THE EFFECTS OF SELECTED ORGANOPHOSPHATES ON THE ELECTROPHYSIOLOGICAL PROPERTIES OF THE TELEOST RETINA

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ABSTRACT

THE EFFECTS OF SELECTED ORGANOPHOSPHATES
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Organophosphates (OP) are classically known to inhibit acetylcholinesterase. Some organophosphates also cause a phenomenon known as delayed neurotoxicity. Rainbow trout, Salmo gairdneri, were exposed to the organophosphates DEF (S,S,S tributyl phosphorotrithioate) and TOCP (triorthocresyl phosphate). Electroretinograms (ERG) were recorded 24 hr post treatment. The ERG recorded from fish treated with OP exhibited decreased latency, enhanced a-wave and depressed b-wave. This action on the a-wave was opposite to the depressed a-wave observed when retinas were exposed to the anticholinesterase agent eserine sulfate. The enhanced a-wave was also seen when the isolated a-wave was recorded following treatment with OP and Na^+ aspartate.

The data support a hypothesis that the OP is acting directly on the noncholinergic photoreceptor and that this action is distinct from any anticholinesterase action the OP may possess. This action may be at the level of the membrane or possibly mediated through actions affecting the metabolism of the photoreceptor.

It is an honor to dedicate this thesis to my parents Paul and Anna Kreft for their patient support and understanding throughout my education and most of all for their unending love which gave me the strength necessary to complete this work.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
BuChE	Butyrylcholinesterase
cGMP	Guanosine 3',5'-cyclic phosphate
CNP	2',3' Cyclic nucleotide 3'phosphate
CNS	Central nervous system
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane
DEF	S,S,S, tributyl phosphorotrithioate
DFP	Diisopropyl phosphorofluoridate
ERG	Electroretinogram
E-605	Parathion
i.p.	Intraperitoneally
nBM	n-butyl mercaptan
NTE	Neurotoxic esterase
OP	Organophosphate
OPIDN	Organophosphate induced delayed neurotoxicity
PNS	Peripheral nervous system
PPA	Phenyl phenylacetate
PPP	Phenyl-3-phenylpropionate
s.c.	Subcutaneous
TEPP	Tetraethyl pyrophosphate
TOCP	Triorthocresyl phosphate

INTRODUCTION

Organophosphate esters have classically been known as inhibitors of cholinesterases. It is because of this property that thay have gained wide use in agriculture as insecticidal agents. Some organophosphates have a second not as well known action which is referred to as "organophosphate induced delayed neurotoxicity" (OPIDN). This delayed neurotoxic phenomenon is not associated with the anticholinesterase activity of the organophosphates. The etiology of this phenomenon and the question of its delayed nature have been extensively studied and are not yet clear. The characteristics of the phenomenon are such that neural lesions occur and become progressively worse until clinical symptoms of ataxia and flaccid paralysis begin to appear.

It was reported to this laboratory that rainbow trout exposed to the organophosphate DEF (S,S,S, tributyl phosphorotrithioate) exhibited severe toxic responses with the retina being especially sensitive. The retina is an excellent model for the study of neural toxicology and in this study was used to investigate the initial insult of organophosphate agents which may lead to the development of delayed neurotoxicity. It is believed that retinal function is indicative of general neural toxicity. This study was conducted to observe and document electrophysiological changes which occur in the rainbow trout retina in response to exposure to organophosphate agents.

LITERATURE REVIEW

The problem of toxic chemicals in the environment is one that man has been facing for years and will have to continue to deal with for centuries to come. With an industrial society becoming increasingly dependent on man-made materials, the chances of chemical contamination of the environment, either by accumulation or by accident are sure to increase. Aside from industry, agriculture has also become extremely dependent on chemicals to increase yields, and decrease losses due to pests and spoilage. The use of chemicals in agriculture pose a particular problem in that the agents used are purposely spread over large areas. This in effect is contaminating millions of acres of land and waterways with thousands of tons of chemicals every year. Currently over 60,000 tons of pesticides are produced yearly in the United States (Casarett, 1980).

Pesticides as a group encompass a wide variety of agents with insecticides and herbicides being the first and second largest uses respectively. Use of these agents has not been without benefit. Their success has been most evident in the control of vector-borne disease. In many areas of the world malaria has been reduced from a rampant killer to virtual nonexistence. Other vector-borne diseases also controlled with pesticides include filariasis, yellow fever, viral encephalitis, typhus, bubonic plague, Rocky Mountain spotted fever and

many others. Estimates of the number of lives which have been saved since the introduction of wide scale DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) usage range in the millions and the number of illnesses prevented range in the hundreds of millions.

For 20 years after its introduction in 1942, DDT was extensively used throughout the world. It was extremely effective and thought to have no adverse ecological side effects. It was not until the third decade of use that evidence of environmental damage began to appear. Now most countries of the world have either eliminated or severely curtailed its use. DDT is a member of a class of insecticides known as organochlorides. Since the ban of DDT the use of organochlorides has generally been reduced and has to a large extent been replaced by organophosphates (OP). The organophosphates were preferred over the organochlorides because they tended in general to have a shorter ecological and biological half-life (Casarett, 1980).

Development of Organophosphates

The history of organophosphates as reviewed by Eto (1979) and Fest and Schmidt (1982) began about 120 years before they were discovered to have pesticide potential. In 1820 alcohol was reacted with phosphoric acid to synthesize the first organophosphate. Development of organophosphate chemistry continued throughout the 19th century. Tetraethyl pyrophosphate (TEPP), which was later to become the first practical OP insecticide, was first synthesized by Moschnine in 1854 and later that year by DeClermont. Both men were unaware of the physiological activity of this compound; in fact DeClermont is said to have tasted the TEPP without realizing its toxicity (Holmstedt, 1963). C.A.A. Michaelis in Germany and A.E. Arbusov in Russia have been called

the founders of classical phosphoric ester chemistry (Eto, 1979). Their work greatly advanced the understanding of the chemistry of phosphoric esters. The biggest boost to organophosphate chemistry came after 1937 when Schrader synthesized Sarin, which was the first of the nerve gas agents to be developed. This development was tightly concealed by the German government because of its great military potential. Schrader is also responsible for the development of compound E-605 (diethyl p-nitrophenyl phosphorothionate) also known as Parathion (Fest and Schmidt, 1982). Parathion and its derivatives have been very heavily used as insecticides throughout the world. Parathion is the pesticide involved in the greatest number of fatal poisonings (Casarett, 1980).

Physiochemistry of Organophosphates

The anticholinesterase action of organophosphates was first noted by Adrian, Feldberg and Kilby (1947). Balls and Jansen (1952) attributed this inhibition to the phosphorylation of the esteratic site on AChE as demonstrated by the reaction of diisopropyl phosphorofluoridate (DFP) with chymotrypsin. A scheme of substrate and inhibitor interaction with AChE is given in Figure 1. In general the interactions can be illustrated by considering the organophosphate as a substrate.

$$\underset{\text{(Enzyme)}}{EOH} + \underset{\text{(Substrate ACh or OP)}}{AX} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{(Enzyme complex)}}{EOH^{\cdot}AX} \qquad (1)$$

$$EOH^{\cdot}AX \xrightarrow{k_2} X^- + H + EOA \xrightarrow[H_2O]{k_3} EOH + A^- + H^+ \qquad (2)$$

Figure 1. Scheme of hydrolysis of acetylcholine by acetylcholinesterase and reactions of the anticholinesterase insecticides Paraoxon and Carbaryl. A: Reaction with acetylcholine. B: Reaction with Paraoxon, reaction k_3 may be replaced by aging process. C: Reaction with Carbaryl. Modified after Casarett (1980).

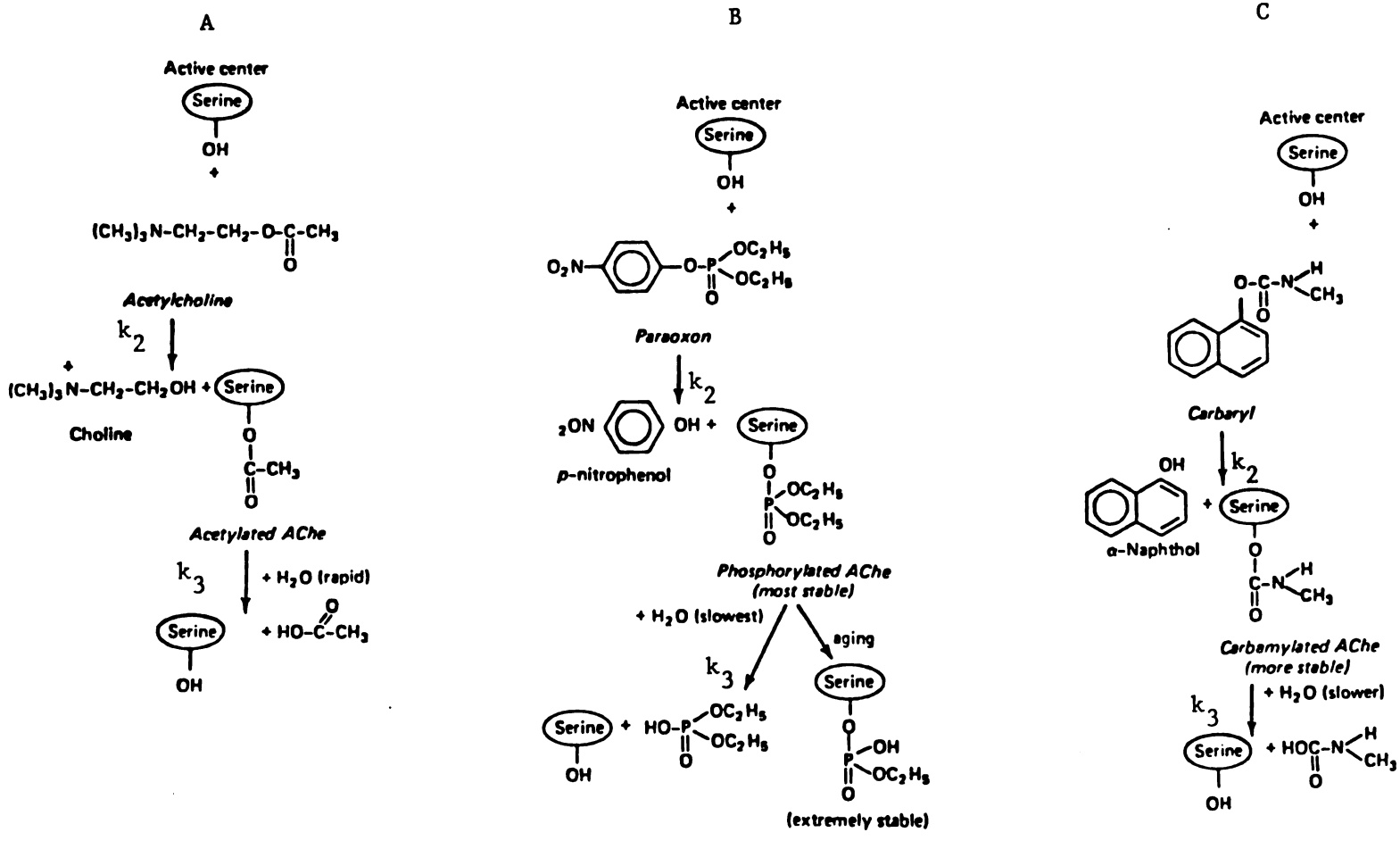
A
Active center
Serine
OH
(CH3)3N-CH2-CH2-O-C-CH3
O
Acetylcholine
k2
(CH3)3N-CH2-CH2OH + Serine
Choline
O
C-CH3
O
Acetylated AChe
k3
+ H2O (rapid)
Serine
OH
+ HO-C-CH3
O
B
Active center
Serine
OH
O2N
O-P
OC2H5
OC2H5
O
Paraoxon
k2
2ON
OH +
Serine
p-nitrophenol
O
P
OC2H5
OC2H5
O
Phosphorylated AChe
(most stable)
+ H2O (slowest)
aging
k3
Serine
OH
+ HO-P
OC2H5
OC2H5
O
Serine
O
P
OH
OC2H5
O
(extremely stable)
C
Active center
Serine
OH
O-C-N
H
CH3
O
Carbaryl
k2
OH
+ Serine
α-Naphthol
O
C-N
H
CH3
O
Carbamylated AChe
(more stable)
+ H2O (slower)
k3
Serine
OH
+ HOC-N
H
CH3
O

Figure 1

The formation of the complex (EOH•AX) is governed by the ratio of $k_1/k\text{-}1$, which is very high for both substrates (i.e., acetylcholine and organophosphates). Thus the formation of the enzyme-substrate or enzyme-inhibitor complex (EOH•AX) is favored (Eq. 1). The reactions governed by k_2 and k_3 are very fast for ACh and the active enzyme (EOH) is rapidly reformed (Eq. 2). For organophosphates, k_2 is slower than for acetylcholine, but not rate limiting. The rate limiting reaction is k_3, such that EOA accumulates while the concentration of EOH•AX is low at any given time. Estimates of the number of molecules of ACh hydrolyzed per minute by one molecule of enzyme are 300,000 and 0.008 for molecules of organophosphates, which is almost 38,000,000X slower, thus the enzyme is essentially irreversibly inhibited (Casarett, 1980). Some organophosphates can cause a process called "aging" (Figure 1) by which the enzyme becomes permanently inhibited. In this case the hydrolyzed enzyme phosphate complex (EOA) is dealkylated to form a very stable complex that does not allow for reactivation of the enzyme via reaction k_3 in Eq. 2.

Several compounds are available which can increase the rate of enzyme reactivation. The most widely used is 2-pyridine aldoxime methiodide (Pralidoxime, 2-PAM) which will accelerate the hydrolysis of the phosphorylated enzyme (k_3, Eq. 2) and thus reactivation of the enzyme. Pralidoxime will not aid in the hydrolysis of a complex that has gone through the aging process and that enzyme must be replaced via denovo synthesis. The effectiveness of Pralidoxime is dependent on the amount of enzyme which has gone through the aging process.

Adrian et al. (1947) were the first to note the anticholinesterase action of organophosphates. It is primarily because of this property

that OP have gained such wide use in agriculture, pest control and potential use as chemical warfare agents. This action of OP has been well studied and documented. In general, it can be characterized by massive stimulation of all cholinergic synapses resulting from the accumulation of the neurotransmitter ACh. The cholinesterase which normally hydrolyzes the choline ester is effectively inhibited by the organophosphate. The systemic effects of elevated ACh are wide and varied. As described in Gilman, Goodman and Gilman (1980), they include tightness of the chest and hypoxemia due to bronchoconstriction and increased bronchial secretions, increased salivation, nausea, anorexia, vomiting, cramps, diarrhea, bradycardia, dyspnea, muscle fatigue, and blurred vision. Effects mediated by accumulated ACh in the CNS include slurred speech, tremor, depression of respiratory and cardiac centers, anxiety, restlessness and insomnia. All of these actions are highly dependent on dose, efficacy and lipid solubility of the particular organophosphate. In cases of severe poisoning, death can result anywhere from 5 min to 24 hr after exposure, and is normally due to paralysis of the respiratory muscles with secondary cardiovascular complications. Poisoning can be effectively treated with atropine sulfate to counteract the accumulation of ACh at muscarinic receptors and Pralidoxime can be administered as a cholinesterase reactivator.

Delayed Neurotoxicity

Some organophosphates have a second action which is not related to the inhibition of a cholinesterase. This action has come to be known as "organophosphate induced delayed neurotoxicity" (OPIDN) or delayed neurotoxicity. Delayed neurotoxicity cannot be blocked by atropine or 2-PAM. Not all OP produce OPIDN even if they are strong inhibitors of

AChE and some OP which cause OPIDN are only weak inhibitors of AChE. The phenomenon of OPIDN is species specific, with chickens and man being very susceptible and common lab rodents being less susceptible or showing uncharacteristic symptomatology. As the name implies the effects of OPIDN are delayed in onset. In avians and mammals the delay in onset has ranged from 8 to 14 days after a single exposure. The characteristics of OPIDN have been classically described as axonal degeneration with secondary demyelination which can lead to paralysis and death. Clinical symptoms appearing after 8 to 14 days include ataxia and flaccid paralysis, both of which are the result of slowly degenerating motor axons. The degeneration of axons occurs both in the CNS and PNS. In the CNS, the spinal cord and medulla appear to be the primary areas affected, with the higher brain centers showing little susceptibility. Once the axonal degeneration has advanced damage can sometimes be reversed in the PNS, generally by the development of colateral innervation. Damage to the spinal cord and medulla is irreversible.

Etiology of Delayed Neurotoxicity

Many theories have been forwarded to explain the etiology of OPIDN. Cavanagh (1964) offered an explanation based purely on observations of the morphologic changes in degenerating spinal-cerebral and cortical-spinal tracts of OP exposed cats. Cavanagh suggests that the organophosphate esters interfere with mechanisms of energy synthesis, storage and transference. The apparent selective vulnerability of the largest and longest fibers implies the loss of what Cavanagh refers to as a "work factor" responsible for maintaining the integrity of large volume fibers. Sensory nerve terminals, with their high mitochondrial

content, are the first to show swelling and fragmentation, one of the earliest signs of neurointoxication. More recently, Harvey and Sharma (1980) have shown, using neuroblastoma tissue culture preparations, that organophosphate esters cause a dose dependent reduction in the metabolism of ^{14}C glucose which they interpret as a measure of cytotoxicity. They also have reported an inhibition of the uptake of ^{14}C leucine. These results indicate that organophosphate compounds depress the rate of protein synthesis, a factor which may in part be responsible for the degenerative syndrome.

Reichert and Abou-Donia (1980) observed marked inhibition of fast axoplasmic transport in the rat optic nerve by Leptophos and triorthocresyl phosphate (TOCP), OP known to produce OPIDN. Parathion, an OP which does not cause delayed neurotoxicity, did not cause significant change in the rate of axoplasmic transport. Austin (1957) reported a 40% drop in lipid synthesis by the sciatic nerve of the chicken 1 day after treatment with diisopropyl phosphorofluoridate (DFP). Austin stated that most of the DFP was "removed" from the tissue as early as 20 min after its administration, whereas the first signs of chronic poisoning took 8-14 days to appear. He hypothesized that the biochemical change which would ultimately lead to nerve degeneration must take place shortly after DFP absorption.

