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EFFECTS OF LONG-TERM LOW-DOSE EXPOSURE TO ORGANOPHOSPHATES ON MOUSE SPERM QUALITY

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EFFECTS OF LONG-TERM LOW-DOSE EXPOSURE TO ORGANOPHOSPHATES ON MOUSE SPERM QUALITY

by

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ABSTRACT

EFFECTS OF LONG-TERM LOW-DOSE EXPOSURE TO ORGANOPHOSPHATES ON MOUSE SPERM QUALITY

By

Michael David Oswalt

Effects of long-term dietary exposure to organophosphorus insecticides on sperm quality were studied. Male mice were fed a mixture of organophosphates consisting of diazinon:dimethoate: malathion:parathion = 16:8:250:4 at the dose of 0, 0.4, 4.0, or 40.0 μg/kg BW/day from 3 weeks to 3 months of age, and 0, 0.15, 1.5, or 15 μg/kg BW/day from 3 months to 8 months of age. The lowest exposure levels were designed to mimic the infant and the adult human dietary exposure. Sperm were examined for motility, capacitation and the acrosome reaction and fertilizing ability. The brain acetylcholinesterase activity was also measured. No effect on sperm motility and the progression through capacitation and the acrosome reaction was observed. Nor was there any effect on in vitro fertilizing ability of the sperm. The brain acetylcholinesterase activity showed an inhibitory response to the organophosphate exposure.

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LIST OF ABBREVIATIONS

Ach	Acetylcholine
AchE	Acetylcholinesterase
ADI	Allowable Daily Intake
BMOC-3	Brinster's Medium for Oocyte Culture
ChAT	Choline Acetyltransferase
CTC	Chlortetracycline
DFP	Diisopropyl Fluorophosphate
DB	Dark Banded
F	Fresh
FDA	Food and Drug Administration
hCG	Human Chorionic Gonadotropin
LB	Light Banded
NAD	Nicotinamide Adenine Dinucleotide
OP	Organophosphate
PBS	Phosphate Buffered Saline
PMSG	Pregnant Mare Serum Gonadotropin
TEPP	Tetraethyl Pyrophosphate
ZP1	Zona Protein 1
ZP2	Zona Protein 2
ZP3	Zona Protein 3

CHAPTER I

INTRODUCTION

Reproduction is one of the most sensitive biological systems to environmental insults. Its proper functioning is essential for the propagation of species. The process of gamete production and eventual fusion and syngamy required for proper fertilization reveals a multitude of chemical toxicity targets. This vulnerability to toxic insult makes reproduction not only one of the most important systems in the field of biology but also in the field of toxicology.

Animal reproduction plays a key role in animal agriculture and the ecological balance of wildlife species. The exposure of the animal kingdom to synthetic chemicals over the past few decades has increased dramatically. This exposure will continue to increase as the production and use of chemicals inevitably rises in the coming years. This could lead to increased concerns over effects of synthetic chemicals on reproduction (Gibbons, 1991).

Given the extent of human and animal exposure to chemicals in our environment and the fertility problems experienced by many species due to unknown factors, the use of sperm cells in *in vitro* toxicity testing can prove to be a

practical and sensitive method of studying toxic effects of chemicals on male reproduction. Sperm cells as biomarkers could also prove invaluable in understanding the vast chemically-linked male infertility problems experienced by humans and animals in our current chemically dependent environment (Gibbons, 1991). The use of sperm cells in in vitro testing could also lead to a better understanding of the ultrastructure and biochemistry of the spermatozoa as it proceeds through the critical physiological steps between ejaculation and sperm egg fusion.

One class of synthetic chemicals that has been extensively used since the end of World War II is the organophosphate (OP) pesticides. Their value as an insecticide has been well documented (Eto, 1974, Hayes, 1975, Derache, 1977). Their selective toxicity in insects has made them one of the most widely used insecticides in the world.

The subtle, detrimental effects of OPs on mammalian reproduction have been implicated (Chou, 1987). This could have a vast economical impact on the animal industry. In addition, toxic effects on reproduction in wildlife species could have far reaching impacts on species perpetuation in an already stressed wildlife environment.

The human exposure to OPs has been well documented (FDA Total Diet Study, 1982-1986). The realization of the impact of long-term, low-dose OP exposure on reproduction is essential in taking a step forward in elucidating the possible

causes of decreased reproductive performance in humans and animals coincidental to the rise in the use of environmental chemicals (Odenwald, 1989). A study of the toxic effects of OPs on reproduction could also expand our understanding of the mechanisms of fertilization as well as our understanding of the mechanisms of OP toxicity.

CHAPTER II

LITERATURE REVIEW I:

ORGANOPHOSPHATE INSECTICIDES

History

The first known OP insecticide was tetraethyl pyrophosphate (TEPP) and was synthesized by Lippe De Clermont in France in 1854. Gerhard Schrader, a German chemist, studied OP extensively for their use as insecticides as well chemical warfare agents in the early 1930's. Some other OP compounds synthesized in these early days were diisopropyl fluorophosphate (DFP), TEPP, and the thio- and thiono-phosphorus compounds including paraoxon and parathion. During World War II, synthesis and toxicity of OPs was classified as top secret by the German government. The widespread use of these compounds as insecticides was, therefore, delayed until after World War II (Holmstedt, 1963).

Parathion is an OP insecticide with a chemical name of O,O-Diethyl O-p-nitrophenyl phosphorothioate (EPA, 1975). Parathion and the methyl homolog, parathion-methyl, have been the most widely used OP insecticides since World War II. Their use, however, is now prohibited in some countries

because of their high mammalian toxicity (Eto, 1974). Parathion, however, has a high insecticidal activity with a wide spectrum of action, and is often used as a measure of effectiveness for the evaluation of other insecticides (Eto, 1974).

Environmental and Accidental Exposure

After parathion was first on the market and became a commonly used insecticide, the incidence of parathion poisoning increased with it's broadening production and usage. Ninety six cases of parathion poisoning with 13 deaths were reported in 1950 alone from a small portion of the cotton-raising section of Brazil (Hayes, 1975). There were over 1,500 poisoning cases and hundreds of deaths from parathion per year in Japan during 1953 and 1954 (Hayes, 1975). In Mexico, there were 1,150 poisoning cases in 1965. OPs have also been used frequently as a method of suicide in the past. In Japan between 1955 and 1960 there was an average of 500 suicides annually using parathion. Between 1955 and 1957, there were 219 suicides with parathion reported in Finland (Toivonen et al., 1959).

Humans are exposed to relatively high concentrations of OP compounds in two distinct ways. One is occupational exposure in the manufacture of pesticides and pesticide products, in their packaging and transport, and in their use

in agriculture or horticulture. The other is the use of pesticides in large quantities for public health purposes including the use of OPs to treat head lice in humans. This increases the risk of exposure to high concentrations of pesticides over relatively short periods which may lead to acute poisoning (Derache, 1977).

The routes of exposure to OPs includes oral, dermal, and respiratory. Although only 1.23% of the parathion on the skin of orchard sprayers was found to be available for absorption, this route may be of critical importance because exposure of this kind may be much greater than that by other routes (Durham et al. 1972). Durham et al.(1972) also found that dermal exposure accounted for a minimum of 80.4 to 90.7% of the absorption of parathion in men who sprayed orchards with that compound.

The general population is exposed to low concentrations of pesticides (and their metabolites) which occur in the environment and in food. The exposure is long term and all sections of the population are involved. The general population is also frequently exposed, for short periods, to low concentrations of OP formulations used to kill household or garden pests. Such exposure would be of a chronic type (Derache, 1977).

The Food and Drug Administration (FDA) conducts annual "basket studies", or Total Diet Studies, to monitor chemical contaminants in foods. These studies are designed to

determine the dietary intake of pesticides and other chemicals from a normal diet and to compare these intakes with Acceptable Daily Intakes (ADI) in humans (FDA, 1986). According to the studies from 1982-1986, a 25-30 year old man consumes a total of 0.08 mg of various OPs per day. Four of the commonly used OP insecticides were found in the human diet in that study, although individually they were all below the ADI's. These frequently found OPs were Diazinon, Dimethoate, Malathion, and Parathion.

Metabolism

The biotransformation of parathion involves two distinct phenomena, activation and deactivation (detoxification) (EPA, 1975). This is performed by a mixed function oxidase and involves an oxidative desulfuration (Davison, 1955).

The metabolic pathway of parathion varies with species. In humans and rodents, the major pathway involves the formation of paraoxon followed by hydrolysis to p-nitrophenol, or the direct hydrolysis of parathion to p-nitrophenol which is then glucuronidated and excreted in the urine (O'Brien, 1960).

Unlike mammals, insects can not readily deactivate many of the most commonly used OP insecticides due to a lack of the degradative enzymes required for deactivation, such as carboxylesterase for malathion and dimethoate. This results

in a much longer half life of the activated, more toxic forms of OPs resulting in a high insecticidal activity. This difference in metabolism between mammals and insects has made OP insecticides effective yet relatively safe pesticides (Eto, 1974).

The conversion of parathion to paraoxon in the environment and in the body leads to a potent inactivator of acetylcholinesterase (AchE) and other β-esterases. AchE is an enzyme responsible for terminating the transmitter action of actylcholine at the junction of cholinergic nerve endings with their effector organs or postsynaptic sites (Jacobson and Saier, 1988).

Organophosphate pesticides are generally short-lived in the environment and are degraded through hydrolysis and other reactions into nontoxic and water soluble compounds. They are also hydrolyzed readily in plants (Hayes, 1975). Organophosphate pesticides, therefore, do not represent a serious environmental problem.

Mechanism of Action

The apparent points of acute attack of OPs are the respiratory system, central nervous system, cardiovascular system, eyes, skin, and blood cholinesterases (ChE). In the cholinergic system, nerve impulses must be transmitted between nerve cells or from nerve cells to muscle or glandular tissues

in order for their effects to take place. To achieve this, the neuro-transmitter acetylcholine (Ach) is released from the presynaptic cell into the synaptic cleft and is then bound by Ach receptors on the postsynaptic cells (Jacobson and Saier, 1988). This causes various but specific physiological effects at the postsynaptic cells depending upon the type of postsynaptic cell. In the case of muscular tissue, it causes muscular contraction and, in the case of nerve cells, it causes further propagation of the action potential. Ach must then be rapidly degraded by the AchE in the synaptic cleft to restore the resting potential of the postsynaptic membrane (Jacobson and Saier, 1988).

Acetylcholinesterase contains two binding sites. The anionic site, which contains a glutamate residue, interacts with the positively charged nitrogen atom of Ach, while the esterase site is associated with the cleavage of the ester bond of Ach. The esteratic site contains a serine residue. This serine residue is acetylated during Ach hydrolysis and is then deacetylated to restore the enzyme activity (Derache, 1977). This mechanism of cholinergic action and Ach inactivation is essentially the same in insects and vertebrates (Derache, 1977).

Parathion inhibits AchE by forming a stable covalent intermediate with the active-site serine in the enzyme. Because of the long half life of the phosphorylated enzyme, the AchE inhibition by OPs is considered irreversible

(O'Brien, 1960).

Neuromuscular junctions exposed to AchE inhibitors are paralyzed because the persistent presence of Ach prevents repolarization of the postsynaptic membrane. This overstimulation eventually desensitizes the Ach receptor and the membrane of the neuron remains closed to ion flow for long intervals even in the presence of the neurotransmitter. The desensitization of the receptor leads to partial recovery from OP toxicity (Gaines, 1960).

The symptoms of OP poisoning vary in intensity with dosage. They include, in mammals, muscle fasciculation, excessive salivation, tremor, miosis, partial paralysis, labored breathing, lachrymation, diarrhea, involuntary urination, convulsion, depression and death (Gaines, 1960). Death is primarily caused by respiratory failure, blocking of the respiratory center, bronchospasm and paralysis of the respiratory muscles (Derache, 1977).

Treatment of OP poisoning consists of intravenous infusion of atropine sulfate. Atropine sulfate effectively competes with Ach for receptor sites but elicits no physiological response. It, therefore, prevents excessive Ach from binding to the Ach receptors on the postsynaptic membrane.

Poison victims should be checked for a clear airway and for cyanosis and should receive 2 to 4 mg of atropine sulfate intravenously. This dose should be repeated at intervals of

5 to 10 minutes until signs of mild atropinization appear and at less frequent intervals thereafter. The patient should be kept on the verge of atropine intoxication until recovery from OP poisoning (Hayes, 1975).

Teratogenicity in Avian Species

OP compounds have been shown to cause some teratogenic effects in early development in avian species. A study of 36 OP compounds and 12 methylcarbamate insecticides injected into chicken embryos between the doses of 0.003 to 5 mg/egg (0.06 to 100 mg/kg) showed teratogenic effects of these compounds. The teratogenic effects included micromyelia (abnormally small size of spinal cord), abnormal beak development, reduced body size, retarded down development, gross edema, and wryneck (an abnormal contracted state of one or more muscles of the neck producing an abnormal position of the head) (Proctor et al., 1976).

Upon measurement of nicotinamide adenine dinucleotide (NAD) levels in treated chicken embryos, it was found that embryos with NAD levels inhibited to less than 45% of controls showed mild to severe teratogenic effects. In embryos with NAD levels inhibited to the range of 52-63% of controls, the teratogenic effects varied from normal to slight or moderate. In embryos with NAD levels inhibited to the range of 69 to

100% of controls, only mild teratogenic effects were seen (Proctor et al., 1976).

Nicotinamide adenine dinucleotide is a biological carrier of reducing equivalents. The most common function of NAD is to accept two electrons and a proton (H^- equivalent) from a substrate undergoing metabolic oxidation to produce NADH, the reduced form of the coenzyme (Jacobson and Saier, 1988). To date, the involvement of NAD in the appearance of teratogenic signs in chicken embryos is not fully understood. However, studies with embryonic chicken limbs and cultures of their mesodermal cells indicate an important role of embryo NAD levels in the control of muscle and cartilage development (Proctor and Casida, 1975).

Acetylcholinesterase activity in chicken embryos treated with OP compounds is also inhibited during early development. Chicken eggs treated with 200 μg Diazinon, an OP pesticide, per egg on day 3 of incubation showed 90% inhibition of AchE at days 6-8 of incubation (Misawa et al., 1981).

Kynurenine formamidase, an enzyme which converts tryptophan to essential pyridine nucleotide cofactors in the yolk sac membrane and possibly in the embryonic liver at later stages, was also shown to be inhibited by OPs. Inhibition of kynurenine formamidase could lead to the decreased availability of NAD in chicken embryos (Hoffman and Eastin, 1981).

This lead to the recognition of two different types of

teratogenic mechanisms for OPs in the early development of chicken embryos. Type I is associated with inhibition of kynurenine formamidase and leads to micromyelia, parrot beak, and abnormal feathering. Type II is associated with inhibition of AchE (Hoffman and Eastin, 1981).

Organophosphate Effects on Reproduction in Mammals

Relatively few studies have been done on the effects of OPs on reproduction in mammalian species. Beck (1953) did, however, find that boar sperm treated with parathion lost all motility 120 minutes after treatment. Parathion also had an inhibitory effect on glycolysis of the boar sperm. Studies in mammals have not shown the same teratogenic responses to OPs as seen in chicken embryos.

Organophosphate Production in the United States

The OP production in the United States has fluctuated in the past 20 years (Table 1). Since 1970, production reached a high in 1975 and a low in 1983. However, the production of OP insecticides has steadily risen in recent years regardless of the ongoing public protest over the use of chemicals in the environment. This trend suggests that OP insecticides will continue to be a major pesticide, and the concern over long

term OP effects on human and animal health will not cease in the foreseeable future.

Table 1. Organophosphate production in the United States[1] .

Year	Production (1,000 lbs.)
1970	132,496
1971	138,185
1972	160,642
1973	172,605
1974	186,587
1975	209,795
1976	189,879
1977	204,045
1978	207,878
1979	203,203
1980	146,644
1981	156,450
1982	122,429
1983	108,074
1984	135,261
1985	157,485
1986	125,727
1987	135,077
1988	138,518
1989	143,545

[1] USDA, Emergency Preparedness Branch, "The Pesticide Review", USDA State and County Emergency Records, (1970-1991).

Organophosphates Frequently Found in the Human Diet

Diazinon is a widely used insecticide in the United States today. It has a relatively low mammalian toxicity, with an acute oral and dermal LD50 in rats of 150-220 mg/kg and 500-900 mg/kg, respectively. Geese and ducks are very

susceptible to diazinon poisoning. The inability of insects to completely metabolize Diazinon gives it a high insecticidal activity. This makes it one of the most potent anticholinesterases in insects (Matsumura, 1975). The ADI for Diazinon in humans is 0.002 mg/kg/day (USDA, 1988).

