. Smears were taken daily from group A for eight days prior to sacrifice after their reproduction data had been collected. Histological sections were then made of their major organs including ovaries. Smears were taken from group B for three weeks prior to mating. Vaginal smears were also taken from females prior to sacrifice, if their ovaries were to be sectioned.

Histological Studies

Any major alterations of tissues were determined by examining histological sections of the following organs: Liver, kidneys, brain, spleen, pituitary, ovaries, uterus, testes and adrenals. Kepone-fed and control animals were examined.

Two staining techniques were utilized including the standard hematoxylin-eosin method and an azocarmine-orange G-methylene blue stain combination. Two methods were utilized in hope that one might disclose something that the other would not. The details of these methods are presented in the appendix.

Each ovary was completely sectioned in a serial manner allowing the entire organ to be examined. The objectives for sectioning the ovaries were to check follicle development and corpus luteum formation. The degree of follicle development would be a reflection of FSH activity and corpus luteum formation would indicate ovulation and sufficient LH activity.

Each ovary was microscopically examined and follicle counts were made of all follicles 270 microns or more in diameter. The diameter measurements were made from the exterior margins of the theca externa of the follicle by means of a calibrated ocular micrometer. Corpora lutea from the last estrous cycle were also counted. Due to their intensive staining, these luteal bodies were easily differentiated from older corpora lutea or other ovarian structures.

Median sections of the testes were prepared to determine if normal spermatogenesis was occurring and if normal amounts of interstitial cell tissue were present. The normal condition was determined by comparison with

untreated fertile males. If the seminiferous tubules were well developed and contained the various stages of spermatogenesis, it was considered a normal and active testis.

Median sections were prepared of the other organs plus sections from abnormal appearing areas. Some adrenals, pituitaries and brains were completely sectioned in a serial manner.

Serological Studies

Since some blood tests might yield valuable information concerning some important physiological actions of Kepone, determinations were made of the following blood components: Calcium, sodium, glucose, and protein-bound iodine (PBI). The blood tests, with the exception of the PBI tests, were conducted by the Clinical Laboratory, College of Veterinary Medicine, The Ohio State University. The PBI determinations were made by the Arlington Laboratories, Columbus, Ohio.

One of the major problems with serology studies involving mice concerns the micro-volume quantities of blood which must be used from such small mammals. Only in recent years have micro-volume procedures been developed, so that little information is available in the literature concerning serology tests with mice.

The blood was obtained by means of a heart puncture from mice anesthetized with diethyl ether. A two or five-ml syringe with a 20-gauge needle was used to draw the blood from the heart. The syringe and needle were rinsed with a physiological saline solution to prevent hemolysis. One-half ml of whole blood was the average volume obtained from an individual mouse and the animal would survive if the heart was punctured properly.

Calcium and sodium determinations were made by flame photometry.[a] Calcium determinations were made from six samples, each pooled from 4 six to eight-month-old females weighing 22-29 grams each. Two samples were from females fed 40 ppm for five months, two samples from females fed 100 ppm for two weeks and two samples from control females. The maximum error is 0.2 mg/100 ml, which is based on two standard deviations of the controls. Normal calcium values in humans and rats range between 8.5-11.5 mg/100 ml.

Sodium determinations were made from individual samples of 12 females weighing 22-29 grams each and five to six-months-old. Four samples were drawn from control females, four samples from females fed 40 ppm for four

---

[a]Determinations of calcium and sodium were made with a Model 21 Coleman Flame Photometer. The details of these methods can be found in the Manual of Operating Directions for the Model 21 Coleman Flame Photometer, 1961. The Coleman Instrument Company, Maywood, Illinois.

months, and four samples from females fed 100 ppm for two weeks. The maximum error is 3 mEq/liter, which is based on two standard deviations of the controls. Normal sodium values for humans and rats range between 130 to 150 mEq/liter.

Blood glucose was determined by the Saifer and Gerstenfeld (1958) method. Basically, the method consists of adding specific enzymes to the prepared blood samples which react only with glucose. These reactions produce a yellow color, the intensity of which is proportional to the glucose concentration. The color intensity is measured by means of a colorimeter at a wave length of 415 mμ. The maximum amount of error is 6 mg/100 ml based on two standard deviations of the controls. The normal fasting glucose for humans and rats ranges between 80-100 mg/100 ml.

Blood glucose determinations were made from the individual samples of 24 female mice weighing 22-29 grams each and five to six-months-old. Eight samples were from control females, eight samples from females fed 40 ppm for four months, and eight samples from females fed 100 ppm for two weeks. Four of the eight samples from each of the three different groups were drawn from mice which had been fasted for 16 hours.

Protein-bound iodine was determined by the Barker et al. (1951) dry ash method. Normal values for humans

range from 4-8 mEq/100 ml. The PBI was determined in three samples, each sample being pooled from four females weighing 22-29 grams each and five to six-months-old. One sample was drawn from control females, the second from females fed 40 ppm for four months, and the third from females fed 100 ppm for two weeks.

Hormone Bioassays

In an attempt to elucidate one of the possible reasons for the reproductive inhibition observed in females fed 40 ppm Kepone, assays of the FSH and LH gonadotrophins were undertaken. There is very little literature available concerning the assay of hormones in mice. Therefore, it was necessary to establish the amount of pituitary tissue needed to obtain measurable activity of the gonadotrophins and to find out if a linear relationship existed between the number of pooled pituitaries and the amount of response of the indicator organ.

The augmentation method of Steelman-Pohley (1953) and the ventral prostate method of Greep et al. (1941) were selected to assay FSH and LH, respectively. The specificity and precision of both methods had been confirmed by Stevens (1962) and many other workers. Both methods allowed crude pituitary extracts to be used on the assay rats.

A trial test on LH was conducted with pituitary extracts from five-month-old virgin females weighing 22-25 grams each. The entire extraction procedure was performed in a constant temperature cold room maintained at 40-43° F. The details of the method are presented in the appendix.

Pituitary extracts containing the hormones of one, two and four pituitaries were prepared. Three samples at each concentration were prepared plus controls and blanks. Each sample was placed in a numbered vial and refrigerated at a temperature of 38-40° F. All samples were prepared within 24 hours of the first injection.

Hypophysectomized 24-day-old Sprague-Dawley male rats weighing 45-50 grams each were used as the assay animals. All rats were weighed before and after the test in order to determine whether variability in the size of the rat affected the dose response.

The hypophysectomized rats were injected twice daily, approximately eight hours apart, with the prepared extracts for four days. A 2.0-ml syringe with a 27-gauge needle was used to make the subcutaneous injections on the dorsal side of the lower neck region. The injection volume was kept constant at 0.5 ml.

The ventral prostate gland was used as the indicator organ of LH activity since this organ is specific to LH and its degree of response is not altered by the presence of FSH. Seminal vesicle and testicle weights were also

recorded. These organs are not specific in their response to LH, but do indicate total gonadotrophic response.

The rats were sacrificed 120 hours after the first injection. The ventral prostate was immediately dissected out and weighed to one tenth of a milligram on a single-pan analytical balance. The seminal vesicles and the testes were then dissected out and weighed in a similar manner.

The actual assay of LH activity in the pituitaries of control and Kepone fed mice was conducted as described above. Sample extracts were pooled from four pituitaries since such a quantity gave the most satisfactory results in the test trials. Seven sample extracts were prepared from five-month-old virgin females weighing 22-25 grams each. These females had been fed 40 ppm Kepone for three months. Seven samples from control females of the same age and weight group were also prepared plus controls and blanks. A control was a sample identical to the test sample except that it contained no pituitary extract. Blanks were un-injected rats.

A trial run on the assay of FSH was conducted with pituitary extracts from five-month-old virgin females weighing 22-25 grams each. The method of procedure was that followed in the LH assays except that the extracts were fortified with 50 International Units (I.U.) of human chorionic gonadotrophin (HCG). Since the pituitary contains considerably more FSH than LH (Stevens, 1962),

only samples containing the extract of one or two pituitaries were assayed. Three samples were employed at each concentration plus controls, blanks, and samples fortified with HCG, but containing no pituitary extract. This last mentioned control was necessary in order to measure the response of the indicator organ to this added hormone.

Intact, 21-day-old, virgin Sprague-Dawley rats weighing 40-45 grams each were employed as the assay animal in the FSH tests. The increase in weight of the ovary was used as the indicator organ of FSH activity since the relationship between administered FSH and ovarian weight was linear when augmented with chorionic gonadotrophin.

According to Stevens (1962), the reason that LH does not alter the ovary response is because HCG is an LH-like substance, and the amounts of HCG given under assay conditions are quite large, so that the ovaries are "overwhelmed" by LH activity and the addition of exogenous pituitary LH does not produce any measurable response.

The 21-day-old rats were injected twice a day, approximately 8 hours apart, for three days. As in the LH assays, 0.5 ml was used. The rats were sacrificed 72 hours after the first injection. The ovaries were immediately dissected out, freed from any fat, and weighed to one tenth of a milligram on a single-pan analytical balance. The

uteri were also dissected out and weighed in order to find out if any relationship existed between their weight and the dosage of pituitary extract administered.

Following the trial tests, the actual assays on treated mice were conducted in which the pooling of two pituitaries per sample were employed in the FSH assays. The procedure followed was that described in the trial assays.

Seven samples were prepared from five-month-old virgin females weighing 22-25 grams each which were fed 40 ppm Kepone for three months. Seven samples were also prepared from a group of control females of the same age and weight plus controls, blanks and fortified controls. All samples were prepared within 24 hours of the first injection.

The sample sizes used in these assays were determined in part by the number of treated mice available. A total of 114 pituitaries were extracted in the FSH and LH bioassays.

Insecticide Residues

Insecticide residues of Kepone in mice were measured for seven major reasons: (1) to measure the relative concentration of Kepone in the various organs; (2) to determine if Kepone is accumulated at a constant sublethal level of ingestion; (3) to determine if Kepone

is excreted following the withdrawal of it from the animal's diet; (4) to discover if it passes through the placenta of a pregnant female; (5) to discover if it passes through the milk of a nursing female; (6) to determine if any metabolite could be detected; and (7) to find out if any pertinent information could be obtained which might help to explain Kepone's mode of action.

A gas-liquid chromatograph[a] with electron capture (E.C.) detector was used for the analysis of prepared samples. The E.C. detector is extremely sensitive to chlorinated compounds, but it is not sensitive to many other organic materials present in animal extracts. This avoids some of the laborious clean-up procedures otherwise necessary (Anonymous, 1962).

The E.C. detector measures the loss of current rather than a positively produced current. As the nitrogen gas flows through the detector, a radium source ionizes the nitrogen molecules and forms electrons. Collectively, these electrons produce a steady current to the electrometer. If a sample is then introduced containing electron absorbing molecules this current will be reduced. The loss of current is a measure of the amount and electron affinity coefficient of the compound.

---

[a]A Barber-Coleman Model-15 gas chromatograph manufactured by the Barber-Coleman Company, Rockford, Illinois, was used for the residue analyses.

For the analysis, a three foot long, 0.25 inch inside diameter Pyrex glass column was employed containing Gas Chrom 60/80 mesh coated with 3% SE-52. High purity nitrogen, at a pressure of 14 PSI, served as the carrier gas at a flow rate of 100 ml/minute through the column. The voltage plateau ranged from 42.5 to 65 volts at a sensitivity of 10 and an attenuation of one. The injector, column and detector units were maintained at 250, 225 and 250° C, respectively. Under these conditions, Kepone had a retention time of seven minutes.

Samples were prepared for analysis by a method slightly modified from that described by Taylor (1963). The details of this method can be found in the appendix. A 10 µl syringe was used to inject the samples into the gas chromatograph. Injection volumes were usually one or two µl depending on the peak area obtained. A greater injection volume was required with some samples from very small organs such as the pituitary.

A series of standards was established by the use of samples containing known amounts of Kepone. Peak area was used as the unit of measurement in the quantitative studies. Unknown samples were then calculated from the standard curve. The calculated recovery rate from fortified samples ranged from 75 to 90% with an average of 85%.

In the preparation of the test samples, the weight of each organ and the volume of solvent which contained the extract were recorded. This information permitted the ppm concentration to be computed for each sample analyzed. Test samples were prepared from Kepone-fed mice and controls. Samples were prepared from the following organs: Liver, brain, pituitary, adrenals, kidneys, ovaries, uterus, testes, spleen, heart, body fat, and muscle tissue. Samples were prepared from embryos and placentae of Kepone-fed mice. Specific parts of suckling young including the brain, liver and stomach contents and whole suckling young were also analyzed.

RESULTS

Mortality and Tolerance Studies

To evaluate the relative toxicity of Kepone to the laboratory mouse, selected groups of juveniles and adults of both sexes were fed various dietary levels of Kepone. In all tests, controls were run simultaneously. Mortality tests were not conducted at concentrations below 40 ppm since no adult mortality occurred below this level in the reproductive tests. The following paragraphs summarize the mortality tests at each dietary level evaluated. A summary of these tests can be found in Table 2.

Controls

During these tests, little mortality occurred within the control groups. From the beginning of this project in January of 1962 until its completion in June of 1963, approximately 300 control animals were used for various lengths of time. During this 18 month period, only 10 individuals died representing a mortality rate of less than 4%. This figure represents only adult mortality.

Forty ppm

Four adult females and four adult males were fed 40 ppm for six months with no mortality. Very slight tremors,

Table 2. Summary of Kepone Mortality Studies with the Laboratory Mouse

Diet (ppm)	Age	Number	Initial Death (days)	Cumulative Percent Mortality at Indicated Days of Treatment			
				25%	50%	75%	100%
50	Adults	8	70	--	--	--	--
	Juveniles	4	--	--	--	--	--
60	Adults	8	107	--	--	--	--
	Juveniles	4	75	75	87	92	98
70	Adults	16	17	19	21	42	?
	Juveniles	8	5	5	9	12	15
80	Adults	8	5	10	14	15	25
	Juveniles	8	5	9	11	13	17
100	Adults	70	6	12	16	20	32
	Juveniles	8	2	5	10	13	19

noticeable only when the mouse was held by the tail, appeared within 14 days. The intensity of tremor would increase and reach a maximum within two months. It would remain constant for one to two months and then decline to a fine, constant tremor or fluctuate in intensity for as long as the animal was maintained on the diet. Following the withdrawal of the treated diet, tremors would decrease within seven days and be hardly noticeable within three weeks. After four weeks, no sign of tremor was present.

No mortality occurred among two female and two male juveniles, 21-24-days-old weighing 8-13 grams each. The test lasted two months but these individuals were maintained on a 40 ppm diet for six to nine months at which time they were sacrificed. A tremor pattern similar to that expressed by the adults was demonstrated.

The majority of data collected in this project were from mice fed 40 ppm. During the entire project over 200 animals were treated at this concentration for various periods of time. Only 12 of these died which represents a mortality rate of less than 6%. Neither sex appeared to be more susceptible than the other to Kepone. Nearly 100 of the more than 200 treated animals were maintained on a dietary 40 ppm for eight or more months and a few up to 18 months without significant mortality.

Fifty ppm

Of the four adult females and four adult males tested at this concentration for six months, only one adult male died which succumbed after 70 days of treatment. No mortality occurred among the two females and two males, 21-24-days-old, weighing 8-13 grams each, during their two months of treatment. These juveniles were actually maintained on this treatment for six months before they were sacrificed.

These animals demonstrated a tremor pattern similar to the 40 ppm treated animals. Tremors would first appear at approximately 14 days after treatment, and become constant within four weeks. The maximum intensity of tremor would occur within two months after which they would remain constant or vary in cycles, sometimes declining to a constant, fine intensity tremor.

Sixty ppm

A dietary level of 60 ppm was lethal to juveniles but not to adults. Only one adult of the four females and four males tested for six months died. This female succumbed after 107 days of exposure.

In the juvenile trials, two females and two males weighing 8-13 grams each were fed 60 ppm following weaning. Their food and water consumption were greater than the controls, but their growth rate was normal.

