NEUROTOXICITY OF ORGANOPHOSPHATES:

SYNERGY AND INTERACTIONS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Janie Clodagh Axelrad

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DECLARATION

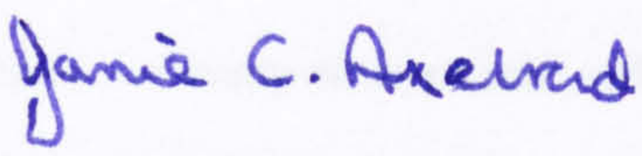
I declare that all the work documented in this thesis was my own, apart from the following:

Preparation of microglia was by Dr. Paul Kingham

Microsome preparation was by Brian Gibbins

Help with cell maintenance and culture came from Emma Court and Alex Holmes

Signed



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Janie Axelrad

Abstract

The primary aim of this thesis was to develop and use an assay involving the inhibition of neurite outgrowth of differentiating neuroblastoma cells as an endpoint, to detect synergistic interactions between organophosphate pesticides and other substances. A variety of interactions were observed between these pesticides, either as pure compounds and proprietary formulations, or their constituents. Although many of the synergistic increases in neurotoxicity were small, some combinations, especially involving formulation constituents, increased the toxicity many hundred-fold. Since pesticides are rarely encountered as pure compounds, this increase in neurotoxicity resulting from the simultaneous exposure to a variety of other components is discussed in relation to the ill health of occupationally exposed individuals. Although attempts are made to determine possible mechanisms for this synergistic increase in neurotoxicity, no consistent effects were found which would explain the differences.

In addition, the thesis describes the development of a cell line that had been chronically pre-exposed to the organophosphate diazinon, and its use to detect differences between chronic and acute exposure to pesticides. The neurotoxicity to these cells was found to differ on many occasions from that of cells that had not been pre-exposed, after acute exposure to various pure or formulated pesticides.

The thesis also investigated the potential for interaction between organophosphate pesticides and the intrinsic cell molecules, prion protein, β-amyloid protein and tau protein, all of which are associated with neurodegeneration. The organophosphate phosmet was found to increase the protease-resistance of the prion protein, a property suggestive of a conformational change in structure of the protein. In addition a synergistic increase in the toxicity of the prion protein, as well as the β-amyloid protein was observed in the presence of phosmet and on some occasions diazinon. Chronic pre-exposure to diazinon increased the expression of tau protein, and some isoforms of the β-amyloid precursor protein, suggesting an interaction had occurred which may have consequences for neurodegeneration. The possible involvement of microglia in organophosphate toxicity was also briefly studied, and it was found that a large increase in neurotoxicity resulted from neuronal exposure to the conditioned medium from these cells after they had been incubated with diazinon and phosmet.

The interactions of organophosphates detailed in the thesis suggest that these products may play a role in neurodegeneration by methods that have not been previously considered, and it is hoped that further investigation will identify more accurately the mechanisms involved.

PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS

Published work:

Axelrad, JC, Howard, CV and McLean, WG. Synergism of phosmet and pirimiphos methyl to inhibit neurite outgrowth from differentiating neuroblastoma cells. Neurotoxicology 21, 634, 2000.

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Axelrad, JC, Howard, CV and McLean, WG. The effects of acute pesticide exposure on nerve cells chronically exposed to diazinon. Toxicology

Awaiting submission:

Axelrad, JC, Howard, CV and McLean, WG. A new but not novel hypothesis on the function and structure of prion protein in cells.

Axelrad, JC, Howard, CV and McLean, WG. Immunodetection of the protease-resistant prion protein after incubation with the organophosphate phosmet.

CONTENTS

ABBREVIATIONS USED IN THE THESIS

AChE	acetylcholinesterase
AD	Alzheimer's disease
ADI	advisable daily intake
ApoE	apolipoprotein E
β-APP	β-amyloid precursor protein
BSA	bovine serum albumen
BSE	bovine spongiform encephalopathy
Ca	calcium
CaM	calmodulin
cAMP	cyclic AMP
cGMP	cyclic GMP
ChAT	choline acetyltransferase
CJD	Creutzfeldt Jakob disease
CNS	central nervous system
COPIND	chronic organophosphate-induced neuropsychiatric disorder
CSF	cerebrospinal fluid
dbcAMP	dibutyryl cyclic AMP
DDT	dichlorophenyltrichloroethane
DEET	N,N-diethyltholumide
DFP	diisopropyl fluorophosphates
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethyl sulphoxide
DTNB	dithiobisnitrobenzoic acid
EPN	O-4-nitrophenyl phenylphosphonothioate
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GPI	glycophosphatidylinositol
GST	glutathione s-transferase
hGF	human growth factor
IC_{50}	inhibition concentration (50%)
IGF-1	Insulin-like growth factor
IgG	immunoglobulin G
IL-2	Interleukin 2
IL-6	Interleukin 6
kDa	kilodalton
KPI	Kunitz-like protease inhibitor
LD_{50}	lethal dose (50%)
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NADP	nicotinamide adenine dinucleotide phosphate
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NOAEL	no observed adverse effect level
NTE	neuropathy target esterase
OP(s)	organophosphate(s)
OPIDN	organophosphate-induced delayed neurotoxicity
PBS	phosphate-buffered saline
PHF	paired helical filaments
PIPLC	phospholipase C
PKC	protein kinase C
PMSF	phenylmethylsulphonyl fluoride
PNS	peripheral nervous system
PON	paraoxonase
ppm	parts per million
ppb	parts per billion

PrP	prion protein
PrP^{c}	cellular prion protein
PrP^{sc}	scrapie-like prion protein
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulphate
SOD-1	Cu/Zn superoxide dismutase
TNF-α	tumour necrosis factor α
TBS	tris-buffered saline
TOCP	tri-*o*-cresylphosphate
tPA	tissue plasminogen activator
TSE	transmissible spongiform encephalopathy

CHAPTER 1

INTRODUCTION

1.1 Aims and background for the investigations in this thesis

The hazards associated with pesticide exposure have long been of concern, both in respect to the operator and the consumer. All pesticides must undergo a series of standardised safety tests before marketing, and increasingly vigorous protective measures have been introduced with the intention of protecting the individual from the neurotoxic effect of exposure. Concerns for the effects on wildlife and the environment are similarly important in the development of novel products, so that products considered to be less harmful have gradually replaced the more hazardous pesticides.

Unfortunately humans are invariably exposed, not to individual pesticides, but to mixtures of large numbers of different chemicals. The individual toxicity of each substance may have been assessed but the hazards resulting from combination with so many other products has not generally been considered.

Standard toxicity testing is unable to address the potential for interaction between the thousands of chemicals in common use, and the toxicity of mixtures is usually assumed to have an additive effect of the toxicities of the known components. However, between the components of a mixture, interactions can manifest in a number of ways (Groten *et al.*, 2001). There may be no interaction, in which case the additivity assumption would be justified. However the presence of one chemical may enhance the toxicity of another chemical, resulting in synergistic increase in toxicity. This synergy or potentiation of effect is increasingly of concern with respect to pesticides, and has been suggested as playing a role in the unexplained ill health of a group of Gulf War veterans (Haley *et al.*, 1997) and increasingly in farmers who have been exposed to a variety of different products on a regular basis (Jamal, 1997). Synergism is commonly seen in drug interactions, although acceptance of the phenomenon with respect to pesticides is far from universal.

Synergistic interactions are not the only hazard arising from mixtures of two or more different chemicals. Antagonism or inhibition of one product in the presence of another may also occur in mixtures. This effect is perhaps of greater concern when combinations of therapeutics interact to negate the beneficial effects of one or other drug, but may potentially reduce the desired toxicity of pesticides so that the product is rendered ineffective (Marinovich *et al.*, 1996).

This thesis is concerned with the possible synergism and interactions of pesticides, and in particular the organophosphates, in combination with other chemicals. The primary aim is to identify and quantify any such synergy by the development of an *in vitro* assay for neurotoxicity. Pesticides are generally insoluble in water, so are formulated in a wide range of solvents and associated products to assist in application (Cremlyn, 1978), all of which have the potential for synergistic interaction between the pesticide and the remaining components of the formulation. This may occur even if the product is not itself neurotoxic. In addition, synergistic interaction can occur with other products either as a result of simultaneous exposure or after repeated exposure over a longer period.

The possibility that chronic exposure to low levels of these products could result in a cumulative toxicity or in differences in susceptibility to future acute pesticide exposure, either involving the same or different products, is also of increasing concern, and in recent years a variety of enquiries have attempted to investigate the potential hazards associated with mixtures and suggest possible mechanisms to identify these hazards. Although discussions are still continuing, no method to assess these problems has so far proved consistently successful or acceptable to both manufacturer and toxicologist. There are many difficulties associated with detection of synergistic interaction or effects of chronic exposure, not least the almost infinite number of different combinations of all the chemicals in current use (Howard, 1997). This thesis will also attempt to address the problems of long-term exposure to low levels of organophosphates and the consequences of this exposure on the interaction with other pesticides.

However it is not only synergistic interaction of pesticides with other chemicals that is of concern. If organophosphates are found to be able to interact with other substances, there is a possibility that they could also interact with intrinsic cell proteins and thereby interfere with normal cell function. Neurotoxicity can result from exposure to a variety of substances, and organophosphates have been tentatively linked with neurodegenerative diseases such as Parkinson's disease (Bhatt *et al.*, 1999; Muller-Vahl *et al.*, 1999). A role for organophosphates in the neurodegenerative prion diseases has been hypothesised (Purdey, 1994), although few investigations have been undertaken to substantiate this. This thesis will therefore also aim to investigate whether there is any evidence for direct or indirect effects of organophosphates on the prion protein fragment PrP106-126, as well as other intrinsic cell proteins, β-amyloid and tau.

Finally, the possible involvement of microglia in the subsequent neurotoxicity of organophosphates will be investigated. Activated microglia release proinflammatory products which may contribute to the neurotoxicity of substances *in vivo*, but which would be undetected in isolated neuronal cultures.

The working hypotheses tested in this thesis are as follows:

a) Combination of pairs of pesticides or their constituents leads to neurotoxicity that differs quantitatively from that expected from an additive effect.
b) The neurotoxicity of pesticides to cells that have been chronically pre-exposed to the organophosphate diazinon differs quantitatively from that observed in cells that have not been pre-exposed to diazinon.
c) The synergistic effect of combinations of pesticides is related to their synergistic action as inhibitors of acetylcholinesterase, or to synergistic effects on cell viability.
d) Simultaneous exposure to organophosphates alters the neurotoxicity of the constituent cell protein fragments PrP106-126 or β-amyloid.
e) Exposure to organophosphates alters the protease resistance of the prion protein.

f) Acute or chronic exposure to organophosphates alters the expression or transcription of the intrinsic cell proteins, prion, β-amyloid precursor protein or tau.

g) The presence of microglia alters the neurotoxicity of organophosphates.

1.2 Neurotoxicity

Neurotoxicity is a destructive effect on cells of the central or peripheral nervous system as a result of the actions of natural or synthetic products. Neurotoxic effects can be produced either directly or indirectly. Direct effects are observed when the neurotoxin itself causes the death of the cells. Indirect effects are seen as the result of the neurotoxin producing a sequence of events culminating in cell death.

Evidence of neurotoxicity can be observed as changes in cell number, functional changes or death either by apoptosis or necrosis. Accumulation of the products of neurones may disrupt feedback mechanisms of normal cell functions, leading to the subsequent neurotoxicity. The accumulation of excess acetylcholine at nerve synapses due to the inhibition of acetylcholinesterase is one example of this (Sultatos, 1994).

Neurotoxic effects can be produced by an enormous array of naturally occurring and synthetic products, and each group of neurotoxins may affect totally different or related areas of neuronal function. Small effects on metabolism can dramatically affect neuronal integrity and function. Changes in the balance of molecules essential to the working of the cells can produce dysfunction. For example Ca^{2+} is an essential molecule for numerous mechanisms of nerve action (Nicotera *et al.*, 1992). A disruption in the balance of Ca^{2+} in the cell disrupts a whole array of related activities such as neurotransmission, arrangement and manufacture of cytoskeletal proteins or the metabolism of other chemicals, any of which can subsequently prove deleterious to the cell.

Neurotoxicity can target different parts of the neurone, such as the cell body, the dendrites or the proximal or distal axon. Direct damage to the cell body may cause indirect damage to the

axons. Neurones in one area of the body may be spared and toxicity confined exclusively to another area. Toxicity often affects either the Central Nervous System (CNS) or the Peripheral Nervous System (PNS) but not both (Spencer, 1980).

The co-operation of non-neuronal cells such as astrocytes or microglia also has a direct or indirect effect on the function of the nerve cells and may play a role in the instigation of neurotoxicity in mammals. After activation by excitotoxins these cells produce cytokines such as TNF-α and Interleukin 1β (Rogove and Tsirka, 1997), which are responsible for the initiation of apoptosis. These cells may themselves be the targets of toxicity (Hollensworth *et al.*, 2000). Such a complicated picture obviously presents the investigator of neurotoxicity with a difficult task.

1.3 Pesticide neurotoxicity

Pesticides are chemicals developed to counter attack by various pests on agricultural and horticultural crops (Cremlyn, 1978). They fall into three main groups, insecticides, herbicides and fungicides that are designed to attack insects, weeds and fungi respectively. There are two main types of pesticide action: systemic and non-systemic or contact. The difference between them is that systemic pesticides penetrate through the cells of the target organism whereas contact pesticides do not.

Until the 1930s pesticides were derived from natural products including common poisons like cyanide and arsenic. From then on the era of the synthetic organic pesticides rapidly led to the development of products such as the organochlorines, organophosphates and carbamates. The function of pesticides is predominantly dependent on their neurotoxicity. Insecticides target the nervous system of insects, thereby causing damage and death. They are in universal use despite their known toxicity, because either they are considered to be much less toxic to mammals or they are degraded so rapidly they are not considered to pose a threat to mammals.

Concerns about the neurotoxicity of pesticides began in the 1960's especially with regard to the organochlorines, and in particular DDT (dichlorodiphenyltrichloroethane) (Carson, 1963). Although DDT was considered of low mammalian toxicity (oral LD_{50} rat 300mg/kg), it was found to be extremely persistent in the environment, leading to bioaccumulation problems in mammals including humans. DDT concentrated up the food chain resulting in effects such as the thinning of eggshells in birds of prey, which almost caused the extinction of species such as the Bald Eagle (Carson, 1963).

The search for replacement pesticides resulted in the development of the organophosphates, a group of chemicals that are very toxic to invertebrates. Despite being generally more acutely toxic to mammals than the organochlorines, they are degraded relatively quickly so are considered less of an environmental hazard (Casida *et al.*, 1983). More recently natural and synthetic pyrethroids have been developed as pesticides. In general these exhibit lower mammalian toxicity than organophosphates and although they are extremely toxic to invertebrates they degrade rapidly.

Population exposure to low levels of a large number of pesticides as well as a variety of other chemicals is an accepted hazard in present times. Although all pesticides are rigorously investigated for toxicity before licensing, little work has been performed on the synergistic effects of pesticides, despite the potential for health problems. Attention is focused on maximum advisable daily intake (ADI) calculated from the no observed adverse effect levels (NOAEL) of individual products.

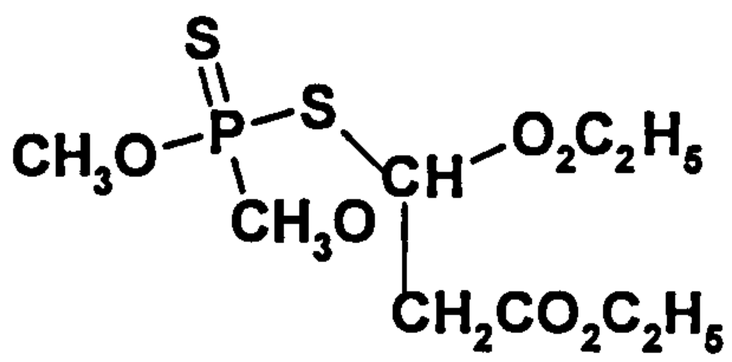
1.4 Organophosphates

Organophosphate (OP) pesticides are esters of pentavalent or trivalent phosphorus acids, incorporating various combinations of oxygen, carbon, sulphur and nitrogen. This results in up to 12 different subclasses including phosphates, phosphonates, phosphorothiates, phosphorodithioates, phosphorothiolates and phosphoroamidates (Chambers, 1992a). They form the most toxic group of insecticides, with respect to mammalian toxicity. Modes of action are similar to World War II nerve gases, such as soman or sarin, although levels of neurotoxicity are much less than with these products. Organophosphates (OPs) are generally more chemically unstable than other pesticides and this lack of persistence suggested their suitability for agricultural use.

OPs are divided into three groups: aliphatic, phenyl or heterocyclic, depending on their configuration.

Aliphatic organophosphates

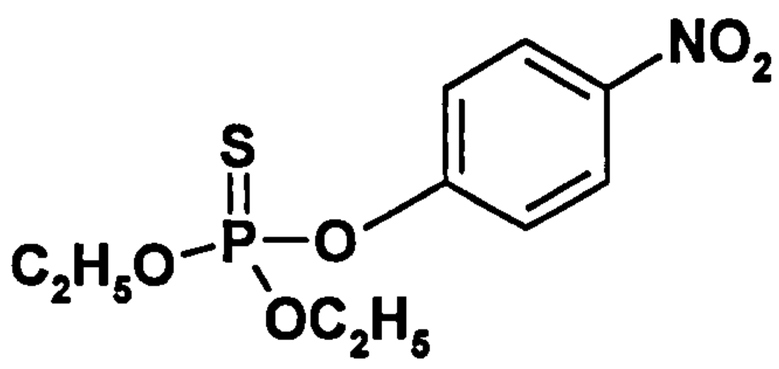
Aliphatic organophosphates are carbon-chain-like structures, and include tetraethyl pyrophosphate (TEPP), the first OP embraced by agriculture, and malathion.



Structure of malathion

Phenyl derivatives

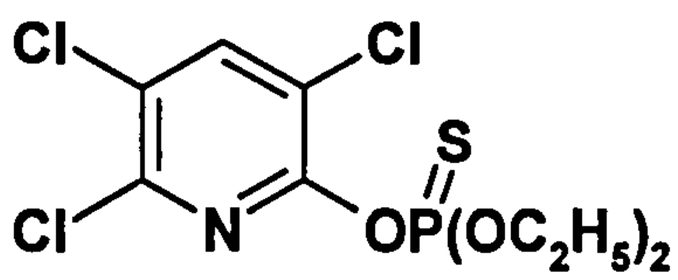
These contain a phenyl ring with one of the ring hydrogens displaced and the phosphorus moiety attached. Other hydrogens are frequently substituted by chloride, nitrite, methyl, cyanate or sulphur groups. Phenyl organophosphates are generally more stable than aliphatics and consequently more persistent. Examples of this group include parathion or fenitrothion.



Structure of parathion

Heterocyclic organophosphates

Heterocyclic organophosphates have a ring structure composed of different (or unlike) atoms, such as oxygen, nitrogen or sulphur. These include most of the popular OPs used today, such as diazinon or chlorpyrifos.



Structure of chlorpyrifos

1.5 Metabolism of organophosphates

The toxicity of organophosphates is generally considered to arise due to their ability to inhibit the enzyme acetylcholinesterase. However many OPs have little or no capacity to inhibit acetylcholinesterase themselves. Instead these 'protoxicants' must undergo metabolic activation by biotransformation to their corresponding oxygen analogues or oxons (Sultatos, 1994). These oxons may be strong inhibitors of acetylcholinesterases. However not all organophosphates inhibit these enzymes. Some bicyclic phosphorus esters such as 4-(R)-1 phospha 2,6,7-trioxabicylclo(2,2,2)octane have no acetylcholinesterase activity but are extremely toxic to mammals (Bowery *et al.*, 1976). Glyphosate, which has very low acute toxicity, was not considered to be able to inhibit cholinesterase until recently (Marrs, 1993; El-Demerdash *et al.*, 2001).

Depending on the chemical structure of the OPs, both the parent compound and the oxon may undergo detoxification through various pathways.

Cytochrome P-450 metabolism

The metabolic activation of organophosphates, usually to the oxon form such as paraoxon or diazinonoxon, is principally catalysed by cytochrome P-450 enzymes (Josephy, 1997a), which are concentrated in the liver of the organisms. These enzymes play a central role in the metabolism of many foreign and potentially toxic compounds, catalysing both detoxification and bioactivation reactions. They are all monooxygenases, so catalyse reactions in which one oxygen atom is inserted into the substrate, while the other is reduced to water in the following way:

$$NADPH + O_2 + S(Substrate)H + H^+ \Rightarrow NADP^+ + SOH + H_2O$$

For all mammalian cytochrome P-450s the reducing factor is the coenzyme NADPH. Cytochrome P-450s are membrane-bound and highly concentrated in the liver, with measurable amounts in the lung, gut and kidney and little or no detectable activity in other tissues. One such enzyme, cytochrome P-450 2B1, catalyses the oxidative activation and detoxification of parathion, probably by donating an oxygen atom to the sulphur of parathion (Sultatos, 1994). This results in the formation of various amounts of diethyl phosphorothionate and diethyl phosphate. Similar reactions occur in the detoxification of other OPs, such as diisopropyl fluorophosphate (DFP) (Laaksonen *et al.*, 1995).

In addition to oxidation reactions, cytochrome P-450 enzymes can catalyse reduction reactions, including the reduction of carbon tetrachloride (Guengerich, 1991). They can catalyse hydroxylation of unactivated hydrocarbons such as octane or cyclohexane bonds. The hydroxylation of N-H bonds is also common, and they are able to catalyse hydroxylation of aromatic rings (Josephy, 1997b).

Exposure to a small amount of xenobiotic can markedly enhance the ability to metabolise the same compound (Josephy, 1997a). Thus exposure to a small dose of toxin protects against a later larger dose. This elevation of biotransformation is known as induction and can result in a two-fold increase in particular P-450 forms. It greatly enhances the detoxification system but takes hours or days to reach maximum and depends on the continued presence of the inducing agent. Typical inducers are ethanol and phenobarbital. Some vegetables such as sprouts can induce P-450 enzymes (Josephy, 1997a), whereas a low protein diet or vitamin deficiencies can cause a reduction in detoxification in the liver (Ehrich *et al.*, 1984). Although metabolism results in a decrease in xenobiotic levels, enzyme induction can also result in increased formation of toxic metabolites.

A-esterases and aliesterases

These are groups of enzymes that hydrolyse organophosphate esters but are not inhibited by them (Mutch *et al.*, 1992). A large proportion of some oxons generated in the liver are themselves metabolised further by these esterases. This reduces the amount of oxon leaving the liver. The A-esterase paraoxonase in the serum is responsible for detoxifying some oxons, such as paraoxon, methyl paraoxon, pirimiphos methyloxon, diazinonoxon and chlorpyrifos oxon (Costa *et al.*, 1990).

Flavin monooxygenase system

A further group of detoxification enzymes, the flavin monooxygenase system is also involved in a variety of pesticide oxidations including sulphoxidation or N-oxidation (Levi and Hodgson, 1988). They are responsible for activation of OPs containing at least one C-P bond, e.g. fenfos, or a thioester linkage e.g. phorate. Such pesticides are often substrates for both flavin monooxygenase and P-450 enzymes. Oxidation of phorate to the sulphoxide by the flavin monooxygenases produces little change in acetylcholinesterase inhibition, but oxidative desulphation to the oxon by the P-450 enzymes increases acetylcholinesterase inhibition 1000-fold (Levi and Hodgson, 1988).

Glutathione S-transferase metabolism

It is not thought that glutathione S-transferase (GST) plays a major role in OP detoxification (Chambers *et al.*, 1994) although some pesticides such as malathion are known to be inactivated by glutathione-dependent O-methylation (Josephy, 1997b).

Individual variation in enzyme levels

Because the impact of neurotoxins depends largely on the rate of metabolism, levels of the various detoxification enzymes are of importance in any potential neurotoxic exposure.

Variation in enzyme levels depends on age and gender (Moser *et al.*, 1998) with young rats being more sensitive to chlorpyrifos than adults; a reduction of cholinesterase inhibition occurs with increasing age. Young rats also have less activity of liver and plasma carboxyesterases and A-esterases than adults. Thuerl *et al* (1997) however found twice as much P-450 activity in neurones from embryonic brains than from post-natal brains. Females generally have reduced liver carboxyesterase activity compared to males. This may be due to lower P450-mediated dearylation in females than males (Chambers *et al.*, 1994). Sex differences are probably hormone-related, since detoxification in ovariectomised female rats resembles more closely that of male rats (Overstreet *et al.*, 1981).

The various systems of hepatic detoxification of organophosphates, involving dearylation, aliesterase phosphorylation and A-esterase hydrolysis all play an important role in acute toxicity. Chambers et al (1994) consider that of all the different pathways involved in organophosphate metabolism, the induction of detoxification reactions is more biologically relevant than is metabolism to more active products. This was suggested because induction of P-450 enzymes reduces the toxicity of some phosphorothionates despite the activity leading to production of the more active metabolite. This is presumed to result from detoxification occurring at a greater rate than the production of the oxon.

Species differences in enzyme levels are common and reflect sensitivity to acute toxicity (Callahan and Kruckenberg, 1967). For example cats have very low levels of acetylcholinesterase compared to monkeys. In humans there appears to be a wide difference in inter-individual human serum paraoxonase activity, with 10-40-fold (Davies *et al.*, 1996) or 6-fold differences (Mutch *et al.*, 1992) being reported, attributed to a DNA polymorphism in the PON1 gene. This polymorphism is considered to confer greater susceptibility to the effects of environmental toxins on a proportion of the population. Unlike mammals, birds have little or no serum A-esterase activity. This may explain their greater susceptibility to toxicity from active OP derivatives (Mackness *et al.*, 1987; Costa *et al* 1982a).

Mutch *et al* (1992) investigated many enzymes involved in organophosphate metabolism in humans, and also found a 2-fold inter-individual variation in serum cholinesterase in addition to the 6-fold variation in human serum paraoxonase. Large variations in enzyme levels, reflecting increased susceptibility have also been observed in sheep and cattle (Anderson *et al.*, 1969). None of the enzymes tested showed a normal distribution. A low paraoxonase level would suggest that after first pass detoxification in the liver higher levels of the more toxic oxon compound would reach the site of action, resulting in greater toxicity.

1.6 Toxicity of organophosphates

Acute toxicity and inhibition of acetylcholinesterase

The acute toxicity of organophosphate compounds has been understood for many decades, and results from the inhibition of acetylcholinesterase by irreversible phosphorylation. Cholinergic neurones use acetylcholine as a neurotransmitter. This is synthesised from the two precursors, acetyl CoA which is derived from energy metabolism in all cells, and choline. Once released from the presynaptic neuronal terminal, acetylcholine may interact with either the muscarinic or nicotinic receptors. It is then rapidly inactivated by hydrolysis by the enzyme acetylcholinesterase. This involves attachment of the acetyl group from acetylcholine to a serine residue on the enzyme, followed by hydrolysis to produce acetate and active enzyme. If acetylcholinesterase is itself inactivated, this will inevitably result in accumulation of the neurotransmitter acetylcholine at the neuromuscular junction, causing excessive activation of the acetylcholine receptors (Sultatos, 1994).

The desired action of organophosphorus pesticides results from their ability to inhibit acetylcholinesterase. OPs inhibit acetylcholinesterase by phosphorylating a serine hydroxyl group within the enzyme active site, yielding a hydroxylated leaving group (Sultatos, 1994). This process inactivates the enzyme and blocks the action of acetylcholine. The phosphorylated enzyme may then either undergo hydrolysis to reactivate it, or may undergo a process of aging, which is irreversible. Aging involves loss of the alkyl moiety from the

phosphorylated enzyme, converting either the methoxy or ethoxy group to an oxygen anion (O-), which stabilises the enzyme (Sultatos, 1994).

As described earlier, not all organophosphate compounds directly inhibit acetylcholinesterase, but instead may be metabolised to their corresponding oxygen analogues or oxons which themselves may be potent acetylcholinesterase inhibitors (Sultatos, 1994). This is especially important in the phosphorothionate and phosphorodithionate organophosphates containing sulphur attached by a double bond to the phosphorus atom.

Spontaneous reactivation of acetylcholinesterase depends on which substrate phosphorylated it. Brain cholinesterases phosphorylated by methyl parathion regenerate 5-10 times quicker than those phosphorylated by paraoxon (Sultatos, 1994). Chambers (1992b) reported that those pesticides with the greatest *in vivo* toxicity were in general those most activated by brain enzymes. He considered that although brain activation was of little importance in severe poisoning due to excess production of intrahepatically generated oxons, it might have increased significance in situations of continuous exposure or chronic low-dose effects. There are also differences in reactivation between brain and plasma cholinesterases (butyrylcholinesterases). Plasma cholinesterase reactivates much quicker than brain so levels of plasma acetylcholinesterase do not accurately reflect acute poisoning (Jamal, 1997).

Spontaneous reactivation of acetylcholinesterase is not possible once 'aging' has occurred. In aging one alkyl side chain is removed non-enzymatically, leaving a hydroxyl group in its place. This is thought to prevent access of water needed for dephosphorylation (Sultatos, 1994). The rate of aging appears to be a function of the structure of the pesticide and the type of cholinesterase inhibited. Without reactivation, recovery of acetylcholinesterase activity can only occur through new enzyme synthesis (Sultatos, 1994).

Impurities may be present or produced as a result of metabolism, which have much greater toxicity than the parent compound. Sulfotepp is one such impurity known to contaminate diazinon. It has levels of toxicity many times greater than diazinon (Dennis *et al.*, 1980) and

therefore may produce acute toxicity of unexpected severity from exposure to diazinon products.

Acetylcholinesterase structure and function

Acetylcholinesterase, together with butyrylcholinesterase, forms the group of cholinesterases. Both can be inhibited by organophosphates. These enzymes exist in multiple molecular forms. Acetylcholinesterase is primarily localised at synapses associated with nerve and muscle. Butyrylcholinesterase synthesis is mainly in the liver and from there it appears in the serum. The function of butyrylcholinesterase is unclear but may have a role in the detoxification of ingested plant esters (Taylor, 1991) or in the control of neurite outgrowth (Layer *et al.*, 1993).

Acetylcholinesterase is an enzyme of the cholinergic nervous system. However it is now apparent that it may have a multi-functional role. It carries novel cell surface antigens that may play a morphogenetic role in cell-cell communication with links to cell adhesion (Johnson and Moore, 1999). A direct role of acetylcholinesterase in neuronal differentiation has also been postulated (van den Beukel *et al.*, 1998) because transient bursts of activity are associated with axonal growth in maturing brains. Thus it is possible that organophosphates may interfere with or influence the possible developmental functions of acetylcholinesterase.

According to Bon *et al* (Bon *et al.*, 1979) the molecular forms of acetylcholinesterase are divided into two classes. Asymmetric forms are composed of one, two or three catalytic tetramers linked to a collagen-like tail by disulphide bridges. These are associated with the neuromuscular junction. The other class consists of the globular forms, which are molecules without collagen tails. These may be monomeric, dimeric or tetrameric and occur in cells involved with cholinergic transmission as well as other neuronal and non-neuronal cells. Globular forms may be subdivided into soluble and detergent soluble forms. These are secreted from cells, although the physiological role of this secretion is unknown (Bon *et al.*, 1979).

Acetylcholine receptors

There are two classes of acetylcholine receptors: nicotinic and muscarinic. Of these there are at least two types of nicotinic receptors and 5 or more types of G-protein-associated muscarinic receptors (Sultatos, 1994). Nicotinic receptors are found in central nervous system synapses, the neuromuscular junction and the autonomic ganglia. Muscarinic receptors are also present at CNS synapses, as well as between parasympathetic postganglionic nerves and their effector organs. The muscarinic receptors mediate a variety of biochemical cell reactions including stimulation of phosphoinositide hydrolysis (Abou-Donia and Huff, 1995).

Inhibition of acetylcholinesterase by OPs leads to a rise in acetylcholine levels at the receptors, which results in continuous activation. Depending on where the stimulation occurs, the presenting symptoms reflect the type of stimulation (Marrs, 1993). Symptoms of muscarinic receptor stimulation include bradycardia, bronchial constriction, incontinence, gastro-intestinal disorders, salivation or blurred vision. Symptoms of nicotinic receptor stimulation include ataxia, convulsions or paralysis. Stimulation at both receptor types can lead to lethargy, tremor or depression (Marrs, 1993).

As well as effects caused by acetylcholinesterase inhibition, OPs can directly affect the receptors themselves. Muscarinic receptor ligand binding is reduced in PC12 cells exposed to OPs (Viana *et al.*, 1988). This does not appear to be the result of a toxic effect of excess acetylcholinesterase because the reduction in binding is due to a reduction in receptor numbers rather than a decreased binding affinity of the existing receptors (Dawson and Jarrott, 1981). The likely explanation is that the organophosphate in some way inhibits the synthesis of these receptors.

Chlorpyrifos oxon is seen to interact with the muscarinic receptors and can also inhibit their effect on adenylate cyclase (Abou-Donia and Huff, 1995). The effect on binding is not receptor-dependent and may be a direct interaction of the oxon with the adenylate cyclase molecule.

Inhibition of receptors differs with different products. Parathion is 50 times more potent at blocking nicotinic receptors than its acetylcholinesterase-inhibiting metabolite paraoxon (Kondo and Yamamoto, 1998).

Tolerance in acute organophosphate toxicity

Repeated exposure to OPs sometimes produces tolerance. This may be connected to the down regulation of receptors, particularly muscarinic, which may even be internalised (Sultatos, 1994). However Sivam *et al.* (1983) showed that the reduction in the number of muscarinic receptors is a gradual process not related to the degree of acetylcholinesterase inhibition. Other possible mechanisms of tolerance are a reduction in choline uptake or release, or an increased production of acetylcholine (Overstreet and Schiller, 1992).

Organophosphate-induced delayed neurotoxicity (OPIDN)

OPIDN has been defined as 'a delayed onset of prolonged locomotion ataxia resulting from a single or repeated exposure to an organophosphorus compound' (Abou-Donia and Lapadula, 1990). This serious and possibly irreversible effect usually occurs 2-3 weeks after exposure to organophosphates and manifests as symmetrical peripheral neuropathy, often with ataxia or paralysis (Johnson, 1982). It occurs mainly in the lower limbs although severe poisoning may also involve the upper limbs. It is unrelated to effects on acetylcholinesterase. Weak cholinesterase inhibitors often produce the most severe OPIDN effects (Marrs, 1993) but many OPs do not produce this effect.

On a neuropathological level, OPIDN is observed as a neurodegenerative disorder characterised by swellings of the distal parts of large axons and subsequent axonal degeneration leading to paralysis (Abou-Donia, 1995). The neuropathological changes have been classified as Wallarian degeneration of axons and myelin of the long sensory and motor pathways of spinal cord and the large fibres of the PNS.

OPIDN was initially described as the premature 'aging' of an enzyme in nerve cells known as neuropathy target esterase (NTE) (Pope *et al.*, 1993). A physiological role for NTE has not yet been found, and loss of NTE function does not affect the health of neurones (Moretto *et al.*, 1994).

Aging is achieved by the inhibition through phosphorylation, probably of a hydroxyl group of serine residues, and subsequent dealkylation of NTE. This dealkylation removes the alkyl side chain from the phosphate moiety resulting in an ionised acid group attached to the phosphorus atom (Sultatos, 1994). Both processes may be necessary for OPIDN induction, and inhibition and aging of 70-80% of NTE activity is correlated with OPIDN development (Johnson, 1990). Abou-Donia *et al* described two groups of OPIDN (Type I and II) as a result of the discovery that certain triaryl phosphites produce a different delayed neuropathy from other phosphates (Abou-Donia and Lapadula, 1990).

However there is accumulating evidence that aging is not necessarily essential for OP neuropathy. NTE activity can return to nearly normal levels in the brain before the onset of OPIDN, and the esterase is present and inhibited by OPs in young animals, usually relatively insensitive to the effects of OPs (Carrington and Abou-Donia, 1984). However, the rate of NTE activity is depressed for longer at the site of the neuropathy than in other areas of the brain. On a molecular level, OPIDN is associated with enhanced autophosphorylation of Ca^{2+}/calmodulin-dependent kinase II, with phosphorylation of various cytoskeletal proteins and neurofilaments. These accumulate due to decreased transport down the axon (Gupta and Abou-Donia, 1995).

Not all species exhibit OPIDN, but it is reproducible in humans and hens. This is why the 'hen test' has been used for many years as the standard test for neurotoxicity (Johnson, 1977). Hens are treated with the potentially neuropathic agent and then observed for ataxia and other signs of neuropathy. OPIDN in hens is considered to predict the problem in humans (Johnson, 1977). Unfortunately direct correlation between the results of this test and the situation in humans is now seriously under debate (Pope *et al.*, 1993). Humans appear to be

more susceptible to a form of OPIDN following repeated small doses of OP pesticides which can be missed by the hen test (Jamal, 1997), and neuropathy can be induced in humans by OPs which have been shown to be non-neuropathic with the hen test (Lotti *et al.*, 1993).

The idea that only those OPs that age NTE can cause OPIDN has been further refuted with the discovery that phosphines, which are not NTE inhibitors, produce a combination of CNS and peripheral nerve damage (Abou-Donia *et al.*, 1996b). Some phosphoroamidate OPs, which have no ageing effect on NTE, are nevertheless neuropathic (Johnson *et al.*, 1991). Moretto *et al* (1994) demonstrate a phosphorothionic acid that promotes OPIDN without NTE inhibition, and suggest a target other than the NTE catalytic site. This site is considered important in the phenomenon of 'promotion' of OPIDN.

Promotion occurs when a non-neuropathic NTE inhibitor exacerbates neuropathy when given after a neuropathic OP (Pope *et al.*, 1993). Work has been performed on pre- and post treatment of the organophosphate DFP with phenylmethylsulphonyl fluoride (PMSF), a non-neuropathic NTE inhibitor, which indicates that another target other than NTE may be producing the interactions (Pope *et al.*, 1993). Animals given the OP before PMSF have extensive signs of nerve degeneration that is greater than with the OP alone. If given in reverse order however, no signs are seen. Since the inhibition of NTE activity is already 90% or more in the situation when the PMSF is given second, few NTE molecules can remain for binding by the PMSF. The extensive pathology that results cannot therefore be explained by additional aging of NTE alone. The same is true for PMSF given first and would not explain the lack of OPIDN in the 'PMSF given first' situation (Pope *et al.*, 1993).

Both the acute effects on acetylcholinesterase and the phenomenon of NTE aging to produce OPIDN have been relatively well studied. However OPs are known to have many more effects of a neurotoxic nature, which so far have received little attention. These may contribute to varying degrees to the neurotoxicity of this group of pesticides. Some of these effects are discussed below.

