

**DEVELOPMENT OF A MODEL CELL CULTURE SYSTEM IN WHICH
TO STUDY EARLY EFFECTS OF NEUROPATHY-INDUCING
ORGANOPHOSPHATES**

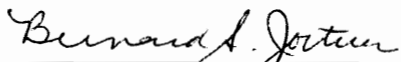
by

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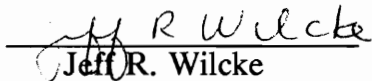
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DEVELOPMENT OF A MODEL CELL CULTURE SYSTEM IN WHICH TO STUDY EARLY EFFECTS OF NEUROPATHY-INDUCING ORGANOPHOSPHATES

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(ABSTRACT)

Certain organophosphorus (OP) compounds produce a delayed neuropathy in man and susceptible animal species after early inhibition and aging of the enzyme, neurotoxic esterase (neuropathy target esterase, or NTE). In this study, the human neuroblastoma cell line, SY-5Y, was examined for its potential to serve as a nonanimal model for the study of the early effects of neuropathy-inducing OPs. For these investigations, the time course of inhibition and aging of NTE after toxicant treatment in neuroblastoma cells was compared to that in brain tissue from the adult chicken, which is the recognized animal model for organophosphorus ester-induced delayed neuropathy (OPIDN). Concentrations of toxicants to be used for treating neuroblastoma cells were determined after observing viability of treated differentiated SY-5Y cells incubated for 24 hours with a range of concentrations of an OP (mipafox) that induces neuropathy, an OP (paraoxon) that does not induce neuropathy, a carbamate (aldicarb), a neurotoxicant (β , β' -iminodipropionitrile, or IDPN) that acts by a different mechanism than the OPs, and a cholinergic agonist (carbachol). Treatment concentrations were chosen that caused less than 30% loss of viability over the 24 hour period.

The time course and extent of detrimental effects of mipafox, which induces OPIDN in hens, and the carbamate, aldicarb, on NTE were similar in the SY-5Y cells to those observed in homogenized chicken brain tissue after the same treatments. Mipafox produced rapid inhibition and aging of NTE, with maximal effects occurring within 10 minutes of exposure. Aldicarb inhibited

NTE but did not age the enzyme. Instead, spontaneous reactivation was observed both in SY-5Y cells and in brain tissue. None of the other negative control compounds (paraoxon, IDPN, carbachol) affected NTE activity in either SY-5Y cells or chicken brain tissue.

To determine if the neuroblastoma cells could be used to study early events that could lead to modification of OPIDN, NTE inhibition and aging were determined in the differentiated SY-5Y cell line after mipafox was removed. Removal of mipafox from the cell culture medium at 5 minutes after exposure, or earlier, resulted in essentially no NTE inhibition or aging. NTE inhibition and aging were also determined after treatment of the SY-5Y cells with the neuropathy-inducing OP, mipafox, and representatives of 2 classes of compounds (carbamates and calcium channel blockers) previously demonstrated to modify OPIDN in hens. The modifiers (aldicarb and verapamil) were used as a 5 minute pre-treatment, simultaneous treatment, and a 2 minute post-treatment. Significant prevention of most of mipafox-induced NTE inhibition and aging was observed. Effects on NTE inhibition and aging in differentiated SY-5Y cells after each mipafox-aldicarb combination and mipafox-verapamil combination of treatments were similar to those in chicken brain homogenate. These results indicated that both aldicarb and verapamil protected NTE against the early biochemical effects of mipafox that are thought to initiate OPIDN in vivo.

The temporal relationship of NTE inhibition and aging to other detrimental effects on neuroblastoma cells was assessed by the capability of mipafox to cause changes in free intracellular calcium ion concentration, measured using fluorescent calcium probes, and by its capability to alter cell morphology as assessed by phase contrast microscopy. NTE inhibition and aging preceded these changes. Capability to inhibit activity of the neural enzyme, acetylcholinesterase, was also determined.

The results of these studies indicate that the SY-5Y model system shows promise for use in the determination of initial mechanisms contributing to the development of organophosphorus-induced delayed neuropathy.

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ABBREVIATIONS

ACh:	acetylcholine
AChE:	acetylcholinesterase
$[Ca^{2+}]_i$:	free intracellular calcium ion concentration
CANP:	calcium-activated neutral protease, or calpain
CNS:	central nervous system
DFP:	di-isopropyl fluorophosphate
HBSS:	Hank's balanced saline solution
IDPN:	β,β' -iminodipropionitrile
INAP:	isonitrosoacetophenone
NTE:	neurotoxic esterase, neuropathy target esterase
OP:	organophosphate
OPIDN:	organophosphorus-induced delayed neuropathy
PMSF:	phenylmethylsulfonyl fluoride
PSP:	phenyl saligenin phosphate
TOCP:	tri-ortho-cresyl phosphate
TOTP:	tri-ortho-tolyl phosphate

PART I

INTRODUCTION

HYPOTHESIS

The hypothesis tested in this research is that early neuronal changes resulting from exposure to neuropathy-inducing organophosphorus esters (OPs) occur in cell culture, and that a cell culture system can be used to monitor the series of events that occur early after exposure to these compounds. The goal of this research was to examine the aging of the bond between neurotoxic esterase (NTE) and a neuropathy-inducing OP, and the temporal relationship of aging to changes in free intracellular calcium ion concentration, or $[Ca^{2+}]_i$. Aging is defined as the dealkylation of the OP covalently bound to NTE, and is known to precede and correlate with the incidence of axonal degradation associated with organophosphorus-induced delayed neuropathy (OPIDN) in susceptible species of animals.

Study of early biochemical changes that occur in neural cells in an attempt to elucidate some mechanisms associated with damage induced by exposure to neuropathy-inducing OPs first required a system in which events in intact cells could be studied. Therefore, the first goal of this research was to establish a model system using a neuronal cell culture. An *in vitro* system allows for isolation of the target tissue of this syndrome and for rapid and early manipulation of that tissue in order to monitor early events. Knowledge of critical steps in the initiation or pathogenesis of OPIDN that may be selectively inhibited could eventually be useful in preventing the neuropathy in exposed individuals.

A series of experiments were performed to first choose a model cell line in which inhibition and aging after exposure to a neuropathy-inducing OP could be measured. The choice of the model was validated by using it to examine inhibition and aging of NTE after treatment with a neuropathy-inducing OP and a series of control compounds, and by comparing those effects to those seen in

similarly treated chicken brain homogenate. Determination of the time course and magnitude of those changes were made. Next, modification of those changes was observed after treatment with compounds known to protect against the development of OPIDN in vivo. Finally, the time course and magnitude of changes in $[Ca^{2+}]_i$ after all toxicant treatments was examined and related to changes in NTE and cellular morphology.

As a whole, this work should help to establish a cellular model for mechanistic studies with neuropathy-inducing OPs and provide information on the role of increased $[Ca^{2+}]_i$ in the development of OPIDN. Once the nature of that relationship and its degree of reversibility over time are determined in the cellular model, more effective means of prevention and treatment of detrimental effects of neuropathy-inducing OPs may be developed and studied, first in vitro and then in vivo.

LITERATURE REVIEW

Historical background

Organophosphate (OP) chemicals may be defined as compounds which contain carbon and are derivatives of phosphorus acid. The synthesis of chemicals of this highly diverse group was first reported over 75 years ago, but significant interest in them did not develop until the early years of World War II. German investigators developed the first OP insecticide, which was first marketed in 1944. Although highly effective, its high toxicity to mammals and ease of hydrolysis led to the development of more stable agricultural compounds and also to the discovery of the OP "nerve gases". Since 1945, a large number of organic triesters of phosphoric acid and phosphorothioic acid have been registered for use as insecticides, and other OP's have been used as plasticizers, stabilizers in lubricating and hydraulic oils, flame retardants, gasoline additives, and anthelmintics [1,2].

Organophosphate toxicity

The most well known toxic effect of OP esters is the direct and acute inhibition of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of acetylcholine (ACh), the neurotransmitter acting at the endings of postganglionic parasympathetic nerve fibers, somatic motor nerves to skeletal muscles, preganglionic fibers of the autonomic nervous system, and certain synapses in the central nervous system (CNS). Clinical signs may be divided into 3 categories: muscarinic, nicotinic, and CNS effects. Muscarinic receptors for ACh are found primarily in smooth muscle, producing bronchoconstriction, increased salivation, lacrimation and sweating, vomiting, diarrhea, involuntary micturition, and bradycardia. Nicotinic signs result from accumulation of ACh at motor nerve endings and autonomic ganglia and include fatigue progressing to generalized muscle fasciculations, dyspnea, pallor, increased blood pressure, hyperglycemia, and a tachycardia which may

overcome the muscarinic effect of OP's on the heart. Restlessness, anxiety, headaches, confusion, memory loss, and tension have been attributed to OP effects at CNS synapses [1,3]. The onset of signs is usually within 12-24 hours of contact. Death is due to respiratory failure [1].

Chronic effects include progressive myopathy beginning at the motor endplate and persistent CNS effects, both of which are considered to be cholinesterase related. Noncholinesterase related effects include teratogenicity and organophosphorus ester induced delayed neurotoxicity (OPIDN) [1].

Clinical signs of OPIDN

OPIDN can be described as a distal axonopathy developing 8-14 days after exposure to the toxicant. The first signs, which may or may not follow cholinergic signs, occur in the lower limbs as a numbness or fatigue, progressing to weakness and paralysis. Involvement of the upper extremities is variable. The most outstanding features of the syndrome are the long latent period before the onset of signs, the bilaterally symmetrical paralysis seen as a high stepping gait in ambulatory cases, and the absence of pain. Improvement may occur over a period of months to years, but the patient is usually left with some impairment [1,2,4].

Cases of paralysis in humans contacting OP's have been recorded since the late 19th century. However, the potential danger of these compounds was not realized until the 1930's, when approximately 20,000 persons in the U. S. were afflicted with the paralysis characteristic of OPIDN. It was found that all of these cases were preceded by ingestion of "Ginger Jake", an alcoholic beverage substitute sold in drugstores. Tri-ortho-cresyl phosphate (TOCP), the primary component of the oil used in the ginger extraction process, proved to be the offender. Since that incident, numerous other cases were reported due to contamination of food products or occupational exposure to TOCP or other OP's [1,2,4].

Pathological aspects of OPIDN

Early histological investigations erroneously concluded that OPIDN was primarily a demyelinating disease. It has since been demonstrated that peripheral and central nervous system lesions are the result of a "dying-back" process or Wallerian degeneration of axons with demyelination being a secondary effect. In experimentally treated hens, the most severe effects were seen in the distal portion of the longest fibers in the peripheral nerves, both motor and sensory. The process was described as a Wallerian degeneration of the distal axon followed by loss of the myelin sheath, proliferation of Schwann cells and macrophage accumulation [5-7]. Similar neurotoxic changes were present in the spinal cord and portions of the medulla and cerebellum. These changes were not seen by light microscopy until after the development of clinical signs [1,2,4].

In more recent studies using a "teased-fiber" technique on the nerves of cats poisoned with a delayed neuropathy-inducing OP, axonal degeneration first suspected as beginning at the most distal end of the axon was actually focal and nonterminal, spreading to involve the entire axon. The lesion is essentially a "chemical transection;" the OP ester induces a focal, distal but not terminal, axonal degeneration which precipitates a Wallerian degeneration. The additional finding of focal varicosities in the nerve fiber led to expansion of the "teased-fiber" technique to ultrastructural studies. The varicosities proved to be associated with large electron-lucent vacuoles located either within the axon, within the myelin sheath, or both. It was speculated that the vacuoles were the result of a breakdown in normal regulatory mechanisms for transmembranous gradients. The varicosities were believed to evolve into focal areas of axonal degeneration [8-10].

Central nervous system lesions have been documented in the cat, monkey, rat, and chicken. These include degenerative changes in the ventral and lateral tracts of the spinal cord, including the dorsal columns, particularly the fasciculus gracilis, the spinocerebellar and corticospinal tracts, and lesions in the cerebellum and medulla [11-13].

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Regeneration of myelinated axons has been observed in peripheral nerves of chickens treated with tri-ortho-tolyl phosphate (TOTP). Myelinated nerve fiber degeneration noted in the spinal cord white matter tracts, however, did not undergo regeneration in that study [14].

The chicken as an animal model for OPIDN

During the initial investigations of TOCP intoxication, it was found that the adult hen developed clinical signs closely analogous to those of patients involved in the "Ginger Jake" paralysis. An oral dose of 0.5 mg TOCP/kg of body weight regularly produced paralysis beginning in the legs approximately 9-10 days after administration. The wings were affected a few days later, and when paresis was at its peak, the hen moved with a slapping, unsteady gait [1,4]. The hen has since become the primary experimental animal used in testing OP esters and has been named by the U. S. Environmental Protection Agency as the required test species for OPIDN [15].

NTE and the initiation of OPIDN

Martin Johnson used radiolabelled neurotoxic OPs to identify the target site of such compounds in the brain. This target protein, neurotoxic esterase (NTE), has been the center of a great deal of research. The ability of other acylating inhibitors to significantly depress NTE without production of paralysis made it apparent that phosphorylation of the protein alone was an insufficient explanation for the axonopathy of OPIDN. The "aging" theory was developed to explain this phenomenon. This theory suggests that, following phosphorylation of the enzyme, a side-chain is lost from the phosphorus atom resulting in a charged phosphoryl substituent remaining at the esteratic active site that cannot be removed from the enzyme [16]. Thus, there are two types of compounds that can bind to and inhibit NTE, those that undergo the "aging" process and produce OPIDN, and those that do not undergo "aging" and do not produce OPIDN. Compounds that do not age NTE are protective against OPIDN if administered prior to a known neurotoxic OP ester,

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because they prevent compounds that age NTE from binding the esteratic active site [17]. No normal physiologic role has been demonstrated for NTE. Nor has inhibition of NTE without aging for prolonged periods by a phosphinate or sulfonate compound produced adverse effects in hens. However, Johnson proposed that aging at the esteratic site could cause disturbance of a normal physiologic function at a nearby second site, or, transference of the side group to another area could cause such a process [1,2,13,18]. Such secondary sites could include enzymes related to receptor-mediated phosphorylation pathways or ion channels, etc. The processes in the neural cell which are affected by NTE inhibition and aging that do lead to OPIDN are at present unknown. One goal of the present research was to establish a system in which to investigate such effects and to begin to examine these effects in vitro soon after OP exposure.

It would appear that NTE inhibition and aging is an initial step in a chain of events that lead to the development of OPIDN. Several types of events have been proposed to be involved.

The suggestion that NTE inhibition and aging is crucial to the initiation of OPIDN is further supported by the fact that prevention of NTE aging prevents the development of OPIDN in vivo. Intramuscular injection of phenylmethylsulfonyl fluoride (PMSF), a compound that inhibits but does not age NTE, 1 to 24 hours prior to oral administration of TOCP, or 4 hours prior to subcutaneous injection of di-isopropyl fluorophosphate (DFP), in hens proved to be effective in protecting the treated birds from developing the clinical signs of OPIDN. If given 24 hours after TOCP or more than 4 hours after DFP, PMSF was ineffective in prevention of paralysis due to organophosphate inhibition and aging of NTE [19]. Recent studies have demonstrated that post-treatment with PMSF will potentiate, rather than prevent the development of OPIDN [20,21].

NTE recovers normal levels of activity in brain before symptoms of OPIDN develop. Recovery in peripheral tissue appears to lag behind initially. Sciatic nerve NTE, inhibited by a single oral dose of TOCP in hens, began to regenerate 2 - 3 days after that in brain, but then appeared to regenerate faster [22]. This may be because distal parts of the nervous system contain significantly

less NTE than proximal [22,23]. When recovery is examined on a percentage basis of control levels, it may appear faster in peripheral nerve than in brain when the absolute rate of synthesis of new enzyme in the two tissues is the same.

The assay for NTE developed by Johnson [24] has undergone many modifications. The method currently used is a differential procedure utilizing phenyl valerate as a substrate for the enzyme, with NTE defined as the activity resistant to paraoxon (not neuropathy-inducing) but sensitive to mipafox (neuropathy-inducing) included in the assay system. Activity is determined by spectrophotometric measurement of the phenol liberated in the process. Adult hens are used as the source of the enzyme in the standard NTE assay. A single maximally-tolerated dose is administered on day 0 and sacrifice of the birds and determination of NTE is done in 24-48 hours. The peak inhibition of the enzyme during this time correlates well with the development of the ataxia seen on day 10-15 in clinical trials. Inhibition of NTE by 70-80% is considered the threshold value for acute exposure to a positive neurotoxic OP compound [1,13]. NTE inhibition after chronic exposure to low doses of a neuropathy inducing OP appears to reach a maximal value that is lower than that considered to be the threshold value for acute exposure [25]. The threshold value for NTE inhibition after chronic exposure is generally considered to be 45 - 65% [26].

NTE has been characterized as a membrane enzyme belonging to that group of esterases capable of hydrolyzing carboxylesters of aliphatic and aromatic alcohols. It is present in brain, spinal cord, peripheral nerve, and in a wide variety of non-neural tissues including spleen, splenic lymphocytes, and peripheral lymphocytes in the hen and in blood platelets, lymphocytes and the placenta in man. Ninety percent of the brain NTE activity is located in the microsomal fraction. Axon fragments contain a significant amount of NTE not associated with myelin. Purified myelin preparations appear devoid of NTE activity [1,18].

OP effects determined using in vitro systems

The NTE assay can be used in vitro for testing the ability of a foreign compound to alter NTE. One in vitro test for examining the interaction of OPs and NTE utilizes neuroblastoma cell culture; this method has great potential for future use [27,28] and was used in the experiments described in this dissertation.

Investigations using a differentiated neural cell line would seem to be a reasonable alternative to in vivo systems for mechanistic studies and screening procedures. Such a system has the potential to effectively mimic the target tissue of OPIDN in vivo, and biochemical effects in cell culture may be compared to the stable population of neural cells in vivo for validation. A significant advantage of neural cells in vitro is that they may be manipulated early after exposure to an OP or other compound, allowing evaluation of early changes that may be critical to the development of OPIDN but are impossible to detect in vivo due to the delay incurred by killing the animal and removing the tissue. Early examination of OP treated cells for effects related to the initiation and development of OPIDN was performed as part of the research for this dissertation.

The cell lines used for this research all were capable of differentiation upon exposure to nerve growth factor (NGF) to a mature adrenergic neuronal cell type [29-31]. Although the neurons affected in OPIDN are cholinergic motor and sensory neurons, such cell types are not available in continuous cell culture. Our studies showed that the cells used here express NTE activity in the undifferentiated state. Differentiated cells in culture expressed still higher activities as a mature neuronal cell form. Additionally, these differentiated cells in vitro have electrically excitable membranes and have exhibited $[Ca^{2+}]$ influx in response to receptor activation as do mature neurons in vivo [31,32] and would presumably utilize the same mechanisms for normal $[Ca^{2+}]_i$ regulation that all mature neurons in vivo do.

The use of in vitro systems to define aspects of neurotoxicity and cytotoxicity is widespread. Specific biochemical lesions produced by OP's and other neurotoxicants may be observed in cell

culture systems. Some examples are discussed below which indicate the usefulness of a cell culture system for mechanistic studies of the effects of neurotoxicants.

Cell culture systems have been used to examine the acute effects of OPs on acetylcholinesterase (AChE) and cholinergic stimulation. For example, the effects of one OP, soman, on muscarinic receptor binding in PC12 cells have been investigated [33]. Differentiation with nerve growth factor (NGF) increased muscarinic receptor binding twofold; in these cells, treatment with soman resulted in a decrease in this binding that was maximal at 24 hours. Other OPs and carbamates tested also caused a decrease in binding by 24 to 48 hours after treatment. In none of the OP treatments was the effect reversible by atropine, indicating that the mechanism of this effect was not mediated by agonist-like activity. For comparison of the in vitro study with events in vivo, research has demonstrated that a single oral exposure of hens to tri-ortho cresyl phosphate (TOCP) resulted in a selective decrease in muscarinic receptors in forebrain neurons [34].

