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EVALUATION OF PESTICIDE EFFECTS ON PARAMETERS OF  
MALE REPRODUCTION IN THE MOUSE

A Dissertation  
Presented for the  
Doctor of Philosophy  
Degree  
The University of Tennessee, Knoxville

Alfred Raymond Martin  
December 1981

**3055641**

## DEDICATION

It is with affection and great pleasure that I dedicate this dissertation to some very special people for their love, encouragement and support during my long college career. It is dedicated to the memory of my father, Raymond Martin (1915-1976) who instilled in me a love for reading and a good bit of academic curiosity. This dissertation is also dedicated to my mother and my sister Suzanne who are always ready with understanding and a kind word. Without them, the world would sometimes seem to be a very lonely place indeed. Finally, this work is dedicated to Rachel Carson, the naturalist and author who first alerted the world to the dangers of synthetic pesticides.

## ACKNOWLEDGMENTS

I wish to express my thanks to the Department of Zoology of The University of Tennessee, Knoxville, for the use of their facilities and equipment. My appreciation is expressed to Dr. John Kennedy, Dr. Gerald Vaughan, Dr. Hanspeter Witschi and Dr. Lena Brattsten for editing the manuscript and for their valuable suggestions for this research. I also wish to thank Mr. Dick Williams for teaching me the use of the electron microscope and Dr. Stephen Handel for his most helpful advice on statistics. Special thanks are extended to my major professor, Dr. Mary Ann Handel, for her extremely helpful assistance and advice in conducting the research and preparing the manuscript of this dissertation. As adviser and friend, she helped me through the many difficult times one encounters in graduate school.

## ABSTRACT

The purpose of this study was to determine the effects of the insecticides Kepone and carbaryl (Sevin<sup>R</sup>) and the nematocide 1,2-dibromo, 3-chloropropane (DBCP) on parameters of male reproduction in the mouse. Mice were given 10 mg/kg kepone every 3 days for up to 44 days, 150 mg/kg carbaryl every 2 days for up to 68 days, or 10 mg/kg DBCP every 3 days for up to 44 days. Pesticides were dissolved in corn oil and administered by gavage. Controls received a dose of corn oil alone to match the volume used as a solvent.

The effects of these three pesticides were evaluated relative to testicular weight, percent of abnormal sperm, epididymal sperm counts and testicular histology. Body and liver weights, plasma acetylcholinesterase levels in carbaryl-treated mice and tissue levels of pesticide in kepone and DBCP-treated mice were measured in order to document absorption of the pesticides. The major findings were as follows: (1) Kepone caused an increase in percent of abnormal sperm by day 36 of treatment and a rise in liver weight by day 16 of treatment. After suspension of treatment on day 44, percent of abnormal sperm returned to normal but liver weight did not. (2) Carbaryl had no effects on any of the parameters of male reproduction studied in this research. (3) DBCP caused a decrease in testis weight by day 44 of treatment and a lowering of epididymal sperm counts by day 36, with an elevation of liver weights. (4) Corn oil, which contains estrogen-like compounds, induced densely staining vacuolated bodies

in the seminiferous tubules of the testis. These dense bodies were observed to occur in all three pesticide treatments as well as in corn oil treated controls but were not observed in mice which did not receive corn oil, suggesting that the corn oil was responsible for the presence of these dense bodies. The occurrence of dense bodies was not significantly different in pesticide-treated mice compared to corn oil treated controls. The data presented here suggest that absorption of kepone and DBCP may lead to adverse effects on male reproduction but do not demonstrate such effects from carbaryl.

## PREFACE

The purpose of this study was to determine what effects the insecticides kepone and carbaryl and the nematocide dibromochloropropane (DBCP) have on male reproductive function in the mouse. This investigation consisted of examination of epididymal spermatozoa to determine sperm count and to examine for morphological anomalies of the sperm and of examination of testicular weight to measure atrophy or edema. Testes were examined with light and electron microscopy to detect abnormalities of sperm-forming tissue. Weight of liver, gas chromatographic analysis of tissues of mice exposed to DBCP and kepone and acetylcholinesterase assay of plasma of carbaryl-treated mice were examined in order to document that the pesticides were being absorbed.

Although the literature is inconclusive, these three pesticides have been implicated as causative agents in disorders of the male reproductive system in humans and other mammals. DBCP was reported to produce oligospermia and sterility in workers who had been exposed to it in a pesticide factory (Anonymous, 1977; Whorton, et al., 1977). Several Russian investigators (Vashakidze, et al., 1966; Vashakidze, 1967; Shtenberg and Rybakova, 1968; Orlova and Zhalbe, 1968) have reported decreased reproductive function in male rats exposed to carbaryl. Other investigators, studying rats fed carbaryl for three generations (Weil, et al., 1972) and pesticide plant workers exposed to carbaryl (Whorton, et al., 1979) were unable to demonstrate any

reproductive abnormalities. Kepone has been demonstrated to cause a decreased sperm count in factory workers (Sterret and Boss, 1977). Abnormalities of germinal epithelium of Japanese quail were observed with kepone treatment (McFarland and Lacy, 1969; Eroschenko and Wilson, 1975; Eroschenko, 1978).

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## I. INTRODUCTION

The year 1939 was marked by an event which has had a profound effect on all living things on this planet. In that year, a Swiss scientist, Dr. Paul Müller, introduced the first synthetic pesticide, 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane, also known as DDT. Until 1940, only arsenicals, petroleum oils, cyanide, nicotine and a few other compounds were available as weapons in the ancient war against insects and other pests. Besides having limited effectiveness, these compounds are extremely toxic to vertebrates, including man. Many of them are in fact, more highly toxic to vertebrates than to the insects against which they were employed. DDT was found to be very effective against some of the worst insect pests such as the gypsy moth, spruce budworm, the Colorado potato beetle and malaria-carrying mosquitoes while being much less acutely toxic to vertebrates. DDT was dubbed the "superinsecticide" and quickly gained spectacular popularity and wide distribution. In 1948, Dr. Müller was awarded the Nobel Prize in Medicine for his discovery which was said to have saved millions of human lives around the world.

Between 1940 and 1973, more than four billion pounds of DDT were used in the United States alone to protect crops and fight insect-borne diseases (Ware, 1978). DDT was only the first of a large number of synthetic pesticides. The invention of chlordane, aldrin, dieldrin, parathion, malathion and other chemicals gave man a powerful arsenal in his fight against insect pests. Unfortunately,

like so many other technological advances, synthetic pesticides have created serious problems which were not immediately obvious. These magnificent chemicals which are often used so indiscriminately around humans were found to be considerably more dangerous to man and economically beneficial species than was originally thought.

In 1962, the conservationist Rachel Carson alerted the world to the dangers of DDT and other synthetic pesticides with her book Silent Spring. She focused popular attention on the virtual indestructability of the DDT molecule, its biomagnification or concentration along the food chain up to and including man, its toxicity and its detrimental effects on the reproduction of birds and other animals. Much controversy ensued following the revelations of Silent Spring and in January of 1973, the Environmental Protection Agency (EPA) banned DDT use in the United States.

Scientists, legislators and the courts are now faced with a seemingly unsolvable dilemma regarding the use and regulation of pesticides. On the one hand, there is increasing evidence that these "elixirs of death" as Carson called them are toxic to humans and possibly mutagenic or carcinogenic and may be slowly poisoning us all. As Rachel Carson points out, for the first time in history, humans and animals are subjected to poisonous substances throughout life. Probably everyone has residues of these toxic chemicals stored in his body and there is much uncertainty as to what the prolonged effects may be.

On the other hand, feeding the world's population has become a critical problem. Fifty-six percent of the world's population is

undernourished. In underdeveloped countries, the figure is as high as 79% (Ware, 1978). To exacerbate the problem, the population of the world is expected to increase from 3.57 billion in 1970 to over 5 billion by 1990. Worldwide, a third of the world's food and fiber crops are ruined by insects and other pests at a loss of \$75 billion per year. Clearly, pesticides have allowed us to raise the carrying capacity of the earth. If pesticide use were banned, loss of food crops would rise greatly above this figure. Banning the use of pesticides would tip the balance in favor of the insects (Bergmann, 1972) and the carrying capacity of the earth, which has been raised by our technology, would drop. Many more millions of people would die of disease and starvation. One can envision a scenario in which global warfare erupts between developed and underdeveloped nations as starving hordes fight over possession of greatly limited food supplies.

Obviously neither mass starvation and war nor poisoning and environmental destruction are acceptable outcomes to the pesticide dilemma. Several alternatives to the present synthetic pesticides have been suggested and some of them show promise. Replacement of the present pesticides with "newer" chemicals would probably not improve the situation since any chemical pesticide would probably present the same problems as those presently used. Two alternatives which may prove feasible are chemical or radiological sterilization of insects and the use of chemicals to control the life cycle of insects. One such technique consists of sterilizing males of a pest species and releasing them to mate with fertile females so that no offspring are produced. The use of hormones to offset the life cycle of insects

also shows promise. Larval or pupal insects treated with juvenile hormone fail to become adults or may be kept at an immature stage until environmental conditions no longer favor survival of the adults. However, both of these techniques are still of relatively minor importance when compared with the practicality of insecticides, largely due to the logistics involved. For example, the sterilization technique was used to eliminate the screw worm from Florida. However, in order to accomplish this, three and a quarter billion sterile flies had to be released over a period of 17 months (Bergmann, 1972). At the present, insecticides are obviously indispensable if global catastrophes are to be averted.

Unfortunately, even if we are willing to accept the environmental and health risks posed by pesticides, the present synthetic insecticides may not be the ultimate solution to pest control. Synthetic pesticides have been in use for only four decades but many strains are developing a resistance to them. For example, houseflies and some strains of mosquitoes are now resistant to DDT (Carson, 1962; Graham, 1970); the green leafhopper, the sheep blowfly and some strains of houseflies and mosquitoes have developed resistance to carbamate or organophosphate insecticides (Schuntner and Roulston, 1967; Winteringham, 1969; Georgiou and Hawley, 1971; Hama and Iwata, 1971; Iwata and Hama, 1972; Tripathi and O'Brien, 1973; Sawick, 1974). Insects have a high reproductive rate and are polymorphic regarding susceptibility to insecticides. When a population of insects is exposed to an insecticide, the population is decimated and for a while, may no longer be a pest. However, a few pre-adapted individuals will

survive to mate and eventually the population will again reach an optimal level. The frequency of the genes which allow resistance will have increased, resulting in a population which is less susceptible to the control agent. This necessitates using more insecticide, combinations of insecticides or different vehicles to maximize absorption of the insecticide. Despite these measures, the adaptable insects will likely develop further resistance leading some authorities to fear that we are essentially causing the evolution of "superbugs." The mechanisms by which insects develop resistance include increased metabolism of the insecticide by increased levels and activities of mixed function oxidases and other enzymes, alterations of acetylcholinesterase and decreased ability to absorb the insecticide (Wilkinson, 1976).

While much is known about pesticide toxicity in general, such as mechanism of acute toxicity, relative acute toxicities to insects and mammals, metabolism and synergism with other substances (see Ware, 1978, for a general review or Wilkinson, 1976, for a detailed presentation of this information), there has been relatively little work done on the evaluation of the effects of pesticides on reproduction and specifically the male reproductive system. (See Thomas, 1975 and Lucier, et al., 1977 for reviews.) This information is vital in properly evaluating the risks of pesticides relative to their obvious benefits. The most extensively studied pesticides, including those studied for effects on reproductive function, are the chlorinated hydrocarbons, especially DDT and its analogs. DDT and DDE have long been known to decrease reproductive success in birds by thinning the

egg shells which causes them to crack (Carson, 1962; Heath, et al., 1969; Peakall, 1970). DDT at 100 ppm was added to the diet of male leghorn chickens for 32 weeks (Arscott, et al., 1972). A half milliliter of semen from these animals was artificially inseminated into untreated hens and the eggs were incubated for 8 days, then candled to determine fertility. Fertility of the eggs, packed sperm volume (as determined by hematocrit), body weight and testis weight for treated animals were found not to be different from the same criteria obtained from untreated control roosters. Therefore, DDT does not appear to affect male reproduction in this species. These pesticides have been shown also to affect mammalian reproduction. In a study by Bernard and Gaertner (1964), pairs of male and female mice were caged together and fed 100 to 300 ppm of DDT in their food. The mice tolerated these high doses well, almost all of them surviving to the end of the 60 or 90 day experimental periods though exhibiting tremors. The numbers of pups produced per litter was not different from that of controls (given food without DDT) but the number of females producing litters declined significantly. Only four of nine pairs of mice fed 200 ppm DDT produced first litters while only two pairs produced second litters. Almost all of the control mice produced litters. While this experiment does indicate that high doses of DDT in the diet inhibit overall reproductive function in mice, it does not indicate whether males, females or both sexes were affected by the pesticide nor does it elucidate a mechanism by which DDT acts on reproduction. In another study (Gellert, et al., 1974), DDT (p,p'-DDT) and its analogs, o,p'-DDT, DDA, p,p'-DDE and methoxychlor,

one milligram (mg) per animal, were dissolved in dimethyl sulfoxide (DMSO) and injected into male rats. Controls received DMSO only. When the animals were killed after 203 days, testis and ventral prostate weight and sperm motility were unchanged from controls. However, luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels were significantly reduced.

The data from these experiments appear inconclusive with respect to effects of DDT on reproduction. The paper by Bernard and Gaertner (1964) indicates some adverse effects on reproduction in a mammal (mouse) while the work of Gellert, et al. (1974) demonstrates almost no effect of DDT or analogs on the mammalian male reproductive system. These data could indicate that chlorinated hydrocarbons adversely influence the female system primarily or that sperm motility and weights of the reproductive structures are not accurate criteria for measuring the effects of DDT in the male. Furthermore, Arscott, et al. (1972) failed to demonstrate that DDT influences reproductive ability in birds. However, since DDT has been shown to decrease avian reproduction by causing thinning of egg shells, a criterion not examined by Arscott, et al. (1972), these data are not necessarily contradictory. Of course, different results may be obtained even in similar experiments if there are differences in species or strain of animal used, agent tested, route of administration, vehicle and dose.

Dikshith and Datta (1972a) have examined the testicular morphology of rats exposed to DDT and parathion. Three groups of male rats given single i.p. injections of one milliliter saline, 50% ethyl parathion or 10% methyl parathion plus 30% DDT were killed at

intervals and testes and liver were fixed, paraffin-embedded, and sections stained with hematoxylin and eosin. Testes and liver weights were not different from controls but pathological changes were noted in seminiferous epithelium. After 1 week, testes of all pesticide-treated mice had some abnormal tubules exhibiting edema, pyknotic spermatocytes and necrotic cells. After 15 days, giant multinuclear cells were observed. By 30 days after treatment, pyknotic resting spermatocytes, abnormal spermatids, necrotic cells and vacuolization of cytoplasm of germinal cells were reported. At the termination of the experiment at 45 days, spermatocytes were reported to have been "fused to form a line of binucleate and tetranucleate spermatocytes" (Dikshith and Datta, 1972b). These were presumably different from the normal condition in which daughter spermatocytes maintain cytoplasmic continuity since the later phenomenon cannot be observed with light microscopy. Liver sections demonstrated hypertrophy and nuclear pyknosis indicating that parathion and DDT possibly induce a general cellular degeneration and are not testis-specific in their effects. In a second experiment (Datta and Dikshith, 1973), rats were given the same pesticides in repeated doses (three times per week) and the same histological anomalies were found.