Olajos, Shopp and Rosenblum (1982) measured the effects of Leptophos on brain 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP). This enzyme catalyzes the hydrolysis of 2',3'-cyclic nucleotides to their corresponding 2'-nucleotides and is essential for maintenance of the myelin sheath. They reported a significantly lowered CNP activity after Leptophos treatment.

All of these examples point to a metabolic action of the organophosphate. Whether the nerve cell body is not synthesizing enough energy substrates or the substrates are not being transported down the length of the axon, or both, has not yet been determined. Any of these metabolic alterations would compromise the distal portions of the axon and lead to eventual degeneration.

Abou-Donia (1978a and b) has suggested a role of acid phosphatase in the etiology of OPIDN. He noted dose dependent increases of plasma acid phosphatase activity in hens treated with Leptophos or TOCP. This increased enzymatic activity points to possible in vivo breakdown of the lysosomal membranes with the subsequent release of acid phosphatase from the damaged liver. These findings are in agreement with Ntiforo and Stein (1967) who found that Malathion released arylsulfatase from rat liver lysosomes. Nerve tissue lysosomes may also be involved as the findings of Glees suggest (1966). He found increased acid phosphatase activity in nerves and neuroglia of chickens after treatment with TOCP. Joseph (1973) reports that membrane premeability of lysosomes of injured cells undergoes changes during Wallerian degeneration.

Aldridge (1954) and others have suggested that the initial event in delayed neurotoxicity was phosphorylation of esterases. Bloch and Hottinger, as reviewed by Abou-Donia (1981), hypothesized a model for OPIDN in which brain AChE is inhibited. This was refuted by Earl and Thompson (1952a and b) who showed that while hen brain pseudocholinesterase (butyrylcholinesterase) was selectively inhibited by TOCP, AChE was unaffected. They hypothesized that butyrylcholinesterase (BuChE) was the primary target in delayed neurotoxicity. Davison (1953) has shown that some OP which do not cause OPIDN also inhibit

butyrylcholinesterase in the same way as TOCP. Aldridge and Barnes (1966a) have shown that tri-4-ethylphenyl phosphate and other compounds are capable of inducing delayed neurotoxicity but do not inhibit hen brain butyrylcholinesterase activity. The BuChE theory has since been dropped.

Poulsen and Aldridge (1964) noted the structural similarity between both phenyl-3-phenylpropionate (PPP) and phenyl phenylacetate (PPA) and the neurotoxic metabolite of TOCP, saligenin cyclic o-tolyl phosphate. They originally suggested that the brain esterases which hydrolyze PPP and PPA may be selectively inhibited by neurotoxic organophosphates. Later work by Aldridge and Barnes (1966b) using a variety of OP compounds revealed no correlation between inhibition of either enzyme and the development of OPIDN. Johnson (1969) was able to show that the enzymes referred to by Poulsen and Aldridge accounted for only 80% of the total brain PPA-hydrolyzing activity. Of the remaining 20%, he has distinguished one fifth (i.e., 4% of total) to be what he calls "neurotoxic esterase" (NTE). The endogenous substrate for this esterase has yet to be identified but its activity can be measured in vitro by the hydrolysis of PPA. Johnson (1974) has shown that simple inhibition of NTE is not enough to cause delayed neurotoxicity. Some carbamates and sulfonyl fluorides inhibit NTE but do not cause OPIDN; in fact these compounds protect hens from the neurotoxic actions of subsequent doses of OP known to produce OPIDN (Johnson and Lauwerys, 1969; Johnson, 1970). This protective action continues until almost 70% of the enzyme is again free to be phosphorylated by the organophosphate. For a compound to be neurotoxic the enzyme inhibitor complex must go through an "aging" process. This process, similar to that explained above for

cholinesterase inhibition, results in the hydrolysis of one of the esters (R-O-P) or amide (R-N-P) bonds of the OP. This aging process would thus yield a stable ionized acidic group on the phosphorus. Johnson (1974) proposes that this charged group causes a deleterious metabolic effect leading to the symptoms of delayed neurotoxicity.

The whole scheme of the NTE hypothesis, though it sounds promising, is not without its problems. Baron (1981), in an excellent review article points out that there is not always a clear correlation betwen NTE inhibition and development of OPIDN. In a study by Sprague and Castles (1979) hens were administered TOCP at neurotoxic and nonneurotoxic levels in a single dose or in smaller doses over a 20 day period. They were unable to draw any correlation between NTE inhibition and clinical or histological signs of delayed neurotoxicity. Even with these controversial observations, Johnson's theory of neurotoxic esterase is becoming widely accepted as a possible mechanism of delayed neurotoxicity. It still remains to be determined if this model, alone or in concert with other models, is a feasible explanation of delayed neurotoxicity.

Clinical History of Delayed Neurotoxicity

The occurrence of delayed neurotoxicity dates back to the late nineteenth century when tubercular patients developed neuropathies following treatment with a mixture derived from phosphoric acid and coal tar phenols known as phosphocreosote. These neuropathies were not associated with the phosphocreosote until years later (Johnson, 1975).

A major outbreak of delayed neurotoxicity occurred in the United States during the late 1920's and early 1930's. During the years of prohibition, a ginger extract from Jamaica was widely used to circumvent

prohibition statutes. This nonpotable extract, containing 60-80% alcohol, was widely marketed as a headache remedy, carminative and general aid to digestion. Although it was indeed nonpotable in its original state, when highly diluted it produced a drink with the same potency as whiskey. One manufacturer substituted TOCP for castor oil in a step in the ginger extraction leaving an extract with a 2% concentration of TOCP. This product was marketed widely and resulted in the paralysis of around 20,000 people with some estimates going as high as 60,000 (Abou-Donia, 1981; Baron, 1981; Morgan and Tulloss, 1976) In late 1959 in Meknes, Morocco cooking oil was diluted with outdated aviation oil. The aviation oil was a man-made mixture designed to withstand the very high temperatures of jet engines which, upon analysis, was found to contain 3% of cresyl phosphates. As a result of its use over 10,000 people developed various levels of paralysis (Svennilson, 1961; Smith and Spaulding, 1959). As recently as 1971, in Egypt, 1300 water buffalo were paralyzed due to exposure to Leptophos (Metcalf, 1984).

S,S,S-Tributyl Phosphorotrithioate (DEF)

The organophosphate DEF (S,S,S-Tributyl phosphorotrithioate) was first evaluated by the Ethyl Corporation as a defoliant in 1954. It is currently produced and marketed as a cotton defoliant by Mobay Chemical Corporation of Kansas City, MO. DEF may be synthesized by the reaction of butyl mercaptan with phosphorus oxychloride in the presence of base,

$$3n\text{-}C_4HgSH + POCl_3 \xrightarrow{\text{base}} (n\text{-}C_4HgS)_3P{=}O \qquad (3)$$

or by oxidation of tributyl phosphorotrithioite (Merphos) which is also a defoliant

$$(n\text{-}C_4HgS)P \xrightarrow{[O]} (n\text{-}C_4HgS)_3P{=}O \qquad (4)$$

(Eto, 1979; Fest and Schmidt, 1982).

The delayed neurotoxic effects of DEF have been well documented. Baron and Johnson (1964) found that intraperitoneal administration of DEF (100 mg/Kg daily for 10 days) to white leghorn chickens produced central and peripheral neurologic lesions accompanied by clinical signs of ataxia. Oral administration of DEF did not induce signs of peripheral weakness but severe lesions of the spinal cord and sciatic nerve were both observed. In general, clinical symptoms were both dose dependent and irreversible.

The implication that oral administration of DEF involves alterations of the mechanism of toxicity was addressed by Abou-Donia, et al. (1979). They found that DEF was rapidly metabolized in the gastrointestinal tract, and hydrolyzed to form n-butyl mercaptan (nBM). Administration of nBM to chickens resulted in general weakness, malaise and loss of balance. They later exhibited loss of appetite, development of tremor and finally paralysis and death. This syndrome was referred to as "late acute" toxicity. The anticholinesterase activity normally encountered with OP poisoning was not observed in these animals, and atropine was

ineffective in preventing this toxicity. In field studies by Wilson, et al. (1980) atropinized (20 mg/Kg s.c.) scaleless chickens developed symptoms of delayed neurotoxicity following aerial spraying with DEF.

The mechanism of the delayed neurotoxicity is unknown and "early warning" tests for it are nonexistent. Wilson, et al. (1980) suggest that there is initial damage to the peripheral nervous system (involving focal lesions in axons and neuromuscular junctions) which could be detectable by the biochemical and electrophysiological tests of the type used clinically in the study of neuromuscular disorders.

Teasley (1967) reported that the organophosphate S,S,S,-tributyl phosphorotrithioite (Merphos) was dumped into the Ashley River, a tributary of Charleston Harbor, SC, as an industrial effluent. This moiety then underwent an oxidation reaction to form DEF. It was later determined that the DEF was responsible for extensive fish kills in these waters. Field studies have shown that DEF induced lenticular abnormalities (cataracts), retinopathy and central nervous system edema in fingerling rainbow trout (Dr. J.B. Hunn, Columbia Nat'l Fishery Res. Lab., Columbia, MO; personal communication). Hunn has also reported similar lesions observed under laboratory conditions. Palawski, Buckler and Mayer (1983) observed significant reductions in growth rate and increased mortality after exposure of rainbow trout fry to DEF.

Effects of Organophosphate Esters on Visual Mechanisms

Exposure to organophosphates has been reported to produce a number of different effects upon the visual system. Ishikawa, et al. (1971) recorded abnormal ERG during the early stages of Fenthion intoxication in rats. Histopathological damage to canine optic nerve and retina treated with ethylthiometon was noted by Uga, Ishikawa and Mukuno

(1977). Pathological changes of the optic nerve consisted of a reduction in the number of myelinated axons and thinning of the myelin sheath of surviving axons. Retinal histopathology was characterized by partial necrosis of the pigment epithelium and Müller cells, with the cytoplasm of the affected cells becoming severely vacuolated. Edema of the optic disk and resulting enlargement of the blind spot was also reported. All of the changes noted by Uga, et al. (1977) were dose dependent. These authors presented morphological evidence that the axoplasmic transport occurring within the fibers of the optic nerve is blocked by organophosphates. Ishikawa and Miyata (1980) present an epidemiological study of the effect of long term organophosphate intoxication in the human and the dog. They identified specific ocular and systemic syndromes in individuals from agricultural regions of Japan where Parathion and Malathion were used extensively. Patients exhibited reduced visual acuity, narrowing of the visual field and optic neuritis. Formation of cataracts and the development of myopia in young children have been suggested as a possible consequence of chronic exposure to organophosphates used widely in Japan since the end of World War II.

Revzin (1980) reported that southern Michigan fruit farmers exposed to chronic low doses of organophosphates demonstrated functionally significant visual disorders. These included diminished peripheral visual functions, involving loss of control of the eye movements mediated by the superior colliculus. Revzin found that the superior colliculus is an important target for the toxic actions of organophosphate esters and related cholinergic drugs, and that certain superior collicular functions are extremely sensitive to agents affecting cholinergic systems. Mevinphos, a commonly used agricultural

organophosphate was shown capable of producing superior colliculus dysfunction at doses substantially lower than those required for eliciting gross parasympathomimetic responses.

The Electroretinogram (ERG)

The electroretinogram (ERG) is the recordable mass response of the retina following photostimulation. It can be recorded in vitro by placing an active electrode in the vitreous humor and a reference electrode at the back of the eye. The ERG is a relatively slow response and as such it usually requires a D.C. coupled recording preparation. The recording is characterized by an initial negative (downward) deflection referred to as the a-wave followed by a positive deflecting b-wave. These may be followed by a slowly developing c-wave which can be positive or negative depending on the species and recording conditions (Armington, 1974).

According to Ottoson and Svaetichin (1952) Holmgren recorded the first ERG in 1865. This response is a summation of various cell types in the retina. Granit (1933) attributed the ERG to the summation of three components which he labelled PI, PII and PIII based on the order of their disappearance under ether anesthesia.

The PI wave, which was the first to disappear under ether anesthesia, corresponds to the c-wave. The c-wave, when present, is thought to occur as a result of interaction between the pigmented epithelium and the rod outer segments. Schmidt and Steinberg (1971), were able to record light induced intracellular responses from pigmented epithelium cells which were identical to the c-wave.

The PII component has been roughly correlated to the b-wave. Various authors as reviewed by Brown (1968) have demonstrated, using intraretinal recording techniques, that the b-wave arises from the inner nuclear layer of the retina. Miller and Dowling (1970), using intracellular recordings, have shown that the only cells which respond to photostimulation with potentials corresponding to the b-wave are the structural glial cells of the retina known as Müller cells. The Müller cells do not act to transmit the visual signal through the retina. They do, however, respond to increases in extracellular K^+ concentrations produced as the visual signal traverses the neural retina. The major source of the increased K^+ is thought to be bipolar cells (Armington, 1974) but other cell types are probably also involved. Although the Müller cells are responsible for a major portion of the b-wave, Tomita (1976) points out that it is still unknown whether or not they are exclusively responsible for the b-wave.

The PIII component has been almost exclusively associated with the a-wave. Penn and Hagins (1969) conducted experiments on signal transmission along the rods which provided evidence that the photoreceptor hyperpolarization in response to a photostimulus accounts for a major portion of the a-wave (PIII). Potts, Madrell and Kingsbury (1960) treated newborn animals with relatively high doses of sodium glutamate. This treatment produces animals whose retinas are essentially a culture of pure receptor cells. Electroretinograms recorded from these animals consisted entirely of the a-wave. The PIII component of the ERG can be further broken down into two parts. An early, rapid, hyperpolarization referred to as the fast PIII and a later and slowly developing hyperpolarization referred to as the slow PIII.

According to Armington (1974) the fast PIII is clearly associated with the response of the photoreceptors whereas it is still unclear whether or not the slow PIII is entirely the result of the receptor cells. The a-wave is unique in electroretinography in that it can easily be isolated pharmacologically. Treatment with sodium aspartate results in isolation of the photoreceptor response (Sillman, Ito and Tomita, 1969a,b). Later studies of Cervetto and MacNichol (1972) produced intracellular recordings which show that aspartate eliminates horizontal cell response and thus leaves the response of receptors essentially normal, except that "negative feedback" from the horizontal cells is no longer present. The ease with which this method of a-wave isolation can be conducted has led to its wide use in the study of the functional integrity of photoreceptors.

Phototransduction

The photoreceptor response to light is characterized by a hyperpolarization of the cell from a normal (dark) relatively depolarized state (approximately 30 mV) (Kandel and Schwartz, 1981). Though this phenomenon has been extensively studied it is still unclear about the exact mechanism by which phototransduction is amplified to a neurological response. It has been well documented that the photopigment rhodopsin undergoes conformational changes in response to light. This is an extremely sensitive system which has been shown to respond to as little as a single photon of light. This response is then amplified to cause the photoreceptor to hyperpolarize. The hyperpolarization is caused by a decreased inward flux of Na^+ ions (dark current) which drives the membrane potential toward the K^+ equilibrium. The steps between the conformational change of rhodopsin and the

decreased dark current are still unclear. The excellent review by Hubbell and Bownds (1979) is summarized as follows. Currently there are two hypotheses for the mechanism of phototransduction and amplification. In the first hypothesis, cGMP is involved in an enzymatic amplification. In the dark, the Na^+ channels of photoreceptors are maintained in an open position via a phosphorylated protein. The protein is maintained in the phosphorylated state by a cGMP-dependent protein kinase. With photostimulation, rhodopsin splits and this activates a phosphodiesterase which in turn cause a reduction in the level of cGMP. The lower level of cGMP results in the dephosphorylation of channel proteins leading to a subsequent closure of Na^+ channels and decrease in the dark current (Figure 14).

The cGMP hypothesis is supported by the fact that a conformational change of one rhodopsin molecule can lead to the disappearance of $5x10^4$ molecules of cGMP. This large response to photostimulation makes cGMP a likely candidate for the amplification mechanism. The decline in cGMP concentration increases with greater illumination and varies with the logarithm of light intensity (Goridis, 1977).

The second hypothesis involves the role of Ca^{++} in the amplification mechanism. In this model, Ca^{++} is continuously sequestered in the lumen of the disks in the receptor outer segment. It is thus postulated that rhodopsin exists as a disk transmembrane protein which upon exposure to light is converted via conformational changes to a Ca^{++} channel. The released Ca^{++} then flows from the disk lumen, down its concentration gradient to the cytoplasm. The increased free Ca^{++} binds to and consequently blocks the Na^+ channels (Figure 2).

Figure 2. Schematic representation of Ca^{++} for phototransduction hypothesis. A: Ca^{++} gradient between disk lumen and cytoplasm, B: Na^{+} gate in plasma membrane of rod outer segment, C: Na^{+}-K^{+} pump in plasma membrane of rod inner segment, D: location of AChE at amacrine cell synapses.