Mouse brain reaggregate cultures have been used to examine acetylcholinesterase (AChE) activity before and after OP treatment [35]. After treatment with diisopropyl fluorophosphate, AChE had recovered to 31% of control activity by 24 hours and had required protein synthesis, while cultures treated with the reversible inhibitor, physostigmine, recovered full AChE activity by 24 hours.

Inhibition of AChE as a result of OP exposure results in the exposure of neural cells to increased amounts of acetylcholine (ACh). The effects of excess ACh have also been determined using in vitro systems. Human neuroblastoma cells (SY-5Y) were induced to differentiate and were tested for response to exogenously applied acetylcholine (ACh). Both differentiated and undifferentiated cells exhibited an increase in calcium ion influx in response to ACh, apparently as a result of muscarinic receptor binding [30].

Although a limitation of the use of cell culture is the inability to evaluate neuropathology as is possible in vivo, morphological changes in cultured cells may be examined and used as evidence of

a toxic process. The studies discussed below demonstrate means of evaluating cell morphology to compare that of normal cultured cells with that of toxicant-treated cells.

For morphological studies, neuroblastoma 2-A cells treated with TOCP or with DFP, both organophosphate compounds that produce OPIDN *in vivo*, were observed by phase-contrast microscopy. Both treatments caused the neurites to have a shrunken, rough, and irregular appearance. Swellings were apparent along the length of the neurites, and were a more prominent feature of DFP treatment [36]. In comparison, electron microscopic studies of peripheral nerves from animals experimentally treated with TOCP demonstrated axonal swelling, accumulation and condensation of neurofilaments and neurotubules, and proliferation of granular endoplasmic reticulum [37].

Neuropathological effects of OPs on *in vitro* systems has also been studied in cultures of chick dorsal root ganglion cells. Cells were examined after treatment with the AChE inhibitor, fenthion, and compared with those treated with other cholinesterase inhibitors (paraoxon and neostigmine) [38]. Fenthion and paraoxon both produced significant changes in morphology under both light microscopy and scanning electron microscopy, including vacuolization and loss of tubular structures in the neurites, retraction of pseudopodia, cell membrane disruption at the growth cone, and lipid accumulations in the cytoplasm of treated cells. Those changes were not seen with neostigmine exposure.

Morphological changes have been observed as well in cultured neuronal cells exposed to neurotoxicants other than the OPs. In one study, scanning and transmission electron microscopy were employed in the examination of ultrastructural changes in cultured cells exposed to the neurotoxicant, methylmercury [39]. After cells were exposed to methylmercury, cytoskeletal architecture was particularly affected, micrographic evidence of a specific microtubular lesion was noted.

Another study reported morphological alterations in PC12 cells examined after exposure to cyanide and prevention of those changes by pretreatment of cultures with diltiazem, a calcium channel blocker [40]. Scanning and transmission electron microscopy of cyanide-treated cells revealed depletion of secretory granules, alignment of remaining granules along the plasma membrane, mitochondrial swelling, loss of microvilli, and bleb formation.

Development of OPIDN and the role of calcium in neurotoxicity

While inhibition and aging of NTE may be considered the initiation step of OPIDN, and axonal degeneration and paralysis its ultimate expression, little is known of the developmental steps involved that lead from the initial biochemical lesion to the clinical expression of delayed neuropathy. The changes associated with the aged NTE-OP bond may trigger a sequential cascade of events or may produce some alteration at an adjacent site in the neuronal plasma membrane that eventually lead to the dysfunction [26].

Current theories regarding the mechanism of neurotoxic events include perturbation of normal calcium concentrations with secondary effects on enzyme systems within the neural cell [41-52]. These effects are detectable in vitro and much of the research on the role of calcium in cell injury has been conducted using cell cultures. Calcium ion regulation of normal physiological processes is well documented [53-61]. Intracellular calcium concentration is actively maintained at a concentration 10,000 times lower than that of the extracellular fluid, in normal cells [49,62]. As a second messenger, calcium mediates cytoskeletal rearrangements, phospholipid metabolism, and protease and endonuclease activity. Derangements in the regulation of intracellular calcium ion concentration can result in derangements in these processes, which can progress to the morphological and functional changes that are associated with toxicity. For example, the characteristic appearance of plasma membrane blebbing seen with toxic and ischemic cell injury can be mimicked in vitro by exposure to calcium ionophores or other compounds that are known to

cause increases in free intracellular calcium. Cytoskeletal changes appear to be mediated by two mechanisms, direct dissociation of microfilaments by high calcium levels and calcium activation of proteases that disrupt the plasma membrane anchor to the cytoskeleton. The result is sites of weakness in the cell membrane that may lead to the production of surface blebs [61,63].

Perturbation of normal calcium homeostasis after exposure to neurotoxicants may activate phospholipase enzymes. Phospholipase activation by calcium may be an important mechanism in cytotoxicity. Accelerated phospholipid breakdown by phospholipase A₂, which is calcium and calmodulin dependent, can cause cell damage. Arachidonic acid, which is generated by phospholipase activity, is metabolized to known mediators of inflammation and allergic responses, and lysophospholipids, also generated by phospholipase A₂, are cytotoxic [61].

Neurotoxicant-induced changes in normal calcium concentrations may also affect activities of other enzyme systems, such as the proteases. Extralysosomal proteolytic systems that function optimally at neutral pH include calcium dependent proteases, or calpains. These enzymes are found associated with cell membranes in virtually all mammalian cells and are associated with a specific inhibitor, calpastatin. They take part in cytoskeletal and cell membrane remodelling, receptor cleavage and turnover, enzyme activation, and modulation of cell division. Two isoenzymes exist, calpain I, which requires micromolar concentrations of calcium ion, and calpain II, which is the lower affinity form requiring millimolar concentrations of calcium. Appropriate calcium ion concentrations precipitate an autolytic activation of the respective isoenzyme under normal physiological conditions; they then act upon cytoskeletal components and proteins integrated into the cell membrane. These proteins may be targets for calcium activated proteases in neurotoxicity [54,56,61].

Changes in calcium concentration and in calcium regulated enzyme activity have been implicated in the development of delayed neuropathy. Administration of calcium channel blockers to hens also treated with neuropathy-inducing OP's moderated the development of clinical signs and

axonal lesions of OPIDN [50-52]. In TOCP and PSP treated birds, calpain activity in brain and peripheral nerve was observed to increase. This and a rise in calcium concentration in sciatic nerve was prevented for a time by treatment with calcium channel blockers, beginning 1 day prior to OP treatment [51].

TOCP treatment of chickens in vivo and of chicken synaptosomes in vitro produced an increase in calcium dependent protein phosphorylation, specifically involving the cytoskeletal components α - and β -tubulin, microtubule-associated protein (MAP-2), and the neurofilament triplet proteins, and cytoskeletal protein degeneration [43,64,65]. These effects correlated well with the development of OPIDN in vivo.

Although they do not cause the same signs of toxicity in animals as do the OPs, changes in intrasynaptosomal calcium concentration have been examined after organochlorine insecticide treatment in vitro. These neurotoxic compounds were shown to produce a dose and time dependent increase in $[Ca^{2+}]$ in a calcium containing medium, indicating that the source of the additional calcium was, at least partially, from outside of the synaptosomal membrane. The effect was blocked by verapamil, a calcium channel blocker, indicating the involvement of voltage-dependent calcium channels [44].

The effects of lindane, another neurotoxic organochlorine insecticide, on synaptosomal calcium concentration has been investigated as well [46]. Lindane was shown to cause an increase in intrasynaptosomal $[Ca^{2+}]$ which was attenuated by the absence of calcium in the incubation medium. In neurohybridoma cells, lindane also produced a dose-dependent increase in $[Ca^{2+}]_i$ [66].

Organophosphates, too, appear to have the potential to alter $[Ca^{2+}]_i$. The organophosphates parathion and azinophos and their respective -oxon derivatives were shown to stimulate calcium pump activity in fragmented sarcoplasmic reticulum. That pump system, which contributes to the maintenance of stable intracellular calcium concentrations, is modulated by membrane lipids which may be altered by lipophilic insecticides [41].

INTRODUCTION

AChE inhibition and the resultant increase in the availability of ACh, may further interfere with calcium homeostasis by excessive stimulation of muscarinic and nicotinic receptors. Calcium influx and mobilization has been demonstrated in tissue culture as a result of treatment with either muscarinic or nicotinic receptor agonists. Carbachol, a muscarinic agonist, appeared to cause an increase in free intracellular calcium in primary cultures of rat forebrain neurons, 60% of which could be prevented by omission of calcium from the medium [32]. In SK-N-SH human neuroblastoma cells, a dose dependent, transient increase in free intracellular calcium could be evoked by treatment with either nicotine or potassium chloride. Calcium ion concentration increases could be induced sequentially by these two treatments, indicating separate mechanisms, and could be blocked independently by nicotinic receptor blockers, such as tubocurarine, and by voltage dependent calcium channel blockers, such as verapamil. No response was observed to either treatment in a calcium free medium; the source of the calcium influx appeared to be extracellular only [67].

Calcium channel antagonists or agonists could be of use in investigating the role of calcium in OP toxicity in an in vitro test system. The calcium channel blocking drugs, verapamil and nifedipine, have somewhat different structures and modes of action; nifedipine blocks voltage-sensitive calcium channels in the plasma membrane and inhibits calcium ion release from intracellular stores, while verapamil blocks the membrane channels only. Both compounds have been shown to protect against the development of OPIDN in vivo [52]. The calcium channel agonist, Bay K 8644, selectively activates one of three identified types of calcium channel (L), and has been used in neuronal cell culture in experiments dealing with calcium conductance and neurotransmitter release [68].

With regard to the evidence that NTE inhibition and aging are the initiating steps of OPIDN, and that calcium ion homeostasis appears to be disturbed and can precipitate a number of regulatory defects in cellular metabolism, the relationship between these events was noted to deserve

investigation. In the following studies, a cell culture model was developed to be used for such work. The investigations carried out by other laboratories discussed above have made use of in vitro techniques for similar work and have explored and found evidence for the involvement of calcium in neurotoxicity. The experiments for this project were designed to establish first, a model in cell culture and second, a sequential relationship between NTE inhibition and aging and perturbations in calcium ion concentration in order to establish some part of the sequence of events involved in the development of OPIDN.

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PART II

MATERIALS AND METHODS

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Maintenance of cell lines

The following cell lines were maintained for examination for expression of NTE, acetylcholinesterase (AChE), normal $[Ca^{2+}]_i$, and morphological characteristics after differentiation with nerve growth factor (NGF) in order to choose the most appropriate cell line for further study: SK-N-SH, a neuroblastoma cell line of human origin, SY-5Y, a subclone of SK-N-SH, and PC12, a rat pheochromocytoma cell line which differentiates into a neuronal cell type. Differentiated cells would be expected to better express phenotypic characteristics of mature neuronal cells, and were chosen for this study because OPIDN is a disorder of adult animals while immature animals seem to be less sensitive [1]. All cells were checked daily and subcultured as cell growth required. Each cell line was maintained in appropriate media, as follows: SK-N-SH cells were kept in minimal essential medium (MEM) with sodium pyruvate and nonessential amino acids in Earle's BSS (90%) and fetal calf serum (10%); SY-5Y cells were kept in Dulbecco's modified Eagle's medium (DMEM) (45%), F12 (45%), and fetal calf serum (10%); and PC12 cells were maintained in RPMI 1640 (85%), 10% horse serum, and 5% fetal calf serum.

Differentiation of neural cell cultures

NGF concentrations used were: 1 μ g/ml NGF in medium for SK-N-SH and SY-5Y cells [2], and 50 μ g NGF/ml of medium for PC12 cells [3]. By 5 days after introduction of NGF to the culture medium, SK-N-SH and SY-5Y cells extend processes with numerous varicosities and appear to form "pseudoganglia," small aggregates of cells interconnected by thick fibers with varicosities. These cultures revert to the undifferentiated form within 3 days of removal of NGF from the

medium [2]. By 7 days after exposure to NGF, PC-12 cells cease to multiply and extend neuronal-like processes, which continue to grow in number, length, and density for an additional 2 weeks of treatment. These fibers morphologically resemble those produced by cultured primary sympathetic neurons; they are 500 - 1000 μm long, branch profusely, have numerous varicosities, are very fine, and form fascicles [4]. The previously reported changes typical of neuronal differentiation were also observed in this study.

Choice of a model cell line

The differentiated SY-5Y cell line was chosen for further study on the basis of superior and less variable control activity of NTE and AChE as compared to the other cell lines tested. They did exhibit morphology in the differentiated state typical of mature neurons. Additionally, they loaded the fluorescent calcium probe fura-2 most consistently of the 3 cell lines examined and exhibited normal $[\text{Ca}^{2+}]_i$ of approximately 100 nM as would be expected in normal cells. It was possible to maintain large numbers of these cells with little difficulty, as this study required.

Choice of toxicant treatments and their concentration

The following toxicants were chosen for study: the neuropathy-inducing OP mipafox; paraoxon, an OP that does not inhibit NTE in vivo and does not produce delayed neuropathy; aldicarb, a carbamate that inhibits NTE in vivo, but presumably does not age the enzyme and does not produce delayed neuropathy; β,β' -iminodipropionitrile (IDPN), an agent that produces a peripheral neuropathy by another mechanism involving neurofilaments in the proximal segments of affected axons [5], but should have no effect on NTE; and carbachol, a cholinergic receptor agonist which should also have no effect on NTE, and was included to separate effects on AChE from those on NTE. All are soluble in aqueous solution or can be diluted in aqueous solution after dissolution in an organic solvent (acetone) so as to result in minimal loss of viability. All toxicants are

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capable of producing toxicity after a single dose in vivo and do not require metabolic activation to do so. Viability was determined by trypan blue exclusion after 24 hour incubation of differentiated SY-5Y cells with a range of concentrations of each test compound. Toxicant concentrations exhibiting acceptable viability (>70%) were screened for NTE and total esterase activity. The goal in choosing test concentrations was to arrive at one that would not overwhelmingly inhibit NTE and total esterase, but that would, in the case of the neuropathic OP mipafox, inhibit NTE to a degree at or near that considered to be the threshold for development of OPIDN in vivo. Equimolar concentrations of all toxicant treatments were chosen (5×10^{-5} M), with the exception of aldicarb, which, due to spontaneous reactivation of aldicarb-inhibited NTE, was used at a concentration 100 times higher than the other toxicants (5×10^{-3} M).

Treatment of tissues

For NTE inhibition and aging studies, cells were suspended at 2×10^7 cells per ml in Hank's balanced saline solution (HBSS). Chicken brain tissue was homogenized and diluted as for NTE assay. Both tissues were treated by addition of the appropriated amount of concentrated stock solution of each toxicant to achieve the final treatment concentration as described above. Treated cell suspensions and brain homogenates were incubated in a 37°C water bath for 2, 5, or 10 minutes, for examination of NTE inhibition and aging at those time points.

Treatment for morphological and calcium experiments was accomplished by incubating cells in culture dishes or 96-well microtiter plates with media with NGF to which an appropriate amount of toxicant stock solution had been added to achieve the same final concentrations described above. When media for cells undergoing morphological examination was changed, the toxicant treatment was replaced as well.

For investigation of effects on acetylcholinesterase (AChE), cells were suspended at 10^7 cells per milliliter and were homogenized by 5 freeze-thaw cycles in liquid nitrogen. Brain tissue was

prepared as for NTE assay. Forty μ l of cell or brain tissue homogenate was placed in each well of a 96-well microtiter plate. Ten μ l of diluted stock toxicant solution was added to achieve the appropriate test concentration in 50 μ l. The plate was incubated in a water bath at 37°C for 10 minutes, then removed, and the AChE assay was performed as described below.

Neurotoxic esterase (NTE) assay

NTE activity was determined according to the method described by Sprague, *et al* [6], modified for microassay [7]. The cells were suspended in HBSS at a concentration of 10^7 cells per milliliter. Cell suspensions were homogenized by 5 freeze-thaw cycles in liquid nitrogen. Chicken brains were homogenized in 6.5 volumes of 50 mM Tris-0.2 mM EDTA buffer (pH 8.0) and frozen at -70°C. Prior to treatment and assay, brain homogenates were thawed and diluted 1:20. Fifty μ l of the cell homogenate was placed in each well of a 96 well microtiter plate and incubated for 20 minutes at 37°C with 50 mM Tris 0.2 mM EDTA pH 8.0 buffer, 25 μ l paraoxon (0.2752 mg/ml), or 25 μ l paraoxon plus 25 μ l mipafox (0.02332 mg/ml), for determination of total esterase activity, esterase activity resistant to inhibition by paraoxon, and esterase activity resistant to inhibition by paraoxon and mipafox, respectively. All volumes were brought up to 150 μ l with buffer. Fifty μ l of phenyl valerate (53.2 mg/ml acetone, diluted 1:50 in 0.03% Triton X-100 in saline) was added as enzyme substrate, and the reaction mixture was incubated for an additional 30 minutes at 37°C. The reaction was stopped by addition of 50 μ l 10% SDS 0.04% 4-aminoantipyrine 1 M Tris solution. Potassium ferricyanide (0.4%) was added at a volume of 50 μ l for development of color. After each addition, the microtiter plate was mixed 2 - 3 times on the automatic mixer of the microtiter plate spectrophotometer. The microtiter plate was incubated at room temperature for 15 minutes for color development and absorbance was then read at 510 nm on the microtiter plate spectrophotometer. The assay was performed in triplicate and absorbances averaged for enzyme activity calculations. Coefficients of variation did not exceed 10%. NTE activity was determined as

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esterase activity that is paraoxon resistant, but mipafox sensitive. NTE activity was calculated as follows:

$$R = \text{Abs}_{\text{px}} - \text{Abs}_{\text{px+mpx}}/0.0017/[\text{protein}], \text{ where}$$

Abs_{px} = absorbance of paraoxon treated well,

$\text{Abs}_{\text{px+mpx}}$ = absorbance of paraoxon and mipafox treated well,

0.0017 = the slope of the standard phenol curve

= absorbance unit/nmoles phenol/ml, and

$[\text{protein}]$ = sample protein concentration in mg/ml.

This calculation gives NTE activity in units of nmoles phenol generated per 30 minutes per mg protein.

NTE aging assay

Determination of that portion of NTE that has been bound to the OP inhibitor and aged was done by performing the NTE aging assay of Johnson, *et al* [8,9], modified for reduced tissue volume and for microassay [7]. Treatment was accomplished by incubation of 1 ml of suspended cells or brain homogenate with the appropriate concentration of toxicant in HBSS (cells) or 50 mM Tris NTE buffer (brain). The reaction was stopped by dilution with 2.5 ml of ice-cold NTE buffer. Brain tissue was centrifuged at 27,000 x g for 30 minutes at 4°C and cells were centrifuged at 1000-1200 rpm for 10-12 minutes at 4°C. Brain tissue was resuspended in NTE buffer and rehomogenized using a glass hand homogenizer. Cells were resuspended in NTE buffer and homogenized by 5 freeze-thaw cycles in liquid nitrogen. Homogenates of treated and control cells and brain were split and each half was incubated for 60 minutes at 37°C in 25 mM isonitrosoacetophenone (INAP), for reactivation of inhibited, unaged NTE, in 50 mM Tris 0.2 mM EDTA buffer (pH 8.5, maintained at 37°C to avoid crystallization) or in a nonreactivating solution, 25 mM KCl in the same buffer. The reaction was stopped by putting the samples on ice and

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allowing them to cool. Samples were then centrifuged at 30,000 x g for 1 hour at 0°C. The pellets were resuspended in cold 50 mM Tris 0.2 mM EDTA pH 8 buffer and rehomogenized with a glass hand homogenizer. The final cell concentration should be the equivalent of 10⁷ cells per ml of buffer. The standard NTE assay was then performed as described above. Aged NTE was determined to be that portion of inhibited NTE activity (determined by that part of the sample incubated in the nonreactivating solution) that is not restored to activity by reactivation with INAP.