Studies of the effects of toxicants on reproductive function involve two levels of inquiry. The toxicologist may first study the cytological, histological and biochemical alterations of sperm, germinal epithelium and other tissues to detect morphological or chemical changes which may have been induced by the toxicant. This reveals how the toxicant may interfere with normal reproductive

function and it can yield information suggesting mechanisms by which the toxicant works. However, these data alone do not indicate definitively that the toxicant actually inhibits reproductive performance. To determine that the toxicant in question does indeed affect reproductive performance, whole animal mating studies or in vitro fertilization studies are required. In essence, treated animals must be placed with treated or untreated mates to see if offspring are produced. Such criteria as litter size, frequency of littering, fertility, viability (proportion of implantations that produce live offspring) and teratogenic effects may be evaluated and compared with data from untreated controls.

Two useful criteria may be obtained from examination of epididymal sperm cells. First, the toxicologist may examine the numbers of epididymal sperm in treated animals relative to controls to determine if there is any significant difference in sperm counts. Another criterion is to estimate the number of abnormal epididymal sperm cells in treated animals relative to control animals. Bruce, et al. (1974) were the first to study sperm abnormalities as a quantitative index of damage induced by an environmental agent. They subjected mice to testicular X-irradiation and then examined sperm cells for abnormalities. Sperm judged to be abnormal were placed into one of seven categories of abnormalities such as misshapen heads, abnormal tail attachment or abnormalities of tail number or coiling. Based on these criteria, the number of "abnormal" sperm were significant in X-ray treated animals when compared with controls. Wyrobeck and Bruce (1975) subjected mice to subacute doses of 25

different chemicals and examined these animals for elevated levels of sperm abnormalities. All but six of the chemicals tested (DDT being one of the six) resulted in elevated abnormal sperm titers. Finally, Soares, et al. (1979) treated mice with the mutagen triethylene-melamine (TEM) and examined for sperm abnormalities. These authors report that their judgment of "normal" became more conservative after extensive examination of sperm cells. They recognized 22 categories of abnormalities.

The authors cited above have arrived at the conclusion that any sperm cell which is morphologically different in any way from the accepted shape or size of a "typical" mouse sperm with curved, pointed acrosome, rounded base and relatively straight tail must be abnormal. However, cells that they consider to be abnormal may be seen in abundance in untreated control mice of many strains, certainly suggesting that mouse sperm may be normally polymorphic.

An examination of epididymal spermatozoa of mice of strains BALB/c, C57BL/6J (wild types) and several  $t^X$  locus-bearing mutant strains revealed that animals from the wild-types and the mutant strains all contained exactly the same types of morphological effects (Hillman and Nadijcka, 1978a). Mutant mouse sperm exhibited no unique abnormalities. Handel and Dawson (1981) also report no unique abnormalities in their study of a male sterile mouse mutant. Likewise, Bruce, et al. (1974), Wyrobek and Bruce (1975) and Soares, et al. (1979) reported essentially the same types of sperm abnormalities (misshapen or abnormally small heads and coiled or multiple tails) with no unique morphological variants. Furthermore, in their

comparison of sperm of wild and mutant mice, Hillman and Nadijcka (1978) demonstrated that the incidence of specific abnormalities varied with the strain. Bruce, et al. (1979), Wyrobek and Bruce (1975) and Soares, et al. (1979) offer no indication that the sperm they consider to be abnormal are in any way functionally abnormal and the ability to fertilize an egg is, in the final analysis, a most important criterion for functional normality. In fact, mice heterozygous for  $t^X$  locus mutations have been shown to transmit the mutant gene at a frequency higher than 50%, indicating that these mutant cells have some selective advantage over sperm bearing wild-type genes (Yanagisawa, 1965; Hillman and Nadijcka, 1978a).

The ideas of what is abnormal are perhaps given credence to some degree by the fact that treatment with radiation or a known mutagen increases the number of "abnormalities." However, it is conceivable that treatment with chemicals or radiation could increase the degree of polymorphism without significantly altering ability to fertilize.

In order to determine if animals with high levels of abnormal sperm do differ from controls in actual reproductive function, these animals would have to be subjected to whole animal breeding studies or in vitro fertilization studies. In the in vivo breeding studies, treated or mutant males, as well as normal control mice, are mated to untreated females. Litter size, frequency of littering and fetal abnormalities are measured to determine reproductive ability (Heywood and James, 1979). In in vitro fertilization studies, sperm cells from treated or mutant and control mice are added to ova from normal females under highly controlled conditions of nutritional composition, pH and

atmosphere (Brackett, 1978). Ova are then examined for signs of fertilization, such as presence of sperm pronuclei or two-celled zygotes. However, even the results of these tests may be questionable since animals with high titers of abnormal cells still have very large numbers of normal sperm cells, perhaps enough to bring about reproductive successes in most cases while masking the proposed inability of abnormal cells to bring about fertilization. It is possible that the question of whether or not morphologically abnormal sperm cells can fertilize an egg will not be answered definitively until some method of preparing pure abnormal cell fractions or strains of mice with all abnormal sperm can be developed.

Probably a better indication that a chemical agent induces abnormalities in sperm production is obtained by direct examination of testicular histology. This technique is of great importance in determining what specific cellular abnormalities occur and what stages of spermatogenesis are affected. Abnormalities in germinal epithelium have been found in animals treated with industrial pollutants, metals, pesticides and other substances.

The abnormalities induced in germinal epithelium are often rather much the same regardless of the toxic agent employed. For instance, testes of rats exposed to DDT and parathion or to parathion alone (Dikshith and Datta, 1972; Datta and Dikshith, 1973) exhibited necrotic cells, vacuolization of cytoplasm, pyknotic nuclei, giant multinuclear bodies and tubular edema. Intratesticular injection of the insecticides lindane and endrin in rats resulted in testicular atrophy and hypertrophy, degeneration of germinal cells and large

multinuclear cells (Dikshith and Datta, 1972b). Boron, a heavy metal pollutant, administered at 1000 ppm or higher induced a decrease in normal spermatids and spermatocytes, and an increase in degenerating, multinucleated cells (Lee, et al., 1978). Hexachlorocyclohexane put into mouse food at 500 ppm for 10 months produced tubules containing large numbers of degenerating, multinuclear giant cells (Nigram, et al., 1979).

Flickinger (1977) obtained some good electron micrographs of testicular histology of rats exposed to the antiandrogenic compound Provera<sup>R</sup> (medroxyprogesterone acetate), a synthetic progesterone. He described vacuolated cells, irregularly-shaped nuclei and disrupted membranes. Other effects were decrease in number of cells in the later stages, misshapen acrosomes, necrotic spermatids and abnormal arrangement of tail fibers. Seminiferous epithelial cells were observed to contain large fat droplets which indented or displaced the nucleus or other organelles. Sertoli cells, while not greatly damaged, were observed to contain increased numbers of lysosomes and fat droplets, presumably phagocytosis of degenerating germinal epithelium.

Hillman and Nadijcka (1978b) studied abnormal spermiogenic figures in mice of strains BALB/c and C57BL/6J (wild types) and in several mutant  $t^X$  strains. As with epididymal sperm cells, these authors report that abnormal spermiogenic cells are to be found in all animals and strains examined, control as well as mutants. Furthermore, the mutant strains (or the control strains, for that matter) exhibited no unique abnormalities. Again, the highest occurrence of abnormalities was found in the control strains. This work and the

preceding one (Hillman and Nadijcka, 1978a) are examinations of just a few strains of wild type and mutant mice. The findings do not necessarily apply to all strains or to mice treated with various toxicants. However, nowhere in the literature have I encountered any reports of unique anomalies in epididymal sperm cells or in spermatogenic cells in any treated animals. Perhaps one reason for the paucity of abnormal spermatogenic cells unique to a given strain or treatment is the fact that most studies of testicular histology of toxicant-treated mice at least are conducted at the light microscopic level where ultrastructure cannot be examined. Hillman and Nadijcka (1978a and 1978b) and Handel and Dawson (1981) conducted both light and electron-microscopic examinations.

Other histological criteria which may reflect damage to the male reproductive system by environmental agents are diameter of seminiferous tubules and germ cell/Sertoli cell ratios. Other useful morphological data may be derived from weights of testes, seminal vesicles and other organs.

In the present research, I have studied the effects of the insecticides carbaryl (Sevin<sup>R</sup>) and chlordane (Kepone<sup>R</sup>) and the nematocide 1,2 dibromo, 3 chloropropane (DBCP) on spermatogenesis in the mouse. These pesticides have been used extensively in contact with humans and all have been implicated as causative agents in sterility. Workers exposed to DBCP in pesticide plants were shown to be either oligospermic or sterile (Whorton, et al., 1977; Potashnik, et al., 1978). Kepone became a major pollution problem in Hopewell, Virginia in the early 1970's and workers involved in its

manufacture were also shown to exhibit sterility or oligospermia (Murray, 1976; Sterrett and Boss, 1977). The connection between carbaryl and sterility is still inconclusive. Vashakidze (1967) and Shternberg and Rybakova (1968) reported adverse effects of carbaryl on reproductive function in male and female rats and suggested that these effects are due to interaction of carbaryl with hormonal control mechanisms. Orlova and Zhalbe (1968) reported spermatogenic damage and reduced sperm motility in rats exposed to carbaryl. However, in a later investigation, rats fed carbaryl (99.8% pure) for three generations failed to show any adverse effects on reproductive function (Weil, et al., 1972). Likewise, sperm counts of individuals who worked in carbaryl manufacture were found to be no different from those of control subjects (Whorton, et al., 1979).

I have examined the effects of these pesticides on the histology of seminiferous epithelium, epididymal sperm counts and percent of abnormal epididymal sperm cells in mice which have received specific doses of pesticide. My studies did not document whole animal reproductive performance because of the availability of this information in the current literature. The ultimate goal of my studies, as well as those in the literature which employed mice, rats or other non-human species, is to determine just what effects these pesticides potentially have on the human reproductive system. There are difficulties with using rodents as models for determining effects of pesticides or other toxicants on humans. Non-human mammals may differ from humans in their physiology and thus in their response to toxicants. Furthermore, very few humans are constantly exposed to the high doses to which

laboratory animals are subjected. However, rodents are routinely studied in toxicology as models of the human system.

The mouse was chosen as the test organism partly because mice are relatively inexpensive and can be kept in large numbers. Mice are a standard test mammal used in toxicological studies, and information regarding the mouse reproductive system is used to infer what the effects of the pesticides on the human will be. Mice are readily obtainable in highly inbred strains so that individual differences in response to a toxicant can be minimized.

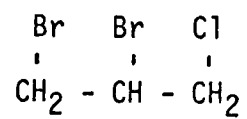
The emphasis of the present investigation is on various parameters of male reproduction (epididymal sperm number, percent of abnormal sperm cells, testis weight and quantitation of abnormalities of spermatogenic tissue, primarily at the light microscopic level). The body of literature examined seems to indicate that almost all environmental agents, pesticides and non-pesticides which affect germinal epithelium have similar morphological effects as if these agents perhaps all act by a common mechanism which is unlikely. Some toxic agents probably do act to upset the balance of hormones to some degree but the more probable explanation is that, since none of the agents tested are testis-specific in their effects, they probably produce general cellular degenerative changes in all tissues where they accumulate.

## II. THE PESTICIDES

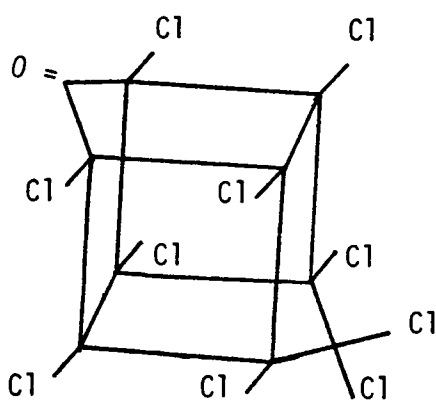
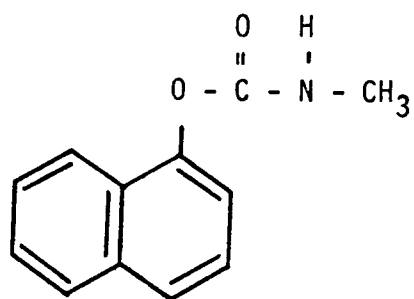
### Dibromochloropropane

DBCP (Fig. 1) (1,2 dibromo- 3 chloropropane, Nemagon, Fumazone, Nemaflow; Merck Index, 1976) is a soil fumigant and nematocide. This pesticide was introduced in 1955 and was used extensively until it was banned (except for use in extreme cases) by the Environmental Protection Agency (EPA) in 1977 following reports of sterility and oligospermia in workers who had been exposed to the substance (Anonymous, 1977b; Whorton, et al., 1977). DBCP, which exerts its toxic effects by acting as an alkylating agent (Rosenkrantz, 1975), is a highly volatile substance, as are all fumigants, and it is therefore easy for workers to be exposed to it by inhalation.

DBCP (oral LD<sub>50</sub> of 260 mg/kg; Merck Index, 1976) has been implicated as a causative agent in several pathological conditions, notably in sterility, carcinogenicity and mutagenicity. Examination of semen samples and serum hormone levels of 25 non-vasectomized men who had been exposed to DBCP in a pesticide factory revealed five individuals to be oligospermic and nine to be totally azoospermic (Whorton, et al., 1977). All abnormalities increased with exposure time. These researchers reported significantly elevated levels of serum FSH ( $11.3 \pm 1.8$  mIU/ml) and LH ( $28.4 \pm 3.3$  mIU/ml) in individuals who had worked in the factory for seven to nine years as compared with workers who had been exposed to DBCP for only three to five weeks ( $2.6$  and  $14$  mIU/ml, respectively). Testosterone levels did not



Dibromochloropropane

Kepone<sup>R</sup>

Carbaryl

Fig. 1. The pesticides.

change significantly (~460 ng/dl) regardless of exposure time. An increase in abnormal sperm was also noted. The authors suggest that symptoms of acute toxicity, such as headache, nausea, weakness and upper respiratory tract infection experienced by some of the workers could have been related to their work in the manufacture of thiocarbamate pesticides. They fail to mention that the above-mentioned abnormalities of spermatogenesis could have been caused or influenced by the thiocarbamates, or even by other, unnamed pesticides manufactured at the plant. Furthermore, the lack of control subjects in the experiment (individuals who did not work in the plant or live in the immediate area) made it impossible to evaluate possible effects of exposure to DBCP on workers who had been employed at the plant only a short time.