Intermediate Syndrome

De Bleeker (1995) first described a novel syndrome of patients who had suffered high exposure to OPs. This Intermediate Syndrome arises after an acute cholinergic crisis, usually but not exclusively after prolonged cholinesterase inhibition, but before any manifestation of delayed neuropathy caused by the inhibition of NTE, to which it is not related. The exact mechanism is not understood but it is thought that any OP compound has the potential to produce it. It manifests with myasthenia-like symptoms and affects proximal limb muscles, neck flexors and respiratory muscles, leading to weakness or paralysis and often death. It is not responsive to atropine, the usual therapy for OP exposure. Senanayake (1987) postulates that it may be due to post-synaptic dysfunction.

Chronic organophosphate-induced neuropsychiatric disorder

Jamal (1997) discusses a further chronic effect of OP agents, which he names chronic organophosphate induced neuropsychiatric disorder (COPIND). This seems to be unrelated either to acetylcholinesterase or NTE enzyme inhibition, and can follow acute exposure, or small, sub-clinical doses. It manifests as a persistent decline in neuropsychological function. Much of the evidence has accumulated from studies on patients who had previously reported an acute cholinergic poisoning episode. Poisoned groups appeared to have deteriorated intellectual functioning, academic skills and often motor skills. Even single episodes of OP toxicity have been associated with persistent decline in neuropsychological function (Rosenstock *et al.*, 1991).

Jamal cites a large number of papers containing anecdotal evidence of long-term neurological dysfunction following OP toxicity. Following recent investigations in farmers (Pilkington *et al.*, 1999) and individuals regularly exposed to pesticides (Bosma *et al.*, 2000), increased awareness may be given to such problems. Jamal also suggests that there is a second group of sufferers of COPIND acquired through long term OP exposure but often with

no acute attack. Instead the syndrome is caused by small repeat doses of a variety of chemicals.

The effects observed may be the result of a variety of interactions such as phosphorylation of neuronal proteins or genetic differences in detoxification enzyme levels, resulting in subtle problems such as personality changes, memory deficiencies or visual disturbances. Jamal (1997) states that the 'essential message is that the mechanisms for production of ill-health from long term exposure to OPs need to be sought not from their acetylcholinesterase effects but rather from an understanding of the effects of alkylphosphorylation of other enzymes and receptors and the way these interact, and also the effects on the second messenger system'.

GABA and cyclic GMP Involvement

One group of bicyclic organophosphate compounds often used as fire retardants, such as 4-(R)-1 phospha 2,6,7-trioxabicylclo(2,2,2)octane, are extremely toxic yet do not have an effect on brain cholinesterase activity (Bowery *et al.*, 1976). It appears that their action is related to the ability to antagonise the inhibitory neurotransmitter GABA (Sivam *et al.*, 1983). This activity depends on the alkyl group of the OP molecule.

These GABA antagonists are also interesting in that they have structural similarities to cyclic AMP (Bellet and Casida, 1973). They up regulate cyclic GMP (cGMP) concentrations in the cerebellum, although they have no effect on cyclic AMP (Mattson *et al.*, 1977). The OPs sarin, tabun and soman, although known for acetylcholinesterase inhibition, increase cGMP levels in rat striatum (Liu *et al.*, 1986). This indicates that other neurotransmitters play a role in OP toxicity. Cyclic GMP appears to have a function in GABAergic transmission in the cerebellum, as it is greatly elevated at the onset of OP-induced seizures such as those produced by soman (Lundy and Magor, 1978). It is known that alteration in GABA metabolism has an effect on cGMP levels. Paraoxon reduces GABA concentration in the brain and increases the excitability that contributes to seizures (Kar and Matin, 1972). Sivam *et al*

(1983) suggest that OPs that cause convulsions but do not inhibit acetylcholinesterase have effects on GABA distribution, possibly due to regulation of GABA synthesis by acetylcholine.

Neuronal cytoskeleton and protein synthesis

Protein phosphorylation represents the main post-translational modification of proteins by which neurones integrate the effects of extracellular signals (O'Callaghan, 1994). Protein phosphorylation can control neurotransmitter receptors, neurotransmitter release and regulate transcription through phosphorylation of transmitter factors. This is often achieved by specific kinase activation that may be dependent on cAMP (Saitoh *et al.*, 1991). Autophosphorylation can also dramatically affect the activity of an enzyme. The CNS contains a wealth of phosphoproteins including many structural elements such as neurofilament triplet proteins, microtubule proteins and microtubule-associated proteins (MAPs) (Abou-Donia *et al.*, 1988). Multiple kinases can phosphorylate the same or distinct substrates and phosphorylation can be achieved at different residues on the same phosphoprotein. Altered phosphorylation of cytoskeletal proteins may be a potential mechanism through which OPs have neurotoxic effects in adult and developing CNS.

So many processes are controlled by protein phosphorylation in the cell that the potential for disruption to neural development by inappropriate phosphorylation is large (O'Callaghan, 1994; Saitoh *et al.*, 1991). OPs, probably by calmodulin (CaM) kinase activation, cause hyperphosphorylation of the cytoskeleton, including the neurofilament triplet, β-tubulin and MAP-2 (Abou-Donia *et al.*, 1988). Aggregations of these aberrantly phosphorylated cytoskeletal parts can be seen after OP treatment (Jensen *et al.*, 1992).

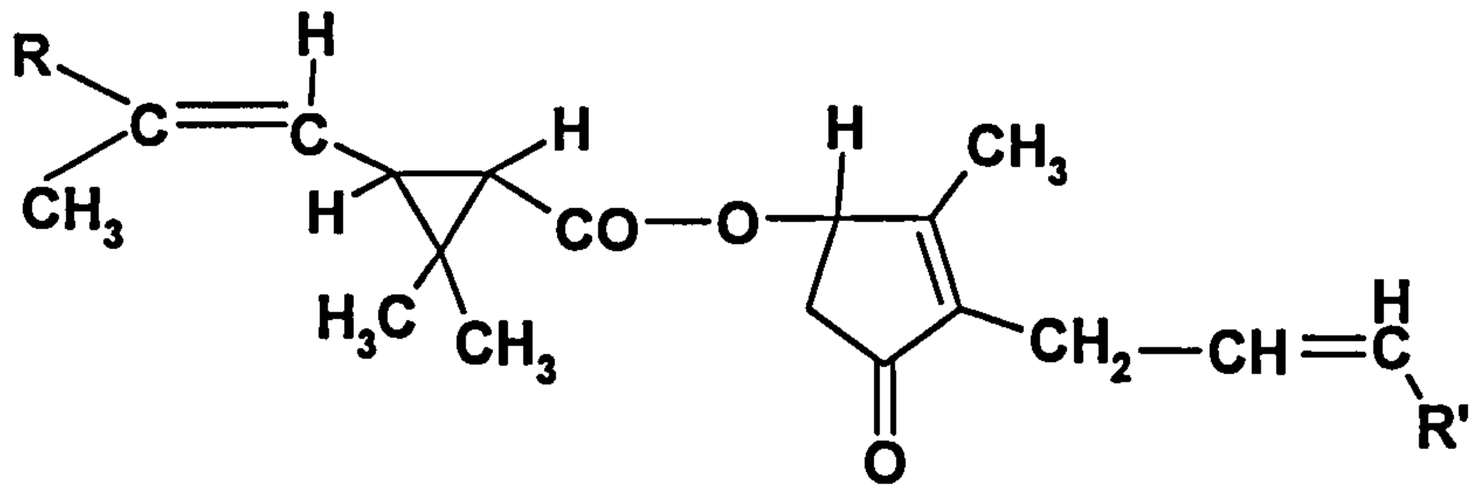
A single injection of DFP increases protein kinase-dependent phosphorylation of cytoskeletal proteins (Abou-Donia *et al.*, 1993) associated with calmodulin-binding by Ca^{2+}/calmodulin-dependent protein kinases (Gupta and Abou-Donia, 1995). Inhibition of axonal-like processes and disruption of neurofilaments has been observed in cultured NB2a and PC12 cells after OP treatment (Flaskos *et al.*, 1998). The tau protein, associated with many

neurodegenerative diseases, can also be phosphorylated by OPs (Gupta and Abou-Donia, 1998; Gupta and Abou-Donia, 1999).

Other areas in which organophosphates may have a disruptive effect on cell structure or function include damage as a result of prolonged receptor stimulation (Karalliede and Senanayake, 1989), or influence on a variety of proteases (Mantle *et al.*, 1997). Tuler suggests that OPs, unlike other acetylcholinesterase inhibitors, may have a direct action involving change in membrane integrity (Tuler and Bowen, 1989). Lipid vacuoles are observed after activity of OPs on nerves but are not seen in response to other neurotoxic compounds. Changes seen *in vitro* are consistent with the inhibition of enzymes or other proteins involved in maintenance and regulation of functional integrity of cell membranes.

1.7 Pyrethroids

Pyrethroids are either natural products extracted from the flowers of Chrysanthemum cinerariaefolium, or synthetic analogues developed as a result of the high costs involved in extraction of the natural product. They are lipophilic and neurotoxic, but show good biodegradability (Aldridge, 1990). Pyrethroids are classified as Group I or II depending on the presence or absence of a cyano group (Casida *et al.*, 1983).



Structure of pyrethrum

1.8 Toxicity of pyrethroids

The major biological activity of pyrethroids is mediated through effects on sodium channels. All the evidence suggests that interaction of pyrethroids with the sodium channels is reversible (Aldridge, 1990).

Group I pyrethroids interact with sodium channels on nerve fibres, prolonging opening and initiating repetitive activity at the synapse and neuromuscular junction (Aldridge, 1990). In general this results in the symptoms of hyperexcitation, ataxia and tremor. Group II pyrethroids such as cypermethrin induce depolarisation block of sensory nerve membrane leading to a 'tickling' sensation (Aldridge, 1990).

Pyrethrum and synthetic pyrethroids are axonal toxins affecting the electric impulse transmission along the axons and cause immediate paralysis, although the insect may later recover unless the product is formulated with a synergist (Casida *et al.*, 1983). Pyrethroids are very stable in sunlight with minimal volatility. Type I Pyrethroids have a negative temperature coefficient and are effective at low temperatures against most agricultural pests. Type II pyrethroids have a positive temperature coefficient (Casida *et al.*, 1983).

Although the voltage-sensitive sodium channel is the primary target site, it is unlikely to be the only site involved in intoxication. Pyrethroids can spontaneously release acetylcholine from rat brain synaptosomes (Eels *et al.*, 1992). Muscarinic receptors of the cerebral cortex are also involved (Eriksson and Nordberg, 1990) and this may lead to permanent changes in the cholinergic system in immature animals. A disruption of the neuroendocrine system has been implicated in pyrethroid toxicity and proconvulsive actions mediated through the benzodiazepine receptor may also be important (Soderlund and Bloomquist, 1989). Two specific health side effects, PNS nerve damage and facial paraesthesia, are associated with pyrethroids, especially in people manufacturing these products (Aldridge, 1990). Axonal degeneration only takes place at near lethal doses and no effects on NTE are observed.

As with OPs and DDT, pyrethroids inhibit calmodulin activity and Ca^{2+}-ATPase activity (Ferguson and Audesirk, 1990). Because neurite outgrowth is partly regulated by calcium influx through voltage-dependent channels (Mattson and Kater, 1987), blocking the channels reduces neurite outgrowth. Prolonged opening allows excess Ca^{2+} influx, which also reduces neurite outgrowth. Thus the influx of Ca^{2+} from the depolarisation effect of pyrethroids may increase intracellular calcium to levels greater than the optimum and have potential effects on the organisation of the CNS architecture in developing neurones (Ferguson and Audesirk, 1990).

1.9 Metabolism of pyrethroids

Commercial pyrethroids possess an ester group that is an important region of the molecule (Aldridge, 1990). *In vivo* the primary detoxification step is ester cleavage, followed by hydroxylation by the usual methods. There is a large difference between the oral and intravenous toxicity of pyrethroids because oral and dermal absorption of these products is slow and the pyrethroid rapidly detoxified (Aldridge, 1990). The greater intravenous toxicity indicates that pyrethroids are highly active molecules and the CNS is very sensitive to their effects. The rate of absorption is a crucial factor in determining toxicity.

The lipophilicity of pyrethroids aids rapid access to the tissues including the CNS, but the rapid metabolism renders the pyrethroids less toxic and ensures recovery from poisoning is also very rapid (Casida *et al.*, 1983). However some pyrethroid metabolites persist in mammals long after the parent compound is metabolised and eliminated. The toxicity of pyrethroids is thought to be dependent more on the configuration of the entire molecule than on specific functional groups (Casida *et al.*, 1983).

Synergists

Pyrethroids are generally formulated with a synergist such as piperonyl butoxide, to prevent the extremely rapid metabolism by the liver Cytochrome P-450 enzymes (Casida *et al.*, 1983).

The synergist maintains the insecticidal activity for longer by reversible inhibition of the Cytochrome P-450 enzymes to prevent this rapid detoxification. Piperonyl butoxide is one of a few manufactured synergists. It has no insecticidal properties of its own but can synergise the toxicity of pyrethrum to houseflies by 300 fold (Casida *et al.*, 1983).

Apart from its use in pyrethrum formulations, piperonyl butoxide also inhibits oxidative cleavage of OPs such as parathion and methyl parathion (Levine and Murphy, 1977). This prevents oxidation to the corresponding oxons, and may increase the importance of dealkylation by glutathione-S-dehydrogenase as an alternative pathway when detoxification by oxidative metabolism is inhibited.

A potential problem is apparent from the use of synergists to prolong the toxicity of pyrethroid products, since the inhibition of cytochrome P-450 enzymes would simultaneously prevent the metabolism of OPs. This may produce unexpectedly increased or even decreased neurotoxic effects, depending on whether metabolites or parent molecules have the greater toxicity.

1.10 Solvent involvement and toxicity

Since most organophosphates are insoluble in water they are usually formulated in a variety of different solvents. Formulations may be as dusts or sprays, or even granules. Surfactants or emulsifiable oils may be added to allow dilution in water without the problems of separation of the pesticide (Cremlyn, 1978). However such solvents may also be neurotoxic and contribute to the effects seen with OP neurotoxicity.

Xylene is often used in chlorpyrifos formulations. It is lipophilic, like chlorpyrifos, and thus both the active ingredient and the solvent may be able to penetrate the brain (Wurpel *et al.*, 1993). Toluene (methyl benzene) is another widely used organic solvent. It can produce brain abnormalities and CNS injury, including degeneration and gliosis in ascending and descending long tracts, and diffuse demyelination of subcortical white matter due to primary neuronal and axonal loss with secondary demyelination (Rosenberg *et al.*, 1988).

Ethanol and other alcohols increase neurite outgrowth and branching (Zou *et al.*, 1993) in some areas of the brain, and inhibit it in other areas. This may damage the CNS by disturbing the balance and organisation of synapses. Ethanol enhances the effects of some actions of Nerve Growth Factor (NGF) (Messing *et al.*, 1991). Alcohols with longer carbon chains and greater lipid solubility are more potent than ethanol in producing effects, suggesting that the action of alcohols is dependent on drug partitioning into lipid membranes (Lyon *et al.*, 1981).

Long-term administration of ethanol reduces choline acetyltransferase levels and choline uptake (Charness *et al.*, 1989). This can damage cholinergic projection neurones and induce disturbance of fast axonal transport leading to polyneuropathy. Ethanol and its metabolite acetaldehyde may also directly damage the developing and mature CNS (Charness *et al.*, 1989). Non-oxidative metabolism of ethanol produces fatty acid ethyl esters that accumulate in tissues. Ethanol also has a potentiating effect on GABA-activated inhibition of cerebral cortical neurones and is a potent inhibitor of NMDA-induced calcium currents in some neurones (Charness *et al.*, 1989). All the alcohols including propanol and butanol inhibit cell-cell adhesion (Charness *et al.*, 1994) without affecting cell proliferation or induction and processing of cell adhesion molecules.

The hazards of solvent formulation can be demonstrated by the example of cyclosporin, a drug that was found to be extremely neurotoxic. On investigation however, this neurotoxicity was found to be almost entirely due to the emulsifier Cremophor EL which was used to formulate the product (Brat *et al.*, 1992). Cremophor EL is a mixture of glycerol-polyethylene glycol ricinoleate, polyethylene glycols and their fatty acid esters and ethoxylated glycerol (Brat *et al.*, 1992). It impairs neurite outgrowth and disrupts organellar motility with the initial sign of cytotoxicity being loss of neurite adhesion.

The neurites formed in the presence of Cremophor EL show structural abnormalities with a series of dilatations or beads filled with lipid vesicles (Brat *et al.*, 1992). This beading has a profound effect on the reduction of flux of organelles moving by rapid transport.

Histopathological investigation shows similarities to the dying back neuropathies seen associated with acrylamide or 2,5-hexanedione (Brat *et al.*, 1992).

It is possible that other polyethylene glycol emulsifiers have the same effect. A series of acute poisonings with a glyphosate preparation in Taiwan were later considered to be due to the surfactant in the preparation (Talbot *et al.*, 1991) rendering a product of negligible toxicity into one of high toxicity. A large difference in the oral toxicity of pyrethrum is observed depending on whether it is formulated in corn oil or polyethylene glycol (McCain *et al.*, 1997). It is apparent that the components used in the formulation of a pesticide are critical in determining their mammalian toxicity.

1.11 Health problems associated with pesticide toxicity

Gulf War Syndrome

Following the Iraqi invasion of Kuwait, soldiers from many countries including 697,000 from the USA were sent to fight. Approximately 30,000 of these have experienced unexplained health complaints. These include chronic fatigue, muscle or joint pain, ataxia, rash and problems with concentration and memory. Some of the victims have died. Of soldiers involved from the many other countries, only personnel from Britain and Canada have complained of similar problems (Abou-Donia *et al.*, 1996a).

Despite initial denials from the Governments concerned, it was later admitted (Chemical and Biological Defence Establishment, 1992) that the veterans had been exposed to a combination of biological and chemical environmental toxins. These included multiple immunisations, pesticides, insect repellents such as DEET (N,N-diethyl-*m*-toluamide) and tablets of the prophylactic product pyridostigmine bromide, which were taken every 8 hours as protection against nerve gas attack. Pyridostigmine bromide is a dimethylcarbamate that reversibly inhibits acetylcholinesterase. It is used to confer protection against the irreversible inhibition by organophosphates (Abou-Donia and Wilmarth, 1996). Recent studies have

suggested that the use of these tablets is a significant factor in Gulf War Syndrome. Only troops from the USA, the UK and Canada used this treatment (Abou-Donia *et al.*, 1996a).

A variety of studies have investigated the association of Gulf War Syndrome with the cocktail of chemical exposure, and although no underlying structural or pathological basis for neuronal injury has been shown in Gulf War veterans, findings are consistent with subcortical and brainstem dysfunction (Haley *et al.*, 1997; Hom *et al.*, 1997). No direct relationship to acetylcholinesterase or NTE inhibition has yet been proven in connection with Gulf War Syndrome. However concurrent exposure to specific chemicals along with an increased susceptibility as a result of low levels or increased competition for metabolising enzymes have been suggested as contributing to the syndrome (Abou-Donia *et al.*, 1996a; Abou-Donia and Wilmarth, 1996). Alternatively there may have been an increased access of toxins to tissues of the central nervous system or increased permeability to pyridostigmine bromide by the blood brain barrier in affected individuals, as suggested in relation to the use of DEET as an insect repellent (Haley *et al.*, 1999). Pyrethroids also impair the function of the blood brain barrier (Gupta *et al.*, 1999a; Gupta *et al.*, 1999b). The 'effective concentration' of simultaneous exposure to DEET and pesticides (Abou-Donia and Wilmarth, 1996) may be enhanced by increased competition for hydrolases leading to a diminished breakdown of the products. The steep concentration-response curves for OPs would result in neurotoxicity at reduced concentrations of each of the products, compared to when given individually (Haley *et al.*, 1999).

Haley *et al* (1999) show an association of low levels of PON1 type Q arylesterase activity with Gulf War symptoms. They suggest that in the presence of low levels of combinations of OPs, pyrethroids, DEET and pyridostigmine, serum butylcholinesterases would quickly be covalently bound and inactivated. This may leave the paraoxonase enzyme system as the primary remaining defence system against many of these products. Thus individuals with low PON enzyme activity would be more susceptible to neurotoxic damage.

It is unlikely that a single product is responsible for the vast array of symptoms associated with Gulf War Syndrome. However the synergistic combination of the many products in use may potentially produce symptoms of unexpected duration and severity. Few studies have tried to determine the possibility of synergistic effects, but there is evidence that synergy can increase the toxicity of combinations of pesticides (Abou-Donia *et al.*, 1996a; Abou-Donia and Wilmarth, 1996; McCain *et al.*, 1997) *in vivo*.

Farmers' ill health

Farmers are possibly exposed to pesticides more than any other occupational group. Animals are treated for various health problems, and crops are sprayed with herbicides and pesticides, often many times yearly. Until 1992 sheep farmers had been required to dip twice yearly against sheep scab. Often the pesticide of choice for dipping was the OP diazinon. Many incidences of subsequent ill health have suggested a link between the use of OPs and health problems (Jamal, 1997) and this has been the subject of numerous inquiries (Psychiatrists, 1998; Official Group on OPs, 1998; Behan, 1996). A recent study has shown that 1 in 5 farmers involved in sheep dipping suffered neurotoxic effects (Pilkington *et al.*, 1999), especially those most exposed to concentrates, despite the protection of masks, gloves and other protective clothing.

Official reports have concentrated on the study of the effects of sheep-dips, while ignoring a proportion of farmers whose ill health is not directly related to these products. There is much evidence (Sultatos, 1994; Rosenstock *et al.*, 1991; Jamal, 1997) that other problems exist however, which may be concerned with repeat exposure to a variety of different products.

Chronic Fatigue Syndrome

Behan (1996) discusses the similarities between Chronic Fatigue Syndrome and the delayed reaction to chronic low dose organophosphate exposure (Jamal, 1997), and concludes that they are identical. Chronic Fatigue Syndrome is a debilitating disorder that is increasingly

common. The predominant feature is fatigue, often accompanied by myalgia. Other symptoms include a variety of psychiatric and autonomic problems such as depression, concentration and memory problems, sleep pattern disturbances: all suggestive of hypothalamic dysfunction. Investigation of patients with Chronic Fatigue Syndrome often demonstrates this abnormality, including up-regulation of 5-HT receptors (Behan, 1996). Nearly all patients initially present with influenza-like symptoms, with the fatigue developing later. Hence it was originally called 'Yuppie flu', which mirrors the 'dippers flu' common amongst farmers exposed to organophosphate pesticides. An increased sensitivity to acetylcholine is generally found in these patients (Behan, 1996).

1.12 Toxicity and Synergy

Despite the fact that mixtures of chemicals are in common use, little is known about the toxicity of the combination of different products. In general, toxicity testing is performed on individual products. Synergy occurs when a relatively non-toxic product potentiates a weak effect in another compound to produce a more dramatic effect (Feron *et al.*, 1998, Groten *et al.*, 2001), and is a useful phenomenon in some situations such as when piperonyl butoxide is added to pyrethroids to increase the toxic effects (Casida *et al.*, 1983) as previously mentioned (p. 26). Although sometimes a distinction is attempted between synergy and potentiation, (which is suggested as an increase in toxicity of a substance which already demonstrates a toxic effect by the presence of a second substance), for the purpose of this thesis the two terms are considered as interchangeable.

The accepted method of assessment of the toxicity of combinations of compounds is to assume the combined toxicity is equal to the sum of the individual effects. Abou-Donia *et al* (1996a) have studied the interactions *in vivo* of chlorpyrifos, DEET and pyridostigmine bromide, all of which were used during the Gulf War. Effects on a variety of enzymes as well as changes in bodyweight and survival in the hen were studied. Toxicity of two products given together was found to vastly exceed the sum of the constituents. Combination of the three products produced even greater neurotoxicity.

Similar results were seen with combinations of pyridostigmine bromide, DEET and permethrin (Abou-Donia and Wilmarth, 1996). The authors suggest that test compounds may compete for xenobiotic metabolising enzymes in the liver and blood and that these esterases play an important buffering role in preventing neurotoxicity in the population. Deficits in plasma esterase activity would predispose individuals to neurological effects produced by certain chemical mixtures. McCain *et al.* (1997) studied pyridostigmine bromide, permethrin and DEET *in vivo* by oral dosing of rats, and found significant synergy.

In vitro investigations of synergy have been attempted by a few groups. Marinovich (Marinovich *et al.*, 1996) investigated acetylcholinesterase inhibition and protein synthesis by single pesticides and mixtures of up to five different pesticides in human SY5Y cells. Although acetylcholinesterase action in the mixture was equivalent to that of the most potent, enhanced inhibition of protein synthesis indicated potentiation or synergism.

Acetylcholinesterase would be expected to have an effect threshold as it reached the point where all available enzyme was inhibited. Protein synthesis is not receptor-dependent, so that synergistic effects are more likely. Marinovich (1996) also showed that a combination of benomyl and pirimiphos methyl reduced toxicity, indicating that inhibition interactions also can occur, and suggesting that it is not possible to predict toxicity of pesticide mixtures on the basis of the toxicity of single products.

Synergy is not only seen with combinations of two or more OPs. In kindling studies xylene has been found to act synergistically with chlorpyrifos on seizure production (Wurpel *et al.*, 1993). Abou-Donia *et al* (1991) have also demonstrated synergy with inhalation of vapours of industrial solvents: the aliphatic hydrocarbon n-hexane, or methyl isobutyl ketone increases the toxicity of the OP O-4-nitrophenyl phenylphosphonothioate (EPN). They concluded that the increased neurotoxicity resulted from induction of cytochrome P-450 enzymes that produced more neurotoxic products by metabolic activation. N-hexane is normally chemically unreactive but can be oxidised by microsomal enzymes to other more toxic metabolites. EPN

is also metabolised to more toxic intermediates in this way. Thus variations in the induction and effects of metabolising enzymes can have unexpected effects on combinations of different products, and these effects cannot be dismissed from consideration in the health implications of Gulf War Syndrome victims and farmers.

Potentiation of effects does not always require simultaneous exposure. Pre-treatment with diazinon, the OP most commonly used in sheep dip causes an increase in cocaine toxicity (Kump *et al.*, 1996). In a similar but opposite manner, pre-treatment with phenobarbital induces cytochrome P-450 and reduces toxicity on subsequent pesticide exposure (Chambers *et al.*, 1994).

A major problem with synergism is that the number of potential chemicals that could cause effects is so vast. Howard (1997) calculates that just to test the commonest 1000 chemicals in unique combinations of three would require 166 million different experiments. This would be an impossible task even in an *in vitro* assay, and would be unthinkable using *in vivo* methods.

1.13 In *vitro* detection of neurotoxicity

A variety of different *in vitro* methods have been investigated in an attempt to demonstrate a correlation with *in vivo* toxicity, with varying success. Because the acute toxicity of organophosphates is primarily the consequence of their property as acetylcholinesterase inhibitors, many investigations have used the inhibition of this enzyme as an end point. A variety of different cell types have been used including human SY5Y neuroblastoma cells (Marinovich *et al.*, 1996; Rowles *et al.*, 1995) and various mouse and human neuroblastoma cells (Veronesi and Ehrich, 1993). The results have shown no correlation with *in vivo* toxicity, although Rowles suggested that the esterase inhibition might prove useful for assessing organophosphate pesticides (Rowles *et al.*, 1995). Rowles also investigated inhibition of NTE and considered it useful for neuropathic but not non-neuropathic organophosphates.

Another commonly investigated parameter is the detection of cytotoxicity, as assessed by changes in viability. Veronesi and Ehrich (1993) found mouse NB41A3 neuroblastoma cells to be more sensitive to organophosphates than human SY5Y neuroblastoma cells using the neutral red assay, and the sensitivity was more pronounced with protoxicants than with metabolically activated compounds. However no correlation was apparent between the relative cytotoxic effects and other parameters. Morphological change and cytotoxicity in chick dorsal root ganglia have also been extensively investigated without apparent correlation with known *in vivo* toxicity (Sharma and Obersteiner, 1981; Tuler and Bowen, 1989; Watanabe and Sharma, 1975). Various other potential markers of *in vitro* toxicity have been suggested (Manzo *et al.*, 1996; Fielder *et al.*, 1997) and are being assessed, including the use of brain organotypic spheroid systems for the evaluation of complex events relying on cell-cell interactions in the CNS (Atterwill, 1989).

The measurement of neurite outgrowth inhibition has been successfully used to assess neurotoxicity in organophosphates (Henschler *et al.*, 1992; Flaskos *et al.*, 1994) and other products (Abdulla and Campbell, 1993), and found to be equally promising for each. In our laboratory, rat PC12 pheochromocytoma, human SY5Y neuroblastoma and mouse NB2a neuroblastoma cells have been used to demonstrate neurotoxicity of organophosphates (Flaskos *et al.*, 1994; Flaskos *et al.*, 1998) and other products including other pesticides (McLean *et al.*, 1998) and antimalarial drugs (Fishwick *et al.*, 1998). Elsewhere organophosphates in rat superior cortical ganglia (Jerkins and Kauffman, 1984), excitatory amino acids in mouse NB41A3 neuroblastoma cells (Abdulla and Campbell, 1993) and mercuric oxide in human SKNSH neuroblastoma cells have been investigated for inhibition of neurite outgrowth.

The inhibition of neurite outgrowth at non-cytotoxic concentrations is considered to be related to disruption of organisation and structure of cytoskeletal elements, particularly neurofilaments (Flaskos *et al.*, 1998; Abdulla *et al.*, 1995), although the exact mechanisms are not known.

1.14 Neurotoxic agents and neurodegenerative diseases

The nervous system is not able to repair itself by replication as is possible with other systems when subjected to injury. Thus small cumulative neurotoxic insults have a potential to result in permanent neuronal loss. This may not manifest until adulthood as a result of small cumulative doses from a much earlier age. In this way, OP toxicity may play a role, either directly or due to a synergistic interaction, in the development of neurodegenerative diseases.

Other neurotoxins are heavily implicated in such diseases. The neurotoxin β-N-methylamino-L-alanine (BMAA) found in cycad nuts is thought to play a major role in the production of Alzheimer's Disease, Parkinson's Disease and Amyotrophic Lateral Sclerosis on the island of Guam (Spencer *et al.*, 1987), all three diseases occurring with high incidence and associated with the consumption of these nuts. Neurodegenerative effects and symptoms of such diseases are observed in monkeys fed on this neurotoxin (Spencer *et al.*, 1987). The different diseases are thought to result from different concentration levels or exposure times (Lewin, 1987).

Infusion of another neurotoxin, ibotenic acid, into the nucleus basalis of the brain of rats (Arendash *et al.*, 1987) results in a significant decrease in cerebral cortex choline acetyltransferase activity and acetylcholine synthesis. Fourteen months after treatment, loss of neurones leads to a spongy appearance due to vacuolation in the lateral cortex and hippocampus. These effects are considered to be due to transneuronal degeneration due to loss of cholinergic activity.

Treatment of rats with cuprizone results in spongiform degeneration of the brain, which is considered to result from a direct toxic effect that may involve enzyme inhibition (Love, 1988).

Similar toxic encephalopathies, often presenting with ataxia, convulsions or even death, have been associated with the use of the insect repellent DEET (Zadikoff, 1979; Roland *et al.*, 1985), and with organophosphates (de Reuck *et al.*, 1979).

The OP phosmet has also been hypothesised as being involved in the causation of BSE (Purdey, 1998), although no research has been performed in this area to determine whether there is a link between organophosphates and transmissible spongiform encephalopathies. Although the evidence for this hypothesis is not experimental, it is based on epidemiological observations that areas of high BSE incidence are those subjected to more intensive, compulsory use of phosmet against warble fly infestation (Purdey, 1996, Phillips, 2000). Accidental exposure of sheep to organophosphates produces scrapie-like symptoms (Boffey, 1968).

Before considering this further, I shall describe the nature and characteristics of Transmissible Spongiform Encephalopathies.

1.15 Transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies (TSEs), which include bovine spongiform encephalopathy (BSE), scrapie in sheep and the human Creutzfeldt-Jakob Disease (CJD), are a group of diseases that cause brain degeneration characterised by a striking pathology including progressive vacuolation until the brain resembles a sponge. TSEs can include any or all of the following pathology: spongiform appearance of the brain, astrocytosis and gliosis and deposits of plaque or fibrillary bundles consisting of a protein called the prion or PrP.

TSEs are all associated with an abnormality in the cellular prion protein (PrP^{c}). Until the recent BSE epidemic TSEs were classified as:

a) Inherited TSEs: includes Fatal Familial Insomnia and Gerstmann-Straussler Scheinker Disease in humans.

b) Sporadic TSEs: includes scrapie in sheep, CJD in humans and Chronic Wasting Disease (CWD) in deer. Sporadic TSE is the most common type although it is still very rare. CJD occurs at a rate of 1 in a million in humans.

c) Iatrogenic diseases: caused by surgical intervention such as the injection of growth hormone to increase height, or brain surgery especially involving the use of dura mater.

The advent of BSE has added a fourth category: infective TSEs. The emergence of what appears to be a new type of CJD affecting young people (Collinge *et al.*, 1996) led to a suggested link between eating beef and CJD.

The prion protein: structure and function

The normal cellular prion protein (PrP^c) is a cell surface, glycophosphatidylinositol (GPI) lipid-anchored protein (Borchelt *et al.*, 1993) that is expressed in most cells including both neurones and glia (Kretschmar *et al.*, 1986; Moser *et al.*, 1995). In fact the only cells that have not been found to contain PrP^c are erythrocytes and granulocytes. The PrP gene maps to the short arm of chromosome 20, and is designated PRPN in the human.

Prion protein appears relatively late in embryonic development (Manson *et al.*, 1992). The molecule consists of around 209 amino acids with a molecular weight of 33-35kDa (Weissmann *et al.*, 1996). Its tertiary structure involves a high proportion of alpha helix with a proline-glycine rich repeat area. Prion protein continuously cycles between the membrane surface and a lysosome or endosomal compartment within the cell (Shyng *et al.*, 1993), a passage that takes about one hour. Usually no structural changes occur throughout the cycle, but any such changes, such as cleavage of its anchor will be corrected on passage through the endoplasmic reticulum or Golgi body (Lehmann and Harris, 1997).

The prion molecule possesses two cleavage sites, one on the anchor itself, which is susceptible to cleavage by phosphoinositol-specific phospholipase C (PIPLC) and is the usual site of cleavage. The second site is located at the proline-glycine rich peptide repeat area where a pair of cysteine residues brackets two N-glycosylation sites (Shyng *et al.*, 1993). This site is susceptible to serine protease digestion.

Mice lacking PrP are seen to develop and function without signs of abnormality (Bueler *et al.*, 1992). The function of PrP is not yet fully understood, but there is evidence to suggest that PrP may be involved in resistance of cells to toxicity by oxygen free radicals. Expression of PrP is related to that of the antioxidant enzyme Cu/Zn-superoxide dismutase (SOD-1), and PrP-deficient cells are more resistant to oxidative stress (Brown *et al.*, 1997c).

Many other functions for prion protein have been suggested. Lymphocyte PrP^{c} surface abundance is increased by cell activation, suggesting a role as a surface molecule participating in cell activation (Cashman *et al.*, 1990). The presence of the GPI anchor also suggests this role because such anchors are associated with T-cell activation (Robinson, 1991). Other possible functions include a role as a nerve cell receptor or cell adhesion molecule that directs and maintains the cytoarchitecture of the CNS, due to its location on the nerve cell membrane (Manson *et al.*, 1992). A role in embryogenesis is also considered (Manson *et al.*, 1992), as is the possibility that it is a cell receptor for molecules in the extracellular medium (Shyng *et al.*, 1993). It may also help in the regulation of chemoreceptor numbers at the neuromuscular junction (Harris *et al.*, 1991).

The Infective theory of prion diseases

It is widely considered that a misfolded form of the prion protein is the transmissible agent in these diseases (McKinley *et al.*, 1983). Prion diseases are usually characterised by the presence of an isoform of the cellular prion protein, which has identical composition but with a different conformational shape. In prion diseases the normal cellular PrP^{c} form is converted to a protease-resistant form PrP^{sc} (Bolton *et al.*, 1982; Jendroska *et al.*, 1991) consisting of about 142 amino acids (Weissmann *et al.*, 1996). Conversion to the abnormal form involves the unwinding of the alpha helix and restructuring to produce a structure with a high proportion of beta sheet. This change in conformation is designated as PrP^{sc}, and is a post-translational change because the accumulating PrP^{sc} is observed without any increase in mRNA. The molecule is thought to acquire different characteristics due to this conversion, becoming more insoluble and resistant to digestion by proteinase K. The protease-resistant

PrP^{sc} has the ability to aggregate and can polymerise into amyloid-like fibrils (Singh *et al.*, 1997).

The PrP^{sc} is hypothesised to cause conversion of normal PrP^{c} by associating with normal PrP^{c} and producing an alteration in tertiary structure to the misfolded PrP^{sc} configuration, by a mechanism involving seeding (Kocisko *et al.*, 1994). The most unusual property of the 'infective' nature of the PrP^{sc} is the apparent lack of any associated nucleic acid with the molecule (Prusiner, 1982). Thus a completely novel method of infectivity, without DNA involvement is widely accepted as the method of production of prion diseases.

The TSEs are so called because inoculation of brain and other tissues from infected donors produces the disease in healthy host animals. This feature is often used to determine the presence of prion disease in the donor. The apparent transmission is often given as evidence of the infectivity of PrP^{sc}. However TSEs are not the only diseases that can be produced in this way. Brains from Alzheimer's disease patients inoculated into monkeys can produce Alzheimer's disease in the host (Baker *et al.*, 1994). The experimental disease Experimental Allergic Encephalomyelitis (EAE) is the normal result of injection of brain homogenate including the antigenic myelin basic protein (MBP) (Leibowitz and Hughes, 1983).

Injection of the buffy coat from the blood of Alzheimer's disease patients into hamsters produces TSE-like symptoms with spongiform brain vacuolation and astrocytosis (Manuelidis *et al.*, 1988). Extension of this work showed that buffy coat from normal individuals also produces symptoms and pathogenesis similar to that of prion disease, in the host (Manuelidis and Manuelidis, 1993). In a repeat of the original study by a group in Japan, no spongiform vacuolation was observed, but a proliferation of fibrils developed which was similar to that produced by aluminium (Takeda *et al.*, 1991).

There is additional evidence that PrP^{sc} may not be the cause of prion diseases (Manuelidis and Manuelidis, 1993; Bendheim *et al.*, 1988; Manuelidis *et al* 1987b). TSE transmission has been achieved with no detectable PrP^{sc} in the host (Lasmezas *et al.*, 1997; Hill *et al.*, 1997a).