Acetylcholinesterase (AChE) assay

AChE determinations were made according to the method of Ellman, *et al* [10], modified for microassay [7]. The 10⁷ cell/ml suspension and the diluted brain homogenate used for NTE assay was used for AChE assay. Fifty µl of tissue was used per well. One hundred fifty µl of pH 8.0, 0.1 M phosphate buffer was added to each tissue sample well. Tissue and reagent blanks were included; the reagent blank contained 200 µl buffer. At the start of the assay, 50 µl of DTNB (5,5'-dithiobis-2-nitrobenzoic acid)/NaHCO₃ (24mg/9mg in 10 ml of pH 7.0, 0.1 M phosphate buffer) was added to all wells and the plate was mixed on the automatic mixer on the microtiter plate spectrophotometer. Then 50 µl of acetylthiocholine solution (13 mg ACTCH/ 10 ml H₂O) was added to sample and reagent blank wells. The plate was again mixed and read (time = 0) at an absorbance of 412 nm. The plate was incubated in a 27°C water bath for 30 minutes and then was read as before (time = 30). AChE activity was calculated as follows:

$$R = \{[(A_{T=30} - A_{T=0})/30 \text{ minutes}]/1.36 \times 10^4\} \\ * [(1/\text{sample volume})/\text{total cuvette volume}] * 1/C_0$$

where R = rate, in moles, of substrate hydrolyzed per minute per cell,

$(A_{T=30} - A_{T=0})/30 \text{ minutes}$ = change in absorbance per minute,

C_0 = concentration of tissue sample added to well in cells/l, and

1.36×10^4 = molar extinction coefficient for ACTCH iodide.

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When corrected for protein concentration instead of cell concentration, [protein] in mg/ml was substituted for C_0 and the equation reduced to:

$$R = (A_{T=30} - A_{T=0}) * 14.7 / [\text{protein}],$$

where R = rate in nmoles of substrate hydrolyzed per minute per mg protein.

[Ca²⁺]_i determination

Intracellular free calcium was measured for initial screening using the membrane permeable acetoxymethyl ester of the fluorescent calcium probe fura-2 (am). The compound was stored frozen at -20°C at a concentration of 1 mg/ml in DMSO. Three ml of cells at a concentration of 10⁶ cells per ml were incubated with 3 µl fura-2 per ml of cells (3 µM final concentration) at 37°C for 20 -30 minutes. A blank tube containing 3 ml of cells without fura-2 was included. A ten fold excess of HBSS (30 ml) was then added and the solution was incubated for an additional 30 -45 minutes. The tubes were then centrifuged at 1000 - 1200 rpm for 10 - 15 minutes and the pelleted cells were resuspended in 3 ml of buffer without fura-2. The fluorescence of the loaded fura-2 was using a spectrofluorometer. The cuvette chamber was heated to 37°C, the emission wavelength was set at 510 nm, and the excitation wavelengths was set at 340 and 380 nm. The instrument was zeroed at 380 nm with buffer only. The blank cells and the fura-2 loaded cells were read at both excitation wavelengths and the readings of the blank were subtracted from all other readings at the respective wavelength. Free intracellular calcium concentration was calculated as follows:

$$[\text{Ca}^{2+}]_i = Kd * (R - R_{\min}/R_{\max} - R) * (S_{f2}/S_{b2})$$

where Kd, the dissociation constant for fura-2 = 224 nM at 37°C,

$$R_{\min} = 0.768,$$

$$R_{\max} = 35.1,$$

$$S_{f2}/S_{b2} = 15.3,$$

and R is calculated as F_{340}/F_{380} [11].

MATERIALS AND METHODS

This determination was performed for initial screening of cell lines.

The following method was used to examine treated differentiated SY-5Y cells for changes in $[Ca^{2+}]_i$. Treated and control cells in 96-well microtiter plates were loaded with the cell permeant probe, fluo-3 am, at a final concentration of 25 μ M in fresh media, incubated at 37°C for 1 hour, washed 3 times in Hank's balanced saline solution (HBSS), and incubated for an additional 30 minutes at 37°C to allow for hydrolysis of the acetoxymethyl ester. Plates were read on a Cytofluor 2300 at an excitation wavelength of 485 +/- 20 (505) nm, an emission wavelength of (526) nm, and a sensitivity setting of 6. $[Ca^{2+}]_i$ was expressed as the percent of control fluorescence, corrected for background fluorescence, for treated cells compared to untreated cells loaded with fluo-3 at the same time point.

Morphological examination

Cells in culture flasks were examined by phase-contrast microscopy twice weekly and on days 1, 3, 7, 10, and 14 after toxicant treatment, for physical characteristics indicative of differentiation, i.e. cell shape and neurite outgrowth and for effects of toxicant treatment.

Statistical methods

Each experiment was conducted as a completely randomized design and was analyzed by the appropriate analysis of variance (ANOVA) for that design. Means were compared by Duncan's multiple range test for comparison of SY-5Y cells to brain and for comparison among treatments.

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MATERIALS AND METHODS

PART III

RESULTS

RESULTS

The following chapters present the data collected during the steps taken to validate the differentiated SY-5Y cell culture model for investigation of the earliest effects of neuropathy-inducing organophosphate (OP) compounds on neural cells. First, this cell line and others were evaluated for suitability as a model for organophosphorus-induced delayed neuropathy (OPIDN) by establishing the presence of the critical enzymes, neurotoxic esterase (NTE) and acetylcholinesterase (AChE), and by demonstrating normal neuronal morphology in the differentiated state. The SY-5Y cell line proved superior to the other cell lines examined. Next, in order to demonstrate the usefulness of this cell line in the examination of early effects of neuropathy-inducing OPs, the effect of organophosphate and control compounds on the inhibition and aging of NTE activity was demonstrated. Effects on inhibition and aging of NTE were as expected based on current knowledge of the compounds tested, and these effects were reproduced in brain homogenate from normal chickens, providing validation for the model. Additionally, the time course of NTE inhibition and aging was established in the first minutes after tissue exposure to mipafox, a neuropathy-inducing OP.

Subsequent experiments were performed in order to determine if the inhibition and aging of NTE by a neuropathy-inducing organophosphate could be modified in this in vitro model by compounds shown to modify the clinical and pathological expression of OPIDN in vivo. A series of treatments were performed in which protective compounds either preceded, were administered with, or followed treatment with a neuropathy-inducing OP. Protection of NTE from inhibition and aging was observed in these experiments. Again, results were reproduced in chicken brain homogenate, further validating the model.

The final group of experiments were designed to examine effects of neurotoxicants in the SY-5Y cells on parameters other than NTE activity. This work was performed in order to attempt to establish a profile of toxicological effects that could differentiate OPs from other neurotoxicants and to attempt to further elucidate some part of the mechanism by which OPIDN develops. Acetylcholinesterase activity was examined in control and treated cells and brain homogenate. Effects of toxicants on this enzyme in cells were compared to those in chicken brain homogenate and were also found to be similar. Additional effects of the toxicant treatments on SY-5Y cells that could not be measured in homogenized tissue were investigated. These included effects of neurotoxicants on cellular morphology, intracellular calcium ion concentration, and viability. The effect of continuous toxicant treatment for up to 2 weeks on differentiated cell morphology was monitored and noted to differ between toxicants inducing neuropathy in vivo and toxicants which do not have this effect. Changes in free intracellular calcium ion concentration as a result of treatment were investigated as such changes may represent some part of the mechanism of development of OPIDN.

CHAPTER I

DEVELOPMENT OF A MODEL CELL CULTURE SYSTEM IN WHICH TO STUDY EARLY EFFECTS OF NEUROPATHY-INDUCING ORGANOPHOSPHORUS ESTERS

**Development of a model cell culture system
in which to study early effects
of neuropathy-inducing organophosphorus esters**

SUMMARY

Certain organophosphorus (OP) compounds produce a delayed neuropathy in man and susceptible animal species after early inhibition and aging of the enzyme, neurotoxic esterase (NTE). In this study, the human neuroblastoma cell line, SY-5Y, differentiated to a mature neuronal cell form, was used to examine the time course of inhibition and aging of NTE after toxicant treatment. The time course and extent of detrimental effects on this enzyme were similar in the SY-5Y cells to those observed in homogenized chicken brain tissue after the same treatments. The results indicate that the SY-5Y model system shows promise for use in the determination of initial mechanisms contributing to the development of organophosphorus-induced delayed neuropathy.

INTRODUCTION

Cell culture systems are being used with ever-increasing frequency to study the effects of toxicants. These model systems have been particularly useful in the study of toxicants that target a specific cell type [1]. In this study, a cell culture system was used for study of effects caused by neuropathy-inducing OPs. The toxic effects of certain organophosphorus esters (OP's), in addition to the well known acute inhibition of acetylcholinesterase, include a distal, central-peripheral neuropathy which develops 8-14 days after exposure to the toxicant. The first signs of organophosphorus-induced delayed neuropathy (OPIDN), which may or may not follow cholinergic signs, occur in the lower limbs as a numbness or fatigue, progressing to weakness and paralysis. The

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most outstanding clinical features of the syndrome are the long latent period before the onset of signs, bilaterally symmetrical paralysis, and the absence of pain [2-4]. Cases of paralysis in humans contacting OP's have been recorded since the late nineteenth century [2,3].

The exact biochemical mechanism by which OPIDN develops is presently unclear. The initiating event is generally considered to be the inhibition and aging of the enzyme neurotoxic esterase (neuropathy target esterase, or NTE) within the neuronal cell. Aging of the neurotoxic esterase-organophosphate bond is defined as a dealkylation of the covalently bound OP, leaving a charged phosphoryl substituent at the esteratic active site. The aging step is irreversible, and thus can be determined to have occurred when the enzyme can no longer be reactivated. Neuropathy-inducing OP's, such as mipafox, bind to and inhibit NTE; subsequently that bond is irreversibly aged. OP's that do not produce delayed neuropathy, such as paraoxon, do not inhibit NTE, while compounds such as carbamates and phosphinates will reversibly inhibit NTE, but will not age and will not produce delayed neuropathy [5]. Inhibition of NTE by 70-80% is considered to be the threshold value required for development of OPIDN in vivo after a single, acute exposure to a neuropathy-inducing OP [6].

The earliest biochemical events associated with the interaction of neuropathy inducing OP's and neural cells are difficult to study in the intact test animal. Investigations using a differentiated neuronal cell line as a model system could facilitate examination of the events taking place in the first minutes to hours after OP exposure that may precipitate neuropathy.

The present study was designed to determine if NTE inhibition and aging could be measured in a neuronal cell culture system and to compare these events in cell culture with those that occur in the nervous system of the chicken, which is the animal model for this disorder [4].

MATERIALS AND METHODS

As OPIDN is a result of damage to neurons, neuronal-type cell lines were investigated as potential cell culture models of this neuropathy. The following cell lines were examined after differentiation for potential use as a model cell line: SK-N-SH, a neuroblastoma cell line of human origin, SY-5Y, a subclone of SK-N-SH, and PC12, a rat pheochromocytoma cell line which differentiates into a neuronal cell type. SK-N-SH cells were maintained in minimum essential medium (MEM) with sodium pyruvate and nonessential amino acids in Earle's Balanced Saline Solution (90%) and fetal calf serum (10%). SY-5Y cells were kept in Dulbecco's modified Eagle medium (DMEM) (45%), F12 (45%), and fetal calf serum (10%). PC12 cells were kept in RPMI 1640 (85%), horse serum (10%), and fetal calf serum (5%). Differentiation to a mature neuronal cell form was accomplished by inclusion of nerve growth factor (NGF) in the culture medium of SK-N-SH and SY-5Y cells at a concentration of 1 μ g NGF/ml medium. Undifferentiated SY-5Y cells are rounded and resemble fibroblasts in culture. By 5 days after introduction of NGF to the culture medium, SK-N-SH and SY-5Y cells extend processes with numerous varicosities and appear to form "pseudoganglia" [7]. PC12 cells were differentiated by inclusion of 50 μ g NGF/ml medium [8]. By 7 days after exposure to NGF, PC12 cells are reported to cease to multiply and to extend neuronal-like processes, which continue to grow in number, length, and density for an additional two weeks of treatment. These processes are reported to closely resemble the morphological appearance of those produced by cultured primary sympathetic neurons [9]. Cells in culture flasks were examined by phase-contrast microscopy at least three times weekly for physical characteristics indicative of differentiation, i.e. cell shape and neurite outgrowth. The differentiated SY-5Y cell line (incubated for at least 6 days with NGF) was chosen for use in later experiments based on activities of NTE and acetylcholinesterase (AChE), the two primary enzymes affected by OP's. For these assays, the cells were suspended in Hank's balanced saline solution (HBSS) at a concentration of 10^7 cells per

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milliliter. NTE activity was determined according to the method described by Sprague, et al. [10], modified for microassay [11], and using a 30 minute incubation period of cells or chicken brain homogenate with phenyl valerate, the substrate for the enzyme. This assay was also used to determine total carboxylesterase activity in cultured cells and brain homogenate by including aliquots not incubated with paraoxon or mipafox as part of the assay procedure. AChE determinations were made according to the method of Ellman, et al. [12], also modified for microassay [11].

Sublethal concentrations of test compounds were chosen by determination of cell viability by trypan blue dye exclusion after 24 hour incubation with a range of concentrations of each compound. Treatment concentrations were selected on the basis of inhibition of neurotoxic esterase and total esterase in intact differentiated SY-5Y cells by test compound concentrations exhibiting acceptable nonlethality (greater than 70% viable cells at 24 hours). The goal in choosing test concentrations was to arrive at one that would not overwhelmingly inhibit NTE and total esterase, but that would, in the case of the neuropathic OP mipafox, inhibit NTE to a degree at or near that considered to be the threshold for development of OPIDN in vivo. Evaluation of sublethal concentrations of mipafox for NTE inhibition yielded values that were used to determine the concentration of mipafox that produced 50% inhibition (I_{50}) of this enzyme in the SY-5Y cells. The I_{50} value for mipafox was used to qualitatively compare NTE in these cells with that of hen brain, and was used for comparison to previously reported values for other cell lines.

The time course of NTE inhibition and aging was determined for the neuropathic OP mipafox and for four negative control compounds: paraoxon, an OP that does not inhibit NTE in vivo and does not produce delayed neuropathy; aldicarb, a carbamate that inhibits NTE in vivo, but presumably does not age the enzyme and does not produce delayed neuropathy; β,β' -iminodipropionitrile (IDPN), an agent that produces a peripheral neuropathy by another mechanism involving neurofilaments in the proximal segments of affected axons [13], but should have no effect

on NTE; and carbachol, a cholinergic receptor agonist which should also have no effect on NTE, and was included to separate effects on AChE from those on NTE. Treatment was accomplished by incubating suspensions of SY-5Y cells or chicken brain homogenate with sublethal concentrations of toxicants at 37°C for times between 2 and 10 minutes.

Determination of that portion of NTE that was bound to the OP inhibitor and aged was done by performing the NTE aging assay of Johnson, et al. [14], modified for reduced tissue volume and for microassay. Isonitrosoacetophenone (INAP) was used to reactivate inhibited enzyme to which toxicants had not covalently bound.

Each toxicant treatment in differentiated SY-5Y cells and chicken brain homogenate was performed in duplicate. All assays were performed in triplicate, using average absorbances for enzyme activity calculations. Results for all treatments in differentiated SY-5Y cells were compared to those in chicken brain homogenate treated and incubated with equivalent toxicant concentrations for the same time intervals, as the chicken is considered to be the animal model for OPIDN [4].

The experiment was performed as a completely randomized design and was analyzed by the appropriate analysis of variance (ANOVA) for that design. Means for NTE inhibition and aging at each time point in SY-5Y cells and brain tissue were compared by Duncan's multiple range test.

RESULTS

The human neuroblastoma cell line, SY-5Y, was selected for use in further experiments to establish the model system based on relatively high activities of neurotoxic esterase and acetylcholinesterase in the differentiated form when compared to the other cell lines examined (Table 1.1). The NTE activity in SY-5Y cells was nearest the activity of that enzyme in chicken brain homogenates (331.7 +/- 19.6 nmoles phenyl valerate hydrolyzed/30 min/mg protein, n=10). Differentiated SY-5Y cells took on a morphological appearance similar to mature neurons. They

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became more flattened and stellate in shape, and extended long processes or neurites upon exposure to NGF.

Viable cells were necessary for study of the effects of neurotoxicants on esterase enzymes. The sublethal concentrations of toxicants chosen, using criteria described in the Materials and Methods section, were: 5×10^{-5} M mipafox, 5×10^{-5} M paraoxon, 5×10^{-3} M aldicarb, 5×10^{-5} M IDPN, and 5×10^{-5} M carbachol. Cell viability was greater than 70% after incubation for 24 hours with these concentrations of toxicants. The attempt was made to use equimolar concentrations of each treatment compound in subsequent studies, however, a 100-fold higher concentration of aldicarb was necessary to prevent complete spontaneous reactivation of NTE by the end of the assay procedure. None of these compounds caused morphological alterations of the SY-5Y cells when exposed for the 10 minute interval used in these experiments.

NTE inhibition and aging could be measured in differentiated SY-5Y cells. Values for NTE inhibition and aging are shown in Table 1.2. Results using SY-5Y cells corresponded to those using brain homogenate. There was no significant difference between values for NTE inhibition and aging for SY-5Y cells and brain homogenate at corresponding time points for each treatment ($\alpha = 0.05$). As expected, no inhibition or aging of NTE was observed with the compounds paraoxon, IDPN, and carbachol. After mipafox treatment, NTE was inhibited rapidly, exceeding 25% inhibition of control activity by 2 minutes after exposure and reaching near threshold values for development of OPIDN in vivo by 10 minutes (Figure 1.1). Aging was initiated between 2 and 5 minutes after exposure, and most of inhibited NTE was aged by 10 minutes. After aldicarb treatment, NTE inhibition also appeared rapidly, peaked by 5 minutes, and then dropped, presumably due to spontaneous reactivation of the enzyme (Figure 1.2). No aging of NTE was observed in aldicarb treated SY-5Y cells or chicken brain tissue.

NTE activity was compared to total carboxylesterase activity in SY-5Y cells and in chicken brain homogenate. NTE comprised 22% $\pm$ 1.8 (mean $\pm$ SEM, n = 10) and 16% $\pm$ 0.9 of the total carboxylesterase activity in cells and brain, respectively. The concentration of mipafox producing 50% inhibition of NTE (I_{50}) was 5 μ M.

DISCUSSION

This study demonstrated that the differentiated SY-5Y neuroblastoma cell line has NTE activity similar to that of chicken brain and that this cell line can be used to distinguish among esterase inhibitors that are capable and that are not capable of inducing OPIDN in vivo. It also demonstrated that the portion of total carboxylesterases exhibiting NTE activity was similar in the differentiated neuroblastoma cells and in chicken brain. Additionally, the I_{50} for mipafox in SY-5Y cells was similar to the 7.3 - 9.6 μ M previously reported for hen brain [15-17]. This system, therefore, shows promise for use in the further investigation of early cellular events in OPIDN.

The use of cultured cells has previously been proposed for examination of inhibition of NTE after treatment with OP esters. Previous studies have documented that NTE activity in a differentiated neural cell line of human origin comprised approximately 25% of the cell line's total esterase activity [18], which was similar to observations made in the present study in SY-5Y cells. NTE activity has also been measured in undifferentiated neuroblastoma cell lines of human (SK-N-BE2) and of mouse (N115) origin [19]. NTE activity in that study was found to be 14.3 $\pm$ 1.2 nmoles phenyl valerate hydrolyzed per mg protein per minute in the human neuroblastoma line and 3.8 $\pm$ 0.1 in the murine neuroblastoma line. NTE activity in the human neuroblastoma cell line in that study was approximately double the NTE activity expressed on a per minute basis of 7.6 $\pm$ 2.1 in differentiated SY-5Y cells in the present study. Cells of murine origin are probably not a good choice for studies of compounds inducing OPIDN, however, since murine NTE is not easily inhibited

by these toxicants, and morphological damage in mice given such compounds is not consistent with that seen in humans and chickens [20].