The above results were verified by Potashnik, et al. (1978) who examined semen samples, plasma hormone levels and testicular biopsy specimens from six individuals who had been exposed to DBCP in a pesticide factory. Radioimmunoassay revealed normal levels of testosterone (4 to 10 ng/ml) as compared to previously examined control subjects who had 4 to 12 ng/ml concentrations. FSH levels were elevated at 13.4 to 21.5 mIU/ml compared with control values of 3 to 10 mIU/ml while LH at 3.7 to 6.8 mIU/ml was considered normal. Examination of the results of the paper by Whorton, et al. (1977) compared to the control subjects examined by Potashnik and his coworkers (1978) reveals that workers exposed to DBCP for only a few weeks had normal testosterone and FSH levels but had elevated levels

of plasma LH (14 mI U/ml as compared to the control values of 3.7 to 7 mI U/ml).

Histological examination of hematoxylin and eosin-stained sections from testicular biopsy of four subjects all revealed complete atrophy of seminiferous epithelium (Potashnik, et al., 1978). Most of the tubules examined were lined with Sertoli cells only and no active spermatogenesis was seen. Spermatogonia were not specifically mentioned, but the implication was that they were usually absent from tubules.

DBCP has also been implicated as a carcinogen. DBCP was dissolved in corn oil and administered to male and female mice by intubation five times per week for up to 54 weeks (Olson, et al., 1973). Dosage began at 160 mg/kg for males and 120 mg/kg for females and was increased until week 28 when dosage was leveled off at half the maximum tolerated dose (260 mg/kg). After 14 weeks, several female mice had mammary tumors. A sample of five mice killed at week 40 all had stomach carcinomas. At 54 weeks, 17 of 24 mice examined had stomach carcinomas.

Several researchers (Rosenkrantz, 1975; Prival, 1977; Biles, et al., 1978; Kapp, et al., 1979) have studied DBCP for possible mutagenicity. DBCP at 21 mg/plate was exposed to agar plates containing a strain of Escherichia coli deficient in the enzyme DNA polymerase I (Rosenkrantz, 1975). DNA pol. I functions in repair of damaged DNA. The rationale was that if an agent alters (damages) the DNA in this strain (i.e., is mutagenic), the cells would be unable to make repairs and the kill area around the point of exposure to the

DBCP would be greater than in the culture of normal DNA pol. I+ E. coli. This was observed.

The same researcher also tested DBCP for mutagenicity using the Ames Salmonella test (Ames, et al., 1973). Salmonella strain TA1530 is known to revert from histidine dependent to histidine independent by a base-substitution mutation (see again Ames, et al., 1973) while strain TA1538 reverts by a frameshift mutation. Rosenkrantz was able to demonstrate that DBCP caused strain TA1530 to revert to histidine-independence but had no ability to revert cells of strain TA1538 indicating that, in Salmonella, DBCP causes mutation by base substitution. On this basis, he concluded that DBCP is an alkylating agent, that is, that it attaches alkyl groups to DNA, RNA and protein. In the case of DBCP, the alkyl group involved would be a propyl group ( $-C_3H_7$ ).

The question was subsequently raised as to whether substances like DBCP were themselves mutagenic or if some impurity is responsible for mutagenicity (Donahue, et al., 1978). Technical grade DBCP (96% pure) and "pure" DBCP (99% pure) were tested for mutagenicity with the Ames Salmonella test (Biles, et al., 1978). The major contaminants of the technical grade pesticide were 1% epichlorohydrin and 0.6% allyl chloride while the remainder consisted of related hydrocarbons. The "pure" DBCP contained virtually no epichlorohydrin. Technical grade DBCP at doses of 50 to 1000  $\mu\text{g}/\text{plate}$  caused more than 100 revertant colonies above control levels while "pure" DBCP resulted in little or no mutagenic effects. Both grades of the pesticide were toxic to colonies at doses of more than 1600  $\mu\text{g}/\text{plate}$ .

When technical grade DBCP was distilled, it caused the same low incidence of revertants as did the "pure" DBCP, while the distillate which was primarily epichlorohydrin still caused a significant number of revertants to histidine independence. However, when pure DBCP was exposed to liver microsomes, it was found to have a strong mutagenic effect at 20 to 200  $\mu\text{g}/\text{plate}$ . Therefore, while DBCP itself is not mutagenic, both the contaminants in DBCP and its metabolic products were shown to be mutagenic. The products of metabolic activation of DBCP were not identified. These experiments demonstrated that, in bacteria at least, a component of DBCP is mutagenic. This indicates that in bacteria, the impurities in DBCP are more responsible for mutagenicity than is the pesticide itself.

Sperm of workers exposed to DBCP were examined for quinacrine-fluorescent spots which are indicative of the Y-chromosome (Kapp, et al., 1979). Presence of two such spots in a normal sized cell was considered to be an indication of non-disjunction involving the Y-chromosome, a form of chromosomal mutation. Sixteen out of 18 DBCP-exposed workers had greater than 2% of sperm cells with two quinacrine fluorescent spots, while all of the 15 unexposed individuals had less than 2%. It appears that, if presence of two such spots is indeed an indication of Y-chromosomal mutation, then up to 2% of sperm in normal individuals are spontaneously mutant with respect to Y-chromosome behavior and DBCP increases this occurrence.

The previously cited papers do indicate that DBCP, or at least its contaminants and metabolites, are mutagenic in bacteria. However, mutagenicity in a bacterial system does not necessarily imply

mutagenicity in a mammalian system. Therefore, the work of Kapp, et al. (1979) is particularly important since it does indicate that DBCP exposure may cause chromosomal abnormalities in humans. Furthermore, while many or all carcinogens have mutagenic effects, mutagenicity does not necessarily indicate that a chemical is carcinogenic or that it will adversely affect development of germinal epithelium.

In light of findings that exposure to DBCP induces sperm abnormalities and reproductive dysfunction (Torkelson, et al., 1961; Anonymous, 1977b; Whorton, et al., 1977; Potashnik, et al., 1978; Sandifer, et al., 1979), the present study was undertaken to determine what abnormalities in various parameters of male reproduction are induced by DBCP. Previous studies of germinal epithelium have been done with hematoxylin and eosin-stained thick sections embedded in paraffin (Potashnik, et al., 1978; Sandifer, et al., 1979) and "degeneration" of germinal epithelium was observed. The present research extends this to an investigation of the ultrastructural effects of DBCP.

### Kepone

Kepone or chlordecone (Fig. 1, page 18) decachlorooctahydro-1, 3,4-methano-2H-cyclobuta [cd] pentalen-2-one) was developed by the Allied Chemical Company in 1958 as an agent effective against leaf-eating insects. It belongs to the class of organochlorine compounds called the cyclodienes which includes chlordane, aldrin, dieldrin, heptachlor, endrin and mirex. The cyclodienes are all persistent insecticides which are stable in the environment. Kepone is only

slightly soluble in water, being more soluble in polar solvents such as alcohols, ketones, acetic acid (Merck Index, 1976) and lipids. Kepone is not metabolized. Being lipid-soluble, it is stored in fat and in the liver (Boylan, et al., 1978) and is therefore subject to biomagnification or concentration in biological systems.

Kepone achieved national notoriety in July, 1975 when the kepone plant in Hopewell, Virginia was closed by the Virginia Department of Health following acute poisoning of plant workers and discharge of large amounts of the insecticide into the James River (Murray, 1976; Sterrett and Boss, 1977). Acute kepone toxicity is characterized by tremors, muscle spasms, excitability, lack of coordination and hyperactivity. Workers in the kepone plant in Hopewell also exhibited loss of memory, blurred vision, pain in the chest and joints and sterility (Sterrett and Boss, 1977).

Kepone is a central nervous system toxicant which exerts its acute toxicity by acting on the membrane of the neuron to interfere with ion fluxes necessary for normal neuron function. It disrupts energy production in the mitochondria by uncoupling oxidative phosphorylation and electron transport (Carmines, et al., 1979). Kepone has been shown to inhibit the function of the enzymes adenosine triphosphatase which removes a phosphate group from ATP and lactate dehydrogenase which catalyzes the reduction of pyruvate to lactate in glycolysis (Sterrett and Boss, 1977).

Kepone has a mammalian (rat) oral LD<sub>50</sub> of 95 mg/kg and a dermal LD<sub>50</sub> of 345 mg/kg. Besides its acute toxicity, kepone is known to induce cellular abnormalities in various organs, especially in the

liver where the pesticide is most concentrated (Boylan, et al., 1978). Kepone, given by intubation to rats for 3 days at a dose of 50 mg/kg in corn oil, induced abnormalities in cells of the liver, adrenal glands and diaphragm (Klein, et al., 1976). Liver cells were characterized by proliferation of smooth endoplasmic reticulum with corresponding disassembly of rough endoplasmic reticulum into smaller particles. This might imply an interference with protein synthesis, though no biochemical assessment of protein synthesis was made. Mitochondria were elongated or irregular by comparison to controls which were more rounded. Cells of the adrenal medulla contained elongated or flattened granules of unknown composition. Mitochondria of the diaphragm cells in treated animals contained swollen cristae and the mitochondria themselves were frequently swollen. These ultrastructural findings probably indicate some cytotoxic effects of kepone but these authors do not speculate as to what cellular functions were impaired by the pesticide. Mitochondrial swelling is a common pathological change in mitochondria in response to a toxicant that is thought to alter osmotic pressure of the cells resulting in absorption of water (Brown and Bertke, 1974).

Kepone has also been implicated in carcinogenesis. Kepone in the diet at a daily dose of 10 ppm was found to induce liver cancer in mice (Jaeger, 1976). However, studies of kepone carcinogenicity are incomplete and it is not yet possible to determine whether or not the compound is responsible for human cancers. Aside from studies of acute toxicity and carcinogenesis in laboratory animals and the

sterility studies, kepone appears to have been subjected to relatively little toxicological study.

Due to its extremely low water solubility, kepone is not readily excreted in the urine. In fact, the major role of the mixed function oxidases is to act on exogenous or endogenous substrates to convert them to molecules which are more readily excreted and thus eliminated from the body. Since kepone is apparently not metabolized by these enzymes, it is concentrated and stored in the liver and secreted through the biliary tract into the intestine. Unfortunately, instead of being eliminated with the feces, kepone is resorbed by the intestine and therefore remains in the body permanently. Since prolonged exposure to kepone has been implicated in cancer and acute toxicity, medical researchers at the Medical College of Virginia have devised a means of eliminating kepone from the bodies of the exposed kepone workers. Administration of Cholestyramine, a compound which decreases lipid absorption by the intestine, also prevents kepone absorption (Cohn, et al., 1978; Boylan, et al., 1978; Anonymous, 1977). However, it is still not known if kepone had been stored in the body long enough to cause irreversible damage.

There is evidence that kepone has adverse effects on reproduction. Good, et al. (1965) added various levels of kepone to the diet of mice. Ten to 37.5 ppm of kepone was fed to male and female mice for 1 month. After the treatment period, treated mice were paired and fed a normal diet without kepone. Numbers of litters, average number of young per litter and time required to produce litters were recorded for mice exposed to each dose. All dosage groups were able to

reproduce but reproductive success decreased with increased dosage. In a second experiment, the same researchers administered 3 ppm kepone for 128 days. The test animals produced 15.9% fewer first litters and 28.2% fewer second litters. This was a much lower dose than in the first experiment, but the drop in percentage of second litters was probably due to a prolonged period of exposure to kepone.

In a study of the physiological effects of kepone on mice, Huber (1965) replicated the results of the previous experiment. Females fed 30 ppm kepone showed normal development of ovarian follicles but almost no corpora lutea. Vaginal smears of these animals showed disturbed estrous cycles. Hormone assays indicated that females fed kepone had normal levels of FSH but had 25% less LH.

McFarland and Lacy (1969) put kepone at 400 ppm into food of male Japanese quail. The birds were kept at a photoperiod of 18 hours of light to 6 hours of dark which stimulates spermatogenesis. When tremors developed, the birds were killed and testis, body and liver weight were recorded. Some testes were dilated, often greater than twice the size of testes of untreated controls, while other testes were atrophic. Examination by light microscopy showed testes to be almost devoid of spermatids and spermatozoa. Liver weight was almost twice that of controls and liver sections revealed lipid vacuoles. This experiment indicates that kepone is getting into the tissues and is being processed in the liver. Degenerative changes observed in the testes are apparently not confined to the male reproductive system but occur in the liver where the pesticide is concentrated, indicating that kepone effects are not specific for reproductive tissue.

These results were verified by Eroshenko and Wilson (1975) who fed 200 ppm kepone to male Japanese quail. Birds fed kepone for 6 and 27 weeks had distended, fluid-filled seminiferous tubules with disquamated, degenerating cells. The lumina of many tubules contained germ cells in various stages of maturation. The numbers of sperms in the lumen were thought to be reduced because the sperm clumps were sparse compared with clumps of sperm in lumina of controls. In a second experiment (Eroshenko, 1978) Japanese quail were fed 200 ppm kepone for 42 days. The animals were then placed on a normal diet and at least 6 birds were killed at 10 day intervals over a period of 60 days. Testes of treated males were thick sectioned and examined by light microscopy. Birds killed immediately after 42 days of kepone treatment had testes which were hypertrophic or atrophic. Reduced numbers of germinal epithelial cells were observed. Germ cells often contained large vacuolated areas. Sperm cells were found to be curled and many had bulbous heads. Ten days after the birds were taken off the kepone diet, testicular morphology as examined by light microscopy, appeared unchanged. Twenty days after removal from the kepone diet, the germinal epithelium appeared taller and sperm clumps were present within Sertoli cells, signs that active spermatogenesis was beginning to recur. Thirty days after kepone treatment, epithelium was taller and spermatogenesis was increased. Testes of all kepone-treated animals, even of those which had been removed from kepone for 60 days contained dense, giant cells. The effects of kepone are not specific to the testis. Although no cellular changes were induced in the ovaries of treated females, the oviducts of immature females had

increased cellular proliferation, cytodifferentiation and formation of tubular glands. Laying quail, which have fully mature reproductive systems exhibited no changes of the oviduct. These data would indicate that kepone functions to cause maturation of parts of the female reproductive system; that is, that it has estrogenic activity. Kepone also caused hypertrophy of cortical and medullary cells of the adrenal and vacuolization of liver cells.

These experiments indicate that kepone does adversely affect spermatogenesis although its effects are not specific for the male reproductive system. The work of Eroschenko (1978) indicated that kepone stimulates maturation of the oviduct in Japanese quail. This estrogenic activity may explain the effects of kepone on the male system since estrogens act as anti-androgens. Further evidence for the estrogenic activity of kepone is offered by Hammond, et al. (1979). These authors did estrogen-receptor binding assays in which kepone competed with diethylstilbestrol (DES) for the estrogen receptor. Affinity of kepone for the estrogen receptor was only 0.01 to 0.04% that of estradiol. However, estradiol is a short-lived molecule compared to the unmetabolizable kepone. Hammond, et al. (1979) also measured uterine weight of rats exposed to kepone. Uteri of rats injected with kepone or estradiol at doses ranging from 4 to 40 mg/kg increased in weight (uterine weight/body weight) as dosage was increased.

### Carbaryl

Carbaryl (Fig. 1, page 18) (1-naphthalenol methylcarbamate, 1-naphthyl N-methyl carbamate, Arylam, Seffein, Sevin; Merck Index, 1976) is a commonly used broad spectrum insecticide for control of about 150 species of insects. It is effective against gypsy moths and certain agricultural pests such as those that attack cotton, apples and pears. It is not effective against houseflies, spider mites and aphids (Matsumura, 1975). Carbaryl is a carbamate, a group that includes methomyl, useful in controlling worms on vegetables and Baygon or propoxur which is very effective against cockroaches and is frequently found in household insect killers (Ware, 1978).