Wild type PrP^c does not become insoluble and proteinase K resistant like PrP^{sc} when co-expressed with PrP^{sc} in cultured cells (Lehmann *et al.*, 1997). The presence of both types of PrP shows no signs that the misfolded PrP^{sc} has infected the wild type isoform. Protease-resistant and detergent-insoluble PrP is not necessarily associated with prion infectivity (Shaked *et al.*, 1999) and the *in vitro* production of protease-resistant PrP^{sc} does not ensure production of infectivity (Hill *et al.*, 1999).

Toxicity of the prion protein

Biophysical analysis of the structure of the abnormal forms of PrP^c have led to the identification of a 20 amino acid fragment of the molecule (PrP 106-126) that is toxic to neuronal cells *in vitro* (Forloni *et al.*, 1993). This is thought to involve a mechanism that at least partially involves oxidative stress (Brown *et al.*, 1998b): Anti-oxidants can block PrP106-126 toxicity in cerebellar cells (Brown *et al.*, 1996). However the neurotoxic peptide also forms ion-permeable channels in lipid bilayer membranes (Lin *et al.*, 1997), which are freely permeable to common physiological ions, and large enough to mediate apoptosis through a discharge of membrane potential and an influx of calcium, and which may also participate in the cytotoxicity produced by PrP106-126.

PrP106-126 also induces proliferation of astrocytes in culture (Forloni *et al.*, 1994); this stimulation depends on factors released by activated microglial cells (Brown *et al.*, 1998b) since it does not affect astrocytes in the absence of microglia. Only neuronal or glial cells that express PrP^c are susceptible to PrP106-126-induced toxicity or proliferation, respectively (Brown *et al.*, 1996), although it is not clear whether this is a direct result of PrP106-126 interacting with PrP^c on the cell surface. It is more likely that the toxicity of PrP106-126 is directed at the microglia, which in turn have an effect on the neurones, since this fragment is shown to require microglia for the toxicity to be apparent (Brown *et al.*, 1996).

The complex relationship between intracellular and extracellular functions of prion protein is still poorly understood, but the results to date imply that susceptibility of nervous tissue to

prion disease may be regulated by the level of expression of cellular prion protein (Buhler *et al.*, 1993). Animals engineered to produce twice as much PrP^c succumb to disease much quicker than normal genotypes (Weissmann *et al.*, 1996). Prion knockout animals are resistant to PrP transmission, and those with half the normal level of PrP also show enhanced resistance (Buhler *et al.*, 1993).

Post-translational changes in prion protein

Post-translational modification of prion proteins may also affect their function. The protein cycles between the cell surface and an endocytic department with almost all the internalised PrP^c returning to the cell surface intact (Shyng *et al.*, 1993). PrP^{sc} however, accumulates inside the cell. PrP^c contains two consensus sites for glycosylation (Lehmann and Harris, 1997) at asparagine residues 181 and 197 in the human. N-glycosylation of PrP^c at these specific sites protects against conversion to the PrP^{sc}-like form and permits efficient cycling between plasma membrane and intracellular compartments, and although it is not essential (Lehmann and Harris, 1997), the absence of glycosyl groups may increase the efficiency of conversion. Action by the enzyme mannosidase II enables these oligosaccharides to become resistant to digestion by endoglycosidase-H (endo-H), which the molecule encounters on its passage back to the surface. Absence of glycosyl groups at the appropriate sites for whatever reason, will compromise the passage of the molecule to the surface. The degree of effect on cycling of the molecule depends on whether one or both glycosylation sites are present. Different proportions of glycosylated molecules are postulated as being responsible for strain differences in CJD and other diseases (Collinge *et al.*, 1996). The bovine genetic disease mannosidosis causes symptoms that in many ways resemble spongiform disease (Hocking *et al.*, 1972), and is caused by a lack of the enzyme mannosidase responsible for protein glycosylation.

A small quantity of surface membrane prion protein is found to be unglycosylated or only partially glycosylated (Lehmann and Harris, 1997). It is likely, given the difficulty of cycling without the presence of the glycosyl groups as discussed above (Lehmann and Harris, 1997)

that this loss of oligosaccharides occurs after expression on the membrane. Mutations in PrP that produce a loss of the anchor (Singh *et al.*, 1997) also have a noticeable effect on cycling within the cell.

Despite our knowledge of the above, the other molecular mechanisms involving the prion protein and their effects on neuropathology are still poorly understood (Phillips *et al.*, 2000). Any post-translational modification of PrP^c may alter its ability to regulate resistance to oxidative stress and so alter the susceptibility of cells to toxicity. To date, little information is available about post-translational modifications of prion protein, although Gordon *et al* (Gordon *et al.*, 1998) have reported post-translational changes in cells treated with the organophosphate phosmet. This may be relevant to the incidence of BSE, which, as has been argued above, correlates with high-dosage systemic use of phosmet on cattle (Purdey, 1996).

Investigation has discovered 4-6 sites on the PrP molecule that can bind copper (Brown *et al.*, 1997b). The binding of copper appears to promote the conformational shift of PrP from predominately alpha helix to beta sheet (Stockel *et al.*, 1998). Cuprizone, a chelating agent, has long been known to produce TSE-like pathology when fed to animals (Pattison and Jebbett, 1971). The effects are likely to be due to the depletion of copper from the cells (Hornshaw *et al.*, 1995; Stockel *et al.*, 1998) and would be assumed to include any bound to the prion molecule. Astrocytosis accompanies these effects (Love, 1988) in the same way as is seen in TSEs. Cuprizone is a disubstituted hydrazine and is known to inhibit monoamine and cytochrome oxidase, and succinate dehydrogenase, as well as reducing the copper content of the brain (Love, 1988). Prion knockout animals have a reduced brain copper content, as well as reduced SOD-1 activity (Brown *et al.*, 1997c) leading to reduced ability to deal with oxidative stress. Similar reductions in SOD-1 are seen in cuprizone-treated animals (Love, 1988; De and Subramanian, 1982) although dietary-deficient animals do not seem to be affected in the same way.

Immune response

A lack of immune involvement in prion diseases has been accepted because of an apparent lack of standard inflammatory response (Porter *et al.*, 1973). However the immune system may be implicated in TSEs. Animals without functioning T or B-cells (nude mice) cannot develop TSEs (Klein *et al.*, 1997) and mice with severe combined immune deficiency (SCID) do not develop TSEs after intraperitoneal injection of prion protein, although they do succumb after intravenous injection (Klein *et al.*, 1997). Although antibody does not appear to play a role in TSEs, B-cells have an undoubted role (Brown, 1997), because mutations that disrupt B-lymphocytes prevent development of TSEs (Klein *et al.*, 1997).

Involvement of growth factors and cytokines

Growth factors and cytokines are also implicated in the passage of prion diseases. In PC12 cells, as a model for neurones, expression of PrP^c mRNA increases after treatment with Insulin-like Growth Factor (IGF-1) or human Growth Factor (hGF) (Lasmezas *et al.*, 1993), IL-2 and TNF-α (Satoh *et al.*, 1998) or IL-6 (Lazarini *et al.*, 1994) *in vitro*. Expression of PrP^c mRNA also increases after differentiation induced by Nerve Growth Factor (NGF) (Mobley *et al.*, 1988), and removal of NGF from differentiating PC12 cells results in rapid loss of PrP (Rubenstein *et al.*, 1990).

Scrapie-induced neuronal loss is attenuated after treatment with basic fibroblast growth factor (Fraser *et al.*, 1997). IL-6 immunoreactivity together with IL-1β and TNF-α is heightened in astrocytes and microglia around amyloid deposits of brains infected with prion diseases (Williams *et al.*, 1994). Similar levels have been found in Alzheimer's disease brains. There is also a marked up-regulation of prostaglandins. All these cytokines are associated with areas of vacuolation, suggesting that damage to astrocytes results in the production of cytokines, which then leads to further damage.

Because cytokines are so intrinsically associated with these diseases, it is possible that PrP expression and susceptibility to PrP-induced neurotoxicity or prion disease may in a similar way, be regulated by externally introduced factors such as environmental toxins or drugs.

Astrocyte involvement

There is considerable evidence that TSEs begin with effects on astrocytes and microglia, as a result of the toxic effects of the PrP106-126 fragment. Effects on neurones, although producing the more obvious pathology of TSEs, may be a secondary reaction in response to glial-derived cytokine production (Forloni *et al.*, 1994). Microglia are thought to undertake this neurotoxic role in response to cytotoxic injury in the CNS (Rogove and Tsirka, 1997), and this may involve production of tissue plasminogen activator (tPA).

PrP appears to prime astrocytes to respond to proliferation signals released by microglia (Brown *et al.*, 1998b). These signals are not specific but are mitogenic factors normally released by microglia. Astrocytosis is only seen in the presence of microglia. Impairment of astroglial function, or an imbalance between astrocytic and microglial activity has been suggested as contributing to neurodegenerative diseases including the TSEs (Mucke and Eddleston, 1993). PrP^{sc} appears to accumulate first in the astrocytes in some TSEs (Diedrich *et al.*, 1991). This occurs before astrocytosis, and accompanying morphological transformations are succeeded by the progression of PrP^{sc} from the astrocyte cell bodies to pericellular sites and then throughout the neuropil.

In the cerebellum, sites of astrogliosis coincide with the distribution of PrP^{sc}. Even when there is a high concentration of PrP^{sc} there is no pathology, although there is a corresponding rise in the expression level of glial fibrillary acidic protein (GFAP) (Moser *et al.*, 1995). The fact that PC12s and NB2a cells can support PrP^{sc} replication without ill effect is further evidence for significant glial involvement (Moser *et al.*, 1995). It is possible that many of the clinical and histopathological signs associated with spongiform diseases are due to secondary cytokine production following initial induction of astrocytosis.

Possible influence of organophosphates in prion diseases

In so far as the mechanism of neurotoxicity of prion proteins is understood, there is some evidence that phosphorylation processes are involved. Prion protein fragment-induced glial cell proliferation involves increases in intracellular calcium and, although there are clear differences between the cytotoxicity produced by prion protein fragments and β-amyloid peptide (Hope *et al.*, 1996), some forms of syndrome associated with a prion gene mutation also lead to formation of hyperphosphorylated tau proteins (Tranchant *et al.*, 1997). Such hyperphosphorylation of tau is commonly seen in Alzheimer's disease (Selkoe, 1994). Phosphorylated neurofilament epitopes have been found in cases of CJD (Nakazato *et al.*, 1990).

Although further experimental evidence of this is still lacking, many factors do indicate a potential link between organophosphates and TSEs. As previously discussed, scrapie-like symptoms have been observed in sheep killed by accidental spraying of nerve gas agents (Boffey, 1968). In addition TSE-like pathology including vacuolation has been found in some victims poisoned by overexposure to OPs (de Reuck *et al.*, 1979). Prion protein is concentrated in cholinergic neurones (Harris *et al.*, 1991), themselves the targets for OP toxicity. Increased levels of choline acetyltransferase (ChAT), the enzyme responsible for catalysing the formation of acetylcholine from its precursors choline and acetyl-CoA, is also associated with increased PrP (Mobley et al., 1988; Wion *et al.*, 1988). Kitamoto *et al.* (1992) has shown that PrP^{sc} accumulates in synapses during disease progression, although whether this accumulation was at the presynaptic or postsynaptic region was not determined.

An increase in GABA is observed early in the brains of scrapie-inoculated animals (Lu *et al.*, 1995), which suggests an interaction of PrP with the GABAergic system. As discussed, some OPs have a similar effect on GABA (Brimijoin and Koenigsberger, 1999).

There is some further circumstantial evidence of possible OP involvement in TSEs. Mayer *et al.* (1978) suggests that a cluster of CJD in Czechoslovakia may have an environmental trigger or an inherited susceptibility. Similarly there is a recognised high incidence of CJD amongst farmers in the UK and elsewhere (Hill *et al.*, 1997b), and farmers are among those occupationally exposed to greatest levels of pesticides. Solvents and pesticides are known to produce encephalopathies in some individuals (Charness *et al.*, 1989; Roland *et al.*, 1985; de Reuck *et al.*, 1979). Thus the suggestion of possible interactions between organophosphates and prion protein is not without foundation.

1.16 Alzheimer's disease

Alzheimer's disease is another neurodegenerative disease involving the deposition of plaques and tangles with loss of neurones leading to degeneration of specific areas of the brain, and OPs may influence this disease in a similar way. Alzheimer's disease is characterised by gradual loss of cognitive and psychomotor abilities. It has been studied for much longer than the prion diseases and greater knowledge has accumulated about the disease, although the causes of the degeneration and involvement of various biochemical pathways are still uncertain.

The manifestation of Alzheimer's disease

Alzheimer's disease is associated with the deposition of β-amyloid, a length of 39-43 amino acid residues cleaved from a larger molecule, the β-amyloid precursor protein (β-APP). β-APP, unlike the GPI-anchored prion protein, is a transmembrane protein comprising 770 amino acids with a single membrane-spanning domain at amino acids 700-723. The β-amyloid begins 28 residues inside the transmembrane domain and extends 11-15 residues inside it (Selkoe, 1994). β-APP is released in soluble form in high concentrations by apparently normal intact cells. It is detected as a circulating peptide in the plasma and cerebrospinal fluid (CSF) of mammals (Haass *et al.*, 1992).

Cleavage of the β-amyloid precursor protein is usually performed by the protease α-secretase, which results in secretion of the large soluble ectodomain into the medium and retention of an 83-residue C-terminal fragment in the membrane, which can then be further cleaved to release other small peptides. Because the cleavage is within the β-amyloid region, it is likely that an attenuated or abnormal processing must occur to release the β-amyloid (Selkoe, 1999). This is thought to occur in the lysosomes after β-APP is internalised and targeted to late endosomes/lysosomes. This second processing pathway for β-APP does not involve cleavage within the β-amyloid area. Instead it involves cleavage by the β-secretase enzyme and results in a slightly truncated version of the β-APP molecule and the retention of a C99 peptide. This is then further cleaved by γ-secretase at residue 711 to release β-amyloid-40 or at 713 to release β-amyloid-42 peptides (Selkoe, 1999).

Alzheimer's disease is characterised by distinctive plaques of two main types, as well as by neurofibrillary tangles (Selkoe, 1994). The classical mature plaques are spherical deposits of dystrophic (dilated) neurites, with associated activated microglia and reactive astrocytes around the periphery of the plaque. Numerous other proteins are also associated with these structures, including the cytoskeletal protein tau and ubiquitin. Similarities to the plaques of TSEs are obvious.

Most plaques however do not form this classical neuritic type, which are found predominantly in the hippocampus, amygdala and entorhinal cortex. Far more abundant are the amorphous, roughly spherical and less dense deposits of β-amyloid in the diffuse or pre-amyloid plaques. Very few if any structurally altered neurites, astrocytes or microglia are associated with diffuse plaques. Amyloid filaments are also sparse or absent. Instead the plaques are composed almost exclusively from the 42-residue β-amyloid, which is produced in much lower quantities, consisting of about 10% of the total secreted β-amyloid (Selkoe, 1999).

Phosphorylation in Alzheimer's disease

Stimulation of phosphorylation in β-APP processing, such as by activation of protein kinase C (PKC) results in increasing secretory cleavage of β-APP (Saitoh *et al.*, 1991). The β-APP molecule is phosphorylated on serine residues, and the secreted molecule is detected as phosphorylated β-APP. Stimulation by phorbol esters however results in increased secretion, which occurs independently of direct phosphorylation of β-APP, as there is no increased phosphorylation of the holoprotein. PKC activation substantially decreases β-amyloid production, independently of β-APP phosphorylation. This indicates that PKC does not mediate β-APP secretory processing directly, but involves additional protein messengers (Selkoe, 1994).

Function of β-Amyloid Precursor Protein

The first function of β-APP was described after identification of a Kunitz protease inhibitor (KPI)-like motif within the molecule, now known to be identical to protease nexin II. This is a KPI-like inhibitor of some serine proteases such as α-chymotrypsin (Van Norstrand *et al.*, 1989).

It is also postulated that β-APP possesses growth–promoting or autocrine functions which lead to proliferation. This is controlled by a functional determinant located just beyond the KPI domain that has neurite promoting properties. β-APP also confers neuroprotection on cells by down regulating levels of calcium in neurones (Selkoe, 1994).

Both direct and indirect evidence suggests other possible functions for β-APP as a mediator of cell-cell or cell-substrate adhesion (Schubert *et al.*, 1989). Enhanced neurite outgrowth is seen after β-APP addition to cultures of neurones. A distinct region of β-APP appears to interact with an unknown molecule on the neuronal surface to increase neuronal adhesion and lead to neurite extension and this is related to a further role in binding of molecules such as heparin and certain metallic cations such as zinc. The neurotrophic role of β-APP in cell

adhesion may act by mediating the trophic effects of NGF (Milward *et al.*, 1992). A role of β-APP as a glycosylated cell surface receptor is also suggested (Kang *et al.*, 1987) because of structural similarities of this protein with other receptors.

Studies by Yankner *et al* (1990b) have shown that the addition of β-amyloid to cultures of cells results in a neurotrophic neurite-promoting activity, possibly by interacting with the extracellular molecules in the nervous system. This suggests that an inappropriate trophic stimulus causes aggregations. However this neurotrophic effect is age-dependent, as the same concentration of β-amyloid added to more mature cell cultures produces increased toxic effects involving reduction and degeneration of neurites leading eventually to cell death (Yankner *et al.*, 1990b).

Like PrP, loss of β-APP does not lead to detrimental effects *in vivo*. AD patients do not appear to lose a fundamental cellular function of β-APP. The β-APP mutation acts by a gain in function i.e. the increased production of the potentially cytotoxic β-amyloid fragments.

β-Amyloid - cause or effect?

The processing of β-APP and its cleavage to produce β-amyloid probably has a role both as a cause of AD and as an effect of aberrant metabolism elsewhere in the brain. Patients with one specific molecular cause of AD produce a missense mutation in β-APP (Selkoe, 1999). β-APP also appears to be responsible for the AD-like phenotype seen in patients with trisomy 21. An increased expression of β-APP is likely to produce early formation of diffuse β-amyloid deposits. However in many autosomal dominant cases of AD β-amyloid deposits cannot be said to be causative, but the morphology, distribution and immunochemical reactions are indistinguishable in β-amyloid and β-APP-linked cases.

β-Amyloid is generated continually as a soluble peptide during normal cellular metabolism and does not require cellular injury or aberrant proteolysis of β-APP (Seubert *et al.*, 1993). This raises the possibility that β-amyloid has a physiological function throughout life. The

cellular release of β-APP derivatives can be controlled by cell surface and neurotransmitter receptor activity (Nitsch *et al.*, 1992). Activation of muscarinic acetylcholine receptors m1 and m3 stimulates β-APP release, whereas activation of m2 and m4 does not. Stimulation of m1 and m3 receptors activates protein kinase C by increased diacylglycerol formation, as a product of phosphoinositol hydrolysis. Although PKC phosphorylates β-APP, there may be other proteins involved in its release. Secreted β-APP lacks a COOH terminus (Shoji *et al.*, 1992). Thus any alterations in the receptor-mediated release could lead to the generation of amyloidogenic or toxic APP fragments.

Analysis of cerebrospinal fluid (CSF) of normal and AD patients shows that β-APP is normally cleaved at both cleavage sites (Shoji *et al.*, 1992). Thus normal cleavage is able to release β-amyloid fragments. The amount of amyloid deposited depends on the rate of production, the rate of removal and the rate at which it forms insoluble fibrils.

Toxicity of β-amyloid

β-Amyloid deposition probably plays a role in the early pathology of all AD cases. Dystrophic neurites, reactive astrocytes and activated microglia are more in evidence in plaques associated with fibrillar than non-fibrillar β-amyloid. This implies a toxic role, either directly from the fibrillar β-amyloid or from increasing toxic effects involving other biologically active proteins.

In a similar manner to the toxicity of PrP106-126, the fragment β-amyloid25-35 is neurotoxic to cultured neurones. This may result either from direct toxicity or from an indirect mechanism that makes the neurone more susceptible to neurotoxicity from excitatory amino acids or other neurotoxins (Mattson *et al.*, 1992). β-amyloid is not toxic to astrocytes (Yankner *et al.*, 1990b), and the toxicity observed with neurones appears to be due to an elevation in calcium levels in the cells.

The presence of β-amyloid increases the sensitivity of neurones to glutamate toxicity (Mattson *et al.*, 1992). Combinations of β-amyloid with NMDA or kainate are much more neurotoxic that the products singly and this seems to depend on calcium influx. Combination of β-amyloid with glutamate also markedly increases neuronal immunoreactivity compared with each component separately. This suggests that environmental factors may determine whether a neurone is vulnerable or not to β-amyloid toxicity (Mattson *et al.*, 1992).

Although both PrP106-126 and β-amyloid25-35 are toxic and have many similarities, they appear to produce their effects in different ways. Both peptides cause free radical production in other cells but the toxicity of β-amyloid is not attenuated by anti-oxidants (Brown *et al.*, 1997a) and does not induce proliferation in microglia like the PrP106-126-mediated toxicity. β-Amyloid is toxic to microglia (Korotzer *et al.*, 1993b), which produce toxic free radicals and TNF-α in its presence (Meda *et al.*, 1995), and both β-amyloid25-35 and PrP106-126 induce cell death by activation of voltage-sensitive calcium channels. The effect by PrP106-126 can be blocked by NMDA receptor antagonists suggesting that calcium influx is through NMDA receptors, whereas such treatment has no effect on β-amyloid toxicity (Brown *et al.*, 1997a). Forskolin inhibits the toxicity of β-amyloid25-35 whereas it does not have the same inhibition on PrP106-126 toxicity.

Just as conformational changes in the prion protein are instrumental in TSEs, changes in the β-amyloid appear to be associated with the neurotoxicity of Alzheimer's disease (May *et al.*, 1993). Classic features of β-sheet structure are present in aggregating toxic β-amyloid peptides but not in non-aggregating, non-toxic peptides. Similar protein fragments such as human amylin as well as the PrP106-126 also cause neuronal degeneration (May *et al.*, 1993; Pike *et al.*, 1995). The self-assembly of the plaques and tangles is driven by the conformational properties, producing increased β-pleated sheet and suggests that this factor may be involved in β-amyloid as well as PrP toxicity

The widespread distribution of dystrophic and degenerating neurites around neuritic plaques suggests that β-amyloid can produce more distant effects, such as disruption of axonal

transport or transynaptic degeneration (Paudel, 1997). This could result in altered cytoskeletal elements including the production of paired helically bound 10nm filaments (PHF), which are also a morphological characteristic of AD. The subunit of PHFs is the microtubule-associated protein tau, but is a hyperphosphorylated and insoluble form of the normally highly soluble protein (Busciglio *et al.*, 1995). Often it is conjugated with ubiquitin, suggesting an unsuccessful attempt at removal (Bancher *et al.*, 1989). Ubiquitin is not specific to neuritic plaques, being found in many other types of neurodegeneration, although its role in this respect remains unknown. The PHF tangles are also found in many other neurodegenerative diseases without neuritic plaques of β-amyloid deposits (Wisniewski *et al.*, 1979). Aggregated and hyperphosphorylated tau is more likely to be accompanied by adverse effects on function and stability of the cytoskeleton (Busciglio *et al.*, 1995). These PHFs may therefore be responsible for neuritic dysfunction or may directly produce cell death.

Exposure of hippocampal neurones to very low concentrations of β-amyloid alone up-regulates NGF receptors, while addition of combination of NGF with low concentrations of β-amyloid results in neurodegenerative changes in NGF-positive neurones (Yankner *et al.*, 1990a). Despite the fact that NGF treatment improves age-related memory impairment in rats, this striking potentiation of NGF on the neurotoxic effects of β-amyloid involves an increase in the toxicity by a factor of 100,000. Other growth factors do not have this effect (Mobley *et al.*, 1988). As well as increasing PrP, injection of NGF into the brain increases mRNA for β-APP in regions containing NGF-responsive cholinergic neurones, accompanied by an increase in choline acetyltransferase (Mobley *et al.*, 1988). Many other proteins accumulate within the mature plaques, including kinases (Saitoh *et al.*, 1991; Clayton and George, 1998), proteases and esterases (Small *et al.*, 1991). There may be complex alterations in secondary feedback mechanisms that aggravate the initial damage, and an inflammatory response is present (Eikelenboom *et al.*, 1994)

Because β-amyloid plaques are located mainly in the grey matter, like the PrP plaques in TSEs, this suggests that neurones are the most likely source of the peptide. However multiple isoforms of β-APP are found in astrocytes (Siman *et al.*, 1989), and β-amyloid can be

detected as fibrils inside microglia (Wisniewski *et al.*, 1989). Thus all these cells may contribute to β-amyloid deposits to some degree. Even the plasma has been found to contain soluble β-amyloid (Seubert *et al.*, 1993) and has a potential role in plaque deposition.

Genetic effects

Alzheimer's Disease is associated with 4 genetic mutations. One normal polymorphism of the apolipoprotein-E (Apo-E) gene is responsible for increased susceptibility and lowers the age of late onset AD to the 60s and 70s. There is some evidence that a different Apo-E allele confers some protection against AD (Selkoe, 1999).

Further genetic involvement is seen in the presenilin-1 and presenilin-2 genes. These are two genes implicated in aggressive early onset AD, usually inducing the disease between the ages of 40 and 60. There is some evidence that these presenilins are actually the γ-secretase enzymes that produce the β-amyloid molecules, but this needs further confirmation (Selkoe, 1999). One further gene, responsible for coding β-APP, is implicated in AD production, although this accounts for less than 0.1% of all AD cases. Inheritance of any of the four genes leads to increased production or increased accumulation of β-amyloid (Selkoe, 1999).

Acetylcholinesterase involvement in Alzheimer's disease

Cholinesterases are heavily involved in the production of AD and OPs have been investigated as a therapy to improve symptoms (Pakaski *et al.*, 2000; Lahiri *et al.*, 1998; Chelliah *et al.*, 1994). Anticholinesterases have a major effect on enzymatic activity of amyloid plaques and tangles, as well as the axonal and perikaryal acetylcholinesterases (Mesulam *et al.*, 1987). In AD patients relatively little acetylcholinesterase remains in normal axons in the cerebral cortex, whereas almost all the cortical acetylcholinesterase activity may be confined to the plaques and tangles. This may, however, be due to *de novo* production arising in response to the degenerative process of AD (Geula and Mesulam, 1989).

The cholinesterases in the plaques seem to have molecular differences to those in normal neurones, and this is probably due to extensive degeneration of cholinergic pathways that produces conformational shifts as the plaques form. Cholinesterases may themselves play a role in the pathogenesis of AD, since they are known to be powerful proteases that may transform a circulatory β-APP into the insoluble subunit of the plaques (Small *et al.*, 1991). They may even contribute to the degenerative changes in cytoskeletal proteins leading to plaque and tangle formation.

The enzymatic properties of the plaque and tangle cholinesterases have been shown to differ from those in normal cells (Wright *et al.*, 1993). These differences include increased affinity for proteases and a greater protease inhibition than cellular cholinesterases.

The protease activity associated with acetylcholinesterase stimulates β-APP release from cells. On release the β-APP is thought to inhibit the protease that released it (Small *et al.*, 1991). Acetylcholinesterase involvement does not just result from degeneration of cholinergic regions. Some areas, such as the neocortex and hippocampus have plenty of acetylcholinesterase-rich neurones, but have no detectable choline acetyltransferase activity because they are not cholinergic cells and do not receive direct cholinergic input. These regions are also depleted in AD, which may be because the protease activity of the enzyme is lost, resulting in localised loss of ability to cleave the β-APP within the β-amyloid sequence which increases the concentration of the full-length form of β-APP (Small *et al.*, 1991).

Brain acetylcholinesterase forms stable complexes with β-amyloid during its assembly into filaments (Alvarez *et al.*, 1998). The toxicity of the complexes is higher than that of the β-amyloid aggregates alone, leading to increased degeneration. The macromolecular complex is very stable, and is only partially broken down by detergent buffers or chaotrophic agents such as guadinine hydrochloride.

Plaque-associated acetylcholinesterase has unique properties. It is resistant to low pH and excess acetylcholine, and is more resistant to inhibition by anticholinesterase agents than free

enzyme (Alvarez *et al.*, 1998), requiring a 5-10 times greater concentration to reach the same level of inhibition as when not in a complex with β-amyloid. This may be due to physical properties such as being enmeshed in a fibrillary environment and therefore difficult for the inhibitor to locate. Only a small amount of acetylcholinesterase is required to promote aggregation into fibrils (Alvarez *et al.*, 1997). The acetylcholinesterase-β-amyloid complex may have a role at the beginning of the amyloidogenic process, acting as a nucleus to increase the rate of fibril formation and stabilize the growing amyloid.

β-amyloid plaques are often found in non-demented individuals suggesting that β-amyloid deposition is not the sole factor in AD production, but the formation of the acetylcholinesterase-β-amyloid complex may be the amyloidogenic factor (Alvarez *et al.*, 1997). The hydrophobic region of the β-amyloid is important in the fibrillogenesis.

Neuronal differentiation is accompanied by a decrease in secreted β-APP and an increase in full-length membrane-associated protein (Bronfman *et al.*, 1996). Neurite outgrowth elicits elevation of total acetylcholinesterase activity, although only of specific molecular forms. In NB2a cells, acetylcholinesterase (but not butyrylcholinesterase) gradually augments with greater levels of cell confluence. Total acetylcholinesterase activity increases 2.8-fold in differentiating, compared to proliferating, cells with the increase due to the enhancement of the G1 and G4 forms of the molecule.

Drugs that block acetylcholine muscarinic receptors have long been known to disrupt higher cognitive functions (Longo, 1966), and cholinergic neurones have a central role in memory. Patients who have died of AD have choline acetyltransferase levels reduced by 60-90%, with cholinergic muscarinic receptors decreased in the cortex of AD patients in parallel to the acetylcholinesterase reduction.

Astrocyte and microglial involvement

In both Alzheimer's disease and TSEs the plaques are associated with reactive microglia and astrocytes, and there is abundant evidence of a microglial-derived inflammatory response in both diseases (Combs *et al.*, 1999). Sustained exposure of the cells to fibrillary β-amyloid (or PrP) prolongs activity of tyrosine kinase-based signaling events. This is regulated not by an influx of extracellular calcium but by intracellular calcium changes (Combs *et al.*, 1999). This is also a factor necessary for activation of MAP kinase, and for protein kinase C activity. Microglia release proinflammatory products, including IL-1 that increase the production of β-amyloid (Goldgaber *et al.*, 1989).

An up-regulation of plasminogen activator inhibitor (PAI) is seen in microglia from AD brains, particularly those associated with plaques. Activation and imbalance of this inhibitor may play a role in the neurite outgrowth and subsequent neurite damage of the disease (Akiyama *et al.*, 1993). β-Amyloid has a marked stimulatory effect on plasminogen activation by tPA (Kingston *et al.*, 1995), especially when it has formed plaques. The presence of β-amyloid deposits may cause the pathogenesis by inappropriate stimulus of plasminogen activation by tPA.

1.17 The role of tau in Alzheimer's disease

β-Amyloid increases cell survival and promotes neuritic branching, with increased arborisation and number of dendrite-like processes (Whitson *et al.*, 1990). It has a trophic effect on hippocampal neurones *in vitro* (Whitson *et al.*, 1989) as well as inducing aberrant branching. Loss of inhibitory factors (Uchida and Tomonaga, 1989) may lead to sprouting and to the massive tau-immunoreactive neuritic network in AD brains. It is not certain whether the sprouting is a response to degeneration or whether the sprouting is induced by a trophic factor which leads to exhaustion and subsequent degeneration (Ihara, 1988).

Tau in AD is phosphorylated at three positions (Saitoh *et al.*, 1991) not normally phosphorylated, and this probably occurs before the formation of the tangles. The

phosphorylation of tau is not limited to AD and TSEs, but is seen in many neurodegenerative diseases (Saitoh *et al.*, 1991). Aging appears to render the brain vulnerable to β-amyloid toxicity. Fibrillar, but not soluble β-amyloid produces phosphorylated tau. However fibrillar β-amyloid does not produce significant neurone loss, tau phosphorylation or microglial proliferation in young monkeys (Geula *et al.*, 1998). All these effects are seen however in elderly animals. Only when the concentration of β-amyloid in the inoculation is 100 times higher do young monkeys succumb to the effects. This age dependent toxicity may be due to the loss of a neurotrophic factor or to the appearance of a neurotoxic cofactor. Additionally microglial reactivity to β-amyloid may increase with increasing age.

1.18 Summary of aims of thesis

In summary, the aim of this thesis is to demonstrate interactions with organophosphates, as detailed in the hypotheses previously stated (1.1). Firstly an *in vitro* assay will be developed and used to identify and quantify any interactions between organophosphates and other substances, including solvents of formulations. This assay will be developed further to detect differences in toxicity resulting from chronic low-level exposure to the organophosphate diazinon. Finally the thesis will investigate whether organophosphates can also interact with the intrinsic cell proteins, prion, β-amyloid and tau as well as with microglial cells, in a way which may interfere with normal cell function and consequently contribute in some way to neurodegenerative diseases.

CHAPTER 2

METHODS

2.1 Materials

All reagents and chemicals were supplied by Sigma Chemical Co. Poole, UK unless otherwise stated.

2.2 Cell Culture

1 Maintenance of NB2a cells

Mouse neuroblastoma cells of the NB2a strain (ECACC cell line: 89121404) were grown in 75 or 150 cm^2 plastic tissue culture flasks (supplied by Falcon/Fred Baker, Runcorn, UK) in a humidified 37°C incubator with a 5% CO_2 atmosphere. They were maintained in a proliferation medium consisting of 4500 mg/L glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL Life Technologies, Uxbridge, UK), supplemented with 5% heat inactivated fetal calf serum, 5% heat inactivated horse serum (Gibco BRL Life Technologies, Uxbridge, UK), 10000 U/ml penicillin plus 100 µg/ml streptomycin and 25 µg/ml gentamycin.

Cells were passaged at approximately three-day intervals, depending on the state of confluence. Cells were removed from the flask by gentle agitation while rinsing in medium with a 5 ml plastic syringe fitted with a plastic syringe extension. The contents of the flask were then transferred to a 20 ml Sterilin pot (Bibby Sterilin Ltd, Staffs., UK) and centrifuged at 1000 rpm for 7 minutes. The supernatant was decanted and the pellet was re-suspended by triturating 5 or 6 times with a 1 ml pipette. Between 50 and 80 µl of cell suspension were transferred to a new flask and approximately 15 ml of proliferating medium were added.

The cells were differentiated by plating cells on plastic culture flasks or in welled plates at the required density. After 24 h, during which time the cells adhered to the plastic, the medium was removed and replaced with serum-free medium containing 1 mM dibutyryl cyclic AMP supplemented with 10000 U/ml penicillin plus 100 µg/ml streptomycin and 25 µg/ml gentamicin. Removal of serum caused the cells to differentiate, leading to the growth of neurites by the cells.

Stocks of NB2a cells were maintained in cryovials in liquid nitrogen. When required they were defrosted quickly by placing in a water bath at 37°C. The contents were transferred from the cryovial to a 1.8 ml Eppendorf tube and centrifuged at 1000 rpm for 7 min. The pellet was re-suspended in 1 ml proliferation medium. A further 10 ml proliferation medium was added and the cell suspension transferred to a Sterilin pot. The suspension was re-centrifuged at 1000 rpm for a further 7 min, to completely wash out the freezing mixture (see below), and the pellet again re-suspended in 1 ml proliferation mixture before transfer to a plastic tissue culture flask in a 15 ml volume of proliferation medium. For this first period of cell growth the normal proliferating medium was supplemented with an extra 5% fetal calf serum. A further 1 ml of conditioned medium (medium from cells during normal growth) was also added to the first passage if necessary to achieve better growth.

To re-freeze the NB2a cultures, cells were plated into plastic culture flasks and grown to confluence. The cells were then removed from the flask as before, and centrifuged at 1000 rpm (400 g) for 7 min. The supernatant was discarded and each pellet was re-suspended in 1 ml of DMEM and re-centrifuged at 1000 rpm for 7 min to wash out the antibiotics from the proliferating medium. The supernatant was again discarded and the pellet was suspended in 1 ml of freezing medium. This consisted of DMEM containing 20% (v/v) fetal calf serum and 5% (v/v) sterile dimethyl sulphoxide (DMSO). Cell aliquots consisted of 10^6 to 10^7 cells/ml.

Each 1 ml cell suspension was transferred into a cryovial and placed in the cell freezing equipment, which gradually lowered the samples into liquid nitrogen over a period of one hour. The cryovials were then transferred into a labelled cryosleeve in the liquid nitrogen.

2 Preparation of cells chronically pre-exposed to diazinon

It has been postulated that exposure to chronic low levels of organophosphate pesticides can have a detrimental effect on the health of individuals, not anticipated from the known acute toxicity of these products. To simulate the effects of chronic exposure to organophosphate pesticides, NB2a neuroblastoma cells were cultured as usual but with the inclusion of 25 μM diazinon (1:1000 dilution from stock of diazinon in methanol) added to the proliferation medium at every passage. Diazinon was used since it is commonly implicated in the health problems reported concerning these products. This concentration was chosen because preliminary studies demonstrated negligible effect on cell growth and neurite extension.

Cells from this culture were used in various studies detailed below in place of the normal cells. A minimum of 30 passages was performed before use of these cells, and most experiments used cells that had been passaged 40-60 times including diazinon.

3 Measurement of neurite outgrowth

Confluent cell cultures were harvested and centrifuged (2.2.1) and the number of cells in the culture was counted on a standard haemocytometer. The calculated volume of cell suspension was added to an appropriate volume of proliferation medium and plated onto the 24 inner wells of a 48-well culture plates to achieve a density of 16,000 cells/ml in a volume of 300 μl/well. After 24 h, during which time the cells adhered to the wells, the medium was removed and replaced with 300 μl serum-free medium containing 1 mM dibutyryl cyclic AMP to induce differentiation. At the same time the substance to be tested was added to the medium in a range of concentrations. Control cells were exposed to the vehicle of dilution only and negative control cells continued to be grown in serum-containing proliferation medium i.e. they were not induced to grow neurites.

Each of the test substances was included at a range of concentrations designed to produce a range of effects on neurite outgrowth from minimal inhibition to maximum inhibition or even

cell death. The test substance was added to the medium at a dilution of 1:200 at each dosage level. Three wells were treated at each concentration and each product was tested a minimum of 4 times in separate experiments.

After a further 24 h, cells were fixed for 20 min in 4% w/v formaldehyde solution (Appendix 1) and stained for 10 min with Coomassie blue stain (Appendix 1). After washing once with phosphate-buffered saline and twice with distilled water, the plates were left to air dry. A minimum of 200 cells from 6-20 different fields, depending on cell spacing, for each treatment were viewed either in a Zeiss Axiovert 35M microscope linked by a video camera to a Kontron Vidas 2.0 image analyser, or in an Olympus A1 microscope linked to a Kontron Ibas 2.0 analyser. A blind trial was performed initially which verified that results obtained from either system were comparable.