These animals, as the adults, demonstrated a tremor pattern similar to that discussed in the 40 and 50 ppm dosages, and seemed to tolerate the treatment until the third month of treatment. One female died after 75 days of treatment and the second female after 92 days. The two males died after 87 and 98 days.

Seventy ppm

The 70 ppm concentration represented the line of demarcation between lethal and sublethal levels for the adult laboratory mouse. Some adults tolerated this level but the majority could not. Eight adult males and eight adult females were tested at this concentration and all died with the exception of two males which tolerated this concentration for six months before being withdrawn from treatment.

Slight tremors, noticeable when holding the mouse by the tail, appeared within 10 days after treatment. Tremors became constant within three weeks and increased to the convulsive stage in those animals that died early. The intensity of tremor fluctuated in the survivors and usually increased prior to death. In the two adult males that survived, the tremor pattern eventually became a constant fine tremor somewhat more exaggerated than that displayed at lower concentrations with irregular

fluctuations of intensity. Upon withdrawal of treatment, all tremors disappeared within four weeks.

The 8 females tested died 18, 20, 29, 29, 30, 33, 38 and 38 days after the beginning of treatment. The six males, which failed to survive, died after 17, 19, 22, 32, 36, and 42 days of treatment.

Twenty-one to 24-day-old juveniles weighing 8-13 grams each succumbed quickly to this dosage. The four juvenile females died after 5, 9, 12, and 12 days of treatment. The four males died 5, 6, 13, and 15 days after treatment began.

The juveniles which died within seven days developed slight tremors within three days which became severe in one or two days prior to death. Those juveniles which survived for nearly two weeks did not develop tremors so quickly. In these animals, slight tremors first appeared after five to seven days of treatment and became more severe in the following days eventually ending with death.

Eighty ppm

A level of 80 ppm was lethal to all adults and juveniles tested. Four adult males died after being exposed for 10, 15, 22 and 25 days. Four adult females died after 5, 13, 14 and 18 days of ingestion.

Four juvenile females, 21-24-days-old died after 9, 9, 11 and 12 days of treatment. The four juvenile males died after 5, 13, 16 and 17 days of treatment.

Slight tremors developed within three to seven days in the adults, becoming constant and quite noticeable within 7-10 days. Severe tremors developed rapidly ending with convulsions and death. In most animals, severe tremors appeared within 10-14 days and frequently these animals would remain in severe tremors for many days before death with no great weight loss. Juveniles developed a similar tremor pattern except that tremors usually developed one to two days earlier.

One Hundred ppm

A concentration of 100 ppm Kepone was lethal to all animals. As this concentration represented the first attempt at finding the chronic lethal dosage, it was tested several times on different test groups to confirm the earlier data. After establishing that 100 ppm was a lethal concentration, the other levels between 40 and 100 ppm were evaluated to find the maximum sublethal dosage for adults and juveniles.

At 100 ppm, all adults died within 32 days after the beginning of treatment with the initial death coming within 8-10 days. In the juveniles, all died within 19

days with the first death occurring after two to five days. No difference in response was found between the sexes.

The tremor pattern in the adults and juveniles was similar to that described at the 80 ppm concentration. Animals that died within 21 days usually developed serious tremors in 10-14 days. Animals that persisted longer frequently did not develop serious tremors until a week later or else showed a fluctuation in tremor intensity before death. In all cases, severe tremors developed one to several days before death with some animals remaining in a state of severe tremors for as long as 14 days prior to death.

After withdrawal of the treated diet, severe tremors subsided to a mild intensity within four to seven days. Within two weeks only slight tremors could be detected and no tremors were visible after four weeks.

In one test, three adult females were alternated every two days from an untreated diet to one containing 100 ppm Kepone. Two females died after 10 days and the third after 13 days. The first two females at the time of their death had been exposed to a treated diet for six of the 10 days. The tremor pattern and time of initial death were similar to previous tests at this concentration which had not been alternated. The third female was placed on an untreated diet at the time of the death of

the other two females and was in serious tremors. This female died three days later even though it ingested no more Kepone.

This suggests that animals which die after exposure to Kepone, may have ingested the necessary lethal amount several days prior to death. If the animal has not ingested a lethal quantity, it usually responds quickly to the removal of the toxicant from its diet.

Food and Water Consumption

In the initial screening test, five control adult males weighing 25-30 grams each, ingested an average of 4.2 to 5.0 grams of chow per day. Five adult males of the same age and weight group ingested an average of 6.5 to 7.1 grams of chow per day after their chow had been treated to contain 40 ppm Kepone. Water consumption was not recorded.

In a more extensive test with adult females, the food consumption of 20 control females weighing 20-25 grams each, individually averaged 3.6 grams per day over a 31 day period. Water consumption, measured for 48 days, averaged 5.1 grams per day. A similar group of 20 females, which had been fed 40 ppm for two months, averaged 5.1 grams of treated chow per day per individual and 7.0 grams of water per day. The weekly consumption rates can be found in Tables 3 and 4. Calculated on a

Table 3. Average Daily Food Consumption of 20 Kepone-Fed and 20 Control Adult Virgin Females

Diet (ppm)	Grams/Day/Individual during Indicated Day Intervals				
	0-8	9-15	16-24	25-31	Average
0	4.0[a]	3.7	3.6	3.8	3.8
0	3.5	3.6	3.5	3.7	3.6
0	3.6	3.6	3.5	3.7	3.6
0	3.7	3.9	3.3	3.6	3.6
0	3.4	3.4	3.2	3.0	3.3
40	5.8	5.7	5.1	5.5	5.5
40	4.4	4.5	4.1	4.7	4.5
40	5.8	6.4	5.5	6.2	5.9
40	4.3	5.2	4.2	4.4	4.5
40	5.2	5.5	4.8	5.0	5.1

[a]Each figure represents the average derived from four females housed in one cage.

Table 4. Average Daily Water Consumption of 20 Kepone-Fed and 20 Control Adult Virgin Females

Diet (ppm)	Grams/Day/Individual during Indicated Day Intervals						
	0-8	9-17	18-25	26-32	33-41	42-48	Average
0	5.4[a]	4.7	5.1	5.2	4.6	4.8	5.0
0	6.4	4.9	6.0	5.5	4.9	5.3	5.5
0	6.1	5.1	4.9	5.2	4.8	4.8	5.2
0	5.8	4.4	4.7	5.1	4.2	4.4	4.8
0	6.1	5.3	5.1	5.1	4.8	4.4	5.2
40	8.0	6.9	7.6	7.3	6.4	6.9	7.1
40	6.3	5.4	5.6	5.7	5.2	5.5	5.6
40	9.5	9.1	9.2	9.3	8.1	8.5	8.9
40	7.0	6.5	6.4	6.6	5.8	6.2	6.4
40	7.8	7.4	7.3	7.9	6.1	6.2	7.1

[a]Each figure represents the average derived from four females housed in one cage.

percentage basis, the treated group consumed 42% more food and 37% more water than the controls.

The food and water consumption of weanlings, 21-24-days-old weighing 8-13 grams each, were measured for two months when fed 0, 40, 60, 70, 80 and 100 ppm. The weekly consumption rates of these groups can be found in Tables 5 through 9.

In summary, the control group averaged 3.9 grams of chow per day per individual and 5.1 grams of water. The mice fed 40 ppm averaged 5.5 grams of chow per day per individual and 6.7 grams of water. The mice fed 60 ppm averaged 5.4 grams of chow per day per individual and 7.6 grams of water. In every case individuals confined separately ate and drank more than the average for an individual in a group, although the additional amount was slight in many cases and greatest in relation to water consumption.

In the 70, 80 and 100 ppm tests, the mice fed 70 ppm averaged 3.4 grams of chow per day per individual and 4.5 grams of water. The mice fed 80 ppm averaged 3.5 grams of chow per day per individual and 4.8 grams of water. The mice fed 100 ppm averaged 2.8 grams of chow per day per individual and 4.4 grams of water.

In the 70, 80 and 100 level tests, the majority of the juveniles did not live longer than 10 days and therefore food and water consumption comparisons with other levels

Table 5. Average Daily Food Consumption of Kepone-Fed and Control 21-24-Day-Old Juveniles

Diet (ppm)	Sex[a]	Grams/Day/Individual during Indicated Day Intervals					
		0-6	7-18	19-30	31-39	40-54	55-67
0	M & F	3.7	3.9	4.5	3.4	3.5	3.7
	M	4.2	4.3	4.1	4.3	4.1	4.0
	F	2.7	3.6	4.0	3.6	3.9	4.1
Average		3.6	3.9	4.3	3.7	3.7	3.9
40	M & F	4.5	5.5	5.0	5.2	4.5	4.9
	M	4.3	7.7	7.2	6.2	5.9	7.1
	F	4.2	5.8	6.0	5.0	5.0	5.5
Average		4.4	6.1	5.8	5.4	5.0	5.6
60	M & F	3.3	6.7	5.5	4.5	5.0	5.5
	M	3.3	5.0	5.5	6.3	6.2	6.3
	F	4.2	6.5	6.7	4.5	5.4	5.1
Average		3.5	6.2	5.8	5.0	5.4	5.6

[a]One male (M) and one female (F) were housed together, while another male and female were housed separately.

Table 6. Average Daily Water Consumption of Kepone-Fed and Control 21-24-Day-Old Juveniles

Diet (ppm)	Sex[a]	Grams/Day/Individual during Indicated Day Intervals[b] 30-39	40-54	55-67	Average
0	M & F	4.2	4.7	4.3	4.5
	M	6.2	5.6	6.0	5.9
	F	5.6	5.2	5.5	5.4
Average		5.1	5.0	5.1	5.1
40	M & F	5.6	6.4	6.0	6.1
	M	7.3	7.9	7.3	7.5
	F	6.5	7.5	7.1	7.1
Average		6.3	7.0	6.6	6.7
60	M & F	6.1	7.4	6.7	6.8
	M	8.0	9.1	8.4	8.6
	F	7.5	8.3	8.0	8.0
Average		6.9	8.1	7.4	7.6

[a]One male (M) and one female (F) were caged together while another male and female were caged separately.

[b]Water measurements were not begun until 30 days after the beginning of treatment.

Table 7. Longevity, Food and Water Consumption and Total Kepone Consumed by 21-24-Day-Old Juveniles Fed 70 ppm Kepone

Sex[a]	Longevity Following Initiation of Treatment (days)	Food (grams)	Water (grams)	Kepone (mg)
M	13	36.5	53.0	2.60
M	6	17.0	30.0	1.20
M	5	14.0	20.0	1.00
M	15	69.0	84.0	4.80
Average	9.7	34.1	46.7	2.40
Ave./Day	--	3.5	4.8	0.24
F	5	12.0	14.0	0.80
F	9	19.0	27.0	1.30
F	12	50.0	67.5	3.50
F	12	49.5	65.5	3.50
Average	9.7	32.6	43.5	2.30
Ave./Day	--	3.4	4.5	0.23
Total Average	9.7	33.5	45.0	2.40
Ave./Day	--	3.5	4.6	0.24

[a]M = male; F = female.

Table 8. Longevity, Food and Water Consumption and Total Kepone Consumed by 21-24-Day-Old Juveniles Fed 80 ppm Kepone

Sex[a]	Longevity Following Initiation of Treatment (days)	Food (grams)	Water (grams)	Kepone (mg)
M	5	16.0	28.0	1.30
M	13	39.0	61.0	3.10
M	16	65.0	82.0	5.20
M	17	56.0	85.0	4.50
Average	12.7	44.0	64.0	3.50
Average/Day	--	3.5	5.0	0.28
F	9	28.5	38.5	3.10
F	9	26.0	36.0	2.90
F	11	37.5	50.0	4.50
F	12	46.0	58.0	5.10
Average	10.3	34.5	45.5	2.80
Average/Day	--	3.4	4.4	0.27
Total Average	11.5	40.0	55.0	3.20
Average/Day	--	3.5	4.8	0.28

[a]M = male; F = female.

Table 9. Longevity, Food and Water Consumption and Total Kepone Consumed by 21-24-Day-Old Juveniles Fed 100 ppm Kepone

Sex[a]	Longevity Following Initiation of Treatment (days)	Food (grams)	Water (grams)	Kepone (mg)
M	19	55.0	95.0	5.50
M	16	47.0	69.0	4.70
M	5	19.0	24.0	1.90
M	10	25.0	45.0	2.50
Average	12.5	36.5	58.0	3.70
Average/Day	--	2.9	4.6	0.29
F	13	40.0	56.0	4.00
F	8	25.0	34.0	2.50
F	11	22.5	47.0	2.30
F	2	6.0	7.0	0.60
Average	9.0	23.5	36.0	2.40
Average/Day	--	2.6	4.0	0.26
Total Average	10.8	30.0	47.0	3.00
Average/Day	--	2.8	4.4	0.28

[a]M = male; F = female.

are not valid. The majority of juveniles treated at these concentrations had growth impairment almost immediately. The food and water consumption averages at the 70, 80 and 100 ppm levels are a reflection of very early mortality. The average longevity of 21-24-day-old juveniles after the initiation of treatment at the 70 ppm level was 9.7 days, at 80 ppm it was 11.5 days, and 10.8 days at 100 ppm.

The growth rate and longevity of 21-24-day-old juveniles were not affected at the 40 and 60 ppm concentrations. Calculated on a percentage basis, juveniles fed 40 ppm averaged 41% more chow and 24% more water intake than controls. Juveniles fed 60 ppm had 38% greater food intake and 49% greater water intake than the controls.

In a test involving four adult males weighing 30-38 grams each, their average individual food intake per day for 14 days was 5.5 grams of chow and 7.0 grams of water. They were then fed a dietary 100 ppm. Food and water consumption increased in all tested animals. This increased consumption occurred approximately three to seven days after starting the treated diet. The average individual amount of food and water consumed by these males until their death was 7.5 grams of treated chow and 9.0 grams of water. This represented a food consumption increase of 36% and a 28% increase in water intake.

Whether there is any correlation between the level of treatment and the degree of increase in food and water intake cannot be stated at this time. Precise measurements were not made of adults of the same age and weight groups at the various levels of treatment. Calculations made from the total amount of food prepared and the total number of mice on each level of treatment during the project indicates that there is some relationship between intake and level of treatment although not in direct proportion. The following figures represent these calculations.

Control Animals: 130 animals consumed 100 pounds of chow in 67 days for an average of 4.3 grams per day per individual.

40 ppm: 95 animals consumed 33,000 grams of treated food in 60 days for an average of 5.8 grams per day per individual.

50 ppm: 4 animals consumed 1000 grams of treated chow in 44 days for an average of 5.7 grams per day per individual.

60 ppm: 2 animals consumed 650 grams of treated chow in 44 days for an average of 7.4 grams per day per individual.

80 ppm: 3 animals consumed 750 grams of treated food in 25 days for an average of 10.0 grams per day per individual.

100 ppm: 3.5 animals consumed 500 grams of treated chow in 17 days for an average of 8.4 grams per day per individual.

These figures indicate some degree of relationship between concentration and consumption up to the 80 ppm level. At the 100 ppm level there occurs a decrease which proves interesting if the following calculations are made. A mouse eating 10 grams per day of food containing 80 ppm Kepone would ingest 0.8 mg Kepone per day. A mouse eating 8.4 grams per day of food containing 100 ppm Kepone per day would ingest 0.84 mg Kepone per day. This may be the explanation for the similar mortality rates between these two levels of treatment on mice.

Growth Measurements

Growth was measured for two months from 21-24-day-old juveniles of both sexes fed 0, 40, 60, 70, 80 and 100 ppm Kepone. No growth impairment was observed at the 40 and 60 ppm concentrations, but growth was severely affected at higher levels. Actually the 40 ppm treated animals demonstrated a slightly greater growth for the first five weeks, after which there was no major difference between any of the 0, 40 and 60 ppm groups. The growth measurements of the juvenile groups are presented in Tables 10, 11, 12 and 13.