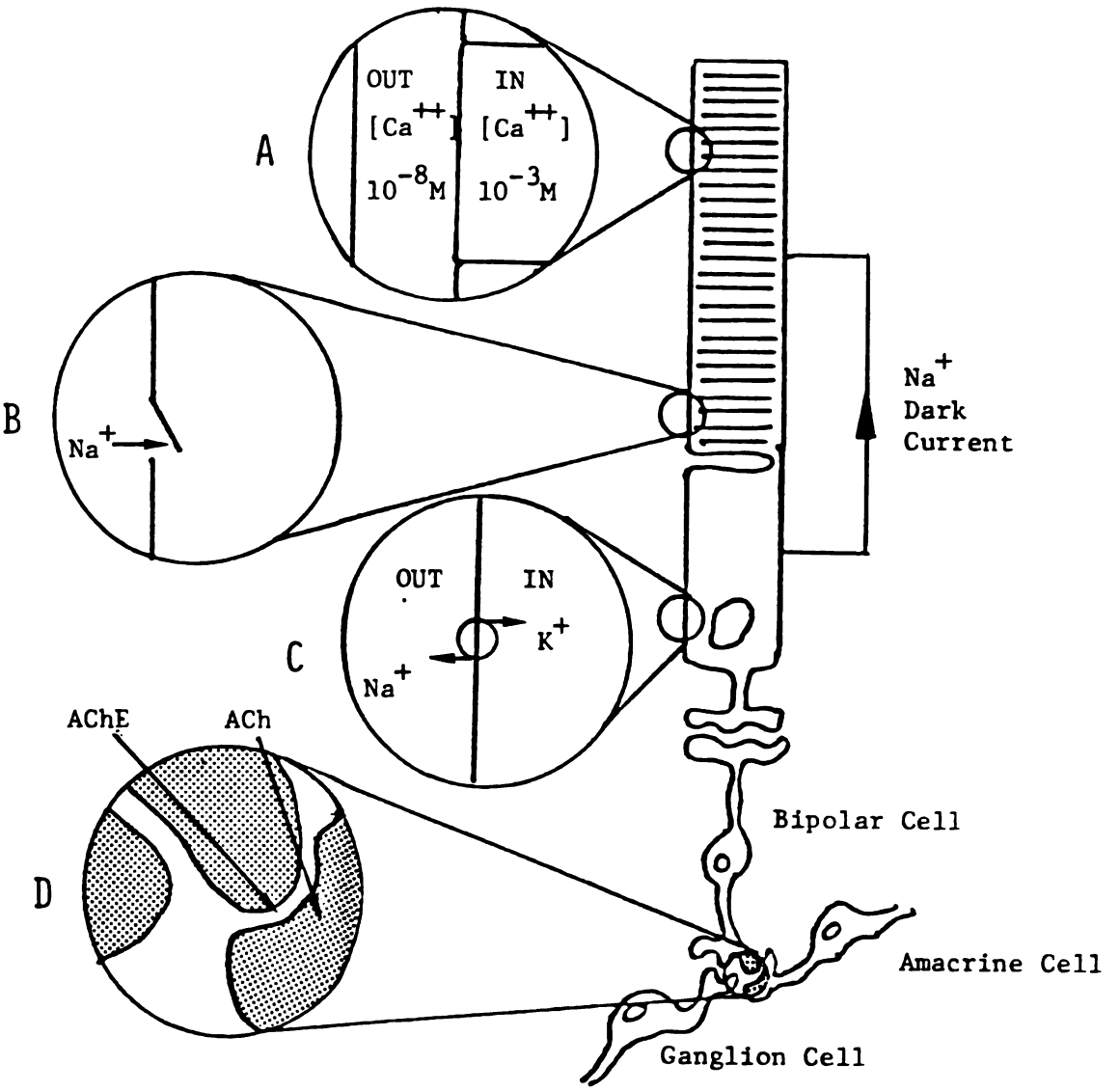
A
OUT
[Ca++]
10-8M
IN
[Ca++]
10-3M
B
Na+
Na+
Dark
Current
C
OUT
IN
K+
Na+
AChE
ACh
D
Bipolar Cell
Amacrine Cell
Ganglion Cell

Figure 2

The Ca^{++} hypothesis receives support from the fact that intracellular injection of Ca^{++} mimics the action of photostimulation by producing hyperpolarization of the photoreceptor. It has also been demonstrated that buffering of cytoplasmic levels of free Ca^{++} with chelating agents affects the photoresponse in quantitative agreement with predictions of the Ca^{++} hypothesis. Both of these experiments suggest that Ca^{++} plays a prominent role either directly or indirectly in modulating the membrane potential in response to light. Although extensive studies have been conducted, it has not been directly demonstrated that Ca^{++} is released from the disk upon photostimulation. It must also be demonstrated that Ca^{++} release results in a sufficient amplification, i.e,, each rhodopsin molecule must release at least 100-1000 Ca^{++} ions within about 1000 msec.

It is still unclear which of these models best represents the true amplification mechanism in the photoreceptor. Cohen (1984) presented both models objectively without any conclusion as to which is more valid. Hubbell and Bownds (1979) allude to a possible combination of these two models pointing to the wide action of Ca^{++} on enzymatic activity.

EXPERIMENTAL RATIONALE

Experiments were designed to study the effect of organophosphate agents on the in vitro scotopic electroretinogram. Since the ERG is a good monitor of retinal functional integrity, the actions of the organophosphates should be reflected by alterations in the ERG.

Retinal function can be an excellent indicator of the onset of general toxicity to both the peripheral and central nervous systems. Alterations to the ERG can be measured and evaluated long before classical systemic symptoms of delayed neurotoxicity begin to occur. This work can also aid in evaluating the mechanism of the delayed neurotoxic phenomenon.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri) (75-200 g) from Balders Fish Farms (Big Rapids, MI) were maintained in 180 gallon fiberglass tanks at 9±1C. Each tank was provided with a slow continuous flow of filtered tap water which was aerated by compressed air filtered through charcoal. The photoperiod was 16 hr light:8 hr dark.

Tissue Preparation

In vitro electroretinograms were recorded from isolated eyecups prepared as follows. After dark adapting the fish for 1 hr, the fish was killed by cervical section and the eyes were quickly enucleated under dim red illumination . A small incision was made at the junction of the cornea and sclera with an 18 gauge needle, following which an iris scissors was used to cut around the circumference of the eye allowing for removal of the cornea and iris. The lens was extracted with a pair of forceps and filter paper strips were used to remove as much aqueous and vitreous humor as possible without physically disturbing the retina. This entire procedure was completed in 3-4 minutes.

The eyecup was placed in a Plexiglass holding chamber through which 100% oxygen flowed. The holding chamber was light tight and maintained at 12C. Eyes were held in this chamber no longer than 30 min before use.

Electroretinogram Recording Chamber and Circuitry

To record an ERG, the eyecup was placed on a narrow shelf in a clear Plexiglass recording chamber containing a thin layer of normal saline (Figure 3). The eyecup was positioned under an opening in the top of the chamber through which the active recording electrode was lowered with the aid of a micromanipulator into electrical contact with the anterior retina. The sclera made electrical contact with a filter paper wick. The reference electrode and the filter paper wick both make electrical contact via the saline solution, thus completeing the circuit. The entire preparation was grounded by a Ag-AgCl electrode placed in the saline.

The electrodes used in these experiments were hand drawn from 2 mm i.d. soft glass tubing (0.2 ml Prothombin pipette, Dade, Miami, FL) to form a tip diameter of approimately 0.5 mm. The electrodes were filled with 4.0 g% nutrient agar (Difco Laboratories, Detroit, MI) in 0.154 molar NaCl solution. Nutrient agar was preferred over nonnutrient agar because it formed a smoother gel when used at 12C and was less likely to crack or cause noise in the recordings due to poor connections. The electrodes were made fresh daily to avoid bacterial growth in the nutrient agar.

The active and reference electrodes were connected to a Grass P16 AC-DC preamplifier (Grass Instruments, Quincy, MA) by an agar bridge (agar filled Polyethylene tubing) and a saturated NaCl filled calomel electrode (S-30080-17 minature reference electrode, Sargent-Welch Scientific Co., Detroit, MI). Due to the slow response time of the ERG a direct-coupled output from the P16 preamplifier was used. The rise time was set at 3 ms and the 1/2 amp high frequency was set at 15 Hz.

Figure 3. Diagram of ERG recording chamber.

A-Eyecup resting on shelf
B-Filter paper wick
C-0.9% saline in light tight containers
D-Light shielded Calomel electrode
E-Active and reference electrodes to P16 preamplifier
F-100% oxygen input
G-Ag-AgCl ground
H-Agar bridge
I-Active glass electrode
J-Reference glass electrode

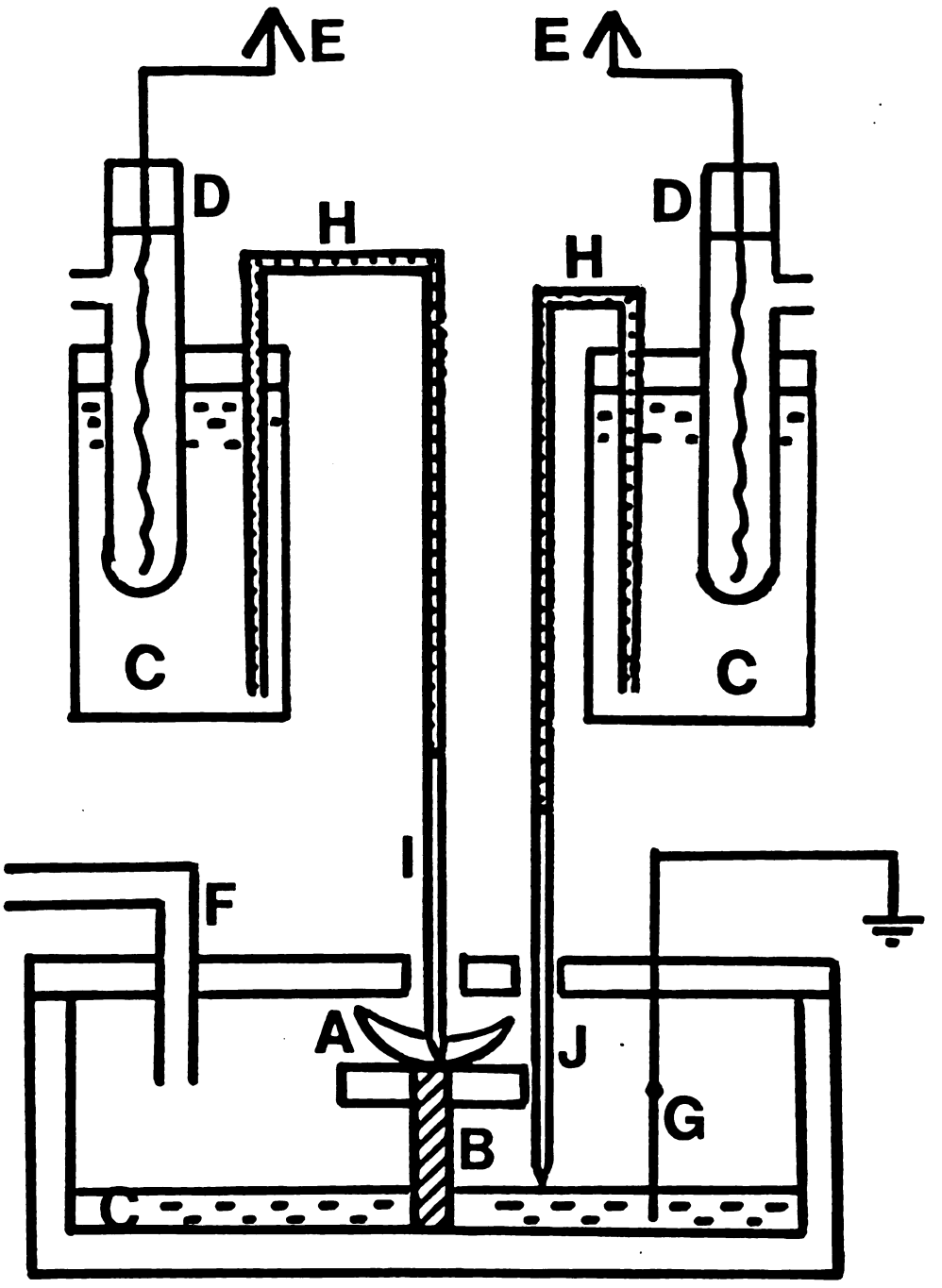

Figure 3

In order to minimize extraneous (i.e., 60 Hz) signals, the electrodes were connected in a standard push-pull configuration. The recordings were made in a light tight, thermally insulated, and electrically shielded faraday cage connected to common ground. The cage was cooled to 12±1C for all ERG recordings.

Prior to recording the ERG, the eyecup was allowed to equilibrate for 10 min following which 10 ERG were consecutively recorded at 1 min intervals. From the time the fish was dark adapted up to placement of the electrode in the eyecup the preparation was kept under dark red illumination. The scotopic ERG was recorded on Channel 1 of a Grass Model 5D polygraph (Grass Instuments, Quincy, MA) or by an on line computer as decribed below.

Photostimulation

Photostimulation was provided by a Grass PS2 photostimulator (Grass Instruments, Quincy, MA). In order to minimize the intensity of the photostimulation the photostimulator was set on an intensity of one and flashed off the white ceiling of the recording chamber to give a diffuse stimulation of low intensity (1.72 lux). A photocell was used to simultaneously record the photostimulus on Channel 2 of the Grass polygraph. For a discussion of the illumination by this instrument see Fonner (1973).

Computer Configuration

The on line computer system consisted of the following pieces of hardware: an Apple II+ computer with 48K RAM, 2 disk drives and joy stick, Apple Computer, Inc. Cupertino, CA; Mountain Analog to Digital and Digital to Analog converter and CPS Multifunction Card, Mountain Computers, Inc., Scotts Valley, CA., PKASO Interface Board model ID12,

Interactive Structures Inc., Bala Cynwyd, PA; Language Card for Apple II+ with 16K RAM, Advanced Logic Systems Inc., Sunnyvale, CA; IDS Prism 132 Printer with graphics, sprint mode and sheet feed, Integral Data Systems Inc., Milford, NH.; Hewlett Packard Digital Graphics Plotter Model #HP 7470A, Hewlett Packard Inc., Palo Alto, CA; and a Heathkit Single Trace Oscilloscope Model 10-4530, Heath Inc., Benton Harbor, MI.

These recordings were made as described above with the following exceptions. The signal from the P16 preampliphier was fed into an operational amplifier which boosted the signal 0,25,50,75 or 100X so that the signal was large enough to fill the $\pm$5 volt range of the A/D converter. The software (Teyler et al., 1982) as provided was designed to measure the latency and amplitude of waveform peaks and modified to the extent that it could be used for analyzing the appropriate points on the digital ERG. The digital ERG could be stored on a floppy disk, displayed and analyzed graphically on the monitor of the Apple computer, displayed on the oscilloscope or a hard copy produced on the Hewlett Packard digital plotter. Because of the long duration of the ERG, resolution of the time axis was greatly reduced. Further modification of the software will be necessary to overcome these limitations.

Electroretinogram Analysis

The curvilinear recordings from the Grass polygraph were computer (PDP-11/24, Digital Electronic Corp., Maynard, MA) adjusted to yield equivalent rectilinear plots from which 6 coordinates were determined (Figure 4). These points were utilized to calculate 19 distinct quantitative parameters (Figure 5) which were statistically analyzed using a two-way ANOVA with replications ($p = 0.05$). Using these 19 parameters an average ERG could be reconstructed as seen in Figure 4C.

Figure 4. Computer transformation from Grass polygraph recording of an ERG. A: Curvilinear recording of ERG showing six points digitized for computer analysis, B: Rectilinear transformation of curvilinear recording, C: Graphic representation of mean ERG parameters, S: Stimulus.

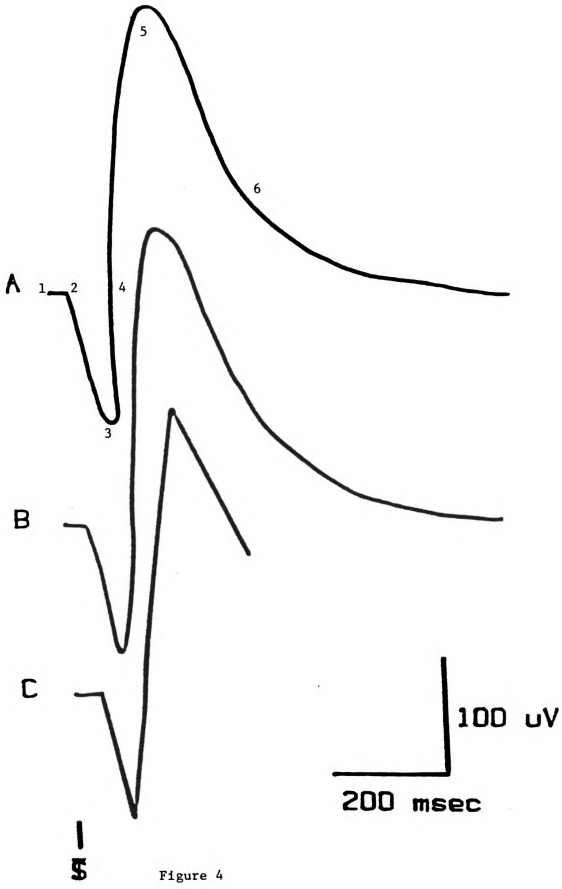

Figure 4

Figure 5. Schematic representation of the nineteen parameters calculated from the digitized ERG.