An automated image analysis program (written by Dr. W.G. McLean, University of Liverpool, U.K.), performed a segmentation and skeletalisation of the images of the cells in each chosen field. After capturing the field the cell bodies were counted and highlighted, and then eroded according to whether each pixel was surrounded by an octagon of highlighted pixels. The resulting cell perimeter that remained was then removed leaving only neurites. These were thinned by serial erosion to single pixel width, and small objects (less than 6 pixels) removed before calculating the average neurite length per cell for each treatment. The number of neurites per cell was also recorded at the same time. This method is described in more detail by Smith (1999), and Fishwick (1997) performed a validation of the method. To eliminate bias when analyzing the fields of cells, methods of unbiased stereology for uniform random sampling in 2-D were followed (Howard and Reed, 1998)

Toxicity was measured in terms of reduction in neurite length for treated cells compared to differentiating controls, with a baseline level of no neurite outgrowth represented by the cells grown in serum-containing medium.

2.3 Samples investigated in these studies

All samples were supplied by Greyhound/Chemservice, Birkenhead, Liverpool, UK, unless otherwise stated.

Pyrethrum, (Agropharm Ltd., High Wycombe, UK)

Piperonyl butoxide, 5-[2-(2-butoxyethoxy) ethoxymethyl]-6-propyl-1,3-benzodioxole, (Agropharm Ltd., High Wycombe, UK).

Diazinon, *O,O*-diethyl *O*-(2-isopropyl-6-methylpyrimidin-4-yl) phosphothioate.

Phosmet, *O,O*-dimethyl *S*-phthalimidomethyl phosphorodimethioate.

Pirimiphos methyl, *O*-(2-diethylamino-6-methylpyrimidin-4-yl) *O, O*-dimethyl phosphorothioate.

Chlorpyrifos, *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate.

Malathion, diethyl (dimethoxythioxophosphoryl-thio) succinate.

Carbaryl, 1-naphthyl methylcarbamate.

Glyphosate, N-(phosphonomethyl) glycine.

Commercial Formulation 1

- a pesticide formulation declared to consist of pyrethrins 0.04%, diazinon 0.5%, piperonyl butoxide technical 0.1% and inert ingredients 99.36%, although chemical analysis revealed over-formulation of ingredients, as follows: diazinon, 0.565%, pyrethrins, 0.062%, piperonyl butoxide, 0.155%.

Regular Mineral Spirit and

Odourless Mineral Spirit for Commercial Formulation 1.

-these spirits made up all or part of the formulation of Commercial Formulation 1.

Rycovet Nupor Warblecide Pour On, 20% (w/v) formulation of phosmet, (Rycovet Ltd., Glasgow, UK), Lot No. 0000015 (purchased from an agricultural supplier).

Roundup Weedkiller containing 0.72% (w/v) glyphosate acid as a 9.7g/l IPA salt of glyphosate in a soluble concentrate, (Solaris, Garden Division of Monsanto, High Wycombe, UK), Lot No. C8K2767 (purchased from an agricultural supplier).

Tough Weed Killer, 5% (w/v) formulation of glyphosate trimesium, (Miracle Gardencare, UK), coded Dec97294, (purchased from an agricultural supplier).

All technical samples were stored at 4°C and formulations were maintained at room temperature. Samples were freshly prepared prior to dilution in medium, as stock solutions in methanol (pyrethrum, diazinon and malathion) or DMSO (all other samples). Glyphosate did not fully dissolve in either vehicle so was prepared as a suspension in DMSO.

2.4 Determination of neurite inhibition of cells exposed to test substances

1 Determination of the effect on neurite outgrowth of methanol and DMSO

To ensure that the vehicles used in these studies were not wholly or partly responsible for the effects on neurite outgrowth observed, the same techniques as described above (2.2.3) were used to assess the effect of methanol and DMSO, both alone and in combination.

Cells treated as described above were exposed to a 0.5% dilution of methanol or DMSO in serum-free medium containing 1 mM dibutyryl cyclic AMP. The vehicles were assessed on 6 occasions and the effect on neurite outgrowth assessed as in 2.2.3.

Further investigations into the effect of methanol on neurite growth was then performed using the following concentrations:

methanol (0.5%); methanol (0.25%); methanol (0.25%) plus DMSO (0.25%).

The experiment was again performed six times, and the effect on neurite length assessed as before. Statistical analysis (one way ANOVA including Bonferroni's correction for multiple comparisons) compared the neurite length of the cells exposed to the vehicle to control cells without such exposure.

2 Determination of the inhibition of neurite outgrowth (IC_{50}) produced by individual pesticide products/formulations

Every sample in 2.3 was assessed for its ability to inhibit neurite outgrowth, according to the methods given above (2.2.3). Each sample was tested a minimum of 4 times. The arbitrary neurite length in pixels at each concentration was transformed to a percentage of the length achieved by control cells differentiating in the absence of pesticide subtracted from a baseline of cells grown in serum-containing medium. Individual IC_{50} values (the concentration at which 50% reduction in neurite outgrowth was achieved) were estimated by plotting (Excel or Prism 3.0) the concentration against the % inhibition of neurite outgrowth achieved. A line of best fit for the points was drawn by the program and the individual IC_{50} value was calculated from the equation of this line. This method was repeated for each individual experiment. An example of the individual results and the corresponding concentration response curve for one experiment with piperonyl butoxide is given in Figures 2.1 and 2.2.

concentration	average neurite length	neurite length control	neurite length after treatment	% neurite length	% neurite length inhibition
positive control	121.2				
negative control	45.3	75.9			
10 nM	120.0		74.7	98.5	1.5
100 nM	119.5		74.2	97.8	2.2
1 µM	82.5		37.2	49.1	50.9
10 µM	44.4		-0.9	-1.2	100
100 µM	0		0	0	100

Figure 2.1: *Individual results for one experiment with piperonyl butoxide. The positive control is the neurite length of cells differentiating in the absence of piperonyl butoxide and the negative control is the neurite length of cells proliferating in serum (arbitrary pixel numbers). The neurite length control is the difference between the positive and negative controls. The neurite length after treatment is the average neurite length minus the negative control, and % neurite length calculates the length as a percentage of the neurite length control. Subtracting this length from 100 produces the % neurite length inhibition, which is plotted against the concentration in Figure 2.2. If cells were absent due to death (100 µM) or inhibition was greater that negative controls (10 µM) a maximum 100% inhibition was assumed.*

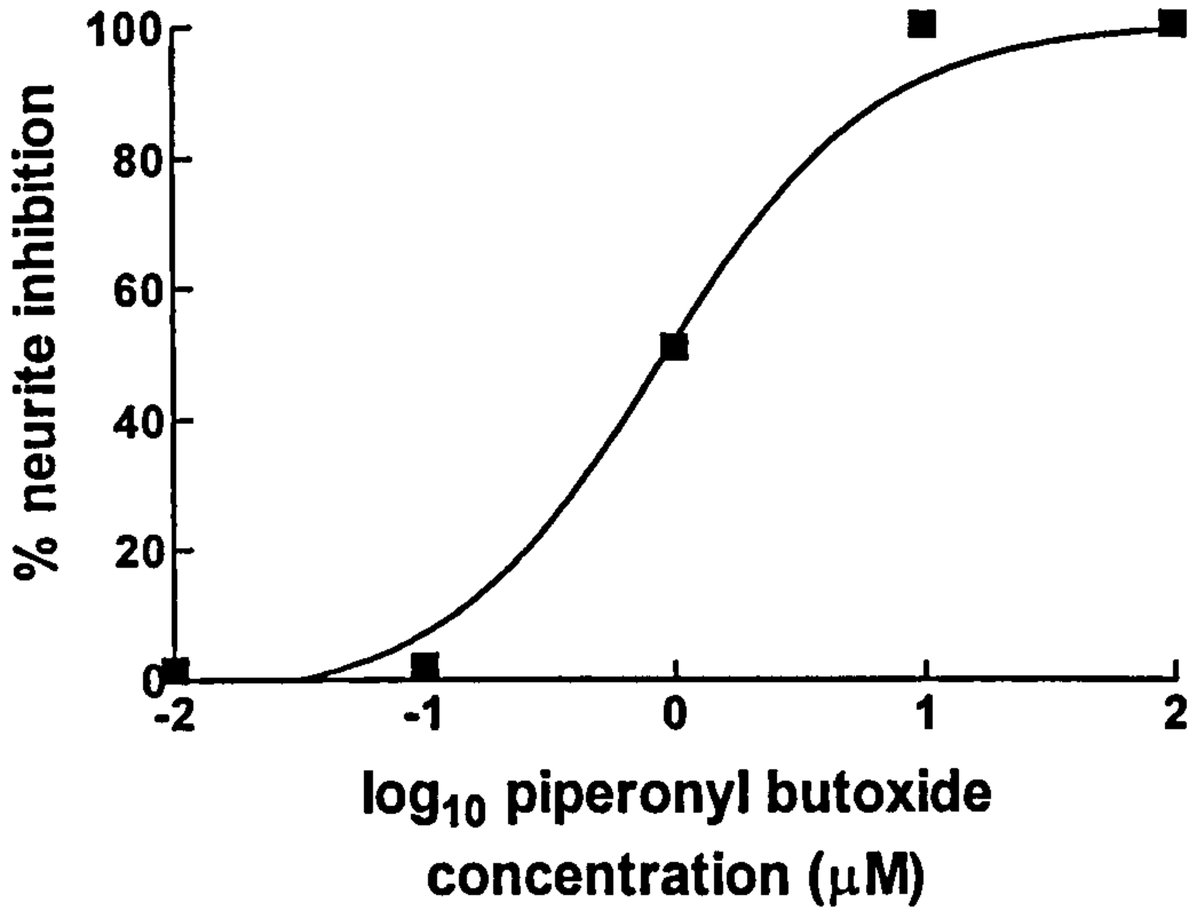


Figure 2.2: *Concentration response curve from the results given in Figure 2.1. The IC_{50} was calculated to be 866 nM.*

Statistical comparisons between treatments were performed on the IC_{50} values from all the individual experiments.

In order to better display these results, a mean IC_{50} value was also determined, where possible, for each sample. The data from all the individual experiments was analysed using non-linear regression performed by computer program (Prism 3.0). Each replicate y value was considered as an individual point with no weighting, to minimise the absolute distances squared. A mean dose response curve (usually a Sigmoid curve) was produced from the data and the IC_{50} and its 95% confidence limits, was calculated by the program. If a sigmoid curve was drawn the following equation was used:

Y=bottom + (top-bottom)/1+10^((logEC50-X)))

where X = log concentration and Y = response. A top value of 100% was set as constant, but the bottom value was defined by the curve.

In addition to the IC_{50} a concentration producing minimal or no inhibition of neurite outgrowth was also assessed from the dose response curve, for use in experiments to assess synergism (2.5).

Proprietary products consisting of formulations of various pesticides in known or unknown constituents were also tested in the same way. The IC_{50} was determined for the formulation, and the equivalent value for the active ingredient as if it was responsible for all the toxicity observed, was calculated. The individual IC_{50} values for the formulation were compared to those of the pure pesticide, and any difference analysed for statistical significance using Student's two-tailed t-test.

3 Determination of the IC_{50} of individual pesticides after activation with rat liver microsomes

Preparation of microsomes

Pesticides are metabolised by liver enzymes, and metabolites may be more or less toxic than the parent compound. In an attempt to simulate this metabolism, studies were performed using liver microsomes, which contain the enzymes responsible for this metabolism *in vivo*.

Three male Wistar rats, weighing approximately 250 g were killed humanely, and their livers were immediately excised. These were washed three times in ice-cold sterile 70 mM phosphate/potassium chloride buffer (Appendix 1). The liver was divided into small (10 g) amounts and each portion was roughly chopped, before homogenising in 5 volumes of buffer by passing 3-4 times through a close-fit glass Teflon automatic homogeniser, followed by a second homogenisation through a closer-fitting homogeniser.

Samples of the homogenate were centrifuged at 900g for 10 min at 4°C. The pellet was discarded and the supernatant re-centrifuged at 8,000g for 10 min at 4°C.

The supernatant was re-suspended in an equal volume of 70 mM phosphate buffer pH 7.4 (Appendix 1) and centrifuged at 105,000g for 65 min at 4°C. The pellet was transferred to a manual homogeniser and divided into clean centrifuge tubes before suspending in 5 ml 70 mM phosphate buffer pH 7.4 (Appendix 1). After further centrifugation at 105,000g for 65 min at

4°C, the supernatant was discarded and the pellet re-suspended in cold 70 mM phosphate/potassium chloride buffer pH 7.4 (Appendix 1).

The concentration of microsomes in the sample was determined by the protein analysis method described in 2.10.2. Samples were maintained as 1 ml aliquots at -80°C until required.

Determination of IC_{50} after microsome activation

NB2a neuroblastoma cells at a density of 30,000 cells/ml were plated in serum-containing proliferation medium into 5 wells of 24-well plates. After 24 h, to allow the cells to adhere to the plastic, the medium was removed and replaced with 600 µl serum-free medium, containing 1 mM dibutyryl cyclic AMP, with the exception of one well, which was replaced with fresh proliferation medium.

Plastic inserts (Costar UK Ltd., High Wycombe, UK) were prepared for each well. Each insert contained the NADPH generating system (Appendix 1) necessary to activate the microsomes. In addition, 27.5 µg/ml microsome homogenate and/or test substance was added to the inserts to produce a total volume of 400 µl as follows:

1 **Control serum** - microsomes in serum-containing medium only.

2 **No microsome control** - no microsomes in serum-free medium.

3 **Microsome control** - microsomes in serum-free medium

4 **Microsomes and test substance** - in serum-free medium

5 **Test substance** - in serum-free medium without microsomes.

The test substance was one of three pesticide substances, phosmet (3 µM), diazinon (41 µM) or pirimiphos methyl (1.5 µM), added to the insert where required. The concentration was that previously determined to be the approximate IC_{20} of the sample in the absence of microsomes under the conditions of this investigation.

After addition of the inserts into the wells, the plates were incubated at 37°C for 24 h. The inserts were then removed and the cells were fixed and stained as before (2.2.3). Neurite outgrowth, as a percentage of the appropriate control was determined for each well, and the degree of neurite inhibition of each test substance in the presence or absence of microsomal activation was compared using Student's two-tailed t-test. Three separate experiments were performed for each of the pesticide substances.

4 Determination of the effects of chronic pre-exposure to diazinon on the inhibition of neurite outgrowth by pesticides

The methods as described in 2.2.3 were used to determine the IC_{50} for neurite inhibition of some of the substances but substituting cells chronically pre-exposed to 25 µM diazinon as described in 2.2.2. Concentrations were based on those chosen for the assessment of neurite outgrowth in normal cells, but generally included one lower concentration. Diazinon (25 µM) was omitted from the medium when the cells were plated into the wells, immediately prior to induction of differentiation.

The substances tested in this way were:

phosmet, pirimiphos methyl, diazinon, pyrethrum, glyphosate, Roundup Weedkiller, Tough Weed Killer.

IC_{50} values obtained with these cells were compared with values from experiments on cells grown without diazinon, and the difference in IC_{50} values analysed for statistical significance using Student's two-tailed t-test. In addition, where appropriate, neurite inhibition at individual concentration points were also analysed (one-way ANOVA including Bonferroni's correction for multiple comparisons) for differences in response between normal cells and those pre-exposed to diazinon.

5 Comparison of the effects of chronic pre-exposure with acute pre-exposure to diazinon

In order to demonstrate that any differences in toxicity observed in cells chronically pre-exposed to diazinon were not simply an additive effect of the substance being tested and any diazinon that remained in the cells during differentiation, the methods described above (2.4.4) were used, but substituting the cells chronically pre-exposed to 25 μM diazinon with normal NB2a cells exposed for only 24 h to 25 μM diazinon. Diazinon was then again excluded from the medium when the cells were plated into the wells.

Glyphosate at the same range of concentrations used in 2.4.4 was similarly tested and an IC_{50} value was obtained from 6 different experiments. Glyphosate was used because exposure to this substance produced significantly more neurotoxicity in cells chronically exposed to diazinon compared to normal NB2a cells. The IC_{50} value obtained was compared to the value for glyphosate in normal cells and in cells chronically pre-exposed to diazinon. The differences were analysed for statistical significance using one-way ANOVA including Bonferroni's correction for multiple comparisons.

2.5 Determination of the interaction between combinations of pesticides on the inhibition of neurite outgrowth

To determine any interactions with combination of these substances, it was necessary to use a concentration substantially below the IC_{50} so that synergism could be detected without the inhibition of neurite outgrowth reaching a maximum. Either the IC_{20} i.e. the concentration that produced a 20% inhibition of neurite outgrowth or an even lower concentration was used. Dilutions from this level were then combined as follows:

Combination	1	2	3	4	5
% Substance A	100	75	50	25	0
% Substance B	0	25	50	75	100

Methods were as for individual substances (2.2.3), maintaining a total dilution of substance in medium of 1:200 for the combination of two substances. In the situation where two different vehicles were used to dilute substances, both vehicles were included in the positive control, and the appropriate alternative vehicle was also added to each of the 100% levels instead of a second substance. Each combination was performed at least 4 times.

If the effect of a combination of any pair of substances were simply additive, then the inhibition of neurite outgrowth at any proportion of the two substances would be expected to be identical to that of each substance on its own.

The difference between the inhibition with each proportion of paired substances and the expected value was determined by calculating the signed and squared difference between the observed and expected inhibition of neurite outgrowth at each proportional combination. Statistical analysis of this difference was inferred using the Student's two-tailed t-test. A significant increase was indicative of synergy or potentiation whilst a significant decrease suggested inhibition.

1 Determination of the interactions of pesticides encountered by farmers

In order to determine whether there are any interactions between those pesticides often encountered by farmers, the following pesticides were assessed for interactions between all paired combinations:

diazinon (10 µM), pyrethrum (500nM), glyphosate (500 µM), phosmet (10 µM) pirimiphos methyl (10 µM)

Stated concentrations were the maximum used (100% of the substance).

Two pesticide formulations were also combined with various individual pesticides. Tough Weed Killer (10 ppm), (5% formulation of glyphosate trimesium), was tested for interaction with phosmet, diazinon and pirimiphos methyl. Nupor (5 ppm), (20% formulation of phosmet) was tested for interaction with pirimiphos methyl.

Nupor and Tough Weed Killer were also assessed together for any interaction.

2 Determination of the interaction with a commercial formulation

Commercial formulation 1 was investigated in this way to indicate whether the individual components of formulated products could influence the inhibition of neurite outgrowth. Commercial Formulation 1 contained diazinon, pyrethrum and the potentiator piperonyl butoxide in a mixture of vehicles consisting mainly of 'regular spirit' and 'odourless spirit'. It was combined as described above (2.5) to determine any interaction with chlorpyrifos, an organophosphate commonly found in pesticide formulations. Further combinations were separately studied with the constituents of the commercial formulation, as follows:

Substance A	Substance B
Chlorpyrifos (10 µM)	Commercial Formulation 1 (10 ppb)
Chlorpyrifos (10 µM)	Diazinon (10 µM)
Chlorpyrifos (10 µM)	Pyrethrum (500 nM)
Chlorpyrifos (10 µM)	Piperonyl butoxide (10nM)
Chlorpyrifos (10 µM)	Regular mineral spirit (100 ppb)
Chlorpyrifos (10 µM)	Odourless mineral spirit (100 ppb)
Chlorpyrifos (10 µM)	Mixture of diazinon, piperonyl butoxid and pyrethrum (10 ppb)
Diazinon (10 µM)	Pyrethrum (500 nM)
Diazinon (10 µM)	Piperonyl butoxide (10nM)

Stated concentrations were the maximum used (100% of the substance).

3 Determination of the interactions of pesticides used in anti-head louse treatments

Three pesticide substances are routinely used in the treatment of head-louse infestation. These are the organophosphate malathion, the carbamate carbaryl and pyrethrum. These pesticides were used in combinations with each other to determine the potential for interaction when using these products. Maximum concentrations used were as follows:

Malathion : (10 µM)

Pyrethrum : (500 nM)

Carbaryl : (1 µM)

Effects on many aspects of cell function could result in synergistic effects on neurite outgrowth. In order to investigate further the mechanisms underlying any synergism, three aspects were studied: the effects of combinations of pesticides on acetylcholinesterase activity, since inhibition of this enzyme is the primary effect of organophosphate and carbamate pesticides, the effects of pesticides on cell adhesion and the general cytotoxicity of the combination of pesticides.

2.6 Determination of the interactive effects on acetylcholinesterase activity of cells exposed to mixtures of pesticides

Ellman (1961) describes the use of a colorimetric measurement for determining the rate of acetylcholinesterase activity in a sample. This method relies on the increase in yellow colouration produced when thiocholine reacts with the dithiobisnitrobenzoate ion. The change in colour results from the following reaction:

$$\text{acetylthiocholine} \xrightarrow{\text{acetylcholinesterase}} \text{thiocholine + acetate}$$

$$\text{thiocholine + dithiobisnitrobenzoate} \longrightarrow \text{yellow colour}$$

Acetylthiocholine has been found to be a suitable substitute for the natural substrate of acetylcholinesterase, acetylcholine. Both organophosphate and carbamate pesticides interact with acetylcholinesterase.

The principle of the method involves determination of the rate of production of thiocholine as acetylcholine is hydrolysed. The method was adapted in the following way.

Cells were plated in serum-containing medium into the central wells of 96-well plates, at a density of 100,000 cells/ml with a volume of 200 µl per well. A total of 5 wells were used at each concentration. After a 24 h incubation period to allow the cells to adhere to the wells, the proliferation medium was removed and replaced with serum-free medium containing 1 mM dibutyryl cyclic AMP. Combinations of two pesticides at concentrations and proportions identical to those used in the studies on the inhibition of neurite outgrowth (2.5) were included in the medium. Cells incubated in serum-free medium containing 1 mM dibutyryl cyclic AMP, both with and without the vehicle of dilution, were also included as negative controls, and cells exposed to the carbamate physostigmine, (10 µM), constituted a positive control. This

concentration is known to have a significant inhibitory effect on acetylcholinesterase activity in these cells.

On the same plate a higher (usually 20 μM) and lower (usually 1 μM) concentration of one of the pesticides was also included, to assess any concentration-dependent effects on acetylcholinesterase activity by the pesticide alone.

All combinations were diluted 1:200 in medium. When only a single pesticide was used or for controls, an equivalent volume of the vehicle of dilution was substituted for the pesticide(s).

Plates were incubated for 24 h at 37°C in the presence of 5% CO_2 during which time the cells differentiated and extended neurites. At the end of this time medium was removed and replaced with 200 μl of 100 mM phosphate buffered saline pH 8.0. The plate was then transferred to an automated plate reader (Dynatech Mr600, Dynatech Labs., Billingshurst, UK).

A 20 μl aliquot of freshly prepared acetylthiocholine iodide, (15 mM in 100 mM phosphate buffered saline pH 8.0) was added to each well and a base-line reading was taken. The reagent, dithiobisnitrobenzoic acid (DTNB) (20 μl of 2.5 mM solution in 100 mM phosphate buffered saline (pH 7.0) including 18.75 mM sodium bicarbonate) was then added and the wells mixed thoroughly by agitating 3 or 4 times through a pipette tip. Readings of the absorbance at 405 nm were then taken at 1 min intervals for 6 minutes.

The rate of conversion of acetylthiocholine at each dose level was determined in a minimum of 4 separate experiments, using the following equation:

Moles of substrate hydrolysed /min per cell = $\Delta A/n\ 1.36 \times 10^{-4}$

where 1.36×10^{-4} was the extinction coefficient of the substrate, ΔA was the rate of change in absorbance (per minute) and n was the total number of cells in each well.

If the effect of a combination of a pair of substances were simply additive, then the rate of acetylcholinesterase activity at any proportion of the two substances would be expected to be identical to that of each substance on its own. Synergism between the paired substances was thus measured as the signed and squared difference between the observed and expected rate of acetylcholinesterase activity. Statistical analysis of this difference was inferred using the Student's two-tailed t-test.

The following pesticide combinations, which had been shown to interact synergistically in their inhibition of neurite outgrowth, were assessed using this method:

Phosmet (10 μM) with pirimiphos methyl (10 μM)

Diazinon (10 μM) with glyphosate (500 μM)

Malathion (10 μM) with carbaryl (1 μM).

In addition the following combination, which had not been shown to interact synergistically in their inhibition of neurite outgrowth, was assessed:

Diazinon (10 μM) with chlorpyrifos (10 μM)

Stated concentrations were the maximum used (100% of the substance).

A further 4 plates were treated in the same way, but with the rate of conversion measured after only 1 h, when the inhibition of enzyme would be expected to be at or near its maximum.

Identical studies were also performed, with both a 24 h and 1 h exposure period, using the cells chronically exposed to diazinon (2.2.2). Differences between the rate of acetylcholinesterase activity of the two cultures were compared for statistical significance using the Student's 2-tailed t-test.

Studies were also performed to detect acetylcholinesterase activity in proliferating cells. In order to eliminate the detection of enzyme activity arising from the serum in the medium, the medium was removed and cells were rinsed three times in phosphate-buffered saline before addition of the reagents. The methods used failed to detect any acetylcholinesterase activity in these cells.

2.7 Determination of the interactive effects on cytotoxicity from pesticide combinations using the MTT assay

Cytotoxicity as indicated by reduction in cell viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.

NB2a cells were plated into wells of 96-well plates at a density of 100,000 cells/ml in serum-containing proliferation medium at a volume of 200 µl. After 24 h to allow adhesion of the cells to the wells, the medium was removed and replaced with serum-free medium including 1 mM dibutyryl cyclic AMP and containing combinations of pesticides as included for the assessment of acetylcholinesterase activity (2.6). Following incubation with the samples for a further 24 h during which time the cells differentiated and extended neurites, 120 µl medium were removed from each well and replaced with 20 µl of sterile-filtered MTT (5 mg/ml in DMEM). MTT is converted to an insoluble blue formazan product by living cells, but not by dead cells or lysis products. The optical density is therefore proportional to the number of viable cells.

After incubation for a further 4 h, the coloured formazan product formed was solubilised by addition of 20% (v/v) Triton-X-100 in 0.5M HCl (50 µl/well) and the plates shaken on a microtitre plate shaker for 10-20 minutes. Absorbance was measured at 570 nm on an automatic microplate reader. If necessary, excess bubbles were pricked with a sterile 23G 1-inch needle prior to reading, since such bubbles interfere with the accurate reading of the wells.

A minimum of 4 plates was treated in this way for each combination tested.

Toxicity was measured as a changed survival of treated cells as indicated by difference in absorbance (reflecting the metabolism of MTT) in pesticide-treated cells compared to untreated controls. If the cytotoxic effect of a pair of compounds were simply additive, then the absorbance at any proportion of the two compounds would be expected to be identical to that of each substance on its own. Synergism between the paired compounds was thus measured as the signed and squared difference between the observed and expected absorbance readings. Statistical analysis of this difference was inferred using the Student's two-tailed t-test.

The following pesticide combinations, which had been shown to interact synergistically in their inhibition of neurite outgrowth, were assessed using this method:

Phosmet (10 µM) with pirimiphos methyl (10 µM)

Diazinon (10 µM) with glyphosate (500 µM)

Malathion (10 µM) with carbaryl (1 µM).

In addition the following combination, which had not been shown to interact synergistically in their inhibition of neurite outgrowth, was assessed:

Diazinon (10 µM) with chlorpyrifos (10 µM)

Stated concentrations were the maximum used (100% of the substance).

Identical studies were performed using the cells chronically exposed to diazinon (2.2.2).

2.8 Detection of the effects on cell adhesion of organophosphate pesticides

NB2a cells were grown in serum-containing proliferation medium in a 75 ml cell culture flask until confluent. The cells were removed from the flask by gentle agitation in medium. The contents of the flask were then transferred to a Sterilin pot, and centrifuged at 1000 rpm for 7 min. The supernatant was discarded and the pellet re-suspended in 1 ml of serum-containing proliferation medium. This was then divided into equal volumes (each containing approximately 10^6 cells) and transferred to four 75 ml culture flasks containing 5 ml fresh proliferation medium. The flasks were incubated for 24 h to allow the cells to adhere to the plastic.

The medium was removed and replaced with 5 ml serum-free medium containing 1 mM dibutyryl cyclic AMP. At the same time diazinon, phosmet or chlorpyrifos was added to the medium at a dilution of 1:400 to achieve a final concentration of 10 µM for diazinon or phosmet and 1 µM for chlorpyrifos. A further flask contained cells incubated in medium containing DMSO only, which acted as a control. The concentrations were chosen from preliminary studies to produce minimal toxicity to the cells.

Flasks were incubated for 24 h at 37°C. At the end of this time, the medium was removed from each flask and kept. A further 2 ml medium was added to the flask, which was then gently shaken on an automatic shaker for 5 min. This was also decanted and pooled with the initial medium. This combined medium comprised the non-adhesive cell fraction.

A further 5 ml medium was added to the flask and the adherent cells were gently suspended in it by agitation with rinsing, using a 5 ml syringe fitted with a syringe extension, as in 2.2.1. The medium was decanted and a further 2 ml added to completely rinse all cells from the flask. This medium was combined with the previous sample and comprised the adhesive fraction of cells. All medium added to the flask had been previously incubated to 37°C.

The number of cells in the adhesive and non-adhesive fractions for each pesticide or control was counted separately on a standard haemocytometer. The proportion of adhesive to non-adhesive cells was determined for each treatment.

The experiment was repeated a further three times. Further experiments were performed in the same way, but the serum-free medium was replaced with serum-containing proliferation medium.

Statistical analysis of results (Student's 2-tailed t-test) was performed to compare the effects of organophosphates on cell adhesion in proliferating and in differentiating cells.

2.9 Interaction of prion protein or β-amyloid with organophosphates

1 Sample preparation

a) PrP106-126

A fragment of the prion protein, consisting of amino acids 106-126 (PrP106-126), has been found to be neurotoxic. The effect has been demonstrated at a concentration of 80 μM and in the presence of microglia (Brown *et al.*, 1996). This peptide was obtained from BACHEM (UK) Ltd., Saffron Walden, UK. It consists of the following structure:

H-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly-OH

The peptide was suspended in DMEM as a 1 mM solution and was thoroughly vortexed before aliquoting into smaller volumes for easier use. Aliquots were maintained at -4°C until required. Before use, aliquots were slowly defrosted at room temperature and then vortexed vigorously to resuspend thoroughly.

b) β-amyloid25-35

A fragment of the β-amyloid protein consisting of amino acids 25-35 (β-amyloid25-35) has also been shown to be neurotoxic (Yankner *et al.*, 1990b). This peptide was also obtained from BACHEM (UK) Ltd., Saffron Walden, UK. It consists of the following structure:

H-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-OH

The peptide was suspended in DMEM as a 1 mM solution and thoroughly vortexed before aliquoting into smaller volumes to be maintained at –4°C until required. Before use the aliquots were slowly defrosted at room temperature and again vortexed vigorously to resuspend thoroughly.

c) β-Amyloid35-25

β-Amyloid35-25 was also supplied by BACHEM (UK) Ltd., Saffron Walden, UK. The molecule consists of the same amino acids as in β-amyloid25-35 but in the reverse order as follows:

H-Met-Leu-Gly-Ile-Ile-Ala-Gly-Lys-Asn-Ser-Gly-OH.

This molecule was treated in an identical manner to β-amyloid25-35 in many of the following experiments, and acted as a control.

2 Determination of interactions from combination of PrP106-126 with organophosphate in differentiating cells

Methods used were essentially those described for the determination of cytotoxicity using the MTT assay (2.7). NB2a neuroblastoma cells in serum-containing proliferation medium were plated at a density of 100,000 cells/ml in a volume of 200 µl into wells of 96-well plates. After 24 h to allow the cells to adhere to the wells the medium was removed and replaced with

serum- free medium including 1 mM dibutyryl cyclic AMP to promote differentiation. Phosmet, at a concentration of 1, 10 or 20 µM, with or without 10 µM PrP106-126 was added to each of 5 wells. A further 5 wells of cells were exposed to 10 µM PrP106-126 alone and 5 wells were maintained in medium containing DMSO only, and acted as a control.

The plate was incubated for a further 24 h before assessment of cell viability as before (2.7). Each experiment was repeated on at least 4 different occasions. The viability was measured with the MTT assay as described in 2.7. Differences between treatments were analysed for statistical significance using one-way ANOVA including Bonferroni's correction for multiple comparisons.

3 Determination of interaction from combination of β-amyloid25-35 with organophosphate in differentiating cells

Further studies were performed as above, substituting β-amyloid25-35 for PrP106-126. In addition β-Amyloid35-25 was also studied in the same way and acted as a control.

4 Further investigations

These studies were also repeated with the following modifications:

a) Serum-containing medium replaced the serum-free medium to investigate the effect in proliferating cells.

a) Diazinon was substituted for phosmet in serum–containing medium.

2.10 Immunodetection of proteins

1 Western blot method

Acrylamide gels were made in a gel caster for a minigel electrophoresis apparatus (SE250 minigel apparatus (20 mA/gel) and TE Transfer Unit 400 mA for 1 h, Hoefer Scientific Instruments, UK). Various different concentrations of acrylamide were used as appropriate (Appendix 1). Each gel was overlaid with 1 ml distilled water and covered with plastic film wrap to prevent evaporation before use. When set (either overnight at 4°C or approximately 1 h at room temperature) the water was decanted and the gels were placed in the electrophoresis apparatus. Appropriate stacking gels were added (Appendix 1) and allowed to set at room temperature. For gels and stacking gels, the mixtures were degassed for 2-3 minutes using a vacuum apparatus to remove air bubbles, before polymerisation.

Samples were prepared (see individual preparation methods) and suspended in SDS sample buffer (Appendix 1) at concentrations calculated to contain the same amount of protein in each sample. After addition of sample buffer the samples were boiled for 5 min before use to denature the proteins. The electrophoresis apparatus was filled with running buffer (Appendix 1) and the attached coolant system switched on. Identical volumes of each sample were loaded into the wells (usually 10 or 15 µl). A range of molecular weight standards (5 µl) was also loaded into one well, and a control lane containing one of the samples was also prepared. The apparatus was allowed to run with maximum voltage at a current of 30 mA per gel, until the running edge of the samples reached the bottom of the gel.

The gel was then removed, the stacking gel was gently scraped away and the gel was transferred onto a 'waffle', consisting of nitrocellulose paper (Protran BA83, Schleicher & Schuell UK Ltd., London, UK) cut to the size of the gel, in a filter paper sandwich backed with synthetic sponge. At all times the gel, nitrocellulose and waffle were kept submerged in transfer buffer (Appendix 1). Before closing the waffle, air bubbles were removed by rolling a glass pipette over the filter paper enclosing the gel.

The waffle was placed into the transfer tank and positioned so that the proteins would transfer from the gel onto the nitrocellulose towards the positive electrode. The apparatus was filled with transfer buffer (Appendix 1) and allowed to transfer for one or two hours at maximum voltage and current.

The nitrocellulose paper was removed from the waffle and placed in a dish containing a blocking medium of either 5% (w/v) bovine serum albumin or 5 or 10% Marvel dried milk powder in Tris-buffered saline. The dish was gently shaken for 1 h at room temperature. An appropriate primary antibody, diluted in either 5% bovine serum albumin or 5% Marvel dried milk powder in Tris-buffered saline, according to preliminary investigations to an optimum concentration to detect the desired protein under these conditions, was placed in another dish. The nitrocellulose with the transferred proteins was incubated face down in the antibody overnight at 4°C with gentle shaking, and the dish was covered with plastic film wrap to prevent evaporation. The control lane was excised from the nitrocellulose prior to antibody incubation, and remained in the blocking medium overnight at 4°C. This control ensured that protein bands subsequently revealed resulted from the binding of primary to secondary antibody, and not due to the secondary antibody binding non-specifically to the nitrocellulose.

After removal of the primary antibody or blocking solution, the nitrocellulose was rinsed three times for 10 min each with 0.05% (v/v) Tween 20 in Tris-buffered saline, before addition of an appropriate secondary antibody. Approximately 10 ml of appropriate secondary antibody labeled with horseradish peroxidase was added to each gel, and blots were incubated with gentle shaking at room temperature for 1 or 2 hr.

Excess antibody was removed by rinsing three times for 10 min each in 0.05% (v/v) Tween 20 in Tris-buffered saline. Enhanced chemiluminescent reagents (Supersignal chemiluminescent substrate stable peroxide solution and chemiluminescent substrate luminol/enhancer solution, Pierce & Warriner (UK) Ltd., Chester, UK) were mixed in equal proportions, before saturating the blots (approximately 10 ml) and shaking vigorously for 5 min. The immunoblot was removed from the solution, placed onto a glass plate, blotted dry with filter paper and covered

with plastic film wrap. An appropriate size of Kodak photographic film was placed over the immunoblot and exposed for a suitable time period (usually 1-5 minutes) to achieve detectable images of protein bands. The film was then developed for images using standard developing chemicals.

At least four experiments were performed, and digital images were taken of protein bands revealed by the procedure (Nikon Coolpix 950 digital camera). The images were analysed for band density using a computerized densitometric programme (Scion Image Beta 3b 7/23/98, National Institutes of Health, USA). Arbitrary densitometric values were compared using one-way ANOVA including Bonferroni's correction for multiple comparisons.

2 Protein analysis of samples prepared for immunoblotting

Protein from cellular sample preparations was analysed according to the Lowry method using a protein analysis kit (Sigma Chemical Co., Poole, UK).

A suitable aliquot of each protein sample (usually 5 or 10 µl) was placed into a flip top tube and made up to 0.5 ml in distilled water. The same volume of protein standards containing known quantities of bovine serum albumin (50, 100, 200, 300 and 400 µg/ml) were also made up according to manufacturer's instructions. A further tube contained water only, as a blank. A 0.5 ml aliquot of Lowry Reagent Solution was added to each vial of sample and standards before thorough vortex mixing. Following a 20 min incubation period, 0.25 ml Folin & Ciocalteu's Phenol Reagent Working Solution was added to each vial and mixed well before incubating for a further 30 min. All procedures were carried out at room temperature.

The absorbance at a wavelength of 570 nm was determined in a spectrophotometer (Cecil CE 1011, Scientific & Medical Products Ltd., Manchester, UK). A calibration curve for the standard protein samples was produced and the quantity of protein in each test sample calculated from a computer-generated equation of the curve. The samples were prepared by dilution

according to the results of each protein analysis, so that each of the samples in an individual experiment contained the same amount of protein.

Samples were prepared in phosphate buffered saline for use in dot blot analyses and in SDS sample buffer (Appendix 1) for use in Western blotting.