The first carbamates to be employed as insecticides were the dimethyl carbamates which were introduced in 1951 by the Geigy Chemical Company. These agents were largely unsuccessful due to their relative expense and limited effectiveness. Carbaryl, an N-methyl carbamate which was developed in 1956, was the first successful carbamate pesticide. It has a high vertebrate selectivity ratio (is of low vertebrate toxicity) and thus is widely used around humans and economically beneficial and domestic species.

Carbaryl is fat soluble, with a slight water solubility of 40 ppm (Matsumura, 1975) and has a mammalian oral LD<sup>50</sup> of 500 to 700 mg/kg (Merck Index, 1976). Carbaryl is quickly oxidized by the mixed function oxidases or converted in the environment under alkaline conditions to the non-toxic compounds 1-naphthol-N-hydroxymethylcarbamate, 4 hydroxycarbaryl, 5 hydroxycarbaryl, 5,6 dihydroxydihydrocarbaryl, 5,6 dihydroxydihydro-1-naphthol and primarily to 1-naphthol

(Dorough, et al., 1963; Dorough and Casida, 1964; Leeling and Casida, 1966).

Carbaryl is a neurotoxicant. The acute toxicity is caused by its inhibition of the vital enzyme acetylcholinesterase and much of the toxicological study of this insecticide is focused on this function (Singh and Agarwal, 1979). The degree of inhibition of acetylcholinesterase by carbaryl is dependent on the intensity and duration of the dose (Pipy, et al., 1978a; Pipy, et al., 1978b) and is reversible. Inhibition of the reticuloendothelial system which consists of cells containing MFO enzymes slows the reversibility of acetylcholinesterase inhibition and enhances carbaryl toxicity (Pipy, et al., 1979).

There is no clear consensus in the literature as to whether carbaryl affects the reproductive system. Vashakidze, et al. (1966) reported that large doses of the insecticide given to male rats over a prolonged period inhibited spermatogenesis. Carbaryl given orally to female rats at doses of 10 and 20 mg/kg for 6 months increased ovarian and uterine weight, elevated gonadotropin levels and changed the duration of the estrous cycle (Vashakidze, 1967). Orlova and Zhalbe (1968) reported reduced fertility, decreased sperm motility and changes in enzymatic activity of testes and ovaries of rats treated with carbaryl. Vashakidze, et al. (1966), Vashakidze (1967), Shtenberg and Rybakova (1968) suggested that the insecticide does not act directly upon the reproductive system, but that it interacts with hypothalamohypophyseal control functions.

A three generation study of rats fed technical carbaryl (99.8% pure) in food failed to show any effects on reproductive

function (Weil, et al., 1972). In this experiment, carbaryl was added to food at estimated doses of 2.5 and 10 mg/kg of body weight. Female rats were fed this diet until 7 to 11 days after weaning and were mated at 109 days of age. When the animals littered, no significant difference was found in fertility (ratio of pregnancies to number of females mated), gestation (ratio of litters with live pups to number of pregnancies), viability (ratio of pups alive at 4 days to number of pups born alive) or lactation (ratio of pups alive at 21 days to number of pups alive at 4 days, an index of the mother's ability to feed the pups). Third generation mothers were killed before parturition and the fetuses were examined. The numbers of live and dead fetuses and numbers of resorption sites were counted. No significant differences were found when these data were compared with controls. The mean number of fetuses per female and number of viable fetuses per female were actually slightly higher in mothers receiving 10 mg/kg/day carbaryl but these data were found to be insignificant also.

In a study of the effects of carbaryl on human male reproduction, semen samples for sperm counts were taken from 49 males who were employed at a carbaryl plant and from a control population of 90 unexposed men who did not work or live in the area (Whorton, et al., 1979). The test subjects were classified according to their jobs within the plant, number of years of exposure to carbaryl, and, in the case of those who no longer worked in the plant, number of years since exposure. No relationship was found between sperm counts and length of exposure to carbaryl. The median sperm count for exposed individuals was 92 million cells per ml while that of controls was

81 million cells per ml. Sperm counts were highly variable, ranging from a few million to over 600 million per ml in the control population. Furthermore, no difference was found between sperm counts of men whose jobs caused them to be heavily exposed and those of men with minimal exposure (Whorton, et al., 1979). Thirty-six of the exposed men provided blood samples for hormone radioimmunoassay. All but four of these men were within the normal levels of FSH ( $< 6$  MI U/ml) and all subjects tested were within normal levels of LH ( $< 15$  mI U/ml) and testosterone (300 to 1000 ng/100 mls). Thus, Whorton, et al. (1979) were unable to demonstrate that even men who had been heavily exposed to carbaryl had developed any adverse effects on reproductive function as exemplified by sperm count and hormone levels.

In the literature, only the studies conducted in the Soviet Union (Vashakidze, et al., 1966; Vashakidze, 1976; Shtenberg and Rybakova, 1968 and Orlova and Zhalbe, 1968) demonstrated any adverse effects of carbaryl on reproductive function. There were several possibilities for this discrepancy. Mode of administration may be a key factor. The Soviet investigators administered carbaryl by oral intubation while Weil, et al. (1972) mixed the toxicant into food. These authors state that a test agent should be given to laboratory animals by a route comparable to that by which humans would be exposed. With intubation or gavage, a large dose is placed into the stomach of the animal at one instant followed by high, sporadic blood levels as the material is absorbed. When lower doses are given with food, absorption of toxicant occurs more slowly and more evenly concurrent with nutrient absorption. The disadvantage of giving the toxicant in

food is that it is impossible to give each animal the same dosage (dose per body weight) since amount of food consumed is not necessarily related to weight and furthermore, precise dose cannot be determined. Each animal would in effect receive a different dosage of carbaryl. In the study of workers who had been exposed to carbaryl in a plant, these individuals were probably taking in the substance by oral, dermal and inhalation contact, but no reproductive abnormalities were observed (Whorton, et al., 1979).

A second factor which may explain the different results obtained by various experimenters is the purity of the carbaryl. Weil, et al. (1972) used technical carbaryl which was 99.9% pure. Purity of carbaryl used by the Soviet investigators was not given. Since these papers were available only in abstract or were briefly discussed in review papers (the original papers were unobtainable), source and manufacturer of the carbaryl used by the Soviet investigators is not known. Perhaps an impurity or a metabolic product of an impurity was responsible for the observed effects on reproductive function.

The strain of laboratory animal used in these tests could possibly be a factor. One inbred strain of animal may have a greater tendency to develop reproductive abnormalities than another. If the Soviets were working with such a strain (their strain was not identified) spontaneous abnormalities may be misinterpreted as being caused by the carbaryl. This problem should, however, be corrected for by use of control animals of the same strain. Finally, there is the human factor involving the knowledge and technique of the investigators. Tissues for microscopy, for example, must be properly fixed

and stained in order to derive any meaningful information from them and sperm counts must be done on unclumped semen specimens. Whorton, et al. (1979) eliminated one test subject from their sperm count experiment because of excess clumping of cells. An example of possible misinterpretation involves a test for teratogenicity which was done by a group of Soviet investigators (Orlova and Zhalbe, 1968). It was observed that pups of mothers given 5 and 15 mg/kg/day of carbaryl developed a condition in which their tails became necrotic and dropped off within 10 days of birth. This was interpreted as a teratogenic effect of carbaryl. However, Farris (1950) and Flynn (1959) were of the opinion that this was actually a condition called "ringtail" which may be associated with low humidity and was not an effect induced by carbaryl.

Thus, it seems that the differences in techniques and interpretation of criteria examined, purity of carbaryl and mode of administration may explain the differences in observed results. That is, the experiments were not equivalent.

### III. EXPERIMENTAL METHODS

#### Animals

The animals utilized were male ICR mice (Cumberland View Farms, Clinton, Tennessee). Animals were housed in cages of 10 animals each, and were given water and food ad libitum. Animals were kept at a cycle of 12 hours of light and 12 hours of darkness per day. All animals were 12 to 14 weeks old at the beginning of an experiment.

#### Materials

Dibromochloropropane (98.6% pure, contaminants unidentified) was purchased from Chem Service, Inc., West Chester, Pennsylvania. DBCP was diluted with Mazola<sup>R</sup> corn oil; both oil-diluted and pure DBCP were stored at 0° C to reduce evaporation. DBCP was administered by gavage at a dose of 150 mg/kg every 3 days. Kepone (99% pure, contaminants unknown) was purchased from Chem Service, Inc., dissolved in corn oil and given by gavage at a dose of 10 mg/kg every 3 days. Carbaryl (99% pure, contaminants unknown) was also purchased from Chem Service, Inc., dissolved in corn oil and administered by gavage at a dose of 150 mg/kg every 2 days for two spermatogenic cycles (68 days).

#### Determination of Pesticide Dosage

Pesticide-fed animals were treated with the highest dosage which most of them could survive to maximize tissue levels. Four mice were given DBCP in doses of 300 mg/kg of body weight and all four died within 12 hours. Eight mice given 225 mg/kg and eight given 150 mg/kg

were all alive and apparently healthy after 4 days. Olsen, et al. (1973) gave mice doses of 120 mg/kg and 160 mg/kg by intubation for up to 54 weeks but mortality was high. Therefore, for the present study, a dosage of 150 mg/kg every 3 days was established in an attempt to administer a relatively high dosage with low mortality. Unfortunately, mortality was still high; within 1 week of the experiment, 27 of 63 treated mice (43%) died while none of the controls died. After the more susceptible individuals died, mortality dropped but other mice did occasionally die during the experiment. Thus, a DBCP-resistant population of mice was studied in this experiment. Major reasons for resistance would be individual differences in the rate of absorption and metabolism of the pesticide and relative efficiency at which the toxicant is transported to the site of action.

It has been reported (Good, et al., 1965) that dietary kepone as high as 40 ppm can be tolerated without significant mortality in mice. Since an average mouse eats 4 to 6 grams of food per day (Arrington, 1972) this constitutes a dosage of 5 mg/kg/day, assuming a 40 gram mouse. Rat oral LD<sub>50</sub> of kepone is estimated at 95 mg/kg. For this study, mice were treated with 10 mg/kg every 3 days to allow for accumulation of kepone in the tissues. Only five treated mice died during experimentation while none of the controls died. Since more of the kepone-treated mice survived than DBCP-treated mice, a group was killed on five different dates. After the 44th day, dosage of pesticide was suspended to see if recovery would occur relative to any of the criteria examined as did Eroschenko (1978).

Carbaryl has a rat oral LD<sub>50</sub> of 500-700 mg/kg. It is quickly metabolized and does not accumulate in tissues as do DBCP and kepone. In this study, mice were given various doses of carbaryl (50 mg/kg, 75 mg/kg, 150 mg/kg and 300 mg/kg) and all treated mice survived and seemed healthy. Since the literature reports no effect of carbaryl on reproductive function and since carbaryl has a low vertebrate toxicity, mice in this study were given a high dose of the insecticide (150 mg/kg every 2 days for up to 68 days.

#### Procedure

Food was removed from cages from 2 to 6 hours prior to gavage. Mice were weighed before each treatment in order to gauge a constant dosage. Mice received pesticides in doses specified above. Controls were given a volume of corn oil alone matching the one used as a solvent.

Five or six pesticide-treated mice and an equal number of controls were killed by cervical dislocation at intervals of 9, 16, 36 and 44 days for DBCP-treated mice, at intervals of 9, 16, 36, 44 and 57 days for kepone-treated mice and at intervals of 13, 20, 34, 41 and 68 days for carbaryl-treated mice. The body, testis and liver weights for each animal were recorded at the time of sacrifice. Samples of testis, epididymis, kidney, fat and liver were taken, weighed and frozen immediately for determination of tissue pesticide levels. Testes and epididymides were carefully trimmed of fat before freezing. Before carbaryl-treated mice were killed, blood samples were taken by orbital bleeding with a Pasteur pipette, rinsed in heparin and placed into heparin-rinsed tubes. Samples were centrifuged to

obtain plasma which was stored in a freezer until cholinesterase assay was performed.

Epididymides were dissected free of fat, placed in 2 mls of phosphate-buffered saline (PBS) containing 1 g/l of glucose minced and set on a warming tray at 40° C for 15 minutes. The resulting sperm suspension was then pipetted off and placed into tubes containing 5  $\mu$ l of formaldehyde to kill the cells. A half milliliter of the suspension was diluted 1/10 with PBS and sperm concentration determined by hemocytometer count. Several drops of the undiluted sperm suspension were placed onto slides and the slides tilted to spread the fluid. Slides were air dried and stained with toluidine blue (1% in 1% sodium tetraborate). These slides were examined for sperm abnormalities. Numbers of abnormal sperm were counted blind (slides coded by an individual other than the experimenter). Sperm cells were considered to be abnormal if they exhibited atypical head size or shape, abnormal tail attachment or excessive tail coiling (Fig. 2) in accordance with the criteria of Wyrobek and Bruce (1975) and Soares, et al. (1979). However, in the present research, the determination of abnormality was generally more conservative than that of the above authors.

Testes from two treated and two control mice were removed immediately and processed for electron microscopy (EM) as specified below. For EM, testes were minced and immediately fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate, 0.003 M calcium chloride, pH 7) for 2 hours, in two changes of buffer alone (2 hours each). Testes were then post-fixed in osmium tetroxide (1%)

Fig. 2. Normal and abnormal sperm.

- A. A normal sperm head. Note hook tip and lobed nuclear area.
- B. A slightly abnormal tail attachment, a little too low toward base.
- C-L. Typical examples of abnormal sperm heads observed with pesticide treatment and in controls. (C-D = blunted heads; E-L = gross malformations)
- M-N. Sperm with abnormally coiled tails.