Dimethoate has an acute oral LD50 in rats of 155-500 mg/kg. It's toxicity is selective towards insects due to a carboxyamide group. Mammals detoxify dimethoate with carboxyamidase, an enzyme which insects lack. Carboxyamidase splits amide bonds. Dimethoate is particularly toxic to houseflies, making it a common household insecticide. It is used as a foliar spray to control sucking insects including aphids, red spider mites, and cherry flies (Matsumura, 1975). No insect resistance to dimethoate has yet been reported. The ADI for dimethoate in humans is 0.002 mg/kg/day (USDA, 1988).

Malathion has an acute oral LD50 in rats of 900-5800 mg/kg and is broken down in the mammalian liver by carboxylesterase. This makes malathion one of the safest insecticides available today. It is widely used for the control of many insects. The selectivity of malathion is due to its carboxyl group, which is susceptible to mammalian hydrolysis. The low mammalian toxicity of malathion makes it a favorite for household pest control (Matsumura, 1975). The ADI for malathion in humans is 0.02 mg/kg/day (USDA, 1988).

Parathion is highly toxic to mammals. The acute oral LD50 in rats is 3.6 mg/kg for females and 13 mg/kg for males.

It's acute dermal LD50 in female rats is 6.8 mg/kg and 21 mg/kg in male rats. In recent years, its use has largely been superseded by less hazardous OPs. Parathion is quickly metabolized and excreted and does not accumulate in the body. Parathion can be used to control a variety of insects such as aphids, mites, beetles, Lepidoptera, leaf hoppers, leafminers, and other pests found on fruits, cotton, vegetables, and forage crops. It is also effective in controlling several soil insects such as wire worms, rootworms, and symphilids (Matsumura, 1975). The ADI of parathion for humans is 0.005 mg/kg/day (USDA, 1988).

Significance of the Current Study

The allowable levels for humans, set by the FDA, are based primarily on short-term, high-dose studies. There is growing concern, however, that there could be far reaching consequences of a mixture of chemicals when the exposure is at a low dose, over a long period of time. Furthermore, unlike human food, animal feed has no regulations for the levels of these pesticides. Long-term, low-dose studies, therefore, are extremely important, for humans and animals, in understanding the possible impact of these chemicals on reproduction.

CHAPTER III

LITERATURE REVIEW II:

REPRODUCTION

History

Sperm cells offer a unique model for assessing the toxic effects of chemicals in that [1] they require optimal conditions for acquiring fertilizing ability and [2] they can be cultured *in vitro* while maintaining their biochemical and physiological properties similar to that seen *in vivo*. They are produced in high numbers and can be collected from many large animal species with noninvasive techniques.

As early as 1949, Mann pointed out 5 advantages of using sperm to test toxic effects of chemicals. These advantages are that: 1) Semen contains one cell type, which can be separated from its plasma with little damage. 2) The cells are nonreproducing. 3) Spermatozoa have little cell reserve, and nearly all nutritive materials must be assimilated from the surrounding medium making it possible to study specific parameters of metabolism by manipulating the contents of the incubation medium. 4) Spermatozoa are highly permeable and their fertilizing ability depends on their interaction with

the surrounding environment. 5) Fertility and motility, as well as many parameters of metabolism, may be investigated with existing methods for end-point measurements of toxicity testing. Today, these basic concepts still hold true. Recent developments in biotechnology, such as in vitro sperm capacitation, in vitro fertilization, and automated sperm analysis, further strengthen these concepts.

The development of computer-aided sperm analysis systems in the early 1980's has lead to the identification , tracking, and analysis of the paths of individual sperm heads over successive video frames, as well as providing information about the morphological properties of individual sperm images. These computer systems have enabled sperm analysis to become a component in basic studies of chemical toxicity. They have also enabled prospective, longitudinal fertility screening of potentially exposed populations, as well as retrospective, cross-sectional analyses when exposures are already believed to have occurred (Katz, 1991).

Sperm Membrane Structure

The plasma membrane of sperm, like most biological membranes, is composed of a phospholipid bilayer with both hydrophobic and hydrophilic components (Singer and Nicolson, 1972). The fatty acid chains are located on the interior of the bilayer, maximizing the hydrophobic interactions, while

the ionic and polar portions of the phospholipid molecules are on the outside of the bilayer, maximizing the hydrophilic interactions.

The content of the plasma membrane of the sperm is not uniform over its entire surface. It has been shown to be made of different constituent parts in different regions of the membranes. A study by Naz et al. (1984) showed that antibodies to sperm membrane glycoproteins react with the antigen on the postacrosomal region and the tail, not on the acrosomal region, and, to a limited extent, on the midpiece.

Sperm Capacitation

Sperm capacitation is a sequence of biochemical processes that is not yet fully explained. It is understood, however, that capacitation is required by all mammalian sperm, ejaculated or epididymal, before they can become viable for fertilization. Capacitation has been described as "preparing or sensitizing mammalian sperm in some way, enabling them to undergo the acrosome reaction in response to a specific stimulus" (Bedford, 1970). The phenomenon of capacitation was first demonstrated by Chang (1951) and Austin (1951) independently by showing that rat and rabbit sperm must first reside in the female reproductive tract before they can successfully penetrate the zona pellucida. Since that time

extensive work has been done attempting to identify ultrastructural and biochemical changes in the membrane associated with the process of capacitation.

Davis and Gergely (1979) found that incubation with bovine serum albumin caused substantial changes in the sperm plasma membrane proteins and facilitated capacitation. Findings that sperm incubated with cholesterol saturated albumin prevented capacitation pointed to some involvement of cholesterol in the capacitation event. Davis and Gergely (1979) also found that plasma membrane isolated from rat sperm cells after incubation in vitro had a significantly lower cholesterol/phospholipid mole ratio when the medium contained serum albumin. From these findings, it is believed that serum albumin facilitates capacitation by depleting the cholesterol level in sperm membranes. Other sperm membrane lipid changes associated with capacitation are hydrolysis of sterol sulfates to free sterols (Langlais et al., 1981; Langlais and Roberts, 1985), and formation of lysophospholipid by endogenous phospholipases (Meizel, 1984; Langlais and Roberts, 1985). Changes in the lipid components of the sperm membrane cause a destabilizing effect which leads to membrane fusion (Parks et al., 1987). These membrane changes may be critical in supporting the fusion of the plasma membrane and acrosome membrane required during acrosome reaction.

Sperm Acrosome Reaction

After capacitation, the membrane covering the acrosome of sperm undergoes a process called acrosome reaction in the later stages in the reproductive tract of the recipient female. This acrosome reaction is necessary for sperm-egg fusion. It is initiated by a series of point fusions between the anterior portion of the plasma membrane and the outer membrane of the acrosome underlying it (Barros, et al. 1967) and can be observed ultrastructurally with the electron microscope (Cross and Meizel, 1989). The factors involved in the initiation of this event are still unknown but acrosome reaction is dependent on capacitation for its onset.

One of the factors believed to be involved in the onset of the acrosome reaction is the binding of the sperm to the zona pellucida. The zona pellucida of the mouse is composed of three glycoproteins named Zona Protein 1 (ZP1), Zona Protein 2 (ZP2), and Zona Protein 3 (ZP3) (Bleil and Wassarman, 1980). ZP3 has been shown to be responsible for the binding of the sperm to the egg surface (Bleil and Wassarman, 1980). Mouse sperm must be intact or not acrosome reacted in order to bind to the ZP3 receptor (Florman and Storey, 1982; Saling and Storey, 1979). ZP3 has also been shown to induce the acrosome reaction after sperm are bound (Bleil and Wassarman, 1983; Wassarman et al., 1986). This

evidence suggests a dual role of ZP3 on the mouse zona pellucida: a receptor for sperm-egg binding and an inducer of the acrosome reaction. This, however, varies among species in that guinea pig, Chinese hamster, and rabbit sperm can initiate binding to the zona pellucida whether they are acrosome intact or acrosome reacted (Kuzan et al., 1984; Myles et al., 1987; O'Rand and Fisher, 1987; Yanagimachi et al., 1983).

Extracellular calcium is also known as a requisite for the acrosome reaction. Summers, et al. (1976) found, through the use of a calcium ionophore (A23187), that an increase in the permeability of the sperm head membrane to calcium is a possible functional change associated with capacitation leading to the acrosome reaction. As the plasma membrane and the outer membrane of the acrosome fuse they form ports through which a progressive release of acrosomal content occurs (Bedford, 1970). The acrosome has been shown to contain a variety of hydrolytic enzymes including hyaluronidase and acrosin.

Hyaluronidase is believed to play a key role in cumulus cell dispersion at the time of gamete fusion. This would allow the sperm to pass through the cumulus cells that characteristically surround the oocyte at ovulation. Another enzyme released from the acrosome at the time of acrosome reaction is acrosin. Acrosin has been implicated as a necessary component in the process of penetrating the zona

pellucida (Stambaugh and Buckley, 1969).

Measurement of Capacitation and Acrosome Reaction

The lack of identifiable structural changes associated with capacitation has made the measurement of the capacitation event difficult for many years. Typically, the occurrence of acrosome reaction has been used as an end point of capacitation observed through transmission electron microscopy.

Ward and Storey (1984) developed a technique to observe both capacitation and acrosome reaction in mouse spermatozoa. The fluorescent probe chlortetracycline (CTC) was used to identify the membrane changes of different stages of capacitation and acrosome reaction. Sperm incubated with CTC were scored according to the fluorescent pattern they displayed. Four distinctive fluorescent patterns were described. The pattern designated F1 showed a uniform fluorescence over most of the head with a line of brighter fluorescence across the equatorial region while the brightest fluorescence was on the midpiece. The second pattern, F2, was characterized by uniform fluorescence over the entire sperm head. The third pattern observed had a similar fluorescence on the midpiece as seen in both F1 and F2 with a bright fluorescence on the anterior portion of the sperm head but a lack of fluorescence on the post acrosomal region. This was

designated as pattern B. The final pattern, AR, was characterized by a similar bright fluorescence on the midpiece and an almost complete lack of fluorescence on the entire head region.

The two patterns labeled F1 and F2 comprised over 70% of sperm found in fresh epididymal sperm preparations (Ward and Storey, 1984). The loss of the combined F patterns coincided with the gain of the B pattern over time suggesting that the sperm showing the F pattern progress into the second step or the B pattern. The time period required for this change to take place coincides with the previously determined time required for mouse sperm to capacitate in vitro (Miyamoto and Chang, 1973). This method can be used to quantitatively follow the progression of a population of sperm incubated in vitro from the state containing mostly fresh sperm to the state containing mostly capacitated sperm.

By means of electron microscopy, the B pattern, believed to be capacitated sperm, was observed to be acrosome-intact sperm while the AR pattern, believed to be acrosome reacted sperm was observed to be truly acrosome reacted (Saling and Storey, 1979). This confirms that sperm can be incubated in vitro and quantitated with the CTC method into the functional groups consisting of fresh, capacitated, and acrosome reacted.

The biological nature of the CTC fluorescent patterns on the sperm membrane is not understood in detail. It may be related to the increased fluorescent emission of CTC when it

is bound to membrane proteins in the presence of a divalent cation, such as Ca^{2+}, in a nonpolar environment (Saling and Storey, 1979).

Motility

Although sperm motility does not play much of a role in uterine transport, it is considered to play an essential role in the penetration of the zona pellucida (Bedford, 1970). Measurements of sperm motility can be made with many different techniques. The simple use of the hemocytometer, which is commonly used to quantify blood cell types, is one common method used to assess sperm motility. Another similar method uses the recently developed Makler chamber. The most recent and presumably the most repeatable method measures the motility of sperm, as well as other physical characteristics of movement, with the use of micro-computer image-analysis technology. There are several software companies with different software packages that effectively measure sperm cell movement (Katz, 1991). Sperm motility depends on a sufficient energy source whether that be from the female tubal fluid or the *in vitro* culture medium (Wolf, 1979). Sperm cell motility, along with cell number, is one of the parameters most frequently studied in semen quality assessments for both humans and animals.

Cholinergic Systems in Sperm

In neurons, Ach is formed from choline and Acetyl CoA by the enzyme cholineacetyltransferase (ChAT). Ach is hydrolyzed by the enzyme AchE into choline and acetic acid. It was suggested as early as 1951 that this cholinergic system may play a role in sperm motility or fertilizing ability. Later, AchE was also identified in boar spermatozoa and was believed to be primarily involved in a cholinergic system supporting sperm motility (Sekine, 1951). AchE was then identified in the spermatozoa of the bull (Nelson, 1964), the sea urchin (Applegate and Nelson, 1962), perch (Tibbs, 1960), and the ram (Mann, 1964). AchE appears to be more concentrated in the tail of spermatozoa than in the head. Nelson (1964) showed that the specific activity of the tail fraction is about five times that of the head fraction in bull spermatozoa.

With the established presence of the hydrolytic enzyme of Ach, the studies that followed were to determine whether or not the biosynthetic enzyme of Ach, ChAT, was present in spermatozoa. ChAT was demonstrated in bull spermatozoa (Bishop et. al., 1976) and in ram spermatozoa (Stewart and Forrester, 1978a). Its distribution was similar to that of AchE. The specific activity of ChAT in the tails of bull spermatozoa was about five times higher than that in the heads or midpieces (Bishop et al., 1976). Receptors for Ach were

also identified in ram spermatozoa and determined to be the nicotinic type (Stewart and Forrester, 1978b).

In the neuromuscular transmission cycle, Ach functions at the postsynaptic neuron by producing an increase in the ionic permeability of the postsynaptic membrane to Na^+ and K^+ after binding to the Ach receptor (Krnjevic, 1974). Stewart and Forrester (1978b) hypothesized that, in sperm, Ach may regulate motility by similarly increasing the ionic permeability of the sperm membranes to Ca^{2+}. Ach increases sperm motility while increasing the exchange of Ca^{2+} across sperm membranes (Stewart and Forrester, 1979). The presence of ChAT, Ach receptors, and AchE in the head of sperm lead to investigations into the involvement of the Ach cycle in not only sperm motility but also in capacitation and acrosome reaction in sperm. Fifty μM paraoxon, a known AchE inhibitor, decreased mouse sperm fertilizing ability in vitro to 40.5% of controls. The same concentration of paraoxon also inhibited the ability of mouse sperm to undergo proper capacitation and acrosome reaction (Chou, 1987). More sperm remained in a fresh, uncapacitated state in the presence of paraoxon than that of the controls showing an inability to undergo capacitation at the expected time after incubation.

CHAPTER IV

HYPOTHESIS AND OBJECTIVES

Hypothesis

Paraoxon has been shown to inhibit capacitation of sperm *in vitro*. OPs are present in the human diet and presumably in the animal diet as well at low doses. The exposure to these OPs is of a chronic nature. This study was designed based on the hypothesis that an AchE-like enzyme plays a role in sperm fertilizing ability and that long-term, low-dose exposure to OPs in feed could affect sperm fertilizing ability.

Objectives

1) To study the effect of OPs on AchE levels in mouse and boar sperm *in vitro*.
2) To study the effects of long-term, low-dose exposure to OPs on sperm function in male mice.

CHAPTER V

EXPERIMENTAL DESIGN

Phase I: Effects of Paraoxon on Capacitation In Vitro

Mouse sperm were treated *in vitro* with 0, 50 or 100 μM paraoxon and observed for capacitation and acrosome reaction. Epididymal sperm from three mice were used for each treatment.

Phase II: AchE Activity of Mouse and Boar Sperm in the Presence of Paraoxon

The second phase of the experiments was designed to study the AchE activity in mouse and boar sperm after *in vitro* treatment with paraoxon. Sperm samples from both mouse and boar were treated with 0, 50 or 100 μM paraoxon. For the mice, epididymal sperm from two mice were measured in duplicate for each treatment. For the boar, two ejaculated sperm samples were measured in duplicate for each treatment.

Phase III: Effects of Dietary Mixtures of OPs on Brain AchE Activity and Sperm Quality in Mice

Preliminary Studies

A short term feeding trial was performed to determine the observable effect doses of the OP mixture for brain AchE activity. For this preliminary trial a mixture of OPs (diazinon: dimethoate:malathion:parathion) was used at the following ratio, 160:80:2500:40. This ratio, which mimics the dietary OP exposure of human infants, was to be used for the first three months of the long-term trial. The mixture was fed to three month old male mice at doses of 40, 400, 4,000, and 40,000 μg/kg BW/day. For each dose, brains from two mice were analyzed for AchE activity after seven days of OP exposure.

Long-Term Dietary Exposure

The long-term feeding trial was designed to examine the effects of low-dose exposure to a mixture of OPs on sperm quality in mice. This was accomplished by examining brain AchE activity and sperm quality. Mixtures of four OP insecticides, mixed in ground Mouse Chow #5015, were used in the feeding trials. Male mice received the treatment diet from weaning to eight months of age. Until three months of age the OPs, diazinon:dimethoate:malathion:parathion, were

mixed in the ratio of 160:80:2500:40 and fed the total dose of 0, 0.4, 4.0, or 40.0 ug/kg BW/day. The low dose (0.4 μg/kg BW/day) was designed to reflect the dose that a human child may receive through dietary exposure from birth to puberty. The total dosage and the ratios were determined based on the FDA basket study of human foods for a child. From three months to eight months of age, the OPs, diazinon:dimethoate: malathion:parathion, were mixed in the ratio of 50:70:760:9 and fed the total dose of 0, 0.15, 1.5, or 15.0 ug/kg BW/day. The low dose (0.15 μg/kg BW/day) was designed to reflect the dose that an adult human may receive through dietary exposure.