Table 10. Growth Measurements of Kepone-Fed and Control 21-24-Day-Old Juveniles

Sex[a]	Individual Body Weights (grams) at Indicated Days after Initiation of Treatment					
	0	6	13	18	25	30
			Controls			
M	12.2	17.0	21.0	21.5	23.0	24.0
M	12.7	19.0	22.5	22.5	24.0	24.5
F	11.8	15.0	17.0	18.0	19.5	19.5
F	12.7	16.5	18.5	19.5	21.0	21.5
Average	12.3	16.9	19.7	20.4	21.9	22.4
			40 ppm			
M	13.2	19.5	21.5	23.5	24.5	24.0
M	11.0	17.5	20.5	21.0	24.0	23.5
F	11.7	17.5	21.0	22.5	24.0	23.0
F	11.0	15.5	18.5	19.5	20.5	19.5
Average	11.5	17.5	20.4	21.6	23.2	22.5
			60 ppm			
M	13.7	19.0	21.5	21.5	23.5	23.5
M	12.5	18.0	20.5	21.5	22.0	21.5
F	11.5	16.5	18.5	19.0	22.0	20.5
F	10.0	14.0	16.0	17.5	19.5	18.5
Average	11.9	16.9	19.1	19.9	21.7	21.0

[a]M = male; F = female.

Table 10--Continued

Sex[a]	Individual Body Weights (grams) at Indicated Days after Initiation of Treatment					
	33	39	46	54	62	67
			Controls			
M	24.5	25.0	26.5	27.0	27.5	28.0
M	25.5	26.0	26.3	28.2	28.2	28.7
F	20.0	21.0	21.5	22.2	23.5	24.0
F	21.5	23.0	22.5	24.0	24.2	24.5
Average	22.9	23.7	24.2	25.4	25.9	26.2
			40 ppm			
M	25.5	25.7	26.5	27.7	28.5	28.8
M	25.0	24.5	25.7	24.0	26.5	28.2
F	24.0	24.5	23.8	24.0	25.7	27.0
F	20.5	20.3	21.0	21.7	22.5	22.0
Average	23.7	23.7	24.2	24.3	25.7	26.5
			60 ppm			
M	24.0	25.0	26.0	27.5	28.0	28.0
M	22.5	23.5	23.5	24.5	25.8	25.8
F	22.0	23.0	22.5	23.5	24.0	24.0
F	19.0	20.0	20.0	21.2	20.5	22.5
Average	21.9	22.9	23.0	24.2	25.0	25.0

[a]M = male; F = female.

Table 11. Individual Body Weights of 21-24-Day-Old Juveniles Fed 70 ppm Kepone

Sex[a]	Grams/Individual at Indicated Days after Initiation of Treatment						Death Day	Weight at Death
	0	4	7	9	12	15		
M	13.5	14.2	15.0	16.0	14.3	--	15	13.6
M	8.2	9.5	10.0	10.5	9.5	--	13	9.0
M	12.8	12.0	--	--	--	--	5	11.7
M	10.9	11.5	--	--	--	--	6	11.3
F	8.5	10.5	11.5	12.5	--	--	12	10.8
F	9.4	11.2	12.6	11.9	--	--	12	10.9
F	8.1	9.0	8.7	--	--	--	9	8.6
F	10.2	11.0	--	--	--	--	5	10.8

[a]M = male; F = female.

Table 12. Individual Body Weights of 21-24-Day-Old Juveniles Fed 80 ppm Kepone

Sex[a]	Grams/Individual at Indicated Days after Initiation of Treatment						Death Day	Weight at Death
	0	4	7	9	12	15		
M	11.9	11.6	12.6	12.6	12.6	12.0	16	11.3
M	12.5	12.6	13.0	14.5	14.5	14.0	17	12.7
M	11.6	10.5	11.5	10.5	10.0	--	13	9.2
M	9.5	10.0	--	--	--	--	5	9.4
F	10.2	12.1	12.8	12.8	--	--	12	10.6
F	9.8	11.4	11.1	11.0	--	--	12	9.2
F	9.5	10.5	11.1	--	--	--	9	10.8
F	8.2	9.0	9.6	--	--	--	9	9.1

[a]M = male; F = female.

Table 13. Individual Body Weights of 21-24-Day-Old Juveniles Fed 100 ppm Kepone

Sex[a]	Grams/Individual at Indicated Days after Initiation of Treatment						Death Day	Weight at Death
	0	4	7	12	15	17		
M	13.0	12.5	11.0	11.5	12.3	12.0	19	11.0
M	12.0	14.0	16.0	15.5	14.5	--	16	14.0
M	11.5	9.5	9.7	--	--	--	10	9.0
M	8.0	7.5	--	--	--	--	5	7.0
F	8.0	9.0	10.0	10.8	--	--	13	10.8
F	10.5	11.0	11.0	--	--	--	8	10.5
F	8.0	7.5	7.0	--	--	--	11	6.0
F	8.0	--	--	--	--	--	2	7.0

[a]M = male; F = female.

Growth was affected immediately or within the first few days at the 70, 80 and 100 ppm levels. Even those animals which persisted for more than two weeks showed little increase in size. This can not be attributed to a decrease in food consumption since they ate amounts comparable to control animals of the same age or weight. Some weight loss was characteristic one to several days prior to death even when food consumption did not decrease proportionally.

At lethal dosages, adult animals began losing weight within two weeks. In one test the body weight and the food and water consumption of four adult males were recorded for two weeks. After this two week period, these adult males were fed 100 ppm Kepone. Within two weeks body weights began to decrease gradually even though food and water consumption had increased. This initial weight loss was at least partially caused by a reduction in body fat in the rump region. The body weights of the four adult males at the beginning of treatment were 35.0, 26.0, 37.0 and 29.5 grams each. At death, 19 to 32 days later, they weighed 26.5, 21.0, 26.0 and 25.0 grams each.

In later tests with females, very little body fat could be found in the rump region after they had been fed 100 ppm for two to three weeks. The decrease in body weight these animals experienced was not a large and sudden reduction. Usually such treated animals would maintain

their body weight for one to two weeks after which a gradual reduction would occur prior to death. This weight loss was not extreme, usually only one to five grams depending on the initial weight of the mouse. The fatter the animal, the greater the weight loss prior to death. Although most animals that died from lethal levels of Kepone experienced some body weight loss, the decrease was not nearly great enough to cause death.

At sublethal dosages, adult animals maintained normal body weights. Mice fed 40 ppm for six to eighteen months maintained normal body weights and body fat in the rump region. Animals fed 40 ppm for such long periods usually weighed one to two grams more than comparable controls due to the enlargement of their livers. It should be stressed, however, that these animals consumed more food and water than the control mice, and they probably could not have maintained their normal weights without this increase in food and water consumption.

Major Organ Weights

Groups of individuals from the various tests were sacrificed, examined and their various organs were weighed to detect any major changes which might have occurred. No differences were found in the brain, heart, adrenals, testes and spleen of adult males. The liver was enlarged

greatly, however, and the kidneys were slightly enlarged in Kepone-fed males.

In adult females, no differences were found in the adrenals, pituitary, brain, ovaries, thymus, heart and kidneys. As in males, the liver was greatly enlarged in treated animals, with a varying degree of enlargement of the spleen and uterus. The weights of the female and male organs can be found in Table 14.

No major differences were found in organ weights of mice treated for four or seven months. This indicates that organ enlargement, if it occurs, takes place within the first four months with little change thereafter. The liver data presented in Tables 15 and 16 clearly exemplifies this observation. For this reason, the major organ weights were averaged together in Table 14.

In regard to uterine weights, it is possible that these increased weights reflect the hormonal disturbance which occurs in treated females, to be discussed later. Under the influence of estrogen, the uterus is greatly distended with a corresponding weight increase. Since Kepone-fed females tend to remain in constant estrus, a stage during which estrogen is thought to be continuously secreted, the increased uterine weights may be a reflection of this condition.

The liver was the only organ which increased in weight in every animal treated. Since the liver was

Table 14. Major Organ Weights and Percent Body Weight of Kepone-Fed and Control Adult Mice

Organ[a]	Weight (grams) and Percent Body Weight							
	Control Males		40 ppm-Males[b]		Control Females		40 ppm-Females[b]	
liver	1.600	5.90%	3.300	11.8%	1.500	6.00%	3.500	14.00%
brain	0.390	1.40	0.396	1.4	0.385	1.50	0.382	1.50
pituitary	--	--	--	--	0.002	0.01	0.002	0.01
adrenals	0.005	0.02	0.005	0.02	0.006	0.02	0.006	0.02
kidneys	0.413	1.53	0.472	1.69	0.339	1.36	0.346	1.38
testes	0.214	0.79	0.210	0.75	--	--	--	--
ovaries	--	--	--	--	0.008	0.03	0.008	0.03
uterus	--	--	--	--	0.153	0.61	0.211	0.84
spleen	0.105	0.38	0.106	0.38	0.096	0.38	0.136	0.54
heart	0.161	0.60	0.167	0.59	0.135	0.54	0.144	0.58
thymus	--	--	--	--	0.038	0.15	0.036	0.14

[a]Minimum sample size of 20 animals.

[b]Mice fed Kepone 4-7 months and 6-9 months old.

Table 15. Average Body and Liver Weights and Percent Body Weight of Kepone-Fed and Control Adult Male Mice

Diet (ppm)	Days on Diet	Number	Body Weight (grams)	Liver Weight (grams)	Percent Body Weight
0	0	20	27.0	1.6	5.9
40	7-10	4	25.9	1.8	7.0
40	14	4	31.1	2.7	8.7
40	22	2	34.5	3.4	9.8
40	28	2	28.5	2.8	9.8
40	42	2	25.0	2.3	9.2
40	56-60	3	28.4	3.4	12.0
40	90	2	26.0	3.1	11.9
40	120	4	29.8	3.9	13.1
40	180-210	6	28.0	3.2	11.4
40	300	1	32.5	4.0	12.3
60	60	1	24.0	3.4	14.2
60	90	1	21.0	3.0	14.3
70	180	1	22.5	3.5	15.6
100	21-28	3	24.7	2.8	11.3

Table 16. Average Body and Liver Weights and Percent Body Weight of Kepone-Fed and Control Adult Female Mice

Diet (ppm)	Days on Diet	Number	Body Weight (grams)	Liver Weight (grams)	Percent Body Weight
0	0	40	25.0	1.5	6.0
40	5	1	21.5	1.5	6.9
40	15	1	23.5	2.0	8.5
40	30	2	20.5	1.7	8.0
40	90	3	21.7	2.5	11.5
40	150	2	27.5	3.3	12.0
40	210	15	25.0	3.6	14.4
40	240-270	28	26.4	3.5	13.3
40	500	2	26.0	3.9	15.0
100	6-8	8	21.5	2.2	10.2
100	14-16	9	21.0	2.6	12.4
100	21-28	12	23.0	3.2	13.9

unique in this respect, additional data were collected on this organ. During the course of this project, nearly 200 livers were weighed and examined along with the body weight, sex, age and treatment. Summaries of these data are presented in Tables 15 and 16.

On 40 ppm dietary, there was an indication of liver enlargement within the first week of treatment. As shown in Tables 15 and 16, the liver of a treated animal doubled its weight within 60 to 90 days and increased only a small amount in relation to body weight after this period. Upon withdrawal of the treated diet, livers would tend to return to normal. Animals which had been treated for two or more months at the 40 ppm level would require four to six months of untreated diet before their livers would approach a normal size. The longer the initial treatment, the greater the amount of time required for this reduction. On the 100 ppm diet, the liver would double its size within 30 days and in some animals within 16 days.

Measurements were also made of the fat content of livers of animals which had been treated at 40 ppm for various lengths of time. Enlarged livers contained more fat than normal livers, but when expressed as percent of the total liver weight, there was no detectable difference. In fact, when expressed as such, some livers of treated animals contained less fat than the controls. Control

mice averaged 0.1-0.3 gram of liver fat which ranged from 5-13% of the total liver weight. Livers of treated mice averaged 0.1-0.5 gram of fat which was 6-13% of the total liver weight.

Reproduction Data

One of the objectives of this study was to ascertain whether Kepone at sublethal concentrations adversely affects reproduction in the laboratory mouse. As indicated in Tables 17 and 18, Kepone had an adverse effect at every level tested ranging from 10 to 40 ppm.

The reproduction data collected for 100 days from group A are presented in Table 17. The number of young produced compared with the controls was reduced 23.9, 79.3 and 87.0% at 10, 30 and 37.5 ppm, respectively. Second litters only occurred in the control and 10 ppm groups. At 30 and 37.5 ppm, all litters occurred within the first seven weeks of the test. There were no signs of pregnancy in these two groups after this period.

At 10 ppm, percent survival of the young to weaning was normal, but the litter size, pair days per litter and pair days per young were all reduced. All the above categories were severely reduced at 30 and 37.5 ppm. Pair days per litter were calculated by dividing the number of days during which reproduction data were collected from the eight pairs of each treatment level (8 x 100) by the number

Table 17. Average Body and Liver Weights and Percent Body Weight of Adult Mice Fed Control Diets Following 40 ppm Kepone Diets

Days on Kepone Diet	Days on Control Diet	Number	Body Weight (grams)	Liver Weight (grams)	Percent Body Weight
			Males		
40	80	3	25.5	2.4	9.4
150	240	1	32.5	1.9	5.8
210	120	3	29.5	2.3	7.8
210	150	1	29.5	2.2	7.5
280	120	1	29.5	2.5	8.5
Controls	--	20	27.0	1.6	5.9
			Females		
60	120	8	28.0	2.0	7.1
90	14	1	24.0	2.7	11.3
90	24	1	22.0	2.0	9.1
50	150	1	28.0	1.6	5.7
210	120	6	28.0	1.6	9.3
210	150	1	28.5	2.1	7.4
Controls	--	40	25.0	1.5	6.0

Table 18. Reproduction Data for 100 Days of Kepone-Fed and Control Mice of Group A

Diet (ppm)	Pairs	Number First Litters	Number Second Litters	Average Young per Litter	Survival of Young (%)	Pair Days[a] per Litter	Pair Days[b] per Young
0	8	7	5	7.7	89	66.7	8.7
10	8	6	4	7.1	87	80.0	11.3
30	8	4	0	4.7	26	200.0	42.1
37.5	8	3	0	4.0	42	266.7	66.7

[a]Number of reproduction days times number of pairs divided by number of litters.

[b]Number of reproduction days times number of pairs divided by number of young.

Table 19. Reproduction Data for 100 Days of Kepone-Fed and Control Mice of Group B

Diet (ppm)	Pairs	Number First Litters	Number Second Litters	Average Young per Litter	Survival of Young (%)	Pair Days[a] per Litter	Pair Days[b] per Young
0	14	14	14	7.1	89	50.0	7.0
40	14	0	0	--	--	--	--

[a]Number of reproduction days times number of pairs divided by number of litters.

[b]Number of reproduction days times number of pairs divided by number of young.

of litters produced. The pair days per young were calculated by dividing the pair days by the number of young produced at each treatment level.

The reproduction data from group B are presented in Table 18. In this experiment only controls and a dietary 40 ppm treatment were compared for 100 days. Preceding this test, the experimental mice were treated for two months before mating. The reproduction of the control group was favorable during this period with all 14 pairs producing two litters. No litters were produced among the 14 pairs of Kepone-fed mice.

To determine if Kepone affected the reproductive capacity of both sexes equally, the following two experiments were conducted. In these experiments, treated diets were given to all animals during the mating period after which they were returned to their previous diet. In this way, reproduction would not be due to cessation of treatment. As described in the Methods and Materials section, one mating test was with mice from group A, the second test with mice from group B.

In the first test with mice from group A, only five litters were borne by 20 treated and 20 control females after a two week mating period. All litters were borne by control females, two of which were sired by treated males, the other three by control males. Two of the litters died within 48 hours after parturition and the other three were

weaned successfully. One of the two litters which died was sired by a treated male, the other by a control male. Litter sizes averaging 5.0 young per litter, were smaller than comparable controls which averaged 7.1. Percent survival of the young from birth to weaning was also below normal, being only 67% in contrast to 89% for control pairs. The small sample size of litters must be taken into consideration when comparing these figures. One of the females previously fed a control diet, died during pregnancy.