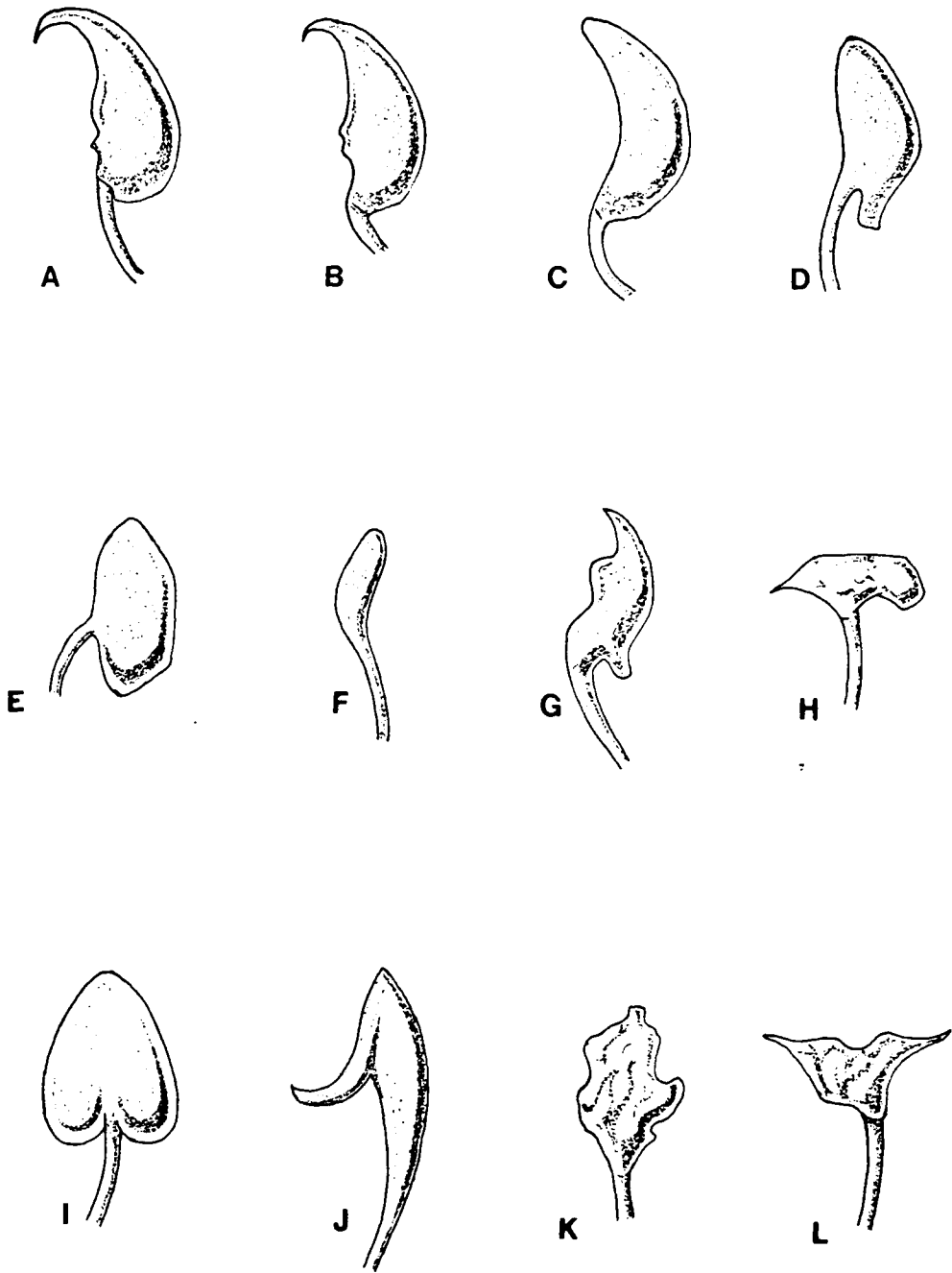


Fig. 2

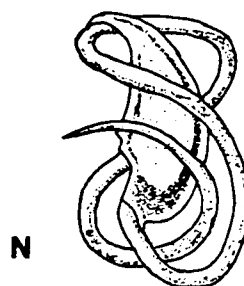
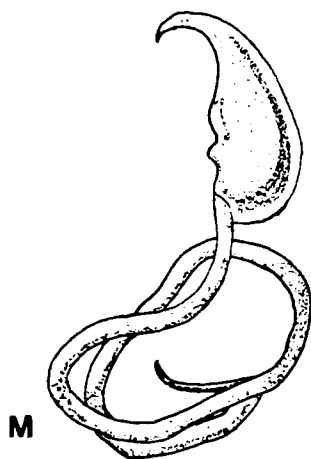


Fig. 2 (Continued)

in cacodylate buffer (0.1M) for 1 hour on ice, dehydrated in a graded series of ethanol and propylene oxide and embedded in Epon 812. Blocks were sectioned using a Reichert UM-2 ultramicrotome. Thin sections for electron microscopy were stained in uranyl acetate and lead citrate. Thick sections for light microscopy were stained with toluidine blue.

#### The Acetylcholinesterase Assay

In order to determine if carbaryl was absorbed, plasma cholinesterase assays were conducted. Since carbaryl is an inhibitor of acetylcholinesterase, lowered levels of this enzyme may be taken as an indicator of its presence. Plasma samples were analyzed using a Sigma Cholinesterase Kit (Sigma Technical Bulletin No. 420, 1977, details below). This assay is based on the procedure of Rappaport, et al. (1959) which employs the acid-base indicator m-nitrophenol to measure the acetic acid produced when cholinesterase hydrolyzes acetylcholine. Acetic acid lowers the pH in the tube causing a subsequent loss of color of the indicator. Background absorbance of the sample is eliminated by determining the difference between absorbances of the enzyme-inactivated blank and the sample.

The amount of plasma obtained was usually 0.04-0.05 ml per sample. For each plasma sample, two tubes were prepared, one a sample for assay and the other a blank. To the blank tube, 0.3 ml NaCl and 0.3 ml of plasma were added and the tube was heated to 60° C for 10 minutes to inactivate the enzyme, then centrifuged to remove turbidity. To the assay tube, 0.2 ml of both NaCl and plasma were added. Three mls of water, 2 mls nitrophenol solution and 0.2 ml of acetylcholine chloride solution were placed into each tube which was subsequently

incubated in a water bath at 25° C for 30 minutes. Absorbance of each tube was read at 420 nm using a Hitachi 100-10 spectrophotometer and the absorbance of the assay tube was subtracted from that of the control. This corrected absorbance of each sample was read against a standard curve to determine enzyme activity in Rappaport Units (Sigma Technical Bulletin No. 420, 1977). Fig. 3 represents a flow chart of this procedure.

#### Gas-Liquid Chromatography of Kepone and DBCP-Treated Tissues

In order to determine if kepone and DBCP were absorbed, tissue samples were analyzed for these pesticides by gas-liquid chromatography (GLC). Both pesticides are lipid-soluble and thus could be expected to be stored primarily in adipose tissue. Samples of liver, epididymal fat, testis, epididymis and kidney were removed and frozen as detailed earlier. After thawing, tissues were rinsed in physiological saline, blotted dry and weighed. The samples were homogenized in 2X 2 mls of 95% ethanol and the combined homogenates were centrifuged for 15 minutes at 2000 g. The alcohol-extracted pesticide-containing supernatant was diluted appropriately in hexane. GLC was performed using a Shimadzu GC-3BE Chromatograph. The liquid phase consisted of a 76.2 cm, 80-100 mesh Gas Chrom Q Supelco column coated with 3% SE 30; the carrier gas was nitrogen. The following conditions were maintained: oven temperature was 219° C while injection temperature and detector temperature were both 230° C; paper speed was 2.5 cm/min. Under these conditions, the kepone peak occurred at 3.8 cm from the hexane peak, indicating a retention time of 1.52 minutes and the DBCP peak appeared at 1.8 cm, a retention time

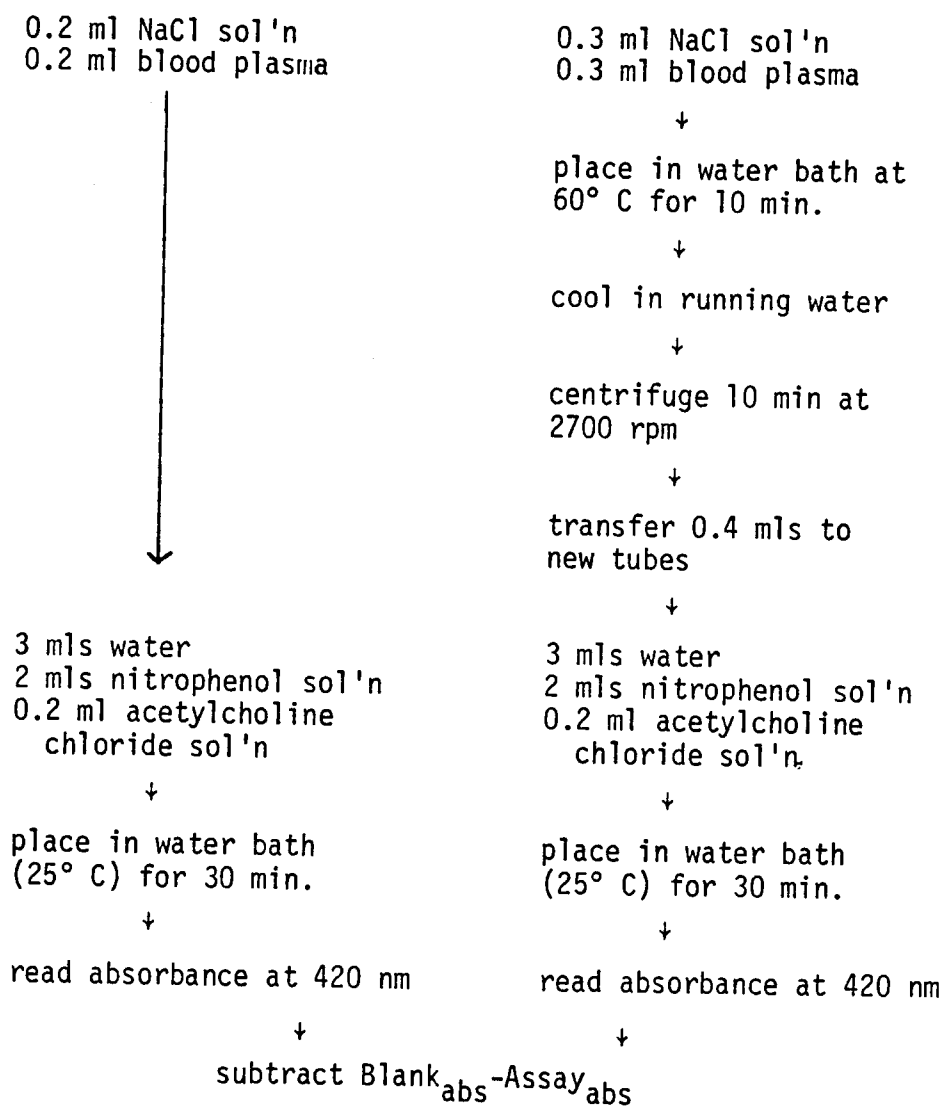


Fig. 3. Acetylcholinesterase assay procedure.

of 0.72 minutes. The size of each tissue peak was read against a standard curve and multiplied by the dilution factor to determine pesticide concentration.

### Statistical Analyses

The Student's t-test was used to test for significant differences between pesticide-treated mice and controls (body weight, liver weight, testis weight, sperm count and percent abnormal sperm). An analysis of variance was used to detect differences throughout treatment period. A chi square test was used to determine any differences in the presence of dense bodies in histological samples.

#### IV. RESULTS

Data were collected relevant to two biological questions: absorption and effect of pesticide. First, it was necessary to document pesticide absorption since an absence of effects on sperm-forming tissue with pesticide treatment could otherwise have been attributed to a paucity of toxicant in the body. Therefore, body and liver weights and tissue concentrations of pesticides were measured. Second, to determine pesticide effects on male reproductive function, the testes and sperm cells were examined for abnormalities of histology, sperm morphology and sperm number.

##### Documentation of Absorption

DBCP. Liver weights of DBCP-treated animals were consistently higher than those of controls during the entire experiment (significant at a  $p \geq 0.05$ ; Fig. 4 and Tables 1 and 2). This can be correlated with the fact that lipid-soluble pesticides are metabolized in the liver, which has an abundance of mixed function oxidases and similar enzymes involved in converting pesticides to more highly water-soluble forms which can be excreted. Body weight in DBCP-treated mice was unchanged from controls, suggesting that nutritional differences did not exist between the two groups. GLC analysis of DBCP-treated mice shows that there are residues of the pesticide in all tissues examined (Table 3). The highest level of pesticide was found in the epididymis (an average of 86.7 ng/mg of tissue), whereas the measured

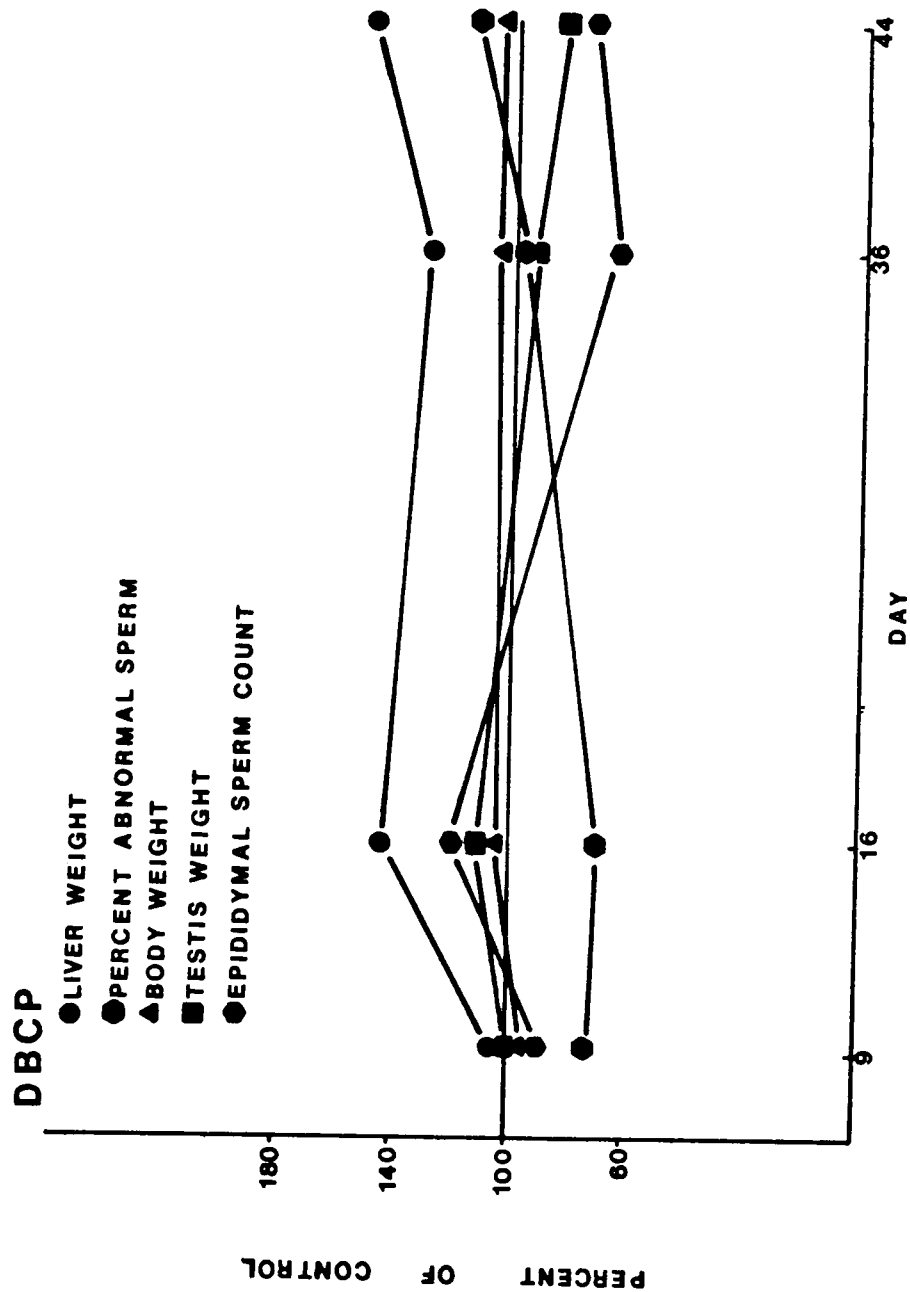


Fig. 4. DBCP effects. Effects of DBCP on liver weight, percent abnormal sperm, body weight, testis weight and epididymal sperm count. Expressed as percent of control values.