2.11 Immunodetection of recombinant prion protein by Western blotting

1 Sample preparation

Recombinant full-length bovine prion protein (Prionics AG, Zurich, Switzerland) was prepared as a 0.001% (w/v) solution in 0.05% (w/v) bovine serum albumin in phosphate-buffered saline. Fourteen vials were prepared with pairs of samples including either 10 or 100 µM solutions of phosmet, pirimiphos methyl or diazinon, or phosphate-buffered saline alone. The organophosphates were initially prepared as 100 mM stock solutions in DMSO or methanol, but further dilutions were made in phosphate-buffered saline only. All vials were then incubated for 1 h at 37°C.

In order to identify possible protease resistance of the prion molecule, proteinase K (10-20 U/mg, Sigma Chemical Co., Poole, UK) was added from a 330 µg/ml stock solution in sterile distilled water to give a final concentration in each vial of 3.3 µg/ml, to one of each pair of the above samples. These were further incubated at 37°C for 1 h. All vials were then treated with phenylmethylsulfonyl fluoride (PMSF) (5mM) to inhibit further protease activity.

Each sample was suspended in an equal volume of double strength SDS buffer (Appendix 1) with 5% (v/v) mercaptoethanol. The samples were boiled in a heating block for 5 minutes.

2. Western blot detection of protein

Samples prepared as above (5 or 10 µl) were loaded into wells on 8% SDS-acrylamide gels (Appendix 1) prepared with 4% stacking gels (Appendix 1), and electrophoresed as described (2.10.1). Gels were then removed and protein transferred from the gel onto nitrocellulose paper (2.10.1)

The nitrocellulose blots were placed in a blocking medium consisting of 5% (w/v) bovine serum albumin in Tris-buffered saline at room temperature for 1 h, before transferring into dishes containing primary antibody (mouse anti-PrP monoclonal antibody 6H4, Prionics AG, Zurich, Switzerland, 1:250 in 5% (w/v) bovine serum albumen in Tris-buffered saline). The blots were shaken overnight at 4°C and then washed three times in 0.05% (v/v) Tween 20 in Tris-buffered saline. The blots were incubated in the appropriate secondary antibody (anti-mouse IgG (Fab specific), horseradish-peroxidase linked; Sigma Chemical Co., Poole, UK), diluted 1:10,000 in 5% (w/v) Marvel dried milk powder in Tris-buffered saline at room temperature for 1 h before developing as described.

The protein bands obtained were assessed for variation in density as before (2.10.1). Protein bands from samples obtained after treatment with organophosphates were compared to those from control samples and analysed for statistical significance using one-way ANOVA including Bonferroni's correction for multiple comparisons.

2.12 Determination of the direct effect of organophosphates on the activity of proteinase K

In order to determine whether any effects observed with proteinase K-treated samples of recombinant prion protein were purely as a result of general proteinase K inhibition by the organophosphates rather than as a direct effect on the prion molecule, purified goat IgG immunoglobulin (reagent grade) was substituted for the recombinant prion protein molecule and analysed for changes by dot blotting according to the following methods.

1 Sample preparation

Goat IgG was prepared in phosphate-buffered saline as a 1 mg/ml solution. This was further diluted to achieve the same concentration (2 µg/5 µl) as for the recombinant prion protein in 2.11.

The sample was then further diluted to achieve a 0.001% (w/v) solution in 0.05% (w/v) bovine serum albumin in phosphate-buffered saline. Eight vials were prepared and pairs of samples were treated with 10 µM phosmet, pirimiphos methyl or diazinon, or with phosphate-buffered saline alone. Preparation was identical to that given in 2.11.1, except that samples were not suspended in SDS buffer.

2 Dot blot detection of immunoglobulin

Equal volumes (4 or 5 µl) of each sample were gently dotted onto nitrocellulose paper (Protran BA83, Schleicher & Schuell UK Ltd., London, UK) from a pipette tip. The paper was allowed to air dry and then placed in a shallow dish containing a blocking medium consisting of 5% (w/v) bovine serum albumin in Tris-buffered saline (Appendix 1). The dish was shaken gently at room temperature for 1 h.

The immunoblot was removed and placed in a dish containing rabbit anti-goat IgG (H&L) horseradish peroxidase-conjugated secondary antibody (supplied by Chemicon International Ltd., Harrow, UK) diluted 1:250 in 5% (w/v) Marvel dried milk powder in Tris-buffered saline. The blot was gently shaken for 1 h at room temperature before rinsing in distilled water, followed by 3 further rinses of 10 min each in 0.05% (v/v) Tween 20 in Tris-buffered saline at room temperature.

Development of the blots by enhanced chemiluminescence was as described previously (2.10.1). Dots revealed were analysed by computerised densitometry methods (2.10.1) and compared using one-way ANOVA including Bonferroni's correction for multiple comparisons.

2.13 Immunodetection of cellular prion protein by Western blotting

1 Sample preparation for detection of cellular prion protein

NB2a neuroblastoma cells were plated into four 75 ml flasks. One flask contained proliferation medium alone, and the remaining flasks contained 1 µM phosmet, diazinon or pirimiphos methyl in proliferation medium. The cells were grown to confluence for 72 h.

The medium was removed and the cells washed with cold phosphate-buffered saline. A 1 ml aliquot of cold lysis buffer (Appendix 1) was added and incubated at room temperature for 10 min. The contents of the flask were then transferred to a Sterilin pot and centrifuged at 1,000 rpm for 10 min at 0°C to remove insoluble material. The pellet was discarded and phenylmethylsulfonyl fluoride (PMSF) 2 mM was added to the supernatant (10 min) before solublising in an equal volume of 2% (w/v) Sarkosyl (N-lauryl-sarcosine) solution for 15 min at room temperature. An equivalent volume of 6 M guanidine hydrochloride (GdnHCl) was then added to the solution, which was incubated at room temperature for a further 10 min.

To precipitate the protein, five volumes of cold methanol were added to each bottle and the samples left overnight in the freezer (-20°C). The precipitated protein was then gently removed from the methanol with a 5 ml pipette, and transferred to a plastic vial. After centrifugation at 10,000 rpm for 5 min at 0°C, to completely remove the methanol, the supernatant was discarded and the pellet was re-suspended in 100 µl lysis buffer or phosphate-buffered saline.

Four further flasks were treated in the same way, but differentiated for 24 h in serum-free medium containing 1 mM dibutyryl cyclic AMP, including one of the three organophosphates as above.

Protein analysis was performed on all samples (2.10.2) and samples were prepared by suspending the calculated amount of protein in SDS sample buffer (Appendix 1) to ensure equal protein in each sample. Mercaptoethanol (5%) was added and the samples were boiled

in a heating block for 5-10 min before use. If they were not to be used immediately they were maintained in the freezer at –4°C until needed.

2 Sample preparation for detection of proteinase K-resistant prion protein in NB2a cells

Flasks were prepared as above (2.13.1). However before the addition of PMSF, proteinase K (3.3 µg/ml) was added to the supernatant after the initial centrifugation, and the samples were incubated for 1 h at 37°C. The reaction was then terminated as usual by the addition of 2 mM PMSF.

3 Sample preparation for detection of PrP in cells chronically pre-exposed to diazinon

NB2a neuroblastoma cells that had been chronically pre-exposed to diazinon (2.2.2) were prepared in an identical manner to those that had not been pre-exposed. Samples from these cells were compared for differences with samples from normal NB2a cells by the Student's 2-tailed t-test.

4 Western blotting of prion protein samples

The methods used were those described in 2.10.1 with the following amendments:

Primary antibody: goat anti-prion protein (PrP_{27-30}) polyclonal antibody (Chemicon International Ltd; Harrow, UK) 1:200 dilution in 5% (w/v) bovine serum albumin in Tris-buffered saline.

Blocking solution: 5% (w/v) bovine serum albumin in Tris-buffered saline.

Secondary antibody: rabbit anti-goat IgG (H&L) horseradish peroxidase-conjugated antibody (Chemicon International Ltd; Harrow, UK) 1:10,000 dilution in 5% Marvel dried milk powder in Tris-buffered saline.

Transfer time: 2 hours

2.14 Immunodetection of cellular β-amyloid by Western blotting

1 Sample preparation

For many immunoblots, the same samples prepared for the detection of prion protein were also used to detect β-amyloid. However samples were also prepared in the following way.

Cells were grown in organophosphates until confluent as for the detection of prion protein (2.13.1). The medium was removed and the cells were rinsed in phosphate-buffered saline. A 1 ml aliquot of lysis buffer (Appendix 1) was added to the flask of cells and the cells were scraped into it. The flask was incubated at room temperature for 10 min, and the contents were transferred into a flip-top vial. The vial was centrifuged at 15,000g for 15 min at 0°C. The pellet was discarded and the supernatant was decanted and analysed for protein (2.10.2) before preparation in SDS buffer.

Four further flasks were treated in the same way, but differentiated for 24 h in serum-free medium containing 1 mM dibutyryl cyclic AMP, including the three organophosphates as above.

Cells chronically pre-exposed to diazinon (2.2.2), although not further exposed to organophosphates, were similarly prepared and the results compared to those from cells that were not pre-exposed, using the Mann Whitney test.

2 Western blotting of β-amyloid samples

The methods used were those described in 2.10.1 with the following amendments:

Primary antibody: rabbit anti-β-amyloid (1-40) polyclonal antibody (supplied by Sigma) 1:250 dilution in 5% (w/v) Marvel dried milk powder in Tris-buffered saline.

Blocking solution: 5% (w/v) bovine serum albumin plus 10% (w/v) Marvel dried-milk powder in Tris-buffered saline.

Secondary antibody: goat anti-rabbit IgG (H&L) horseradish peroxidase-conjugated antibody (Chemicon International Ltd., Harrow, UK) 1:10000 dilution in 5% (w/v) Marvel in Tris-buffered saline.

Transfer time: 1 hour (to eliminate background noise).

2.15 Immunodetection of cellular tau by Western blotting

1 Sample preparation for detection of tau

Samples were prepared according to the methods for preparation of cellular β-amyloid described in 2.14.1 although no organophosphates were added to the medium. Cells chronically pre-exposed to diazinon (2.2.2) were similarly prepared and the results compared to those from cells that were not pre-exposed using one-way ANOVA.

2 Western blotting of tau samples

The methods used were those described in 2.10.1 with the following amendments:

Primary antibody: goat anti-tau (C-17) polyclonal antibody (Autogen Bioclear UK. Ltd., Calne, UK) 1:200 dilution in 5% (w/v) bovine serum albumin in Tris-buffered saline.

Blocking solution: 5% (w/v) bovine serum albumin in Tris-buffered saline.

Secondary antibody: rabbit anti-goat IgG (H&L) horseradish peroxidase-conjugated antibody (Chemicon International Ltd., Harrow, UK) 1:10000 dilution in 5% (w/v) Marvel in Tris-buffered saline.

Transfer time: 2 hours.

2.16 Confirmation that phosmet did not have effects due to a change in pH

In order to discount a change in pH for any effects observed after addition of phosmet to cell cultures, the pH of a solution of phosphate-buffered saline was recorded with increasing concentrations of phosmet. No changes in pH were detected.

2.17 Preparation of microglia

Microglia were prepared according to the methods described by Kingham & Pocock (2000). Four brains were removed from neonate rats onto ice-cold phosphate-buffered saline (140mM NaCl, 5mM KCl, 25 mM Na_2HPO_4, 11 mM glucose, 0.2% bovine serum albumin pH 7.4) and homogenized with 10-15 strokes of a hand-held homogeniser. The suspension was centrifuged at 500g for 10 min and the pellet resuspended in 10 ml of 70% isotonic Percoll (Amersham Pharmacia Biotech, Bucks., UK) (100% stock consisted of 9 parts Percoll to 1 part 10 X phosphate-buffered saline). This was overlayered with 10 ml of 35% Percoll and 10 ml phosphate-buffered saline. The Percoll gradient was centrifuged at 1250g for 45 min and the cells found at the 35/70% interface were removed. These were pooled and washed with phosphate-buffered saline before centrifuging at 500g for 10 min. The pellet was resuspended in DMEM for counting before dilution.

2.18 Investigation of effects of exposure of microglia to organophosphates on inhibition of neurite outgrowth in NB2a neuroblastoma cells

Microglia were counted in a standard haemocytometer and plated in serum-containing medium into 24-well plates at a density of 100,000 cells/ml with a volume of 500 µl/well. One well per treatment was used. After a 24 h incubation period to allow the microglia to adhere to the wells, the serum-containing proliferation medium was removed and replaced with serum-free medium containing 1 mM dibutyryl cyclic AMP. At the same time either 100 nM phosmet, 100 nM diazinon or the vehicle of dilution (DMSO) was added to the medium of three of the wells at a dilution of 1:400. The microglia were incubated in the medium for 24 h.

On the same day, NB2a neuroblastoma cells were plated in serum-containing medium into 8 wells of a 24 well plate at a density of 20,000 cells/ml with a volume of 500 µl/well. The cells were incubated for 24 h to allow the cells to adhere to the wells.

The medium was removed from all the wells of the NB2a-containing plate, and replaced in 4 wells with 500 µl serum-free medium containing 1 mM dibutyryl cyclic AMP to induce differentiation. At the same time, the above organophosphates were added to the medium in the same way as described for the microglia.

The medium was then removed from each microglial containing well and placed in the corresponding well containing NB2a cells. The resulting plate therefore contained for each treatment, one well of NB2a cells directly exposed to the test substance or control and one well exposed to conditioned medium from microglia exposed to the test substance or control for 24 h. The plates were incubated for a further 24 h before fixing and staining as described in 2.2.3. Cells in each well were analysed for neurite length as described (2.2.3) and the average length per cell for each treatment compared for statistically significant difference by Student's 2-tailed t-test for paired means. A total of 8 separate experiments were performed.

The experiments were repeated using cells chronically pre-exposed to diazinon (2.2.2).

APPENDIX 1

Reagents used in cell culture experimental methods

Cell fixative: 4% (w/v) paraformaldehyde, 4% (w/v) sucrose in phosphate-buffered saline (stored at 4°C for no more than 1 week).

Coomassie Blue stain: 0.6% (w/v) Coomassie Brilliant Blue, 10% (v/v) methanol, 10% (v/v) acetic acid in phosphate-buffered saline (stored at room temperature).

NADPH generating system: 0.4 mM NADP, 4 mM glucose-6-phosphate, 2 mM Mg_2Cl_2, 2 U/5µl glucose-6-phosphate dehydrogenase (freshly made from stock solutions).

Stock solutions for NADPH generating system:

100mM NADP: 100mM = 76.54mg/ml in DMEM medium (dilution factor 250).

1M glucose-6-phosphate: 1M = 282.1 mg/ml in DMEM medium (dilution factor 250).

500mM $MgCl_2$: 500mM = 101.65 mg/ml in DMEM medium (dilution factor 250).

2 units per 5µl glucose-6-phosphate dehydrogenase: 2 units/5 µl = 250 units/625 µl. Add 625 µl 1/15 PO_4 buffer to 250 units of enzyme.

Phosphate (PO_4) buffer (70 mM): 9.94% (w/v) Na_2HPO_4, 11.1% (w/v) NaH_2PO_4 in sterile distilled water pH 7.4 (stored at 4°C).

PO_4 and KCl buffer: 1.15% (w/v) KCl in phosphate (PO_4) buffer (70 mM) pH 7.4 (stored at 4°C).

Reagents used in immunocytochemistry methods

Lysis buffer: 100 mM NaCl, 10 mM EDTA, 0.5% (w/v) deoxycholate, 0.5% (v/v) Nonidet-P-40, in 1 mM Tris-HCl pH 7.4-7.8 (stored at 4°C).

SDS Sample buffer either: 8M Urea, 2% (w/v) sodium dodecyl sulphate (SDS), 0.001% (v/v) bromophenol blue solution (10% (w/v) solution) in upper gel buffer (stored at room temperature).

or: 20% (v/v) Glycerol, 8% (w/v) sodium dodecyl sulphate (SDS), 0.005% (w/v) bromophenol blue in phosphate-buffered saline (stored at room temperature).

Double strength SDS sample buffer: 8M Urea, 4% (w/v) sodium dodecyl sulphate (SDS), 0.001% (w/v) bromophenol blue in upper gel buffer (stored at room temperature).

Upper gel buffer: 30 g Tris base, 240 ml 1M HCl made up to 500ml with distilled water, pH 6.8 (stored at 4°C).

Lower gel buffer: 90.75 g Tris base, 130 ml 1M HCl, made up to 500 ml with distilled water, pH 8.8 (stored at 4°C).

Running buffer (10x): 30 g Tris base, 144 g glycine, 100 ml 10% w/v SDS, made up to 1litre with distilled water, pH 8.3 (stored at 4°C).

Transfer buffer: 7.57 g Tris-HCl, 36.03 g glycine, 500 ml methanol, made up to 1litre with distilled water (stored at 4°C).

Tris-buffered saline (TBS) 10x: 12.1 g Tris base, plus 81.76 g sodium chloride made up to 1litre with distilled water, pH 7.4 (stored at 4°C).

Polyacrylamide gels:

Proportions of constituents in gels

[1]Acrylamide- x% (w/v) acrylamide and 8.6/100x (w/v) N,N'-methylenebisacrylamide where x is the percentage acrylamide in the gel, (supplied by Merck, Poole, UK).

[2]AMPS – ammonium persulphate

[3]TEMED- N,N,N',N'-tetramethylethylenediamine

% gel	[1]acrylamide	distilled water	lower gel buffer	10% SDS	[2]AMPS	[3]TEMED
8	3.5	6	3.3	0.13	0.045	0.019
12.5	5.5	4	3.3	0.13	0.045	0.019
15	6.5	3	3.3	0.13	0.045	0.019

Proportions of constituents of stacking gels

% gel	[1]acrylamide	distilled water	upper gel buffer	10% SDS	[2]AMPS	[3]TEMED
4	0.66	2.90	1.25	0.05	0.05	0.001
5	0.83	2.75	1.25	0.05	0.05	0.001
6	1.00	2.60	1.25	0.05	0.05	0.001

CHAPTER 3

DEVELOPMENT OF THE ASSAY AND ITS USE TO DETECT NEUROTOXICITY OF INDIVIDUAL PESTICIDES

3.1 Introduction

The assay used in these studies is the inhibition of neurite outgrowth of differentiating neuroblastoma cells as a marker for neurotoxicity. The mechanism by which neurite outgrowth is inhibited involves, at least in part, the disruption of the neurofilament network (Flaskos *et al.*, 1998; Abdulla *et al.*, 1995). The methods have been used previously to demonstrate neurotoxic potential of various products in this laboratory (Flaskos *et al.*, 1998; Smith *et al.*, 2001) and in others (Abdulla and Campbell, 1993), including organophosphate pesticides (Henschler *et al.*, 1992).

The search for *in vitro* models of neurotoxicity (Fielder *et al.*, 1997), especially in relation to organophosphate pesticides has resulted in investigation of many cellular models with varying success. These include the assessment of cytotoxicity measured by neutral red dye uptake of neuroblastoma cells (Veronesi and Ehrich, 1993), morphological effects on dorsal root ganglia (Tuler and Bowen, 1989), the inhibition of acetylcholinesterase activity (Marinovich *et al.*, 1996) or of protein synthesis (detected as [^{3}H]leucine incorporation) in neuroblastoma cells (Marinovich *et al.*, 1996).

The inhibition of neurite outgrowth of differentiating cells has been used to demonstrate dose-dependent neurotoxicity with pyrethrin and DDT in chick embryo neurones (Ferguson and Audesirk, 1990), and mercuric oxide (Abdulla *et al.*, 1995) and excitatory amino acids (Abdulla and Campbell, 1993) in neuroblastoma cells. Our laboratory in Liverpool has developed these methods to distinguish neurotoxicity of a variety of products including pesticides (Flaskos *et al.*, 1998) and artemesinin derivatives (Smith *et al.*, 2001).

Many other permanent cell lines have been used to assess the inhibition of neurite outgrowth, and in our laboratory human SY5Y (McLean *et al.*, 1998) and mouse NB2a neuroblastoma cells and rat PC12 pheochromocytoma cells (Flaskos *et al.*, 1998) have all produced measurable neurite inhibition in response to neurotoxins. The murine NB2a neuroblastoma cell has been determined to be a sensitive predictor of neurotoxicity (Flaskos *et al.*, 1998), and its relative ease of culture suggested it was suitable for further use and development.

An example of neurite inhibition by the organophosphate chlorpyrifos is shown in Figure 3.1. Within 24h of the removal of serum, and addition of dibutyryl cyclic AMP, NB2a neuroblastoma cells ceased proliferation (as seen in 3.1A) and extended extensive neurites (3.1B). In the presence of neurotoxins, illustrated here by 1 μM chlorpyrifos, the outgrowth was inhibited (3.1C). This inhibition is measurable by light microscopy using a computerized digital imaging system, as described earlier (2.2.3).

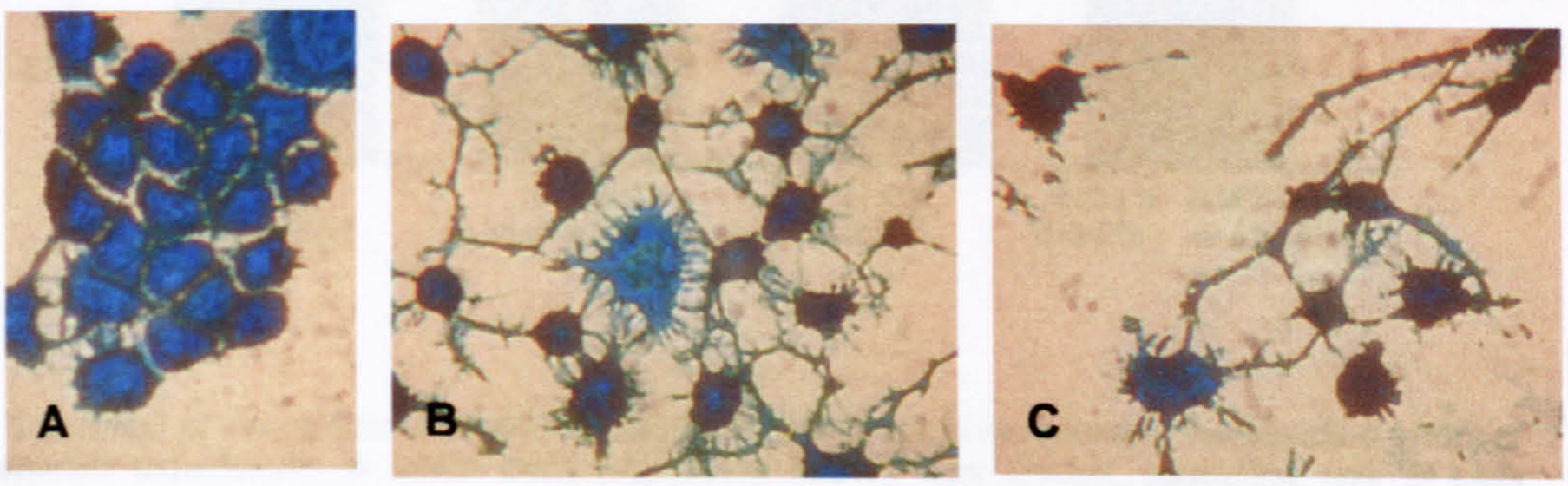


Figure 3.1: *Photomicrographs of NB2a neuroblastoma cells after differentiating for 24 h in the presence or absence of chlorpyrifos. Figure 3.1A shows cells maintained in serum-containing medium. 3.1B demonstrates neurite outgrowth when the serum is removed and 1 mM dibutyryl cyclic AMP is added. 3.1C shows cells differentiating in the presence of 1 μM chlorpyrifos. All cells were fixed in 10% formalin and stained with Coomassie Blue stain.*

Before further development of this assay to detect synergy between individual products, it was necessary to quantify neurotoxicity of individual pesticides and related products, and compare these results to data generated elsewhere *in vivo*.

3.2 Determination of the effects of the vehicles of dilution

Initially the methods described in 2.4.1 were used to ensure that the vehicles used to dilute the test substance were not themselves responsible for significant neurotoxicity. Since all experiments used either methanol or DMSO to dissolve test substances, these vehicles were investigated at various concentrations for their ability to inhibit neurite outgrowth. The results are given in Figure 3.2.

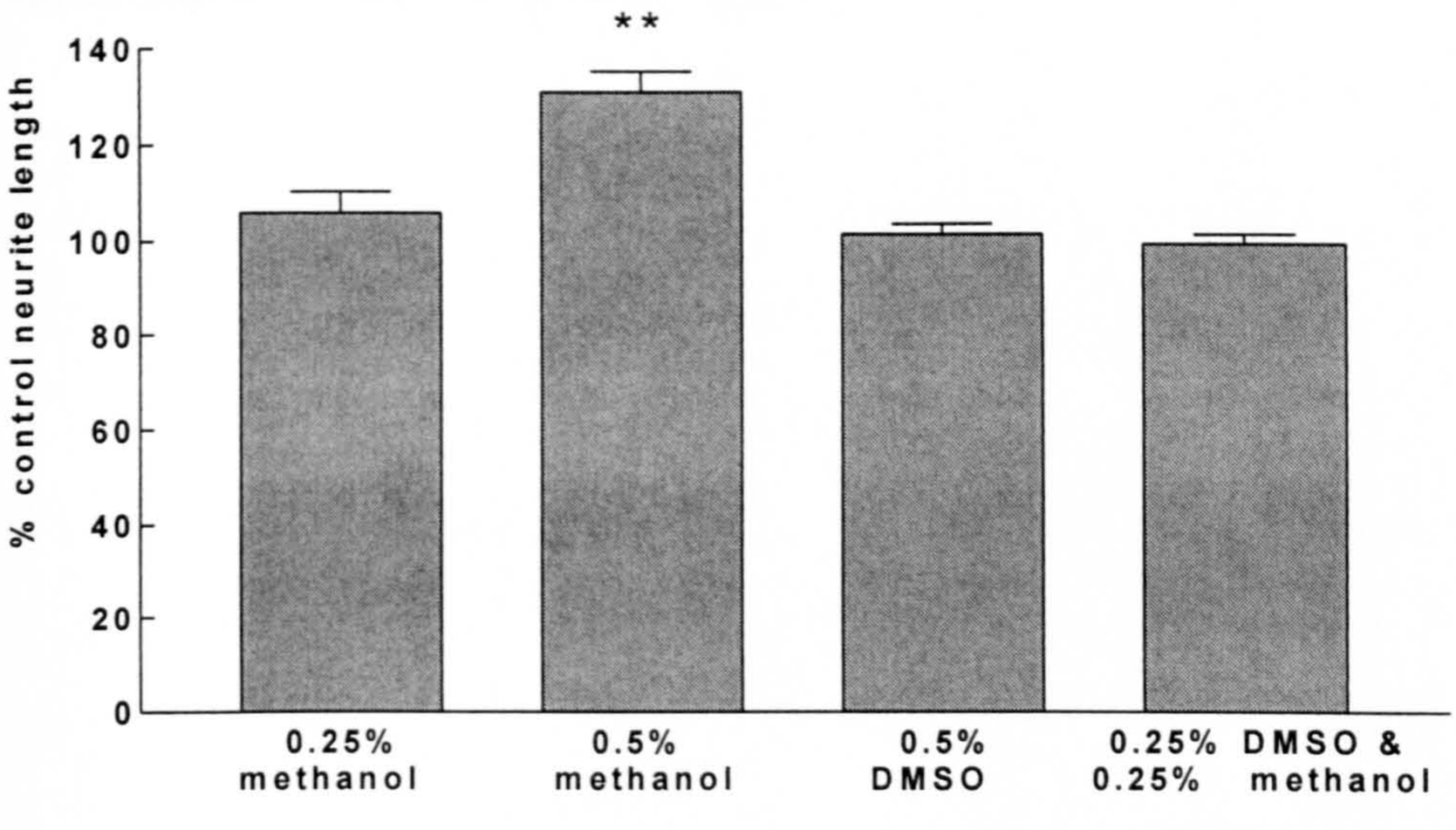


Figure 3.2: *Effect of the vehicle of dilution on neurite length. Error bars represent the standard errors of 6-12 separate experiments in which each experiment had its own control, consisting only of cells differentiating in serum-free medium containing 1mM dibutyryl cyclic AMP. Statistical analysis compared cells differentiating in serum-free medium containing DMSO, methanol or a mixture of both, to the control, using one-way ANOVA including Bonferroni's correction for multiple comparisons. ** Represents statistically significant difference ($p<0.005$).*

Methanol at a concentration of 0.5% induced a statistically significant increase in neurite length compared to serum-free controls. Methanol 0.25%, DMSO 0.5% and a mixture of 0.25% methanol and 0.25% DMSO did not produce any difference in neurite length compared to controls. These results stress the importance of including the vehicle of dilution in control samples, particularly in the case of methanol, in any experiments involving measurement of changes in neurite length.

3.3 Determination of individual IC_{50} values of test products

The methods described in 2.4.2 were used to determine individual IC_{50} values, where possible, for all products detailed in 2.3.

Organophosphate pesticides

The relationship between the concentration of various pesticide substances and the inhibition of neurite outgrowth produced is shown in Figure 3.3.

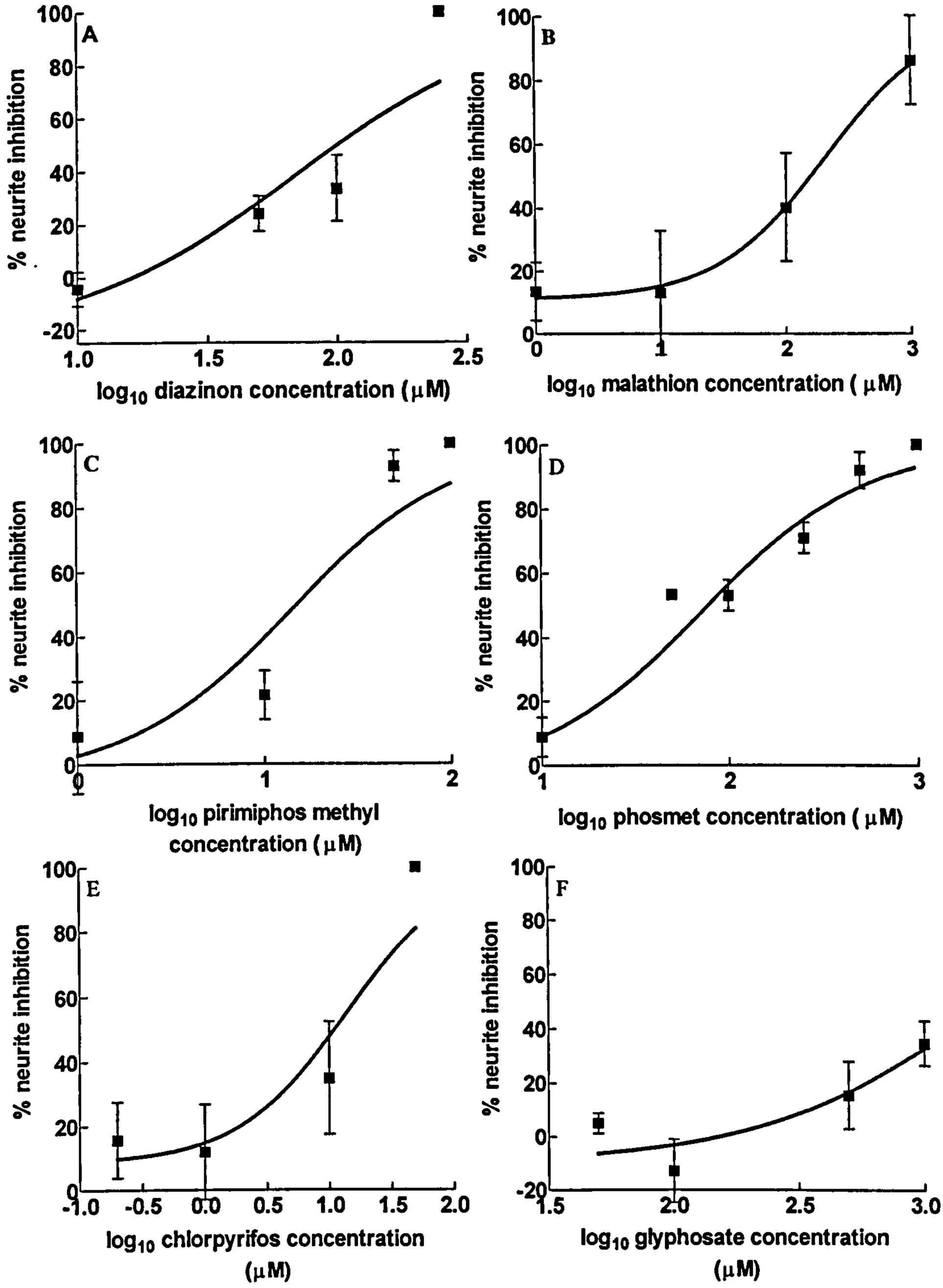


Figure 3.3: *Relationship between the concentration and the inhibition of neurite outgrowth observed in NB2a neuroblastoma cells differentiating for 24 h in A) diazinon, B) malathion C) pirimiphos methyl D) phosmet E) chlorpyrifos and F) glyphosate. Error bars represent standard errors of at least 4 different experiments.*

The mean IC_{50} values and 95% confidence intervals were determined to be as follows:

a) diazinon : 68 µM (33-142 µM);

b) malathion: 203 µM (41-1001 µM);

c) pirimiphos methyl: 14 µM (5-37 µM);

d) phosmet: 72 µM (46-114 µM);

e) chlorpyrifos: 13 µM (4-45 µM);

f) glyphosate: 1587 µM (650-3878 µM).

A summary of these results, compared to known oral LD_{50} values is given in Figure 3.4.

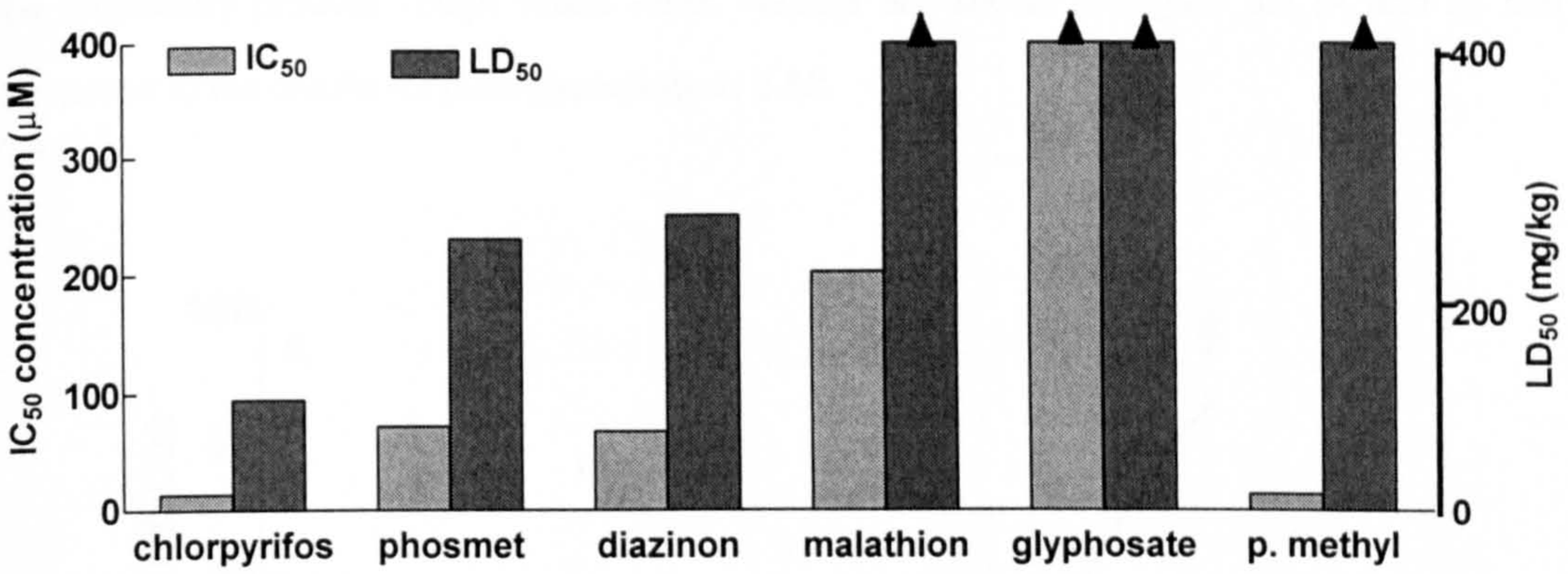


Figure 3.4: *Summary of IC_{50} values for inhibition of neurite outgrowth, compared to known oral LD_{50} values for the same organophosphates. The scale on the right denotes approximate oral LD_{50} (rat) values in mg/kg bodyweight. Arrow signifies values greater than the limits of the appropriate scale.*

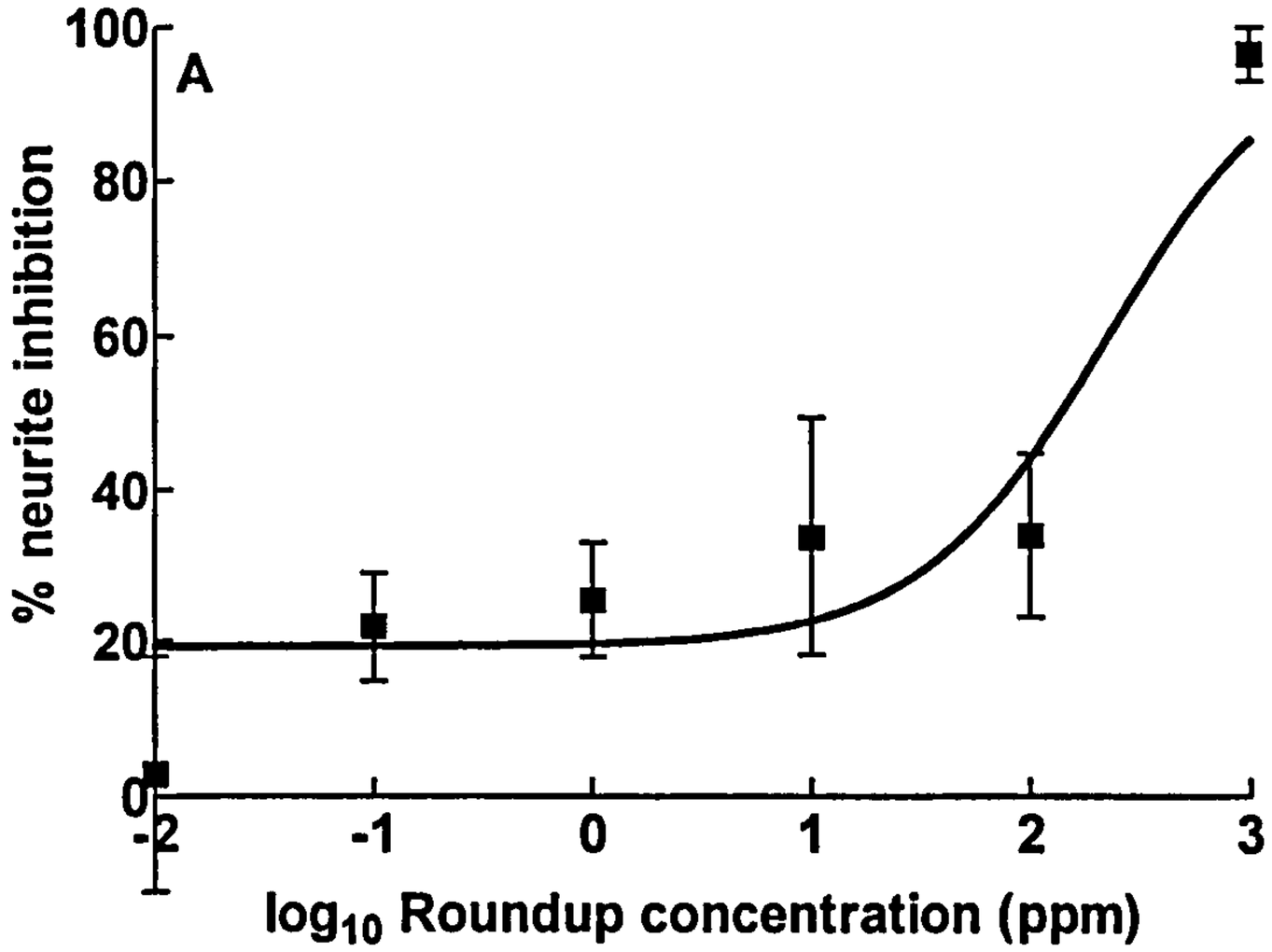
With the exception of pirimiphos methyl, the relative toxicity ranking of the individual organophosphates was comparable *in vitro* and *in vivo*. The LD_{50} value (mg/kg bodyweight) was in general equivalent to 3-7 times the *in vitro* IC_{50} value (µM).