After the completion of the long-term, low-dose feeding, sperm from the treated animals were analyzed for their ability to proceed through capacitation and acrosome reaction. For this analysis, sperm from three mice per treatment were analyzed.

Sperm were also collected and used to inseminate oocytes collected from non-treated females in vitro. Three males from each treatment group were used for the in vitro fertilization assay. For each male, oocytes from two non-treated females were inseminated.

Motility of epididymal sperm was measured at three time points after collection, 0, 45, and 95 minutes. For each treatment, motility of sperm from three mice were scored.

At the end of the eight month exposure, brains from treated mice were then analyzed for AchE activity. Three

brains from each treatment group were analyzed. Six weeks after the withdrawal of the OP treatment, brains from the mice were analyzed for AchE activity for possible enzyme activity recovery. For the control, low, and medium doses, brains from two mice were analyzed. For the high dose, the brain from one mouse was analyzed.

CHAPTER VI

MATERIALS AND METHODS

Animals

For all experiments with mice, B6D2-F1 were used. B6D2-F1 is the first generation of the cross between C57BL-6J females and DBA-2J males (Jackson Labs, Bar Harbor, Maine). The mice were kept on a schedule of 14 hr light and 10 hr dark and at 21°C. Mouse Chow #5015 (Purina Mills Inc., St. Louis, MO) and water were provided *ad libitum*.

Chemicals

Brinster's medium for oocyte culture (BMOC-3), used for *in vitro* fertilization and sperm culture, was from Gibco (St. Louis, MO) (Brinster, 1971). BMOC-3 contains 189 mg/L $CaCl_2$, 356 mg/L KCL, 162 mg/L KH_2PO_4, 294 mg/L $MgSO_4$-$7H_2O$, 5546 mg/L NaCL, 2106 mg/L $NaHCO_3$, 5000 mg/L bovine serum albumin, 1000 mg/L D-glucose, 2253 mg/L DL-sodium lactate, 56 mg/L sodium pyruvate, 3.3 mg/L streptomycin sulfate, and 3.3 mg/L penicillin potassium. For the CTC fluorescence assay of capacitation, modified BMOC-3 was used (Chou, 1987). Modified

BMOC-3 was made from 251 mg/L $CaCl_2$, 356 mg/L KCl, 162 mg/L KH_2PO_4, 294 mg/L $MgSO_4$-$7H_2O$, 6976 mg/L NaCl, 2106 mg/L $NaHCO_3$, 4 g/L bovine serum albumin, and 1000 mg/L D-glucose (Sigma Chem. Co., St. Louis, MO). In mofified BMOC-3, an additional 1.6% bovine serum albumin (Sigma Chem. Co., St. Louis, MO) was included to accelerate the rate of capacitation and acrosome reaction. Dulbecco's phosphate-buffered saline (PBS) was used as washing medium for the sperm AchE assay. PBS was made from 200 mg/L KCl, 200 mg/L KH_2PO_4, 8000 mg/L NaCl, and 1150 mg/L Na_2HPO_4 (Sigma Chem. Co., St. Louis, MO).

Chlortetracycline, Tris, cysteine, glutaraldehyde, pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), Hoechst No. 33258 bisBenzimide stain, Amberlite resin (100-200 mesh), and Ach-iodide were purchased from Sigma Chem. Co., St. Louis, MO. Ethanol was purchased from Quantum Chemical Corp., Tuscola, IL. Acetic acid was purchased from Mallinckrodt Inc., Paris, KY. Acetyl-1-^{14}C-Choline was purchased from E.I. du Pont de Nemours and Co., Billerica, MA. Safety-Solve scintillation cocktail was purchased from Research Products International Corp. (Mount Prospect, IL). Diazinon, dimethoate, malathion, and parathion were purchased from Pfaltz and Bauer, Waterbury, CT.

Feed Preparation

Mouse Chow #5015 was ground fine and used as the base of the diet. The OP mixtures were dissolved in a vehicle consisting of 90% absolute ethanol and 10% corn oil. The control diet was mixed with the vehicle containing no OPs. The OP mixture was dissolved in the vehicle and poured onto the ground feed. The feed was mixed and sifted through a #20 screen (USA Standard Testing Sieve, Sargent-Welch Scientific Company, Skokie, IL) twice to insure thorough mixing. The feed was prepared weekly, stored in a sealed feed can and fed to the mice ad libitum in a feeder jar Unifab Corp.0, Dry-Diet Feeder 7oz. (Kalamazoo, MI).

CTC Fluorescence Assay for Capacitation and Acrosome Reaction

For Sperm Treated With Paraoxon In Vitro

For CTC fluorescence experiments, a modified version of the assay described by Ward and Storey (1984) was used. The sperm cells were collected from mature male B6D2-F1 mice. Both epididymides were removed and placed in a 35x10-mm petri dish (Corning Glass Works, Corning, N.Y.) containing 0.5 ml modified BMOC-3 with the test chemical. Each epididymis was poked with a 25G needle to allow the sperm to pass out into

the medium. 0.45 ml sperm containing medium was then removed and placed in a centrifuge tube (Corning Glass Works, Corning, NY) already containing 0.35 ml modified BMOC-3 for dilution. The medium in the petri dish and culture tube contained either 0, 50 or 100 μM paraoxon. The sperm suspension was incubated at 37° C, 100% humidity, and 5% CO_2 in air for 115 minutes.

For CTC assays, samples were taken at 0, 15, 35, 55, 75, 95, and 115 minutes with 0 time being the first sample taken after the final dilution was completed. This was between 3-4 minutes after sperm were released from the epididymis. At each sampling time, 20 μl of 500 uM CTC solution in a chilled buffer of 20 mM Tris, 130 mM NaCl, and 5 mM cysteine was added to a warmed (37° C) microscope slide. This was followed by adding 20 μl sperm suspension. After allowing 10 seconds for staining, the cells were fixed with 10 μl 12.5% glutaraldehyde in 1 M Tris buffer (pH 7.8), stirred thoroughly, and a coverslip was applied.

Sperm on slides were illuminated with a 100 W mercury bulb and examined with a Nikon Optiphot microscope equipped with a 380-425nm excitation filter, a 520nm barrier filter, and a 510nm dichroic mirror. Sperm were scored in 4 different patterns. Sperm scored as F were considered fresh or uncapacitated and displayed a bright fluorescence over the entire head and midpiece. Sperm scored as DB or Dark Banded were considered capacitated and displayed bright fluorescence over the anterior portion of the head and midpiece with a band

which lacked fluorescence at the posterior portion of the head. Sperm scored as LB or Light Banded were also considered capacitated and were the same as those scored DB except the fluorescence on the anterior portion of the head was fainter than the midpiece. The fourth pattern was designated AR or Acrosome Reacted and had bright fluorescence on the midpiece and a lack of fluorescence over the entire head. At least 100 sperm were examined on each slide.

For Sperm Collected From OP Mixture-Treated Mice

For the in vivo treated mice, the same procedure as above was used except the medium did not contain any test chemicals. The sperm were collected, incubated, and examined the same as for those treated in vitro.

In Vitro Fertilization

Sperm were collected from male mice at eight months of age at the completion of the feeding trial. For each in vivo treated mouse, one epididymis was placed in the inside well of a Falcon Organ Tissue Culture Dish, 60X15mm, containing 0.5 ml BMOC-3. The outside well contained 3 ml BMOC-3 containing 0% BSA for humidification. The epididymis was then poked with a 25G needle to release the sperm into the medium. This dish, with the epididymis in the center well, was incubated at 37° C, 5% CO_2 in air, and 100% humidity for 1 hour before being

which lacked fluorescence at the posterior portion of the head. Sperm scored as LB or Light Banded were also considered capacitated and were the same as those scored DB except the fluorescence on the anterior portion of the head was fainter than the midpiece. The fourth pattern was designated AR or Acrosome Reacted and had bright fluorescence on the midpiece and a lack of fluorescence over the entire head. At least 100 sperm were examined on each slide.

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used for insemination.

Oocytes were collected from non-treated female B6D2-F1 mice, 21-42 days of age. These mice were superovulated with intraperitoneal injections of 10 IU pregnant mare serum gonadotropin (PMSG) followed 50 hours later by 10 IU human chorionic gonadotropin (hCG). Twelve to 15 hours after hCG injections were given, the oviducts were removed and washed in 3 ml BMOC-3 without BSA in the outside well of a Falcon organ tissue culture dish and then placed in the inside well containing 1 ml BMOC-3. The ampulla portion of the oviducts were torn open to allow the cumulus mass to be released into the medium. The cumulus masses were then transferred to a second petri dish and placed in the center well containing 1 ml BMOC-3. Fifty μl of sperm suspension which had been incubated for 1 hour was then placed in this dish with the eggs and incubated for 24 hours before fertilization was examined. The final sperm concentration at insemination was 1-5 X 10^6 cells/ml.

To examine fertilization, 100 μl of 370 μM bisBenzimide Hoechst No. 33258, a DNA stain, was added to the petri dish containing sperm and eggs and allowed to stain the DNA for 1 hr. The Hoechst stain allows simple observation of the nucleus of the cell (Figure 1). The eggs were then washed by transferring them to 1 ml fresh BMOC-3 and then transferred onto a microscope slide to be scored. Eggs were scored on a Nikon Optiphot microscope equipped with a 100W mercury bulb,

365/10nm excitation filter, 400nm dichroic mirror, and 400nm barrier filter. Embryos containing 2 cells with a nucleus in each cell were scored as fertilized (Figure 2). Embryos with one cell but two pronuclei were also scored as fertilized (Figure 3). Oocytes containing only one cell and one nucleus were scored as non-fertilized (Figure 4). Oocytes that had fragmented or lost their cytoplasm were scored as degenerated (Figure 5).

AchE Assay

A method that permits a rapid and accurate determination of the enzymatic hydrolysis of μmolar/min quantities of Ach was used (Reed, 1966). It is a measurement of ^{14}C-acetic acid formed by hydrolyzing Ach with AchE for a defined length of time after unhydrolyzed substrate is bound with an ion-exchange resin (Amberlite; 100-200 mesh). This assay measures AchE, as well as non-specific ChE activities, thus the result could reflect the presence of both acetyl- and psuedo-ChE activities. The known pseudo ChEs activity that will be counted in this assay could include Butyryl-ChE and Propionyl-ChE. There is, however, no existing information that indicates the existence of these pseudocholinesterases in seminal plasma or sperm (Augustinsson, 1963). The assay, based on the reaction shown below, was used to measure AchE

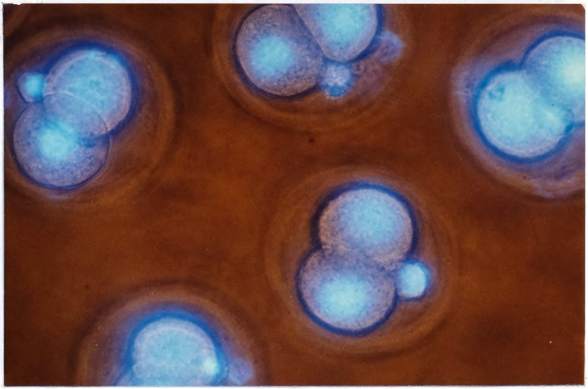

Figure 1. Hoechst staining of mouse embryos (400x, bisBenzimide stain)

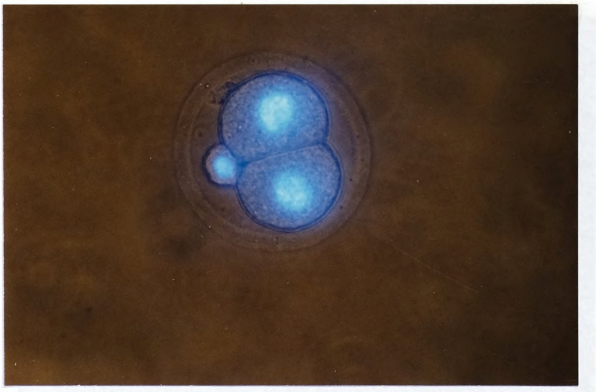

Figure 2. Two cell embryo (400x, bisBenzimide stain)

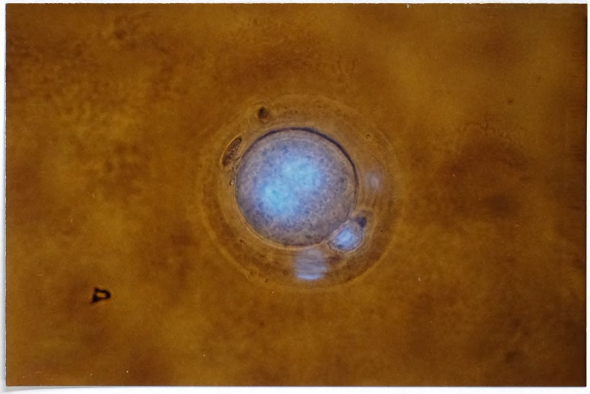

Figure 3. Embryo with one cell and two pronuclei (400x, bisBenzimide)

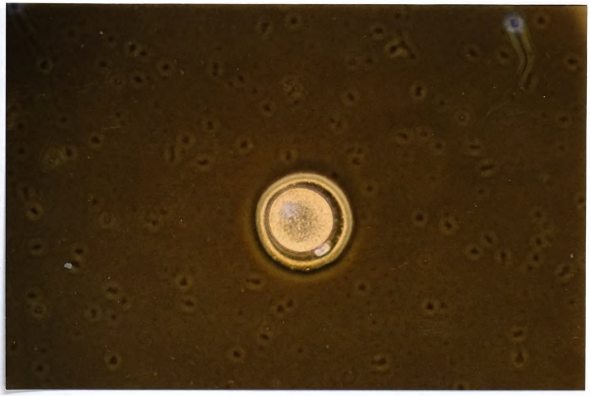

Figure 4. One cell non-fertilized egg (200x, bisBenzimide stain)

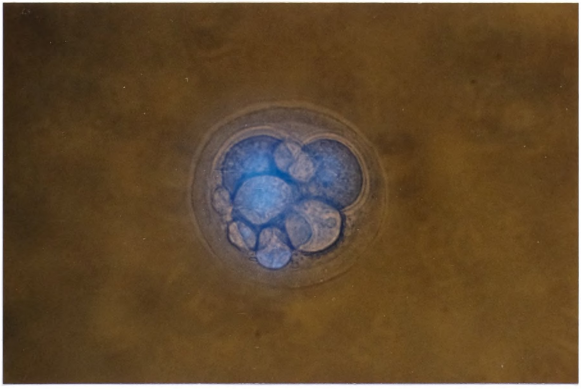

Figure 5. Fragmented egg (400x, bisBenzimide stain)

activity in brains and sperm cells.

Acetyl-1-^{14}C-Choline ------→ ^{14}C-Acetic Acid + Choline

Mouse brains from the long-term, low-dose feeding trial were homogenized in 8 ml sodium phosphate buffer and vortexed. One hundred μl of this solution was then placed in 4.9 ml sodium phosphate buffer and vortexed again. This solution was then kept on ice and used in the subsequent assay. Each brain was measured in duplicate. One hundred μl of the brain homogenate was placed in each of three tubes, A, B, and C. Tube A was boiled for 10 minutes to inactivate the enzyme activity and used as a blank to measure spontaneous hydrolysis of the substrate.

An Ach substrate solution was prepared which contained 5% radioactive (hot) Ach and 95% 3mM non-radioactive (cold) Ach. The hot Ach was made of Ach iodide [acetyl-1-^{14}C] containing 0.05 mCi dissolved in 0.01 N glacial acetic acid. The cold Ach was made of 3 mM Ach iodide dissolved in 0.01 N glacial acetic acid.

One hundred μl of Ach substrate solution was then added to each of the three tubes, A, B, and C and the tubes were incubated at 37°C for 10 minutes to allow the reaction to proceed. The reaction was stopped by adding 300 mg Amberlite resin to each tube.

To extract and count ^{14}C-acetic acid, 5 ml ethanol was

added to each tube and vortexed. Each tube was then centrifuged at 2,100 x g for 5 minutes. Three ml of the supernatant was placed in a scintillation vial containing 10 ml scintillation cocktail and counted by a Searle Isocap/300 scintillation counter.