Table 1. Effects of pesticide treatment--DBCP.

| Parameter                             | Day                                   |       |       |       |
|---------------------------------------|---------------------------------------|-------|-------|-------|
|                                       | 9                                     | 16    | 36    | 44    |
| DBCP                                  |                                       |       |       |       |
| Body Wt.                              | No. Animals                           | 7     | 13    | 5     |
|                                       | Mean (g)                              | 37.13 | 39.29 | 39.22 |
| Liver Wt.                             | S.D.                                  | 2.66  | 3.17  | 4.94  |
|                                       | No. Animals                           | 7     | 13    | 5     |
| Testis Wt.                            | Mean (g)                              | 2.37  | 2.45  | 2.71  |
|                                       | S.D.                                  | 0.23  | 0.36  | 0.15  |
| Epid. Sp.                             | No. Animals                           | 7     | 9     | 5     |
|                                       | Mean (g)                              | 0.25  | 0.23  | 0.19  |
| Percent Abn. Sp.                      | S.D.                                  | 0.03  | 0.04  | 0.02  |
|                                       | No. Animals                           | 7     | 11    | 5     |
| Mean (X10 <sup>6</sup> /Cells/Sample) | Mean (X10 <sup>6</sup> /Cells/Sample) | 2.77  | 1.86  | 1.92  |
|                                       | S.D. (X10 <sup>6</sup> )              | 13.80 | 7.29  | 9.47  |
| Percent Abn. Sp.                      | No. Animals                           | 7     | 11    | 5     |
|                                       | Mean                                  | 16.27 | 15.22 | 18.08 |
| S.D.                                  | S.D.                                  | 8.74  | 4.98  | 2.94  |

Table 2. Effects of pesticide treatment--controls for DBCP and kepone.

| Parameter                 | Day                                  |       |       |       |       |
|---------------------------|--------------------------------------|-------|-------|-------|-------|
|                           | 9                                    | 16    | 36    | 44    | 57    |
| Controls for DBCP, Kepone |                                      |       |       |       |       |
| Body Wt.                  | No. Animals                          | 10    | 10    | 12    | 11    |
|                           | Mean (g)                             | 35.86 | 35.82 | 37.51 | 36.94 |
| Liver Wt.                 | S.D.                                 | 3.15  | 2.56  | 3.12  | 3.60  |
|                           |                                      |       |       |       | 5     |
| Testis Wt.                | No. Animals                          | 10    | 10    | 10    | 11    |
|                           | Mean (g)                             | 1.99  | 1.69  | 1.92  | 1.85  |
| Epid. Sp.                 | S.D.                                 | 0.24  | 0.38  | 0.47  | 0.29  |
|                           |                                      |       |       |       | 5     |
| Percent Abn. Sp.          | No. Animals                          | 8     | 8     | 10    | 9     |
|                           | Mean (g)                             | 0.20  | 0.23  | 0.25  | 0.22  |
| Epid. Sp.                 | S.D.                                 | 0.08  | 0.03  | 0.04  | 0.04  |
|                           |                                      |       |       |       | 5     |
| Percent Abn. Sp.          | No. Animals                          | 10    | 10    | 11    | 9     |
|                           | Mean (X10 <sup>7</sup> Cells/Sample) | 2.74  | 2.34  | 2.91  | 2.63  |
| Percent Abn. Sp.          | S.D.                                 | 8.60  | 7.56  | 13.20 | 12.10 |
|                           |                                      |       |       |       | 5     |
| Percent Abn. Sp.          | No. Animals                          | 10    | 10    | 11    | 10    |
|                           | Mean                                 | 19.57 | 24.28 | 15.47 | 16.04 |
| Percent Abn. Sp.          | S.D.                                 | 13.33 | 11.70 | 5.08  | 9.40  |
|                           |                                      |       |       |       | 5     |
| Percent Abn. Sp.          |                                      |       |       |       | 14.23 |
|                           |                                      |       |       |       | 5.34  |

Table 3. Concentrations of DBCP and kepone in body tissues.

| Organ                                 | Number of<br>Animals Examined | Average Pesticide<br>Concentration |
|---------------------------------------|-------------------------------|------------------------------------|
| <u>Dibromochloropropane Treatment</u> |                               |                                    |
| Liver                                 | 5                             | 10.4 $\pm$ 1.1 ng/mg               |
| Fat                                   | 5                             | 12.7 $\pm$ 2.3                     |
| Kidney                                | 5                             | 25.5 $\pm$ 3.2                     |
| Epididymis                            | 2                             | 86.7 $\pm$ 0.6                     |
| Testis                                | 5                             | 2.1 $\pm$ 0.4                      |
| <u>Kepone Treatment</u>               |                               |                                    |
| Liver                                 | 3                             | 139.7 $\pm$ 12.7 pg/mg             |
| Fat                                   | 4                             | 76.7 $\pm$ 11.4                    |
| Kidney                                | 3                             | 33.1 $\pm$ 5.9                     |
| Epididymis                            | 2                             | 9.5 $\pm$ 0.7                      |
| Testis                                | 2                             | 14.7 $\pm$ 1.1                     |

liver concentration was a mean of 10.39 ng/mg. The kidney also accumulated pesticide at an average of 10.39 ng/mg. Testis showed the least accumulation with a mean of only 2.06 ng/mg. Liver weight was increased by treatment but the presence of high levels in other tissues probably indicates that the liver was unable to process all of the absorbed pesticide. No DBCP residues were detected in tissues of control mice.

Kepone. There was no change in body weights of kepone-treated animals, suggesting that there were no nutritional differences between treated mice and controls. Liver weight increased in treated animals early in the experiment (by day 16) and was significantly elevated ( $p \geq 0.01$ ) above those of controls (Fig. 5 and Table 4). Visual examination of livers revealed very large but normal appearing organs. Liver weight continued to rise even after cessation of treatment. GLC analysis of tissues show that kepone is most concentrated in the liver (mean of 139.7 pg/mg of tissue). The epididymides had the lowest concentration (mean of 9.5 pg/mg). Kepone was present in every tissue of the treated animals which was tested (Table 3). Control tissues had no kepone residues.

Carbaryl. There was no significant change in body or liver weights of carbaryl-treated mice (Fig. 6, Tables 5 and 6). Analysis of plasma samples for acetylcholinesterase was done on 12 different carbaryl-treated mice and 10 control mice over a period of 21 days in which mice were killed (Table 7). Again, no significant difference was observed between treated and control mice. This result is not

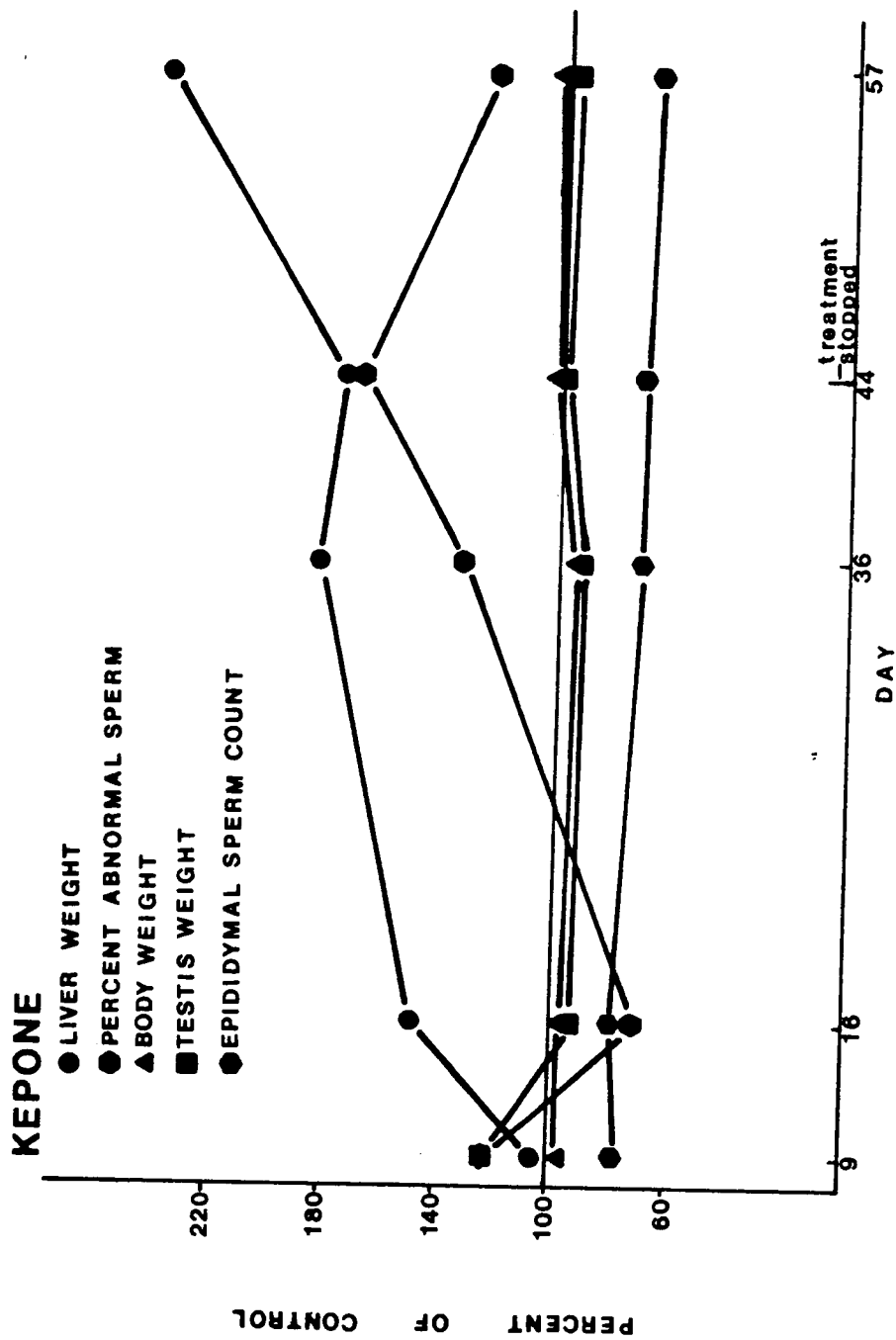


Fig. 5. Kepone effects. Effects of kepone on liver weight, percent abnormal sperm, body weight, testis weight and epididymal sperm count. Expressed as percent of control values.

Table 4. Effects of pesticide treatment--kepone.

| Parameter        | Day                                  |       |       |       |
|------------------|--------------------------------------|-------|-------|-------|
|                  | 9                                    | 16    | 36    | 57    |
| Kepone           |                                      |       |       |       |
| Body Wt.         | No. Animals                          | 9     | 4     | 4     |
|                  | Mean (g)                             | 34.92 | 35.89 | 38.61 |
| Liver Wt.        | S.D.                                 | 2.58  | 3.40  | 3.04  |
|                  | No. Animals                          | 9     | 4     | 4     |
|                  | Mean (g)                             | 2.48  | 3.59  | 3.29  |
| Testis Wt.       | S.D.                                 | 0.17  | 0.44  | 0.59  |
|                  | No. Animals                          | 7     | 3     | 4     |
|                  | Mean (g)                             | 0.24  | 0.23  | 0.22  |
| Epid. Sp.        | S.D.                                 | 0.02  | 0.03  | 0.05  |
|                  | No. Animals                          | 7     | 4     | 4     |
| Percent Abn. Sp. | Mean (X10 <sup>7</sup> Cells/Sample) | 2.04  | 2.05  | 1.89  |
|                  | S.D. (X10 <sup>6</sup> )             | 9.11  | 14.80 | 7.48  |
| Percent Abn. Sp. | No. Animals                          | 7     | 11    | 8     |
|                  | Mean                                 | 23.64 | 20.79 | 27.74 |
|                  | S.D.                                 | 6.83  | 6.44  | 10.53 |
|                  |                                      |       |       | 18.97 |
|                  |                                      |       |       | 4.57  |

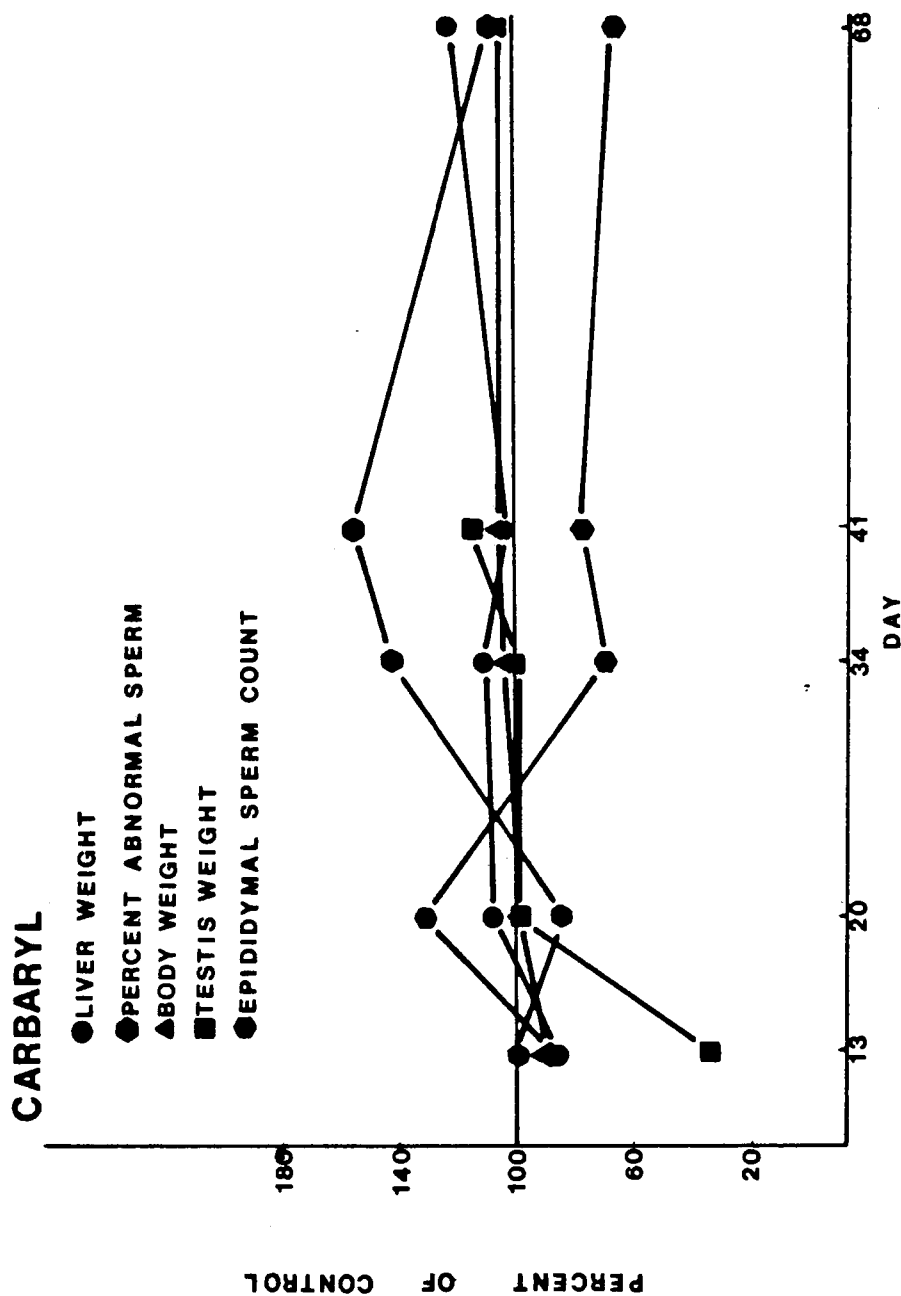


Fig. 6. Carbaryl effects. Effects of carbaryl on liver weight, percent abnormal sperm, body weight, testis weight and epididymal sperm count. Expressed as percent of the control values.