	Title	Calculation
1	a-Wave amplitude (μV)	Y3 - Y2
2	b-Wave amplitude (μV)	Y5 - Y3
3	b-Wave amplitude - isoelec. (μV)	Y5 - Y4
4	ERG latency (msec)	X2 - X1
5	Peak a-wave latency (msec)	X3 - X1
6	Peak b-wave latency (msec)	X5 - X1
7	ERG duration - total (msec)	X6 - X2
8	a-Wave duration (msec)	X4 - X2
9	b-Wave duration (msec)	X6 - X4
10	Pre a-Wave duration (msec)	X3 - X2
11	Post a-Wave duration (msec)	X4 - X3
12	Pre b-Wave duration (msec)	X5 - X4
13	Post b-Wave duration (mse)	X6 - X5
14	Pre a-Wave dy/dt (μV/msec)	(Y3 - Y2)(X3 - X2)
15	Post a-Wave dy/dt (μV/msec)	(Y4 - Y3)(X4 - X3)
16	Pre b-Wave dy/dt (μV/msec)	(Y5 - Y4)(X5 - X4)
17	Post b-Wave dy/dt (μV/msec)	(Y6 - Y5)(X6 - X5)
18	Maximum b-wave (μV)	Y5
19	Minimum b-wave (μV)	Y6

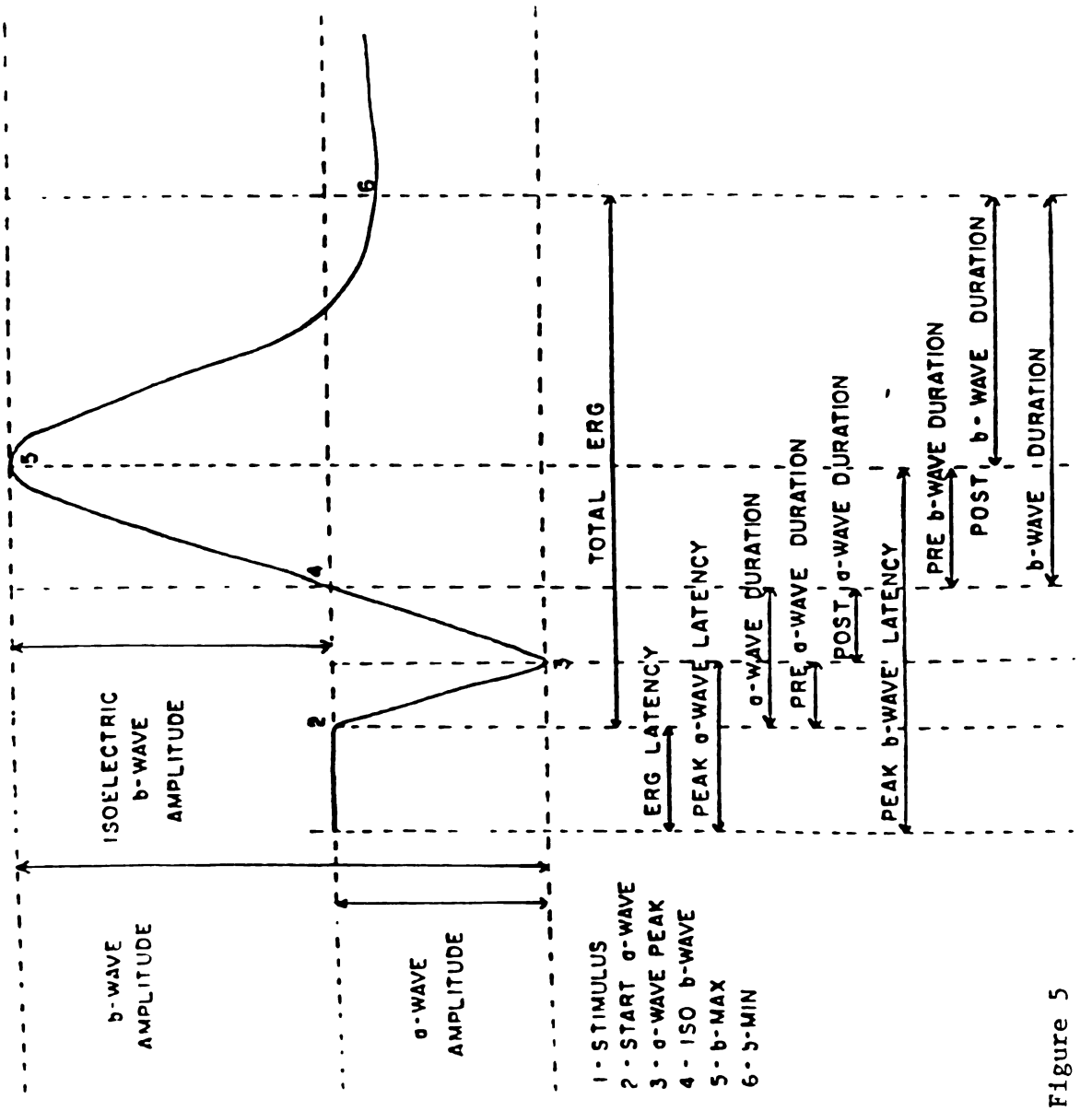
b-WAVE AMPLITUDE
ISOELECTRIC b-WAVE AMPLITUDE
a-WAVE AMPLITUDE
1 - STIMULUS
2 - START a-WAVE
3 - a-WAVE PEAK
4 - ISO b-WAVE
5 - b-MAX
6 - b-MIN
TOTAL ERG
ERG LATENCY
PEAK a-WAVE LATENCY
a-WAVE DURATION
PRE a-WAVE DURATION
POST a-WAVE DURATION
PEAK b-WAVE LATENCY
PRE b-WAVE DURATION
POST b-WAVE DURATION
b-WAVE DURATION

Figure 5

Treatment with Eserine Sulfate and Carbachol

Eyecups were prepared from dark adapted rainbow trout as described above. Prior to recording the ERG, the eyecup was placed in an eserine-Ringer solution aerated with 100% O_2 for a period of 10 min inside a darkened and cooled faraday cage. The eserine-Ringer solution consisted of 100 mM NaCl, 1.8 mM $CaCl_2$, 2.0 mM KCl, 5.0 mM $NaHCO_3$, 5.0 mM $MgSO_4.7\ H_2O$, 20.0 mM glucose and 0.3 mg eserine sulfate per 100 ml of Ringer. To verify inhibition of AChE, eyecups were identically treated prior to assaying for AChE activity (Appendix II). Eyecups treated with carbachol were exposed in the same manner using 66.7 mg carbachol per 100 ml Ringer.

Treatment with Organophosphates

In experiments where fish were exposed to TOCP (Tri-o-tolyl phosphate, Practical grade, Eastman Kodak, Rochester, NY) or DEF (Technical grade, provided by Mobay Chemical Corp., Agricultural Chemicals Div., Research and Development, Kansas City, MO) the animals were selected at random and anesthetized to effect with tricaine methanesulfonate (MS-222, Ayerest Laboratories, N.Y., NY). Each fish was weighed and injected (i.p.) with the drug or control medium. Fish receiving TOCP were injected with 100% TOCP at a dose of 1000 mg/Kg. Control fish in this group received 0.9g% NaCl at a dose of 1 ml/Kg. Fish receiving DEF (100 mg/Kg) were injected with 100 mg DEF/ml of corn oil vehicle (Crisco oil, Procter and Gamble, Cincinatti, OH). Control fish in this group received corn oil 1 ml/Kg live weight.

Treatment with Na-Aspartate

Before recording the ERG each eyecup was placed in an O_2 saturated aspartate-Ringer solution for a period of 10 min in the darkened, 12C,

faraday cage. The aspartate-Ringer solution consisted of 100 mM Na-aspartate, 1.8 mM $CaCl_2$, 2.0 mM KCl, 5.0 mM $NaHCO_3$, 5.0 mM $MgSO_4.7H_2O$, and 20.0 mM glucose. Pretreatment with Na-aspartate produces an isolated a-wave which is useful for identification of the site of action of a drug.

RESULTS

The In Vitro Electroretinogram--General Observations

In vitro electroretinograms (ERG) were recorded as described in the methods section and were normal in that they contained a negative (hyperpolarizing) a-wave followed by a positive (depolarizing) b-wave. Recordings were made on previously dark adapted animals, under scotopic conditions and with sufficient oxygen to eliminate the possible generation of the c-wave. Because of daily variations in recordings (Tables 1-8) it was decided to collect control data with each set of treatment data. Thus each treatment was statistically compared to its own set of controls. The reader should be cautioned that quantitative comparisons of absolute values should not be made between experiments.

The Effect of Eserine Sulfate

Data from eyes treated with eserine sulfate (an anticholinesterase agent) are given in Table 1 and average ERG reconstructed from those data are shown in Figure 6. The data show no significant difference in ERG latency or in peak a-wave latency. There was a significant decrease in the amplitude of the a-wave and b-wave. The b-wave latency was increased significantly from control. Other parameters which were significantly different include a-wave duration, post a-wave duration, both pre and post wave slopes for the a- and b-waves, and the maximum

Table 1. Effect of eserine sulfate (0.3 mg/100 ml) on the in vitro scotopic electroretinogram of the rainbow trout.

Table 1.

	Control	Experimental
a-Wave amplitude (μV)	-32.29± 2.4808(50)	-27.44± 2.1225(50)*
b-Wave amplitude (μV)	110.71± 6.6835(50)	81.04± 5.2094(50)
b-Wave amplitude - isoelectric (μV)	78.65± 5.7141(50)	52.64± 4.9726(50)*
ERG latency (msec)	29.09± 0.9502(50)	29.29± 0.8644(50)
Peak a-wave latency (msec)	70.29± 1.2051(50)	71.27± 1.0684(50)
Peak b-wave latency (msec)	167.22± 2.2798(50)	176.49± 4.0054(50)*
ERG duration (msec)	433.09± 9.1598(50)	425.73± 8.2981(50)
a-Wave duration (msec)	69.06± 1.5698(50)	76.46± 2.2379(50)*
b-Wave duration (msec)	364.03± 9.7837(50)	349.27± 9.0593(50)
Pre a-wave duration (msec)	41.21± 0.9308(50)	41.98± 0.9616(50)
Post a-wave duration (msec)	27.86± 1.1630(50)	34.48± 1.6894(50)*
Pre b-wave duration (msec)	69.07± 2.2604(50)	70.74± 4.3373(50)
Post b-wave duration (msec)	292.95± 8.7712(50)	278.53± 9.0664(50)
Pre a-wave dy/dt (μV/msec)	-0.78± 0.0543(50)	-0.71± 0.0379(50)*
Post a-wave dy/dt (μV/msec)	1.15± 0.0803(50)	0.86± 0.0436(50)*
Pre b-wave dy/dt (μV/msec)	1.11± 0.0743(50)	0.75± 0.0591(50)*
Post b-wave dy/dt (μV/msec)	-0.29± 0.0148(50)	-0.22± 0.0090(50)*
Maximum b-wave absolute (μV)	78.40± 5.7462(50)	51.74± 5.1773(50)*
Minimum b-wave absolute (μV)	-9.08± 2.7200(50)	-8.92± 4.6498(50)

Mean±SE(N) * Different at 5% level based on ANOVA.

Figure 6. Graphical representation of mean in vitro scotopic ERG parameters from eyecups treated with eserine sulfate prior to recording. Control: solid line, Treatment: dashed line. (N = 50).

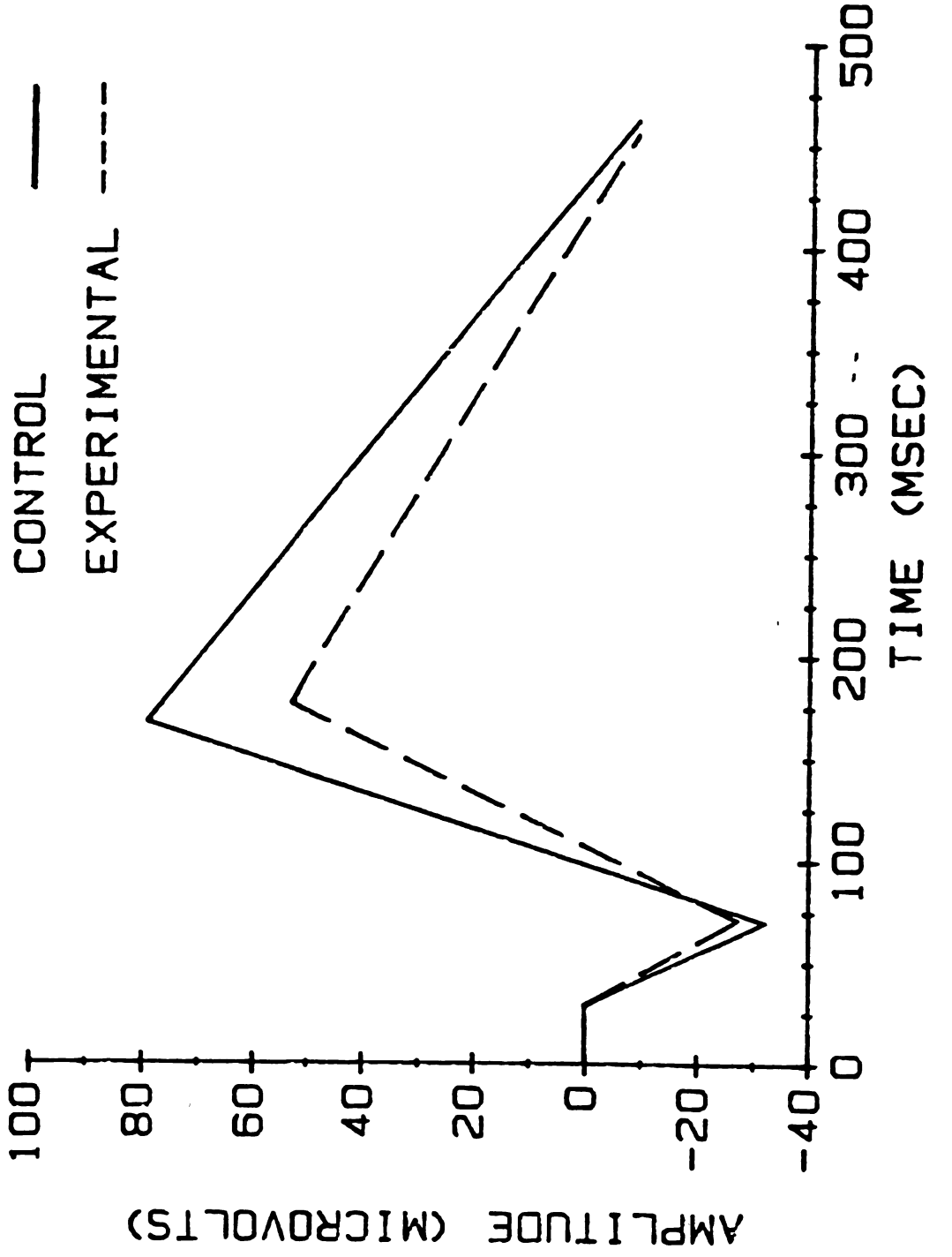
CONTROL
EXPERIMENTAL
AMPLITUDE (MICROVOLTS)
100
80
60
40
20
0
-20
-40
0
100
200
300
400
500
TIME (MSEC)

Figure 6

b-wave amplitude. The remainder of the nineteen parameters were not significantly different from control values.

The Effect of Carbachol

The effect of carbachol (a nonhydrolyzable analog of ACh) on the scotopic ERG (Table 2, Figure 7) showed that there were significant differences from control in each of the 19 parameters. Particular changes of note were a 30% increase in the latency of the ERG from 31.8 msec to 41.3 msec. The latency of the peak a-wave increased 42% from 58.8 msec for control to 83.7 msec for treatment. The a-wave amplitude was depressed by 33% and the b-wave amplitude was depressed by 83%. The latency of the peak b-wave was increased by 23% from 136.1 msec for control to 167.6 msec for treatment. In general the ERG from the treatment group was depressed when compared to its corresponding controls.

The Effect of TOCP after 24 hr

In these TOCP studies the ERG's were recorded 24 hr after i.p. injection of the chemical. These date are presented in Table 3 and Figure 8. An increase in the latency of the ERG (29%) from 40.9 msec to 52.6 msec was observed. The a-wave latency was likewise increased by 24% while there was no significant change in the b-wave latency. The a-wave amplitude for the treatment group was 49% higher than control, 26.1 and 17.5 μV respectively. The b-wave amplitude was 61% smaller in the treatment group than in the controls.

The Effect of DEF after 24 hr

Data from these DEF studies are given in Table 4 and Figure 9. There was a significant difference between treatment and control groups for each of the nineteen parameters described. The most notable of

Table 2. Effect of exogenous acetylcholine analog, carbachol chloride, on the in vitro scotopic electroretinogram of the rainbow trout.

Table 2.

	Control	Experimental
a-Wave amplitude (μV)	-13.47± 1.4561(40)	-9.08± 0.6065(40)*
b-Wave amplitude (μV)	48.53± 3.6633(40)	12.23± 1.3019(40)*
b-Wave amplitude - isoelectric (μV)	35.44± 4.1521(40)	6.21± 0.8517(40)*
ERG latency (msec)	31.75± 1.1106(40)	41.26± 2.1571(40)*
Peak a-wave latency (msec)	58.79± 1.6159(40)	83.67± 5.8088(40)*
Peak b-wave latency (msec)	136.10± 2.8030(40)	167.56± 7.4222(40)*
ERG duration (msec)	379.73± 7.2152(40)	359.10± 6.6286(40)*
a-Wave duration (msec)	49.64± 2.1000(40)	80.64± 5.6719(40)*
b-Wave duration (msec)	330.10± 7.4492(40)	278.47± 7.0013(40)*
Pre a-wave duration (msec)	27.04± 1.2979(40)	42.40± 4.4816(40)*
Post a-wave duration (msec)	22.60± 1.4228(40)	36.30± 2.6347(40)*
Pre b-wave duration (msec)	54.71± 2.6970(40)	45.40± 3.0179(40)*
Post b-wave duration (msec)	275.39± 5.9072(40)	232.80± 7.1072(40)*
Pre a-wave dy/dt (μV/msec)	-0.52± 0.0558(40)	-0.28± 0.0327(40)*
Post a-wave dy/dt (μV/msec)	0.58± 0.0342(40)	0.20± 0.0250(40)*
Pre b-wave dy/dt (μV/msec)	0.60± 0.0548(40)	0.14± 0.0189(40)*
Post b-wave dy/dt (μV/msec)	-0.25± 0.0150(40)	-0.10± 0.0131(40)*
Maximum b-wave absolute (μV)	34.37± 4.4032(40)	2.62± 1.5701(40)*
Minimum b-wave absolute (μV)	-33.46± 4.9207(40)	-20.92± 2.5043(40)*

Mean±SE(N) * Different at 5% level based on ANOVA.

Figure 7. Graphical representation of mean in vitro scotopic ERG parameters from eyecups treated with carbachol prior to recording. Control: solid line, Treatment: dashed line. (N = 40).