The second test involving mice from group B produced similar results. After a four week mating period, only two litters were produced by 10 treated and 10 control females. Both litters died within 48 hours after parturition. There were three other definite pregnancies, one of which ended in the death of the female while pregnant, the other two apparently were consumed by the mothers at birth as no remains of either were found. All litters and pregnancies occurred only in control females. As only treated males were mated with control females in this test, all pregnancies or litters were sired by treated males. Again, litter size and percent survival of the young to weaning were less than comparable controls.

The final reproduction experiment involved withdrawing the treated diet from previously treated males and females and mating them with control females and males.

All treated animals had previously been treated for six months at 40 ppm. These mice were all from group B, whose reproduction data appear in Table 18. All control animals had proved fertile in previous tests. None of the treated females had produced a litter in previous tests although some of the treated males had produced fertile matings with control females in the test just previously described.

Reproduction in previously treated females resumed in six to seven weeks following the withdrawal of Kepone from their diet. This means that fertile conceptions began occurring three to four weeks following the withdrawal of Kepone. Eight of the 10 previously treated females bore first litters and five gave birth to second litters within the 100-day test period.

Initial litters were smaller than comparable controls averaging only 4.3 young per litter. The percent survival of the young was reduced to 64.7% in contrast to 89% for the controls. Second litters were slightly larger (4.6) but the outstanding increase was in the increased survival of young to 87.5%. The number of young produced was only 42.4% of the control reproduction during the 100-day experimental period.

Control females mated with previously treated males began reproducing immediately. Litter size averaged 5.4 compared with 7.1 for the controls. Percent survival of the young was normal for both first and second litters,

Table 20. Reproduction Data for 100 Days Following Withdrawal of Dietary Kepone from Mice in Group B, Previously fed 40 ppm for 6 Months

Category	Control Females and Kepone-Fed Males	Control Males and Kepone-Fed Females	Controls
Pairs	10	10	5
First Litters	10	8	5
Ave. No. Young	5.5	4.3	7.4
% Survival	88.6	64.7	89.1
Second Litters	6	5	4
Ave. No. Young	5.7	4.6	7.0
% Survival	87.9	87.5	86.2
Pair Days/Litter	62.5	76.9	55.6
Pair Days/Young	11.5	17.3	7.6
% Reproduction	65.1	42.4	100.0

being 88.9 and 87.9%, respectively. This group of 10 pairs produced 10 first litters and six second litters in the 100-day test period. The number of young produced was 65.1% of the controls.

Several observations should be pointed out in relation to this last experiment and the two mating experiments between treated and control animals. Females that had previously been fed Kepone experienced some difficulties with their first litters following the withdrawal of Kepone. In the experiment in which Kepone was withdrawn from the diet, two females remained infertile and four of the remaining eight females lost their first pregnancies or litters either before or after parturition. One female died during pregnancy and another was sacrificed for examination after it had lost its litter and no remains were found. No such difficulties were apparent during the period of second litters which was reflected in a normal percent survival of young. Apparently, the detrimental effects of sublethal levels of Kepone are reversible if the treated diet is withdrawn. Since no definite conclusions could be reached, it seems best to sum the results by saying that Keponse residues in the female continued to exert a detrimental effect on the physiology of the reproductive system following the removal of Kepone from the diet. These data indicate that this period may range from 20 to 60 days.

In the two mating experiments between treated and control sexes, two pregnant females died, four of the seven litters died, and no remains of two pregnancies were found. It should be emphasized that these females were previously control animals placed on a 40 ppm diet at the time of mating. No pregnancy occurred in the previously treated females. In other words, control females fed a 40 ppm diet at the time of mating had fertile conceptions, but frequently had trouble either completing their full term of pregnancy or successfully weaning the young produced.

Several females placed on a 40 ppm diet in the late pregnancy stage did not have as much difficulty in completing their pregnancy or weaning their young, although the percent survival of the young was reduced. The initial development period of the embryo is apparently the most critical and sensitive period to undesirable influences including insecticides. The next most critical periods, as indicated by these studies, were the first 48 hours after birth and the time of weaning if a treated diet is continued.

In some cases, sucklings, nursing from mothers on a 40 ppm treated diet, were smaller than controls. As a result, a longer suckling period was sometimes necessary before weaning. In one litter, the young developed tremors during the suckling period. Adversely affected young, if they survived the weaning period, would grow

rapidly and reach normal size with the persistent tremor pattern discussed previously.

At this point the results of two individual tests should be reported. In one case, a female in late pregnancy was put on a diet of 100 ppm. Eight days later a litter of nine was born. The mother took normal care of the young for 10 days. On the eleventh day the young were found dead or dying and some had been chewed badly by the mother. No nest was present and the mother showed no maternal behavior. The following day all were dead. In this test, tremors appeared in the mother within eight days and continued to increase in intensity. The young seemed to be developing normally with hair appearing on the 10th day, but their average weight was only 3.0 grams each in contrast to 6.0 grams for comparable 10-day-old control sucklings. It could not be determined whether the young had died from malnutrition, Kepone poisoning, or if the mother had killed some herself.

In a similar test, a lactating female was put on a 100 ppm diet two days after the birth of her five young. Slight tremors appeared in the mother five days later. On the seventh day all the young were found dead or dying, no nest was present, and the mother was in constant tremors showing no maternal behavior. She was then fed a control diet and three days later the tremors had disappeared.

This same female was mated again and within five weeks gave birth to a litter of seven which she weaned successfully. In November, she gave birth to seven young, her third litter, and was again fed 100 ppm. Five days later the young were dead, no nest was present and the mother was in constant tremors. She recovered quickly following the removal of the treated diet.

She was mated again and gave birth to six young in January, 1963. Three days before the birth of the litter she was placed on 40 ppm. This litter, although less in weight than controls, appeared to be progressing normally until February 18 when two young were found dead and the remaining four were in constant tremors. These four young recovered, although the tremors persisted, and were weaned on March 9. They weighed 5.0 grams at 22 days of age, 9.0 grams at 30 days and 13.5 grams at 40 days when weaned. Controls averaged approximately 5.0 grams more at each age and were usually weaned at 28 days.

This female was maintained on 40 ppm and mated again. No pregnancies occurred. In late April she was fed a control diet and 46 days later gave birth to a litter. As had been observed in several other cases when the female had been recently removed from a treated diet, this litter died within 48 hours.

Vaginal Smears

Vaginal smears were taken from the following three major groups: (1) A group of virgin female adults; (2) the females of group A; and (3) the females of group B. A definite disturbance of the estrous cycle was found in all treated females. In general, treated females tended to develop constant or extended estrus.

The virgin group was divided into two subgroups of 20 females each. During the pretreatment trial both subgroups had similar estrous cycles in relation to estrus occurrence and length of the estrous cycle. After one subgroup had been fed 40 ppm for three weeks, smears were taken for the next three weeks. The daily smears revealed that the treated females had a much higher percent occurrence of estrus and a longer estrous cycle. Four treated females had developed constant estrus. During this three week period, 56.1% of the smears from the treated females were estrus in contrast to 23.9% from the controls. The average estrous cycle length was 7.7 days in the treated females as compared to 4.8 for the controls.

Daily smears were taken from the virgin females after the treated females received 40 ppm for four months. Daily vaginal smears for 30 days revealed that 12 of the 20 treated females had developed constant estrus. During this period over 71% of the smears from the treated females were estrus. The average length of the estrous cycle in

those few treated females having a cycle was 8.0 days. Only 24.8% of the smears from the control females were estrus with an average estrous cycle of 5.4 days. A summary of the vaginal smears from virgin females is presented in Table 20.

Smears taken from group A following their reproduction experiment gave similar results. Nearly 68% of the smears from the treated females were estrus as compared to 17.5% for the controls. Nine of the 16 treated females had developed constant estrus.

Smears taken from group B before their reproduction experiment revealed that 81% of the smears from the 14 treated females were estrus. Ten of these 14 females had developed constant estrus. These females were fed 40 ppm Kepone for two months. Only 23.8% of the smears from the 14 control females were estrus. The control females had a normal estrous cycle of 4.8 days. A summary of the vaginal smear data from groups A and B can be found in Table 21.

It is not known whether females which developed constant estrus were actually in true estrus. If cornified cells were the major cell component of a smear it was designated as estrus. The smears from the control females were very typical and distinct as to the stage of the estrous cycle. Smears from treated females were frequently atypical giving the impression that the regulating mechanism had been disturbed. As an example,

Table 21. Summary of Vaginal Smear Data from 40 ppm Kepone-Fed and Control Adult Virgin Females

Days Fed Kepone	Number Females	Smears	Percent Estrus	Estrous Cycle (days)
0	20	410	24.7	4.87
0	20	410	24.1	4.82
0	20	410	23.9	4.86
21	20	410	56.1	7.69
0	20	600	24.8	5.46
120	20	600	71.3	--

Table 22. Summary of Vaginal Smear Data from Kepone-Fed and Control Females of Groups A and B

Group	Diet (ppm)	Days on Diet	Number Females	Smears	Percent Estrus
A	0-10	240	16	128	17.5
A	30-37.5	240	16	128	67.5
B	0	60	14	294	23.8
B	40	60	14	294	81.0

some leukocytes would appear for one or two days in smears from a female which had developed constant estrus indicating that estrus was receding. But the next day, a typical estrus was present again instead of the expected metestrus.

Undoubtedly this estrous cycle disturbance is a reflection of a disturbed hormonal balance. The female system is sensitive and intricate depending on the proper interplay of the various cycling levels of hormones. Any disturbance of a single cycling hormone level could upset the estrous cycle. The atypical smears of some of the treated females may be an indication that the levels of these hormones are fluctuating to some degree but not enough to initiate the next stage of the estrous cycle.

Histological Studies

Microscopic examination of histoligical sections of the pituitary and thymus glands revealed no alteration in their internal structure. All but one of the 50 adrenal glands examined appeared normal. This lone exception had a layer of fat deposited around the medulla zone, and had been removed from a female fed 100 ppm Kepone for three weeks.

No alteration of structure was found in spleens from treated animals except moderate congestion in those spleens which were enlarged. Uteri removed from treated

females were normal except those in the estrogenic phase, which showed definite cystic hyperplasia of the endometrium. This condition is thought to be due to prolonged or abnormal estrogen stimulation.

The brain tissue of most treated animals appeared normal. There were indications of moderate congestion in a few brains, and some myelin alteration in the nerve tracts.

Testes from treated males appeared normal. The amount and degree of development of the seminiferous tubules, interstitial tissue, and the process of spermatogenesis all appeared normal.

Degenerative changes were commonly found in the kidneys of treated mice. They had varying degrees of glomerulonephritis, disseminated lupus erythematosus, hypertrophy of the tubules and hemorrhage in the distal convoluted and loop of Henle's tubules. Frequently, large exudates were found in the Bowman's capsule and the glomerular membrane was ruptured.

Hyperplasia and congestion were found in all livers taken from treated animals. The degree of other degenerative changes, such as focal necrosis and cellular hypertrophy, varied greatly although the degree appeared to depend on the duration of treatment with Kepone. Such degenerative changes were much more extensive in animals

treated for 10 to 16 months than in animals treated four to six months.

Electron microscopic examination of two livers from mice which had been fed 40 ppm for four months showed two major alterations: Reduced number of mitochondria and formation of liposphereS.

LiposphereS, when first identifiable, occur as small single bodies and are complex lipoprotein structures. As the dosage or duration of treatment is increased, the structures increase in number, size and thickness of the capsule. The structures tend to coalesce to form pleomorphic inclusions up to 25 microns in diameter. As the inclusions increase in size, the capsule acquires a definite fibrillar structure with distinct laminations. Similar inclusion bodies have been reported after exposure to a variety of compounds including carcinogenic azo-compounds and chlorinated naphthalenes, but only in rodents (Ortega et al., 1956).

Although based on a small sample size, more tumors occurred in the livers of animals that had been on a Kepone diet for 12 or more months than in animals treated a lesser period of time. Two of 10 mice treated for more than 12 months developed extensive tumorous growths throughout the liver. This condition was not found again during this study.

Table 23. Summary of Pathological Alterations Found in Histological Studies of Mice Fed 40 ppm Kepone from Two to Eighteen Months

Organ	Animals Examined	Pathological Findings
Pituitary	8	Normal
Spleen	8	Congestion when enlarged
Thymus	6	Normal
Adrenals	25	Normal
Brain	12	Some congestion and myelin alteration.
Uterus	12	Cystic Hyperplasia of endometrium
Kidneys	25	Glomerulonepleritis, tubule hypertrophy, distal tubule and loop of Henle hemorrhage, disseminated lupus erythematosus, large exudates in Bowman's capsule, and glomerular membrane rupture.
Liver	30	Focal necrosis, regenerative lobules, cellular hypertrophy, congestion, hyperplasia, liposphere formation and reduction in mitochondria number.
Ovaries	55	Ovulation and corpora lutea formation inhibited or severely reduced.

Histological examination of ovaries from treated and control females revealed that vesicular follicle development was normal in most treated females, but corpora lutea were usually absent. The average numbers of follicles 270 microns or more in diameter and corpora lutea in ovaries from 20 control females were 6.1 follicles and 3.8 corpora lutea per ovary. The average numbers of follicles and corpora lutea from the ovaries of 30 treated females which had been fed 40 ppm for four to eight months were 5.6 follicles and 0.8 corpora lutea per ovary. Ninety percent of the ovaries from control females contained one or more corpora lutea compared to only 25% in treated females. A summary of the pathological alterations in the organs examined is presented in Table 23.

Serological Studies

Determinations were made of the calcium, sodium, protein-bound iodine (PBI) and glucose levels in the blood of treated and control adult females. A summary of these data is presented in Table 24.

Calcium determinations disclosed no difference between treated and control mice. Values ranged from 8.5 to 10.5 mg/100 ml which are within the normal range established for rats and man. These data indicate that the characteristic tremors of chlorinated organic insecticide poisoning are not due to hypocalcemia. It is

Table 24. Summary of Serological Tests from Kepone-Fed and Control Adult Female Mice

Diet (ppm)	Days on Diet	Number[a] Samples	Average Blood Component Values	Range
			Calcium	
0	0	2	9.0 mg/100 ml	8.5-9.5 mg/100 ml
40	150	2	9.6 "	9.0-10.2 "
100	14	2	10.0 "	9.5-10.5 "
			Sodium	
0	0	4	140 mEq/L	131-156 mEq/L
40	120	4	122 "	111-132 "
100	14	4	134 "	127-144 "
			Protein-Bound Iodine (PBI)	
0	0	1	4.3 mg/100 ml	--
40	120	1	4.6 "	--
100	14	1	5.3 "	--
			Blood Glucose	
0	0	4	150.1 mg/100 ml	97.5-188.0 mg/100ml
40	120	4	133.3 "	107.5-188.0 "
100	14	4	117.9 "	94.0-140.0 "
			Blood Glucose (fasted 16 hours)	
0	0	4	100.5 mg/100 ml	85.0-110.0 mg/100ml
40	120	4	96.2 "	85.0-105.0 "
100	14	4	47.7 "	30.0-68.0 "

[a]Calcium and PBI samples represent pooled blood from four mice. All others were individual measurements.

also an indication that the parathyroid gland was functioning within normal limits in these treated mice.

Sodium values of all twelve tested animals were within normal limits indicating that the zona glomerulosa of the adrenal cortex was secreting sufficient mineralocorticoids to regulate the sodium content of the body and its acid-base balance. The average values for sodium in females fed 40 ppm lies on the lower range of normalcy and may be something to consider further. Possibly these slightly lower values are caused by the greater water intake of treated animals which in effect would dilute the sodium content in the extracellular fluids.