The SY-5Y line is of human origin, making it an optimal choice for a model for study of this disorder, since man is one of the most susceptible species to OPIDN. Additionally, the cell line is neural in origin, and OPIDN follows initiating events in neural cells in vivo. Cells differentiated with NGF were used because they resemble mature neurons in vivo, and because it is the adult, rather than immature, animals of susceptible species that develop OPIDN [4]. Results of the present study demonstrate that the differentiated SY-5Y model system behaves in a manner not unlike that of brain tissue from the normal adult chicken, which is the generally accepted animal model for OPIDN [4].

In vitro systems have a great deal of promise for use in mechanistic studies of neurotoxicity and for use in risk assessment as part of screening batteries [21,22]. Previously, brain homogenates have been used to study effects of neuropathy-inducing OPs on NTE, AChE, and total esterase activities and to compare NTE and other esterases between species [15,17 23,24]. Inhibition and aging of NTE in hen brain homogenates has been shown to occur rapidly, in a matter of minutes [23]. Reactivation of NTE inhibited by mipafox, using potassium fluoride as the reactivating compound, in that study was not accomplished; this was assumed to be due to the phosphorodiamidate structure of this compound. In vitro systems using brain homogenates are limited, however; they cannot be used to study effects of neurotoxicants on the physiology of intact cells.

SY-5Y cells have been shown to acquire characteristics of the specialized neuronal cell. This includes expression of the enzyme activities examined in the present study. SY-5Y cells also demonstrate morphological expression of a neural cell phenotype, properties of excitable membranes, and opioid receptors [7,25,26]. Additionally, cultured cells have already been used in

the study of aspects of OP-induced neurotoxicity, other than NTE inhibition, including morphological response to OP exposure, changes in protein metabolism, release and metabolism of dopamine, changes in muscarinic receptor binding number, and inhibition of acetylcholinesterase activity [27-31].

The use of cell culture model systems in toxicological experimentation has numerous advantages. Such a system allows for rapid, early manipulation of tissue after exposure to toxic compounds. Since differentiated SY-5Y cells and chicken brain homogenate responded similarly in the experiments reported, the SY-5Y model may be representative of events occurring at the level of the target tissue in vivo at tissue concentrations approximating those used in the experiments. Disadvantages of cell culture models also exist. Differentiated SY-5Y cells in culture are still dividing, although much more slowly than do undifferentiated SY-5Y cells. In addition, there are no interactions between diverse tissue types, and there is no consideration of the pharmacokinetics of toxicants in the intact animal. The time course of NTE inhibition and aging could be altered by absorption, distribution, metabolism, and excretion of the offending compound in the live animal.

The prevention of OPIDN in vivo requires pretreatment of the animal with a protective compound; the need for this is obvious in light of the rapidity with which aging occurs. It has long been accepted that NTE inhibition and aging occur within hours after exposure [32]; the present study confirms that concept and indicates that these processes may be taking place with greater rapidity than previously thought. The use of a test system such as the cell culture system described may be of considerable advantage, especially in the identification of methods for prevention of NTE aging after the OP has reached the neuronal cell.

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TABLE 1.1. Relative activities of NTE and AChE in differentiated neuronal cell lines.

Differentiated cell line	NTE activity ¹	AChE activity ²
SK-N-SH	217.42 +/- 14.66	1.13 +/- .07
SY-5Y	226.98 +/- 62.00	2.80 +/- .03
PC12	5.34 +/- 3.78	14.30 +/- .24

¹nmoles phenyl valerate hydrolyzed/30 minutes/mg protein, mean +/- SEM.

²moles x 10⁻¹⁶ acetylthiocholine hydrolyzed/minute/cell, mean +/- SEM.

TABLE 1.2. NTE inhibition and aging in differentiated SY-5Y cells and chicken brain homogenate after mipafox and aldicarb treatments.

Treatment	Tissue	Exposure time	% of control NTE activity inhibited ¹	% of control NTE activity aged ¹
mipafox (5 x 10 ⁻⁵ M)	SY-5Y	2 min	22.55 +/- 3.48	0
		5 min	59.87 +/- 5.21	26.64 +/- 7.96
		10 min	67.62 +/- 9.60	60.00 +/- 9.25
mipafox (5 x 10 ⁻⁵ M)	Brain	2 min	41.73 +/- 1.64	0
		5 min	50.00 +/- 5.34	36.08 +/- 2.47
		10 min	74.16 +/- 7.46	58.88 +/- 0.14
aldicarb (5 x 10 ⁻³ M)	SY-5Y	2 min	14.59 +/- 10.32	0
		5 min	16.19 +/- 6.91	0
		10 min	9.19 +/- 1.96	0
aldicarb (5 x 10 ⁻³ M)	Brain	2 min	8.03 +/- 3.43	0
		5 min	10.33 +/- 0.03	0
		10 min	2.32 +/- 1.64	0

¹mean +/- SEM.

FIGURE 1.1. Percent of control NTE activity inhibited and aged in differentiated SY-5Y cells (a) and chicken brain homogenate (b) after mipafox treatment (mean +/- SEM). The values on the curves for SY-5Y cells did not differ from the values on the curves for brain homogenates ($\alpha = 0.05$).

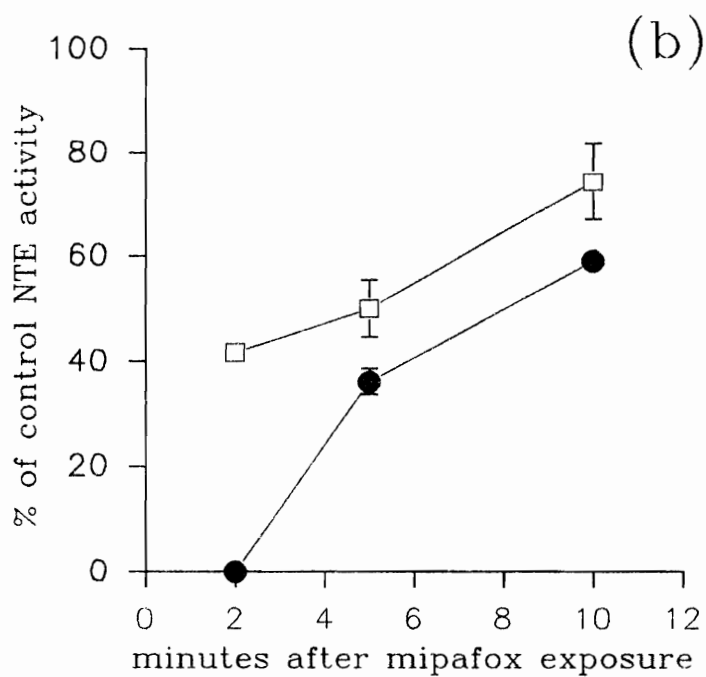
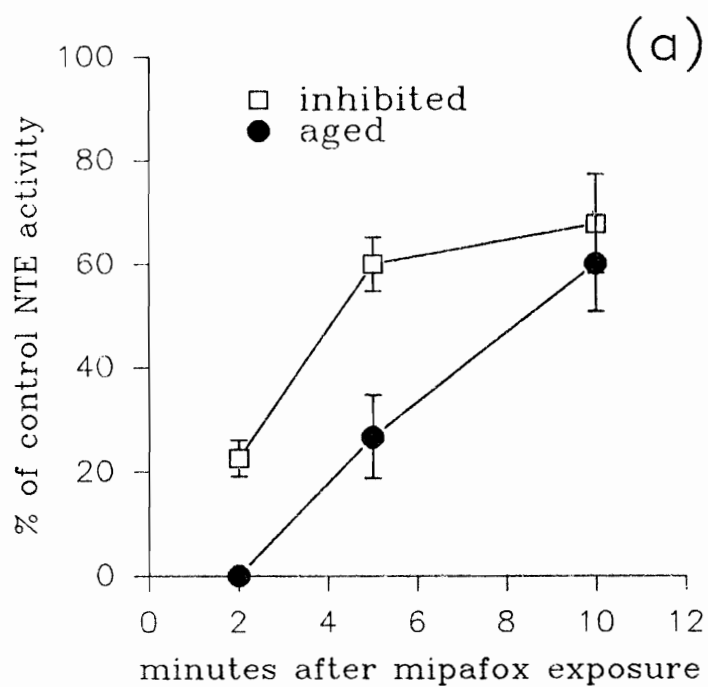
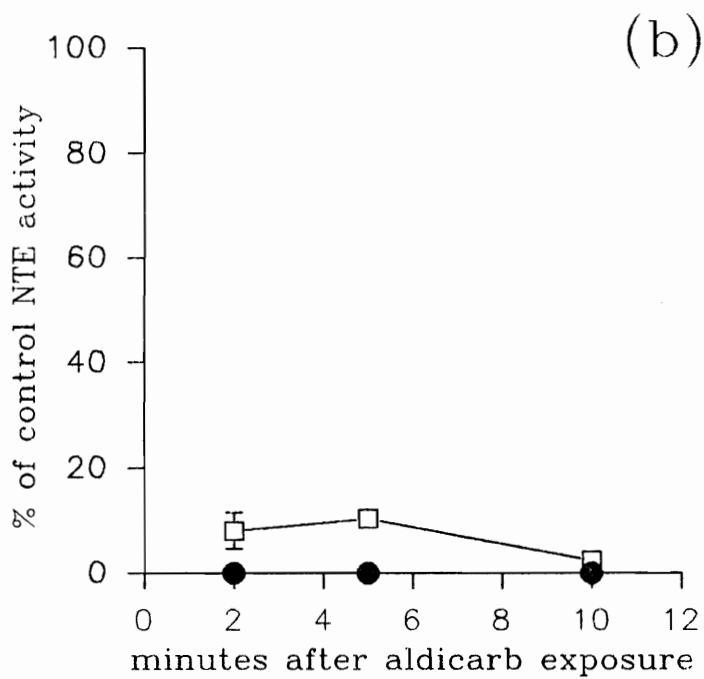
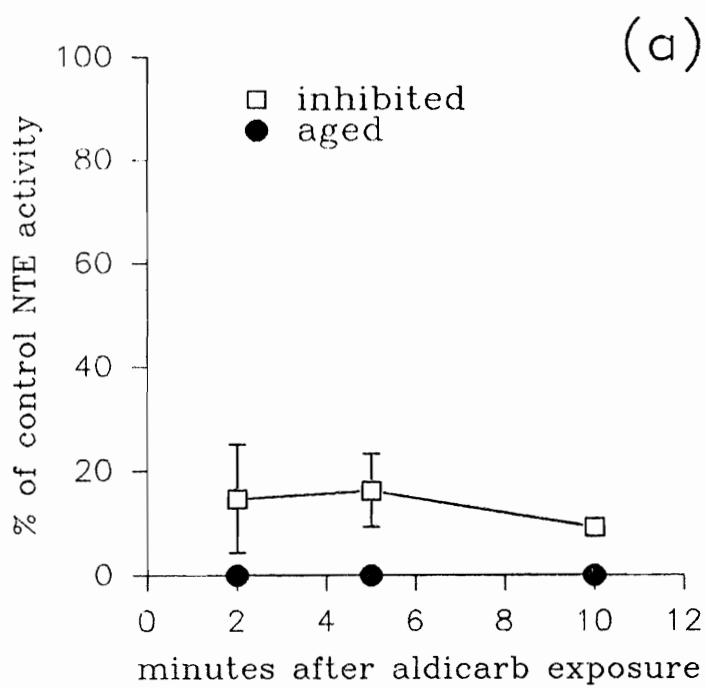


FIGURE 1.2. Percent of control NTE activity inhibited and aged in differentiated SY-5Y cells (a) and chicken brain homogenate (b) after aldicarb treatment (mean $\pm$ SEM). The values on the curves for SY-5Y cells did not differ from the values on the curves for brain homogenates ($\alpha = 0.05$).



CHAPTER II

MODIFICATION OF MIPAFOX-INDUCED INHIBITION AND AGING OF NEUROTOXIC ESTERASE IN NEUROBLASTOMA CELLS

Modification of mipafox-induced inhibition and aging of neurotoxic esterase in neuroblastoma cells

SUMMARY

A neuroblastoma cell line was used as an in vitro model system to examine early effects on inhibition and aging of neurotoxic esterase (neuropathy target esterase, NTE) of compounds previously demonstrated to modify organophosphorus-induced delayed neuropathy (OPIDN) in hens. For this study, NTE inhibition and aging were determined in differentiated SY-5Y cells after mipafox treatment in combination with the carbamate, aldicarb, and with the calcium channel blocker, verapamil. A 5 minute pre-treatment, simultaneous treatment, and a 2 minute post-treatment of aldicarb or verapamil were used relative to mipafox treatment. Significant protection of NTE from inhibition and aging was observed when either aldicarb or verapamil was included in the incubation of SY-5Y cells with mipafox. Effects on NTE inhibition and aging in differentiated SY-5Y cells after each combination of treatments were similar to those in chicken brain homogenate. The results indicate that both aldicarb and verapamil protected NTE in neuroblastoma cells and in chicken brain against the early biochemical effects of mipafox that are thought to initiate OPIDN in vivo.

INTRODUCTION

The initial events of organophosphorus-induced delayed neuropathy (OPIDN) have been accepted for some time to be the inhibition and aging of neurotoxic esterase (NTE) by the offending compound [1]. Previous work in this laboratory has established the time course of NTE inhibition and aging in isolated brain tissue and cultured cells by the neuropathy-inducing organophosphate

(OP), mipafox, and has established that the differentiated SY-5Y human neuroblastoma cell line could be used as a model for examination of effects of this compound on NTE in the first minutes after exposure [2].

A number of compounds have been shown to ameliorate the development of OPIDN in vivo. Among these are those compounds which inhibit neurotoxic esterase (NTE), but do not age the enzyme. Carbamates are one such group of compounds [3]. When administered to adult chickens, the accepted animal model for OPIDN [4], prior to exposure to a neuropathy-inducing OP, this class of chemicals protects the animal from subsequent challenge by neuropathy-inducing organophosphate compounds [3].

Other classes of compounds have also been demonstrated to protect hens from development of OPIDN. These include corticosteroids and calcium channel blockers [5-8]. Treatment of hens with calcium channel blocking drugs prior to exposure to a neuropathy-inducing OP has been shown to significantly attenuate the development of clinical signs and lesions of OPIDN [7,8]. Hens treated with either verapamil or nifedipine and the neuropathy-inducing OP, phenyl saligenin phosphate (PSP), were significantly less ataxic than hens treated with PSP alone. Histological lesions were less severe, and electrophysiological assessment of neuromuscular function supported the protective role of the calcium channel blocking drugs in these experimental animals [7,8]

The mechanisms of development of OPIDN and of protection by the calcium channel blocking compounds are currently unknown. The purpose of the present study was to examine the effects of treatment with a protective compound in combination with a neuropathy-inducing OP on NTE in the first minutes after tissue exposure. Differentiated SY-5Y neuroblastoma cells were used for this study, as this model allows for identification of those early events that occur at the target site in intact, neural derived cells. The early effects of protective compounds on NTE inhibition and aging in SY-5Y cells were compared to those in chicken brain homogenate.

MATERIALS AND METHODS

The differentiated SY-5Y cell line, previously described as a model for examination of early effects of OP compounds on neuronal cells [2], was used in the present study in which the effects on NTE of modifiers of OPIDN were examined. SY-5Y cells were kept in Dulbecco's modified Eagle medium (DMEM) (45%), F12 (45%), and fetal calf serum (10%). Differentiation to a mature neural cell phenotype was accomplished by inclusion of nerve growth factor (NGF) in the culture medium at a concentration of 1 µg NGF/ml medium for 5 to 7 days [9]. For treatment, differentiated SY-5Y cells were suspended in Hank's balanced saline solution (HBSS) at a concentration of 2×10^7 cells per milliliter.

Cell suspensions were treated in the same manner as chicken brain homogenate for comparison of effects and to further validate the cell culture model as an indicator of events that occur in tissues from animals susceptible to OPIDN. Brain tissue from normal, untreated, adult chickens was homogenized in 6.5 volumes of 50 mM Tris-0.2 mM EDTA buffer (pH 8.0) and frozen at -70°C. Prior to treatment and assay, brain homogenates were thawed and diluted 1:20.

Sublethal concentrations of the test compounds, mipafox (5×10^{-5} M) and aldicarb (5×10^{-3} M), were chosen for this study for their capability to cause NTE inhibition without substantial loss of cell viability as previously described [2]. The concentration of verapamil chosen was based on plasma concentrations in humans receiving therapeutic doses of this calcium channel blocking drug. Reported values for plasma concentrations of verapamil following therapeutic doses range from 100 µg/L to 500 µg/L [10]. Since this drug is 90% plasma protein bound [11], tissue concentrations may be assumed to equilibrate with free drug concentration in the plasma, which in this case would be 2×10^{-8} M (10 µg/L) to 1×10^{-7} M (50 µg/L). SY-5Y cell viability, as determined by trypan blue dye exclusion after 24 hour incubation with a range of concentrations of verapamil, was greater than 90% for concentrations ranging from 1×10^{-8} M to 5×10^{-7} M. A verapamil concentration of 10^{-7} M was chosen for use in this study.

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The time course of OP-induced NTE inhibition and aging, and their alteration in the presence of compounds that ameliorate OPIDN, was determined for the neuropathic OP mipafox in addition to each of the following co-treatments: 5 minute pre-treatment with aldicarb, simultaneous treatment with aldicarb, 2 minute post-treatment with aldicarb, 5 minute pre-treatment with verapamil, simultaneous treatment with verapamil, and 2 minute post-treatment with verapamil. The effect of verapamil alone was also examined. Effects of mipafox alone and of aldicarb alone on NTE have previously been described [2]. Treatment was accomplished by incubating suspensions of SY-5Y cells or chicken brain homogenate with each set of compounds at 37°C for times between 2 and 10 minutes.

NTE activity was determined according to the method described by Sprague, et al. [12], modified for microassay [13], and using a 30 minute incubation period of cells or chicken brain homogenate with phenyl valerate, the substrate for the enzyme.

Determination of that portion of NTE that was bound to the OP inhibitor and aged was done by performing the NTE aging assay of Johnson, et al. [14], modified for reduced tissue volume and for microassay [2]. Isonitrosoacetophenone (INAP) was used to reactivate inhibited enzyme to which toxicants had not covalently bound.

Decreasing the time of exposure to neuropathy-inducing OPs has potential to reduce the opportunity for NTE inhibition and aging to occur. Therefore, the effect of mipafox removal was investigated. For these experiments, cells were treated in culture flasks with 5×10^{-5} M mipafox in media for 2 minutes and for 5 minutes. The mipafox-containing media was removed by pipette, the cells were rinsed with fresh media, and then the cells were replaced in fresh media and allowed to incubate for 24 hours at 37°C before assay for NTE inhibition and aging.

Each toxicant treatment in differentiated SY-5Y cells and chicken brain homogenate was performed in duplicate. All assays were performed in triplicate, using average absorbances for enzyme activity calculations. Results for all treatments in differentiated SY-5Y cells were compared

to those in chicken brain homogenate treated and incubated with equivalent toxicant concentrations for the same time intervals, as the chicken is considered to be the animal model for OPIDN [4].

The experiment was performed as a completely randomized design and was analyzed by the appropriate analysis of variance (ANOVA) for that design. Means for NTE inhibition and aging at each time point in SY-5Y cells and brain tissue were compared by Duncan's multiple range test. Means for each treatment were compared to the means for treatment with mipafox alone in the same tissue also by Duncan's multiple range test.