The assay for enzyme activity in sperm was similar to the procedures described above, except the preparation of the brain homogenate was not performed. Instead, ejaculated boar semen, collected by the gloved hand technique, was washed twice by centrifugation in PBS medium and sperm concentration was counted. This was then placed into three separate tubes, one was treated with 50 μM paraoxon, one was treated with 100 μM paraoxon, and the other was used as a control. One hundred μl of these solutions was then placed in tubes A, B, and C. One hundred μl of the substrate solution was then added to each tube and the procedure described above was followed.

For mouse sperm, six male mice were sacrificed and the epididymides from all six mice were placed in a petri dish containing 1.5 ml PBS medium. The epididymides were then poked with a 25G needle to release sperm into the medium and the concentration was counted. This solution was then split into three separate petri dishes, one was treated with 50 μM paraoxon, one was treated with 100 μM paraoxon, and the other was used as a control. One hundred μl of these solutions was then placed into tubes A, B, and C. One hundred μl substrate solution was then added to each tube and the procedure

described above was followed.

The AchE calculations were as follows:

a) efficiency:

After counting, 100 μl ^{14}C-toluene which contained 40,200 dpm was added to the vials and counted again. The formula for counting efficiency was:

$$\frac{\text{Post } ^{14}\text{C-toluene } \frac{\text{B channel counts}}{\text{time counted}} - \text{Pre } ^{14}\text{C-toluene } \frac{\text{B channel counts}}{\text{time counted}}}{40{,}200}$$

b) dpm/nmole Ach added to each tube

cpm = B channels/time counted
dpm = cpm/efficiency
dpm/nmoles = dpm/total # nmoles Ach in 100 μl Ach substrate solution

c) for brains:

$$\frac{\text{average sample dpm - blank dpm}}{\text{mg brain in the reaction tube}} \times \frac{5/3}{10 \times \text{avg dpm/nmole}}$$

5/3 = counted 3 of 5 ml
10 = counted 10 min. > 1 min.

d) for sperm:

$$\frac{\text{average sample dpm - blank dpm}}{\text{number of cells / ml}} \times \frac{5/3 \times 10^7}{10 \times \text{avg dpm/nmole}}$$

5/3 = counted 3 of 5 ml
10 = counted 10 min. > 1 min.
10^7 = dilution factor

Statistical Analysis

Statistical analysis was performed using the Statistical Analysis System (SAS, 1982). The overall significance of treatment effects on the AchE assays and the *in vitro* fertilization assays were determined by analysis of variance (Gill, 1978) according to the model:

$$Y_{ij} = \mu + \tau_i + E_{(i)j}$$

where μ = population mean, τ_i = treatment effect (fixed), and $E_{(i)j}$ = error (random). The form of the response to treatment was characterized using orthogonal polynomial contrasts (Gill, 1978).

The overall significance of treatment effects on sperm motility assays and capacitation and acrosome assays was determined by analysis of variance (Gill, 1978) according to the model:

$$Y_{ijk} = \mu + \alpha_i + M_{(i)j} + \beta_k + (\alpha\beta)_{ik} + (M\beta)_{(i)jk} + E_{(ijk)}$$

where μ = population mean, α_i = treatment effect (fixed), $M_{(i)j}$ = mice per treatment effect (random, error 1), β_k = time effect, $(\alpha\beta)_{ik}$ = interaction of treatment with time, $(M\beta)_{(i)jk}$ = interaction of mice per treatment with time not separable from $E_{(ijk)}$ = error 2 (random).

All data are presented as mean ± standard deviation.

CHAPTER VII

RESULTS

Phase I: Effects of Paraoxon on Capacitation In Vitro

The *in vitro* effects of 50 and 100 μM paraoxon on mouse sperm capacitation and acrosome reaction were similar to those found by Chou (1987). The treatment had a significant effect on sperm scored as fresh ($p < 0.002$) and sperm scored as capacitated ($p < 0.0002$). A moderate response to treatment was seen in the sperm scored as acrosome reacted ($p < 0.27$).

At 0 time the percentages of the sperm population showing the CTC fluorescent patterns were not affected by either dose of paraoxon. The percent of sperm scored as fresh (Figure 6, Appendix A) began to differ among treatments between 15 and 35 minutes. Sperm scored as fresh in the control group at 35 minutes was 25.0%±4.3, while those scored as fresh in the 50 and 100 μM treatments were 57.7%±4.8 and 60.0±2.9.

Sperm scored as capacitated (Figure 7, Appendix A) in the control group at 35 minutes was 70.7±4.2 while those scored as capacitated in the 50 and 100 μM treatments were 34.7%±2.9 and 34.3%±2.1. This suggests a shift in the population of sperm cells from an early fresh state to a capacitated state around

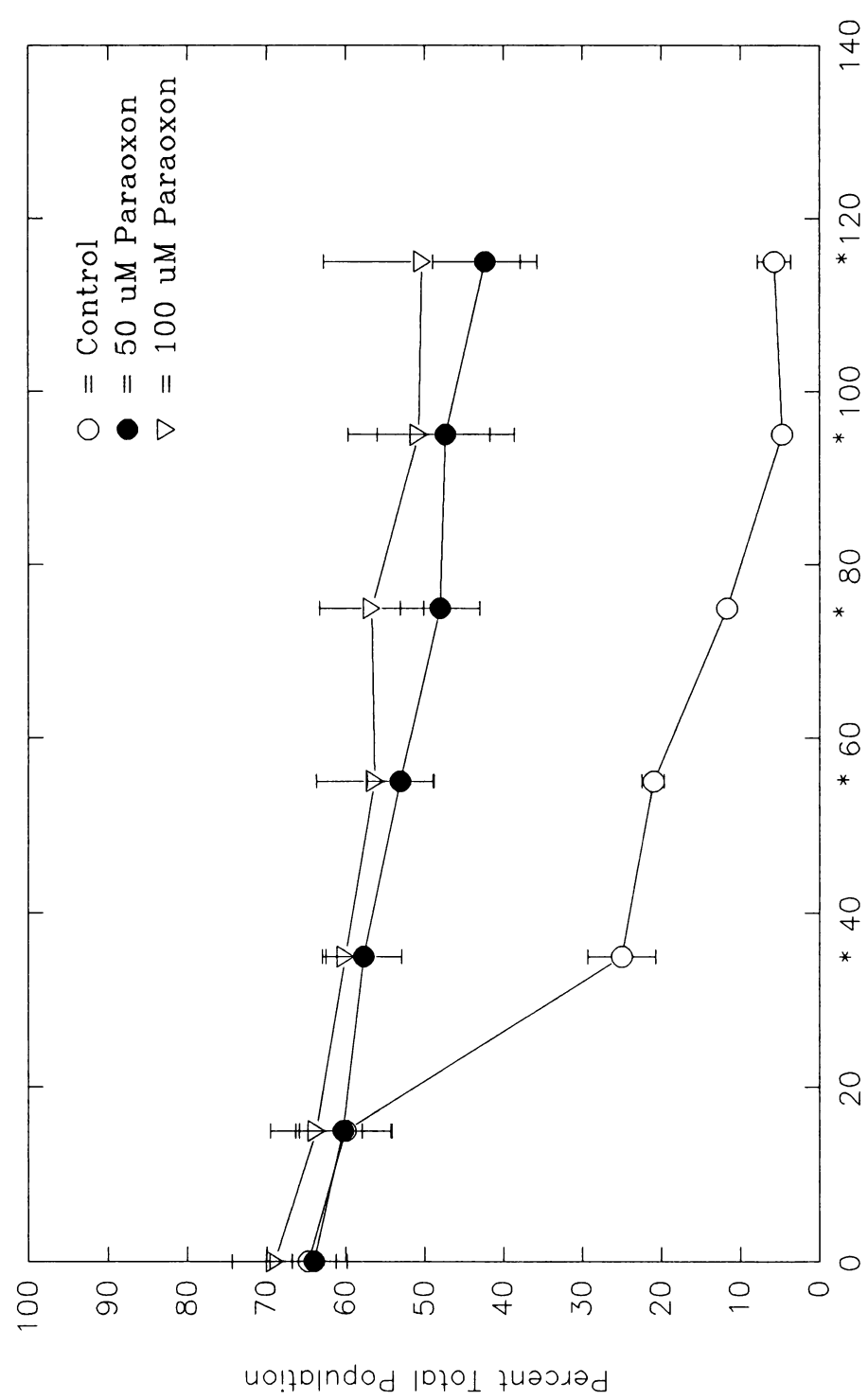

Figure 6. Time course of the changes in the percentage of sperm showing Fresh CTC fluorescence patterns.
* = Significantly different at this time point ($p<0.002$).

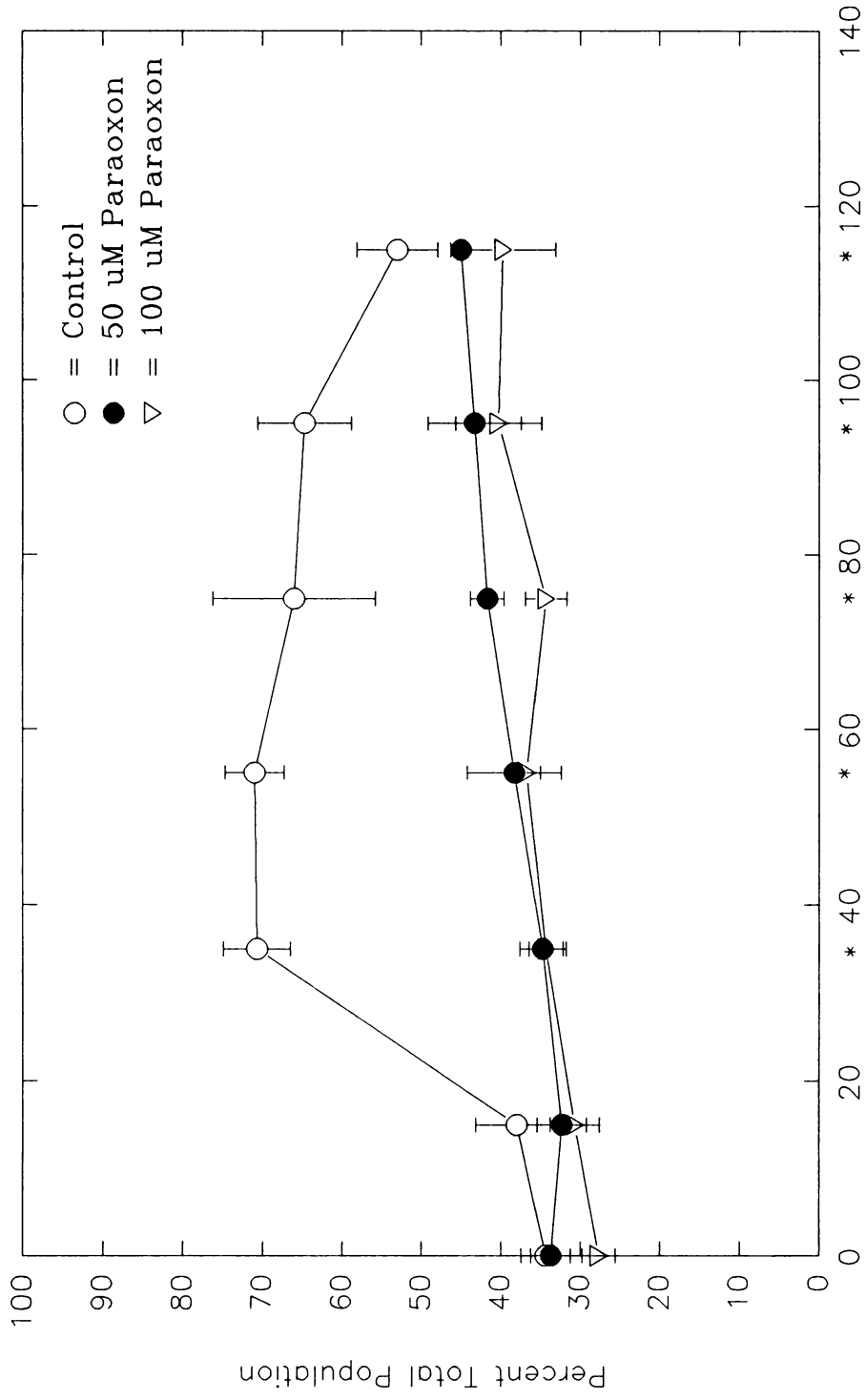

Figure 7. Time course of the changes in the percentage of sperm showing Capacitated CTC fluorescence patterns.
* = Significantly different at this time point ($p<0.05$).

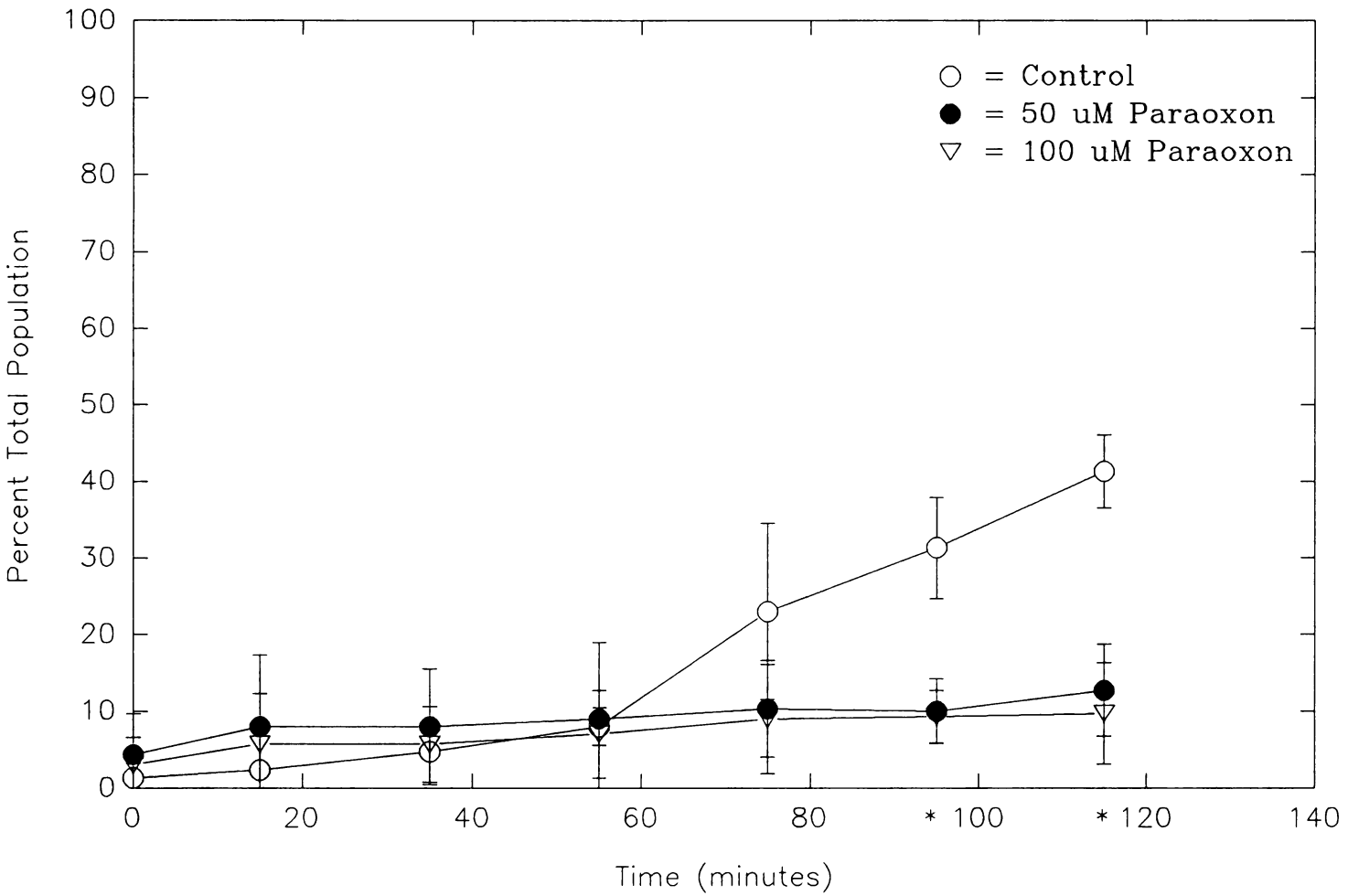

Figure 8. Time course of the changes in the percentage of sperm showing Acrosome Reacted CTC fluorescence patterns.
* = Significantly different at this time point ($p<0.005$).

35 minutes in the control.

The sperm scored as acrosome reacted (Figure 8, Appendix A) in the control reached a high of 41.3%±4.8 at 115 minutes. Sperm scored as acrosome reacted in the 50 and 100 μM treatments groups remained low reaching a high of 12.7%±6.0 and 9.7%±6.6 at 115 minutes. These changes reflect a shift in the population of sperm cells from a capacitated state to an acrosome reacted state in the control. Sperm in both the 50 and 100 μM paraoxon treatments lacked this shift to acrosome reacted cells.