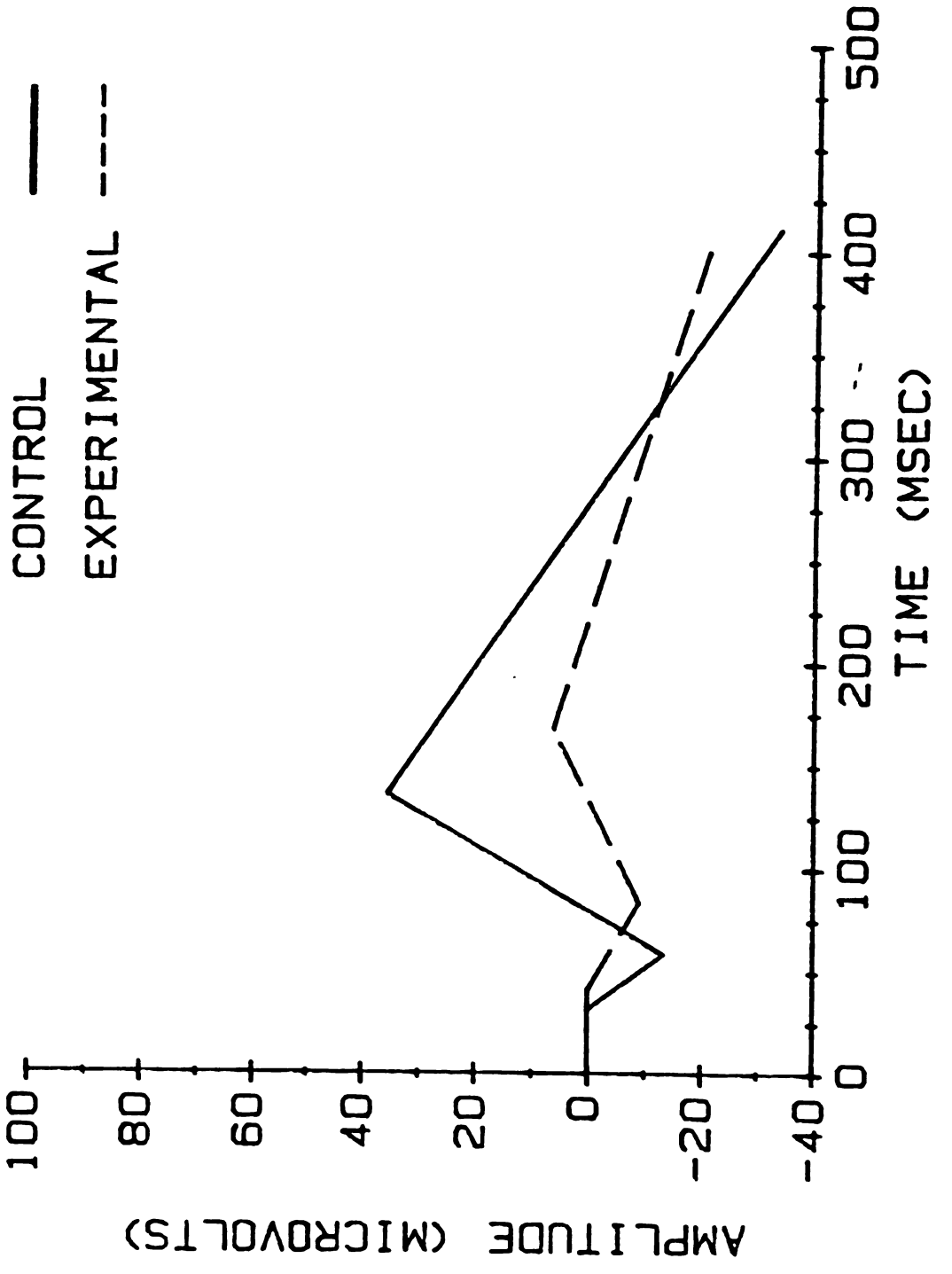
CONTROL
EXPERIMENTAL
AMPLITUDE (MICROVOLTS)
100
80
60
40
20
0
-20
-40
0
100
200
300
400
500
TIME (MSEC)

Figure 7

Table 3. Effect of TOCP (1000 mg/Kg i.p.) 24 hr post injection on the in vitro scotopic electroretinogram of the rainbow trout.

Table 3.

	Control	Experimental
a-Wave amplitude (μV)	-17.51± 0.9252(40)	-26.11± 0.7125(60)*
b-Wave amplitude (μV)	49.51± 2.3439(40)	34.92± 2.7823(60)
b-Wave amplitude - isoelectric (μV)	33.31± 2.0152(40)	12.99± 1.7715(60)*
ERG latency (msec)	40.91± 1.3513(40)	52.60± 1.3670(60)*
Peak a-wave latency (msec)	81.18± 2.4852(40)	100.56± 1.9348(60)*
Peak b-wave latency (msec)	207.50± 3.8246(40)	200.89± 2.8778(60)
ERG duration - total (msec)	463.08± 6.7649(40)	427.51± 7.6415(60)
a-Wave duration (msec)	82.89± 2.9356(40)	101.75± 2.3098(60)
b-Wave duration (msec)	380.66± 7.2921(40)	330.74± 9.1630(60)
Pre a-wave duration (msec)	40.27± 1.6560(40)	48.09± 1.6092(60)
Post a-wave duration (msec)	40.92± 1.1338(40)	53.78± 2.2066(60)
Pre b-wave duration (msec)	83.71± 3.1565(40)	46.54± 2.8869(60)*
Post b-wave duration (msec)	296.95± 5.0217(40)	283.03± 7.1663(60)
Pre a-wave dy/dt (μV/msec)	-0.44± 0.0180(40)	-0.60± 0.0315(60)
Post a-wave dy/dt (μV/msec)	0.41± 0.0200(40)	0.41± 0.0328(60)
Pre b-wave dy/dt (μV/msec)	0.38± 0.0244(40)	0.22± 0.0224(60)
Post b-wave dy/dt (μV/msec)	-0.15± 0.0063(40)	-0.18± 0.0107(60)
Maximum b-wave (μV)	33.08± 2.0173(40)	8.67± 2.4185(60)
Mimimum b-wave (μV)	-34.04± 0.8588(40)	-39.69± 3.5599(60)

Mean±SE(N) * Different at 5% level based on ANOVA.

Figure 8. Graphical representation of mean in vitro scotopic ERG parameters from fish treated with TOCP (1000 mg/Kg i.p.) 24 hr prior to recording. Control: solid line, N = 40; Treatment: dashed line, N = 60.

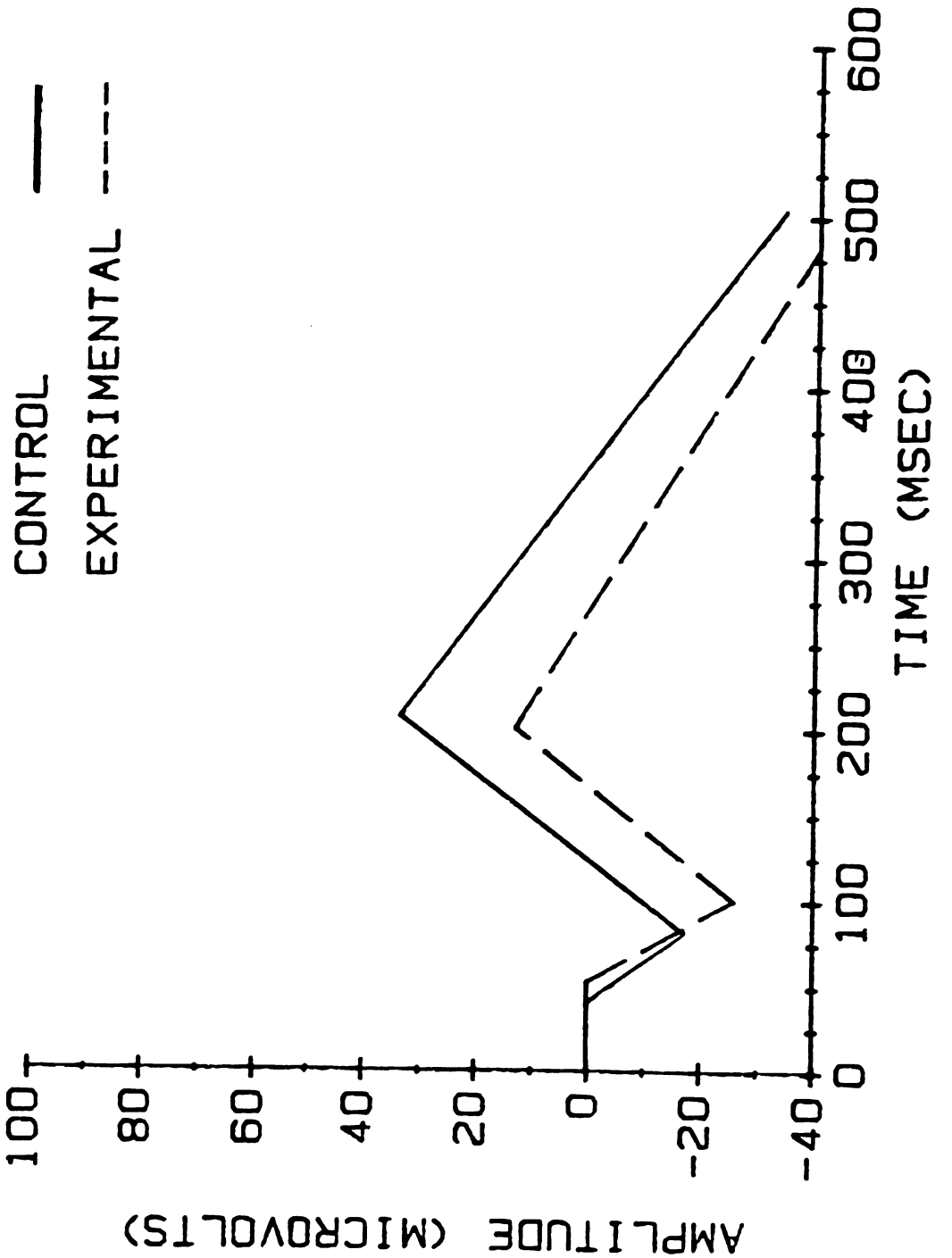
CONTROL
EXPERIMENTAL
AMPLITUDE (MICROVOLTS)
100
80
60
40
20
0
-20
-40
0
100
200
300
400
500
600
TIME (MSEC)

Figure 8

Table 4. Effect of DEF (100 mg/Kg i.p.) 24 hr post injection on the in vitro scotopic electroretinogram of the rainbow trout. N=90 for all groups.

Table 4.

	Control	Experimental
a-Wave amplitude (μV)	-24.82± 1.5252	-30.28± 1.0753*
b-Wave amplitude (μV)	92.72± 4.0701	61.66± 3.5734*
b-Wave isoelectric (μV)	68.69± 2.7402	33.57± 2.7675*
ERG latency (msec)	40.35± 1.3497	23.27± 1.1917*
Peak a-wave latency (msec)	74.00± 1.3621	58.04± 1.4979*
Peak b-wave latency (msec)	176.96± 2.6896	131.05± 2.5356*
ERG duration - total (msec)	688.44±28.1516	446.90± 6.9715*
a-Wave duration (msec)	61.92± 0.9663	67.34± 1.2671*
b-Wave duration (msec)	626.52±28.4254	379.56± 7.1692*
Pre a-wave duration (msec)	33.06± 0.7945	35.11± 0.8099*
Post a-wave duration (msec)	28.36± 0.8402	32.23± 0.9433*
Pre b-wave duration (msec)	75.76± 1.4505	40.78± 2.1350*
Post b-wave duration (msec)	550.76±28.3890	342.11± 6.4433*
Pre a-wave dy/dt (μV/msec)	-0.72± 0.0421	-0.88± 0.0320*
Post a-wave dy/dt (μV/msec)	0.96± 0.0746	0.89± 0.0420*
Pre b-wave dy/dt (μV/msec)	0.94± 0.0427	0.67± 0.0468*
Post b-wave dy/dt (μV/msec)	-0.13± 0.0094	-0.21± 0.0070*
Maximum b-wave absolute (μV)	68.49± 2.7445	31.19± 3.0696*
Minimum b-wave absolute (μV)	10.26± 1.0671	-39.37± 1.3708*

Mean±SE(N) * Different from control at 5% level based on ANOVA.

Figure 9. Graphic representation of mean in vitro scotopic ERG parameters from fish treated with DEF (100 mg/Kg i.p.) 24 hr prior to recording. Control: solid line, Treatment: dashed line. (N = 90).

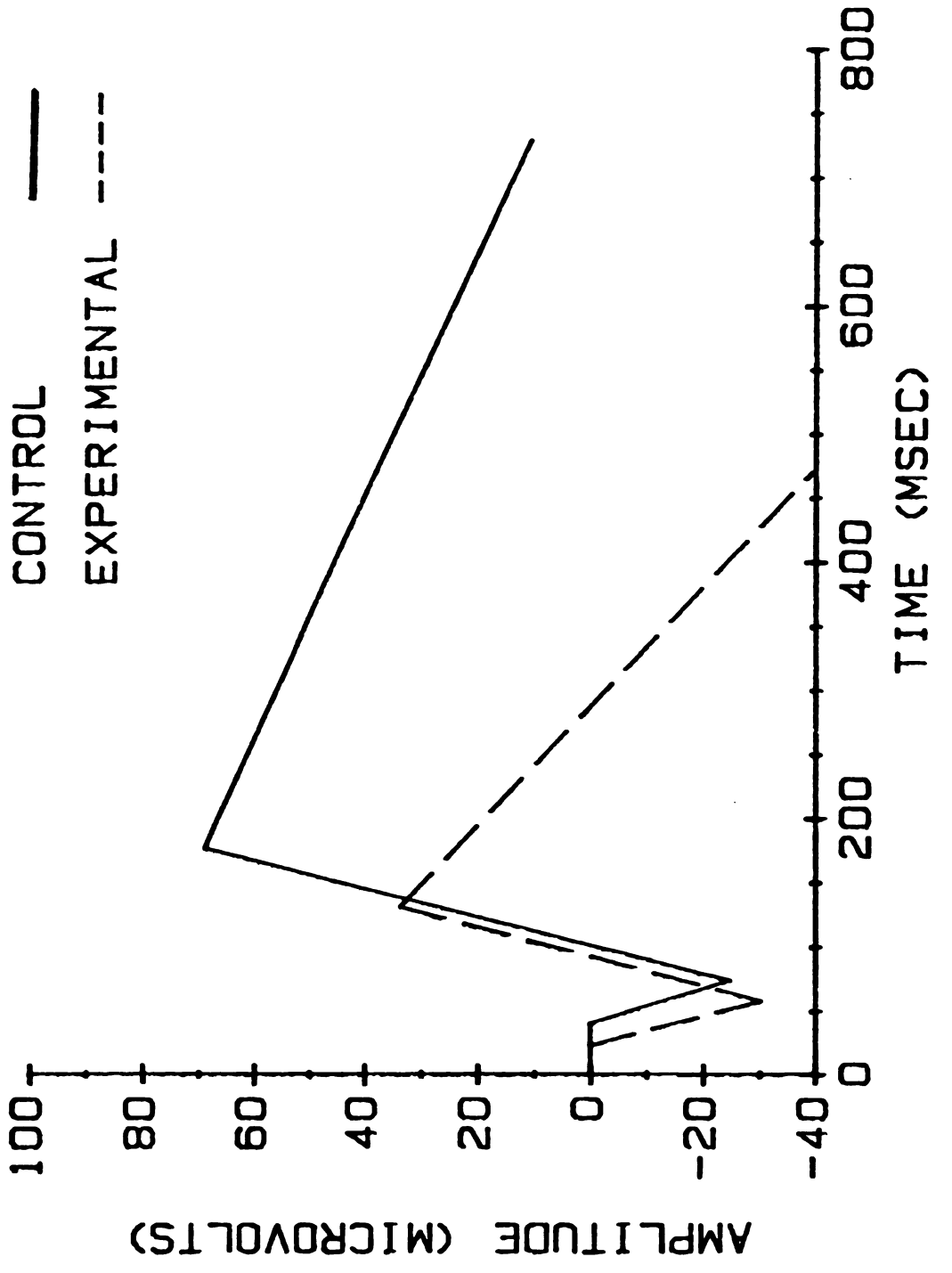
CONTROL
EXPERIMENTAL
AMPLITUDE (MICROVOLTS)
100
80
60
40
20
0
-20
-40
0
200
400
600
800
TIME (MSEC)

Figure 9

these changes include a 42% decrease in the latency of the ERG. The treatment ERG latency was 23.3 msec as compared to 40.4 msec for the control group. The latency of the peak a-wave was 22% less than controls and the amplitude of the a-wave was 22% greater in the treatment group than in the controls. The effects on the b-wave include a 26% decrease in latency and a 51% decrease in amplitude.

Effect of Organophosphates on the Isolated a-wave

Recordings of isolated a-wave (following aspartate treatment) were made from animals previously treated with TOCP or DEF. Data from the TOCP experiment are given in Table 5 and Figure 10. These show significant changes in eight of the nine parameters of the isolated a-wave. The amplitudes of both the fast and slow PIII were significantly increased. Duration of the fast PIII was increased while the duration of the slow PIII was significantly decreased. The peak latency for the fast PIII was increased while the peak latency for the slow PIII was decreased. The latency of the ERG was not changed from control. The data from the isolated a-wave of eyes treated with DEF are given in Table 6 and Figure 11. With DEF there was an increase in amplitude of both the fast and slow PIII. The duration of the fast PIII was decreased and the duration of the slow PIII was increased. The latency of both the ERG and the peak fast PIII was decreased. The latency of the peak slow PIII was unchanged from control. In general the isolated a-wave of the DEF-treated eyes exhibited an increased response to photostimulation.

Table 5. Effect of TOCP (1000 mg/Kg i.p.) 24 hr post injection on the in vitro scotopic isolated a-wave of the rainbow trout.

	Control	TOCP
Fast PIII amplitude (μV)	-56.12± 2.6452(50)	-83.82± 5.4020(50) *
Slow PIII amplitude (μV)	-60.79± 3.8312(50)	-72.42± 5.9620(50) *
PIII maximum amplitude (μV)	-116.93± 5.8719(50)	-156.24±10.3339(50) *
Fast PIII duration (msec)	104.34± 3.6307(50)	121.99± 2.7777(50) *
Slow PIII duration (msec)	639.11±59.4026(50)	299.41± 5.7221(50) *
Fast PIII dy/dt (μV/msec)	-0.58± 0.0360(50)	-0.71± 0.0468(50) *
ERG latency (msec)	53.22± 1.8735(50)	49.49± 1.5857(50)
Fast PIII latency (msec)	155.49± 5.4758(50)	171.49± 3.2513(50) *
Slow PIII latency (msec)	833.80±63.4509(50)	473.30± 7.7386(50) *

Mean±SE(N)

* Different at 5% level based on ANOVA.