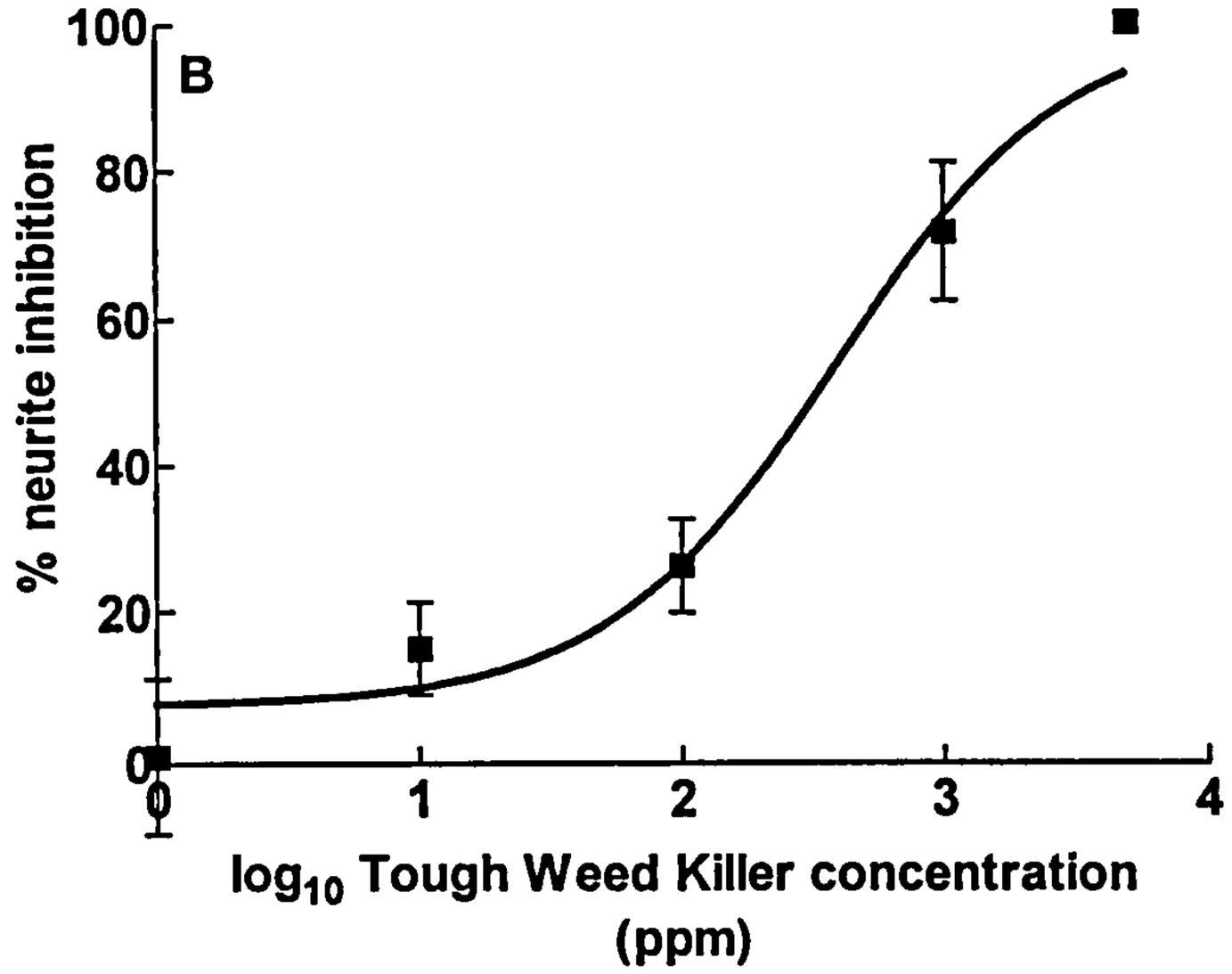
Pesticide formulations

Once I had established that this assay was able to detect activity of different organophosphates, and that this activity reflected the known toxicity of the products as determined by standard *in vivo* toxicity testing, the investigation was extended to demonstrate any differences when these products were included in formulations. IC_{50} values were obtained for various proprietary pesticide products. These were compared to the IC_{50} of the constituent pesticide ingredient.

Formulations of glyphosate

The experimental methods were used to determine the IC_{50} for one formulation of glyphosate as the proprietary product Roundup and one formulation of the glyphosate trimesium salt as the proprietary product Tough Weed Killer. Results are shown in Figure 3.5 (A and B) and compared to the results for pure glyphosate in 3.5C.





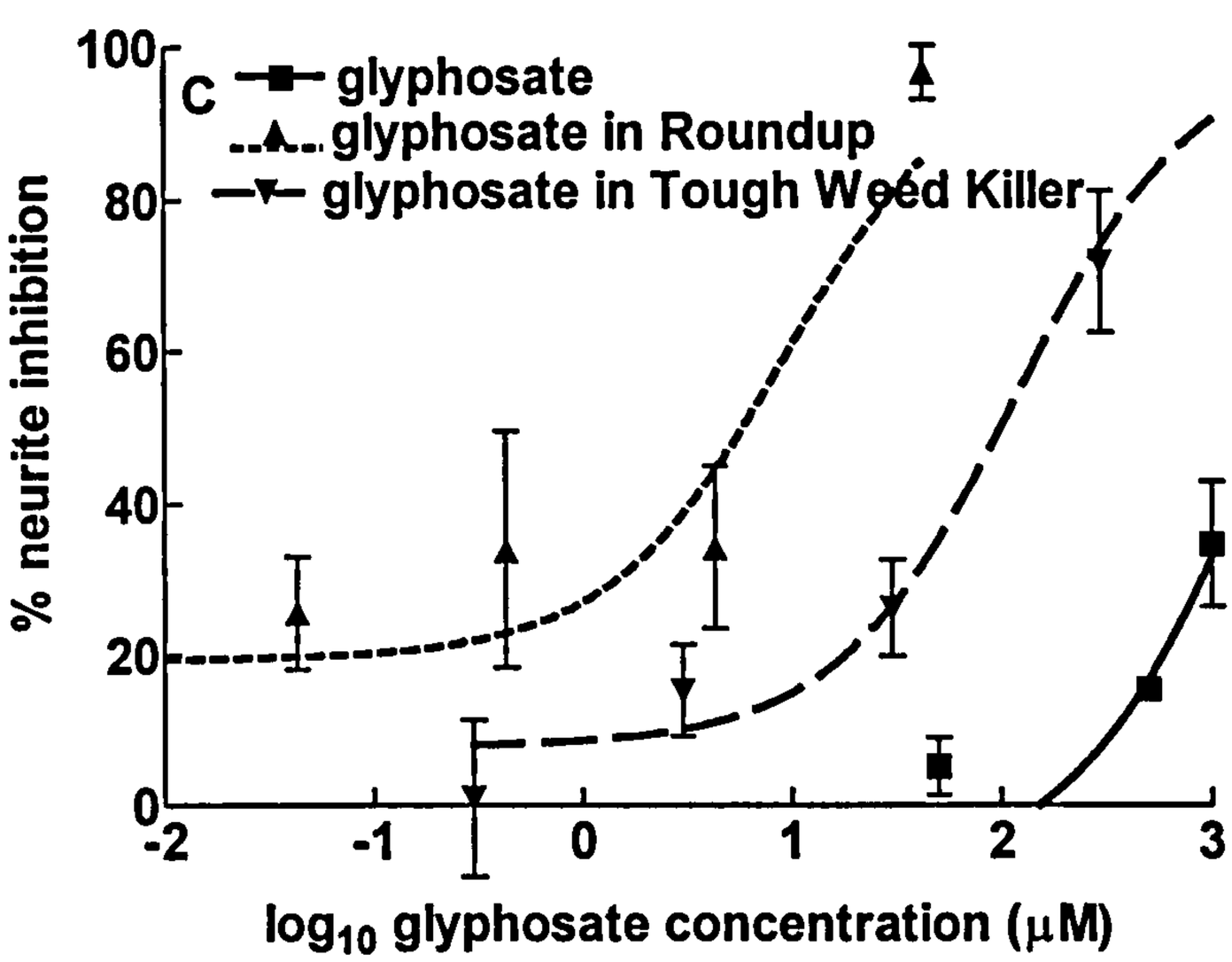


Figure 3.5: *Relationship between the concentration and the inhibition of neurite outgrowth observed in NB2a neuroblastoma cells differentiating for 24 h in A) glyphosate formulated as Roundup, B) glyphosate trimesium formulated as Tough Weed Killer. The results from formulations are summarized in C) to compare the equivalent neurite inhibition of the active ingredients when formulated in the two products with that of pure glyphosate. Error bars represent standard errors of 4-6 separate experiments.*

The mean IC_{50} values and 95% confidence limits were determined to be 223 ppm (73-679 ppm) for Roundup, equivalent to 10 µM (3-29 µM) of glyphosate alone and 392 ppm (202-761 ppm) for Tough Weed Killer, equivalent to 118 µM (61-228 µM) of glyphosate alone. This compared to the mean IC_{50} value for pure glyphosate of 1587 µM (650-3878 µM). The equivalent neurotoxicity of glyphosate was therefore apparently increased 165 times in the product Roundup and 13 times when formulated as glyphosate trimesium in the product Tough Weed Killer.

Due to the inability to determine individual IC_{50} values for all the separate experiments with the two formulations, statistical analysis to compare these values could not be performed. Instead, the point at which the response curve crossed the x-axis in each of the individual experiments was ascertained, and the equivalent glyphosate values at this point were compared to those for pure glyphosate for each of the two formulations, using one-way ANOVA including Bonferroni's correction for multiple comparisons. Statistically significant differences were observed for Roundup compared to glyphosate ($p<0.01$) and for Tough Weed Killer compared to glyphosate ($p<0.01$).

Formulation of phosmet

The above experimental methods were used to determine the IC_{50} value for Nupor, a 20% w/v formulation of phosmet. Results are shown in Figure 3.6A and compared with the values for pure phosmet in 3.6B.

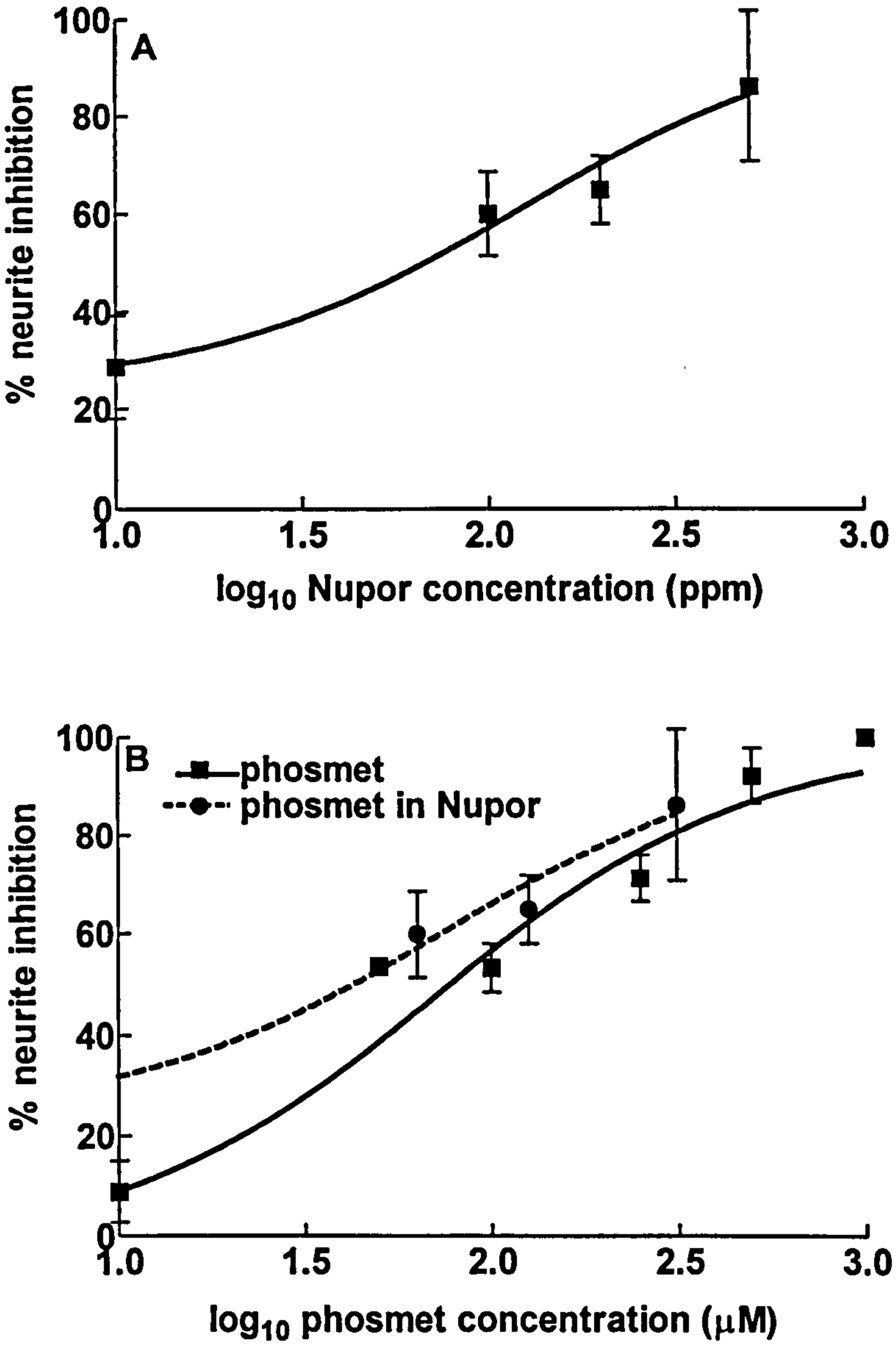


Figure 3.6: *A) Relationship between the concentration and the inhibition of neurite outgrowth observed in NB2a neuroblastoma cells differentiating for 24 h in Nupor (20% (w/v) phosmet). The results are summarized in 3.6B to compare the equivalent neurite inhibition of the active ingredient in the formulation with that of pure phosmet. Error bars represent standard errors of 4-8 different experiments.*

The mean IC_{50} and its 95% confidence limits for Nupor (20% (w/v) phosmet) were determined to be 128 ppm (41-400 ppm), equivalent to an IC_{50} of 81 μM (26-252 μM) for the phosmet alone. This compared to a mean IC_{50} of 72 μM (47-114 μM) for pure phosmet. Statistical analysis compared the IC_{50} of phosmet to the equivalent phosmet in Nupor. No statistically significant difference was found. Formulation of phosmet in Nupor therefore did not affect the neurotoxicity of the active ingredient.

Proprietary product containing a mixture of diazinon, pyrethrum and piperonyl butoxide

The experimental methods were also used to determine the IC_{50} for Formulation 1, a proprietary product consisting of a mixture of diazinon, pyrethrum and piperonyl butoxide (as detailed in 2.3), both as the formulation as sold and therefore including all vehicles and other additives, and as the equivalent mixture of active ingredients prepared in DMSO.

Results are shown in Figure 3.8A and 3.8B and compared in 3.8C.

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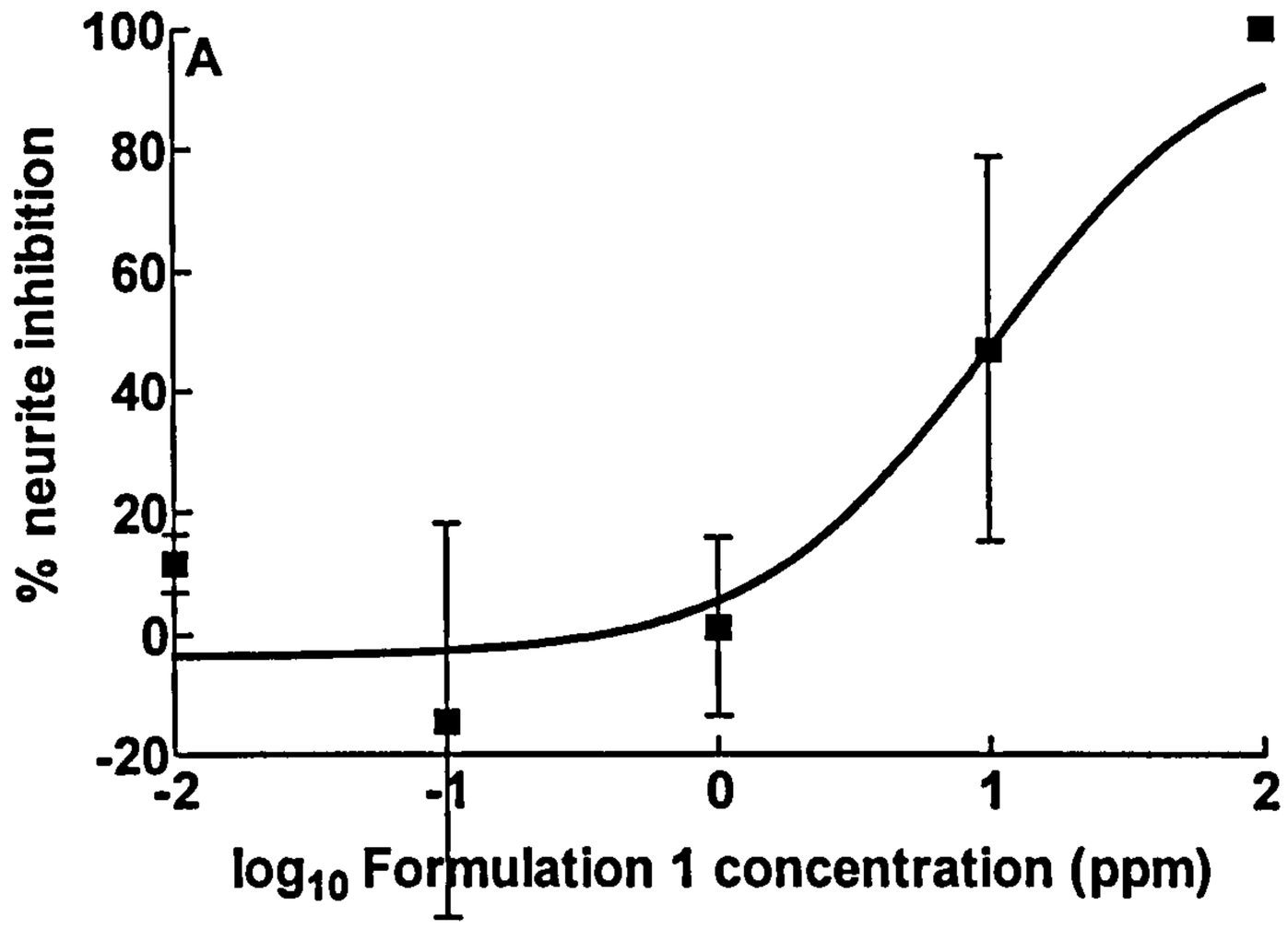
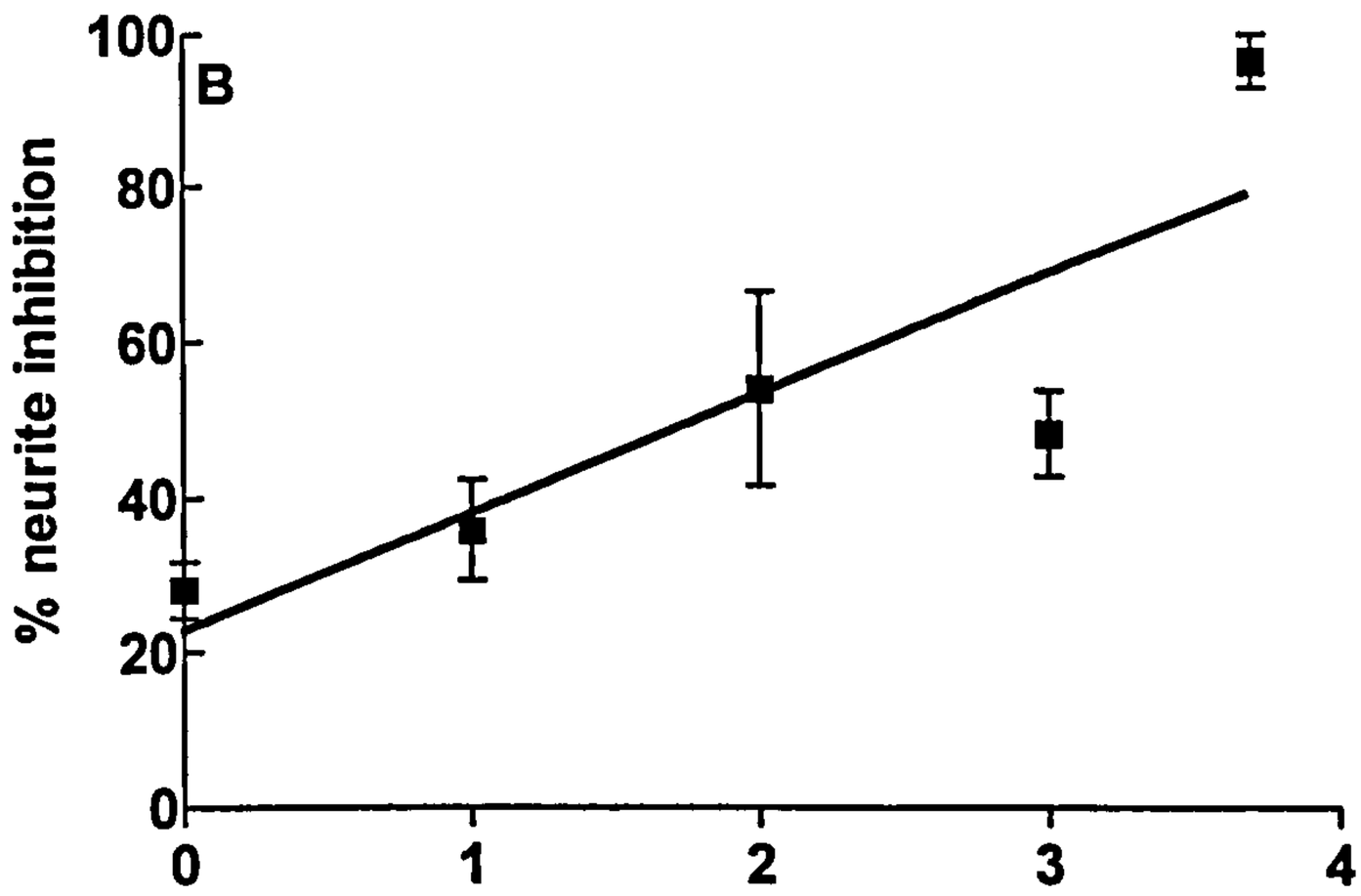




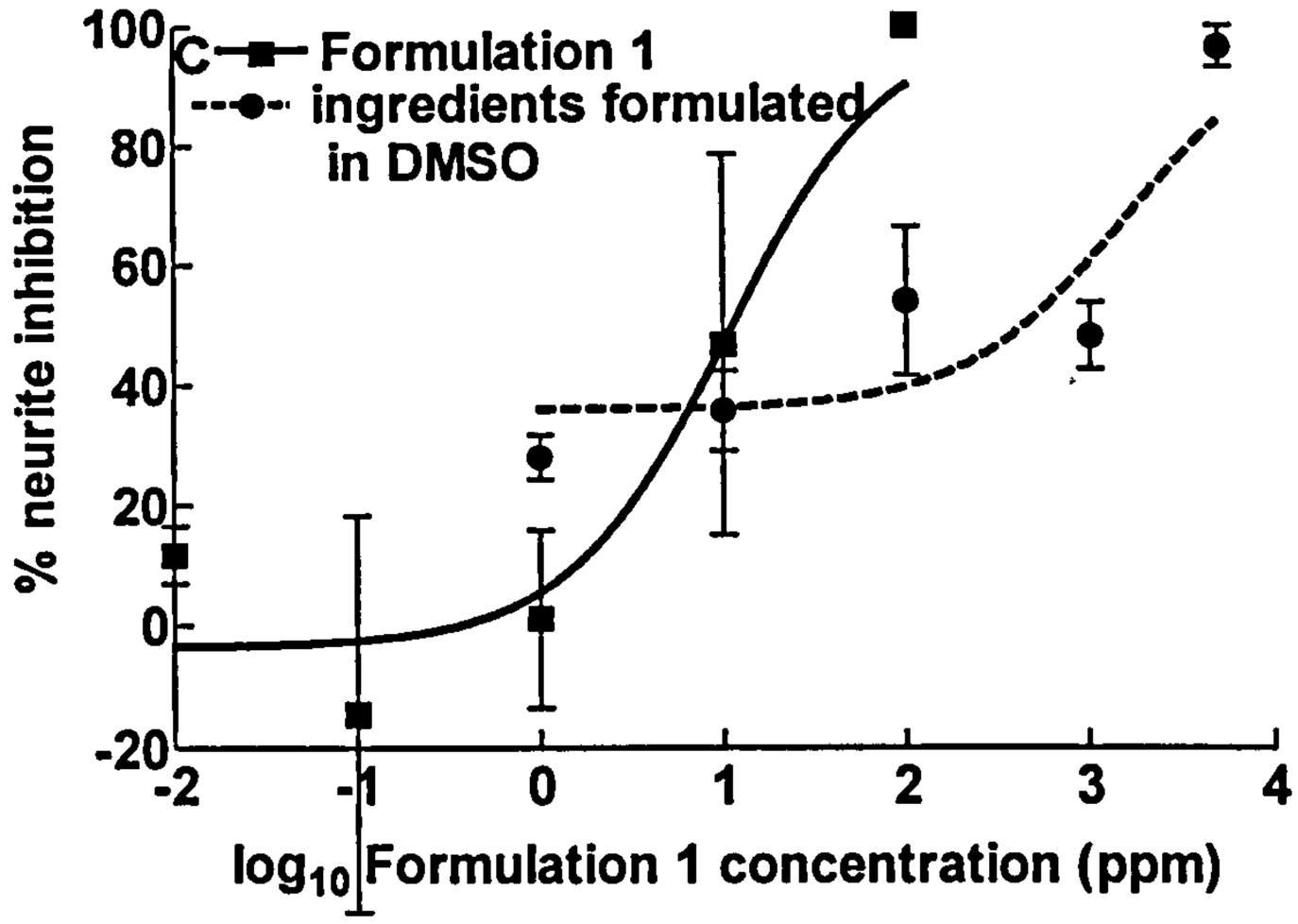


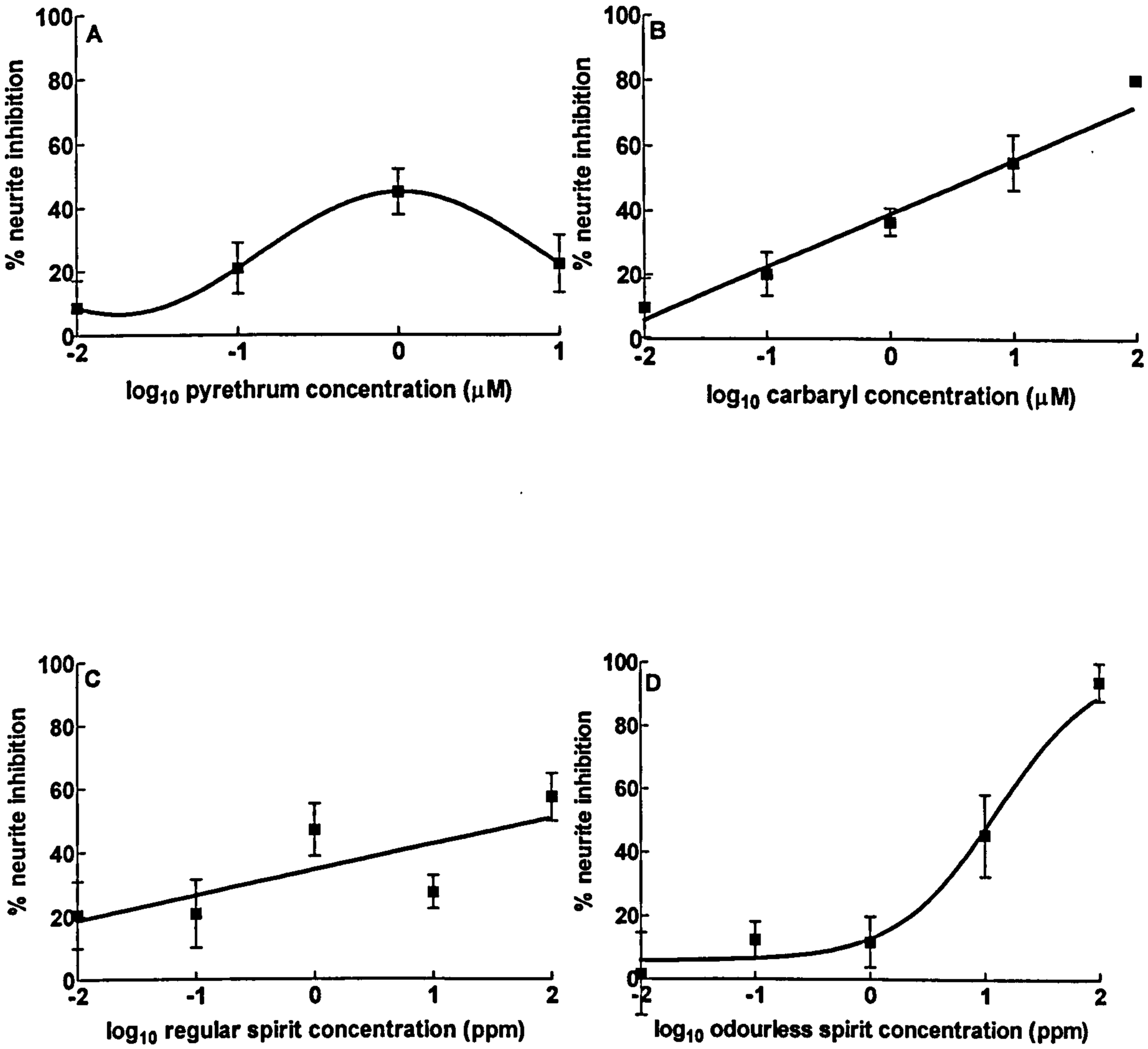
***Figure 3.8**: Relationship between the concentration and the inhibition of neurite outgrowth observed in NB2a neuroblastoma cells differentiating for 24 h in A) Formulation 1 containing diazinon, pyrethrum and piperonyl butoxide and B) the ingredients of the product formulated in DMSO at the same concentrations as in Formulation 1. The results are summarized in 3.8C to compare the equivalent neurite inhibition of the active ingredients in the formulation with that of the ingredients of the formulation only. Error bars represent standard errors of 4-5 separate experiments.*

The mean IC_{50} and its 95% confidence limits for Formulation 1 containing diazinon, pyrethrum and piperonyl butoxide were determined to be 11 ppm (2-66 ppm), whereas the IC_{50} of the ingredients formulated without the vehicles was 1580 ppm (592-4218 ppm).

The IC_{50} value was therefore found to be 474 times greater in the commercial formulation than in a mixture of the same active ingredients without vehicles. Because of a wide variation in individual results, the difference between IC_{50} values was not found to be statistically significant.

Non-organophosphate pesticides and ingredients of pesticide formulations

The same experimental methods were used to determine the IC_{50} for pyrethrum (a natural pyrethroid) and for carbaryl, a carbamate pesticide that is a reversible acetylcholinesterase inhibitor. Furthermore various ingredients of pesticide formulations were investigated viz. two vehicles known to be used in Formulation 1, and piperonyl butoxide, the potentiator used in most formulations containing pyrethrum. The results are shown in Figure 3.9.



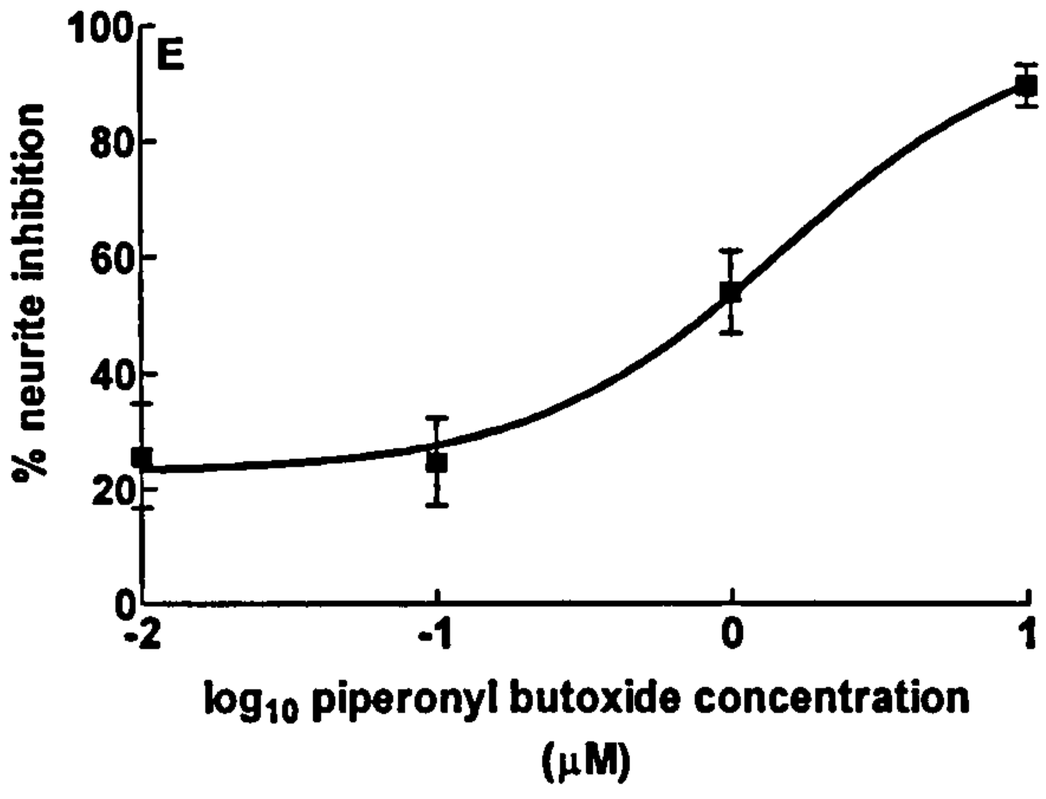


Figure 3.9: *Relationship between the concentration and the inhibition of neurite outgrowth observed in NB2a neuroblastoma cells differentiating for 24 h in A) pyrethrum B) carbaryl C) regular spirit for Formulation 1 D) odourless spirit for Formulation 1 and E) piperonyl butoxide. Error bars represent standard errors of 4-6 separate experiments.*

Experiments with pyrethrum were unable to determine a precise IC_{50} value although it was considered to be in the region of 1 μM. A bell-shaped dose response curve was observed with the range of pyrethrum concentrations used. A maximum neurite inhibition of about 45% was achieved in the region of 1 μM, but thereafter, higher concentrations of pyrethrum resulted in reduced neurite inhibition (i.e. increased neurite extension).

The IC_{50} and its 95% confidence limits for carbaryl were determined to be 12 μM (5-27 μM).

The IC_{50} and its 95% confidence limits for regular mineral spirit were determined to be 151 ppm (45-513 ppm) and those for odourless mineral spirit were determined to be 13 ppm (6-29 ppm). It is clear that the vehicles used in Formulation 1 produced significant neurotoxicity individually, and that the difference in the IC_{50} observed with Formulation 1 compared to the mixture of active ingredients (Figure 3.8) may be explained to some extent by the effects of these vehicles.

The IC_{50} and its 95% confidence limits for piperonyl butoxide were determined to be 1.5 μM (0.7-3.5μM).

3.4 Microsomal activation of phosmet, diazinon and pirimiphos methyl

Three of the organophosphates, phosmet, diazinon and pirimiphos methyl were investigated after activation with microsomes, as described in 2.4.3, to determine any changes in their ability to inhibit neurite outgrowth resulting from their metabolism. The concentration used was chosen at the approximate IC_{20} of each product, as assessed from preliminary response curves. The inhibition of neurite outgrowth was compared for each pesticide in the presence and absence of microsomes. The results are summarized in Figure 3.10.