Phase II: AchE Activity of Mouse and Boar Sperm in the Presence of Paraoxon

After exposure to 0, 50, and 100 μM paraoxon in vitro, the activity of AchE in mouse sperm was measured. In the control, 5.95 nmoles Ach/min/10^8 sperm were hydrolyzed (Figure 9, Appendix B). The sperm treated with 50 μM paraoxon hydrolyzed 2.41 nmoles Ach/min/ 10^8 sperm. The sperm treated with 100 μM paraoxon hydrolyzed 1.54 nmoles Ach/min/10^8 sperm. A linear dose-response relationship was observed in the effect of paraoxon on mouse sperm AchE activity, ($p < 0.01$).

The AchE activity of boar sperm treated with paraoxon was also measured (Figure 10, Appendix C). The control boar sperm hydrolyzed 8.54 nmoles Ach/min/10^8 sperm. The sperm treated with 50 μM paraoxon hydrolyzed 0.27 nmoles Ach/min/10^8 sperm.

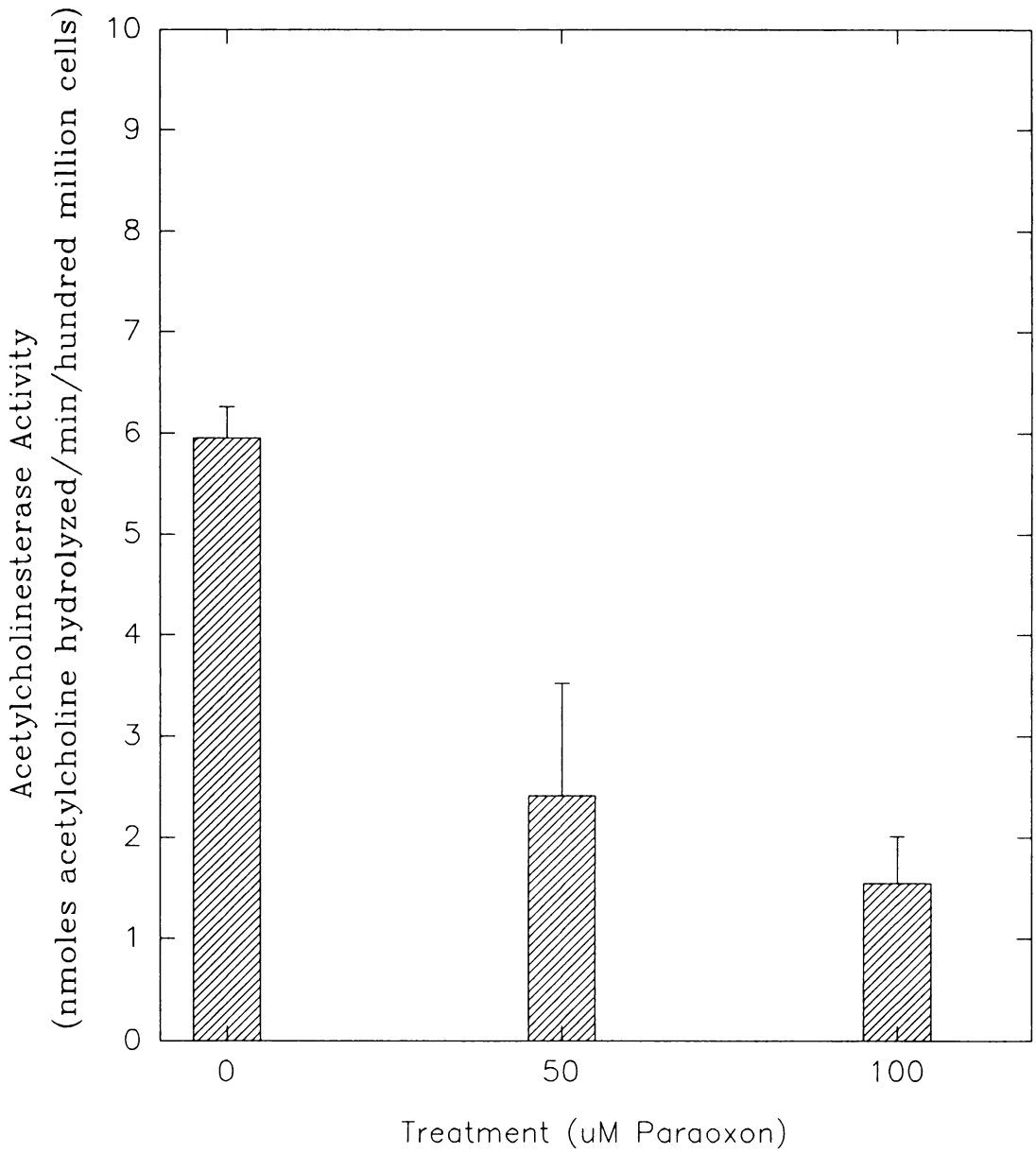

Figure 9. Acetylcholinesterase activity of mouse sperm treated in vitro with 0, 50, or 100 uM paraoxon ($p<0.01$).

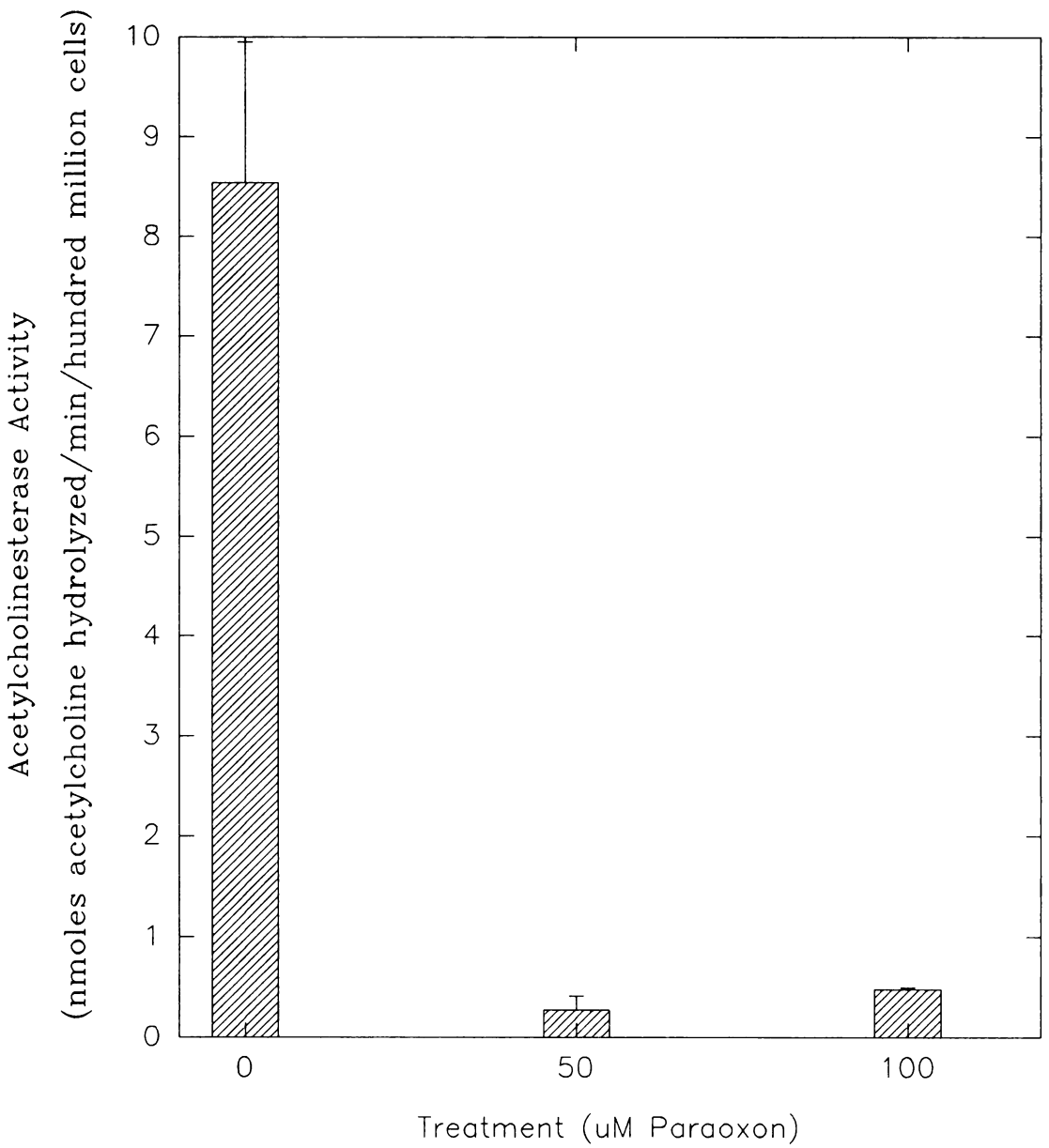

Figure 10. Acetylcholinesterase activity of boar sperm treated in vitro with 0, 50, or 100 uM paraoxon ($p<0.01$).

The sperm treated with 100 μM paraoxon hydrolyzed 0.48 nmoles Ach/min/10^8 sperm. Significant AchE inhibition was observed in boar sperm exposed to both doses of paraoxon. Unlike the mouse sperm dose-response relationship, the boar sperm response was not linear to the doses tested ($p < 0.01$).

Upon measurement of the Ach hydrolysis in the seminal plasma, it was found that the boar seminal plasma contained very high levels of AchE activity. The activity in the seminal plasma was 110.01 nmoles/min/100 μl. On the average, boar semen contains 2.5 X 10^8 sperm cells/100 μl seminal plasma. In comparison to the enzyme activity of sperm cells contained in 100 μl seminal plasma, 5.95 nmoles/min/100 μl, the seminal plasma contains a high proportion of the enzyme activity in the semen.

Phase III: Effect of Dietary Mixtures of OPs on Brain AchE Activity and Sperm Quality

Preliminary Studies

A total of 40, 400, 4,000, or 40,000 μg of the mixture of OPs/kg body weight/day were given in ground Mouse Chow to young mature male mice, two animals/treatment, for 7 days. The animals were then terminated and the brain AchE activity was measured. In the brain of the control mice, the activity was 8.26 ±0.83 nmoles/min/mg brain while in those from the treatment groups the activities were 6.48 ±0.30, 5.30 ±1.53,

3.25 ±0.98, and 2.11 ±0.15 nmoles/min/mg brain from the lowest dose to the highest respectively (Figure 11, Appendix D). This indicated that all doses tested induced an observable effect on the brain AchE activity. The nature of the response to treatment was significant and linear ($p < 0.001$). Based on these results, 40 μg/kg BW/day, which is 100 times the possible adult exposure, was included in the eight month feeding trial as the high dose.

Long-Term Dietary Exposure

The mice fed the OP mixture for eight months at doses of 0, 0.4/0.15 μg/kg BW/day, 4.0/1.5 μg/kg BW/day, and 40.0/15.0 μg/kg BW/day were studied for sperm quality. Capacitation, acrosome reaction, fertilizing ability, and motility of the sperm were measured. Brain AchE levels were also measured.

Capacitation

In the capacitation and acrosome reaction studies the sperm from the OP-treated animals progressed through capacitation and acrosome reaction as seen in the control. The response to treatment of the sperm scored as fresh was not significant ($p > 0.65$), nor was the response to treatment of the sperm scored as capacitated ($p > 0.97$) or of the sperm scored as acrosome reacted ($p > 0.75$). The percent of sperm scored as fresh (Figure 12, Appendix E) reached baseline between 95 and 115 minutes in all treatments, 5.3%±3.1 in the

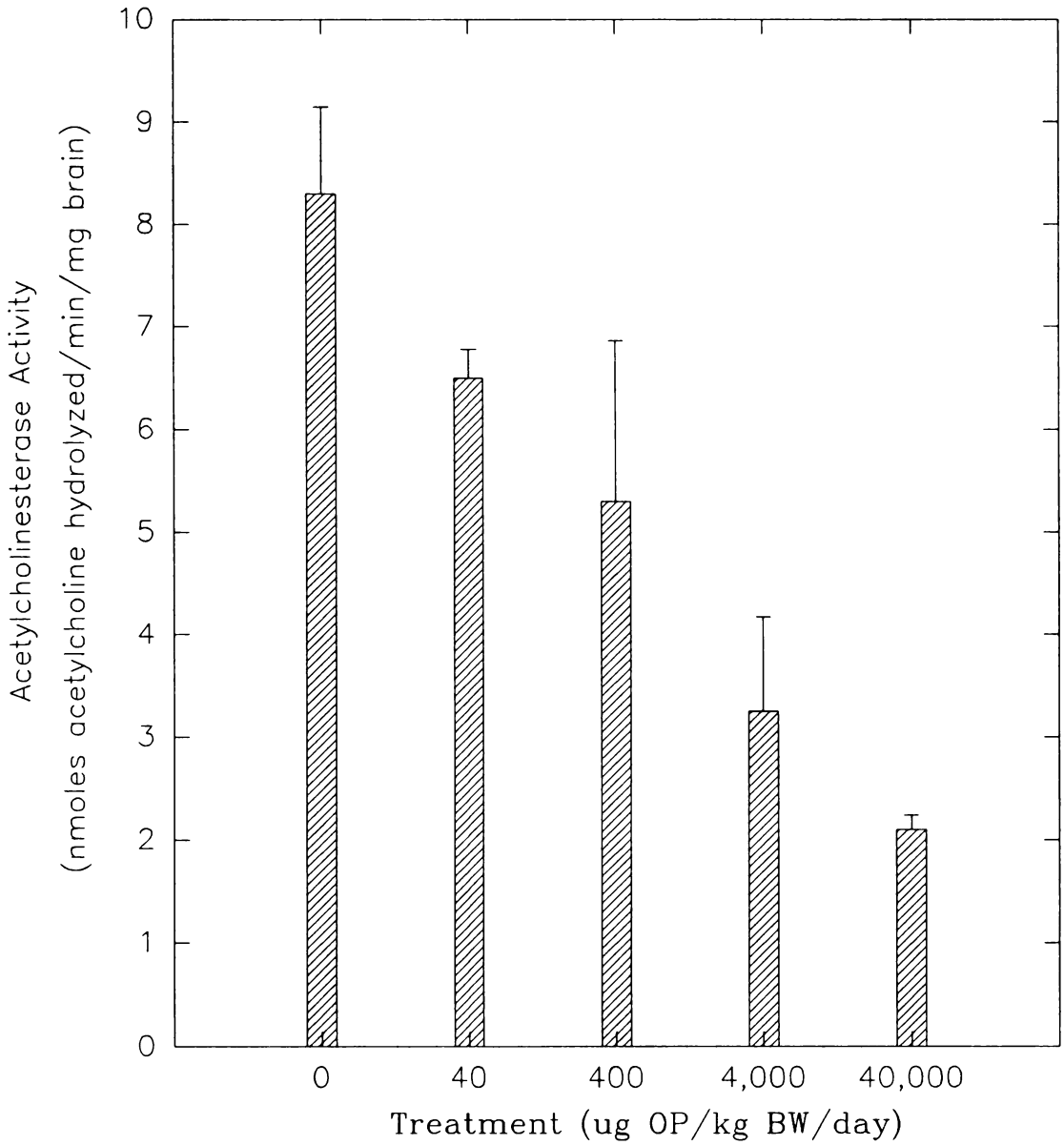

Figure 11. Acetylcholinesterase activity of brains from mice fed with OP mixture for seven days ($p<0.001$).