Table 5. Effects of pesticide treatment--carbaryl.

| Parameter        | Day         |       |       |       |
|------------------|-------------|-------|-------|-------|
|                  | 9           | 16    | 36    | 57    |
| Carbaryl         |             |       |       |       |
| Body Wt.         | No. Animals | 6     | 5     | 4     |
|                  | Mean (g)    | 37.07 | 38.31 | 39.90 |
| Liver Wt.        | S.D.        | 5.99  | 2.26  | 4.31  |
|                  | No. Animals | 6     | 5     | 4     |
| Testis Wt.       | Mean (g)    | 1.88  | 1.90  | 2.17  |
|                  | S.D.        | 0.27  | 0.29  | 0.20  |
| Epid. Sp.        | No. Animals | 6     | 6     | 6     |
|                  | Mean (g)    | 0.16  | 0.23  | 0.24  |
| Percent Abn. Sp. | S.D.        | 0.01  | 0.04  | 0.03  |
|                  | No. Animals | 6     | 4     | 5     |
| Percent Abn. Sp. | Mean        | 1.22  | 0.85  | 2.21  |
|                  | S.D.        | 6.38  | 3.14  | 6.95  |
| Percent Abn. Sp. | No. Animals | 6     | 4     | 5     |
|                  | Mean        | 8.10  | 17.29 | 14.72 |
|                  | S.D.        | 1.98  | 5.31  | 10.77 |

Table 6. Effects of pesticide treatment--controls for carbaryl.

| Parameter             | Day                                  |       |       |       |       |       |
|-----------------------|--------------------------------------|-------|-------|-------|-------|-------|
|                       | 13                                   | 20    | 34    | 41    | 68    |       |
| Controls for Carbaryl |                                      |       |       |       |       |       |
| Body Wt.              | No. Animals                          | 6     | 6     | 5     | 4     | 1     |
|                       | Mean (g)                             | 38.82 | 36.86 | 37.20 | 37.16 | 41.83 |
|                       | S.D.                                 | 3.87  | 5.57  | 4.29  | 4.74  | -     |
| Liver Wt.             | No. Animals                          | 6     | 6     | 5     | 4     | 1     |
|                       | Mean (g)                             | 2.28  | 1.72  | 1.71  | 2.12  | 2.27  |
|                       | S.D.                                 | 0.53  | 0.44  | 0.39  | 0.10  | -     |
| Testis Wt.            | No. Animals                          | 2     | 6     | 6     | 4     | -     |
|                       | Mean (g)                             | 0.45  | 0.26  | 0.23  | 0.21  | -     |
|                       | S.D.                                 | 0.35  | 0.04  | 0.02  | 0.04  | -     |
| Epid. Sp.             | No. Animals                          | 6     | 6     | 5     | 5     | 1     |
|                       | Mean (X10 <sup>7</sup> Cells/Sample) | 1.47  | 1.38  | 1.24  | 2.82  | 2.92  |
|                       | S.D. (X10 <sup>6</sup> )             | 6.93  | 5.35  | 4.52  | 7.71  | -     |
| Percent Abn. Sp.      | No. Animals                          | 6     | 6     | 5     | 5     | 1     |
|                       | Mean                                 | 8.1   | 10.18 | 12.16 | 9.49  | 10.40 |
|                       | S.D.                                 | 1.68  | 6.03  | 5.33  | 5.70  | -     |

Table 7. Plasma acetylcholinesterase activity.

| Day | Mean Cholinesterase<br>(Rappaport Units) | S.D. | No. Animals |
|-----|------------------------------------------|------|-------------|
| 20  | 0.07                                     | 0.03 | 3           |
|     | 0.07 (control)                           | 0.02 | 2           |
| 34  | 0.10                                     | 0.02 | 2           |
|     | 0.13 (control)                           | 0.00 | 2           |
| 41  | 0.09                                     | 0.08 | 7           |
|     | 0.11 (control)                           | 0.06 | 6           |

Source: Sigma Bulletin, No. 420, 1977.

surprising since carbaryl is readily metabolized. It is possible that effects of the pesticides on plasma acetylcholinesterase were eliminated by the time plasma samples were prepared. The data examined to document pesticide absorption indicate that kepone and DBCP were absorbed but they fail to prepare absorption of carbaryl.

#### Effects on the Male Reproductive System

In this research, effects of pesticides of the male reproductive system were studied relative to testicular weight, percentage of abnormal cells, epididymal sperm numbers and testicular histology.

DBCP. Testis weight of DBCP-fed animals did not vary significantly relative to controls until day 44 (Fig. 4, page 48; Tables 1 and 2, pages 49 and 50) by which time testis weight of DBCP-treated mice was significantly lower ( $p \geq 0.05$ ) than that of controls, indicating possible pesticide-induced testicular damage. Furthermore, analysis of variance of testis weight revealed no differences over treatment period. Abnormal cells in DBCP-treated mice are expressed as percent of controls. Sperm morphology appeared to be highly variable in all samples, including controls. Initially, percent abnormal cells were lower in DBCP-treated mice than in controls but this difference was not significant. The percentage of abnormal cells was equivalent to that of controls during the entire experiment (Fig. 4; Tables 1 and 2). Numbers of epididymal sperm cells were equal to those of controls until day 36 at which time the number of epididymal sperm cells is significantly lower ( $p \geq 0.05$ ) in pesticide-fed mice than in controls. However, by day 44, the number does not significantly differ from that

of controls (Fig. 4, page 48). Analysis of variance reveals no significant trend to changes in epididymal sperm cells during treatment.

Since testis weight dropped to a value significantly different from controls by day 44 and epididymal sperm count did the same by day 36 (although day 44 is not different from controls), it is assumed that testis weight is being affected by pesticide treatment. However, to determine this with certainty, a longer treatment period would be necessary. Likewise, the t-tests indicate that epididymal sperm may be affected but again, this conclusion is uncertain. The analysis of variance test was conducted to detect a change in each parameter throughout DBCP treatment without relating the data to controls. Analysis of variance fails to indicate any substantial change in these parameters during experimentation, and significance is found only relative to controls.

Testes of pesticide treated animals were observed with light and transmission electron microscopy. The only observed anomaly occurring frequently enough for the collection of meaningful quantitative data were densely-staining bodies found in the germinal epithelium (Fig. 7). These bodies appear to be abnormally-formed or degenerating spermatogenic cells. They have a dense inner core with a vacuolated periphery which stains lighter than the core. This lighter staining area also is sometimes observed to contain portions of sperm cells, particularly of outer dense fibers. These bodies were observed in both pesticide-treated and in control mice fed corn oil. Of 20 blocks examined from DBCP-treated mice over the entire experiment up to day 44, eight were

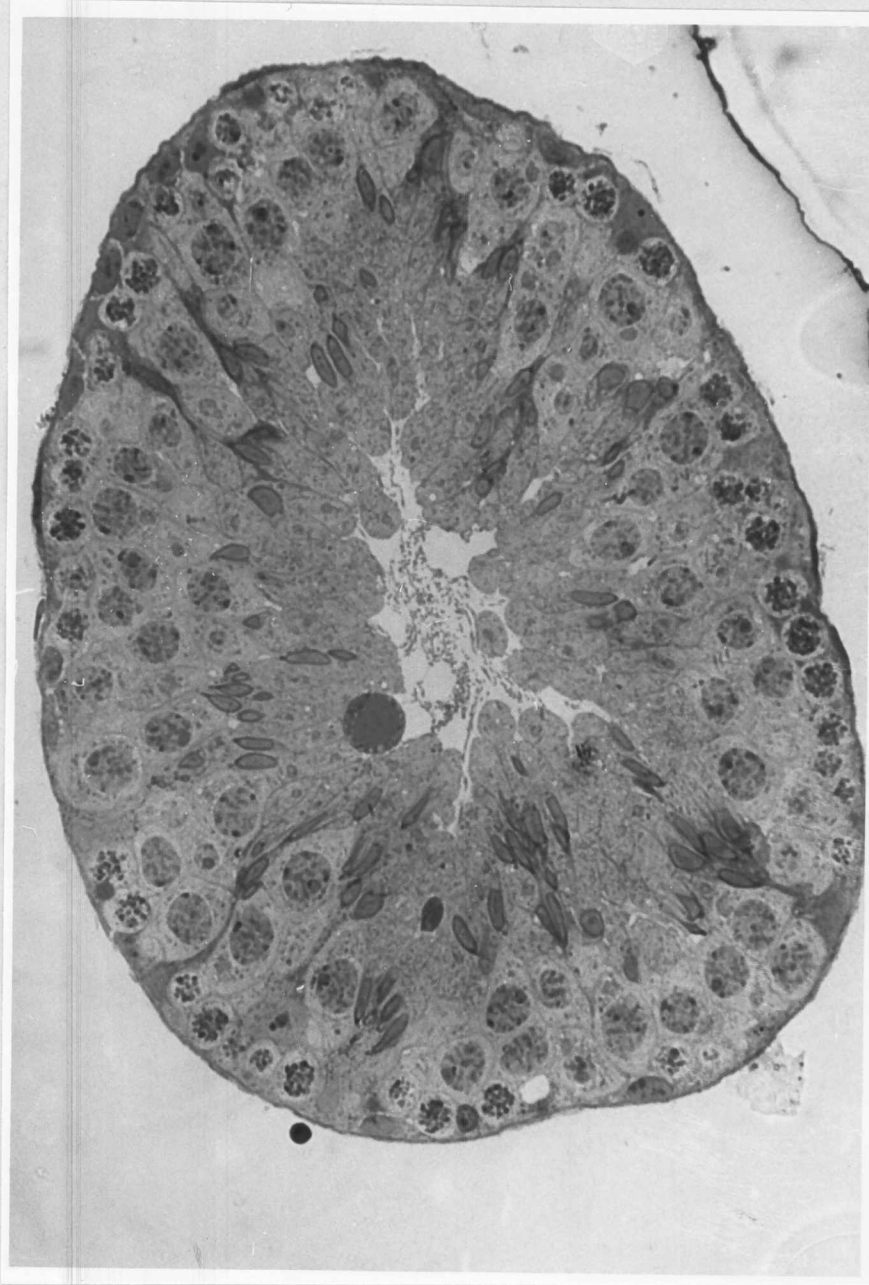


Fig. 7. Photomicrograph of a dense body in the germinal epithelium. X250. Note dense core and location within tubule lumen.

found to contain dense bodies (40%) (Table 8). Of 46 blocks from control mice over the same period, 19 had dense bodies (41%) (Table 8). The occurrence of dense bodies in DBCP-treated mice over the entire course of treatment was 97% that of control value, an insignificant deviation from control ( $p \geq 0.05$ ).

Kepone. Testis weights were unchanged with kepone treatment and did not vary at any time during experimentation (Fig. 5, page 53; Tables 2 and 4, pages 50 and 54, respectively). However, a sharp significant ( $p \geq 0.01$ ) rise in abnormal sperm cells occurred by day 36 of the treatment period. However, an analysis of variance indicates no difference over the treatment period with this parameter. After cessation of treatment, percent of abnormal sperm returned to normal within 13 days (Fig. 5; Tables 2 and 4). Epididymal sperm number did not vary significantly from controls at any time. Histologically, the only anomaly of the testis was the presence of dense bodies which were observed in both treated and control mice. Of 18 blocks from kepone-treated mice during the course of treatment up to day 44, 9 (50%) had dense bodies. Of 46 blocks from control animals during the treatment period, 19 (41%) had dense bodies (Table 8). A Chi Square test indicated that the occurrence of dense bodies was not significant from that of controls.

Carbaryl. At no time during experimentation was there a statistically significant difference in treated mice relative to controls with regard to testicular weight, percent abnormal sperm or epididymal sperm counts (Fig. 6). Histologically, no abnormalities

Table 8. Testicular histology (numbers of blocks with dense bodies).

| Day | Treatment                         | Total Blocks<br>Examined | No. Blocks<br>With D.B. |
|-----|-----------------------------------|--------------------------|-------------------------|
| 9   | DBCP                              | 5                        | 0                       |
|     | Control                           | 11                       | 1                       |
| 16  | DBCP                              | 6                        | 4                       |
|     | Control                           | 15                       | 8                       |
| 36  | DBCP                              | 5                        | 1                       |
|     | Control                           | 8                        | 4                       |
| 44  | DBCP                              | 4                        | 3                       |
|     | Control                           | 12                       | 6                       |
| 9   | Kepone                            | 4                        | 1                       |
|     | Control                           | 11                       | 1                       |
| 16  | Kepone                            | 5                        | 3                       |
|     | Control                           | 15                       | 8                       |
| 36  | Kepone                            | 5                        | 3                       |
|     | Control                           | 8                        | 4                       |
| 44  | Kepone                            | 4                        | 2                       |
|     | Control                           | 12                       | 6                       |
| 44  | Kepone<br>(treatment<br>stopped)  | 6                        | 4                       |
|     | Control<br>(treatment<br>stopped) | 6                        | 4                       |
| 13  | Carbaryl                          | 4                        | 1                       |
|     | Control                           | 4                        | 1                       |
| 20  | Carbaryl                          | 5                        | 5                       |
|     | Control                           | 4                        | 0                       |
| 34  | Carbaryl                          | 4                        | 4                       |
|     | Control                           | 4                        | 4                       |
| 41  | Carbaryl                          | 4                        | 4                       |
|     | Control                           | 4                        | 0                       |

were observed in the testes of treated or control mice other than the presence of the dense bodies. Of 17 blocks from carbaryl-treated mice, 14 (82%) had dense bodies. Of 16 control blocks during the same period, only 5 or 31% had dense bodies (Table 8). Although dense bodies in treatments were over twice as abundant as in controls, this difference was statistically not significant.

## V. DISCUSSION

These data document absorption of pesticide (liver and body weight and tissue analysis of pesticide residues). They also demonstrate the effects of these pesticides on specific parameters of male reproduction (testis weight, percent of abnormal sperm, epididymal sperm count and testicular histology) and these criteria are presumed to be valid indicators of damage to the male reproductive system.