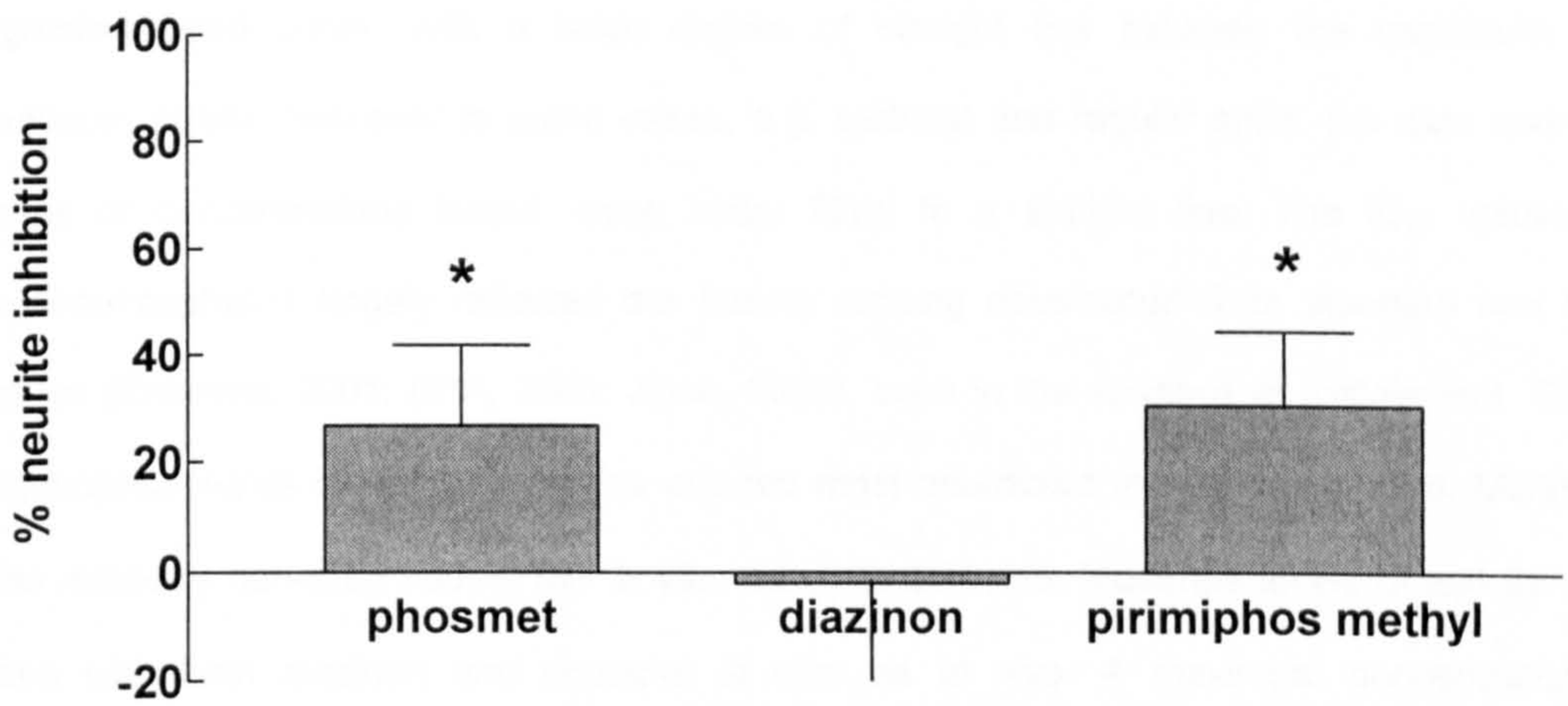


Figure 3.10: *Mean inhibition of neurite outgrowth of NB2a neuroblastoma cells differentiating for 24 h in phosmet (6 μM), diazinon (41 μM) or pirimiphos methyl (1.5 μM) in the presence of microsomal activation. Results are expressed as % change above controls treated with the corresponding organophosphate without microsomal activation. Error bars represent standard errors of 3 separate experiments. Statistical analysis was by Student's t test for paired means. * represents a statistically significant increase in neurite inhibition after microsomal activation of phosmet ($p<0.02$) and pirimiphos methyl ($p<0.03$) compared to controls without activation.*

Microsomal activation of phosmet and pirimiphos methyl produced a small but significant increase in inhibition of neurite outgrowth ($p< 0.02$ and 0.03), compared to the outgrowth observed without microsomal activation. No significant difference in inhibition of neurite outgrowth was observed in the presence or absence of microsomal activation of diazinon.

3.5 Discussion

In order to develop this *in vitro* assay to detect synergistic changes resulting from combination of products, first it was necessary to demonstrate that the assay detected activity in individual products and was able to distinguish between the effects produced by these products. The results demonstrated that both criteria were met.

Justification of assay

The log concentration/response data, with the exception of pyrethrum, generally produced a sigmoid-shaped curve, with a large degree of straight line between the maximum and minimum points. However in some cases, e.g. carbaryl and regular spirit, the data over the range of concentrations tested, were better fitted to a straight line. The IC_{50} values for organophosphates largely reflected the toxicity ranking determined from standard oral LD_{50} values (Extoxnet, 2001; EPA, 2001; Jinno, 2001), even in the absence of metabolism. Of the organophosphates tested, chlorpyrifos was the most neurotoxic *in vitro* and *in vivo*. Malathion was relatively non-toxic both in this *in vitro* assay and *in vivo*. Moderate levels of toxicity were seen with both diazinon and phosmet *in vitro* as *in vivo*. A maximum concentration of glyphosate produced minimal neurotoxicity, reflecting the high oral doses that can be given *in vivo* without ill effect (Extoxnet, 2001; EPA, 2001; Jinno, 2001).

The only exception to this correlation was pirimiphos methyl, which was relatively neurotoxic in this assay, yet is relatively non-toxic *in vivo* (Cremlyn, 1978). It is possible that the LD_{50} value is not as accurately reported for this product, or that the vehicle used in the oral LD_{50} study masked toxicity which would have been apparent with a different vehicle, as seen with some products (Gyrd-Hansen *et al.*, 1993; McCain *et al.*, 1997). Alternatively the action of pirimiphos methyl on neurones may differ from the action on other cells.

The use of this assay can generally be seen to be a good predictor of relative toxicity for organophosphate pesticides, and as such could be used in preference to *in vivo* studies for a preliminary indication of neurotoxicity of these and probably other chemicals. Effects on the

inhibition of neurite outgrowth in neuroblastoma cells predict *in vitro* toxicity more satisfactorily than some other *in vitro* tests. Other methods have been criticized as being inadequate at distinguishing neurotoxicity without being supplemented by other end-points (Schmuck *et al.*, 2000). For example the assessment of morphological changes in isolated dorsal root ganglia (Tuler and Bowen, 1989) produced results that did not generally reflect the relative *in vivo* toxicity of the test substances, besides involving subjective assessment and employing electron microscopy; not practical for a rapid throughput assay. The investigation of relative cytotoxicity in dorsal root ganglia as well as a variety of other cells (Sharma and Obersteiner, 1981) did not produce a meaningful correlation between the parameters observed, including acetylcholinesterase inhibition and lipophilicity, and the effects known *in vivo*.

Henschler *et al.* (Henschler *et al.*, 1992) employed similar methods to those used here in other permanent cell lines (C6 glia and N-18 neuroblastoma cells) to distinguish between organophosphates on the basis of ability to produce delayed neurotoxicity. The cells were exposed to the test substance for 14 days prior to assessment after a further 6 days. Results for the single pesticide tested in common with the studies here, chlorpyrifos, showed an IC_{50} of 44 µM. This compares favourably with the result (13 µM (4-44 µM)) obtained here after only 24 h exposure and 24 h observation. It appears from this single example that nothing was gained by the extension of exposure and observation times.

It is always difficult to relate IC_{50} values *in vitro* to actual exposure risks *in vivo*. Approximations of exposure absorption and subsequent metabolism, and estimates of excretion can be calculated but the results may be criticised on the grounds that they have no relationship to actual exposure, which may be influenced by a variety of different components. However extrapolation from the summary graph (Figure 3.4) indicates that figures for the IC_{50} values (µM) achieved with this methodology are in general 3-7 times lower that the LD_{50} oral values expressed as mg/kg bodyweight. Thus, despite this variation in correlation factor, use of this test could be justified as a better predictor of *in vivo* toxicity since it is based on an actual rather than a hypothesised correlation. Results could produce a figure that is more relevant to the human exposure situation and which is expressed in the accepted terminology

of LD_{50} values, than calculations based on expected metabolism and excretion. The testing of many more organophosphates of known *in vivo* toxicity as well as other substances, could confirm whether this suggested correlation applies more widely.

Although the organophosphates are generally considered to be more neurotoxic than pyrethroids *in vivo* (Casida *et al.*, 1983; Vijverberg and van den Bercken, 1979), this *in vitro* neurotoxicity assay indicated that pyrethrum was more neurotoxic than organophosphates when applied directly to neuroblastoma cells. *In vivo*, organophosphates are metabolized by cytochrome P450 enzymes to their corresponding oxons, which are usually, but not always more toxic than the parent molecules (Sultatos, 1994). However intermediate metabolites may be produced with unknown toxic potential (Manzo *et al.*, 1996; Barber *et al.*, 1999a). Pyrethroids on the other hand are rapidly metabolized to less toxic metabolites (Aldridge, 1990). Although some P450 enzyme activity is known to be present in immortalised cells (Veronesi and Ehrich, 1993; Thuerl *et al.*, 1997), the extent to which the nerve cells contributed any degree of metabolic activation or detoxification of the test substances was not investigated in this study. It was assumed to be minor in comparison with hepatic metabolism *in vivo*.

The inclusion of microsomes to simulate liver metabolism resulted in a greater degree of neurotoxicity associated with phosmet and pirimiphos methyl, presumably from more neurotoxic metabolites (pirimiphos methyl oxon and phosmet oxon), than without microsomal activation. No difference was observed with diazinon, with or without microsomes, suggesting that either diazinon was not metabolised, or that the product of metabolism (assumed to be diazinon oxon), was no more neurotoxic than the parent molecule. Besides, it is by no means clear how metabolic alteration of the anticholinesterase activity of organophosphates, such as that proposed by Barber *et al* (Barber *et al.*, 1999b) is translated into inhibition of neurite outgrowth as measured here, if at all. Organophosphates have numerous effects on cells not related to esterase inhibition or metabolism. For example, it is chlorpyrifos, rather than its oxon, that inhibits DNA synthesis and produces development changes in synaptic function after acute exposure (Dam *et al.*, 1998).

In some circumstances metabolic activity may be inhibited or inefficiently performed. Inhalation of volatile products may permit direct access of protoxicants to the brain (Spencer and Schaumburg, 1985). Simultaneous exposure to other products, such as associated solvents, may inhibit or enhance metabolism (Abou-Donia *et al.*, 1991; Vitarius *et al.*, 1995). Likewise liver metabolism may produce different metabolites to those achieved in the brain. In some individuals the relative or complete absence of serum enzymes such as paraoxonase (Kondo and Yamamoto, 1998), responsible for further metabolism of toxic oxon molecules, would prolong the toxicity of these metabolites *in vitro*. Therefore induction of *in vitro* metabolism may mask the true effects *in vivo*.

It would not be unexpected that, in the absence of hepatic enzymes, a different pattern of relative neurotoxicity would be found not exclusively associated with esterase inhibition. Other effects, such as changes in protein synthesis or phosphorylation may inhibit neurite outgrowth by means independent of the metabolism of the protoxicant. By excluding microsomal activation, separate assessment of protoxicants and metabolites may be performed, which ensures comparison to non-organophosphate products that are not metabolized in the same way.

Similarly, this assay has no representation of the blood-brain barrier. An additional factor that might contribute to synergism *in vivo* would be alterations in the blood-brain barrier known to occur as a consequence of exposure to organophosphates and pyrethroids (Gupta *et al.*, 1999a). The *in vitro* assay is thus designed for the study of mechanisms of neurotoxicity of compounds and their combinations entirely at the cellular level.

Similar toxicity rankings for the IC_{50} and LD_{50} of organophosphates, even in the absence of microsomal activation, justified the further development of this assay, and suggested that it may prove a useful preliminary screen for neurotoxicity. The slope of the response curves should also be considered when relating the toxicity of pesticides to the *in vivo* situation, as demonstrated in Figure 3.11.

Extrapolation from neurotoxic concentrations to predict no-effect concentrations may not always be appropriate. A reduction by a factor of 10 to predict potential no-effect levels from IC_{50} values may be useful for products with steep response curves such as diazinon, but for those with a shallow response curve such as malathion, similar extrapolation would still maintain significant levels of neurotoxicity.

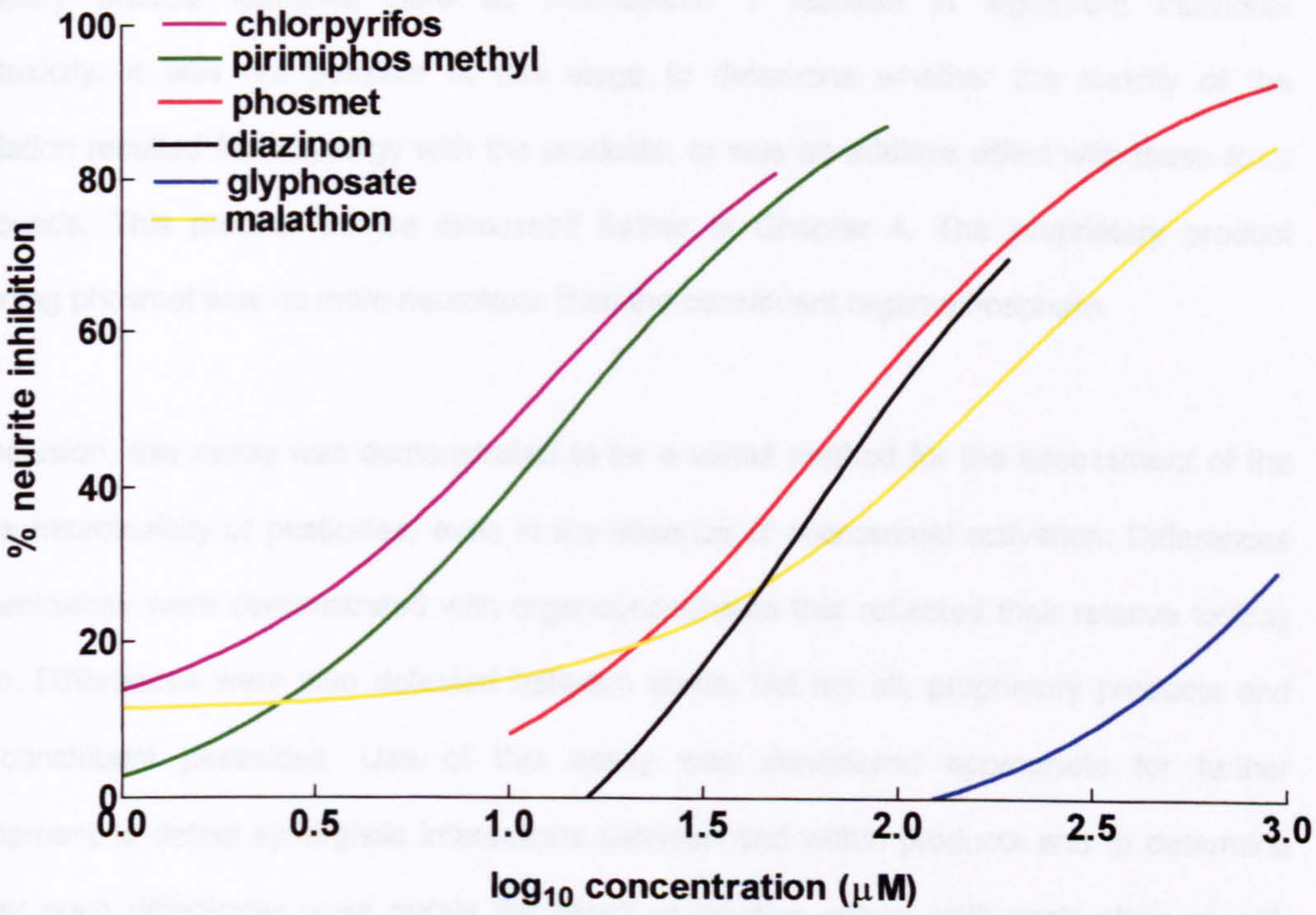


Figure 3.11: *Summary of individual response curves demonstrating inhibition of neurite outgrowth in NB2a neuroblastoma cells differentiating for 24 h in increasing concentrations of various organophosphate pesticides.*

Formulations and related products

Some but not all pesticide formulations resulted in significant increases in neurotoxicity, compared to the constituent pesticides. This was most notable in the case of formulations of glyphosate, where a 165-fold increase in toxicity was observed, compared to the pure product. The equivalent IC_{50} was reduced to approximately 10 µM; similar to the much more toxic substances such as chlorpyrifos. The neurotoxic potential of the other components of these formulations was unknown, so it was not possible to determine the extent of their involvement. However, separate assessment of two constituent mineral spirits from the proprietary product identified here as Formulation 1 resulted in significant individual neurotoxicity. It was not possible at this stage to determine whether the toxicity of the formulation resulted from synergy with the products, or was an additive effect with these toxic compounds. This product will be discussed further in Chapter 4. The proprietary product containing phosmet was no more neurotoxic than the constituent organophosphate.

In conclusion, this assay was demonstrated to be a useful method for the assessment of the relative neurotoxicity of pesticides, even in the absence of microsomal activation. Differences in neurotoxicity were demonstrated with organophosphates that reflected their relative toxicity *in vivo*. Differences were also detected between some, but not all, proprietary products and their constituent pesticides. Use of this assay was considered appropriate for further development to detect synergistic interactions between and within products and to determine whether such differences were purely the result of additive effects with each other or with other neurotoxic products in the formulation, or whether there was evidence of any synergistic interactions at a cellular level.

CHAPTER 4

THE SYNERGISTIC INTERACTION OF PESTICIDES

4.1 Introduction

The study of neurotoxicity in man and animals is complicated by the problems of synergism due to simultaneous exposure to different chemicals. The concept of synergism has achieved particular prominence in connection with the ill health experienced by Gulf War veterans exposed to a cocktail of prophylactic treatments (Abou-Donia and Wilmarth, 1996), and of farmers exposed to sheep dip pesticides (Pilkington *et al.*, 1999; Committee on Toxicity of Chemicals in Food, 1999). Only a few attempts have been made to analyze various combinations of products *in vivo* (Abou-Donia *et al.*, 1996a; McCain *et al.*, 1997; Audegond *et al.*, 1989; Jonker *et al.*, 1993a; Jonker *et al.*, 1993b) and *in vitro* (Marinovich *et al.*, 1996; Roloff *et al.*, 1992; Bianchi-Santamaria *et al.*, 1997; Piatti *et al.*, 1994).

The acute effects of organophosphate pesticides through inhibition of neuronal acetylcholinesterase activity are well known (Sultatos, 1994). Toxicity can also be manifested chronically as organophosphate-induced delayed neuropathy (OPIDN) (Johnson, 1990), which is related to inhibition of the enzyme neuropathy target esterase (NTE), or as the myaesthenia-like Intermediate Syndrome (Senanayake and Karalliedde, 1987). Further actions of organophosphates include effects on GABA receptors (Kar and Matin, 1972), hormones (Smallridge *et al.*, 1991) and other neurochemical pathways (Lundy and Magor, 1978; Liu *et al.*, 1986; Newball *et al.*, 1986; Gupta and Dettbarn, 1987). Other groups of pesticides target different neuronal mechanisms: pyrethroids for example, act primarily to prolong opening of Na^+ channels to produce repetitive discharge (Aldridge, 1990).

Synergism or potentiation is only one result of combination of different substances, and is assumed to be 1+1>2 for the purpose of this study. Although these investigations are concerned with an increase in toxicity greater than an expected additivity (1+1=2), other interactions may result in antagonism (1+1<2) inhibition (1+1<1) or lack of effect (1+1=1),

which may increase in importance in respect of drug interactions. However it is likely that this assay would also detect these interactions if they occurred.

Being generally insoluble in water [Davis, 1980 #451], most pesticides are formulated in a variety of different solvents, and are often used in conjunction with potentiators, such as piperonyl butoxide with pyrethroid formulations as mentioned in Chapter 3. Where pesticides are used in combinations, there is a potential for interactions not only between the pesticides, but also between pesticides, solvents and potentiators. Whole animal studies have been undertaken to demonstrate interactions between compounds resulting from interference with liver metabolism. In that way it has been shown that pre-treatment of mice with diazinon, the organophosphate most commonly used in sheep dips, causes an increase in cocaine immunotoxicity (Kump *et al.*, 1996), which is thought to be due to effects on metabolism by cytochrome P450 enzymes. Rats treated with both n-hexane and the organophosphate tri-*o*-cresylphosphate (TOCP) rapidly develop ataxia which is more severe and persistent than that produced with either product alone (Pellin *et al.*, 1987). Likewise, simultaneous dermal exposure of hens to *o*-ethyl-*o*-4-nitrophenyl phenylphosphonothioate and methyl n-butyl ketone produces a potentiation of neurotoxicity (Abou-Donia *et al.*, 1985).

Where interactions occur at the level of cells and tissues, synergism may be demonstrated *in vitro*. For example, Marinovich *et al.* (Marinovich *et al.*, 1996) have shown that, although the effects on acetylcholinesterase of human SY5Y neuroblastoma cells exposed to a mixture of pesticides is equivalent to that of the most potent pesticide, the effects on protein synthesis can in some cases indicate potentiation or synergism.

Such is the overwhelming number of potentially neurotoxic compounds in daily use (Howard, 1997) that an early *in vitro* screen of potential interactions would be useful to indicate potential problems. This method described here involves the development of the assay detailed in Chapter 3, which uses an indicator of toxicity at the cellular level, viz. outgrowth of neurites from differentiating neuroblastoma cells (Flaskos *et al.*, 1998; Abdulla and Campbell, 1993),

to assess synergistic interactions between combinations of pesticides and associated compounds.

4.2 Research hypothesis investigated in this chapter

Combination of pairs of pesticides or their constituents leads to neurotoxicity that differs quantitatively from that expected from an additive effect.

4.3 Determination of potential synergism of products encountered by farmers

Five pesticide products commonly encountered by farmers were assessed in combinations of two pesticides for possible synergistic reactions, according to the methods detailed in 2.5. The products used were as follows:

phosmet, diazinon, pirimiphos methyl, pyrethrum and glyphosate.

A concentration equivalent to the approximate IC_{20} of each of the pesticides was chosen as the maximum used in these experiments. In the absence of synergistic response, use of a range of proportions of two pesticides would be expected to produce the response curve shown in Figure 4.1.

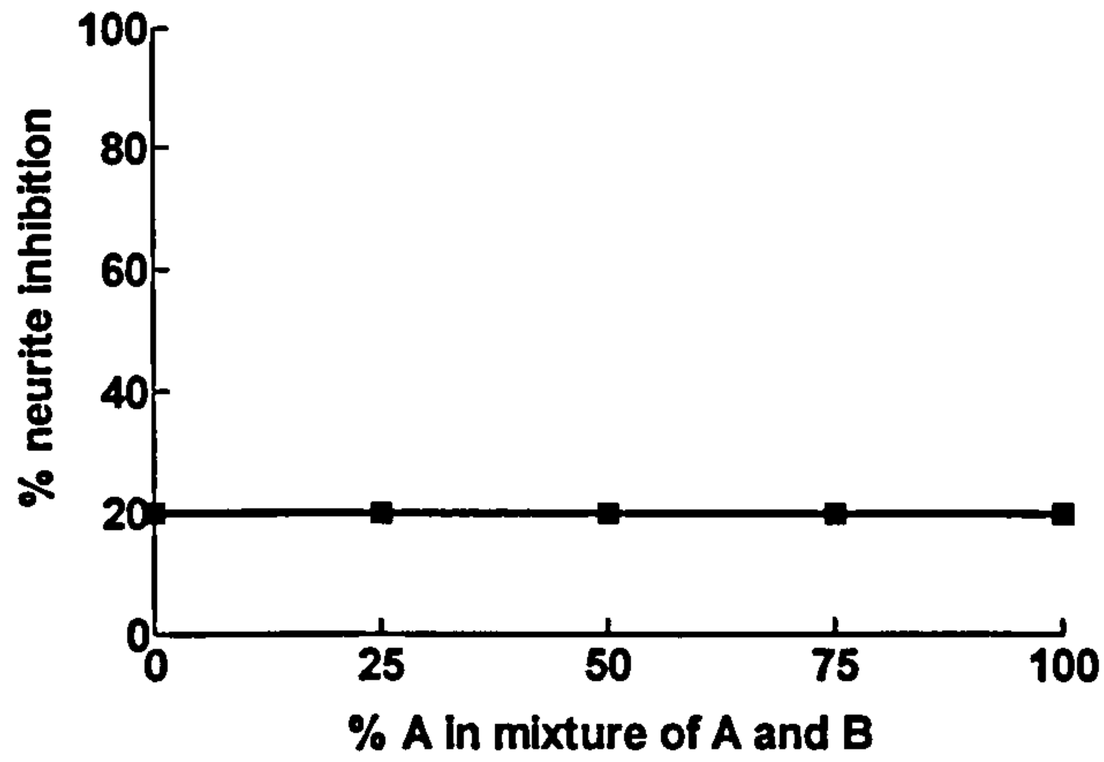


Figure 4.1. *Schematic graph of the expected relationship between various combinations of two products at concentrations equivalent to their combined IC_{20} in the absence of synergistic interactions.*

The expected results illustrated assume a linear relationship between concentration and response. This assumption may be questioned however, particularly since as seen in Chapter 3, the dose response relationships for the individual substances may be different. In order to test this assumption, graphs plotted from the actual expected results derived from the individual sigmoid curves of each substance for two examples are shown in Figure 4.2.

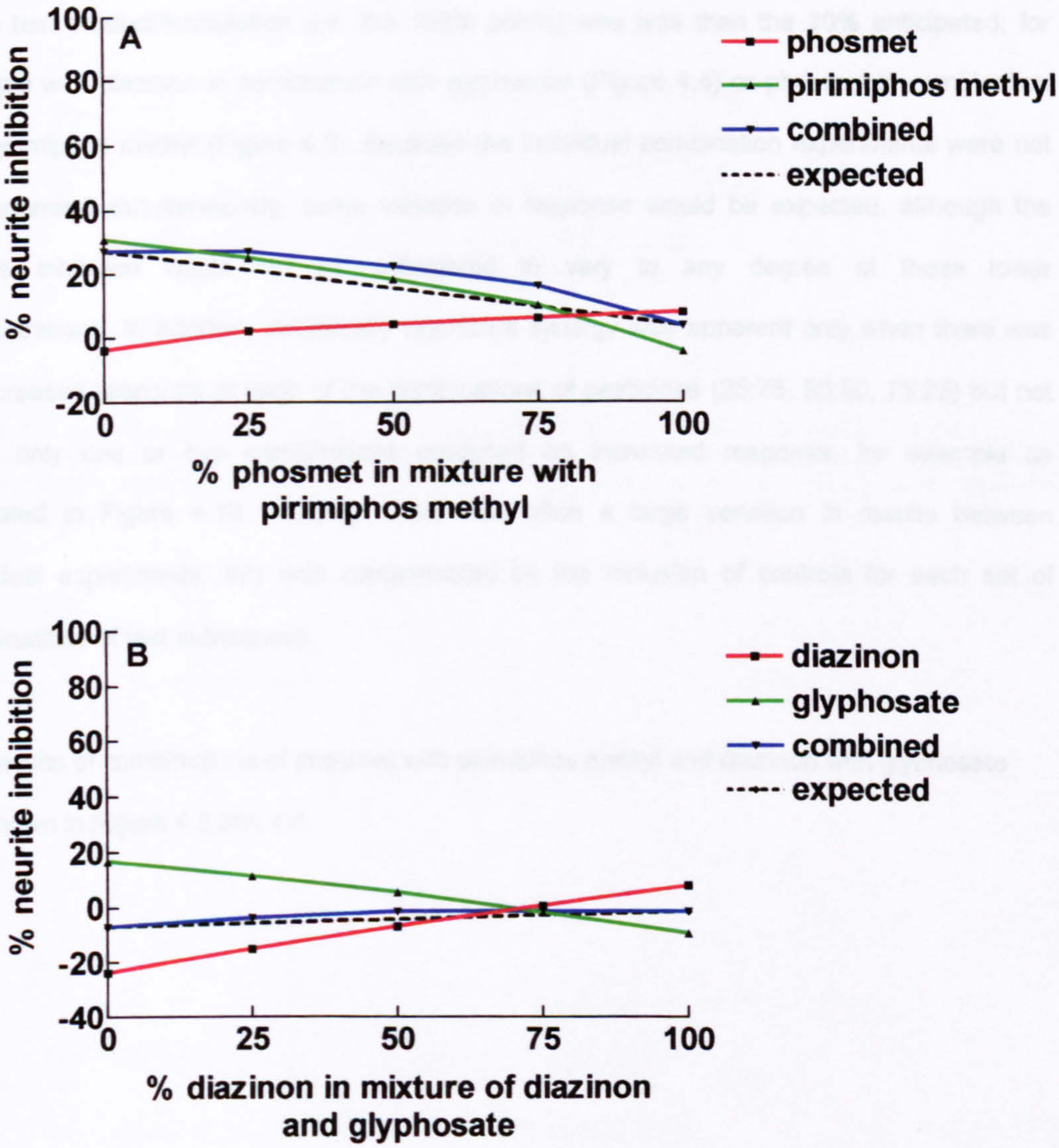


Figure 4.2: *Examples of actual neurite inhibition and combined inhibition of A) phosmet and pirimiphos methyl and B) diazinon and glyphosate. The values for individual pesticides are calculated from the equation of the actual individual sigmoid response curves shown in Figure 3.3 and the combined curve is the sum of these actual values. The expected linear results line is included for comparison, and is derived by a straight line between the values of both of the 100% inhibition points for the two substances.*

As can be seen in Figure 4.2A, the expected curve for phosmet and pirimiphos methyl is not entirely straight. However it is not anticipated that this small divergence from the combined curve would affect the overall demonstration of synergy. The curve for combination of diazinon and glyphosate (4.2B) closely follows the calculated combined curve.

In many cases of the results that follow, the observed inhibition produced by one or both single test product/formulation (i.e. the 100% points) was less than the 20% anticipated; for example with diazinon in combination with glyphosate (Figure 4.4) or phosmet in combination with pirimiphos methyl (Figure 4.3). Because the individual combination experiments were not all performed simultaneously, some variation in response would be expected, although the neurite inhibition would not be anticipated to vary to any degree at these lower concentrations. In addition, statistically significant synergy was apparent only when there was an increased response at each of the combinations of pesticides (25:75, 50:50, 75:25) but not when only one or two combinations produced an increased response, for example as illustrated in Figure 4.18. Although there was often a large variation in results between individual experiments, this was compensated by the inclusion of controls for each set of combinations of test substances.

The results of combinations of phosmet with pirimiphos methyl and diazinon with glyphosate are shown in Figure 4.3 and 4.4.

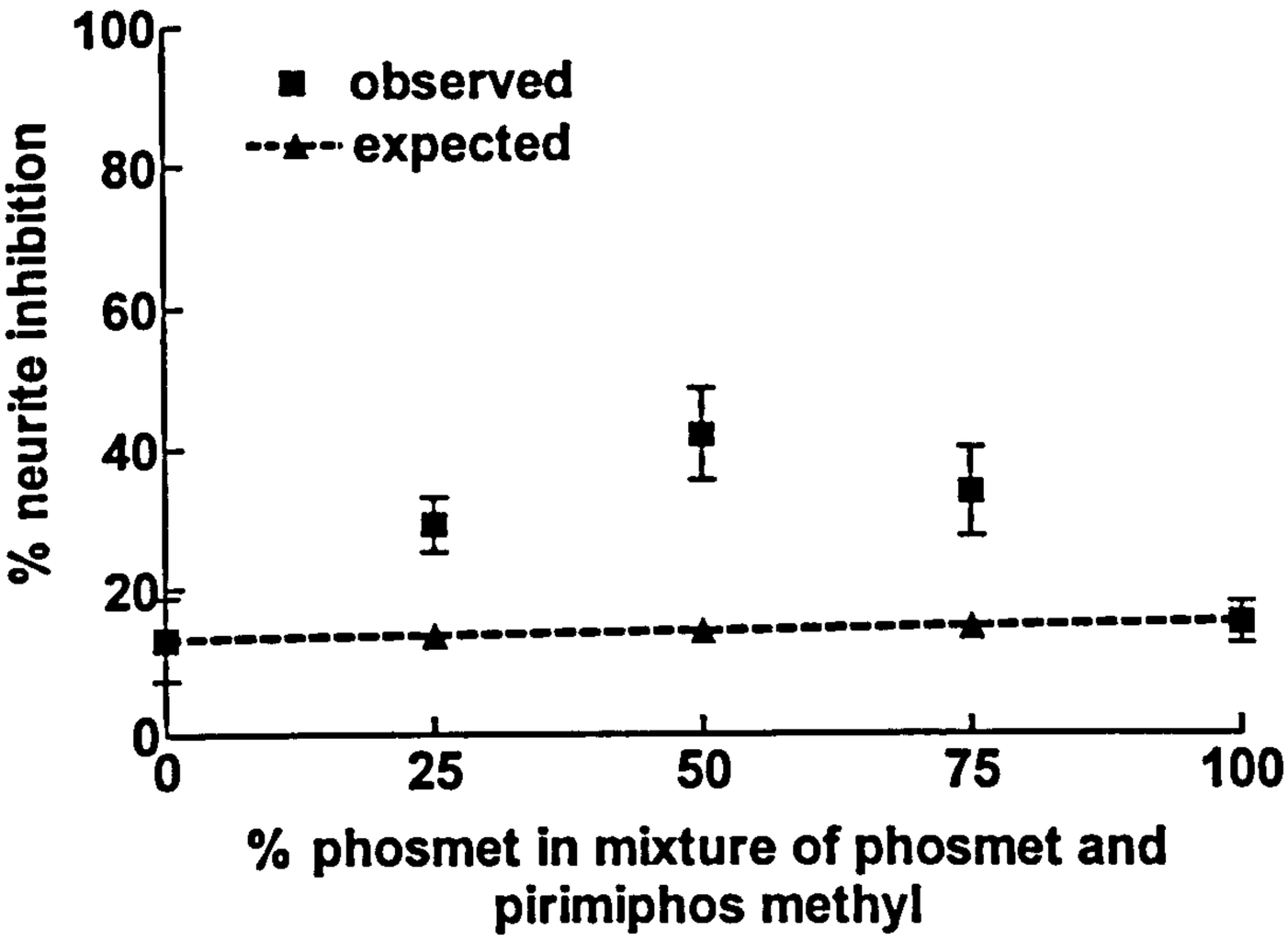


Figure 4.3: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of phosmet and pirimiphos methyl. Each was used at a maximum concentration of 6 µM. Error bars represent standard errors of 6 experiments. Statistically significant synergy was observed (p<0.0008).*

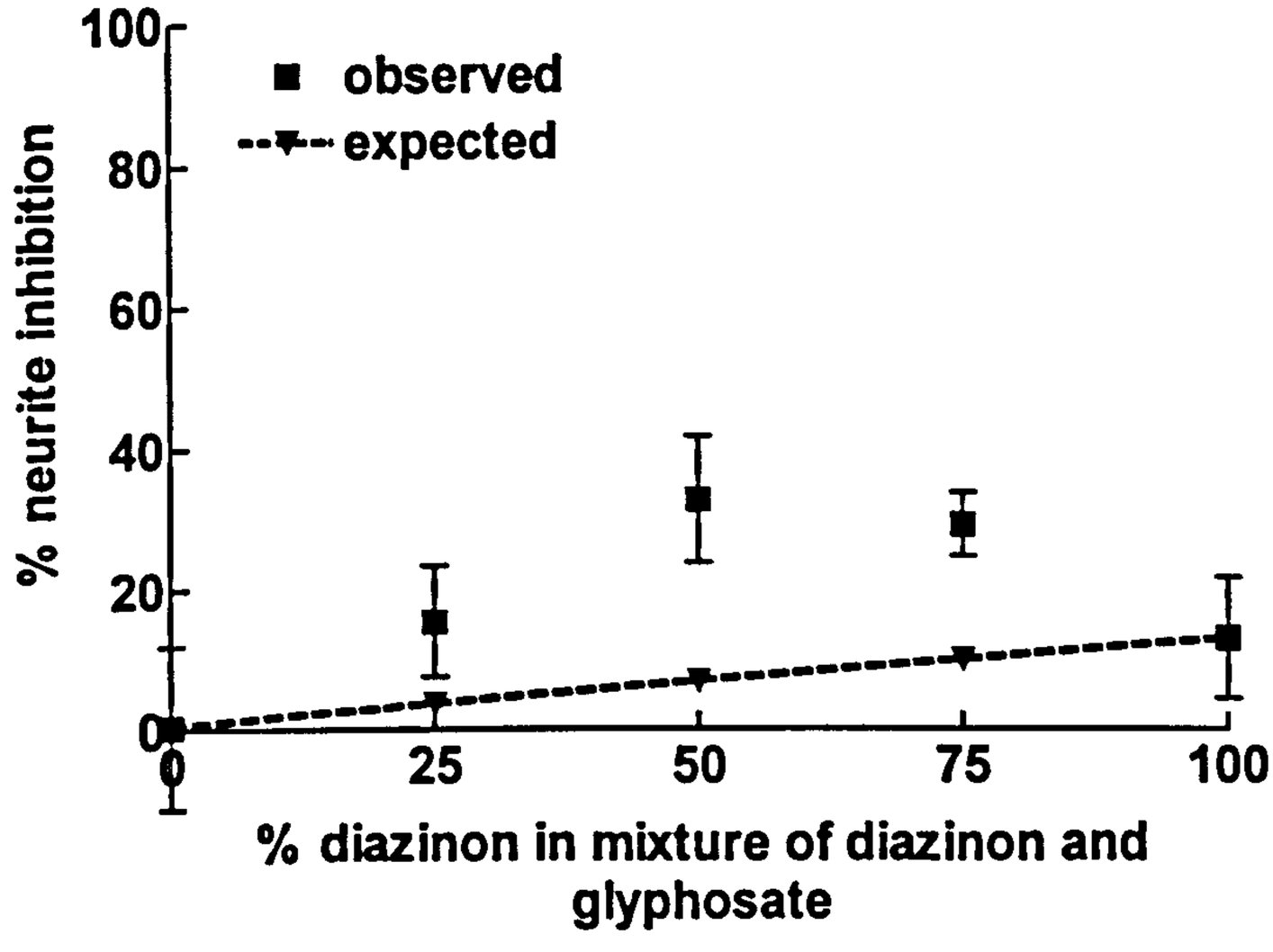
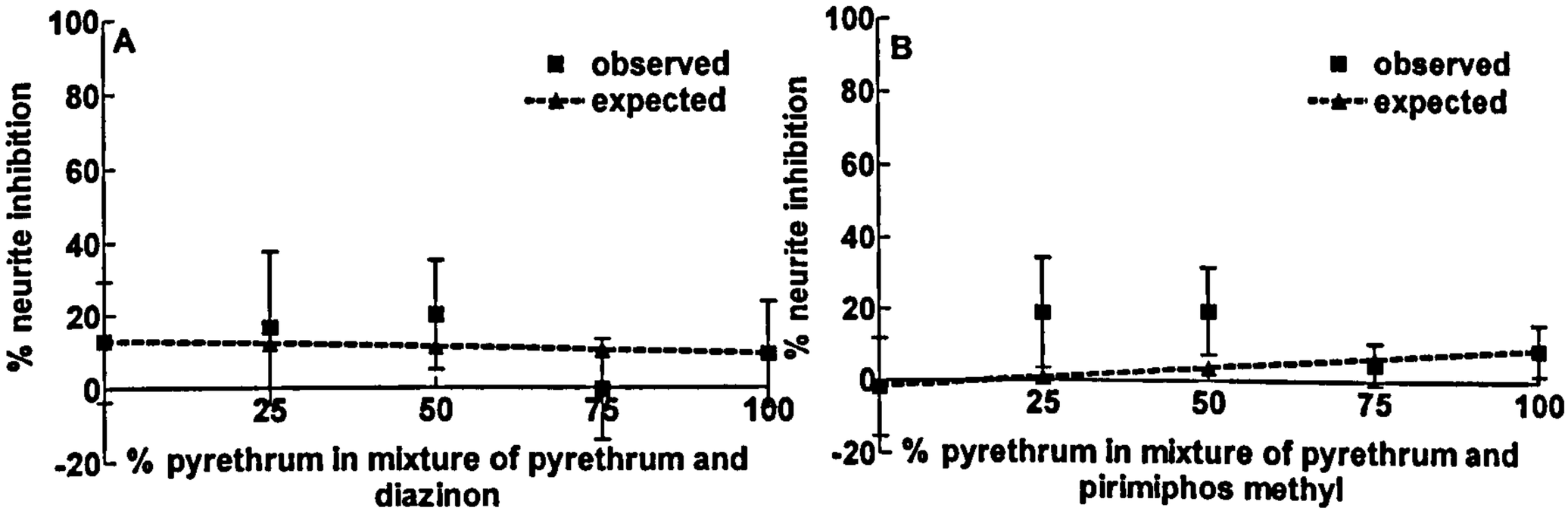


Figure 4.4: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of diazinon and glyphosate. Diazinon was used at a maximum concentration of 10 µM and glyphosate at a maximum of 500 µM. Error bars represent standard errors of 4 experiments. Statistically significant synergy was observed (p< 0.02).*

Combination of phosmet with pirimiphos methyl (Figure 4.3), and diazinon with glyphosate (Figure 4.4) produced statistically significant synergy.