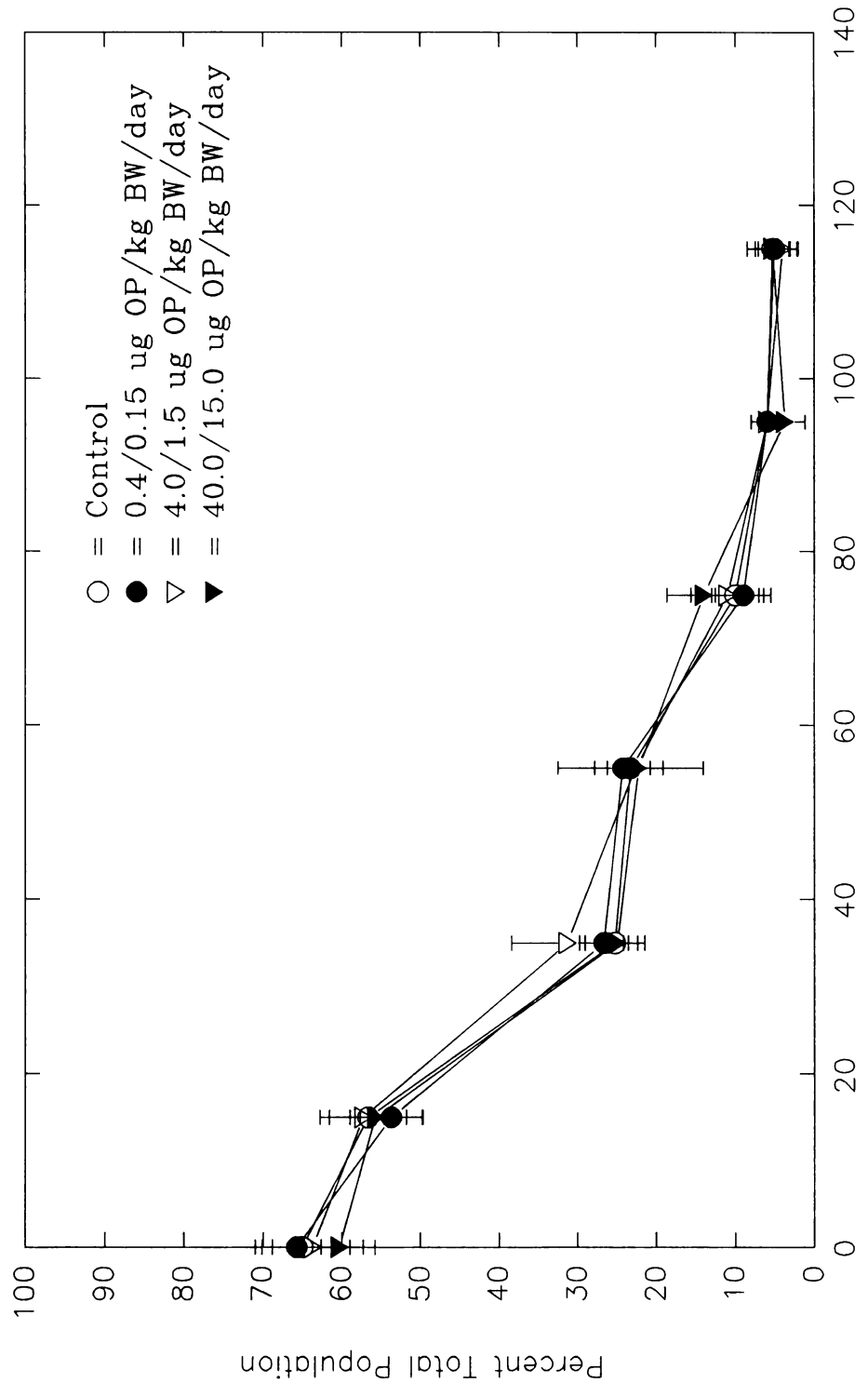

Figure 12. Time course of the changes in the percentage of sperm showing Fresh CTC fluorescence patterns.

control, 5.0%±2.0 in the 0.4/0.15 μg/kg BW/day dose, 4.0%±2.0 in 4.0/1.5 μg/kg BW/day dose, and 3.7%±2.5 in the 40.0/15.0 μg/kg BW/day dose.

The percent of sperm scored as capacitated (Figure 13, Appendix E) peaked between 55 and 75 minutes in the control, 0.4/0.15 μg/kg BW/day, 4.0/1.5 μg/kg BW/day, and 40.0/15.0 μg/kg BW/day doses at 66.3%±2.9, 68.7%±3.8, 68.3%±1.5, and 68.0%±5.0, respectively.

The percent of sperm scored as acrosome reacted (Figure 14, Appendix E) peaked at 115 minutes in the control, 0.4/0.15 μg/kg BW/day, 4.0/1.5 μg/kg BW/day, and 40.0/15.0 μg/kg BW/day doses at 51.0%±4.4, 51.3%±4.9, 51.7%±4.0, and 52.0±7.0%, respectively.

Fertilizing Ability

The number of oocytes harvested from each female and the total number of eggs observed in each treatment group are shown in Table 2. The results of the in vitro fertilization experiments are shown in Figure 15 (Appendix F). Sperm from the control, 0.4/0.15 μg/kg BW/day dose, 4.0/1.5 μg/kg BW/day dose, and 40.0/15.0 μg/kg BW/day dose treatment groups fertilized 82.7% ±4.8, 78.3% ±4.7, 82.0% ±4.9, and 78.0% ±4.9 of the oocytes, respectively. No effects on fertilization by these treatments was observed ($p > 0.30$).

Motility

Motility of the sperm from treated males was measured at

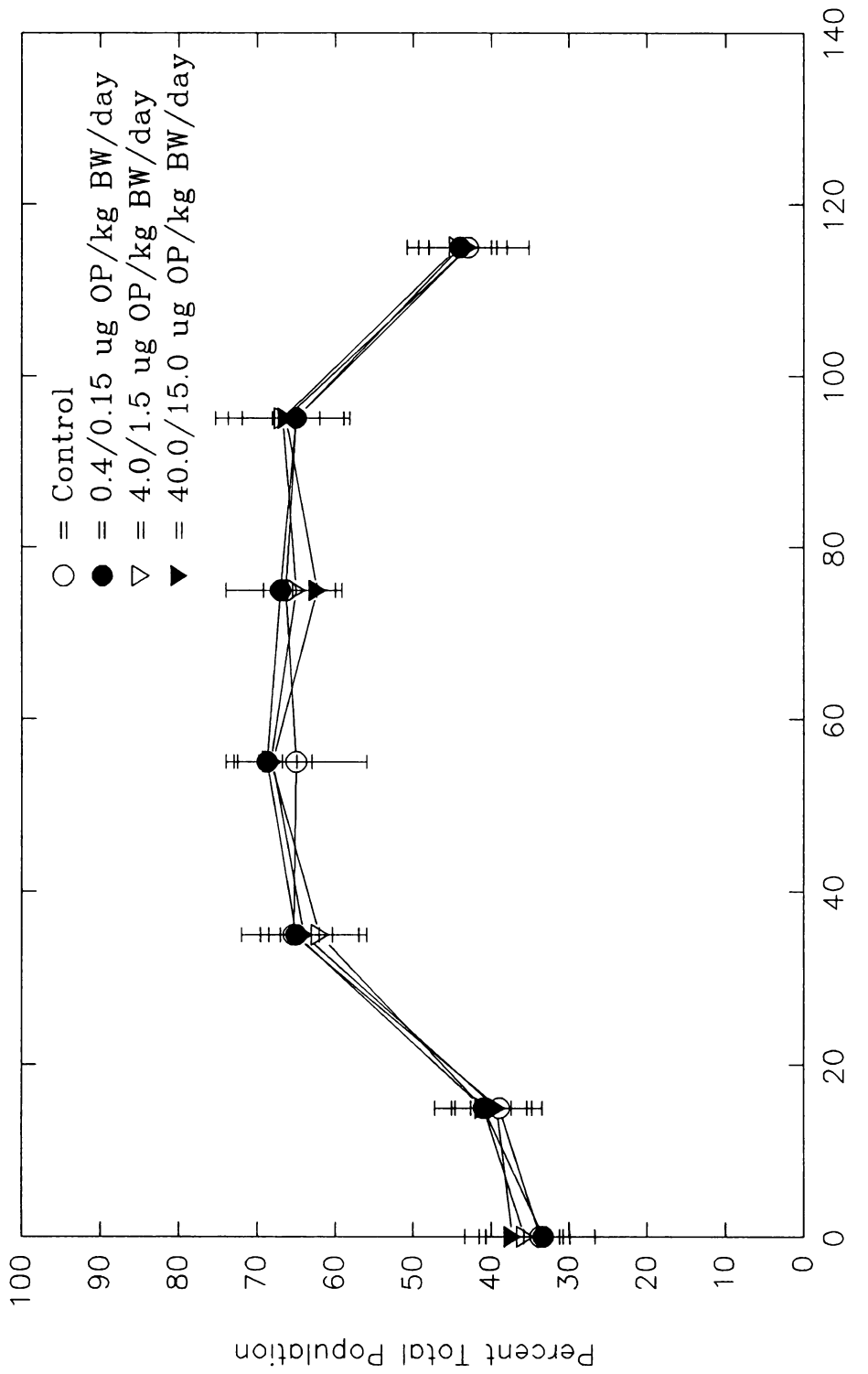

Figure 13. Time course of the changes in the percentage of sperm showing Capacitated CTC fluorescence patterns.

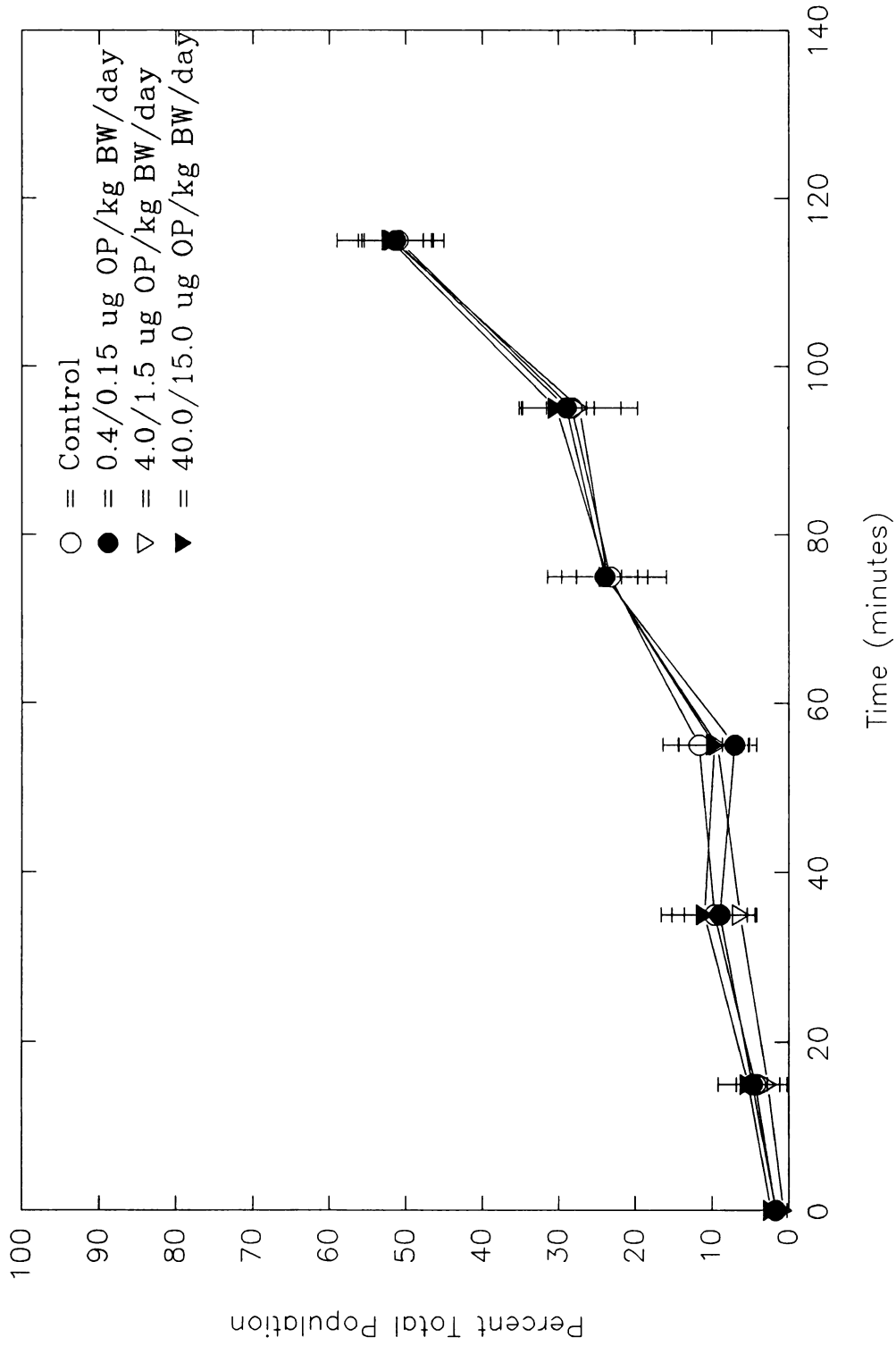

Figure 14. Time course of the changes in the percentage of sperm showing Acrosome Reacted CTC fluorescence patterns.

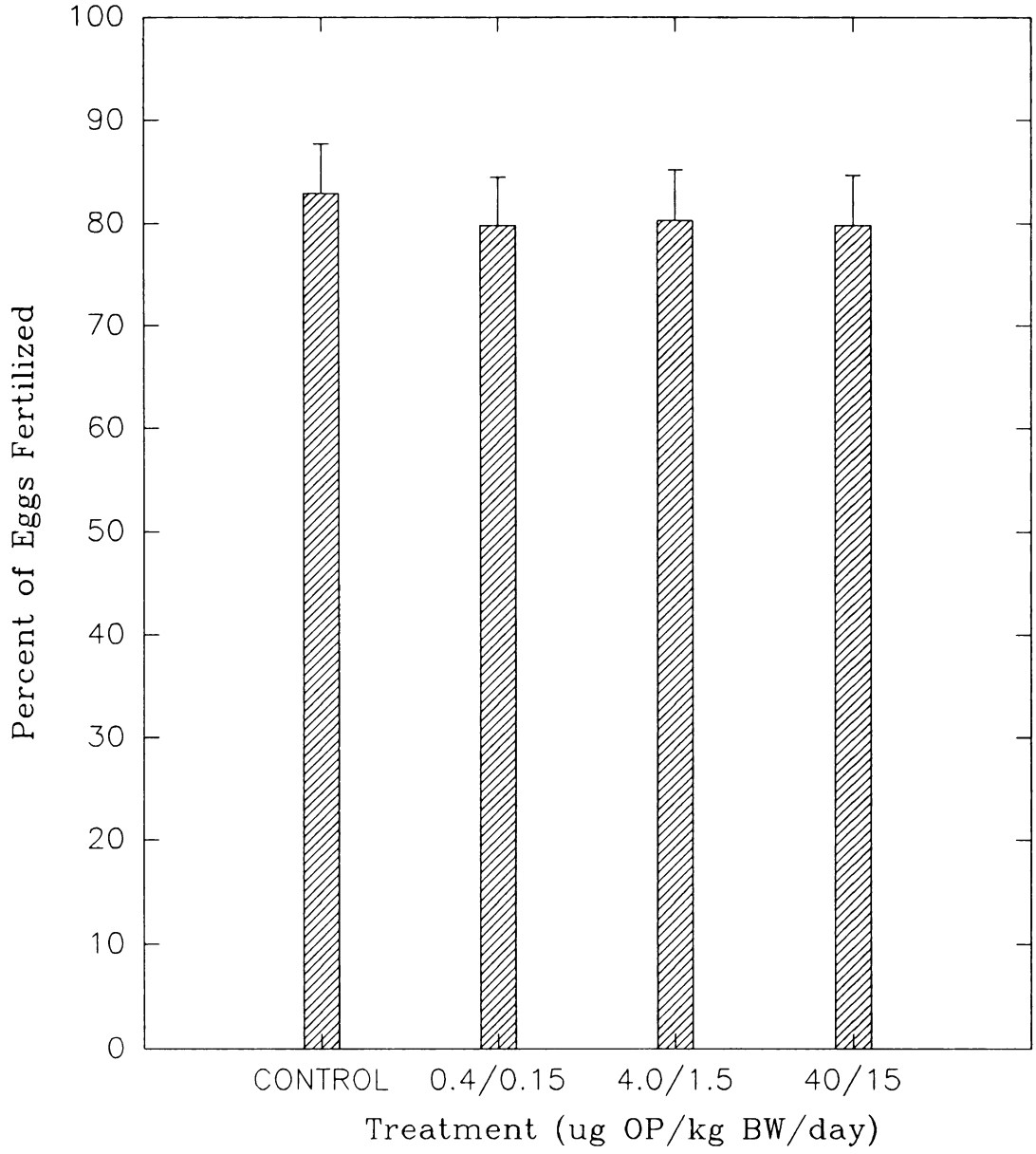

Figure 15. In vitro fertilizing ability of sperm from males fed with OP mixture for eight months.

0, 45, and 95 minutes after collection. The results are shown in Table 3. No effect of treatment on motility was observed ($p > 0.50$).

Table 2. Number of Oocytes Collected from Female Mice[1] in *In Vitro* Fertilization Assays.

Treatment of Males Used to Inseminate Oocytes	# of Oocytes Collected from Each Female Mouse
Control	40
	40
	29
	37
	36
	53
	Total 235
0.4/0.15 µg/kg BW/day	21
	47
	34
	44
	39
	57
	Total 242
4.0/1.5 µg/kg BW/day	27
	35
	49
	35
	26
	43
	Total 215
40.0/15.0 µg/kg BW/day	26
	36
	53
	46
	27
	34
	Total 222

[1] All females are non-treated.

Table 3. Mean sperm motility[1] of the OP-treated males measured *in vitro*.

Treatment	Sampling time (minutes[2]) 0	45	95
Control	81.0±4.4	69.7±11.2	59.7±6.4
0.4/0.15 μg/kg BW/day	78.3±5.9	68.3±10.1	55.0±5.0
4.0/1.5 μg/kg BW/day	75.3±5.1	59.7±6.4	58.3±2.1
40.0/15.0 μg/kg BW/day	80.7±3.8	68.0±2.6	64.3±2.3

[1] Percent of motile sperm ± standard deviation.
[2] Minutes after sperm collection.