Figure 10. Isolated PIII (a-wave) reconstructed from mean parameter values on in vitro scotopic ERG from fish treated with TOCP (1000 mg/Kg i.p.) 24 hr prior to recording. Eyecups treated with Na-aspartate prior to recording. Control: solid line, Treatment: dashed line. (N = 50).

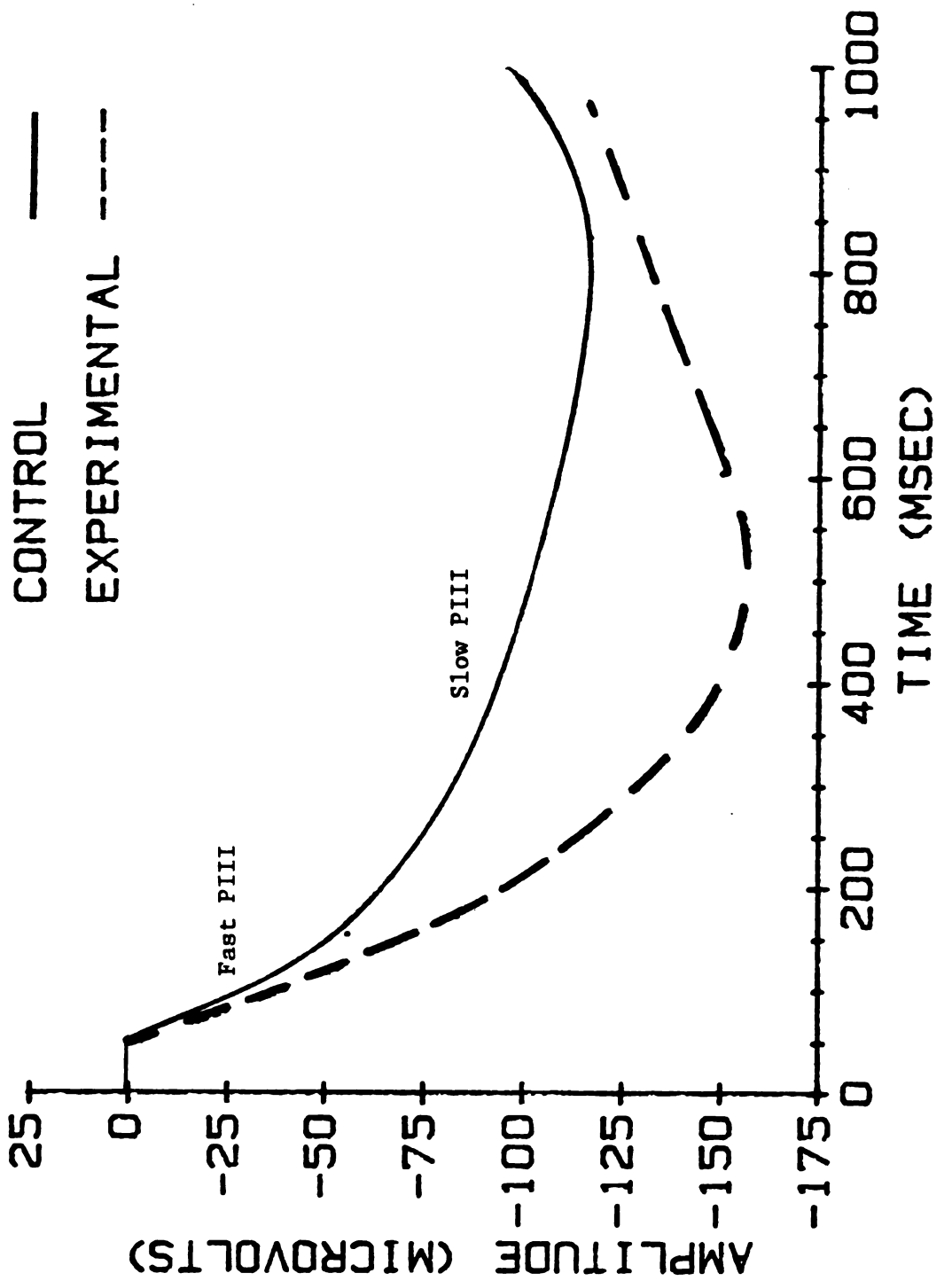
CONTROL
EXPERIMENTAL
Fast PIII
Slow PIII
AMPLITUDE (MICROVOLTS)
25
0
-25
-50
-75
-100
-125
-150
-175
0
200
400
600
800
1000
TIME (MSEC)

Figure 10

Table 6. Effect of DEF (100 mg/Kg i.p.) 24 hr post injection on the in vitro scotopic isolated a-wave of the rainbow trout.

	Control	Experimental
Fast PIII amplitude (μV)	-71.15± 1.6235(100)	-88.65± 4.8275(100)*
Slow PIII amplitude (μV)	-35.81± 1.6975(100)	-50.42± 3.1638(100)*
PIII maximum amplitude (μV)	-106.96± 2.9571(100)	-138.18± 7.5945(100)*
Fast PIII duration (msec)	112.46± 1.8224(100)	101.06± 1.6972(100)*
Slow PIII duration (msec)	175.51± 3.3909(100)	195.63± 3.6309(100)*
Fast PIII dy/dt (μV/msec)	-0.65± 0.0188(100)	-0.91± 0.0533(100)*
ERG latency (msec)	42.44± 1.1329(100)	38.77± 0.9059(100)*
Fast PIII latency (msec)	154.90± 2.4839(100)	139.82± 1.9034(100)*
Slow PIII latency (msec)	330.41± 3.8774(100)	335.45± 2.7882(100)

Mean±SE(N)

* Different at 5% level based on ANOVA.

Figure 11. Isolated PIII (a-wave) reconstructed from mean parameter values on in vitro scotopic ERG from fish treated with DEF (100 mg/Kg i.p.) 24 hr prior to recording. Eyecups treated with Na-aspartate prior to recording. Control: solid line, Treatment: dashed line. (N = 100).

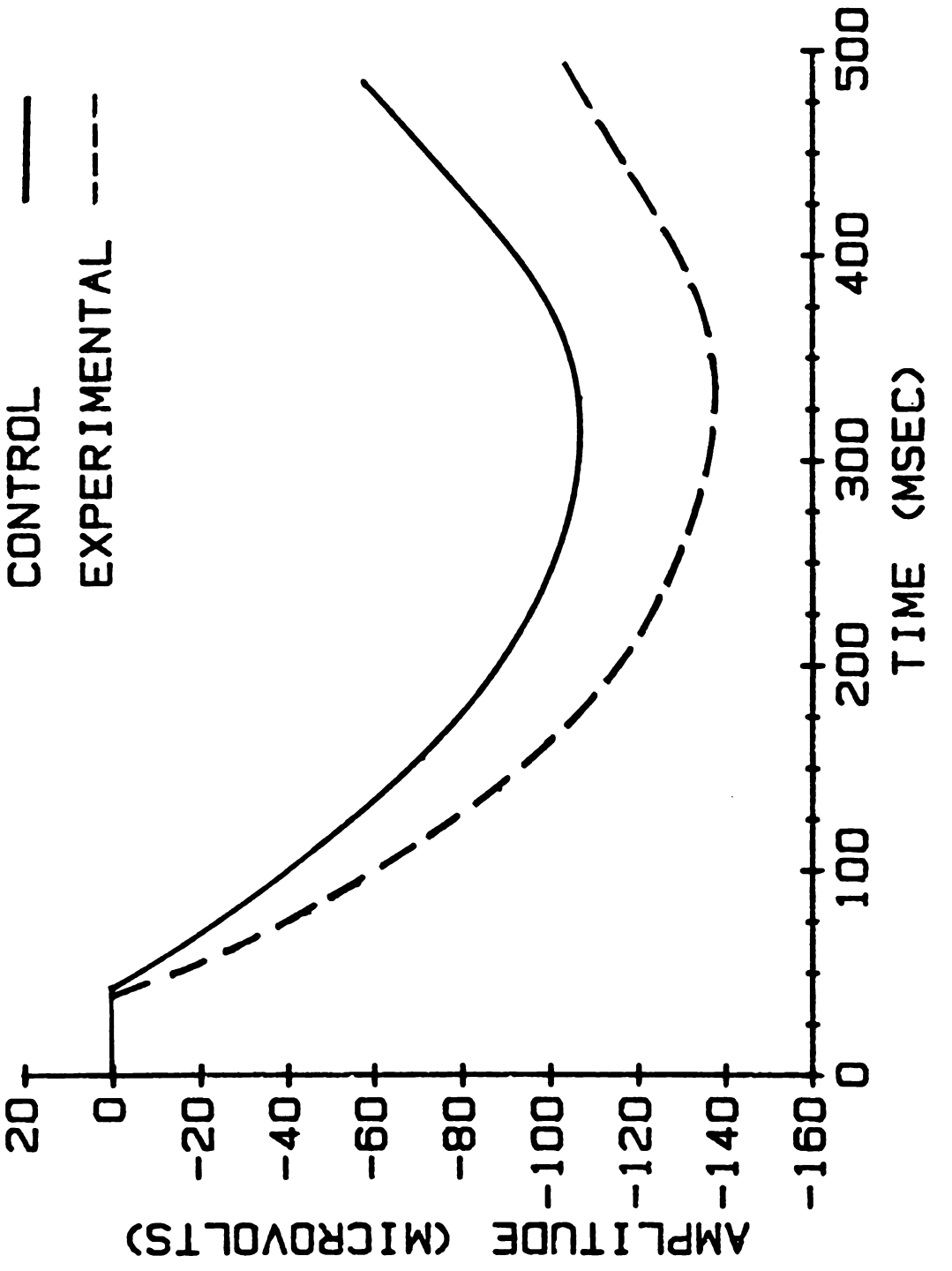
CONTROL
EXPERIMENTAL
AMPLITUDE (MICROVOLTS)
20
0
-20
-40
-60
-80
-100
-120
-140
-160
0
100
200
300
400
500
TIME (MSEC)

Figure 11

The Effect of TOCP after 15 Days

Table 7 and Figure 12 describe the results from fish receiving a single injection of TOCP 15 days prior to recording scotopic ERG. The data show few significant changes from the 15 day control. The a-wave amplitude which had increased with the 24 hr study now shows a 48% decrease from control and the b-wave is still depressed by 64%. It had been depressed 61% in the 24 hr study. In most other respects the treatment and control groups were not significantly different.

The Effect of DEF after 15 Days

The results following a single injection of DEF 15 days prior to the ERG recording were similar to the TOCP data in that very few significant changes were noted. The b-wave was the only component of the ERG which exhibited any significant deviation from the control. Table 8 and Figure 13 list the data which show increased latency of the peak b-wave and an increased duration of the whole ERG.

Acetylchoninesterase Activity

Assays to determine AChE activity were run after treatment with TOCP or DEF at periods of one and 15 days following a single i.p. injection (Table 9). Exposure to TOCP significantly reduced plasma AChE activity after one day. The AChE activity in whole blood, brain, and spinal cord were not significantly changed from control. After 15 days the only significant change was a greater than normal plasma AchE activity while blood, brain and spinal cord was not significantly different from control.

The AChE data from fish treated with DEF (100 mg/Kg) show a significant decrease in whole blood AchE activity after one day (Table 10). The levels for plasma, brain and spinal cord were not

Table 7. Effect of TOCP (1000 mg/Kg i.p.) 15 days post injection on the in vitro scotopic electroretinogram of the rainbow trout.

Table 7.

	Control	Experimental
a-Wave amplitude (μV)	-26.08± 1.1400(160)	-13.57± 0.7618(90)*
b-Wave amplitude (μV)	80.77± 2.8286(160)	32.32± 2.2702(90)*
b-Wave amplitude - isoelectric (μV)	54.87± 2.5948(160)	19.95± 1.6302(90)*
ERG latency (msec)	41.91± 0.7957(160)	42.57± 0.9038(90)
Peak a-wave latency (msec)	82.12± 1.2869(160)	90.30± 1.4863(90)
Peak b-wave latency (msec)	199.01± 2.2505(160)	208.24± 3.8551(90)
ERG duration - total (msec)	450.83± 4.8458(160)	436.58± 8.8210(90)
a-Wave duration (msec)	79.31± 2.2592(160)	97.54± 3.2597(90)
b-Wave duration (msec)	371.52± 5.4516(160)	337.93± 9.2096(90)
Pre a-wave duration (msec)	40.21± 1.0110(160)	47.84± 1.2208(90)
Post a-wave duration (msec)	39.10± 1.5948(160)	49.81± 2.4654(90)
Pre b-wave duration (msec	77.50± 1.6122(160)	68.13± 3.2336(90)
Post b-wave duration (msec)	293.68± 4.7740(160)	269.80± 7.0872(90)
Pre a-wave dy/dt (μV/msec)	-0.66± 0.0264(160)	-0.30± 0.0158(90)*
Post a-wave dy/dt (μV/msec)	0.81± 0.0411(160)	0.28± 0.0203(90)*
Pre b-wave dy/dt (μV/msec)	0.70± 0.0308(160)	0.25± 0.0181(90)*
Post b-wave dy/dt (μV/msec)	-0.20± 0.0078(160)	-0.10± 0.0048(90)*
Maximum b-wave (μV)	54.09± 2.6297(160)	18.24± 1.7913(90)*
Minimum b-wave (μV)	-4.30± 1.2736(160)	-9.64± 1.0939(90)

Mean±SE(N) * Different at 5% level based on ANOVA.

Figure 12. Graphic representation of mean in vitro scotopic ERG parameters from fish treated with TOCP (1000 mg/Kg i.p.) 15 days prior to recording. Control: solid line, N = 120; Treatment: dashed line, N = 90.

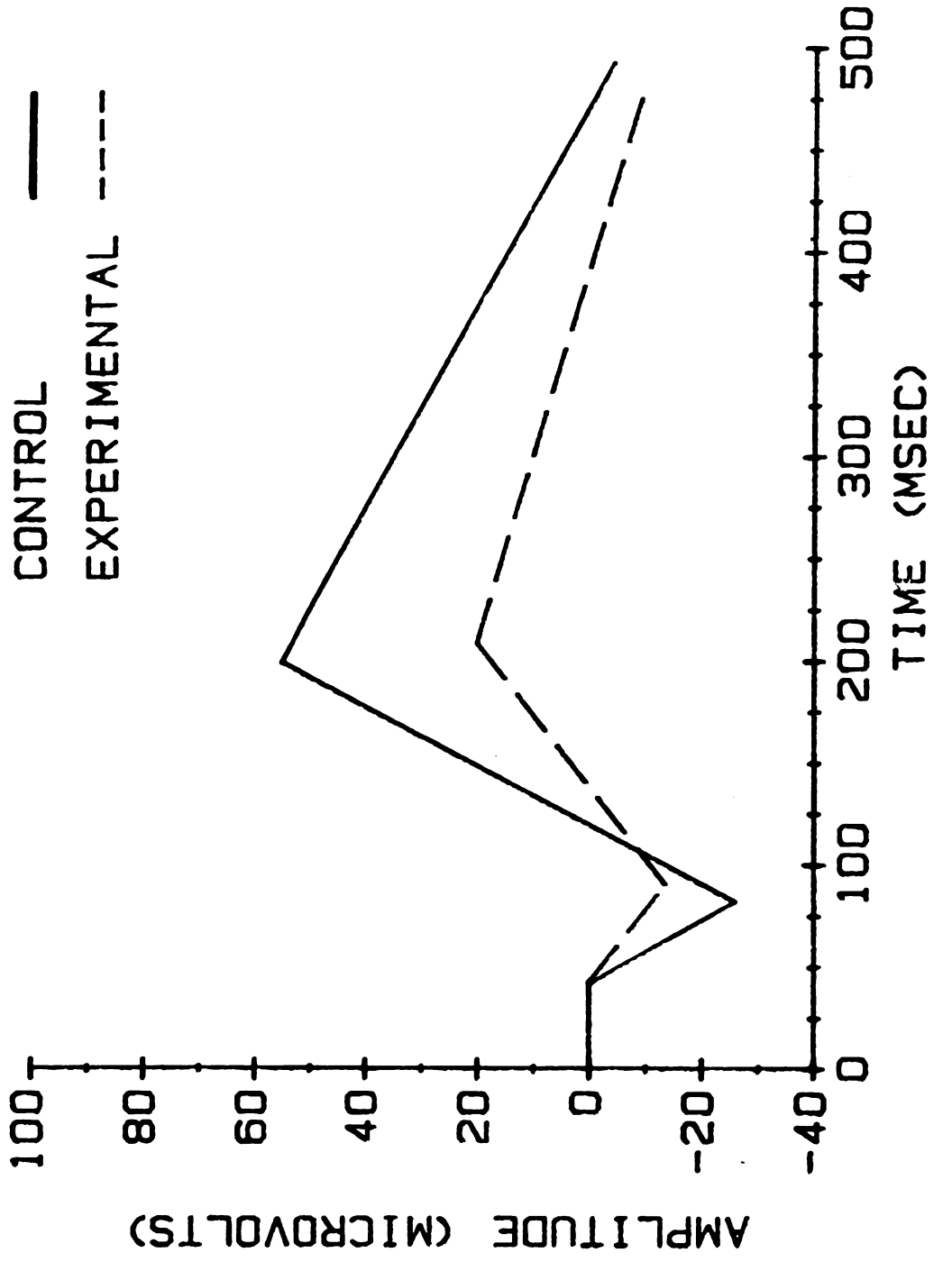
CONTROL
EXPERIMENTAL
AMPLITUDE (MICROVOLTS)
100
80
60
40
20
0
-20
-40
0
100
200
300
400
500
TIME (MSEC)

Figure 12

Table 8. Effect of DEF (100 mg/Kg i.p.) 15 days post injection on the in vitro scotopic electroretinogram of the rainbow trout.

Table 8.