RESULTS

Treatment with the carbamate, aldicarb, and with the calcium channel blocker, verapamil, reduced the inhibition and aging of NTE induced by the neuropathy-inducing OP, mipafox. This effect was observed in both SY-5Y cells and in chicken brain tissue, and with all combinations of compounds to which both of these tissues were exposed. In SY-5Y cells (Figure 2.1), the percentage of control NTE activity inhibited at 10 minutes and aged at 5 and 10 minutes after mipafox treatment and each of the aldicarb co-treatments was significantly less when this carbamate was included in the incubation than when cells were treated with mipafox only ($\alpha = 0.05$). NTE inhibition and aging in chicken brain tissue after mipafox treatment and each of the aldicarb co-treatments was also significantly less than when the incubation did not include aldicarb ($\alpha = 0.05$) at all time points (Figure 1). There was no significant difference in NTE inhibition and aging between SY-5Y cells and brain tissue ($\alpha = 0.05$), with the exception of NTE inhibition at 5 and 10 minutes after mipafox treatment with a 2 minute aldicarb post-treatment.

As expected of carbamates, aldicarb alone could inhibit NTE, but could not age the enzyme. The inhibition of NTE by aldicarb alone in SY-5Y cells was in 14.5%, 16.1%, and 9.1% at time points of 2, 5, and 10 minutes after exposure. NTE inhibition in chicken brain homogenate was 8.0%, 10.3%, and 2.3% at 2, 5, and 10 minutes, respectively, after exposure to aldicarb. When

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exposed to mipafox and aldicarb in combination, the NTE inhibition and aging in chicken brain and in SY-5Y cells was not significantly different from NTE inhibition and aging observed when brain and SY-5Y cells were treated with aldicarb alone ($\alpha = 0.05$). The exception was NTE aging at 10 minutes in SY-5Y cells treated with mipafox when aldicarb was used as a 5 minute pre-treatment.

The calcium channel blocker, verapamil, did not cause inhibition or aging of NTE in either SY-5Y cells or in homogenized chicken brain tissue when used alone as a treatment. Verapamil did, however, affect mipafox-induced NTE inhibition and aging. In SY-5Y cells (Figure 2.2), the percentage of NTE activity inhibited by mipafox in the presence of verapamil was significantly lower than the NTE activity inhibited by mipafox alone at the 10 minute time period. Aging at 5 and 10 minutes after mipafox treatment and each of the verapamil co-treatments was also significantly lower than when cells were treated with mipafox alone ($\alpha = 0.05$). NTE inhibition and aging in chicken brain tissue after mipafox treatment and each of the verapamil co-treatments were significantly decreased from those values in brain tissue treated with mipafox alone ($\alpha = 0.05$) at all time points with two exceptions. The percentage of NTE inhibition at 5 minutes in tissue simultaneously treated with mipafox and verapamil and in tissue treated with verapamil 2 minutes after mipafox exposure was not significantly lower than that in brain tissue treated with mipafox alone. There were no significant differences between inhibition and aging in SY-5Y cells and chicken brain when verapamil was added to the incubation mixture 5 minutes before or at the same time as mipafox ($\alpha = 0.05$).

Inhibition of NTE appeared to be reversible, as removal of the neuropathy-inducing OP, mipafox, from exposed cell cultures prior to such time as discernable aging of NTE had occurred, allowed NTE in these cells to recover. If mipafox was removed within 5 minutes after cells were exposed, essentially no NTE inhibition or aging in cells was evident when they were examined 24 hours later after continued incubation with fresh media.

DISCUSSION

This study used differentiated human neuroblastoma cells to examine the interaction of a neuropathy-inducing OP and inhibitors of the development of OPIDN. Effects of the combined treatments were measured within minutes of tissue exposure. The use of a cell culture model, such as the SY-5Y neuroblastoma cell line, for studies of events occurring in neurons exposed to neuropathy-inducing toxicants has the notable advantage of allowing examination of early changes that take place in live, intact cells. This will permit studies on reversible and irreversible events that occur within neural cells when they are exposed to neurotoxicants such as the neuropathy-inducing OPs. Such studies cannot be performed using tissue homogenates. Once validated by enzyme activities or other parameters that can be examined in tissue from *in vivo* experiments, the model may then, subsequently, be used to explore mechanisms of toxicity and other parameters that require intact cells, such as viability, morphological changes, and changes in ion concentrations and conductances.

The differentiated SY-5Y model used in this study appears to be a good model for events that occur when neurons are exposed to neuropathy-inducing OPs. These cells exhibited little significant difference in NTE inhibition and aging when compared to brain tissue from the chicken, the accepted animal model for evaluation of OPIDN. In addition, control NTE activities were similar, as was the proportion of total carboxylesterases that exhibit NTE activity in each of the two tissues [2]. In this study, NTE in differentiated SY-5Y cells was also demonstrated to behave similarly to that of chicken brain homogenates when exposed to a neuropathy-inducing OP and inhibitors of OPIDN development. Variability of NTE activity in the SY-5Y cells was noted to be higher than that of NTE in brain tissue and may have contributed to the statistically significant differences in NTE activities, when seen between these two tissues. This variability may be a consequence of retention of some undifferentiated characteristics by differentiated SY-5Y cells, because they retained the ability to divide, although to a much reduced extent than do

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undifferentiated SY-5Y cells. If variability proves to be a concern, perhaps alternative methods of differentiation should be explored, such as the removal of serum from the media, in addition to NGF treatment, or treatment with other compounds used to differentiate cultured cells.

When cell cultures are used for studies such as those described here, of early events in neural cells exposed to neuropathy-inducing OPs, it is important that the cells be differentiated to a mature form, since OPIDN is a disorder that occurs in mature, fully differentiated neuronal cells [4]. Differentiated cells in culture have the required morphological attributes for evaluation of neurite degeneration, and they have higher levels of activity of enzymes present in neural cells, such as NTE, than do cells in the undifferentiated state [2,9]. It is necessary that the model cells resemble the in vivo tissue type as closely as possible, rather than the more pluripotent undifferentiated cell, in order to effectively mimic the target of OPIDN. The SY-5Y cell line has the additional advantage in that it is of human origin, and humans, like chickens, are especially susceptible to OPIDN [15].

In vivo, pre-treatment with carbamates and non-aging NTE inhibitors protects chickens from the development of OPIDN [3,16], whereas post-treatment with certain of these compounds has been demonstrated to exacerbate the condition [17,18]. In this study, aldicarb post-treatment of mipafox treated tissue exerted a protective effect, as did pre- and simultaneous treatment. This may be attributed in some part to the short time interval (2 minutes) between mipafox and aldicarb exposure in these studies compared to the longer time period between the administration of OPs and non-aging NTE inhibitors in the hen (≥ 4 hours) [17,18]. It is likely that the affinity of aldicarb for the esteratic active site of NTE is great enough to displace mipafox that has not yet aged, when given within minutes of the time the cells were exposed to the OP. Additionally, the presence of aldicarb at a 100-fold higher concentration than that of mipafox should have favored NTE inhibition by aldicarb rather than mipafox. Furthermore, consideration may need to be made of the fact that aldicarb-inhibited NTE spontaneously reactivates rapidly, especially in this cell culture system [2]. Rapid reactivation of aldicarb-inhibited NTE may permit mipafox to inhibit the newly

reactivated enzyme, but at a time later than the times at which NTE inhibition was measured in the present study. If this carbamate-induced delay in NTE inhibition by mipafox were to occur, aging could occur some time after the end of the 10 minute period observed here. This could account for the apparently higher inhibition of NTE in aldicarb pre-treated tissue exposed to mipafox, as compared to inhibition of NTE observed in tissue exposed to mipafox prior to treatment with aldicarb. Aldicarb-inhibited NTE appears to spontaneously reactivate after the treatment is washed from the tissue. In these experiments, the aldicarb had already been removed before NTE assays were performed, contributing to the low NTE inhibition seen at the time of assay.

In addition to the carbamates, other reversible inhibitors of NTE have been studied for their effects on OPIDN in animal models. For example, phenyl methane sulfonyl fluoride (PMSF) is another inhibitor of NTE that has proven protective in vivo when administered prior to neuropathy-inducing OPs. NTE of hens treated with PMSF returns to control levels more quickly than does NTE inhibited by neuropathy-inducing OPs [16]. The sulfonyl fluorides, such as PMSF, however, once bound, cannot be removed from NTE by reactivating compounds currently in use for aging determinations [14]. This characteristic makes this compound unsuitable for NTE aging studies which are done in vitro until such time as a reactivator is discovered that can restore PMSF-bound NTE to its catalytically active state. Although PMSF is considered to be a reversible inhibitor of NTE in vivo, it has been shown to potentiate the development of OPIDN, rather than protect against it, when administered to chickens after exposure to a neuropathy-inducing OP [17,18]. If the interaction of PMSF and OPs were to be explored in the cell culture model, effects other than NTE inhibition and aging could still be examined in whole, viable cells; such studies could be particularly useful in determining the mechanism by which OPIDN develops.

Calcium channel blockers administered to hens have previously been demonstrated to ameliorate clinical and morphological effects of OPIDN. In this in vitro study, protection of NTE by verapamil was demonstrated. This effect on NTE was not seen in vivo in previous studies, even

though those previous studies did document protection from development of OPIDN in hens given verapamil as a pre-treatment to phenyl saligenin phosphate (PSP) [7]. NTE activity in that study in the hen was measured in brains obtained 72 hours after PSP administration, and not in the first minutes after tissue exposure as in the present study. The time of examination of NTE activity after exposure to neuropathy-inducing OPs and compounds protecting animals from development of OPIDN may be critical in order to identify the effects seen in the present study. It is possible that the in vivo effect of verapamil on NTE may be better monitored in peripheral nerve, which is a more isolated sample of tissue where the neuropathy occurs than is whole brain. The importance of peripheral nerve NTE inhibition has been noted by other investigators [19-21], but NTE inhibition in peripheral nerve was not determined in the previous in vivo studies in which hens were pretreated with verapamil in order to modify OPIDN.

The demonstration here, using an in vitro system, that NTE could be protected from a neuropathy-inducing OP by verapamil, although this was not apparent in hens, may also be due to the pharmacokinetics of calcium channel blockers. Verapamil is metabolized in the liver [11] and this may impose a limitation of its duration of effect in vivo that is not seen in the in vitro model. For this reason, the cell culture system may allow for mechanistic studies that may not be possible in vivo due to pharmacokinetic factors, including studies of competitive and noncompetitive inhibitors of NTE.

The cells culture system was used to advantage in this study because it did show that removal of a neuropathy-inducing OP prior to aging of NTE would allow inhibited NTE to recover. Mipaflox was removed from the culture medium within 5 minutes of exposure, and no NTE inhibition and aging was evident 24 hours later. This indicates that intact cells are likely to recover if a neuropathy-inducing OP is removed early after initial exposure. Recovery of NTE in SY-5Y cells may indicate that a critical mass of NTE must be inhibited and aged for inhibition to be discerned 24 hours later. Pharmacokinetic factors make studies such as this difficult in vivo. In vivo,

exposure of neural tissue to a neuropathy-inducing OP is essentially continuous until such time that these neurotoxicants in the body are metabolized and excreted. Effects seen in differentiated SY-5Y cells may approximate those events occurring when a neuropathy-inducing OP interacts with its initial target, but an additional consideration of the cell culture model is to be noted, as the differentiated cells are still replicating, although at a much slower rate than undifferentiated cells. Therefore, they may also be synthesizing new NTE at a rate faster than would neural tissue in vivo. The cell culture model does behave similarly to tissue from chickens, however, and does exhibit potential for study of the early effects of neuropathy-inducing OPs, which are difficult to examine in animal models of OPIDN. Most importantly, cell culture offers the opportunity to study events that only take place in live, intact cells. NTE activity is lost in unfrozen tissue homogenates over time, making it impossible to examine effects on that enzyme in them over a number of hours.

The present study demonstrates further usefulness of the differentiated SY-5Y cell culture model in the examination of early effects of neuropathy-inducing OPs and the interaction of these toxicants with compounds that modify OPIDN. Additional work may provide valuable insight into the mechanism of development of OPIDN and initiate development of methods of protection or treatment of this condition.

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FIGURE 2.1. Percent of control NTE activity inhibited and aged in differentiated SY-5Y cells and chicken brain homogenate after mipafox treatment with (a) 5 minute pre-treatment, (b) simultaneous treatment, and (c) 2 minute post-treatment with aldicarb. NTE activities in untreated SY-5Y cells and chicken brain homogenates, to which NTE activities were compared after mipafox and aldicarb treatments, were 226.98 +/- 62.00 (mean +/- SEM) and 331.7 +/- 19.6 nmoles phenyl valerate hydrolyzed/30 min/mg protein, respectively. Mipafox alone inhibited NTE in differentiated SY-5Y cells by 22.5% +/- 3.5, 59.8% +/- 5.2, and 67.6% +/- 9.6 after exposure for 2, 5, and 10 minutes, respectively. Aging at these times was 0%, 26.6% +/- 8.0, and 60.0% +/- 9.2, respectively. Mipafox alone inhibited NTE in chicken brain homogenates by 41.7% +/- 1.6, 50.0% +/- 5.3, and 74.1% +/- 7.4 at 2, 5, and 10 minutes, respectively. Aging was 0%, 36.0% +/- 2.5, and 58.8% +/- 0.1 at those respective times. Aldicarb alone was capable of inhibiting NTE activity without aging the enzyme.

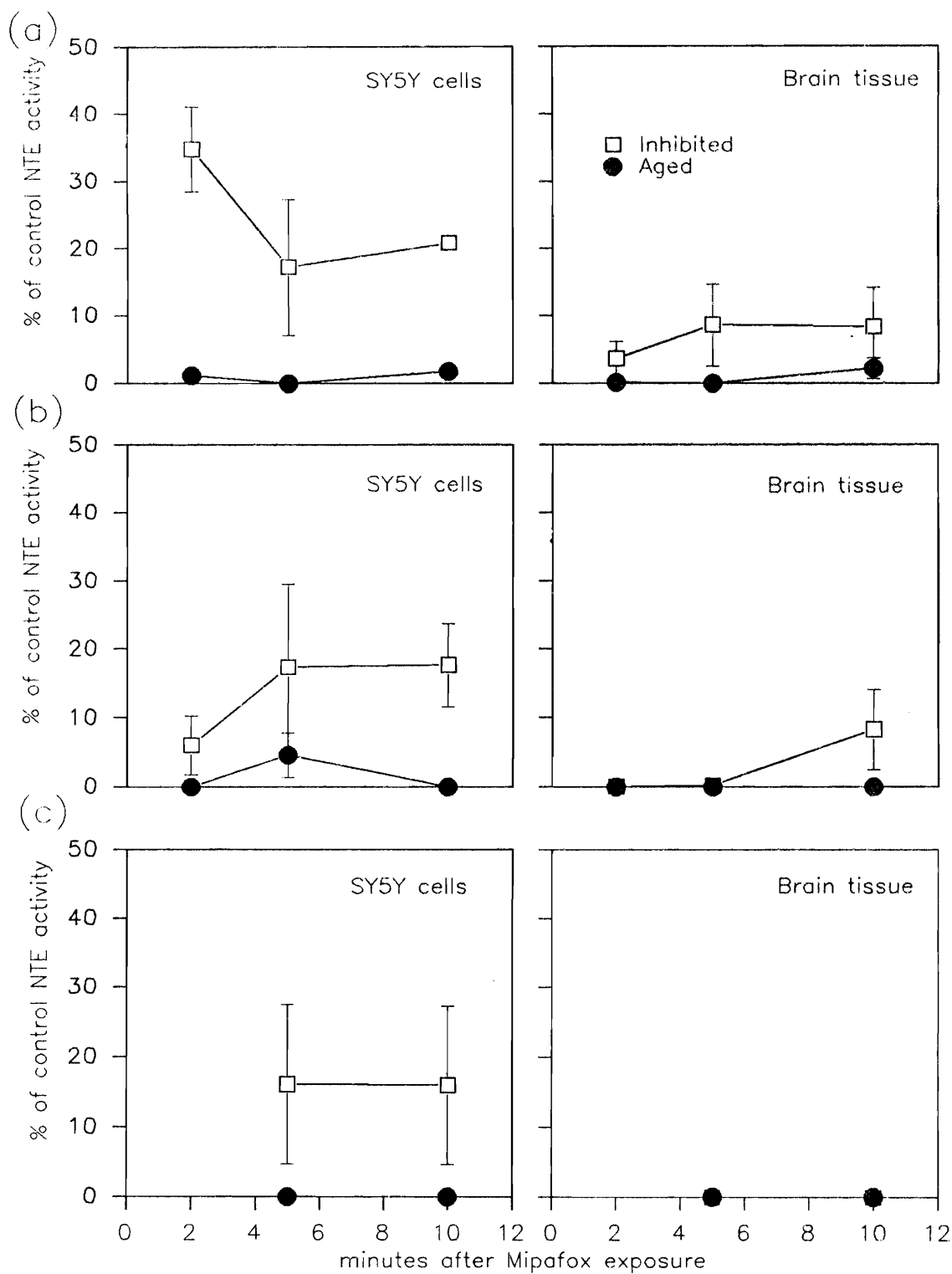
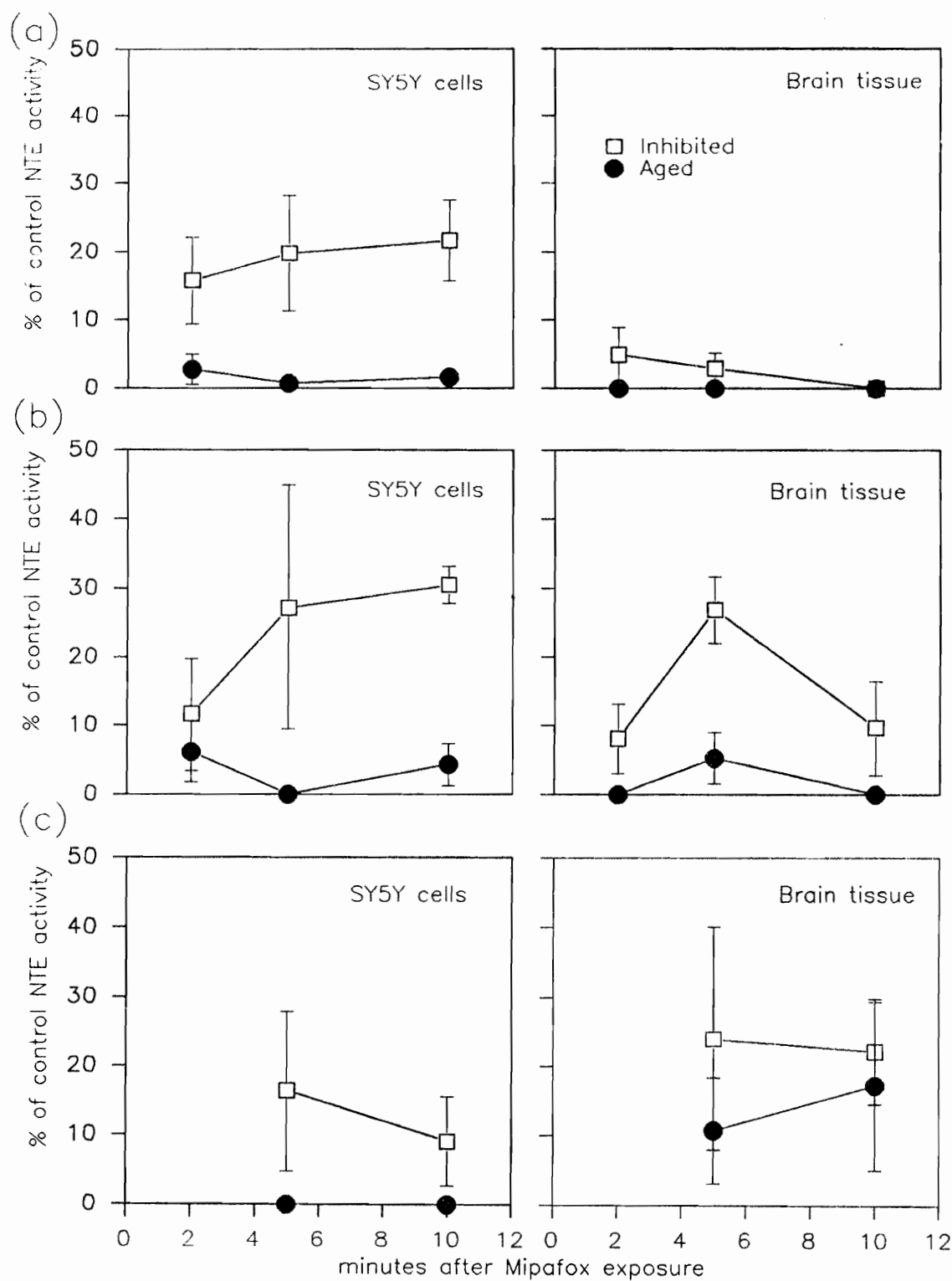


FIGURE 2.2. Percent of control NTE activity inhibited and aged in differentiated SY-5Y cells and chicken brain homogenate after mipafox treatment with (a) 5 minute pre-treatment, (b) simultaneous treatment, and (c) 2 minute post-treatment with verapamil. NTE activities in untreated SY-5Y cells and chicken brain homogenates, to which NTE activities were compared after mipafox and verapamil treatments, were 226.98 ± 62.00 (SEM) and 331.7 ± 19.6 nmoles phenyl valerate hydrolyzed/30 min/mg protein, respectively. The inhibition and aging of NTE resulting from exposure to mipafox alone is given in the legend to Figure 1.