Brain AchE Activity

The brain AchE activity in the animals fed the long-term, low-dose OP diets was measured after the eight month feeding trial. The brains from the control animals showed an activity of 6.20±0.02 nmoles Ach/min/mg brain (Figure 16, Appendix G). The brains from the 0.4/0.15 μg/kg BW/day treatment group showed an activity of 5.27±0.54 nmoles Ach/min/mg brain. The brains from the 4.0/1.5 μg/kg BW/day treatment group showed an activity of 3.68±0.65 nmoles Ach/min/mg brain. The brains from the 40.0/15.0 μg/kg BW/day treatment group showed an activity of 3.89±1.06 nmoles Ach/min/mg brain. The nature of the response to treatment was significant and linear ($p > 0.002$).

Six weeks after withdrawal from the OP treatments the

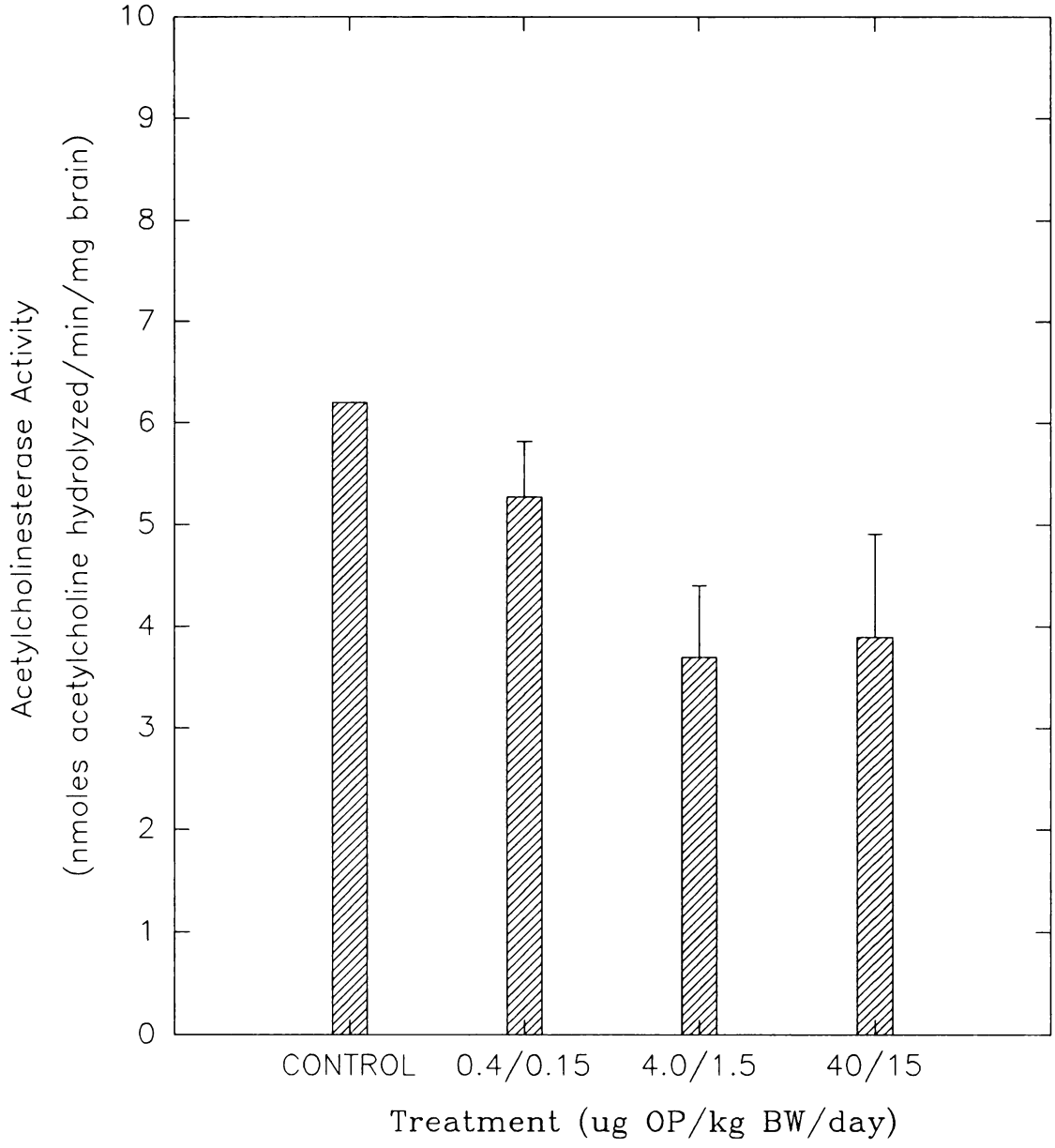

Figure 16. Brain acetylcholinesterase activity of mice fed with OP mixture for eight months ($p<0.002$).

animals were measured for brain AchE activity to determine any possible recovery of AchE activity. The brain enzyme activity appeared to almost completely recover from the inhibition (Figure 17, Appendix H). AchE activity was 8.55±0.16 nmoles Ach/min/mg brain in the control, 8.61±0.60 nmoles Ach/min/mg brain in the 0.4/0.15 μg/kg BW/day treatment group, 7.8±0 nmoles Ach/min/mg brain in the 4.0/1.5 μg/kg BW/day treatment group, and 8.72 (one animal) nmoles Ach/min/mg brain in the 40.0/15.0 μg/kg BW/day treatment group. At the time of the measurement, no difference in enzyme activity was observed among the treatment groups ($p > 0.85$).

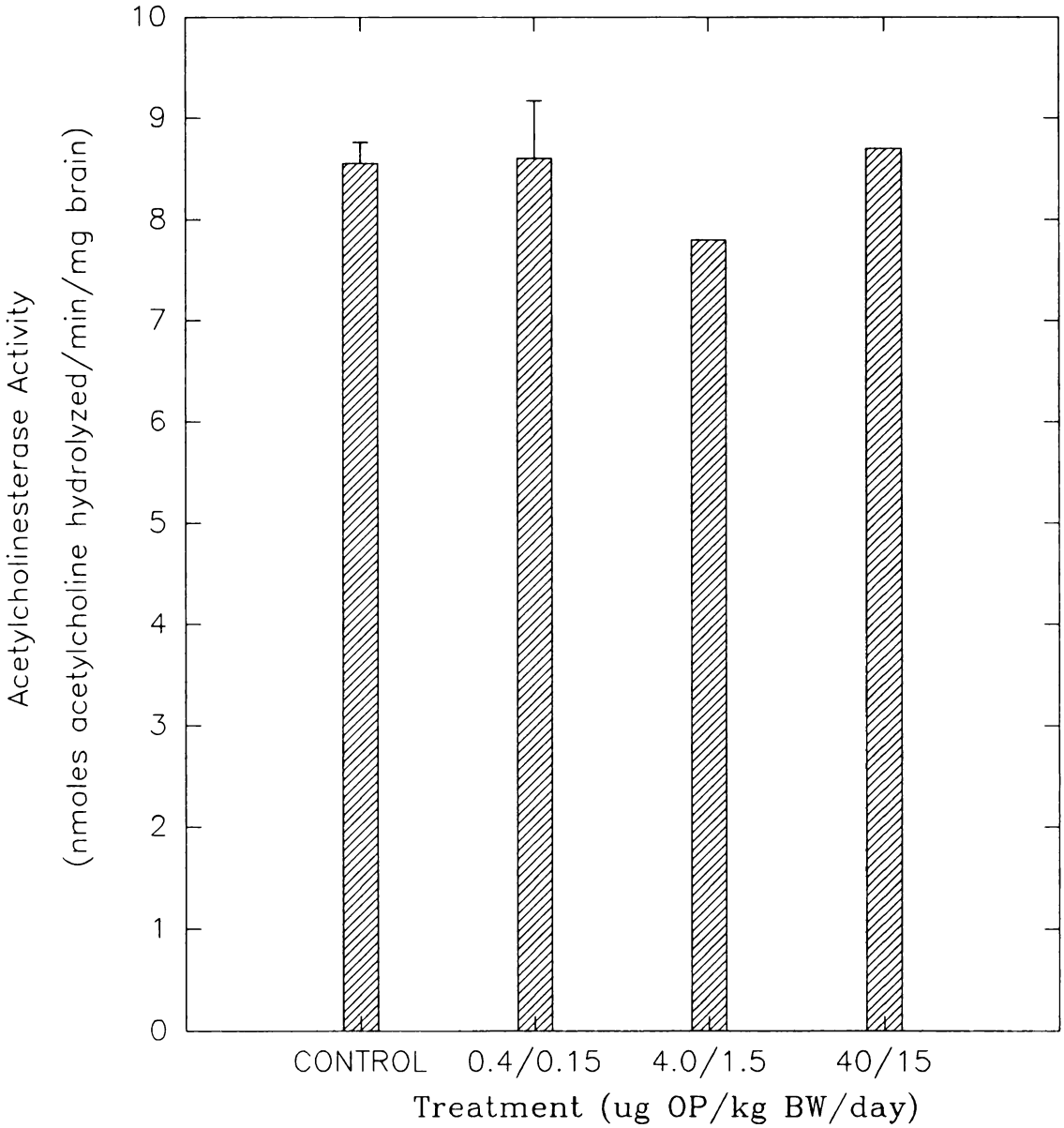

Figure 17. Brain acetylcholinesterase activity of mice six weeks after withdrawal from long-term OP exposure.

CHAPTER VIII

DISCUSSION

Both Chou's (1987) and the current study demonstrated that paraoxon interacts directly with the sperm and prevents the sperm from progressing through capacitation and, in turn, acrosome reaction.

An interesting experiment by Chou (1987) demonstrated that the inhibitory effect of paraoxon on sperm was limited to treatment of the sperm *in vitro* within the first 10 minutes after collection. Sperm treated with paraoxon after 10 minutes of incubation did not show the characteristic inhibition of capacitation as seen in the current study. This indicated that the initiation of capacitation begins before 10 minutes after collection. Once the capacitation is initiated, paraoxon toxicity can no longer be observed.

AchE has been shown to regulate ion transport in non-nervous tissue such as erythrocytes and muscular tissue (Sastry and Asdavongvivad, 1979). In muscular tissue, AchE binding to the membrane causes the depolarization of the membrane leading to eventual muscular contraction. In sperm cells, AchE could play a role in sperm motility, acting on the actin and myosin found in the tail of the sperm cell. This

hypothesis is supported by the fact that the relative activity of AchE in the tail portion of bull sperm is 5 times that found in the head (Nelson, 1964). In addition, the presence of the enzyme in the head portion of sperm along with our laboratory observations suggest that AchE or an AchE-like enzyme may be involved in the capacitation process.

The in vitro toxicity of paraoxon in sperm is very important in that it could have major implications on the understanding of the control of sperm fertilizing ability. It could also be very important in developing methods to increase the effectiveness of reproduction in animal agriculture.

The measurement of AchE activity in mouse and boar sperm in vitro also showed some interesting results. The species comparison, a comparison of AchE activity of controls, revealed 30% more activity in the boar sperm than that in the mouse sperm. The ability of paraoxon to inhibit AchE activity was much greater in the boar sperm than in the mouse sperm, suggesting a higher sensitivity of boar sperm to OP toxicity than mouse sperm. The dose response of boar sperm hydrolytic activity also suggests the presence of isomers of Ach hydrolytic enzymes. This species variation in the susceptibility to OP-induced toxicity in sperm could be a concern in the animal industry when toxicity data in laboratory animals are used to assess chemical risk in livestock.

Results from this study showed that AchE activity is much

higher in the seminal plasma than in the boar sperm. Perhaps, in the boar, additional AchE is introduced to the sperm in the seminal plasma at ejaculation to stimulate motility or capacitation. Withholding the high AchE until ejaculation could be a natural mechanism for delaying the progress of sperm through capacitation and acrosome reaction until mating. If this is the case, the development of a method to delay the AchE activity after ejaculation may be used to extend the boar sperm longevity during storage.

The feeding trial was designed to determine whether the inhibition of capacitation seen in vitro by paraoxon could also been seen in mice fed a mixture of OPs at the dose that humans or animals may receive in their normal diet, 10 times that dose, and 100 times that dose. The long-term, low-dose OP treatments had no inhibitory effect on sperm quality in mice based on the parameters measured in this study. Consumption of the diet did, however, inhibit the AchE activity in the brains of the treated mice, including those in the 0.4/0.15 μg/kg BW/day treatment group, which was equivalent to the dietary exposure reported by the FDA.

In the in vivo study, the parent compounds were used, while in the in vitro study, the more toxic metabolite of parathion, paraoxon was used. The lack of an observable effect on sperm quality from the in vivo treatment could be due to a low concentration of active OP metabolite that reached the male reproductive organ.

The treatment diets for the *in vivo* feeding trial were designed from weaning to 3 months based on a 19.22 g mouse consuming 3 g of feed per day and from 3 months to 8 months based on a 32.56 g mouse consuming 5 g of feed per day. The consumption of feed during the trial, however, was not measured. It should be noted that this could have resulted in the exposure of the animals through their diet to a dose different from those in the original design.

Six weeks after withdrawal from OP exposure, the mice from each treatment group had almost completely recovered from the AchE inhibition. This suggests that long-term, low-dose OP exposure does not have a permanent effect on brain AchE activity at the levels tested. However, the ADI for the most toxic OP in the feeding trial, parathion, is 5 μg/kg BW/day. When the test dose, 10 times less than the ADI, caused inhibition of brain AchE in mice, there should be concern and further studies in long-term effects of OPs in the central nervous system.

CHAPTER IX

CONCLUSIONS

Fifty and 100 μM paraoxon inhibited capacitation and the acrosome reaction *in vitro* in mouse sperm. These doses also inhibited the AchE activity of mouse and boar sperm when treated *in vitro*. An unexpectedly high activity of Ach hydrolysis was observed in boar seminal plasma.

Based on the parameters measured in this study, long-term, low-dose exposure to OPs at the levels tested did not cause any observable effects on mouse sperm quality. Long-term, low-dose exposure, however, inhibited brain AchE activity in the treated mice.

The ability of OPs to inhibit sperm capacitation *in vitro* is an important model in studying mechanisms of sperm capacitation and sperm fertilizing ability. Sperm AchE activity is susceptible to paraoxon inhibition at the concentration that inhibited sperm capacitation and the acrosome reaction. The actual role of AchE in sperm fertilizing ability remains for investigation.

APPENDICES

APPENDIX A

Time course of the changes of sperm showing CTC fluorescence patterns.

Treatment	Time (min)	Fresh	Capacitated	Acr. Reac.
Control	0	64.7±4.9	34.3±3.1	1.3±1.9
	15	60.0±5.9	38.0±5.1	2.3±1.2
	35	25.0±4.3	70.7±4.2	4.7±0.5
	55	21.0±1.4	71.0±3.7	8.0±2.4
	75	11.7±1.2	66.0±10.2	23.0±11.5
	95	4.7±1.2	64.7±5.9	31.3±6.6
	115	5.7±2.1	53.0±5.1	41.3±4.8
50 μM	0	64.0±2.8	33.7±2.5	4.3±5.4
	15	60.3±6.0	32.3±3.1	8.0±9.3
	35	57.7±4.8	34.7±2.9	8.0±7.5
	55	53.0±4.2	38.3±5.9	9.0±9.9
	75	48.0±5.0	41.7±2.1	10.3±6.3
	95	47.3±8.7	43.3±5.9	10.0±4.2
	115	42.3±6.6	45.0±0.8	12.7±6.0
100 μM	0	69.0±5.4	27.7±2.1	3.0±3.6
	15	63.7±5.8	30.7±3.1	5.7±6.6
	35	60.0±2.9	34.3±2.1	5.7±4.9
	55	56.3±7.4	36.7±1.7	7.0±5.7
	75	56.7±6.6	34.3±2.6	9.0±7.1
	95	50.7±9.0	40.3±5.4	9.3±3.4
	115	50.3±12.5	39.7±6.6	9.7±6.6

APPENDIX B

Acetylcholinesterase activity of mouse sperm treated in vitro with 0, 50, or 100 μM paraoxon.

Treatment	AchE Activity (nmoles/min/10^8 sperm)	Mean ± S.D.
Control	6.17 5.73	5.95±0.31
50 μM	1.62 3.19	2.41±1.11
100 μM	1.87 1.20	1.54±0.47

APPENDIX C

Acetylcholinesterase activity of boar sperm treated in vitro with 0, 50, or 100 μM paraoxon.

Treatment	AchE Activity (nmoles/min/10^8 sperm)	Mean ± S.D.
Control	7.54 9.53	8.54±1.41
50 μM	0.37 0.17	0.27±0.14
100 μM	0.46 0.49	0.48±0.02

APPENDIX D

Acetylcholinesterase activity of brains from mice fed with OP mixture for seven days.