	Control	Experimental
a-Wave amplitude (μV)	-37.75± 1.5155(200)	-38.50± 1.1908(180)
b-Wave amplitude (μV)	82.21± 2.4287(200)	95.38± 3.2144(180)
b-Wave amplitude - isoelectric (μV)	46.05± 2.2425(200)	59.90± 2.9871(180)
ERG latency (msec)	41.58± 0.6160(200)	43.37± 0.6878(180)
Peak a-wave latency (msec)	86.72± 0.8797(200)	91.75± 1.1820(180)
Peak b-wave latency (msec)	193.35± 1.4568(200)	208.55± 1.7051(180)*
ERG duration - total (msec)	320.36± 3.2259(200)	351.56± 3.5293(180)*
a-Wave duration (msec)	87.82± 1.7336(200)	92.25± 2.1297(180)
b-Wave duration (msec)	232.52± 2.6725(200)	259.31± 2.8742(180)*
Pre a-Wave duration (msec)	45.13± 0.8113(200)	48.94± 0.9317(180)
Post a-Wave duration (msec)	42.69± 1.2060(200)	43.44± 1.3931(180)
Pre b-Wave duration (msec)	63.91± 1.5270(200)	72.93± 1.6129(180)
Post b-Wave duration (mse)	168.59± 2.2912(200)	185.82± 2.6013(180)
Pre a-Wave dy/dt (μV/msec)	-0.81± 0.0258(200)	-0.79± 0.0205(180)
Post a-Wave dy/dt (μV/msec)	0.87± 0.0304(200)	0.91± 0.0311(180)
Pre b-Wave dy/dt (μV/msec)	0.66± 0.0276(200)	0.75± 0.0312(180)
Post b-Wave dy/dt (μV/msec)	-0.28± 0.0070(200)	-0.26± 0.0074(180)
Maximum b-wave (μV)	44.04± 2.4163(200)	56.31± 3.2924(180)
Minimum b-wave (μV)	-1.89± 2.0514(200)	10.29± 2.5970(180)

Mean±SE(N) * Different at 5% level based on ANOVA.

Figure 13. Graphic representation of mean in vitro scotopic ERG parameters from fish treated with DEF (100 mg/Kg i.p.) 15 days prior to recording. Control: solid line, N = 200; Treatment: dashed line, N = 180.

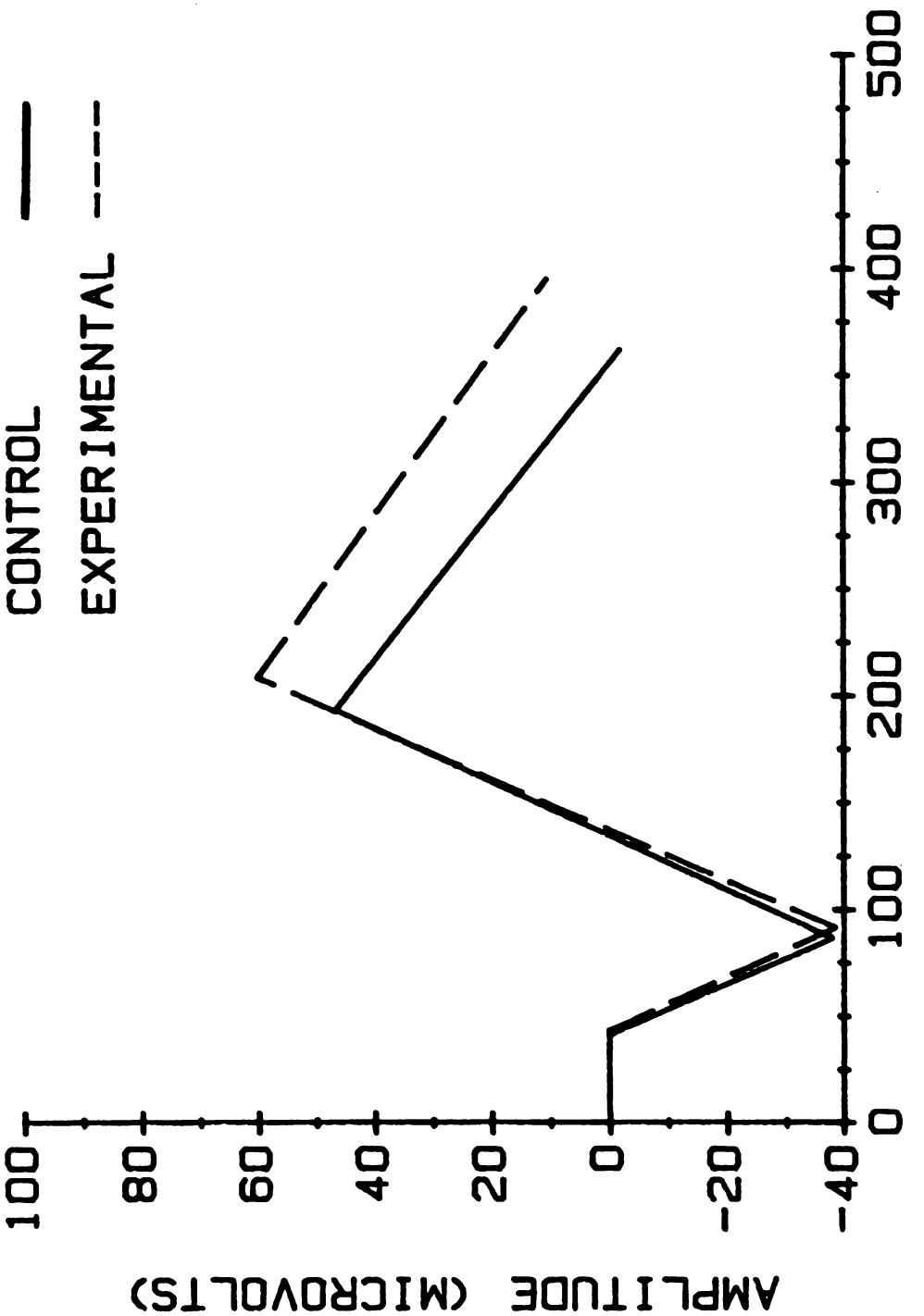
CONTROL
EXPERIMENTAL
AMPLITUDE (MICROVOLTS)
100
80
60
40
20
0
-20
-40
0
100
200
300
400
500
TIME (MSEC)

Figure 13

Table 9. Cholinesterase activity from selected tissue of rainbow trout following exposure to TOCP. Experimental animals were injected with TOCP (1000 mg/Kg i.p.) 24 hr prior to assay. Units are expressed as μM activity/hr/g or ml.

Tissue	Control	Day 1	Day 15
Blood	45.21± 11.61(9)	29.07± 6.61(3)	62.52± 14.96(5)
Plasma	36.34± 9.97(9)	7.39± 7.39(3)*+	67.00± 7.56(5)*
Brain	1335.60± 149.49(9)	1283.85± 41.70(3)	1320.37± 277.22(5)
Spinal Cord	1945.66± 346.55(9)	1458.49± 136.91(3)	1914.01± 379.07(5)

Mean±SE(N)

* Different from the control at 5% level based on ANOVA.

+ Two samples exhibited zero detectable activity.

Table 10. Cholinesterase activity from selected tissue of rainbow trout following exposure to DEF. Experimental animals were injected with DEF (100 mg/Kg i.p.) 24 hr prior to assay. Units are expressed as μM activity/hr/g or ml.

Tissue	Control	Day 1	Day 15
Blood	31.42± 3.66(10)	18.21± 4.87(5)*	25.53± 5.27(10)
Plasma	26.61± 3.55(10)	42.44± 21.49(3)	26.97± 6.86(10)
Brain	1561.04± 110.04(10)	1101.31± 241.79(5)	1435.38± 103.93(10)
Spinal Cord	2006.03± 97.28(9)	1407.56± 393.58(4)	1516.13± 156.59(10)*

Mean±SE(N)

* Different from the control at 5% level based on ANOVA.

significantly different from the controls. After 15 days, blood activity returned to normal levels with plasma and brain levels were not significantly different from control but spinal cord activity was significantly lower than control.

Acetylcholinesterase activity was measured in retinas treated with eserine. The eyecups were exposed to a Ringer solution containing 0.3 mg/100 ml of eserine sulfate. Results show a mean activity of 10.3 μM/mg protein/hr for control and a mean of 5.0 μM/mg protein/hr for the treatment group. This difference was significant using a paired t test, $p = 0.05$, $df = 4$, $(n = 5)$.

Table 11 presents a summary of five main parameters for each of the drug treatments. The data are expressed as a percent of the control for each experiment. This table is intended to give an overview of the study and was not used for quantitative analysis.

Table 11. Summary of the effects of organophosphates on the in vitro scotopic electroretinogram of the rainbow trout expressed as percent of control if significantly different.

TREATMENT	ERG Lat	a-Lat	a-Amp	b-Lat	b-Amp
ESERINE 0.3mg/100ml	N.S.	N.S.	15% dec	6% inc	33% dec
CARBACHOL 66.7mg/100ml	30% inc	42% inc	33% dec	23% inc	83% dec
TOCP (24h) 1000mg/kg	29% inc	24% inc	49% inc	N.S.	61% dec
DEF (24h) 100mg/kg	42% dec	22% dec	22% inc	26% dec	51% dec
TOCP (15 day) 1000mg/kg	N.S.	N.S.	48% dec	N.S.	64% dec
DEF (15 day) 100mg/Kg	N.S.	N.S.	N.S.	8% inc	N.S.

inc = increased
dec = decreased
N.S. = not significant

DISCUSSION

General Observations-In Vitro Scotopic ERG

The ERG was used as an index of retinal function. Significant differences from control, as determined by using a two way ANOVA with replications, were considered to be indicative of a "toxic" response of the retina to the test substance. The isolated eyecup preparations proved ideal for these studies because it separated the retinal response from the possibility of complicating systemic factors. Thus any observed changes were considered to be the result of a direct action of the drug on the retina and not mediated by drug actions on the cardiovascular, respiratory or nervous systems. Isolation of the eyecup was not considered to pose undue metabolic stress on the retina; Ubels (1976) noted that the isolated eyecup preparation could be maintained for up to 6 hr without significant deterioration.

It was observed in this study that there was a large variation in ERG parameters between eyes but very little within replicatons. Several factors could contribute to this variation. Although every attempt was made to maintain constant conditions, it was inevitable that various aspects of the recording conditions changed.

Three conditions of the recording procedure which were difficult to precisely control were 1) electrical properties of the low resistance active and reference agar electrodes (i.e., tip geometry), 2) precise

placement of electrode in the center of the eyecup and 3) maintaining a constant volume conductor by removal of equal amounts of vitreous body in each preparation. The latter two were performed under dark red illumination and hence precise control of these factors was difficult. In the dark adapted teleost retina, retinomotor activity causes the rods to be retracted from the pigmented epithelium thus weakening the structrual integrity of the retina. Dark adaptation is a standard preparatory step for isolating a pigmented epithelium free retina (Hoffert and Ubels, 1978). In preparing the eyecups for these experiments filter paper strips were used to absorb aqueous and vitreous humor. During this process the retina would occasionally adher to the filter paper resulting in the dislodgement of the retina from its normal juxtaposition to the pigmented epithelium. This last factor was the most likely cause of the eye to eye variations observed in these experiments.

The digitized ERG data were compiled into 19 distinct parameters. These parameters thoroughly describe the ERG and allow for a quantitative statistical analysis (Tables 1-8). The figures (Figures 5-13) of the reconstructed ERG's are for conceptual purposes only and were not used as a basis for the quantitative interpretation.

The Effect of Inhibition of AChE on the In Vitro Scotopic ERG

In order to determine the effect of cholinesterase inhibition (i.e., increased endogenous ACh) eyecups were treated with eserine sulfate, a potent inhibitor of AChE. Eserine, also known as physostigmine, is a tertiary amine which readily penetrates lipid barriers and it is well documented that it is not associated with the phenomenon of delayed neurotoxicity (Gilman et al., 1980). Eserine exposed eyecups exhibited

a 50% reduction in AChE activity for the duration of the experiment. This is expected because the main form of elimination of eserine sulfate is by hydrolytic cleavage catalyzed by plasma esterases. Both TOCP and DEF are known to cause delayed neurotoxicity (Casarett, 1980) and although neither are potent inhibitors of AChE, it was felt that the ERG should be measured with AChE inhibited by eserine so that the anti-cholinesterase effects could be separated from the delayed neurotoxic actions of the organophosphates.

Eserine significantly decreased a-wave amplitude but did not affect the ERG latency or peak a-wave latency. The b-wave amplitude was decreased and the initiation of the b-wave (post a-wave duration and slope) showed a much slower development. These results were expected in that AChE has been histochemically identified only in the amacrine cells of the retina (Nichols, Hewitt and Laties, 1972). This corresponds to the proximal region of the retina thought to be primarily responsible for the b-wave. Because the b-wave is a complex response of the Müller cells and not a direct measure of nervous activity it is difficult to determine exactly how the inhibition of AChE at the amacrine cell layer is translated into an alteration of the b-wave. It appears however that transmission of the photo-signal through the neural retina is slowed. Ames and Pollen (1969) have identified an inhibitory role of ACh in the retina which could inhibit the spread of the visual signal and lead to slower development and decreased amplitude of the b-wave. The depressed a-wave amplitude could also be a result of the inhibitory action of the accumulated ACh via a horizontal cell mediated negative feedback to the photoreceptor cells. These data give a good indication of what can be expected if the only action of the organophosphate on the retina was the

inhibition of AChE with resulting increased levels of ACh at the cholinergic synapses.

The Action of Exogenous ACh on the ERG

The inhibitory action of ACh on the ERG was investigated with the use of carbachol. In order to eliminate the problem of rapid hydrolysis of ACh by endogenous AChE, isolated eyecups were treated with a nonhydrolyzable analog of ACh (carbachol). In general the entire ERG was severely attenuated (Table 2). The ERG latency increased and the latency of each of the waves was also increased. The amplitude of both waves was decreased markedly. Although the level of carbachol used was nonphysiological, these results give a dramatic demonstration of the wide action of ACh, even on areas of the retina which have been shown to be free of AChE (i.e., photoreceptors).

The major findings of both of these studies indicate that with increased levels of ACh, whether via AChE inhibition or exogenous addition, the ERG shows general depression of both the a-wave and the b-wave (Tables 1,2,11). The carbachol data exhibited much greater effects of ACh which were probably due to the large dose and that with carbachol the entire retina was saturated with an ACh analog. With eserine the accumulation of ACh was limited to cholinergic synapses of the amacrine cells.

The results of the above experiments will aid in the interpretation of the action of organophosphates on the retina. Although the organophosphate agents used in this study have been shown to be weak inhibitors of AChE, it cannot be assumed that they exhibit no anticholinesterase action on the retina. Therefore the above findings form a very important part of the complete study. Assays for

the level of AChE activity were only able to show a statistically significant decrease in selected tissue after treatment with these organophosphate agents (Tables 9 and 10). Although there was very little anticholinesterase action these studies can be used as a reference point for further work.

Action of TOCP on the ERG

Although DEF was the main agent of interest in this study, TOCP was also tested because it has been documented to produce OPIDN. The fish were able to tolerate large doses of TOCP (1000 mg/Kg) administered i.p. 24 hr before the ERG were recorded. The results from these experiments (Table 3 and Figure 8) show a marked effect on the a-wave. The latency of the ERG was increased 11.7 msec and the latency of the peak a-wave increased by 19.4 msec. At the same time the amplitude of the a-wave rose 8.6 μV. The development rate of the a-wave, as measured by pre a-wave dy/dt, was significantly increased. This increased rate of response combined with the increased amplitude of the a-wave represents a potentiation of the photoreceptor response to light. This action of TOCP is opposite to that observed with physostigmine or with carbachol. It appears that TOCP is affecting the retina such that the hyperpolarization of the photoreceptors is delayed but once initiated it develops more rapidly and to a greater extent. This apparent potentiation of the photoreceptor response must have overcome the otherwise inhibitory action of any accumulated ACh as seen with eserine and carbachol (Tables 1 and 2). These actions of TOCP are at the photoreceptor layer of the retina which has been documented to be noncholinergic. Thus the organophosphate TOCP is exhibiting effects which are independent of any anticholinesterase activity it may have.

Action of DEF on the ERG

The results recorded one day after exposure to DEF (100 mg/Kg i.p.) show significant changes in each of the 19 parameters. Changes of most interest are those parameters associated with the a-wave which include a 42% decrease in ERG latency, a 22% decrease in peak a-wave latency and a 22% increase in a-wave amplitude (Tables 1 and 11). Thus the photoreceptors of the DEF-treated retinas initiated their response faster, and once initiated the response developed faster and to a greater extent than control retinas. This is similar to the response following exposure to TOCP, however DEF treatment resulted in a decreased ERG latency as compared to the increased latency observed with TOCP.

Action of Organophosphates on the Isolated a-wave

Since a large decrease in b-wave amplitude was noted for both groups (TOCP and DEF) it can be argued that the increased rate of development and amplitude of the a-wave was simply due to decreases in the b-wave. This question can be addressed two ways. First, the data from the eserine and carbachol experiments both show a decreased b-wave amplitude, however in both cases the a-wave amplitude was decreased rather than increased. Secondly, experiments were conducted with both TOCP and DEF in which the a-wave was isolated by treating the eyecup with sodium aspartate just prior to recording the ERG. In the group treated with TOCP the results are very similar to those found with the intact ERG (Table 5, Figure 10). The PIII of the treatment group had a greater amplitude and faster rate of development than that of the controls. In particular, the fast PIII, which is the only visible portion of the PIII in the intact ERG, was increased by 49%. The only

parameter which was not significantly different from control was the latency of the ERG, this may be due to a decreased negative feedback from the horizontal cells.

In the eyes treated with DEF the isolated a-wave results are identical to those observed with the intact ERG. The data given in Table 6 and Figure 11 show a greater amplitude and faster rate of development in the treatment group. It can also be noted that the latency of the PIII was significantly decreased with DEF, just as it was significantly decreased with DEF treatment in the intact ERG.

These results are significant because the photoreceptor layer of the retina has been shown histochemically to be free of endogenous cholinesterase activity. The isolated a-wave experiments support a hypothesis that the organophosphates are acting on the retinal photoreceptors directly and independently of any action that may be occurring in the proximal neural retina.