### Documentation of Absorption

Tests for absorption demonstrated that DBCP and kepone were absorbed but failed to prove absorption of carbaryl. With both DBCP and kepone treatment, significantly elevated liver weights were observed and pesticide residues were found in all tissues examined. In carbaryl-treated mice, however, there was no change in liver or body weights relative to controls and analysis of plasma samples for cholinesterase activity demonstrated no effects on this enzyme in plasma. The high levels of DBCP and kepone in livers of treated mice and the elevated weights of these organs were not unexpected findings. The liver is the major location of the metabolism of DBCP to a more highly water soluble form for excretion (Wilkinson, 1976). Kepone, however, is almost entirely unaltered by these liver enzymes. Instead, the liver serves to eliminate this substance into the intestine in the bile although it is reabsorbed by the intestine (Cohn, et al., 1978). The presence of pesticide residues in other organs is possibly due to the inability of the liver to process such

large systemic concentrations of the pesticides at any one time and to the fact that virtually every tissue examined is known to contain some of the same types of enzymes for metabolism of fat-soluble molecules as the liver. Also, pesticide was found in all organs examined possibly because the fatty tissue is inadequate to store the large amounts to which the animals were subjected. The lack of effects of carbaryl on body and liver weights was also not surprising. Carbaryl is readily oxidized by liver enzymes and would not be expected to accumulate in tissues as would the other pesticides. This may also be the reason why no effects were found on plasma cholinesterase activity. Since plasma samples were prepared 1 or 2 days after treatment with carbaryl, it is possible that the effects of the pesticide on cholinesterase had been ameliorated. Another possibility is that the freezing of plasma samples prior to analysis by spectrophotometry may have inactivated the enzyme to some degree. However, acetylcholinesterase in frozen samples is stable for at least several months according to Sigma Bulletin No. 420 (1977).

Another test for absorption which was not done extensively in the present research is the examination of histology of non-reproductive structures. Dikshith and Datta (1972b) examined liver of rats treated with DDT and parathion and found hypertrophy and vacuolization of liver cells. Examination of a few stomachs and livers in the present study (not enough were studied to quantify the data) revealed similar changes in a few livers of DBCP-treated mice. However, I believe that the criteria used in this research are adequate to document absorption. Tissue analysis of pesticide residues is a

direct method of proving absorption and liver weight reflects induction of enzymes involved in pesticide metabolism. The tests did fail to prove absorption with carbaryl treatment, however. Perhaps addition of other tests (histological examination of non-reproductive tissues and examination of body tissues other than plasma for cholinesterase activity) should have been used to complement the criteria used in this research. However, it is possible that absorption of carbaryl was not indicated because there was no carbaryl in the tissues. Either the pesticide was not absorbed or its effects were eliminated by freezing of plasma and experimental manipulation of tissues.

#### Effects on Parameters of Male Reproductive Function

This study indicates that the pesticides DBCP and kepone have effects on male reproductive function but effects of carbaryl are not indicated. DBCP was shown possibly to lower epididymal sperm count by day 36. These data corroborate the findings of Whorton, et al. (1977) who describe oligospermia in men exposed to DBCP in a pesticide plant. Kepone caused a rise in percent of abnormal sperm by day 36 with recovery by 13 days after treatment was stopped. A one-factor analysis of variance indicates that the effect of kepone on percent abnormal sperm was significant but the correlation coefficient did not indicate a specific trend. A variety of sperm abnormalities was found in all treated and control animals and is illustrated in Fig. 2, page 40. The percentage of abnormalities was increased significantly only in kepone-treated mice. There were no unique abnormalities found in this study either in control or treated animals. All of the morphological

variants found here have been described in various mutant mice (Hillman and Nadijcka, 1978a and b; Handel and Dawson, 1981), in X-irradiated mice (Bruce, et al., 1974) and in mice treated with various noxious chemicals (Wyrobeck and Bruce, 1975; Soares, et al., 1979). How these abnormalities arise in normal, mutant, and treated animals is not known. The fact that the same kinds of morphological abnormalities are observed in normal animals argues against interference with the hypothalamic-pituitary-gonad endocrine axis by either mutation or chemicals. The relative ease of inducing the sperm defects suggests that specific and direct genetic effects on sperm morphogenesis are an unlikely cause for these abnormalities. It may well be that the morphogenetic processes of spermiogenesis are quite susceptible to epigenetic interference, perhaps resulting from a disturbance in the local environment provided by Sertoli cells. Pesticides and other noxious chemicals may simply mimic and enhance normally occurring changes in the testicular environment. What is not known, however, is whether morphologically abnormal sperm cells are also functionally abnormal. Analysis of fertilization in vivo and in vitro, and of subsequent development would be necessary to resolve this issue.

In addition to sperm abnormalities, testis weight and morphology were assessed. DBCP lowered testis weight by day 44. The lowered testis weights may be indicative of testicular atrophy, frequently associated with a loss of germinal epithelium (Dikshith and Datta, 1972b, McFarland and Lacy, 1969). However, in the present research, atrophy was not evident when testes were examined with microscopy.

Decrease in testis weight was only significant at the end of the experiment. Perhaps a longer experimentation period would have produced a more marked testicular atrophy. The drop in epididymal sperm number observed after DBCP treatment would indicate that fewer sperm are being produced under the influence of the pesticide. Since epididymal sperm count was lowered significantly by day 36 but was not significantly different by day 44, it is not possible to state with certainty that DBCP had an effect on that parameter.

This study has not demonstrated that carbaryl affects male reproductive function. Either the parameters employed were inadequate to detect the effects or there were actually no effects to be seen. Relevant to this is the possibility that carbaryl was not absorbed by the treated mice. The literature is inconclusive regarding the effects of carbaryl on reproduction. Carbaryl has been reported to cause a decrease in sperm motility and changes in enzymatic activity of the testis (Orlova and Zhalbe, 1968), two parameters not studied in the present research. Vashakidze, et al. (1966) reported that large doses of the insecticide over prolonged periods inhibited spermatogenesis in rats. However, semen samples of individuals working in a carbaryl plant revealed no effects on sperm count (Whorton, et al., 1979) and male and female rats fed carbaryl over three generations exhibited no effects on fertility, gestation, viability of young or lactation (Weil, et al., 1972). It is difficult to compare the present research with that of the above authors since the experiments and parameters used were not equivalent.

While the parameters employed in this research reflect specific effects of pesticides on the male reproductive system, they do not indicate whether the affected individuals are fertile. Even with an increase in morphologically abnormal sperm cells and a decrease in sperm count, the semen could well be adequate for fertilization. Abnormal cells were found abundantly in controls as well as in pesticide-treated mice and sperm count varied from  $0.85 \times 10^7$  to  $2.92 \times 10^7$  cells (Tables 1, 2, 4, 5, and 6, pages 47, 48, 52, 54, and 55, respectively). Microscopy revealed normal germinal epithelium in testes of all weights. It is conceivable that pesticide-treated mice would be unaffected in mating success. In the final analysis, the most important criterion of effects of environmental agents on the reproductive system is the potential for treated and control animals to mate and produce normal sized, viable litters. Therefore, the data presented here would be particularly valuable if coupled with mating studies, in vitro fertilization studies or both. DBCP (Anonymous, 1977b; Whorton, et al., 1977) and kepone (Strett and Boss, 1977) have been shown to cause sterility in workers chronically exposed to these pesticides, but these individuals received unusually high doses of pesticide over a long period of time. Dose-response data, derived from animal studies would be particularly useful data.

### Testicular Dense Bodies

Examination of testicular histology revealed no observable qualitative differences between treatment or controls or among the specific pesticide treatments. The only observed anomaly was the presence of dense bodies in the germinal epithelium in pesticide and

corn oil fed mice. Normal Sertoli cells and all stages of germinal epithelium were found in all mice examined. The dense bodies were found exclusively in or on the periphery of the lumen of the tubules suggesting one of two things: First, the later spermatocytes and spermatids may be more sensitive to damage than earlier stages or damage is not manifest until the later stages. Second, it is possible that a constituent of the treatment is concentrated in the lumen resulting in exposure primarily of the cells bordering the lumen. It is interesting that dense bodies were not found in four mice examined which had not been involved in the experimentation and which had thus not received either pesticide or corn oil. These were fixed in the same manner as the experimental specimens. Examination of controls of other strains and examination of the literature failed to verify that these dense bodies are routinely found in the germinal epithelium.

The dense bodies were found in all pesticide treatments in this research and in the corn-oil fed controls and their frequency in treated mice was not significantly different from that of controls. These data raise an interesting question. Why were dense bodies, which appear to be malformed or degenerating spermatogenic cells found in the controls? There are three possible explanations. First, were the dense bodies fixation artifacts? Examination of testes of four mice not given either pesticide or corn oil failed to reveal any dense bodies although these were fixed in the same manner as the testes in these experiments. Furthermore, other investigators (Dikshith and Datta, 1972a and b; Datta and Dikshith, 1973; Eroschenko and Wilson, 1975; Eroschenko, 1978) have reported similar bodies in pesticide-

treated rodents, indicating that they result from treatment. The second possibility is that dense bodies arise spontaneously in a certain percentage of cells in normal sperm-forming tissue. The literature offers no indication that this occurs. Again, referring to the small sample of four untreated mice and examination of control mice of other strains, no dense bodies were found.

The third possible explanation for the occurrence of dense bodies in control mice is that some constituent of the corn oil itself other than the pesticide was responsible for inducing the dense bodies. This appears to be the most likely explanation. Refined corn oil has been reported to have estrogen-like activity when fed to mice. Booth, et al. (1960) fed several types of vegetable oils to female mice and nearly all elicited an increase in uterine weight. Five female mice fed 10% corn oil in the diet had a mean uterine weight of 15.8 mg as compared with a uterine weight of 9.5 mg for five mice fed the identical diet without corn oil. Four mice injected subcutaneously with 0.8 mls of corn oil had a mean uterine weight of 18.2 mg as compared with four saline-injected controls which had a uterine weight of 10.3 mg. Four uninjected controls had a mean uterine weight of 9.9 mg. During proestrus, the interplay of many hormones is responsible for the observed increase in uterine weight in preparation for the estrus stage but the female sex hormones, most notably estrogen, are primarily associated with this function. Furthermore, it is widely known among endocrinologists that certain plants manufacture and store estrogen-like compounds, lending credence to Booth's et al. (1960) observations. The antispermatogenic effects of the estrogens

result from the alteration of the hormonal environment of androgen-sensitive cells.

Since corn oil contains estrogen-like compounds, why then was it employed in this study? All three pesticides are more highly soluble in oil than in water and corn oil is the least toxic oil for administration of toxicants. Also, most of the other vegetable oils tested by Booth, et al. (1960) exhibited estrogenic activity equal to or greater than the activity found in corn oil. Thus, corn oil was chosen as the best vehicle for use in these experiments.

#### Suggestions for Further Study

The results of this research indicate specific effects of these three pesticides on certain parameters of male reproductive function. The data do not elucidate the mechanisms whereby these effects arise. It has been suggested that the effects of pesticides are indirect, perhaps acting on the hormonal environment of androgen-sensitive cells (Shtenberg and Rybakova, 1969). Kepone has been demonstrated to have an affinity for the estrogen receptor thereby exerting estrogen-like effects. Hammond, et al. (1979) did estrogen-receptor binding assays in which kepone competed with the synthetic estrogen diethylstilbestrol (DES) for the estrogen receptor. Kepone had an affinity for the receptor only 0.02 to 0.04% that of the estrogens but since kepone is not metabolized, it is available for receptor binding longer than estrogens, thereby enhancing its estrogenic effects. A further indication of an endocrine mechanism is the fact that workers exposed to DBCP had elevated levels of serum FSH and LH (Whorton, et al., 1977). However, workers exposed to carbaryl exhibited no such

gonadotropin fluctuations (Whorton, et al., 1979). The present research should be extended by measuring the affinity of each pesticide for the estrogen-receptor and measuring the effects of each pesticide on gonadotropin and testosterone levels in mouse serum. The discovery that kepone binds the estrogen-receptor indicates that it may well act by influencing the hormonal environment of androgen-sensitive cells.

However, the fact that kepone has an affinity for estrogen receptor does not preclude the possibility that it and other pesticides also act by another mechanism, such as general cellular degeneration. Therefore, in future research, examination of the histology on non-reproductive tissues such as liver, kidney and stomach would aid in determining the mechanism of action of these pesticides. The possible effects of pesticides on function of spermatozoa has been alluded to in this dissertation. Any future research should include long-term mating and reproductive studies on treated males coupled with assessment of sperm function utilizing in vitro fertilization tests (Brackett, 1978). Only by such studies can the ability of pesticide-treated sperm to fertilize and initiate normal development be determined.

### Human Implications

A major significance of many toxicological studies is the ability to predict the effects of a toxicant on humans. For such investigations, "model" mammalian systems, typically rodents, are employed. This is because toxicological experimentation on human subjects is greatly restricted on moral, religious and legal grounds. However, extrapolation of data from model mammalian systems to humans

must be made with great caution. Experimental findings in non-human species may or may not apply to humans. According to Rao, et al. (1981),

There is no conclusive evidence that the chemicals which induce adverse reproductive effects in animals actually represent a hazard to humans under normal conditions of use and exposure.

However, as these authors point out, dose-response data regarding effects of toxicants on reproductive function, as generated by animal studies, can be used to extrapolate an approximate safety factor for human exposure. Such extrapolations regarding safe doses and specific effects are presently the best tool which the toxicologist has to predict effects in humans since relatively little human data may be available.

The significant implication of toxicological data for humans is that exposure to these and probably other pesticides as well offers potential undesirable effects other than the acute toxicity associated with each pesticide. Much less is known of the chronic effects of pesticides. The present study has, I hope, contributed in a small way to our knowledge of the chronic effects of pesticides and their potential dangers to humans. Results of this and other studies bear out the fact that the invention of synthetic pesticides has interjected a dangerous and permanent factor into human ecology which may well have far reaching effects in the future. As we increase use of synthetic pesticides, and accumulate these substances in the environment and in our tissues, even small effects on reproductive function may prove significant and fertility of humans and other species may decline or may presently be declining and endanger perpetuation of many life forms on the earth, including humans. It is my personal opinion that the

dangers posed by synthetic pesticides are so great that we would be safer had they never been invented. We are reminded of Rachel Carson's timely caveat that "every human being is now subjected to contact with dangerous chemicals from the moment of conception until death" (Silent Spring, 1962).

## VI. CONCLUSION

Experiments were conducted to determine if the pesticides DBCP, kepone and carbaryl affect parameters of male reproduction in the mouse. It was found that DBCP at a dosage of 150 mg/kg every 3 days for 44 days caused a decrease in testis weight, indicating testicular atrophy by day 44 and a possible reduction in epididymal sperm count by day 36. Abnormal sperm numbers and histology were unaffected. Kepone, given at a dosage of 10 mg/kg every 3 days for 44 days caused an increase in abnormal sperm by day 36 while epididymal sperm counts, testicular weight and histology were unchanged. At a dosage of 150 mg/kg, carbaryl given every 2 days for up to 68 days did not have any effects on histology of the seminiferous epithelium, epididymal sperm counts, abnormal sperm numbers or testicular weight. Corn oil given in large doses induces dense bodies which appear to be abnormal or degenerating spermatogenic cells and may be related to the presence of plant estrogens in the oil.

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## VITA

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