PBI values were all within a normal range of 4-8 mg/100 ml indicating proper functioning of the thyroid gland. It was thought that possibly the basal metabolism of treated mice would be greater than control mice since treated animals consumed greater quantities of food and water with no increase in body weight. If this were so, higher PBI values would be expected in treated animals. Possibly the basal metabolism is not greater in treated animals. Their greater food and water consumption may be due to an inefficient use of the food or decreased intestinal absorption. Less efficient use of consumed food could be caused by an interference at the enzyme level within the metabolite pathways.

Blood glucose values were normal in all non-fasted animals. However, four mice fasted for 16 hours after being fed 100 ppm Kepone for two weeks, did not maintain normal glucose levels. The level of one dropped to 30 mg/100 ml. After the fasting these four mice averaged 47.7 mg/100 ml glucose, compared to 100.5 and 96.2 mg/100 ml for the controls and the four females fed 40 ppm for four months.

Hormone Bioassays

Since many of the earlier studies of this project indicated a disturbance of the hormone levels in the female, bioassays were conducted of the follicle stimulating hormone (FSH) and the luteinizing hormone (LH) of the pituitary gland.

A trial test was conducted with both hormones to ascertain the minimal amount of pituitary tissue necessary to give a measurable effect within the bioassay rats. Such tests would also disclose whether there was a linear relationship between the number of pituitaries pooled and the response of the indicator organ.

The FSH trial test indicated that a pooling of two mouse pituitaries supplied enough extract to give a measurable response of FSH in the rat. The 50 I.U. of human chorionic gonadotrophin (HCG), which were used to fortify the extracts, increased the rat ovary weights

58.9% based on the weight of the ovaries from the control rats. The HCG fortification plus the extract from one pituitary increased the rat ovary weights 112.7%. Two pituitaries pooled plus a fortification of 50 I.U. of HCG increased ovary weights 202.9%.

No relationship was found between uterine weights and the number of pituitaries pooled. The uteri of rats injected with 50 I.U. of HCG alone equalled the uterine weights of rats injected with pituitary extracts plus the HCG. A summary of the FSH bioassays can be found in Tables 25 and 26.

In the actual FSH bioassay, no difference was found in pituitary extracts from treated and control females. This is in agreement with the histological studies which disclosed that follicle development was normal in treated females.

Although these bioassays indicated that the FSH content of the pituitaries from treated and control females were equal, there still remains the possibility that the release of these hormones may vary. In other words, treated mice may have equal amounts of FSH, but it may be a constant secretion rather than a cyclic release of the hormone. Histological and vaginal smear data suggest that Kepone-fed females are under a more constant stimulation of FSH than the control females.

Table 25. Follicle Stimulating Hormone Bioassays in Rats with Fortified Pituitary Extracts from Control Adult Female Mice

Treatment[a]	Number Rats	Initial Weight (grams)	Final Weight (grams)	Ovaries Weight (mg)	Uterus Weight (mg)
Controls	3	49.0	58.0	20.5	35.2
HCG	3	50.0	60.0	34.8	108.6
1-Extracts + HCG	3	45.5	53.5	43.6	117.8
2-Extracts + HCG	3	50.0	60.5	62.1	120.7

[a]Human chorionic gonadotrophin (HCG), 50 I.U., was used in all fortified extracts.

Table 26. Follicle Stimulating Hormone Bioassays in Rats with Fortified Pituitary Extracts from Kepone-Fed and Control Adult Female Mice

Treatment[a]	Number Rats	Initial Weight (grams)	Final Weight (grams)	Ovaries Weight (mg)	Uterus Weight (mg)
Controls	5	42.0	52.0	20.7	49.8
HCG	5	43.0	53.2	35.8	134.7
2-Extracts + HCG Kepone-Fed Females	7	42.2	54.1	53.0	136.6
2-Extracts + HCG Control Females	7	42.5	54.2	54.3	141.5

[a]Human chorionic gonadotrophin (HCG), 50 I.U., was used in all fortified extracts.

The LH trial test indicated that the pooling of four pituitaries was necessary to obtain a definite measurable response of LH. It also indicated a linear relationship between the number of pituitaries pooled per sample and the degree of response of the ventral prostate gland. No relationship was found between the rat seminal vesicle weight and the number of pituitaries pooled, but there was a linear relationship between testes weight and pituitary extract concentration. However, the testes criteria in rats is not LH specific as is the ventral prostate.

As indicated in Table 27, the extract of one pituitary resulted in a 45.8% increase in the ventral prostate if the control prostate weight is equal to one. Samples consisting of two and four pooled pituitaries increased the ventral prostate by 101.7 and 239.0%, respectively. One, two and four pituitary samples increased the weight of the rat testes 21.4, 32.3 and 73.0%, respectively.

The actual LH bioassay revealed a difference in the LH content of pituitaries from treated and control females (Table 28). The ventral prostate gland weights of hypophysectomized male rats averaged 16.2 mg when injected with pituitary extracts from females fed 40 ppm Kepone for three to four months. In comparison, the ventral prostate averaged 20.8 mg in rats injected with pituitary extracts from control adult females.

Table 27. Luteinizing Hormone Bioassays in Rats with Pituitary Extracts from Control Adult Female Mice

Treatment[a]	Number Rats	Initial Weight (grams)	Final Weight (grams)	Ventral Prostate Weight (mg)	Seminal Vesicle Weight (mg)	Testes Weight (mg)
Controls	3	49.2	59.0	5.9	6.0	145.7
1-Extract	3	48.8	58.3	8.6	6.3	176.8
2-Extracts	3	51.3	60.5	11.9	6.7	192.7
4-Extracts	3	52.5	62.3	20.0	9.3	252.1

[a]Each treatment contained the hormone extracts of 1, 2 or 4 mouse pituitaries as indicated.

Table 28. Luteinizing Hormone Bioassays in Rats with Pituitary Extracts from Kepone-Fed and Control Adult Female Mice

Treatment[a]	Number Rats	Initial Weight (grams)	Final Weight (grams)	Ventral Prostate Weight (mg)	Seminal Vesicle Weight (mg)	Testes Weight (mg)
Controls	7	47.1	54.5	6.3	5.4	134.3
4-Extracts Control Females	7	45.9	56.4	20.8	11.2	286.5
4-Extracts Kepone-Fed Females	7	46.9	54.7	16.2	8.9	236.2

[a]Each treatment contained the hormone extracts from four mouse pituitaries.

By interpolation from the LH trial test data, the activity of 4 pituitary extracts from treated females was equal to three normal pituitaries. This would mean that pituitary extracts from treated females contain approximately 25% less activity than pituitary extracts from control females.

This difference in LH content was also exemplified in the weight of the testes. As indicated in Table 28, the testes weights of rats injected with pituitary extracts from treated mice averaged 236.2 mg in contrast to 286.5 mg for rats injected with extracts from control females. By interpolation from the LH test trial data, this indicates an approximate 25% reduction in activity seen in the ventral prostate data. However, testicular response is thought to be due to the combined synergistic action of both FSH and LH and not specific to LH as is the ventral prostate gland.

Insecticide Residues

Residues of Kepone in the various organs of mice were measured by gas-liquid chromatography. Techniques used were such that the data should be taken only as approximations. A summary of these data are presented in Tables 29 through 33.

Mice fed 40 ppm for 150 days contained an average of 462.5 µg Kepone in their liver, brain and kidney samples. Mice fed 40 ppm for 500 days contained an average of

456.3 µg Kepone in similar samples. The fact that mice treated for 500 days contained no more Kepone than mice treated for only 150 days indicates that maximum accumulation occurs within 150 days following the initiation of treatment (Tables 29, 30 and 31).

Within the first 150 days of treatment at a dietary 40 ppm, there is an initial rapid accumulation of Kepone during the first 30 days, a decrease in the accumulation rate for the next 60 days and then another increase in the accumulation rate until maximum storage has occurred. The average ratio of the amount of Kepone accumulated in the liver to the computed daily intake of Kepone was 2.0% during the first 30 days, 0.3% during the next 60 days, and 2.0% for the following 60 days. This is based on an average food consumption of five grams per day.

The liver was the major organ in which Kepone accumulated. When its Kepone content is expressed in total micrograms (µg) instead of ppm, this fact is evident because of its large size. This size relationship becomes important since the liver enlarges tremendously in treated mice.

Certain organs revealed little increase in Kepone accumulation after the first 30 days of treatment. These included the gonads, adrenals, uterus, spleen, heart and muscle. Further Kepone storage took place mainly in the liver, brain, kidney and body fat.

Table 29. Residues of Kepone in Mouse Organs as Measured by Gas-Liquid Chromatography

Sex[a]	Diet (ppm)	Days on Diet	Kepone (ppm)					
			Liver	Brain	Kidney	Fat	Muscle	Adrenals
F	40	5	45	3	7	13	--	--
F	40	15	39	20	30	27	5	--
F	40	30	78	26	27	25	20	67
F	40	30	64	30	32	33	15	25
F	40	90	67	29	27	29	15	15
F	40	90	66	30	39	34	16	37
F	40	150	168	26	74	81	23	45
F	40	150	90	61	37	49	19	86
F	40	500	120	75	88	84	26	72
F	40	500	96	55	35	29	11	20
F	100	5	55	16	15	25	4	28
F	100	15	36	35	49	64	5	--
M	40	100	60	25	25	41	10	25
M	40	300	113	65	49	22	8	--

[a]M = male; F = female.

Table 30. Residues of Kepone in Mouse Organs as Measured by Gas-Liquid Chromatography

Sex[a]	Diet (ppm)	Days on Diet	Kepone (ppm)				
			Testis	Ovary	Uterus	Spleen	Heart
F	40	30	--	15	19	--	33
F	40	30	--	25	18	--	33
F	40	90	--	13	15	--	6
F	40	90	--	26	26	14	23
F	40	150	--	--	7	--	--
F	40	150	--	20	45	13	--
F	40	500	--	18	17	--	--
F	40	500	--	--	9	--	--
F	100	5	--	--	--	--	21
M	40	100	26	--	--	13	7
M	40	300	17	--	--	8	--

[a]M = male; F = female.

Table 31. Total Estimated Kepone in Mouse Organs as Measured by Gas-Liquid Chromatography

Sex[a]	Diet (ppm)	Days on Diet	Kepone (µg)			
			Liver	Brain	Kidney	Total
F	40	5	67.5	1.2	1.8	70.5
F	40	15	77.0	7.2	8.4	92.6
F	40	30	140.0	13.5	6.7	160.2
F	40	30	96.0	10.7	7.7	114.4
F	40	90	160.0	11.5	6.0	177.5
F	40	90	138.7	11.0	8.5	158.2
F	40	150	520.0	8.3	22.8	551.1
F	40	150	335.0	25.5	13.4	373.9
F	40	500	418.0	30.0	29.0	477.0
F	40	500	403.2	23.5	9.0	435.7
F	100	5	66.5	6.4	3.5	76.4
F	100	15	89.3	15.0	13.2	117.5
M	40	100	189.0	10.5	11.7	211.2
M	40	300	450.0	26.0	24.0	500.0

[a]M = male; F = female.

Upon withdrawal of the treated diet, Kepone residues disappeared rapidly from the mouse organs (Tables 32 and 33). One male and one female fed 40 ppm for 210 days retained only a trace of Kepone in their livers after they had been fed a control diet for 150 days. All other organs contained no measurable Kepone.

Two adult females fed 40 ppm for 90 days had reduced amounts of Kepone in their organs when fed control diets for 14 and 24 days. These reductions are based on comparisons with two other females fed 40 ppm for 90 days and then sacrificed (Tables 32 and 33). Considering just the liver, brain, kidney, fat and muscle samples, the two females treated for 90 days averaged 179 µg of residual Kepone. The two females fed a control diet for 14 and 24 days after a 90 day treatment period averaged 134 and 79 µg of residual Kepone. Such reductions expressed on a percentage basis would be equal to 25 and 56%, respectively.

The liver showed the greatest total reduction of Kepone of any organ in these tests. Only 59 µg were found in the liver of the female fed a control diet for 24 days after a 90 day treatment period in contrast to an average of 150 µg in the females fed 40 ppm for 90 days. This represents a decrease of approximately 60%. The brain also showed a rapid loss of accumulated Kepone, equal to 50% after 24 days.

Table 32. Residues of Kepone in Mouse Organs Following Withdrawal of 40 ppm Diet, Measured by Gas-Liquid Chromatography

Sex[a]	Days on Kepone Diet	Days on Control Diet	Kepone (ppm)				
			Liver	Brain	Kidney	Fat	Muscle
F	90	0	67	29	27	29	15
F	90	0	66	30	39	34	16
F	90	14	46	15	4	11	0.5
F	90	24	30	17	25	19	11
M	150	240	0.6	0	0	0	0
F	60	150	0.9	0	0	0	0
F	210	150	0.4	0	0	0	0
M	210	150	4.2	0	0	0	0

[a]M = male; F = female.

Table 33. Total Estimated Kepone in Mouse Organs Following Withdrawal of 40 ppm Diet, Measured by Gas-Liquid Chromatography

Sex[a]	Days on Kepone Diet	Days on Control Diet	Kepone (µg)			
			Liver	Brain	Kidney	Total
F	90	0	160.0	11.5	6.0	177.5
F	90	0	138.7	11.0	8.5	158.2
F	90	14	125.0	5.4	1.0	131.4
F	90	24	59.4	5.4	6.5	71.3
F	60	150	1.4	0	0	1.4
F	210	150	0.8	0	0	0.8
M	210	150	9.4	0	0	9.4
M	150	240	1.2	0	0	1.2

[a]M = male; F = female.

The fact that the liver has such large and rapid reductions of accumulated Kepone following withdrawal of treatment might be an indication that the liver is the major detoxication center of Kepone in the laboratory mouse. Whether the liver is important in the excretion of Kepone from the body is not known. Observations which indicate a much greater urination rate in treated mice might be an indication that the kidney is an important excretory organ of Kepone.

The residue studies gave some indication that the characteristic tremors of chlorinated organic insecticides may be due to Kepone accumulation in the brain. Upon removal of the treated diet, Kepone is removed rapidly from the brain. Such data correlate well with the appearance, persistence, and disappearance of tremors when an animal is initially treated, maintained and then withdrawn from a treated diet.

The possibility that Kepone accumulation in muscle tissue may give rise to tremors is not ruled out, although the data from this study does not support such a theory. Kepone did not accumulate in muscle as rapidly as it did in the brain. The concentration of Kepone in muscle tissue was rather low and actually showed little increase in accumulation after 30 days of treatment. A female fed 100 ppm for 15 days and in serious tremors contained only 5 ppm

Kepone in the muscle tissue in contrast to 35 ppm in the brain. However, it is not known if 5 ppm in muscle tissue would be more adverse to the physiology of the muscle than 35 ppm in brain tissue.

The Kepone content of four brains was measured in a way that the cerebral hemisphere tissues were analyzed separately from the rest of the brain. No consistent difference was found in the two brain divisions. It is not known whether different brain areas vary in their sensitivity to Kepone.

Seven developing embryos, averaging 0.3 gram each, contained 4.5 to 5.5 ppm Kepone. Their mother had been fed 40 ppm for six days. Analysis of four placentae averaged 12 ppm each. The liver and brain of the mother contained 13 and 11 ppm Kepone, respectively.

A mouse born 24 hours prior to analysis and weighing 1.5 grams contained 5.4 ppm Kepone in its entire body. Its mother had been fed 40 ppm for eight days.

The body of a suckling mouse six-days-old and weighing 4.5 grams contained 3 ppm Kepone. The brain (0.21 g), liver (0.14 g) and stomach contents (0.11 g) of a six-day-old litter-mate contained 8.8, 33.6 and 16.8 ppm, respectively. Their mother had been fed 40 ppm since the birth of the litter. The brain (0.29 g) and liver (0.43 g) of another litter-mate 15-days-old and weighing 6.0 grams contained 36 and 47 ppm Kepone, respectively.

During the residue study, Kepone was found in every type of organ or tissue analyzed. Trace amounts were found in one pituitary which only weighed .0015 gram. Trace amounts were also found in the livers of two juveniles born to a mother 43 days after she had been removed from a treated diet. There was no indication of a Kepone metabolite in the tissues analyzed. This might be an indication that Kepone is a very stable compound which may be partially responsible for its rapid accumulation in the mouse, especially in the liver.