Long-term Exposure to Organophosphates

Experiments to determine the long-term (15 day) effects of the organophosphates were conducted. Animals were given single doses (1000 mg/Kg) of TOCP and 15 days later ERG were recorded. These eyecups exhibited a very depressed ERG (Table 7 and Figure 12) as compared with their control. Where in the one day study the a-wave was significantly increased, the data for the 15 day study show a significant decrease in a-wave amplitude. The b-wave amplitude is also significantly reduced as it was in the one day study, despite the fact that body levels of cholinesterase activity at this time were at normal levels or above . Nearly all of the remaining parameters were not significantly different from those of controls. Comparison of these results to those of

carbachol (Table 2) indicate that these findings were the result of an accumulation of ACh. On further investigation, this hypothesis must be rejected. The TOCP data do not show the changes in latency observed with carbachol and the levels of cholinesterase activity (Table 9) for neural tissue are at normal levels. Thus the mechanism involved in the TOCP action cannot be associated with increases in endogenous ACh or inhibition of AChE. This indicates that the action of the organophosphates may be transient in nature with different effects and mechanisms being manifested over the 15 day study.

In the 15 day study using DEF, the data exhibited very few changes from control. The peak b-wave latency was significantly increased and the duration of the b-wave and the total ERG were both greater than controls. Thus by 15 days the ERG had nearly completely recovered from the initial insult with DEF. The remaining effects were limited to the b-wave; these could be the result of lingering effects of DEF. The characteristic odor of DEF was still present in the fish after 15 days and Table 10 shows depression of spinal cord AChE at this time.

Retinal Cells Affected by Organophosphates

It is evident from the data of both the intact ERG and the isolated a-wave that the organophosphates are acting at most layers of the retina. The b-wave amplitude was consistently reduced with every treatment. These data are difficult to correlate with any particular cell type because of the nonspecific nature of the b-wave response. It is difficult to determine whether or not this decrease is due to a direct action on the cell other than inhibition of AChE because of the decreased b-wave amplitude found with eserine and carbachol treatment. Thus, these data cannot be correlated with any specific action of the

organophosphate or with any particular cell type. These limitations do not pertain to the data regarding the a-wave. It has been well documented that the a-wave is primarily the result of the hyperpolarization of the photoreceptors. Thus the a-wave, in both the intact ERG and isolated PIII, lends itself well to study the action of drugs upon the photoreceptors.

Mechanisms of Action of Organophosphates

The data point to a direct action of the OP on the photoreceptor. Within the photoreceptor there are several areas where the OP could be acting. Treatment with DEF resulted in a decreased latency, increased rate of development and increased amplitude of the a-wave. The photoreceptors apparently become hypersensitive to light. This reaction is most likely due to an action of the OP which causes the dark Na^+ current to be decreased earlier, more rapidly and to a greater extent. This would allow the photoreceptor to initiate the a-wave with a shorter latency and to hyperpolarize faster. It was also observed that the a-wave rate of development was faster. This could result from the blockage of a greater number of Na^+ channels in the photoreceptor outer segment.

In the cGMP model for phototransduction the organophosphate could be acting along the amplification cascade to cause this increased response. A likely target is guanylate cyclase. With this enzyme inhibited, the levels of cGMP would be lowered, levels of cGMP dependent protein phosphorylation would be reduced and subsequently fewer Na^+ channels would be open in the resting (dark) state.

This model can be used to explain the three main phenomena observed in the a-wave. The fewer number of channels open would allow for a more rapid initiation of the hyperpolarization because fewer channels would have to be blocked to result in a significant change in dark current. With cGMP levels being initially reduced, the remaining cGMP would be rapidly hydrolyzed by the light activated phosphodiesterase and lead to an accelerated closure of Na^+ channels. With fewer channels open initially the percent of those closing with a given photostimulation would increase and thus result in a greater change in Na^+ dark current and a greater hyperpolarization of the photoreceptor (Figure 14).

In the Ca^{++} model several mechanisms of OP action are possible. One such method would be that the Ca^{++} is sequestered into the lumen of the disks in the photoreceptor outer segment at a faster rate such that a greater than normal Ca^{++} gradient is produced across the disk membrane. Upon photostimulation the Ca^{++} would be driven down its concentration gradient at an increased rate and result in the blockage of a greater number of Na^+ channels at an accelerated rate. A second point of action which could be playing a role in either model is that the Na^+-K^+ pumps in the photoreceptor inner segments have an increased activity. This increased pumping would tend to raise the rate of Na^+ flux into the photoreceptor outer segment. Thus the Na^+ dark current would be greater, and upon photostimulation the blockage of the Na^+ channels would result in a greater change in the dark current. This larger change in the current would cause the photoreceptor to hyperpolarize to a greater extent and thus result in the observed changes in the a-wave.

It is possible that the organophosphates are acting directly on the membrane of the photoreceptors by altering the membrane properties such

Figure 14. Proposed cyclic nucleotide cascade in phototransduction. Abbreviations used are: GTP: Guanosine triphosphate, PDE: Phosphodiesterase, GC: Guanylate cyclase, * indicates activated molecule. After Fung and Stryer, 1980.

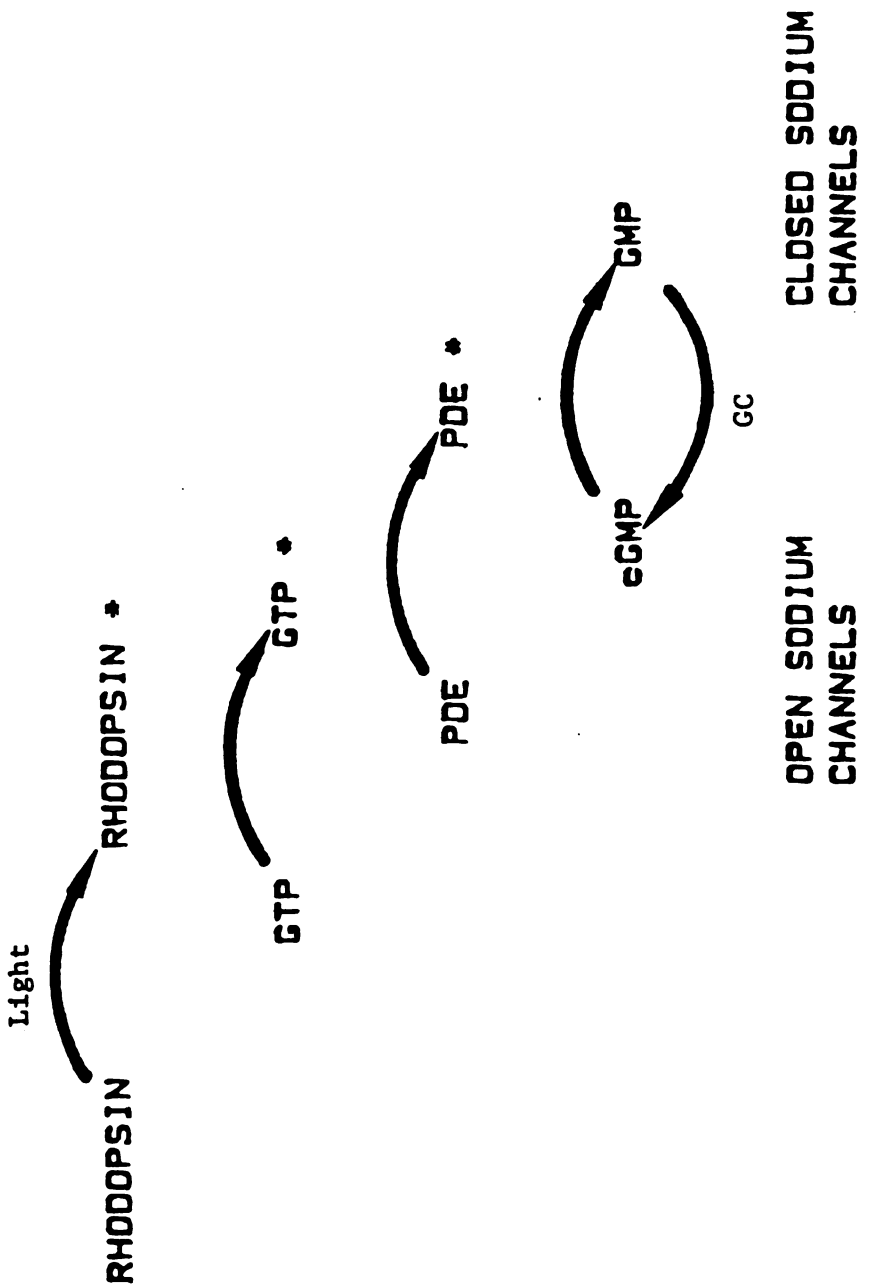
RHODOPSIN
Light
RHODOPSIN *
GTP
GTP *
PDE
PDE *
cGMP
GMP
GC
OPEN SODIUM CHANNELS
CLOSED SODIUM CHANNELS

Figure 14

that the photoreceptors become less stable and thus hypersensitive to photostimulation. Work by Hoffert and Daigle (Appendix I) has demonstrated alterations to the properties of nerve membranes following exposure to DEF. These investigations were intracellular recordings conducted on the giant medial axon of the earthworm. This axon was selected because of its large size and shape and also because of its property of ephaptic transmission which eliminates complicating factors from neurotransmitters (i.e., ACh). They noted significant changes in resting membrane potential, membrane resistance, positive overshoot, threshold voltage and chronaxie. These findings strongly support a direct action of organophosphates on noncholinergic neural tissue and imply a direct action of the OP on the membrane.

It is not clear which of the above mechanisms is responsible for the action of the organophosphates. It is clear however, that the OP do have a direct action on noncholinergic neural tissue which is separate and independent of any anticholinergic action the OP may possess.

SUMMARY AND CONCLUSIONS

1. Through the use of isolated, non-perfused eyecups of rainbow trout it has been shown that the organophosphte agents TOCP and DEF have a direct effect on the retinal cells irrespective of changes mediated by other systemic systems, e.g., the central nervous system.

2. Exposure of eyecups to eserine sulfate resulted in a general depression of the ERG documenting the effect of inhibiting acetylcholinesterase at the cholinergic synapses of the retina.

3. Evidence that ACh affects not only cholinergic synapses but the whole retina is provided by the experiments using carbachol. Treatment of eyecups with the nonhydrolyzable analog of ACh caused a severe depression of both the a- and b-wave components of the ERG.

4. Exposure of fish to the organophosphates TOCP and DEF for 24 hrs resulted in significant alterations of the ERG which differed from the changes caused by eserine sulfate and carbachol.

5. It was demonstrated (using the isolated a-wave preparation) that the increased amplitude of the a-wave caused by treatment with TOCP and DEF was the result of a direct action on the noncholinergic photoreceptors and was independent of the reduced b-wave.

6. Classic symptoms of advanced OPIDN were not observed at 15 days post treatment with TOCP or DEF. Alterations in the electrophysiological response of the noncholinergic photoreceptors may be an indication of the initial changes associated with OPIDN and constitite another example of the species specific nature of the delayed neurotoxic phenomenon.

7. It is suggested that the inhibitory action of organophosphates on guanylate cyclase and the ensuing reduction of the cyclic nucleotide cGMP may be involved in the initiation of delayed neurotoxicity.

RECOMMENDATIONS FOR FURTHER STUDY

1. A study similar to the one reported here using microelectrodes and intracellular recording techniques in order to study more closely the effects of the organophosphates on the individual cell types.

2. Assays to determine the effects of organophosphates on the levels of various components of the cGMP amplification cascade, i.e., guanylate cyclase and cGMP.

3. Intracellular studies on neural tissue outside the retina to determine the effect of the organophosphates on membrane properties.

4. Test the effects of organophosphates on visual pathways in the central nervous system (i.e., visual evoked potentials). Measure the effect of axoplasmic flow in the optic nerve of the rainbow trout.

5. Treat young rodents with high doses of sodium glutamate to cause the development of retinas which are cultures of pure photorecetpors. This model can then be used to better study the direct action of OP on the photoreceptor without the complicating factors of the rest of the neural retina.

6. **Test for the presence and inhibition of neurotoxic esterase in the retina and other selective tissue of the rainbow trout.**

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APPENDICES

APPENDIX I

Actions of the Organophosphate DEF on the Membrane Properties of the Medial Giant Axon of the Earthworm

J. Russell Hoffert, Ph.D. and Mark D. Daigle

Data from treatment of retinas with DEF indicate a possible direct action of DEF on the plasma membrane of the retinal photoreceptors. To test this hypothesis intracellular recordings from the medial giant axon of the earthworm were made. This axon was selected because of its large size, uniform cylindrical shape, and presence of ephaptic transmission. These axons are nonmyelinated but have a highly organized oligodendritic structure and can be related to the outer membrane of the photoreceptor since it is an excitable neuromembrane with analagous Na^+ channels and pump.

Membrane properties, before and after exposure to 100ppm DEF, were evaluated through measurements of the resting potential, resting membrane resistance, action potential characteristics and strength-duration curves. Significant changes do occur within three hours following exposure to DEF (Table 12).

In the resting state, DEF caused a decreased membrane potential and increased membrane resistance which may be explained by a decreased K^+ efflux. In the active state, action potentials observed by passing depolarizing current through the intracellular recording electrode, showed that DEF had no effect on the duration or the slope of the depolarizing and repolarizing processes. Although spike amplitude was decreased, DEF does not appear to affect the "fast" Na^+ and K^+ channels.

Table 12. Electrophysiological observations on the giant axon of the earthworm following acute exposure (100 ppm) to S,S,S,-tributyl phosphorotrithioate (DEF).

Table 12.

	Control	Experimental
Resting potential (mV)	-50.65± 1.1347(160)	-41.58± 1.4719(85)*
Membrane resistance (megohms)	14.05± 1.1518(130)	20.10± 2.5973(29)*
Positive overshoot (mV)	10.20± 1.9917(44)	17.14± 2.1000(64)*
Positive after potential (mV)	9.38± 1.3662(14)	6.40± 1.9157(6)
Threshold (mV)	-17.97± 1.6789(34)	-11.19± 1.7173(54)*
Threshold % resting	61.13± 4.1899(33)	77.44± 6.8472(59)*
Spike duration (mS)	44.92± 5.0626(35)	36.82± 3.3898(41)
Pre-dy/dt (mV/mS)	1.29± 0.1595(33)	1.49± 0.1431(37)
Post-dy/dt (mV/mS)	-3.74± 0.4848(32)	-4.15± 0.4962(37)
Frequency (cps)	4.01± 0.6006(9)	4.99± 0.5962(35)
Rheobase (mV)	0.70± 0.1774(15)	1.12± 0.2257(16)
Chronaxie (mS)	92.83±15.7290(15)	54.02± 5.1341(15)*

Mean±SE(N)

* Significantly different at 5% level.

Average current = 1.13± 0.1255(68) nA

However, the degree of depolarization necessary to reach the point at which the voltage gated sodium channels open (threshold) was increased. A significant decrease in chronaxie following DEF treatment showed increased neuron irritability. It appears that acute exposure to DEF can modify neural function of the resting membrane through a direct action not associated with its anticholinesterase properties. In addition, DEF increased the irritability of the cell, probably due to capacitive membrane changes. Additional measurement of the time constants will allow us to separate the resistive (ionic channels) from the capacitive (lipid bilayer) components. These results are consistent with the increased ERG a-wave amplitude observed following DEF exposures. This is the first time organophosphates have been shown to directly affect the function of the neural membrane. Increased environmental contamination with organophosphates will result in increased incidence of this type of neural pathology.

APPENDIX II

Acetylcholinesterase Assay

Principle

This assay measures the amount of ACh remaining after an incubation period with endogenous AChE. Choline esters react with hydroxyl amine to form a hydroxamic acid. This gives a color reaction with ferric ions.

Reagents

1) Acetylcholine Solution: 11.3 g (1/20 mole of acetylcholine bromine is made up to 100 ml with water. Lyophilized samples are to be diluted to 5.0 ml.
2) Barbital Buffer: dissolve 10.3 g of sodium barbital in 450 ml of water, with vigorous stirring add 5.0 ml of conc. HCl (12N) and then 45 ml water to make a total of 500 ml.
3) Magnesium-Potassium Salt Mixture: dissolve 4.2 g of magnesium chloride (9 g of the hexahydrate) and 200 mg of KCl and add water to 100 ml total volume.
4) ACh Buffer: <u>just before using</u> mix 16 ml of barbituate buffer with 2.0 ml of ACh solution and 2.0 ml of magnesium-potassium salt mixture.
5) Alkaline Hydroxylamine Solution: 14 g of hydroxylamine hydrochloride is made up to 100 ml with water.
6) Sodium Hydroxide: 14 g is made up to 100 ml with water.

7) Mix equal amounts of alkaline hydroxylamine and sodium hydroxide on the day of the experiment.

8) Ferric Chloride Solution: to approximately 200 ml of water add 2.0 ml of conc. HCl (12N). Now add 10 g of $FeCl_3 \cdot 6H_2O$ and make to a final volume of 1000 ml.

Procedure

1) Immerse tubes in 37C water bath (one control, two unknown).

2) Add to each tube 200 ul of ACh-Br buffer.

3) Add 20 ul of tissue to unknown tubes

4) Incubate all tubes at 37C for 60 min.

5) After 60 min add 20 ul tissue to control tubes.

6) **Immediately** add 20 ul of alkaline hydroxyl amine solution.

7) Wait one min (time not critical) before adding 600 ul of 4N HCl (or 600 ul of 5% TCA)

8) Transfer 500 ul aliquot to 15 ml tube.

9) Add 10 ml ferric chloride solution.

10) Mix, centrifuge for 5 min.

11) Read at 540 mn against blank (tube #1) from standard curve.

Standard Curve

1) Two ml of ACh buffer contains 10 ul moles of ACh.

2) Make standard curve up with the following tubes.

#	ul ACh buffer	ul H_2O	ACh conc.
1	0	220	0.00 uM
2	25	195	1.25 uM
3	50	170	2.50 uM
4	100	120	5.00 uM
5	200	20	10.00 uM

3) Run all tubes in duplicate except control tissue.

Reference

Natelson, S. 1961. Microtechniques of Clinical Chemistry. 2nd ed. Charles C. Thomas, Publisher. Springfield, IL. 578p.