Treatment	AchE activity (nmoles/min/mg brain)	Mean ± S.D.
Control	8.85 7.67	8.26±0.83
40 μg/kg BW/day	6.27 6.69	6.48±0.30
400 μg/kg BW/day	4.22 6.38	5.30±1.53
4,000 μg/kg BW/day	3.94 2.56	3.25±0.98
40,000 μg/kg BW/day	2.00 2.21	2.11±0.15

APPENDIX E

Time course of the changes in the percentage of sperm showing CTC fluorescence patterns.

Treatment	Time (min)	Fresh	Capacitated	Acr. Reac.
Control	0	65.0±6.0	33.7±7.0	1.7±1.5
	15	56.7±2.3	39.0±3.6	4.3±1.5
	35	25.3±3.8	65.3±3.2	9.7±5.5
	55	23.3±9.2	65.0±9.0	11.7±4.7
	75	10.0±3.0	66.3±2.9	23.3±1.5
	95	6.0±1.0	65.0±6.9	28.3±6.4
	115	5.3±3.1	43.0±5.0	51.0±4.4
0.4/1.5	0	65.7±3.1	33.3±2.5	1.7±1.2
μg/kg BW/day	15	53.7±4.0	41.0±3.6	4.7±4.5
	35	26.7±3.1	65.0±4.6	9.0±4.6
	55	24.3±3.5	68.7±3.8	7.0±1.7
	75	9.0±3.5	67.0±7.0	24.0±5.6
	95	6.0±2.0	65.0±3.0	29.0±2.6
	115	5.0±2.0	44.0±4.0	51.3±4.9
4.0/1.5	0	63.7±6.4	35.7±5.8	0.7±1.2
μg/kg BW/day	15	57.3±5.5	41.0±6.2	2.7±1.5
	35	31.3±7.1	62.0±5.0	6.3±2.1
	55	22.7±3.5	68.3±1.5	9.3±5.1
	75	11.0±4.6	65.0±2.6	23.7±4.0
	95	6.0±1.0	66.7±8.6	27.3±7.6
	115	4.0±2.0	44.3±5.0	51.7±4.0
40.0/15.0				
μg/kg BW/day	0	60.3±4.5	37.3±6.1	2.3±1.2
	15	55.7±5.9	39.3±5.8	5.3±1.5
	35	25.0±2.6	64.0±8.0	11.0±5.6
	55	22.3±1.5	68.0±5.0	9.7±4.6
	75	14.0±4.6	62.3±3.2	23.7±7.8
	95	3.7±2.5	66.3±7.4	30.3±4.9
	11	5.3±2.1	43.0±7.8	52.0±7.0

APPENDIX F

In vitro fertilizing ability of sperm from males fed with OP mixture for eight months.

Treatment	#fert./total	Mean±SD % fertility/treatment
Control	36/40-90%	82.7±4.8
	34/40-85%	
	22/29-76%	
	29/37-78%	
	31/36-86%	
	43/53-81%	
0.4/0.15	15/21-71%	78.3±4.7
µg/kg BW/day	40/47-85%	
	27/34-79%	
	36/44-82%	
	29/39-74%	
	45/57-79%	
4.0/1.5	23/27-85%	82.0±4.9
µg/kg BW/day	26/35-74%	
	38/49-78%	
	30/35-86%	
	23/26-88%	
	35/43-81%	
40.0/15.0	20/26-77%	78.0±4.9
µg/kg BW/day	28/36-68%	
	44/53-83%	
	37/46-80%	
	21/27-78%	
	28/34-82%	

APPENDIX G

Brain acetylcholinesterase activity of mice fed with OP mixture for eight months.

Treatment	AchE activity (nmoles/min/mg brain)	Mean ± S.D.
Control	6.21 6.21 6.18	6.20±0.02
0.4/0.15 μg/kg BW/day	4.73 5.80 5.27	5.27±0.54
4.0/1.5 μg/kg BW/day	4.35 3.65 3.05	3.68±0.65
40.0/15.0 μg/kg BW/day	3.69 5.04 2.95	3.89±1.06

APPENDIX H

Brain acetylcholinesterase activity of mice six weeks after withdrawal from long-term OP exposure.

Treatment	AchE activity (nmoles/min/mg brain)	Mean±S.D.
Control	8.43 8.66	8.55±0.16
0.4/0.15 μg/kg BW/day	8.18 9.03	8.61±0.60
4.0/1.5 μg/kg BW/day	7.80 7.80	7.80±0
40.0/15.0 μg/kg BW/day	8.72	8.72

LIST OF REFERENCES

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Applegate, A. and Nelson, L. "Acetylcholinesterase in Mytilus Spermatozoa." Biol. Bulletin, 123 (1962), 475.

Augustinsson, K.B. "Classification and Comparative Enzymology of the Cholinesterases and Methods for their Determination." In: Cholinesterases and Anticholinesterase Agents. Ed. Eichler, O., and Farah, A., Berlin: Springer Ver lag, 1963, pp. 89-128.

Austin, C.R. "The Capacitation of Mammalian Sperm" Nature, (1952) 170, 326.

Barros, C., Bedford, J.M., Franklin, L.E., and Austin, C.R. "Membrane Vesiculation as a Feature of the Mammalian Acrosome Reaction." J. Cell Biol., 34 (1967), C1-C5.

Beck, S. D. "Effect of Insecticides on the Metabolism and Motility of Mammalian Spermatozoa." J. Econ. Entomol., 46(4) (1953), 570-574.

Bedford, J.M. "Sperm Capacitation and Fertilization in Mammals." Biol. Repro. Suppl., 2 (1970), 128-158.

Bishop, M.R., Sastry, B.V.R., Schmidt, D.E., and Harbison, R.D. "Occurrence of Choline Acetyltransferase and Acetylcholine and Other Quaternary Ammonium Compounds in Mammalian Spermatozoa." Biochem. Pharmacol., 25 (1976), 1617-1622.

Bleil, J.D., and Wassarman, P.M. "Mammalian Sperm-Egg Interaction: Identification of a Glycoprotein in Mouse Egg Zona Pellucida Possessing Receptor Activity for Sperm." Cell, 20 (1980), 873-882.

Bleil, J.D., and Wassarman, P.M. "Sperm-Egg Interactions in the Mouse: Sequence of Events and Induction of the Acrosome Reaction by a Zona Pellucida Glycoprotein." Dev. Biol., 95 (1983), 317-324.

Brinster, R.L. "In Vitro Culture of the Embryo." In: Pathways to Conception. Ed. Harold C. Mack. Springfield, Il.: Charles G. Thomas Publishing Co., 1971.

Chang, M.C. Fertilizing Capacity of Spermatozoa Deposited Into the Fallopian Tubes" Nature, (1951) 168, 697.

Chou, K.C. "Effects of Some Organophosphates on Fertilization of Mouse Gametes In Vitro." Ph.D. Dissertation, University of Michigan, (1987), 10-75.

Cross, N.L. and Meizel, S. "Minireview: Methods for Evaluating the Acrosomal Status of Mammalian Sperm." Biol. Repro., 41 (1989), 635-641.

Davis, B.K. and Gergely, A.F. "Studies on the Mechanism of Capacitation: Changes in Plasma Membrane Proteins of Rat Spermatozoa During Incubation In Vitro." Biochem. Biophys. Res. Comm., 88(2) (1979), 613-618.

Davison, A.N. "The Conversion of Schradon (OMPA) and Parathion Into Inhibitors of Cholinesterase by Mammalian Liver." Biochem. J., 61 (1955), 203-209.

Derache, R. "The Toxicity of Organophosphorus Compounds in Man." In: Organophosphorus Pesticides. Elmsford, New York: Pergamon Press, 1977, pp. 87-98.

Durham, W.F., Wolfe, H.R., and Elliot, J.W. "Absorption and Excretion of Parathion by Spraymen." Arch. Environ. Health, 24 (1972), 381-387.

Environmental Protection Agency. "Initial Scientific and Minieconomic Review of Parathion." Washington D.C. (1975), 12-45.

Eto, M. Organophosphorus Pesticides: Organic and Biological Chemistry. Ed. Zweig, G. Boca Raton, Florida: CRC Press, 1974, pp. 123-230.

Florman, H.M., and Storey, B.T. "Mouse Gamete Interactions: The Zona Pellucida is the Site of the Acrosome Reaction Leading to Fertilization In Vitro." Dev. Biol., 91 (1982), 121-130.

Food and Drug Administration, "The FDA Total Diet Study Program." In: Environmental Epidemiology. Chelsea: Lewis Publishers, Inc., (1986).

Gaines, T.B., "The Acute Toxicity of Pesticides to Rats." Toxicol. Appl. Pharmacol., 2 (1960), 88-99.

Gibbons, A. "Reproductive Toxicity: Regs Slow to Change." Science, 254 (1991), 25.

Gill, J.L. "Design and Analysis of Experiments in the Animal and Medical Sciences." Vol. 1, 2, and 3, Iowa State University Press, Ames, Iowa, 1978.

Hayes, Wayland J., Toxicology of Pesticides. Baltimore: Waverly Press, 1975.

Hoffman, D.J. and Eastin, W.C. Jr. "Effects of Malathion, Diazinon, and Parathion on Mallard Embryo Development and Cholinesterase Activity." Env. Res., 26 (1981), 472-485.

Holmstedt, B. "Structure-Activity Relationships of the Organophosphorus Anticholinesterase Agents." In: Cholinesterases And Anticholinesterase Agents. Ed. G. B. Koelle. Berlin: Spring-Verlag, (1963), 428-485.

Katz, D.F. "Human Sperm as Biomarkers of Toxic Risk and Reproductive Health." J. NIH Res., 3(5) (1991), 63-67.

Krnjevic, K. "Chemical Nature of Synaptic Transmission in Vertebrates." Physiol. Rev., 54 (1974), 318-540.

Kuzan, F.B., Fleming, A.D., and Seidel, G.E., Jr. "Successful Fertilization In Vitro of Fresh Intact Oocytes by Perivitelline (Acrosome-Reacted) Spermatozoa of the Rabbit." Fertil. Steril., 41 (1984), 766-770.

Langlais J. and Roberts K.D. "A Molecular Membrane Model of Sperm Capacitation and the Acrosome Reaction of Mammalian Spermatozoa." Gamete Res., 12 (1985), 183-224.

Langlais J., Zollinger, M., Plante, L., Chapdelaine, A., Bleau G., and Roberts, K.D. "Localization of Cholesteryl Sulfate In Human Spermatozoa in Support of a Hypothesis for the Mechanisms of Capacitation." Proc. Natl. Acad. Sci., USA, 78 (1981), 7266-7270.

Mann, T. "Metabolism of Semen." Adv. Enz., 9 (1949), 329-390.

Mann, T. The Biochemistry of Semen and of the Male Reproductive Tract. Methuen, London. 1964, 209.

Matsumura, F. Toxicology of Insecticides. New York: Plenum Press, 1975.

Meizel S. "The Importance of Hydrolytic Enzymes to an Exocytotic Event, the Mammalian Sperm Acrosome Reaction." Biol. Rev. 59 (1984), 125-57.

Misawa, M., Doull, J., Kitos, P.A., and Uyeki, E.M. "Teratogenic Effects of Cholinergic Insecticides in Chick Embryos I. Diazinon Treatment on Acetylcholinesterase and Choline Acetyltransferase Activities." Toxicol. Appl. Pharmacol., 57 (1981), 20-29.

Miyamoto, H., and Chang, M.C. (1973) "The Importance of Serum

Albumin and Metabolic Intermediates for Capacitation of Spermatozoa and Fertilization of Mouse Eggs In Vitro." J. Reprod. Fertil., 32 (1973), 193-205.

Myles, D.G., Hyatt, H., and Primakoff, P. "Binding of Both Acrosome-Intact and Acrosome-Reacted Guinea Pig Sperm to the Zona Pellucida During In Vitro Fertilization." Dev. Biol., 121 (1987), 559-567.

Naz, R.K., Alexander, N.J., and Isahakia, M. "Monoclonal Antibody to a Human Germ Cell Membrane Glycoprotein That Inhibits Fertilization." Science, 225 (1984), 342-344.

Nelson, L. "Acetylcholinesterase in Bull Spermatozoa." J. Reprod. Fertil., 7 (1964), 65-71.

O'Brien, R.D. "The Reaction with Cholinesterase In Vitro." In: Toxic Phosphorus Esters. Ithaca, New York: Academic Press, 1960, pp. 73-114.

Odenwald, M. Frankfurter Rundschau, (1989), 18 Feb.

O'Rand, M.G., and Fisher, S.J. "Localization of Zona Pellucida Binding Sites on Rabbit Spermatozoa and Induction of the Acrosome Reaction by Solubilized Zonae." Dev. Biol., 119 (1987), 551-559.

Parks, J.E., Arion, J.W., and Foote, R.H. "Lipids of Plasma Membrane and Outer Acrosomal Membrane from Bovine Spermatozoa." Biol. of Repro., 37 (1987), 1249-1258.

Proctor, N.H., Casida, J.E. "Organophosphorus and Methyl Carbamate Insecticide Teratogenesis: Diminished NAD in Chicken Embryos." Science, 190 (1975), 580-582.

Proctor, N.H., Moscioni, A.D., and Casida, J.E. "Chicken Embryo NAD Levels Lowered by Teratogenic Organophosphorus and Methylcarbamate Insecticides." Biochem. Pharmacol., 25 (1976), 757-762.

Reed, D.J., Goto, K., and Wang, C.H., "A Direct Radioisotopic Assay for Acetylcholinesterase." Anal. Biochem., 16, (1966), 59-64.

Saling, P.M., and Storey, B.T. "Mouse Gamete Interactions During Fertilization In Vitro. Chlortetracycline as a Fluorescent Probe for the Mouse Sperm Acrosome Reaction." J. Cell Biol., 83 (1979), 544-554.

Sastry, B.V. and Sadavongvivad, C., "Cholinergic Systems in Non-nervous Tissues" Pharm. Rev., 30 (1979), 65-132.

Sekine, T. "Choline Esterase in Pig Spermatozoa." J. Biochem. 38, (2) (1951), 171-179.

Singer, S.J. and Nicolson, G.L. "The Fluid Mosaic Model of the Structure of Cell Membranes." Science, 175 (1972), 720-731.

Stambaugh, R. and Buckley, J. "Identification and Subcellular Localization of the Enzymes Effecting Penetration of the Zona Pellucida by Rabbit Spermatozoa." J. Reprod. Fertil., 19 (1969), 423-432.

Statistical Analysis System. SAS User's Guide. Stat. Anal. Syst. Inc., Cary, N.C., 1982.

Stewart, T.A. and Forrester, I.T. "Acetylcholinesterase and Choline Acetyltransferase in Ram Spermatozoa." Biol. Reprod., 19 (1978a), 271-279.

Stewart, T.A. and Forrester, I.T. "Acetylcholine-Induced Calcium Movements in Hypotonically Washed Ram Spermatozoa." Biol. Reprod., 21 (1979), 109-115.

Stewart, T.A. and Forrester, I.T. "Identification of a Cholinergic Receptor in Ram Spermatozoa." Biol. Repro., 19 (1978b), 965-970.

Summers, R.G., Talbot, P., Keough, E.M., Hylander, B.L., and Franklin, L.E. "Ionophore A23187 Induces Acrosome Reaction in Sea Urchin and Guinea Pig Spermatozoa." J. Exp. Zool., 196 (1976), 381-385.

Tibbs, J. "Acetylcholinesterase in Flagellated Systems." Biochem. Biophys. Acta., 41 (1960), 115-122.

Toivonen, T., Ohela, K., and Kaipainen, W.J. "Parathion Poisoning Increasing Frequency in Finland." Lancet, 2 (1959), 175-176.

United States Department of Agriculture, Emergency Preparedness Branch, "The Pesticide Review." For USDA State and County Emergency Record (1970-1991).

Ward, C.R., and Storey, B.T. "Determination of the Time Course of Capacitation in Mouse Spermatozoa Using a Chlortetracycline Fluorescence Assay." Develop. Biol., 104 (1984), 287-296.

Wassarman, P.M., Bleil, J.D., Florman, H.M., Greve, J.M., Roller, R.J., Salzmann, G.S., and Samuels, F.G. "The Mouse Egg's Receptor for Sperm: What is it and How Does it Work?" Cold Spring Harbor Symp. Quant. Biol. 50 (1986), 11-19.

Wolf, D.P. "Mammalian Fertilization." In: The Biology of the

Fluids of the Female Genital Tract. Holland: Elsevier North, 1979, 407-414.

Yanagimachi, R., Kamiguchi, Y., Sugawara, S., and Midamo, K. "Gametes and Fertilization in the Chinese Hamster." Gamete Res., 8 (1983), 97-117.

Jacobson, G.R., Saier, M.H., "The Nervous System: Neurotransmission" In: Biochemistry, (1988), 1193-1211.