DISCUSSION

This study gives definite indications that Kepone, fed at sublethal concentrations, can result in many detrimental physiological effects in the laboratory mouse. Unless otherwise specified, animals designated as treated are mice which had been fed at the 40 ppm concentration.

No mortality occurred among the treated animals. Apparently these individuals were able to establish an equilibrium between the ingestion, storage, excretion and metabolism of Kepone. Residue studies indicated that maximum accumulation is reached within five months after the initiation of treatment. That certain detoxication or excretory processes occur is indicated by the fact that there is no further Kepone accumulation after 150 days of treatment. It seems likely that the enlargement of the liver represents, in effect, a response which would increase the capacity of a mouse to store and detoxify greater amounts of Kepone.

The greater food and water consumption by treated mice was at first attributed to an increase in basal metabolism. PBI tests did not confirm this and possibly such consumption increases are caused by decreased intestinal absorption of Kepone treated food or less efficient use of absorbed foodstuffs. Whether the greater

volume of urine excreted by treated mice is a cause or a result of the greater water intake is not known. The possibility that a relationship exists between the observed increased volume of urination by treated mice and the excretion of Kepone should be explored in future research.

The constant tremors in treated mice seem to be a unique characteristic of Kepone. Sherman and Ross (1961) also report a constant tremor syndrome in Kepone treated chickens, a syndrome which did not occur following the administration of other chlorinated organic insecticides. Similar tremors are produced by other chlorinated organic insecticides prior to death, but a constant tremor syndrome has not been reported at sublethal levels.

The tremor pattern seemed to be correlated with the rapid accumulation of Kepone, the persistence of a high level of Kepone within the brain and liver, and the rapid excretion of Kepone residues following the withdrawal of the treated diet. The stability of this compound may be a factor in this syndrome. Tremors cease within four weeks following the withdrawal of the treated diet. The residue study indicated that approximately 56% of the accumulated Kepone is excreted within a four week period.

The life processes of the treated individual did not seem to be affected as adversely as the reproductive processes. However, histological studies indicated that

the adverse effects on the life processes would increase as the duration of treatment increased. Although the treated animals seemed to be able to reach an equilibrium with the intake of Kepone at 40 ppm, the maintenance of such an equilibrium gradually led to degenerative changes within the organs, especially within the liver and kidney. The extensiveness and degree of degenerative changes were greater in individuals treated for 10-12 months than in individuals treated for 5-6 months. The majority of these pathological alterations were reversible if the treated diet was withdrawn as indicated by reduction in the size of enlarged livers and the resumption of reproduction in females. All mice were maintained under laboratory conditions and it is doubtful whether treated mice could have maintained themselves as well under greater stress. In a study by Good et al., (1963), mice fed 37.5 ppm Kepone could not tolerate stress produced by the removal of their drinking water as well as control mice.

The major physiological effects produced by Kepone involved the reproductive processes. The reproductive capacity of treated animals was inhibited or severely reduced. This effect of Kepone upon the reproduction of mice has been reported by Good et al. (1963).

The females were largely responsible for this reduction in reproduction. Matings between control males

and treated females failed to increase the treated females reproduction rate. Matings between treated males and control females indicated that most treated males were fertile, although litter size was reduced. Although histological examination of testes disclosed normal spermatogenesis within the seminiferous tubules, such studies would not reveal whether the number of fertile sperm was normal or if the final maturation of the sperm in the epididymis was affected. Several samples of sperm removed from the epididymis and the vas deferens indicated that treated males had an equal number of sperm but that more sperm were non-motile.

Data collected by means of vaginal smears, hormone bioassays, histological examinations, and matings gave definite indications that the female hormonal system was disturbed. The occurrence of constant estrus, the presence of large vesicular follicles, the absence of corpora lutea, and the failure to reproduce are indications that the treated females were under a prolonged influence of FSH or some FSH-like compound and only small amounts of LH.

Similar syndromes have been produced by parabiosis between a hypophysectomized female and an ovariectomized female rat (Witschi, 1934). The administration of purified LH to such a female parabiont results in the ovulation of the follicles and corpora lutea formation. A similar

syndrome was also produced by Dey (1943) in the guinea pig and by Barrnett and Mayer (1954), Greer (1953) and Hillarp (1949) in the rat. They produced this syndrome consisting of constant estrus, large follicles, but no corpora lutea formations, by placing lesions in the anterior hypothalamus of the brain. The lesion is believed to have blocked the rhythmic release of LH from the pituitary. As discussed in the literature review, this blocking action is believed to take place between the nervous components of the hypothalamus and the hypophysial portal system which drains the median eminence. In effect, such a block would prevent hormonal substances liberated by nerve endings in the median eminence from reaching the anterior pituitary by way of the portal system. These hormone substances are thought to originate in the hypothalamus and to stimulate or inhibit the release of hormones from the pituitary.

From this aggregation of data, the following hypothesis is formed concerning the reproductive effect of Kepone within the laboratory mouse.

The rapid accumulation and persistence of Kepone within the brain results in an interference in the processes of the brain cells. Because of the fat soluble properties of Kepone, this effect may be in the cell membrane and along the myelin sheath of the nerve tracts. The deposition of Kepone in these areas could affect the permeability of the membranes and result in the

depolarization or the inhibiting of the repolarization of such membranes. In either case, the effects would be constant and would prevent the normal functioning of such cells. This might result in the constant stimulation of an inhibiting center blocking the rhythmic release of LH, or an activating center prolonging FSH stimulation. The tremor syndrome may also be due to such cell disturbances within the brain.

The blocking of the rhythmic release of LH would prevent ovulation and corpora lutea formation and consequently reproduction. This blocking action would upset the normal feedback oscillation of the adenohypophysial-ovarian system. Therefore, the continuous or prolonged secretion of FSH along with small amounts of LH would produce a continuous secretion of estrogen from the vesicular follicles producing a constant estrus and endometrial hyperplasia of the uterus. The amount of LH secreted appears to be large enough in quantity to act with FSH to cause estrogen secretion, but subminimal for ovulation.

Just how these potential hormonal relationships would affect progesterone secretion is unknown since it is not known whether the corpus luteum is important in regulating the estrous cycle in rodents. The secretory activity of the corpus luteum is definitely necessary for a successful pregnancy, but Young (1945) indicates that in

rats the secretion of progesterone from modified follicular tissue may be the source of progesterone important to the regulation of the estrous cycle. According to Young (1945), the secretion of progesterone by follicular tissue begins during proestrus and diminishes markedly a short time after ovulation. If such is the case, a continuous secretion of progesterone might be expected from this source if ovulation does not occur. High levels of progesterone in the blood definitely have an inhibiting effect on the secretion of LH by the pituitary. On the other hand, if progesterone secretion from corpora luteal bodies is important in the regulation of the rodent estrous cycle, the quantity of progesterone secreted would be reduced in the absence of such luteal formations.

As the above discussion only represents a hypothesis, other possible causes of reduced gonadotrophic secretion should not be overlooked. Conceivably the degree of activity of the gonadotrophic hormones on the gonads could be sub-normal. Foreign substances in the blood, malfunction of the liver in its action upon hormones, or insufficient enzyme activity in the target tissue might restrict the full effect of the hormones. Research by Christian (1955) has revealed that stressed mice may have reduced pituitary secretion. This reduction is believed to be caused by abnormal levels of adrenal cortical hormones

or by direct neural inhibition of the pituitary through the hypothalamus.

That accumulated Kepone may interfere with the function of certain vitamins or enzymes is also conceivable, which in effect would cause a vitamin deficiency. Many workers including Meites and Reed (1949) and Albritton (1955) have demonstrated that vitamin deficiencies can affect pituitary activity in mammals.

The presence of Kepone within developing embryos and suckling young indicates that Kepone can be passed through the placentae or the milk of female mice. That such events may affect the offspring was demonstrated by the appearance of tremors in one nursing litter, the greater mortality within litters nursing from mothers on treated diets, and the frequent loss of unborn or newly born litters borne by mothers fed Kepone shortly before or during the early stages of pregnancy.

SUMMARY

Tests with Kepone, the trade name for decachlorooctahydro-1,3,4-metheno-2H-cyclobuta(6d) pentalen-2-one, were conducted on the BALB/cJaxGnMc strain of laboratory mice from January, 1962 through June, 1963. Kepone is a chlorinated organic insecticide with the empirical formula of $C_{10}Cl_{10}O$.

In tests extending up to six months, diets containing Kepone concentrations of 60 ppm or less were found to be sublethal to the adult laboratory mouse. Juvenile mice tolerated 50 ppm for a similar period of time. No significant mortality occurred among juveniles or adults fed 40 ppm for 12 consecutive months. A concentration of 60 ppm was fatal within three months to juveniles fed this diet following weaning at 21-24-days-old. Most adults succumbed within 40 days when fed 70 ppm. At the 80 and 100 ppm levels, all mice died within 32 days. No differences were found between the tolerance limits of the two sexes.

A constant tremor appeared in all mice fed 30 ppm or higher. At sublethal levels, tremors appeared within two weeks, reached their peak of intensity within two months and then remained more or less constant. At 80 or 100 ppm dietary, tremors appeared within seven days,

became severe after 10 to 14 days, and usually terminated in convulsions and death within 32 days. The withdrawal of the treated diet anytime before a lethal quantity of Kepone had been ingested would result in a reduction in tremor intensity within four to seven days and no tremors were visible after four weeks.

Food and water consumption of Kepone-fed mice increased compared to controls. This increase was usually in the range of 25 to 45%, but did not result in increased body weights. The growth of 21-24-day-old juveniles weighing 8-13 grams each was not affected at treatment levels up to 60 ppm. Diets containing 70 ppm or more adversely affected the growth of weaned juveniles. These died within 19 days and experienced little growth during this period. Adults fed 40 ppm maintained normal body weights. Adults fed sublethal levels for several months would frequently weigh one or two grams more than controls because of the enlargement of the liver. Adults fed lethal levels of Kepone demonstrated a gradual decrease in body weight several days prior to death. Such decreases were not great enough to cause the death nor were they due to decreased food consumption since consumption actually increased.

The liver was the only organ that showed an increase in size in all treated animals examined. At 40 ppm, liver enlargement began within the first 10 days of treatment and

usually doubled its weight within 60 to 90 days. Further enlargement was slight or very gradual. The fat content of the liver did not increase in the enlarged livers. The only other organs indicating enlargement were the kidneys of the male and the spleen and uterus of the female. Individual cases of female kidney and male spleen enlargement did occur. There was no indication of any reduction or enlargement in the brain, pituitary, adrenals, testes, ovaries, heart or thymus.

Reproduction by Kepone-fed females of group A was reduced at all concentrations of Kepone tested. With the reproduction of the controls being equal to 100% in terms of the number of young produced, the reproduction was reduced 23.9, 79.3 and 87.0% at the 10, 30 and 37.5 ppm concentrations, respectively. Fourteen pairs of females from group B fed 40 ppm failed to produce one litter in 100 days. No mortality occurred among the mated pairs.

In mating experiments involving treated males with control females and treated females with control males, the females were largely responsible for the reduction in reproduction. Not one treated female produced a litter in these tests while several treated males produced pregnancies in control females.

Reproduction resumed within six to seven weeks in Kepone-fed females following the withdrawal of the treated diet. These females had failed to produce a litter in 100

days while on treatment. This would indicate fertile conceptions within three to four weeks following the withdrawal of the treated diet. The initial litters of these females were smaller with a lower percent survival of young than controls. Second litters were slightly larger, but the percent survival of young was equal to controls. The smaller litter size and the greater mortality among the young born in the first litters may be an indication that the Kepone residues within the female are still capable of exerting a detrimental effect upon the female and her young. This residue effect may persist for 60 days after the withdrawal of the treated diet. Kepone residues were found in the young of such females.

Some treated males fertilized control females immediately indicating that their fertility had not been totally inhibited by Kepone ingestion. Other males did not fertilize their mates until several weeks following the withdrawal of the treated diet. Whether a correlation exists between such males and the withdrawal of the treated diet is not known. Overall, the number of young produced in 100 days following the withdrawal of the treated diet from the males was 34.9% less than controls. This reduction was largely due to smaller rather than fewer litters. Possibly this reduction in litter size is an indication of some effect of Kepone upon the number or motility of sperm produced by Kepone-fed males. Histological examination of

testes revealed normal spermatogenesis in the seminiferous tubules and normal amounts of interstitial tissue. Such examinations would not reveal whether normal maturation of the sperm occurred in the epididymis.

Vaginal smear data indicated a definite disturbance of the estrous cycle within Kepone-fed females. Females fed 40 ppm appeared to be in constant estrus within four weeks after the initiation of treatment. Up to 80% of the smears taken from treated females were estrus compared to an average of only 24% from the controls.

Serological studies, in which determinations were made of the calcium, sodium, protein-bound iodine, and blood glucose levels, indicated no abnormal alterations of these components in treated mice with the exception of blood glucose in fasted animals treated at the 100 ppm concentration for three weeks. These animals did not maintain normal blood glucose values when fasted for 16 hours. Their glucose values dropped as low as 30 mg/100 ml with an average of 47.7 mg/100 ml in contrast to 100.5 and 96.2 mg/100 ml in controls and mice fed 40 ppm for four to six months, respectively.

Histological examination of prepared sections from the organs of treated mice revealed no major degenerative changes in the pituitary, thymus, adrenals and testes. Enlarged spleens showed moderate congestion. Uteri in the estrogenic phase revealed cystic hyperplasia of the

endometrium. There were indications of moderate congestion and myelin alteration in a few brains.

The kidney and the liver were the organs in which degenerative changes were commonly found. The majority of kidneys from treated mice had varying degrees of glomerulonephritis, disseminated lupus erythematosus, hypertrophy of the tubule cells, and hemorrhage within the distal convoluted and loop of Henle's tubules. Frequently, large exudates were found in the Bowman's capsule and occasionally the glomerular membrane was ruptured.

Hyperplasia and congestion were found in the livers of all treated animals. The degree of other degenerative changes, such as focal necrosis and cellular hypertrophy, varied greatly although such variation appeared to depend on the length of treatment. Such degenerative changes were more extensive in animals treated 10-12 months than in animals treated 5-6 months.

Electron microscopic examination of two livers from treated mice indicated a reduction in number of mitochondria and the formation of cytoplasmic inclusions similar in appearance to liposphere s.

Histological examination of ovaries from mice fed 40 ppm for four to eight months revealed normal follicle development but few corpora lutea. Treated females averaged only 0.8 corpus luteum per ovary in comparison to 3.8 for controls. Ninety percent of the ovaries from control

females contained at least one corpus luteum compared to 27% in Kepone-fed females.

Hormone bioassays of the FSH and LH hormones of the pituitary revealed that Kepone-fed females had equal amounts of FSH in comparison with controls, but approximately 25% less LH activity. It is possible that this reduction in LH activity within the treated females may be sufficient to prevent ovulation. This disturbance of the LH hormone may also contribute to the upsetting of the normal feedback oscillation of the adenohypophysial-ovarian system.

The constant estrus of treated females, whose ovaries contained large vesicular follicles but usually no corpora lutea, represents a syndrome which might be caused by the blocking of the rhythmic release of LH. The blocking of sufficient quantities of LH to trigger ovulation may lead to prolonged FSH stimulation and constant estrogen secretion from the large follicles producing constant estrus.

Insecticide residue studies with gas-liquid chromatography revealed that Kepone is accumulated rapidly but that no further accumulation occurs after five months of treatment on 40 ppm. Upon withdrawal of the treated diet, Kepone is excreted rapidly. The analyses of organs from two females indicated that as much as 25 and 56% of the accumulated Kepone was excreted within 14 and 24 days, respectively, following withdrawal of the treated diet.

Only a trace of Kepone was found in mice fed a control diet for 150 days after being fed 40 ppm for 210 days.

The liver was the major organ accumulating Kepone. Its enlargement, in effect, enables treated mice to store and possibly detoxify greater amounts of Kepone. The residue data also suggest that the liver is the major detoxication organ in the mouse.