CHAPTER III

CYTOTOXIC EFFECTS OF ORGANOPHOSPHORUS ESTERS AND OTHER NEUROTOXIC CHEMICALS ON CULTURED CELLS

Cytotoxic effects of organophosphorus esters and other neurotoxic chemicals on cultured cells

SUMMARY

The capability of a neuronal cell line to respond to 5 neurotoxic chemicals was assessed by using viability, morphology, acetylcholinesterase (AChE) activity, and free intracellular calcium ion concentration ($[Ca^{2+}]_i$) as cytotoxic endpoints. Differentiated SY-5Y cells were used as the test system for examination of these effects, as they were particularly useful for the study of early effects of a neuropathy-inducing organophosphate (OP), mipafox. Other compounds studied for their cytotoxic effects in SY-5Y cells were: paraoxon, another organophosphorus ester; aldicarb, a carbamate; β , β' -iminodipropionitrile (IDPN), a compound which induces a peripheral neuropathy, and carbachol, a cholinergic agonist. Results indicated that acetylcholinesterase inhibition was the most sensitive indicator of neurotoxicity of the OPs and the carbamate. Inhibition of AChE was observed within 10 minutes of toxicant exposure and at concentrations of these compounds that did not affect viability at 24 hours, and at concentrations which did not affect morphology or $[Ca^{2+}]_i$ until days later. For compounds that did not have as notable an effect on AChE (IDPN and carbachol), continuous exposures over several days were needed before cytotoxic effects were noted. These results indicate that toxic effects of esterase inhibitors may be more easily detected in the differentiated SY-5Y cell culture system than toxic effects of other chemicals.

INTRODUCTION

Previous work in this laboratory has resulted in the development of a model cell culture system and has established its use in the examination of early effects of neuropathy-inducing

organophosphate (OP) compounds on neural cells [1]. Validation of the model system for detection of early events occurring after exposure to these compounds was accomplished by comparing enzyme activities, specifically activity of neurotoxic esterase (NTE), in neuroblastoma cells with activity in homogenized brain tissue from adult chickens [1], the preferred test species for the investigation of organophosphorus-induced delayed neuropathy (OPIDN) [2]. The model was then further validated by demonstrating similar effects on NTE in neuroblastoma cells and hen brain homogenates after treatment with compounds known to modify the outcome of OPIDN *in vivo* [3]. Since the differentiated SY-5Y cell culture model behaved in a manner similar to brain tissue from normal adult chickens upon exposure to neuropathy-inducing OPs, this cell culture model could have the potential to be used to examine other parameters indicative of toxicity in neural cells, especially parameters that are not possible to observe in homogenized tissue. For example, live, intact cells are required in order to investigate effects of neurotoxicants on intracellular ion concentrations and conductance, metabolic function, viability, and morphology.

The purpose of the present study was to examine some nonspecific effects of neurotoxicants on intact cells (i.e. effects on viability and morphology) and to examine the effect of neuropathy-inducing OPs and other neurotoxic compounds on free intracellular calcium concentration ($[Ca^{2+}]_i$) in live, intact cells. Acetylcholinesterase (AChE) activities in neuroblastoma cell culture and chicken brain homogenate were also evaluated as another possible sensitive indicator of exposure to these neurotoxicants.

MATERIALS AND METHODS

The SY-5Y cell line, a neuroblastoma line of human origin, was used in the present study. SY-5Y cells were kept in Dulbecco's modified Eagle medium (DMEM) (45%), F12 (45%), and fetal calf serum (10%). Differentiation of cells to a mature neuronal form was accomplished by inclusion

of nerve growth factor (NGF) in the culture medium at a concentration of 1 µg NGF/ml medium for 5 to 7 days [4].

Differentiated SY-5Y cells were treated in situ in culture dishes for viability and morphology experiments. The treatment compounds were as follows: mipafox, an OP which produces OPIDN in vivo; paraoxon, an OP that is acutely toxic, but does not produce delayed neuropathy; aldicarb, a carbamate that is acutely toxic in vivo, but does not produce delayed neuropathy; β , β' -iminodipropionitrile (IDPN), an agent that produces a peripheral neuropathy involving neurofilament masses in the proximal segments of affected axons [5]; and carbachol, a cholinergic receptor agonist. Another carbamate, carbaryl, and another neuropathy-inducing OP, phenyl saligenin phosphate (PSP), were also included for viability experiments. Verapamil and atropine were used as non-neurotoxic controls for viability experiments. Solvent controls were also included for these investigations. Concentrations used in viability studies are shown in Table 3.1. Viability was assessed by trypan blue dye exclusion after 24 hours of continuous incubation with a range of concentrations of the compounds.

One optimal sublethal concentration of each compound was chosen for determining the duration of continuous toxicant exposure necessary for morphological changes, changes in AChE activity and changes in $[Ca^{2+}]_i$. The concentrations chosen were as follows: mipafox, 5×10^{-5} M; paraoxon, 5×10^{-5} M; aldicarb, 5×10^{-3} M; IDPN, 5×10^{-5} M; and carbachol, 5×10^{-5} M. SY-5Y cell morphology was assessed by observing cell shape and neurite appearance by phase-contrast microscopy while cells were continuously incubated with one of the toxicants for 2 weeks or until complete cell death, whichever came first. Phase-contrast microscopy of untreated control cells in NGF containing media alone was also performed. Media was changed twice weekly for all differentiated cells. Fresh media for treated cells contained toxicants at the indicated concentrations.

Free intracellular calcium ion concentration ($[Ca^{2+}]_i$) was determined by use of the cell permeant acetoxymethyl ester of the fluorescent probe, fluo-3, fluo-3 am (Molecular Probes).

Differentiated SY-5Y cells were seeded on 96 well microtiter plates and allowed 1 day to attach before treatment. Cells were treated with the concentrations of toxicants listed above and were assayed for $[Ca^{2+}]_i$ at 8, 24, 48, and 72 hours. The addition of atropine was made to a second set of the above treatments in order to control for $[Ca^{2+}]_i$ increase due to cholinergic receptor activation. Additionally, mipafox treatments were included, with and without atropine, with each of the following co-treatments: 5 minute pretreatment with aldicarb, simultaneous treatment with aldicarb, 2 minute post-treatment with aldicarb, 5 minute pretreatment with verapamil, simultaneous treatment with verapamil, and a 2 minute post-treatment with verapamil. These cells were assayed for $[Ca^{2+}]_i$ at 8, 48, and 72 hours. Treatment with aldicarb or verapamil in conjunction with mipafox treatment has been demonstrated previously to ameliorate the early effects of the neuropathy-inducing OP on NTE [3]. Treated cells were loaded with fluo-3 am at a final concentration of 25 μ M in fresh media, incubated at 37°C for 1 hour, washed 3 times in Hank's balanced saline solution (HBSS), and incubated for an additional 30 minutes at 37°C to allow for hydrolysis of the acetoxymethyl ester. Plates were read on a Cytofluor 2300 spectrofluorometric microtiter plate reader (Millipore) at an excitation wavelength of 485 +/- 20 (505) nm, an emission wavelength of 530 +/- 25 (526) nm, and a sensitivity setting of 6. Since calculation of exact $[Ca^{2+}]_i$ requires accounting for cell number when using an amplitude probe such as fluo-3, assay results were expressed as percent fluorescence of untreated control cells loaded with fluo-3 read at the same time point.

For examination of acetylcholinesterase (AChE) activity after treatment with nonlethal concentrations of neurotoxicants, differentiated SY-5Y cells were suspended in HBSS at a concentration of 10^7 cells per milliliter. Cell suspensions were treated alongside chicken brain homogenate for comparison of effects. Each tissue was treated with the chosen concentration of each toxicant for 10 minutes, then assayed for AChE activity by the method of Ellman, *et al* [6], modified for microassay [7]. Each toxicant treatment in differentiated SY-5Y cells and chicken brain

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homogenate for AChE assay included 5 or 6 replicates. Results for all treatments in differentiated SY-5Y cells were compared to those in chicken brain homogenate treated and incubated with equivalent toxicant concentrations for the same time intervals. The AChE experiment was performed as a completely randomized design and was analyzed by the appropriate analysis of variance (ANOVA) for that design. Means for AChE activity in SY-5Y cells and brain tissue were compared by Duncan's multiple range test.

RESULTS

Viability of differentiated SY-5Y cells after 24 hour incubation with neurotoxicants used as test compounds and other compounds and solvents is shown in Table 3.1. Non-neurotoxicants (verapamil and atropine) did not affect viability of differentiated SY-5Y cells. With the exception of mipafox, concentrations of neurotoxicants as high as 10^{-4} to 10^{-3} M did not appreciably affect cell viability if they were soluble in aqueous solution. For example, even concentrations of aldicarb and IDPN as high as 10^{-3} M had minimal effects on the viability of exposed cells at 24 hours. Continuous exposure for 24 hours to test compound concentrations chosen for subsequent experiments (5×10^{-5} M for mipafox, paraoxon, IDPN, and carbachol; 5×10^{-3} M for aldicarb) resulted in viabilities over 80%, with the exception of mipafox, where viability of cells treated with 5×10^{-5} M mipafox was greater than 70%. Viability of SY-5Y cells treated with carbaryl or PSP was affected by those compounds and the acetone in which they were dissolved.

Cells in culture exposed to neurotoxicants were evaluated for the development of morphological changes by phase-contrast microscopy. This procedure permits investigation of the time course of cytopathic effects as well as provides indication of changes that may precede loss of viability. Although not evident as loss of viability within 24 hours, cytotoxic effects were demonstrated in cells treated with paraoxon, aldicarb, or carbachol for 3 to 7 days when examined by phase-contrast microscopy. Cells exposed to these neurotoxicants demonstrated morphological

changes indicative of acute toxicity after 3 days of continuous exposure to concentrations of these compounds that were nonlethal after 24 hours of continuous exposure. Normal differentiated cells are stellate in appearance, with long neurites that extend and attach to the surface of the culture plate or interact with other cells. Cells exposed to paraoxon, aldicarb, or carbachol for 3 days became rounded and eventually detached (Figure 1). Cells treated with mipafox or IDPN showed different morphological effects of toxicant exposure than did those treated with paraoxon, aldicarb, or carbachol. Mipafox-treated cells began to exhibit swellings, or blebs, on the neurites by day 3 post-treatment. By day 10, neurites were attenuated, and some no longer exhibited a normal growth cone. On day 14, a larger proportion of cells were rounded and detached, some cells had refractile areas, and most neurites ended abruptly, rather than ending in a widened, attached growth cone. Cells treated with IDPN appeared similar to untreated cells 3 days after initiation of exposure, and they appeared less affected than those treated with mipafox on day 7. However, on day 10, IDPN-treated cells were exhibiting lesions similar to those exhibited by mipafox-treated cells. By day 14, some of the neurites of IDPN-treated cells were beginning to fragment. Untreated control cells maintained normal differentiated appearance over 14 days.

The effect of toxicant treatment of SY-5Y cells on $[Ca^{2+}]_i$ was investigated in order to determine if this model neuronal cell culture system could be used for evaluation of a biochemical change known to occur when membranes of live, intact cells are perturbed. Results of experiments to examine $[Ca^{2+}]_i$ in cells treated with neurotoxicants were inconclusive. It appeared, however, that change in $[Ca^{2+}]_i$ did not occur within hours of exposure of SY-5Y cells to neurotoxicants. The observed values for $[Ca^{2+}]_i$ in treated cells and a discussion of them are included in the appendix at the end of this chapter.

Three of the neurotoxicants used for this study are classified as cholinesterase inhibitors (mipafox, paraoxon, and aldicarb). It is therefore possible that acetylcholinesterase activity in SY-5Y cells could be a sensitive indicator of neurotoxicity. Acetylcholinesterase activities for differentiated

SY-5Y cells and chicken brain homogenate are presented in Table 3.2. Activities in differentiated SY-5Y cells, expressed as activity per mg protein, were significantly lower than those in chicken brain homogenates. All treatments significantly inhibited AChE activity in SY-5Y cells ($\alpha = 0.05$), but inhibition was most notable after treatment with mipafox, paraoxon, and aldicarb ($\alpha = 0.01$). SY-5Y AChE was more sensitive than chicken brain AChE to inhibition after exposure to equivalent concentrations of mipafox and slightly less sensitive than chicken brain AChE to inhibition after exposure to equivalent concentrations of paraoxon and aldicarb.

DISCUSSION

Cell culture systems have previously been used to examine viability, morphology, $[Ca^{2+}]_i$, and AChE [8-14], but no single system has examined all of these parameters in neuronal cells after treatment with neurotoxins such as the neuropathy-inducing OPs (e.g. mipafox). That issue was addressed in the present study, with the following findings.

Toxicant effects on cell viability at 24 hours were not specific and could not be used to differentiate neurotoxins from compounds that were not neurotoxic. Cell viability in this study seemed most sensitive to solvents (acetone and DMSO) in which toxicants not soluble in water might be dissolved. Therefore, if viability of neuronal cells in culture were used as an indicator of neurotoxicity, the solvent used would need to be evaluated as well, since it could interfere with test results. For this reason, test compounds for subsequent experiments were chosen that were soluble in aqueous buffer at the concentrations used for testing. Of the compounds tested in aqueous solution, only mipafox treatment resulted in more than 20% lethality after 24 hours of continuous exposure to concentrations in the micromolar range. From these studies, it appears that viability is not a sensitive indicator of the effects of neurotoxins in the SY-5Y cell culture model.

In contrast to the relatively indefinite results obtained when viability was used to indicate that neurotoxins were affecting cells in culture, phase-contrast microscopy done over a time span

of days provided better indication of cellular toxicity. Pathological changes occurred in SY-5Y cells after continuous toxicant treatment with the 2 compounds that produce delayed axonopathies, mipafox and IDPN. These changes included formation of blebs and abnormal growth cones. Pathological changes observed under phase-contrast microscopy were similar in both mipafox- and IDPN-treated cells, but they occurred slightly earlier in mipafox-treated cells. Morphological findings reported here for mipafox-treated cells were similar to those reported previously when neuroblastoma 2-A cells were treated with 2 other neuropathy-inducing OPs, tri-ortho-tolyl phosphate (TOTP) and di-isopropyl fluorophosphate (DFP) [15]. In that previous study, the neurites of the cultured cells examined by phase-contrast microscopy exhibited a shrunken, rough appearance with irregular swellings along their length after 24 hours incubation with 8×10^{-6} to 1.5×10^{-4} M TOTP or 2×10^{-4} to 5×10^{-3} M DFP [15]. The similarity of *in vitro* lesions induced by mipafox and IDPN is of interest, since these compounds cause different morphological changes in myelinated fibers of the central and peripheral nervous systems in animals exposed to them. Further studies using ultrastructural techniques are needed before differences in morphological changes of neuroblastoma cells after exposure to mipafox and IDPN can be identified. There is the possibility that cells in culture may respond differently to neurotoxicants that work by different mechanisms. To date, such studies have not appeared in the literature.

Phase-contrast microscopy performed in this study could differentiate between the neurotoxicants. The morphological changes in differentiated neuroblastoma cells exposed to mipafox and IDPN observed using phase-contrast microscopy in this study were different from the changes observed with the neurotoxicants that do not cause neuropathy (paraoxon, aldicarb, carbachol). Paraoxon, aldicarb, and carbachol all cause increases in responses mediated by the neurotransmitter, acetylcholine [16,17]. Cells exposed to these neurotoxicants did not develop blebs, but instead became round and detached between 3 to 7 days of continuous exposure, an indication of detrimental effects on viability [18], presumably as a result of acute, rather than delayed, toxicity.

Mipafox- and IDPN-treated cells were still viable 14 days after initiation of toxicant exposure. The fact that these cells do retain viability would provide opportunity for future studies to examine reversible and irreversible events that occur in this cell culture system after exposure to neuropathy-inducing toxicants.

The potential to measure free intracellular calcium ion concentration ($[Ca^{2+}]_i$) in intact neural-derived cells was of particular interest after exposure of these cells to neuropathy-inducing OPs. Changes in intracellular free calcium concentration, which can only be accurately measured in intact cells, and in calcium regulated enzyme activity have been implicated in the development of delayed neuropathy. This implication comes from studies in which treatment with calcium channel blocking drugs prior to exposure to neuropathy-inducing OPs was shown to significantly attenuate the development of clinical signs and pathological lesions of OPIDN [19,20]. Hens treated with either verapamil or nifedipine and the neuropathy-inducing OP, phenyl saligenin phosphate (PSP), were significantly less ataxic than hens treated with PSP alone. Histological lesions were less severe, and electrophysiological assessment of neuromuscular function supported the protective role of the calcium channel blocking drugs in these experimental animals [19,20]. In hens treated with neuropathy-inducing OPs, the activity of calcium activated neutral protease (CANP), or calpain, in brain and peripheral nerve was observed to increase within 4 days after administration of these compounds. This and a rise in total calcium concentration in sciatic nerve was prevented for a time by treatment with calcium channel blockers, if begun 1 day prior to OP administration [19].

The implication that changes in $[Ca^{2+}]_i$ concentrations could contribute to the development of OPIDN also comes from studies in which tri-ortho-cresyl phosphate (TOCP) administration to hens and TOCP treatment of chicken synaptosomes *in vitro* produced an increase in calcium dependent protein phosphorylation, specifically involving the cytoskeletal components α - and β -tubulin, microtubule-associated protein (MAP-2), and the neurofilament triplet proteins, and cytoskeletal protein degeneration [22-24]. These effects correlated well with the development of

OPIDN in vivo. X-ray microprobe analysis of OPIDN affected axons in TOCP treated chickens revealed slightly elevated calcium concentration in the axoplasm and substantially increased calcium concentration in mitochondria [25]. X-ray microprobe analysis, however measures total calcium concentration and does not differentiate between free and bound calcium [26].

Increases in $[Ca^{2+}]_i$ have been reported in neuronal cell culture as a result of cyanide toxicity and anoxia [11,14,27], and were expected to be seen after exposure to neuropathy-inducing OPs if the cell culture system used here could mimic events noted after hens were exposed to these compounds. These increases did not, however, occur in this study due to inherent problems with the system used for calcium measurements, namely differences in cell number, esterase inhibition as a result of experimental treatments, and subcellular distribution of $[Ca^{2+}]_i$. Those problems are discussed in detail in the appendix at the end of this chapter, as are potential solutions to them. Changes in calcium ion concentration and/or distribution do have a great deal of potential as neurotoxic endpoints in cell culture. Additional work is warranted in systems that can eliminate the problems encountered here.