The brain, kidney, fat and muscle follow the liver in Kepone accumulation. Certain organs including the gonads, adrenals, uterus, spleen, heart and muscle showed little increase in Kepone accumulation after the first 30 days of Kepone ingestion.

The rapid accumulation and the persistence of high levels of Kepone within the brain may be an indication that the tremor syndrome and the hormonal disturbances originate in this organ. Kepone is excreted rapidly from the brain following the withdrawal of the treated diet. This pattern of rapid accumulation, persistence, and rapid excretion correlates with the tremor and reproductive disturbances.

The presence of Kepone in suckling young and in developing embryos indicates that Kepone can pass through the placenta or the milk of Kepone-fed female mice. The appearance of tremors in one nursing litter and the increased mortality of pre-born and post-born young among treated females leaves little doubt that such passage of Kepone can affect the offspring.

APPENDIX

Pituitary Bioassay Procedures

Follicle Stimulating Hormone Bioassay

Equipment and Reagents:

1. 7.0 mm Micro-homogenizer
2. pH Meter, with smallest size electrodes
3. Centrifuge, with at least 5000 rpm capacity
4. Glass stirrer
5. Boiled saline solution, cool to 36-38° F
6. Weak solutions of HCl and NaOH
7. 5-ml Hypodermic syringe and 20-gauge needle
8. 2-ml Hypodermic syringe and 27-gauge needle
9. 15-ml Centrifuge tubes
10. 5-ml Glass beakers
11. 20-ml Glass vials with caps
12. Refrigerator, to store extracts at 36-38° F
13. Refrigerated room between 40-43° F
14. Single-pan analytical balance

Procedure:

1. Remove pituitary from head of mouse just sacrificed by breaking neck. The pituitary may be removed by the following procedure:

    a. Cut the skull bones around the cranium with a scissors and remove roof of cranium with tweezers.

b. Lift the brain to the rear and off the base of the cranium by inserting a probe under the olfactory nerves and gently lifting the brain toward the caudal end.

c. Step (b) will expose the pituitary lying in a small depression of the basisphenoid bone, the sella turcica. Before the pituitary can be removed from this depression, the thin covering of meninges must be carefully removed from the pituitary by means of a sharp-pointed probe.

2. Place the pituitary into the micro-homogenizer containing 0.5 ml refrigerated saline.

3. If sample is to contain more than one pituitary, repeat steps 1 and 2 as often as necessary. Two pituitaries were used in the FSH assays.

4. Macerate the pituitaries with the plunger of the homogenizer.

5. Transfer homogenizer contents to a 5-ml pH beaker.

6. Rinse homogenizer and plunger with remaining 2.5 ml saline; add to pH beaker. A 5-ml hypodermic syringe may be used to measure and apply the quantities of saline needed.

7. Adjust pH to 8.5 with weak HCl and NaOH solutions.

8. Transfer pH beaker contents to centrifuge tubes and stir at least 10 minutes.

9. Centrifuge at least 5 minutes and draw off supernatant liquid and place in a numbered glass vial.

10. Add 50 I.U. of Human Chorionic Gonadatrophin and store in refrigerator.

11. Inject into intact, 21-day-old, virgin Sprague-Dawley rats weighing 40-45 grams each, subcutaneously with a 2-ml syringe and a 27-gauge needle, a total of 6 injections at 0.5 ml per injection.

12. Dissect out the rat ovaries and weigh to 0.1 milligram 72 hours after the first injection.

Luteinizing Hormone Bioassay

The LH procedure is identical to the FSH procedure with the following exceptions:

1. Four pituitaries were used per sample instead of two.

2. A total of 4 ml saline were used per sample instead of 3 ml saline.

3. No Human Chorionic Gonadotrophin was added.

4. Supernatant was injected into hypophysectomized 24-day-old Sprague-Dawley male rats weighing 45-50 grams each, over 4 days, a total of 8 injections of 0.5 ml per injection.

5. Dissect out the ventral prostate and weigh to 0.1 milligram 120 hours after the first injection.

Microtechnique Procedures

Azocarmine G-Orange G-Methylene Blue

Equipment and Reagents:

1. Dissecting instruments
2. Azocarmine G stain
3. Orange G stain
4. Methylene blue stain
5. Distilled water
6. Phosphotungstic acid
7. Fixative (alcholoic and aqueous Bouin's)
8. Xylene
9. Alcohols (35, 50, 75, 85, 95, and 100%)
10. Anilin
11. Paraffin
12. Microtome and knife
13. Glass slides
14. Cover slides (22X50 mm)
15. Egg albumin adhesive
16. Picolyte
17. Alcoholic lamp
18. Oven (to melt paraffin)
19. Slide dryer
20. Slide container boxes
21. Diamond pencil
22. Glacial acetic acid
23. Coplin staining jars
24. Staining rack

Stain and Special Solution Preparations:

1. Azocarmine G Stain
    a. Add 0.1 g azocarmine G to 100 ml distilled water and boil for 5 minutes.
    b. Filter through Whatman's No. 1 filter paper after cooling.
    c. Add 2 ml glacial acetic acid.
2. Orange G-Methylene Blue Counterstain
    a. Mix 10 g phosphotungstic acid, 1.2 g methylene blue, 4.4 g orange G and 1000 ml distilled water.
3. Aqueous Bouin's Fixative
    a. Mix 75 parts saturated aqueous solution of picric acid with 25 parts formalin and 5 parts glacial acetic acid.
4. Alcoholic Bouin's Fixative
    a. Mix 150 parts 75% alcohol, 60 parts formalin, 15 parts glacial acetic acid, and 75 parts saturated aqueous solution of picric acid.
5. Anilin Solution
    a. Add 1 ml anilin to 100 ml of 95% alcohol.

Procedures: Fixation and Preservation

1. Kill the animal to be sacrificed by breaking the neck.
2. Dissect and remove desired tissue as quickly as possible.

3. Rinse in physiological saline if the organ is contaminated with blood, and place immediately into a vial containing the fixative for 6-24 hours.

4. Pour off Bouin's fixative and replace with 75% alcohol for 24 hours. Change alcohol at least twice. If aqueous Bouin's is used as the fixative, precede the 75% alcohol with 50% alcohol for one hour.

Procedures: Embedding

5. Pour off 75% alcohol and replace with 85% alcohol for 24 hours. Tissues may be stored in 85% alcohol for several weeks.

6. Pour off 85% alcohol and replace with 95% alcohol for 30 minutes.

7. Pour off 95% alcohol and replace with 100% alcohol for one hour. Change the alcohol after 30 minutes.

8. Pour off 100% alcohol and replace with a mixture of 100% alcohol and xylene, equal parts. Leave for 30 minutes.

9. Pour off alcohol - xylene mixture and replace with xylene for 30 minutes.

10. Pour off xylene and add one-half volume of xylene. Place in 58° F oven for 30 minutes.

11. Add one-half volume paraffin to xylene and leave for 30 minutes.

12. Pour off xylene-paraffin mixture and replace with second paraffin for 60 minutes.

13. Pour off second paraffin and replace with third paraffin and leave overnight.

14. Embed tissue within paraffin in properly labeled containers.

15. Store paraffin blocks and containers in refrigerator overnight.

Procedures: Sectioning and Mounting

16. Warm blocks to room temperature and section with microtome and knife at 8-12 microns.

17. Place section strips in labeled cardboard containers in serial order and store in refrigerator overnight.

18. Mount serial section strips on slide with egg albumin and dry overnight.

Procedures: Deparaffining and Staining

19. Place slides in staining rack into xylene and leave for 10 minutes.

20. Place slides into second xylene for 10 minutes.

21. Place slides into 100% alcohol for 10 minutes.

22. Place slides into second 100% alcohol for 10 minutes.

23. Place slides into 95% alcohol for 5 minutes.

24. Place slides into second 95% alcohol for 5 minutes.

25. Place slides into 75% alcohol for 5 minutes.

26. Place slides into 50% alcohol for 5 minutes.

27. Place slides into 35% alcohol for 5 minutes.

28. Place slides into distilled water for 10 minutes.

29. Place slides into azocarmine G stain for 15 minutes.

30. Dip slides into distilled water.

31. Dip slides into anilin solution.

32. Dip slides for 2-5 seconds into distilled water until diffusion currents cease.

33. Place slides into orange G-methylene blue counterstain for 15 minutes.

34. Place slides into 100% alcohol for 5 minutes.

35. Place slides into 100% alcohol for 5 minutes.

36. Place slides into 100% alcohol for 5 minutes.

37. Place slides into 100% alcohol for 5 minutes.

38. Place slides into xylene for 10 minutes.

39. Place slides into second xylene for 5 minutes.

40. Mount slides with cover glass and picolyte. Label slides with diamond pencil.

41. Dry slides for several days on 50° C drying oven.

42. Place slides in slide container boxes and store slides in horizontal position.

Hematoxylin - Eosin Staining

Equipment and Reagents:

1. Identical to Azocarmine G-Orange G-Methylene Blue Method with the following exceptions:

a. Delafield's hematoxylin stain (standard)

b. Eosin stain (standard)

c. Alkalized water (0.1% solution of sodium bicarbonate in distilled water)

d. Acidized water (1.0 ml concentrated HCl in 300 ml distilled water)

Procedures: Fixation and Preservation

1-5 Same as previously described.

Procedures: Embedding

6-15 Same as previously described.

Procedures: Deparaffining and Staining

16-28 Same as previously described.

29. Place slides into hematoxylin stain for 30 minutes.

30. Place slides into tap water for 5 minutes until tissue turns blue.

31. Dip slides into acidified water until tissues turn red.

32. Dip slides into alkalized water until tissues turn blue.

33. Place slides into distilled water for 2 minutes.

34. Place slides into 35% alcohol for 5 minutes.

35. Place slides into 50% alcohol for 5 minutes.

36. Place slides into 75% alcohol for 5 minutes.

37. Place slides into 95% alcohol for 5 minutes.

38. Place slides into eosin stain for 1-5 minutes until tissues show a red hue.

39. Dip slides into 95% alcohol for several seconds to get rid of excess eosin.

40. Dip slides into 100% alcohol for 30 seconds or just long enough to complete the dehydration process.

41. Place slides into xylene for 10 minutes.

42. Place slides into second xylene for 5 minutes.

43. Mount slides as described previously.

Sample Preparation for Gas-Liquid Chromatography

Equipment and Reagents:

1. Dissecting instruments
2. Single pan analytical balance
3. Diamond pencil
4. Plastic capped glass vials (30-ml)
5. Mortar and pestle (several)
6. 7.0 mm Micro-homogenizer
7. Whatman's No. 1 Filter Paper
8. Separatory funnels (50-ml)
9. Short stem funnels
10. Distilled water
11. Hexane, reagent grade
12. Acetone, reagent grade
13. Sodium sulfate, anhydrous reagent
14. 5-µl syringe

Procedures:

1. Sacrifice by breaking neck of mouse and dissect out desired tissue.

2. Weigh organ or tissue on analytical balance.

3. Macerate organ in mortar and pestle with sodium sulfate (4 x tissue weight). Use micro-homogenizer without sodium sulfate for mouse pituitary, adrenals and ovaries.

4. Extract macerated organ with four washes of acetone to a total of 25 ml.

5. Filter extract through Whatman's No. 1 filter paper into a separatory funnel.

6. Add 10 ml hexane and 5 ml distilled water to the filtrate of the extract and shake well.

7. Allow aqueous acetone (bottom layer) to separate from hexane and pour off aqueous acetone layer into a 2nd 50-ml separatory funnel.

8. Pour off hexane layer into numbered plastic capped vial.

9. Add 10 ml hexane to remaining aqueous acetone solution and shake well.

10. Allow to separate. Pour off aqueous acetone layer (bottom layer) and add the hexane layer to the vial containing the first hexane wash. Store vial in refrigerator.

11. Before injecting into gas chromatography unit, filter samples through Whatman's No. 1 filter paper and 0.5 grams sodium sulfate.

12. Some samples, such as liver, may have to be diluted while others are concentrated before analysis by gas chromatography in order to obtain a measurable peak area.

13. Inject, by means of the 5-µl syringe, a one or two µl volume depending on the peak area obtained at one ul. In some cases, a greater injection volume will be necessary in order to obtain a peak from very small organs like the pituitary.

LITERATURE CITED

Albritton, E. C. 1955. Standard values in nutrition and metabolism. W. B. Sanders Co., Philadelphia, Pa. 380 pp.

Allen, E. 1922. The oestrous cycle in the mouse. Am. J. Anat. 30:297-371.

Allied Chemical Corporation. 1962. Agricultural development bulletin, experimental insecticide compound GC-1284, 1962. Morristown, N. J.

Ambrose, A. M., H. E. Christensen and D. J. Robbins. 1953. Pharmacological observations on chlordane. Federation Proc. 12(1):298.

American Association of Economic Entomology. 1949. Proc. 4th Ann. Mtg., North Central States Br. 160 pp.

Anderson, R. W., R. W. Blakely and H. I. MacGregor. 1952. The toxicity of aldrin for growing turkeys. Sci. Agr. 32(11): 586-591.

Anderson, W. A. D. (ed.). 1961. Pathology. The C. V. Mosby Co., St. Louis, Mo. 1389 pp.

Anonymous. 1962. Pesticide residue analysis with the aerograph hy-fi and the electron capture detector. Wilkens Instrument & Research, Inc., Walnut Creek, Calif., Mimeo 16 pp.

Apostolakis, M. 1959. Detection and estimation of pituitary gonadotrophins in human plasma. J. Endocrin. 19(4):377.

Ball, W. L., K. Kingsley and J. W. Sinclair. 1953. Observations on toxicity of aldrin. I. Growth and estrus in rats. Arch. Ind. Hyg. Occup. Med. 7(4):292-300.

Ban, J. M., T. J. DeCino, N. W. Earle and Yun Pei Sun. 1956. The fate of aldrin and dieldrin in the animal body. J. Agr. Food Chem. 4(11):937-941.

Barker, S. B., M. J. Humphrey and M. H. Soley. 1951. The clinical determination of protein-bound iodine. J. Clin. Invest. 30(1):55-62.

Barrnett, R. J. and J. Mayer. 1954. Endocrine effects of hypothalmus lesions. J. Anat. Record 118(2):374.

Bates, R. W. and J. P. Schooley. 1942. Studies on the assay of pituitary gonadotropins using the augmentation reaction. Endocrin. 31(1):390.

Black, D. G., J. Getty, H. R. Jameson and H. Pirie. 1950. The effects of continuous ingestion by poultry of benzene hexachloride. Vet. J. 106:386-391.

Borgman, R. 1952. Toxicity to animals of insecticidal compounds aldrin and dieldrin. 16th Biennial Rept. of the Director, Kansas State Coll. 52 pp.

Boving, B. C. 1959. Endocrine influences on implantation, 205-224. In C. W. Lloyd, (ed.), Recent progress in the endocrinology of reproduction. Academic Press, N. Y. 532 pp.

Brown, J. B. 1959. Estrogen excretion in normal and abnormal menstrual cycle, 53-63. In C. W. Lloyd, (ed.), Recent progress in the endocrinology of reproduction. Academic Press, N. Y. 532 pp.

Burlington, H. and V. F. Lindeman. 1950. Effects of DDT on testes and secondary sex characters of white leghorn cockerals. Proc. Soc. Exp. Biol. Med. 74(1):48-51.

Burlington, H. and V. F. Lindeman. 1952. Action of DDT on the blood of the chicken. Federation Proc. 11(1):20-21.

Carson, R. 1962. The Silent Spring. Houghton Mifflin Co., Boston, Mass. 368 pp.

Christian, J. J. 1955. Effect of population size on the adrenal glands and reproductive organs of male mice in populations of fixed size. Am. J. Physiol. 182:292.

Choudhury, B. and V. B. Robinson, 1950. Clinical and pathological effects produced in goats by ingestion of toxic amounts of chlordane and toxaphene. Am. J. Vet. Res. 11:50-57.

Conley, B. E. 1951a. Pharmacologic and toxicologic aspects of DDT. J. Am. Med. Assoc. 145(10):728-733.

Conley, B. E. 1951b. Toxic effects of technical benzene hexachloride and its principal isomers. J. Am. Med. Assoc. 147(6):571-574.

Consumer Reports. 1963. Household Insecticides. Consum. Repts. 28(7):321-327.

Crevier, M., W. L. Ball and K. Kay. 1954. Observations on toxicity of aldrin (S. esterase). Arch. Ind. Hyg. Occup. Med. 9(4):306-314.

Davidow, B. and J. Radomski. 1952. Metabolite of heptachlor, its analysis, storage and toxicity. Federation Proc. 11(1):336.

DeWitt, James B. 1956. Chronic toxicity to quail and pheasant of some chlorinated insecticides. J. Agr. Food Chem. 4(10):863-866.

DeWitt, J. B. and J. L. George. 1960. Bureau of sport fisheries and wildlife pesticide review, 1959. U.S.D.I. Fish and Wildlife Circ. No. 84, Mimeo., 36 pp.

Dey, F. L. 1943. Evidence of hypothalamus control of hypophyseal gonadotropic functions in the female guinea pig. Endocrin. 33(2):75-82.

Doisy, E. A., Jr., and B. C. Bocklage. 1949. Chronic toxicity of gamma isomer of hexachlorocyclohexane in the albino rat. Proc. Soc. Exp. Biol. Med., 71(3):490-493.

Donovan, B. T. and G. W. Harris. 1956. The effects of pituitary stalk section on light-induced oestrus in the ferret. J. Physiol. 131(1)102.

Durham, W. F., C. Cueto and W. J. Hayes, Jr. 1956. Hormonal influences on DDT metabolism in the white rat. Am. J. of Physiol. 187(2):373-377.

Emery, F. E. and E. L. Schwabe. 1936. The vaginal smears of rats as influenced by frequent examinations. Anat. Record 64(2):147-154.

Fevold, H. L. 1941. Synergism of the follicle stimulating and luteinizing hormones in producing estrogen secretion. Endocrin. 28(1):33-36.

Fitzhugh, O. G. 1948. Use of DDT insecticides on food products. Ind. Eng. Chem. 40:704-705.

Fitzhugh, O. G. and A. A. Nelson. 1951. Comparison of chronic effects produced in rats by several chlorinated hydrocarbon insecticides. Federation Proc. 10(1):295.

Fletcher, R. F. and P. S. Brown. 1957. Gonadotrophin excretion and chemical features in secondary amenorrhoea. Clin. Sci. 16:669.

Genelly, R. E. and R. L. Rudd. 1956. Effects of DDT, toxaphene and dieldrin on pheasant reproduction. Auk 73(4): 529-539.

Good, E. E., G. W. Ware and D. F. Miller. 1963. Effects of insecticides on reproduction in the laboratory mouse: I Kepone. Tox. Appl. Pharmacol. (in press).

Gray, C. H. and A. L. Bacharach. 1961. Hormones in blood. Academic Press, N. Y. 655 pp.

Greep, R. O., H. B. Van Dyke and B. F. Chow. 1941. Use of anterior lobe of prostate in assay of metakentrin. Proc. Soc. Exp. Biol. Med. 76:686-689.

Greep, R. O. and R. J. Barrnett. 1951. The effects of pituitary stalk section on the reproductive organs of female rats. J. Endocrin. 49(2):172.

Greer, M. A. 1953. The effects of progesterone on persistent vaginal estrus produced by hypothalamic lesions in the rat. Endocrin. 53(4):380.

Guyton, A. C. 1961. Textbook of medical physiology. W. B. Sanders Co., Philadelphia, Pa. 1181 pp.

Ham, A. W. and T. S. Leeson. 1961. Histology. J. B. Lippincott Co., Philadelphia, Pa. 942 pp.

Harris, G. W. 1959. Follicular ripening ovulation and estrous behavior, 21-44. In C. W. Lloyd, (ed.), Recent progress in the endocrinology of reproduction. Academic Press, N. Y. 532 pp.

Hillarp, N. A. 1949. Studies on the localization of hypothalamic centres controlling the gonadotrophic function of the hypophysis. Acta Endocrinol. 2(1):11.

Hodge, H. C., E. A. Maynard, J. F. Thomas, H. J. Blanchet, Jr., W. G. Wilt, Jr. and K. E. Mason. 1950. Short-term oral toxicity tests of methoxychlor in rats and dogs. J. Pharmacol. Exp. Therap. 99:140-148.

Kitselman, C. H. 1953. Long term studies on dogs fed aldrin and dieldrin in sublethal dosages, with reference to the histopathological findings and reproduction. J. Am. Vet. Med. Assoc. 123:28-30.

Kunze, F. M., A. A. Nelson, O. G. Fitzhugh and E. P. Gang. 1949. Storage of DDT in the fat of the rat. Federation Proc. 8(1):311.

Kunze, F. M. and E. P. Laug. 1953. Toxicants in tissues of rats on diets containing dieldrin, aldrin, endrin and isodrin. Federation Proc. 12(1):339.

Laug, E. P. and O. G. Fitzhugh. 1946. 2, 2-Bis (p-chlorophenyl)-1, 1, 1-trichlorethane (DDT) in the tissues of the rat following oral ingestion for periods of six months to two years. J. Pharmacol. Exp. Therap. 87:18-23.

Laug, E. P., A. A. Nelson, O. G. Fitzhugh and F. M. Kunze. 1950. Liver cell alteration and DDT storage in the fat of the rat induced by dietary levels of 1 to 50 ppm DDT. Jour. Pharmacol. Exp. Therap. 98:268-273.

Lehman, A. J. 1951. Chemicals in foods. A report to the Association of Food and Drug Officials on current developments. Part II. Pesticides. Assoc. Food Drug Off. J. 15(4): 122-133.

Lehman, A. J. 1952a. Chemicals in foods. A report to the Association of Food and Drug Officials on current developments. Part II. Pesticides. Section III. Subacute and chronic toxicity. Assoc. Food Drug Off. J. 16(2):47-53.

Lehman, A. J. 1952b. Chemicals in foods: A report to the Association of Food and Drug Officials on current developments. Part II. Pesticides IV. Biochemistry. Assoc. Food Drug Off. J. 16(3):85-91.

Lillie, R. D., M. I. Smith and E. F. Stohlman. 1947. Pathologic action of DDT and certain of its analogs and derivatives. Arch. Path. 43(2):127-142.

Lloyd, C. W. (ed.). 1959. Recent progress in the endocrinology of reproduction. Academic Press, N. Y. 532 pp.

Long, J. A. and H. M. Evans. 1922. The oestrous cycle in the rat and its associated phenomena. Mem. Univ. Cal. 6:1-148.

Loraine, J. A. and J. B. Brown. 1956. Further observations on the estimation of urinary gonadotropins in non-pregnant human subject. J. Clin. Endocrin. Metab. 16(9):1180.

Markee, J. E., C. H. Sawyer and W. H. Hollingshead. 1946. Activation of the anterior hypophysis by electrical stimulation in the rabbit. Endocrin. 38(6):345-357.

Martin, Hubert. 1961. Guide To the Chemicals Used in Crop Protection. Publication 1093, Research Branch, Canada Dept. of Agriculture, London, Ontario. 387 pp.

McNamara, B., R. J. Bing and F. Hopkins. 1946. Studies on the chronic toxicity of DDT in the dog. Federation Proc. 5(1):67.

McArthur, J. W. 1952. The identification of pituitary interstitial cell stimulating hormone in human urine. Endocrin. 50(3):304.

Meites, J. and J. O. Reed. 1949. Effects of restricted feed intake in intact and ovariectomized rats on pituitary lactogen and gonadotrophin. Proc. Soc. Exp. Biol. Med. 70:153.

Meyer, R. K., C. Biddulph and J. C. Finerty. 1946. Pituitary gonad interaction in immature female parabiotic rats. Endocrin. 39(1):23-31.

Nalbandov, A. V. 1958. Reproductive physiology. W. H. Freeman & Co., San Francisco, Calif. 271 pp.

Neal, L. M., R. C. Barton, E. G. Holmstrom and H. A. Salhanick. 1954. A routine bioassay procedure for gonadotropic extracts. J. Clin. Endocrin. Metab. 14(7):792.

Nelson, W. O. 1939. Atypical uterine growths produced by prolonged administration of estrogenic hormones. Endocrin. 24(1):50-54.

Nelson, A. A., O. G. Fitzhugh and G. Woodard. 1950. Comparison of several chlorinated hydrocarbon insecticides with respect to pathological changes produced in rats by feeding at low levels, 1 to 100 ppm. Federation Proc. 9(1):339.

Nikitovitch-Winer, M. and J. W. Everett. 1957. Resumption of gonadotrophic function in pituitary grafts following retransplantation from kidney to median eminence. Nature 180:1434.

Ortega, P., W. J. Hayes, W. F. Durham and A. Mattson. 1956. DDT in the diet of the rat. Public Health Monograph No. 43, U.S. Dept. of Health, Education and Welfare, Washington, D. C. 27 pp.

Ortega, P., W. J. Hayes and W. F. Durham. 1957. Pathologic changes in the liver of rats after feeding low levels of various insecticides. A.M.A. Arch. of Pathology 64:614-622.

Ortega, P. 1958. Hepatocellular pathology following various chlorinated hydrocarbon insecticides. Federation Proc. 17 (1):451.

Philips, F. S. and A. Gilman. 1946. Studies on the pharmacology of DDT. J. Pharmacol. Exp. Therap. 86:213-221.

President's Science Advisory Committee Report. 1963. Use of pesticides. U.S. Govt. Printing Office, Washington 25, D. C. 25 pp.

Prosser, C. L. and F. A. Brown. 1961. Comparative Animal Physiology. W. B. Saunders Co. Philadelphia, Pa. 688 pp.

Rogers, P. V. and E. Allen. 1937. Epithelial growth caused by stimulation with various smear methods as demonstrated by mitotic stasis with colchicine. Endocrin. 21(5):629-632.

Rosenberg, M. M., T. Tanaka and H. E. Adler. 1950. Toxicity of chlordane to laying pullets. Am. J. Vet. Res. 11:236-239.

Rudd, R. L. and R. E. Genelly. 1956. Pesticides: Their use and toxicity in relation to wildlife. Game Bulletin No. 7. Dept. of Fish and Game, California. 209 pp.

Saifer, A. and S. J. Gerstenfeld. 1958. The photometric microdetermination of blood glucose with glucose exidase. Lab. Clin. Med. 51:448.

Sawyer, C. 1959. Nervous control of ovulation, 1-18. In C. W. Lloyd, (ed.), Recent progress in the endocrinology of reproduction. Academic Press, N. Y. 532 pp.

Sherman, M. and M. M. Rosenberg. 1953. Acute toxicity of four chlorinated dimethanonaphthalene insecticides to chicks. J. Econ. Entomol. 46(6):1067-1070.

Sherman, M. and E. Ross. 1961. Acute and subacute toxicity of insecticides to chicks. Tox. and Appl. Pharmacol. 3:521-533.

Simpson, M. E., G. von Wagenen and F. Carter. 1956. Content of anterior pituitary of monkey (Macacus mulatta) with special reference to gonadotrophins. Proc. Soc. Exp. Biol. Med. 91:6.

Smith, M. I. and E. F. Stohlman. 1944. The pharmocologic action of DDT and its estimation in the tissues and body fluids. Publ. Health Rept. 59(30):984-993.

Smith, P. E. and E. T. Engle. 1927. Ablation and transplantation of the hypophysis in the rat. Am. J. Anat. 40:159.

Snell, G. D. 1941. Reproduction, 55-88. In G. D. Snell, (ed.), Biology of the laboratory mouse. Dover Publications, Inc. N. Y. 497 pp.

Steelman, S. L. and F. M. Pohley. 1953. Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotrophin. Endocrin. 53(6):604-616.

Steelman, S. L. and A. Segaloff. 1959. Recent studies on the purification of the pituitary gonadotropins. Recent Progr. Hormones Res. 15:115.

Stevens, V. C. 1962. A study of certain endocrine and physiological aspects of reproduction in the cottontail rabbit. Ph.D. Dissertation, The Ohio State University, Columbus, Ohio. 203 pp.

Stohlman, E. F., W. T. Thorp and M. I. Smith. 1950. Toxic action of chlordane. Arch. Ind. Hyg. Occup. Med. 1:13-19.

Stormont, R. T. and B. E. Conley. 1952. Pharmacologic properties of toxaphene, a chlorinated hydrocarbon insecticide. J. Am. Med. Assoc. 149(2):1135-1137.

Taylor, A. 1963. The rapid determination of seed-dressing insecticide residues in animal relicta. Analyst 87(1039): 824-826.

Thienes, C. H., C. F. Lombard, F. J. Fielding, A. J. Lesser and M. J. Ellenhorn. 1946. Alterations in reproductive functions of white rats associated with daily exposure to nicotine. J. Pharmacol. Exp. Therap. 87:1-9.

Treon, J. F., F. P. Cleveland and J. Cappel. 1955. Toxicity of endrin for laboratory animals. J. Agr. Food Chem. 3(10):842-848.

Treon, J. F. and F. P. Cleveland. 1955. Toxicity of certain chlorinated hydrocarbon insecticides for laboratory animals with reference to aldrin and dieldrin. J. Agr. Food Chem. 3(5):402-408.

Turner, C. D. 1955. General Endocrinology. W. B. Saunders Co., Philadelphia, Pa. 553 pp.

U. S. Department of Health, Education and Welfare. 1963 Clinical handbook on economic poisons. Public Health Service Pub. 476, Rev. ed. Washington, D. C. 144 pp.

Wade, N. J. and E. A. Doisy. 1935. Cornification of vaginal epithelium of ovariectomized rats produced by smearing. Proc. Soc. Exp. Biol and Med. 32:707-709.

Waud, R. A. 1952. A study of the toxicology of the insecticide aldrin. J. Pharmacol. Exp. Therap. 106:423.

Weikel, J. H., Jr. 1958. Ion movement across the rabbit erythrocyte membrane as affected by chlornated insecticides. Arch. Intern. Pharmacodynamic 113:261-272.

Witschi, E. and W. T. Levine. 1934. Oestrus in hypophysectomized rats parabiotically connected with castrates. Proc. Soc. Exp. Biol. Med. 32:101-107.

Woodard, G. and R. R. Ofner. 1946. Accumulation of DDT in the fat of rats in relation to dietary level and length of feeding. Federation Proc. 5(1):215.

Worthington, W. C. 1955. Some observations on the hypophyseal portal system in the living mouse. Bull. John Hopkins Hosp. 97(4):343.

Young, W. C. and W. R. Fish. 1945. The ovarian hormones and spontaneous running activity in the female rat. Endocrin. 36(3):181-189.

Zavon, M. R. 1961. The toxicology and pharmacology of endrin. The Kettering Laboratory in the Dept. of Preventive Medicine and Industrial Health, College of Medicine, Univ. of Cincinnati, Ohio. 24 pp.

AUTOBIOGRAPHY

I, James Joseph Huber, was born on a small farm in Finneytown (Cincinnati), Ohio, on July 29, 1933. I attended St. Clare grammar school and Roger Bacon high school, both located within the suburbs of Cincinnati. I was employed by the Clopay Corporation from 1951 until 1953. In 1953 I entered The Ohio State University and received my Bachelor of Science degree in 1957. I received a graduate fellowship from the Ohio Cooperative Wildlife Research Unit in 1957 and entered the graduate school of The Ohio State University. In 1958 I married Carol Ann Salamony and was blessed with a son, James Augustine, in 1962. In 1959 I received my Master of Science degree from The Ohio State University. As a graduate assistant in the Department of Zoology and Entomology, The Ohio State University, I began my work toward the Doctor of Philosophy degree in 1960. In 1962 I received a research assistantship from the Natural Resources Institute, The Ohio State University, which carried me through the remainder of my graduate study.