In this study, the effects of neurotoxicants on AChE activity were also determined because 3 of the neurotoxicants tested are classified as cholinesterase inhibitors (mipafos, paraoxon and aldicarb). Inhibition of acetylcholinesterase was found in the present study to be a sensitive indicator of exposure to these neurotoxicants. Cultured neuronal cells have also been used previously to examine OP effects on AChE [9-10]. In one study, AChE activity in mouse CNS reaggregate cultures was found to be nearly completely inhibited by the neuropathy-inducing OP diisopropyl fluorophosphate (DFP). Activity of AChE recovered to 31% of control activity by 24 hours, and was completely recovered by 7 days as a result of synthesis of new enzyme [9]. In another study, mouse embryo neuron cultures were used to compare the relative toxicities of OP inhibitors of AChE and their phosphorylated oximes [10]. Inhibition of AChE in these primary cell cultures by these compounds correlated with that seen in vivo. Although continuous cell culture

systems have previously been reported to have cholinergic neurotransmitter systems [28], previous studies have not used them to examine the effects of OPs and carbamates on AChE activity. The present study indicates that cholinesterase activity in this cell culture system could be a sensitive indicator of the capability of neurotoxicants to inhibit AChE. Previous studies in our laboratory also provided evidence that differentiated SY-5Y cells were also sensitive indicators of OPs and carbamates that inhibit neurotoxic esterase [1,3].

The effects of AChE inhibition on cells in culture reflect a biochemical neurotoxicity. Morphological effects of AChE inhibition and ACh accumulation primarily involve muscle tissue, even though the change in concentrations of ACh are a result of effects of toxicants on innervating axons. However, changes in the appearance of synaptic vesicles of affected neurons of rats killed 5 days after a single exposure to fenitrothion have been reported [29]. Another study, in which rats were killed as long as 15 days after exposure, demonstrated flattening and thickening of the junctional fold at the motor endplate and vacuolations of Schwann cells [29]. Neuronal necrosis and gliosis have been described in the hippocampus of rats treated with parathion [30] or fenitrothion [31]. If a neuronal cell culture system were to be used to examine morphological changes that occur as a result of AChE inhibition and ACh accumulation, an approach using neural cells co-cultured with muscle cells may be suggested.

Effects of the compounds tested here on AChE in SY-5Y cells correlated with those seen in chicken brain homogenates. Although SY-5Y cells had remarkably less AChE activity than brain tissue, inhibitory effects on a percentage basis were similar. This enzyme was a particularly sensitive indicator of treatment with esterase-inhibiting OPs.

The SY-5Y cell culture system has a great deal of potential for the examination of neurotoxic compounds, especially using morphological changes over time or AChE inhibition as indicators of neurotoxicity. It appears to be particularly sensitive to esterase inhibitors. Effects on AChE shown in this study and on NTE in previous studies [1,3] demonstrate the usefulness of the

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system when inhibition of enzyme activities were used as determinants of toxic effects. Initial studies indicate that other compounds are less likely to demonstrate changes in cellular esterase activity; other endpoints need to be explored as indicators of the toxicity of these compounds.

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TABLE 3.1. Viability of differentiated SY-5Y cells after 24 hours exposure to neurotoxicants and solvents.

Compound	Concentration in culture media	% viable cells
Neurotoxicants:		
mipafox	5×10^{-4} M	67
	5×10^{-5} M	71
	5×10^{-6} M	74
paraoxon	5×10^{-4} M	89
	5×10^{-5} M	94
aldicarb	10^{-3} M	87
	10^{-4} M	92
IDPN	10^{-3} M	92
	10^{-4} M	96
	10^{-6} M	97
carbachol	10^{-4} M	81
	10^{-5} M	88
carbaryl	10^{-4} M	2
	10^{-5} M	19
PSP	10^{-4} M	2
	10^{-5} M	10
Non-neurotoxicants:		
atropine	10^{-5} M	93
verapamil	5×10^{-6} M	95
	5×10^{-7} M	99
Solvents:		
acetone	10%	35
DMSO	10%	75

Compounds were dissolved in distilled water (except for carbaryl and PSP, which were dissolved in acetone) and diluted 10-fold in cell culture media to give the listed final concentrations.

TABLE 3.2. Acetylcholinesterase activity in differentiated SY-5Y cells and brain after treatment with neurotoxicants.¹

Tissue	Treatment	AChE activity	% of control
SY-5Y	control	0.498 +/- 0.009	
	mipaflox	0.329 +/- 0.008 ^{2,3}	66.1
	paraoxon	0.098 +/- 0.001 ^{2,3}	19.6
	aldicarb	0.125 +/- 0.005 ^{2,3}	25.1
	IDPN	0.473 +/- 0.009 ²	94.9
	carbachol	0.445 +/- 0.003 ^{2,3}	89.3
Brain	control	187.3 +/- 2.62	
	mipaflox	167.3 +/- 3.66 ^{2,3}	89.3
	paraoxon	0 ^{2,3}	0
	aldicarb	0 ^{2,3}	0
	IDPN	196.9 +/- 2.68 ²	105.1
	carbachol	163.5 +/- 2.32 ^{2,3}	87.3

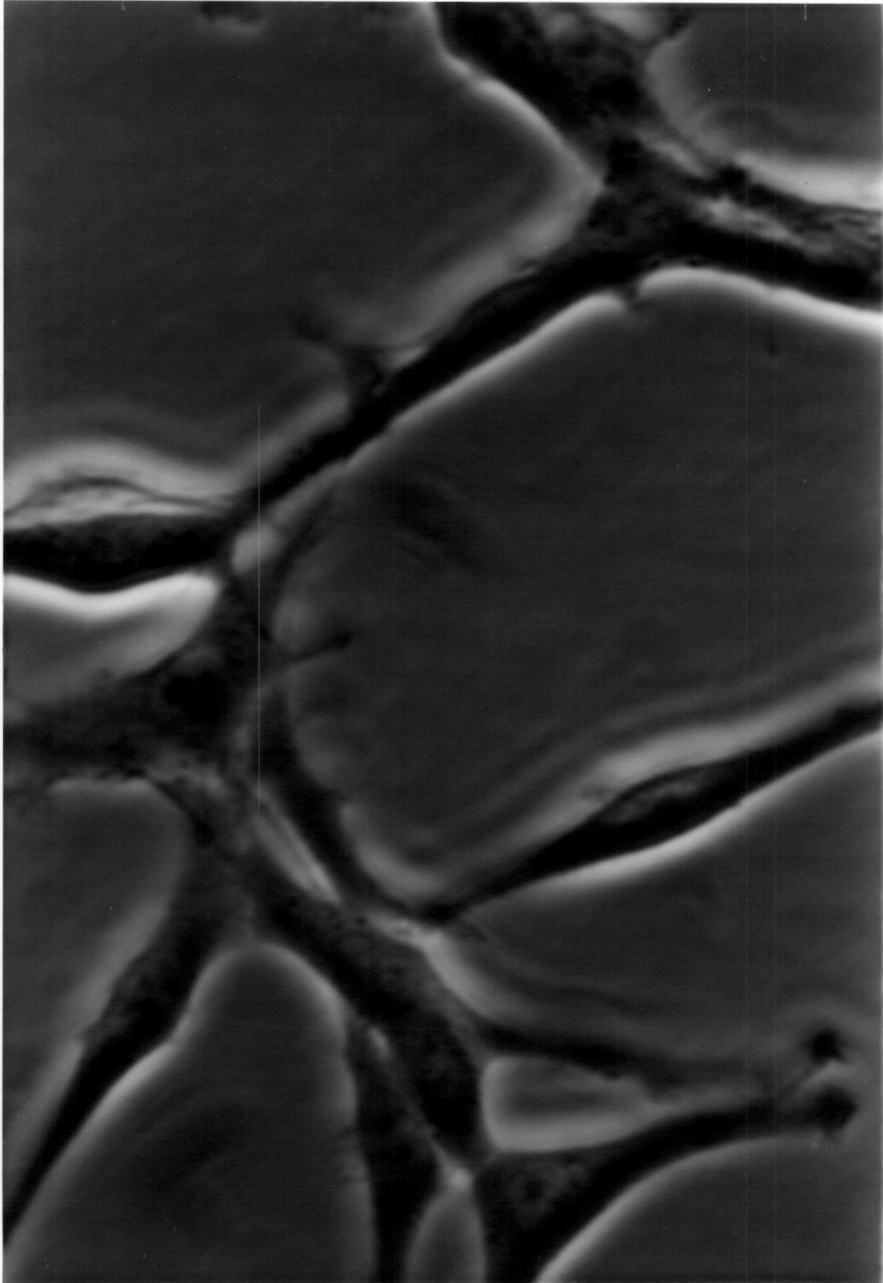
¹ activity, expressed as nmol acetylthiocholine hydrolyzed/min/mg protein +/- SEM, at 10 minutes after exposure to 5×10^{-5} M mipaflox, paraoxon, IDPN, and carbachol, and 10 minutes after exposure to 5×10^{-3} M aldicarb.

² Significantly different from control, $\alpha = 0.05$. Activities in SY-5Y cells were significantly different from those in brain ($\alpha = 0.05$).

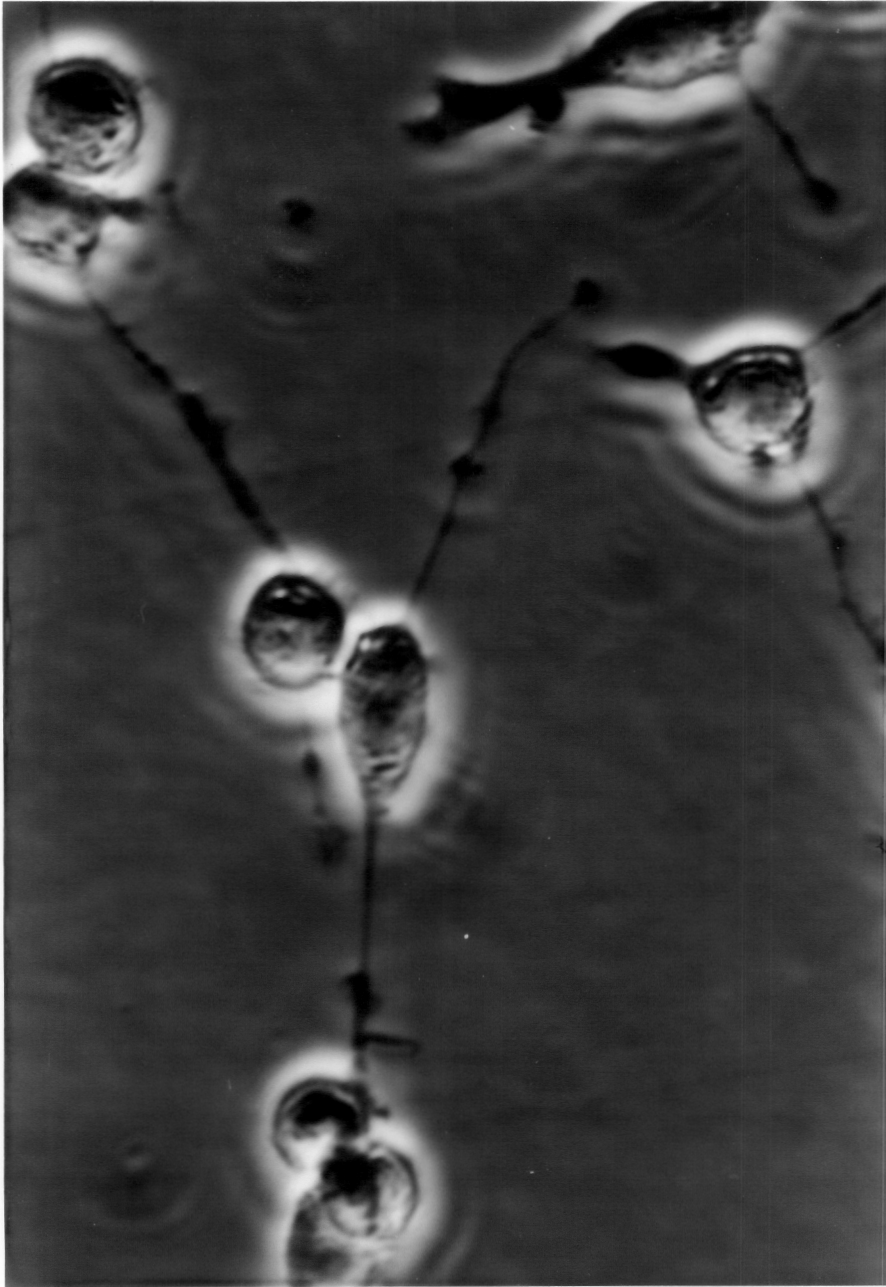
³ Significantly different from control, $\alpha = 0.01$.

FIGURE 3.1. Phase-contrast photomicrographs of control and toxicant-treated differentiated SY-5Y cells. (a) untreated control SY-5Y cells, exhibiting characteristics of differentiation. Note the long neurites and widened ends of the growth cones. (b) differentiated SY-5Y cells after 3 days of continuous treatment with aldicarb. This appearance was also typical of cells treated for the same duration with paraoxon or with carbachol. (c) differentiated SY-5Y cells after 10 days of continuous treatment with mipafox. (d) differentiated SY-5Y cells after 14 days of continuous treatment with IDPN. Note the attenuated appearance of neurites and the lack of a normal growth cone in (c) and (d).

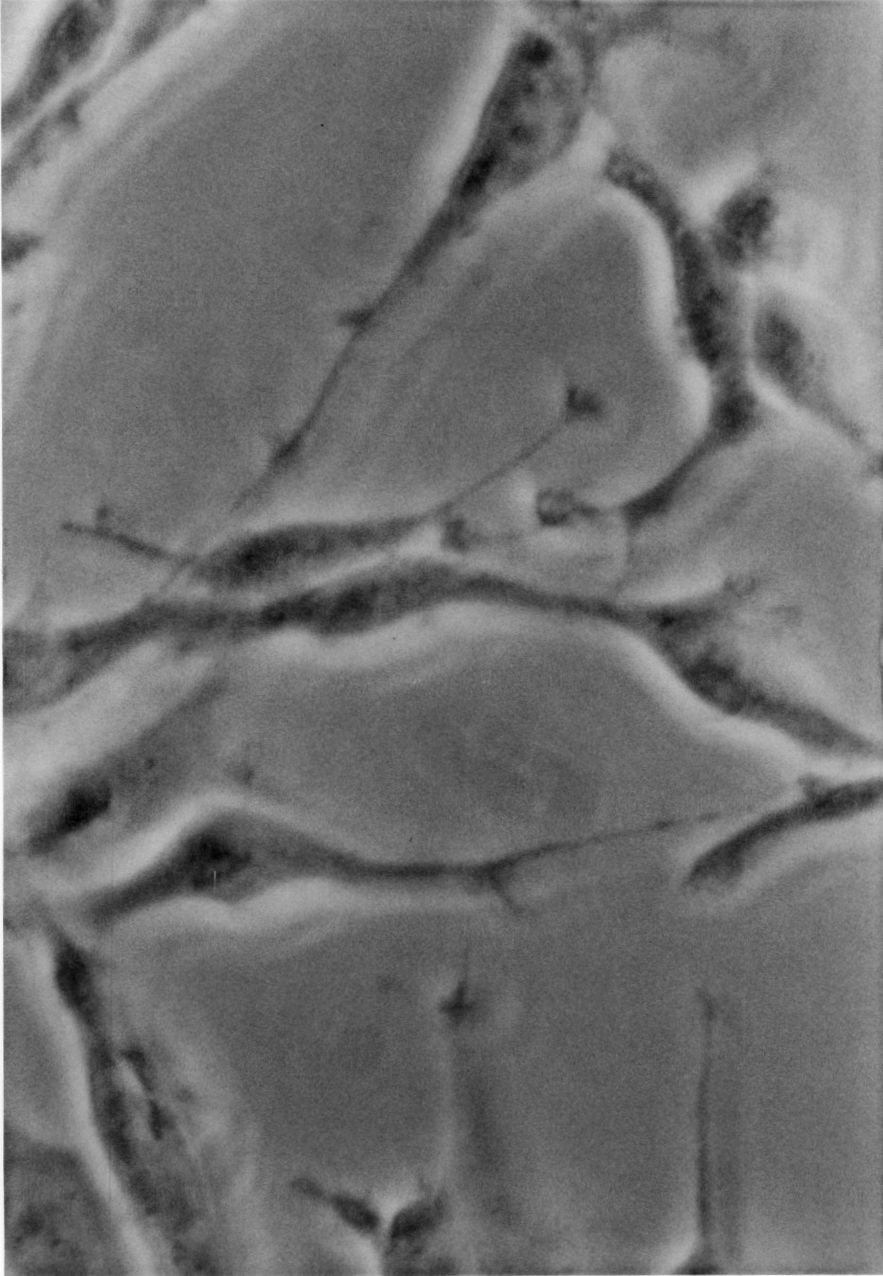
(a)



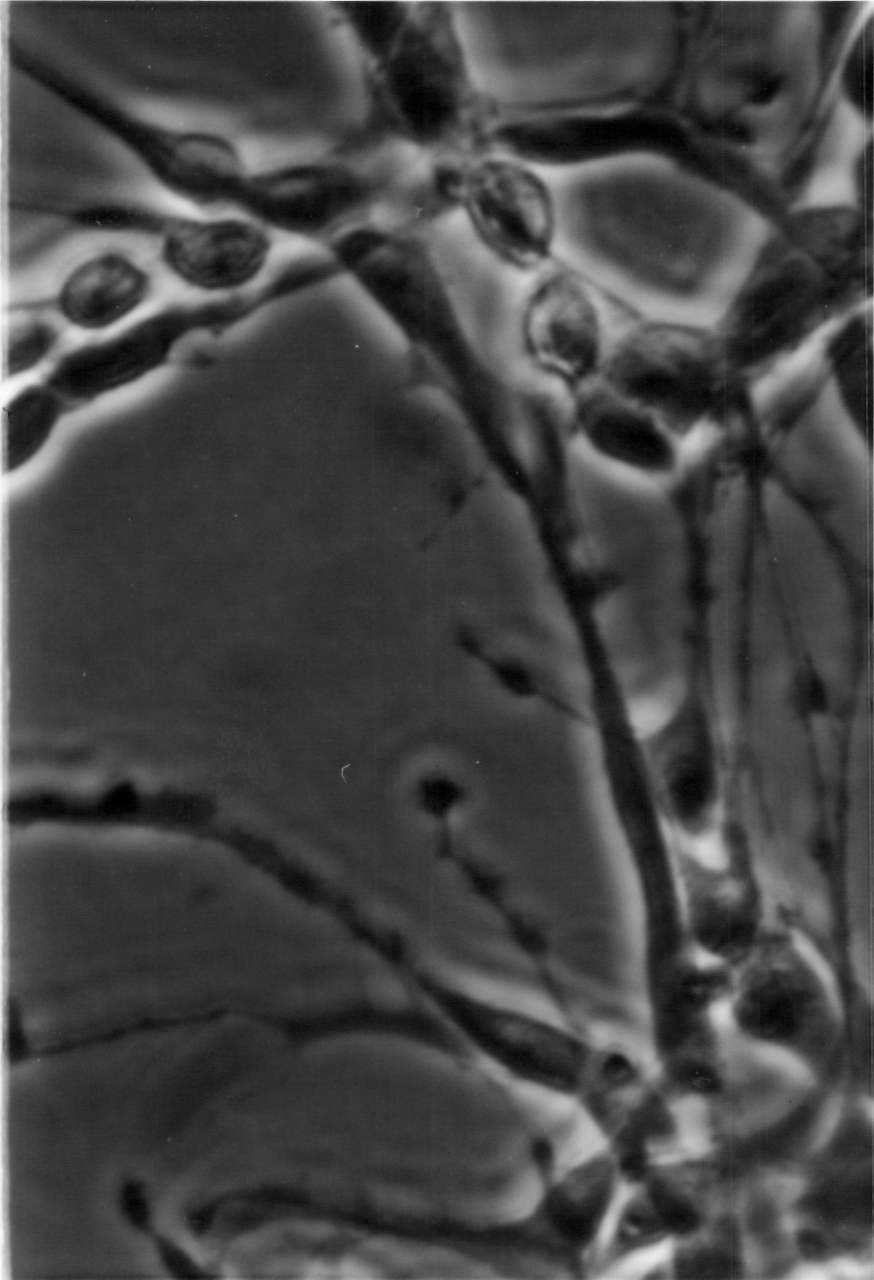
(b)



(c)



(d)



APPENDIX

Effects of Neurotoxicants on Intracellular Free Calcium Ion Concentrations

Free intracellular calcium ion concentration ($[Ca^{2+}]_i$) in cells treated with mipafox alone or pretreated with verapamil before exposure to mipafox did not change appreciably over the 3 day period in which this parameter was measured. However, 3 days after initiation of exposure to paraoxon, aldicarb, IDPN, or carbachol, $[Ca^{2+}]_i$ was less than 70% of values in untreated cells. Exposure to atropine for 3 days also caused decreases in $[Ca^{2+}]_i$. No increases in $[Ca^{2+}]_i$ that were more than 1.5 times that of control were seen at any time point with any of the treatments to which cells were exposed (Table 3.3).

The results of the calcium studies reported here did not indicate changes of large magnitude in free calcium ion within the cell when exposed to mipafox, paraoxon, aldicarb, IDPN or carbachol. Decreases in $[Ca^{2+}]_i$ were noted at 72 hours after initiation of exposure to the latter 4 compounds. Decreases in $[Ca^{2+}]_i$, especially 3 days after exposure to these toxicants, should be viewed with caution since they do, at least in part, reflect differences in cell number between control and treated cells at the same time point. By 3 days, cells exposed to paraoxon, aldicarb and carbachol were rounded and detaching in morphological studies. This was interpreted as a degenerative change associated with loss of the capability to divide and of viability. Subjective assessment of treated wells indicated lower numbers of cells as compared to control wells. This would be reflected as decreases in $[Ca^{2+}]_i$, even though there may be an absence of any change on a per cell basis. To compensate for such changes, one could use a fluorescent probe with which $[Ca^{2+}]_i$ is calculated from the ratio of bound probe fluorescence to unbound probe fluorescence. This determination is independent of cell number, but was not possible under the conditions of this experiment.

Additional problems encountered in this experiment include those of esterase inhibition, and differences in subcellular distribution of calcium ion. It is to be noted that the use of fluorescent

probes requires the use of cell permeable esters of the probes for loading of cultured cells. These esters are hydrolyzed within the cell before being bound to intracellular calcium ions.¹ In this study, SY-5Y cells were exposed to esterase inhibitors before being loaded with the acetoxymethyl ester of fluo-3. The inhibition of nonspecific esterases, included as part of the assay for neurotoxic esterase (NTE),² was 49%, 29% and 21%, respectively, when 5×10^{-5} M mipafox, 5×10^{-5} M paraoxon, and 5×10^{-3} M aldicarb were added to the cells. The potential for inhibition of hydrolysis of esters of fluorescent probes loaded into cells exposed to OPs and carbamates deserves further investigation before ester-containing probes are used for routine analysis of detrimental effects of these compounds on cells in culture. Readings of fluorescence of these probes on a whole cell basis does not reflect changes in distribution of calcium ion within the cell. Calcium ion may be released from intracellular stores and made available for processes associated cytoskeletal degeneration or other effects without resulting in a change in ion concentration in the cell as a whole. Digital imaging of fluorescence in individual cells may be more informative in this regard.

¹ Haugland R.P. (1989) Handbook of fluorescent probes and research chemicals. Molecular Probes, Inc., Eugene, OR.

² Sprague G.L., Sandvik L.L., and Brookins-Hendricks M.J. (1981) Neurotoxicity of two organophosphorus ester flame retardants in hens. J. Toxicol. Environ. Health, 8, 507-518.

TABLE 3.3. Free intracellular calcium ion concentration in neurotoxicant and control compound treated SY-5Y cells.

Treatment	<u>% of control fluorescence¹</u>			
	hours after exposure:			
	8	24	48	72
mipafox	90+/-11	89+/-14	105 +/- 20	83+/-21
paraoxon	84+/-2	63+/-17	76 +/- 5	52+/-6
aldicarb	80+/-6	69+/-15	74 +/- 12	41+/-6
IDPN	79+/-7	118+/-17	83 +/- 10	63+/-6
carbachol	86+/-7	86+/-12	96 +/- 8	67+/-16
verapamil	81+/-7	121+/-11	92 +/- 15	82+/-22
atropine	106+/-15	117+/-17	99 +/- 8	43+/-8
mipafox + atropine	98+/-22	131+/-45	100 +/- 6	33+/-4
paraoxon + atropine	79+/-11	50+/-28	71 +/- 6	45+/-8
aldicarb + atropine	74+/-4	76+/-24	57 +/- 8	24+/-11
IDPN + atropine	80+/-16	86+/-9	106 +/- 1	39+/-10
carbachol + atropine	79+/-13	139+/-31	107 +/- 10	29+/-3

TABLE 3.3 (continued). Free intracellular calcium ion concentration in neurotoxicant and control compound treated SY-5Y cells.

Treatment	<u>% of control fluorescence¹</u>			
	hours after exposure:			
	8	24	48	72
mipaflox + aldicarb:				
5 min pre-treatment	93+/-31	--	60+/-17	57+/-13
simultaneous	97+/-22	--	58+/-15	50+/-8
2 min post-treatment	101+/-27	--	70+/-10	45+/-5
atropine + mipaflox + aldicarb:				
5 min pre-treatment	102+/-16	--	54+/-11	49+/-8
simultaneous	107+/-17	--	66+/-12	5+/-2
2 min post-treatment	95+/-30	--	63+/-6	10+/-3
mipaflox + verapamil:				
5 min pre-treatment	76+/-4	--	92+/-21	121+/-8
simultaneous	73+/-6	--	104+/-14	110+/-9
2 min post-treatment	93+/-8	--	97+/-19	111+/-6
atropine + mipaflox + verapamil:				
5 min pre-treatment	83+/-19	--	77+/-7	106+/-5
simultaneous	84+/-11	--	116+/-17	30+/-4
2 min post-treatment	75+/-12	--	125+/-24	37+/-3

¹ comparison of 4 samples (+/- SD) made to fluorescence in untreated cells loaded with fluo-3, background fluorescence subtracted. Standard deviation of 4 samples of control cells was +/- 14%.

PART IV

DISCUSSION

Discussion

The series of experiments reported in this dissertation provide evidence of the usefulness of the differentiated SY-5Y cell line in the study of cellular effects of neuropathy-inducing organophosphates and other neurotoxicants. The work done here established and validated the model for those parameters investigated. These studies demonstrated firstly that differentiated SY-5Y cells have neurotoxic esterase that is similar to that of chicken brain, and that it, like NTE in chicken brain, is largely inhibited and aged within 10 minutes of exposure to a neuropathy-inducing OP. Secondly, the utility of the model system was demonstrated in the examination of the effects of compounds known to be modifiers of OPIDN in vivo. Finally, it was demonstrated that other reasonable endpoints indicative of neurotoxicity could be explored in this system that may not be possible in homogenates of nervous tissue from experimental animals. In general, the model has been demonstrated to be of potential value in the study of mechanistic aspects of neurotoxicity, as well as in the development of screening systems for the evaluation of potentially neurotoxic compounds.

The SY-5Y line is of human origin, making it an optimal choice for a model for study of this disorder, since man is one of the most susceptible species to OPIDN [1,2]. Additionally, the cell line is neural in origin, and OPIDN follows initiating events in neural cells in vivo. Results of the present study demonstrate that the differentiated SY-5Y model system behaves in a manner not unlike that of brain tissue from the normal chicken, which is the generally accepted animal model for OPIDN [3], with respect to esterase activities and inhibition.

The use of cell culture model systems in toxicological experimentation has numerous advantages. Such a system allows for rapid, early manipulation of tissue after exposure to toxicant compounds. Since differentiated SY-5Y cells and chicken brain homogenate responded similarly in the experiments reported, the SY-5Y model may be representative of events occurring at the level of the target tissue in vivo at tissue concentrations approximating those used in the experiments.

The use of a cell culture model, such as the SY-5Y neuroblastoma cell line, also has the notable advantage of allowing examination of live, intact cells. This will permit studies on reversible and irreversible events that occur within neural cells when they are exposed to neurotoxicants such as the neuropathy-inducing OPs. Such studies cannot be performed using tissue homogenates. A model such as the neuroblastoma cells reported here may be validated by comparison with in vivo data. Enzyme activities or other parameters that can be examined in tissue from in vivo experiments may be used for these comparisons. The model may then, subsequently, be used to explore mechanisms of toxicity and other parameters that require intact cells, such as viability, morphological changes, and changes in ion concentrations and conductances.

This model has the potential for use as part of a screening battery in the examination of chemicals for their potential to induce OPIDN and potentially in screening for neurotoxicants in general. In vitro screening systems offer numerous advantages over whole animal testing systems, including reduced overall testing costs, isolation of tissues of interest, use of tissues of human origin, and detailed control over cell environment and experimental conditions [4]. Additionally, the system may prove useful for mechanistic studies of the development of OPIDN. Many questions are yet unanswered that may be addressed using differentiated SY-5Y cells. More detailed examination of the temporal relationship of known OP effects, enzyme kinetics, competitive and noncompetitive reactions using neuropathy-inducing OPs and protective compounds are possible.

DISCUSSION

Modifications of the system may yield important information. Co-cultures with muscle cells or glial cells may be used to examine interaction of different tissue types as a consequence of OP intoxication. Culture conditions could be manipulated to better mimic conditions in animals. Once toxicokinetic information is available for the neuropathy-inducing OP being tested, concentration of the toxicant in culture media could be adjusted to simulate the changes in plasma toxicant concentration over time. Such information is available for some neuropathy-inducing OPs [5]. Addition of metabolic capability, such as by including S9 fractions, may aid in this regard.

The differentiated SY-5Y model used in this study exhibited little significant difference in NTE activity and its susceptibility to inhibition and aging by the compounds used here when compared to brain tissue from the chicken. However, variability of the NTE activity of the SY-5Y cells was higher than that of brain tissue and most likely was responsible for the statistically significant differences in inhibited and aged NTE activities seen between these two tissues. That variability may be a consequence of retention of some undifferentiated characteristics by the differentiated cells, such as the retained ability to divide. Alternative methods of differentiation could be explored, such as the removal of serum from the media, in addition to NGF treatment, or treatment with other compounds that induce differentiation in cell culture. It is important that the cells be differentiated to a mature form, since OPIDN is a disorder that occurs in mature, fully differentiated neuronal cells. Differentiated cells in culture have the required morphological attributes for evaluation of neurite degeneration, and they have higher levels of activity of enzymes present in neural cells, such as NTE, than do cells in the undifferentiated state [6,7]. It is necessary that the model cells resemble the *in vivo* tissue type as closely as possible, rather than the more pluripotent undifferentiated cell, in order to effectively mimic the target of OPIDN.

Other disadvantages of cell culture models also exist. Differentiated SY-5Y cells in culture are still dividing, although at a rate much slower than that of undifferentiated SY-5Y cells. In

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addition, there are no interactions between diverse tissue types, and there is no consideration of the pharmacokinetics of toxicants in the intact animal. The time course of NTE inhibition and aging could be altered by absorption, distribution, metabolism, and excretion of the offending compound in the live animal.

This study demonstrated that the differentiated SY-5Y neuroblastoma cell line has NTE activity similar to that of chicken brain and that this cell line can be used to distinguish among esterase inhibitors that are capable and that are not capable of inducing OPIDN in vivo. This system, therefore, shows promise for use in the further investigation of early cellular events in OPIDN.

The prevention of OPIDN in vivo requires pretreatment of the animal with a protective compound; the need for this is obvious in light of the rapidity with which aging occurs. It has long been accepted that NTE inhibition and aging occur within hours after exposure [2]; the findings of the present study of changes within minutes confirms that concept and indicates that these processes may be taking place with greater rapidity than previously thought. The use of a test system such as the cell culture system described may be of considerable advantage, especially in the identification of methods for prevention of NTE aging after the OP has reached the neuronal cell.

In vivo, pre-treatment with carbamates and non-aging NTE inhibitors protects chickens from the development of OPIDN [2,8], whereas post-treatment with certain of these compounds has been demonstrated to exacerbate the condition [9,10]. In this study, aldicarb post-treatment of mipafox treated tissue exerted a protective effect, as did pre- and simultaneous treatment. This may be attributed in some part to the short time interval (2 minutes) between mipafox and aldicarb exposure in these studies compared to the longer time period between the administration of OPs and non-aging NTE inhibitors in the hen (≥ 4 hours). Additional work in this system may help to elucidate the paradoxical effects of prevention and promotion of OPIDN by the same compound,

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depending only on the time of administration, relative to the administration of the neuropathy-inducing OP.

In this study, protection of NTE by verapamil was also demonstrated. This effect was completely unexpected, as the mode of protection of the calcium channel blockers was presumed to simply involve calcium ion mobilization at some time after OP inhibition and aging of NTE. A new question arises from this result; is the normal function of NTE in the cell related to calcium channel function or some other aspect of intracellular calcium ion regulation? This effect on NTE was not seen in vivo in previous studies which did document protection from development of OPIDN in hens given verapamil as a pre-treatment to phenyl saligenin phosphate (PSP) [11]. However, brain NTE activity was not examined until a much later time point in these studies. Factors influencing toxicant distribution and metabolism may impose limitations on its duration of effect of verapamil in vivo that are not seen in the in vitro model. In this manner, the cell culture system allows for examination of effects that may not be possible in vivo due to pharmacokinetic factors.

Removal of mipafox from the culture medium within 5 minutes of exposure, resulted in essentially no NTE inhibition and aging evident after 24 hours continued incubation with fresh media. This indicates that intact cells are likely to recover if a neuropathy-inducing OP is removed early after initial exposure, prior to such time as a critical amount of NTE has aged.

Cell culture systems have previously been used to examine viability, morphology, $[Ca^{2+}]_i$, and AChE [12-18], but no single system has examined all of these parameters after treatment with neurotoxicants such as the neuropathy-inducing OPs (e.g. mipafox). These four endpoints were tested in the differentiated SY-5Y system to evaluate their potential for use in these cells as indices of neurotoxicity in screening systems.

Toxicant effects on cell viability after 24 hours of continued incubation were not specific and could not be used to differentiate neurotoxicants from compounds that were not neurotoxic. Cell

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viability in this study seemed most sensitive to solvents (acetone and DMSO) in which toxicants not soluble in water might be dissolved. Of the compounds tested in aqueous solution, only mipafox treatment resulted in more than 20% lethality after 24 hours of continuous exposure to concentrations in the micromolar range. However, upon qualitative examination at times longer than 3 days, mipafox was one of the two compounds of which treatment of cells for prolonged periods of time did not result in complete cell death.

Pathological changes occurred in SY-5Y cells after continuous toxicant treatment with the 2 compounds that produce delayed axonopathies, mipafox and IDPN. Mipafox- and IDPN-treated cells were still viable 14 days after initiation of toxicant exposure. Pathological changes observed under phase-contrast microscopy were similar in both mipafox- and IDPN-treated cells, but they appeared to occur slightly earlier in mipafox-treated cells. Cells treated with mipafox or IDPN exhibited attenuation of neurites and lack of a normal growth cone by 10 days after treatment. Morphological findings reported here for mipafox-treated cells were similar to those reported previously when neuroblastoma 2-A cells were treated with 2 other neuropathy-inducing OPs, tri-ortho-tolyl phosphate (TOTP) and di-isopropyl fluorophosphate (DFP). In the previous study, the neurites of the cultured cells examined by phase-contrast microscopy exhibited a shrunken, rough appearance with irregular swellings along their length [19]. Neuropathy-inducing OPs and IDPN do not cause similar morphological appearance of peripheral nerves from animals exposed to these compounds. Further studies using ultrastructural techniques are needed before differences in morphological changes of neuroblastoma cells after exposure to mipafox and IDPN can be identified. To date, such studies have not appeared in the literature.

The results of the calcium studies reported here were inconclusive due to some inherent problems with the system used for calcium measurement. Apparent decreases in $[Ca^{2+}]_i$ were noted at 72 hours after initiation of exposure to the toxicants. Decreases in $[Ca^{2+}]_i$, especially 3 days after

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exposure to these toxicants, should be viewed with caution since they do, at least in this instance, reflect differences in cell number between control and treated cells at the same time point. To compensate for such changes, one could use a fluorescent probe with which $[Ca^{2+}]_i$ is calculated from the ratio of bound probe fluorescence to unbound probe fluorescence. This determination is independent of cell number, but was not possible under the conditions of the experiment described here. Additionally, the potential for inhibition of hydrolysis of esters of fluorescent probes loaded into cells exposed to OPs and carbamates deserves further investigation before ester-containing probes are used for routine analysis of detrimental effects of these compounds on cells in culture.

Another consideration is the subcellular distribution of calcium ion within the cell. Use of digital imaging systems that can examine a single cell and localize areas of higher or lower $[Ca^{2+}]_i$ could circumvent this problem. Additionally, once changes are determined, incubation in media with higher $[Ca^{2+}]$ or in Ca^{2+} -free media may help to determine if transient changes in $[Ca^{2+}]_i$ are a result of redistribution of calcium from intracellular stores or due to opening of calcium channels in the plasma membrane. The question of possible changes in $[Ca^{2+}]_i$ and effects on calcium-mediated enzyme systems as a mechanism of injury in OPIDN warrants further investigation.

The mechanism of verapamil and other calcium channel blocker protection could be investigated. For example, does verapamil bind to NTE at some site other than the esteratic active site resulting in a conformational change in NTE such that OP binding is sterically hindered and inhibition of the enzyme is no longer favored? Is the normal function of NTE somehow related to calcium channels, perhaps as a regulatory protein? Conductance and intracellular distribution of calcium and other ions, changes in intracellular pH, and activity of enzyme systems governing cytoskeletal regulation may be areas for future study in cell culture.

Also in this study, the effects of neurotoxicants on AChE activity were determined. Effects of the compounds tested here on AChE in SY-5Y cells correlated with those seen in chicken brain

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homogenates. Although SY-5Y cells had remarkably less AChE activity than brain tissue, inhibitory effects on a percentage basis were similar. This enzyme was a particularly sensitive indicator of treatment with an esterase-inhibiting OP.

The SY-5Y cell culture system has a great deal of potential for the examination of neurotoxic compounds, as it is particularly sensitive to esterase inhibitors. Effects on NTE and on AChE shown in this study [6,21,22] demonstrate the usefulness of the system when inhibition of enzyme activities were used as determinants of toxic effects. Initial studies indicate that other compounds are less likely to demonstrate changes in cellular esterase activity; other endpoints need to be explored as indicators of the toxicity of these compounds.

The present study demonstrates usefulness of the differentiated SY-5Y cell culture model in the examination of early effects of neuropathy-inducing OPs and their modification. Additional work may provide valuable insight into the mechanism of development of OPIDN and methods of protection or treatment of this condition.

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CONCLUSIONS

The differentiated SY-5Y cell culture model for examination of early effects of neuropathy-inducing organophosphorus esters appears from this work to be a valid model simulating effects occurring in hen brain. It has merit in investigation of early alteration of neurotoxic esterase and acetylcholinesterase in neuronal cells by OPs and in the examination of other nonspecific indicators of neurotoxicity and cytotoxicity. It is sensitive enough to differentiate between esterase inhibitors that induce delayed neuropathies in vivo and those that do not. Additionally, it can be used to examine modification of early effects of OPs in neuronal cells by compounds demonstrated to modify the outcome of organophosphorus-induced delayed neuropathy in vivo.

PART V
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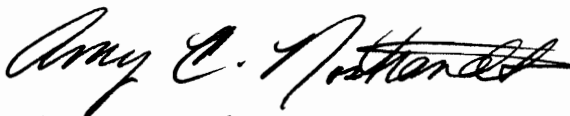
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