The results from the remaining combinations of pyrethrum, phosmet, pirimiphos methyl, diazinon or glyphosate are shown in Figure 4.5.



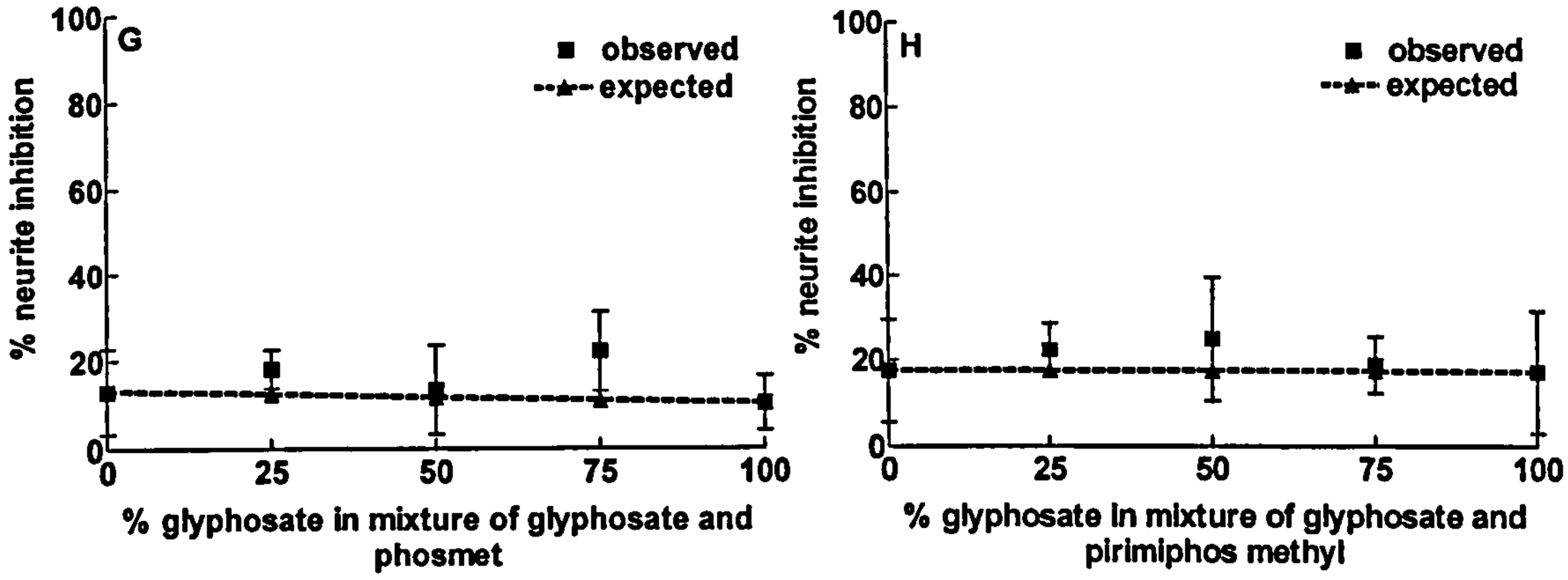


Figure 4.5: *Relationship between the relative concentrations and inhibition of neurite outgrowth of mixtures of A) pyrethrum and diazinon; B) pyrethrum and pirimiphos methyl; C) pyrethrum and phosmet; D) pyrethrum and glyphosate; E) phosmet and diazinon; F) pirimiphos methyl and diazinon; G) glyphosate and phosmet; and H) glyphosate and pirimiphos methyl. Diazinon was used at a maximum of 10 µM, phosmet and pirimiphos methyl at a maximum of 6 µM, pyrethrum at a maximum of 500 nM and glyphosate at a maximum of 500 µM. Error bars represent standard errors of at least 4 separate experiments. No statistically significant synergy was observed with any combination.*

The combinations above did not produce statistically significant synergy.

A summary of the synergistic interactions of the five pesticides is given in Table 4.6.

diazinon	phosmet	pirimiphos methyl	glyphosate	pyrethrum
diazinon	A	A	S	A
	phosmet	S	A	A
		p.methyl	A	A
			glyphosate	A
				pyrethrum

Table 4.6: *Summary of synergistic interactions of five pesticides commonly used by farmers. All five pesticides were investigated in combination with all others. An additive response only is indicated by an A, and S denotes synergistic increase in neurotoxicity.*

4.4 Synergism of farmers' pesticides as formulated products

In order to further investigate the synergy observed with the combinations of phosmet with pirimiphos methyl and diazinon with glyphosate, commercial formulations of phosmet (Nupor, 20% (w/v)) and glyphosate trimesium (Tough Weed Killer 5% (w/v)) were combined with various pesticides. The results of combination of Nupor with pirimiphos methyl are given in Figure 4.7 and of Tough Weed Killer with diazinon in Figure 4.8.

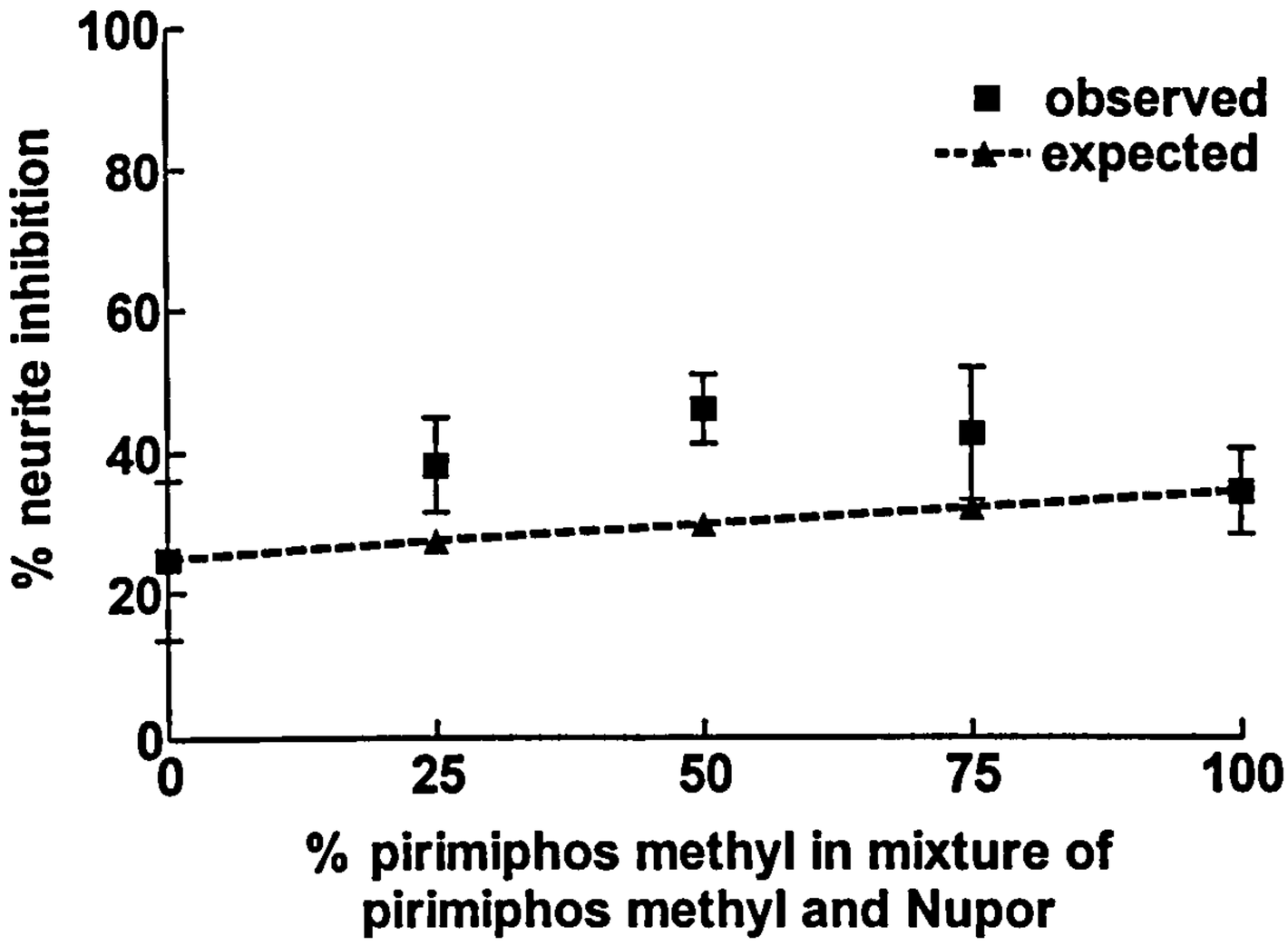


Figure 4.7: Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of pirimiphos methyl and Nupor. Pirimiphos methyl was used at a maximum concentration of 6 µM and Nupor at a maximum of 5 ppm. Error bars represent standard errors of 4 experiments. Statistically significant synergy was observed (p=0.007).

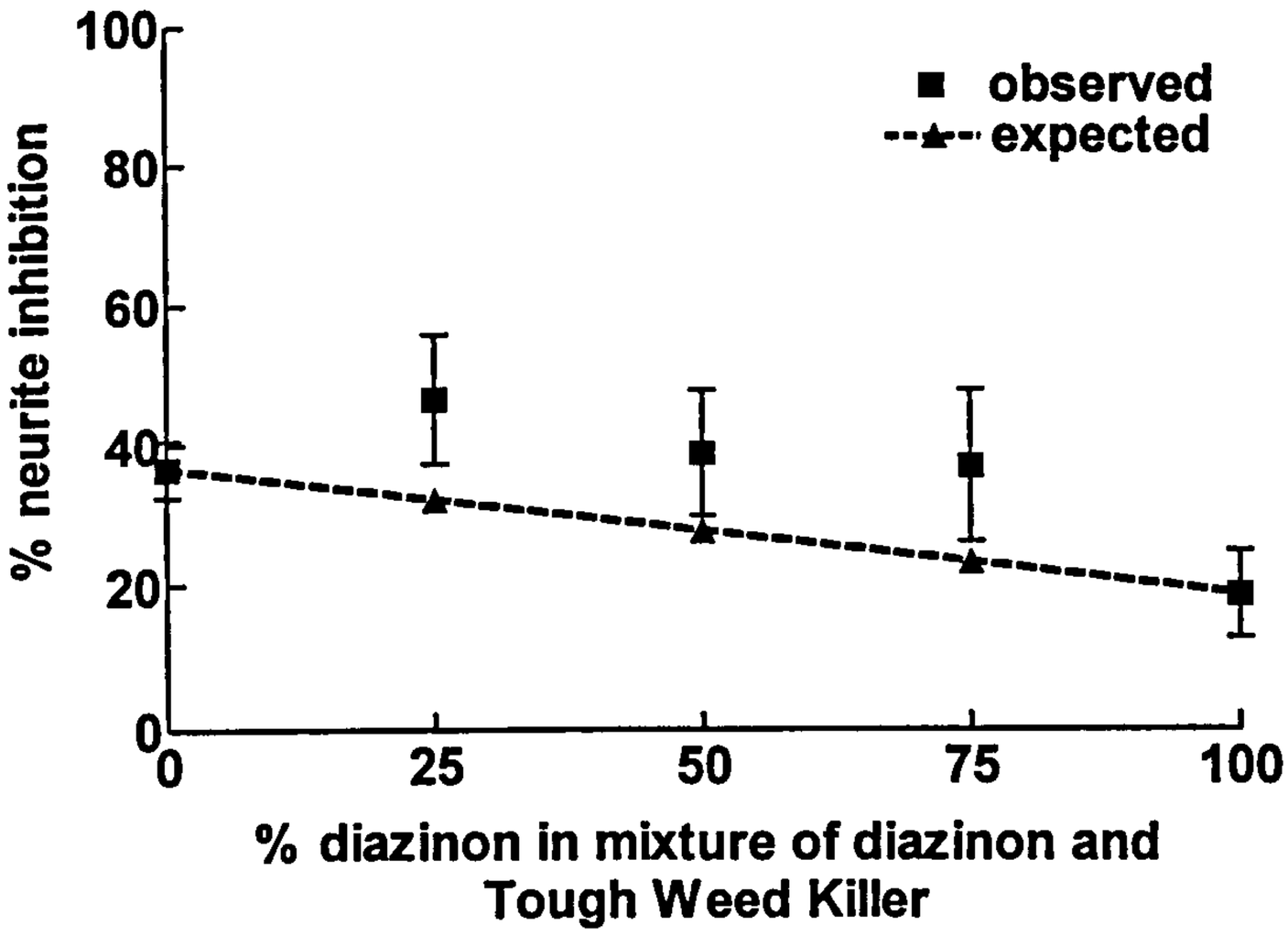


Figure 4.8: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of Diazinon and Tough Weed Killer. Diazinon was used at a maximum concentration of 10 µM and Tough Weed Killer at 10 ppm. Error bars represent standard errors of 4 separate experiments. Statistically significant synergy was observed (p=0.028).*

Combination of Nupor (5% phosmet) with pirimiphos methyl and combination of Tough Weed Killer (5% glyphosate trimesium) with diazinon produced statistically significant synergy.

Tough Weed Killer was also combined with pirimiphos methyl and with Nupor. The results are shown in Figure 4.9 and 4.10.

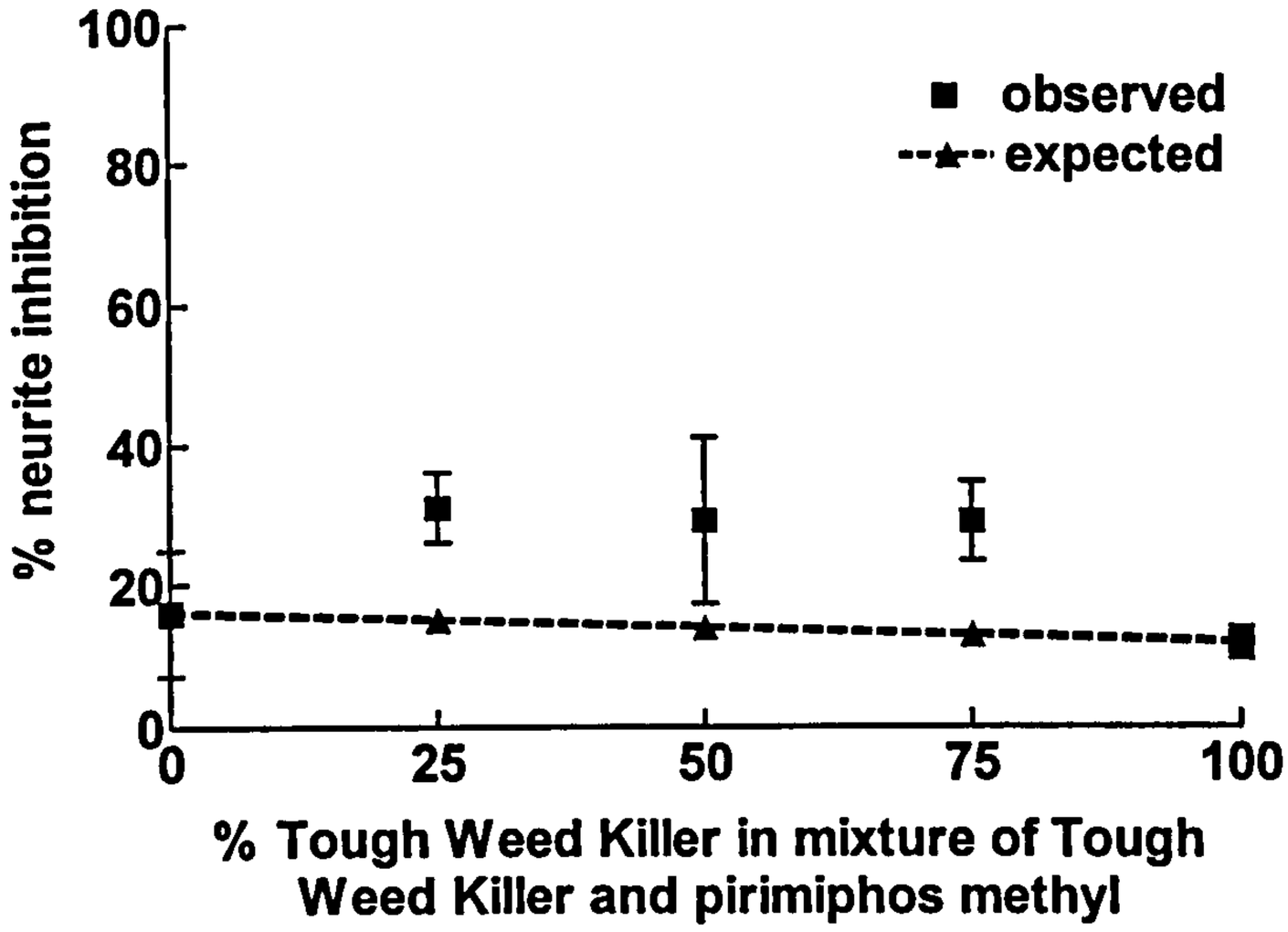


Figure 4.9: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of Tough Weed Killer and Pirimiphos Methyl. Tough Weed Killer was used at a maximum concentration of 10 ppm and pirimiphos methyl at 6 µM. Error bars represent standard errors of 4 separate experiments. Statistically significant synergy was observed (p=0.005).*

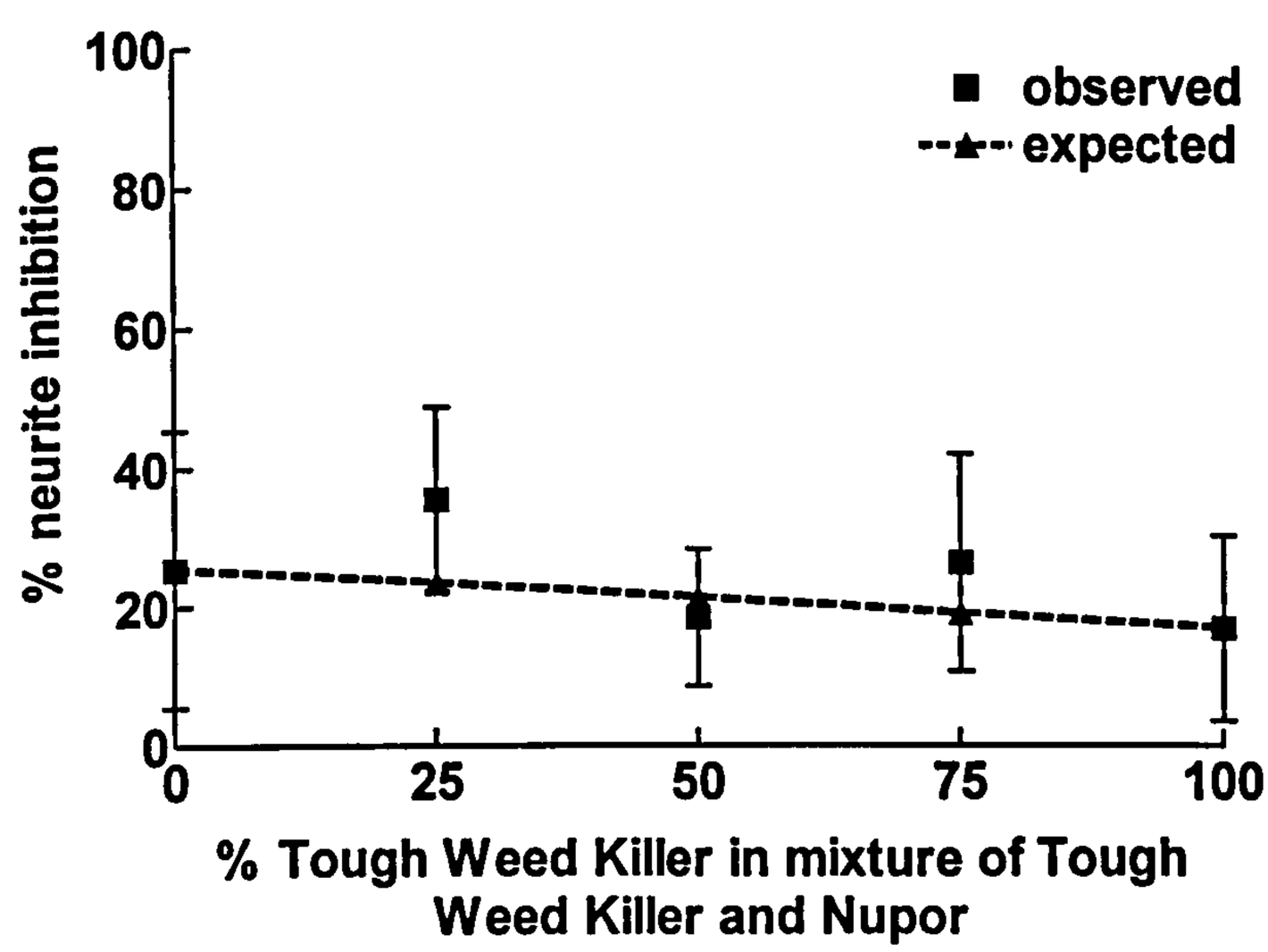


Figure 4.10: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of Tough Weed Killer and Nupor. Tough Weed Killer was used at a maximum of 10 ppm and Nupor at 5 ppm. Error bars represent standard errors of 4 separate experiments. No statistically significant synergy was observed.*

Combination of Tough Weed Killer with pirimiphos methyl (Figure 4.9) produced statistically significant synergy. Combination of Tough Weed Killer with Nupor did not produce statistically significant synergy (Figure 4.10).

Where synergy occurred, the approximate extent was calculated. The level of neurite outgrowth produced by each substance when used in combination was read from the original graph of the relationship between inhibition of neurite outgrowth and concentration of each individual substance on its own, from which the IC_{50} values were calculated (Chapter 3). One could thus obtain the concentration of each substance that would have produced that extent of inhibition if it had been used independently. That concentration was then expressed as a fraction of the concentration when used in combination. This is shown in Table 4.11.

Substance	In the presence of:	Potentiation of toxicity (factor)
Phosmet	pirimiphos methyl	8 - 20
Pirimiphos methyl	phosmet	1.3 - 4.7
Diazinon	glyphosate	4 - 8
Glyphosate	diazinon	1 - 5
Diazinon	glyphosate formulation	5 - 24
Glyphosate formulation	diazinon	46 - 92
Pirimiphos methyl	phosmet formulation	2 - 6
Phosmet formulation	pirimiphos methyl	13 - 48
Pirimiphos methyl	glyphosate formulation	1.4 - 3.8
Glyphosate formulation	pirimiphos methyl	21 - 70

Table 4.11: *Summary of potentiation of toxicity by combination of pesticides associated with farmers' ill health. The relationship between concentration and inhibition of neurite outgrowth from NB2a cells was calculated for each pesticide or formulation on its own. From that relationship, the concentration of each substance that would have produced the same inhibition of neurite outgrowth as that produced when it was used in combination was calculated. This was then expressed as a fraction of the concentration used in the combination. The range of values derives from the maximum and minimum values from the 3 different percentages (25:75; 50:50; 75:25) at which the pesticides were combined.*

4.5 Determination of potential synergism of three pesticide products used in anti-head louse formulations

The active ingredients of three formulations, carbaryl, malathion and pyrethrum, commonly used as anti-head louse preparations were combined in all pairs and assessed for synergy, as described in 2.5. The results are shown in Figure 4.12-4.14.

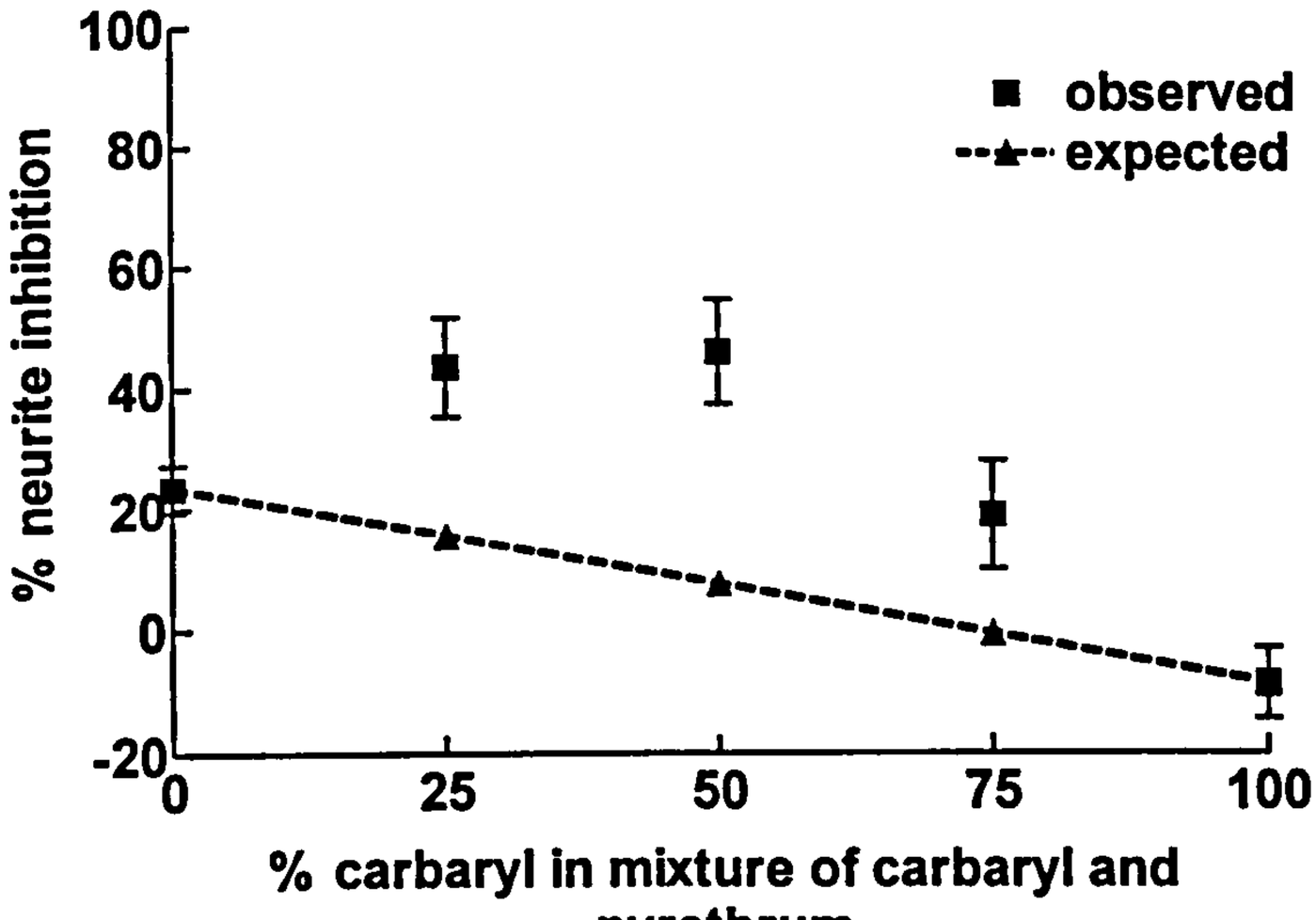


Figure 4.12: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of carbaryl and pyrethrum. Carbaryl was used at a maximum concentration of 1 μM and pyrethrum at 500 nM. Error bars represent standard errors of 4 separate experiments. Statistically significant synergy was observed (p=0.006).*

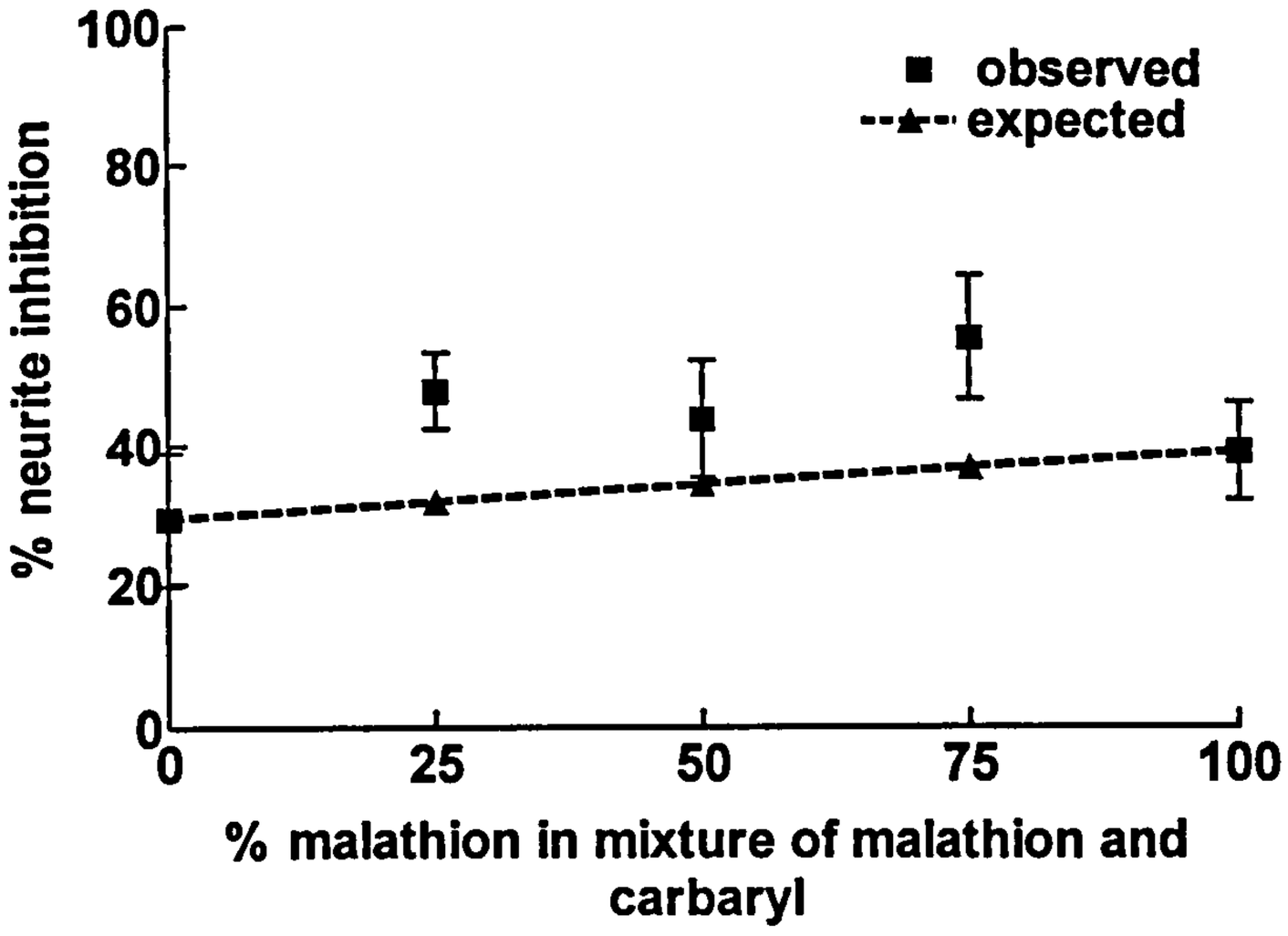


Figure 4.13: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of malathion and carbaryl. Carbaryl was used at a maximum concentration of 1 µM and malathion at 10 µM. Error bars represent standard errors of 4 separate experiments. Statistically significant synergy was observed (p=0.022).*

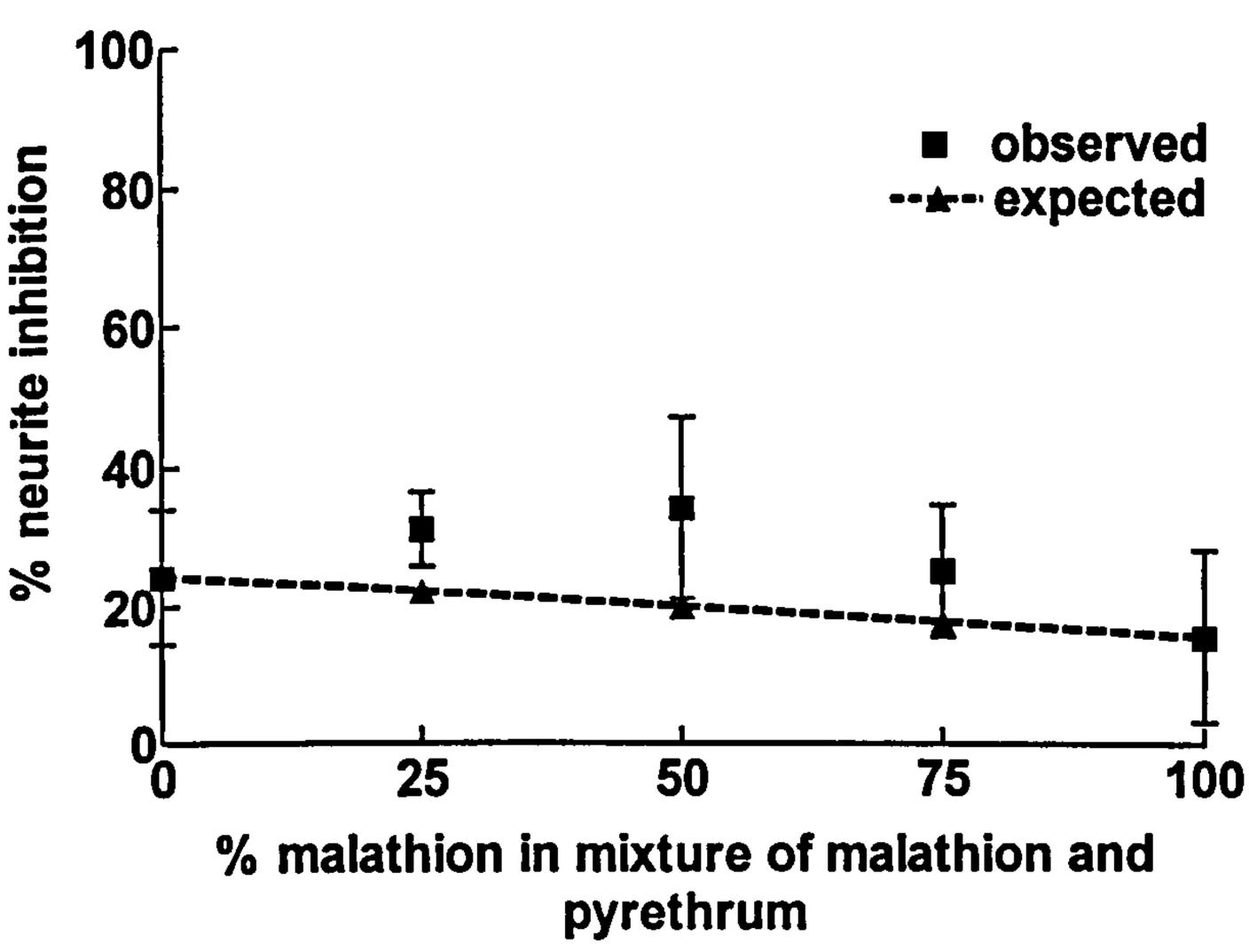


Figure 4.14: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of malathion and pyrethrum. Malathion was used at a maximum concentration of 10 µM and pyrethrum at 500 nM. Error bars represent standard errors of 4 separate experiments. Statistically significant synergy was observed (p=0.0026).*

Each combination of the three products, malathion with pyrethrum, malathion with carbaryl and carbaryl with pyrethrum produced statistical significant synergy.

As detailed for the farmers' synergy results, the degree of potentiation as a result of the second product was calculated and summarized in Table 4.15.

Substance	In the presence of:	Potentiation of toxicity (factor)
Malathion	carbaryl	32 - 75
Carbaryl	malathion	14 - 58
Malathion	pyrethrum	9 - 37
Pyrethrum	malathion	*
Carbaryl	pyrethrum	4 - 36
Pyrethrum	carbaryl	*

Table 4.15: *Potentiation of toxicity by combinations of pesticides involved in anti-head louse treatment. The relationship between concentration and inhibition of neurite outgrowth from NB2a cells was calculated for each pesticide or formulation on its own. From that relationship, the concentration of each compound that would have produced the same inhibition of neurite outgrowth as that produced when it was used in combination was calculated. This was then expressed as a fraction of the concentration used in the combination. The range of values derives from the 3 different percentages (25:75; 50:50; 75:25) at which the drugs were combined. * indicates unable to assess value because of the unusual dose-response relationship of pyrethrum (see Figure 3.9A)*

4.6 Determination of potential synergism of pesticide formulations and their constituent ingredients

To further investigate the interactions between formulations of pesticides, both within formulations and between the ingredients of different formulations, one pesticide formulation was investigated together with chlorpyrifos, a commonly used active ingredient of a variety of formulations. Commercial Formulation 1 consisted of a mixture of pyrethrum, piperonyl butoxide and diazinon in various solvents as detailed in 2.3. Each combination of ingredients within the formulation was tested in pairs. The results are shown in Figure 4.16, apart from that for diazinon with pyrethrum which was shown in Figure 4.5.

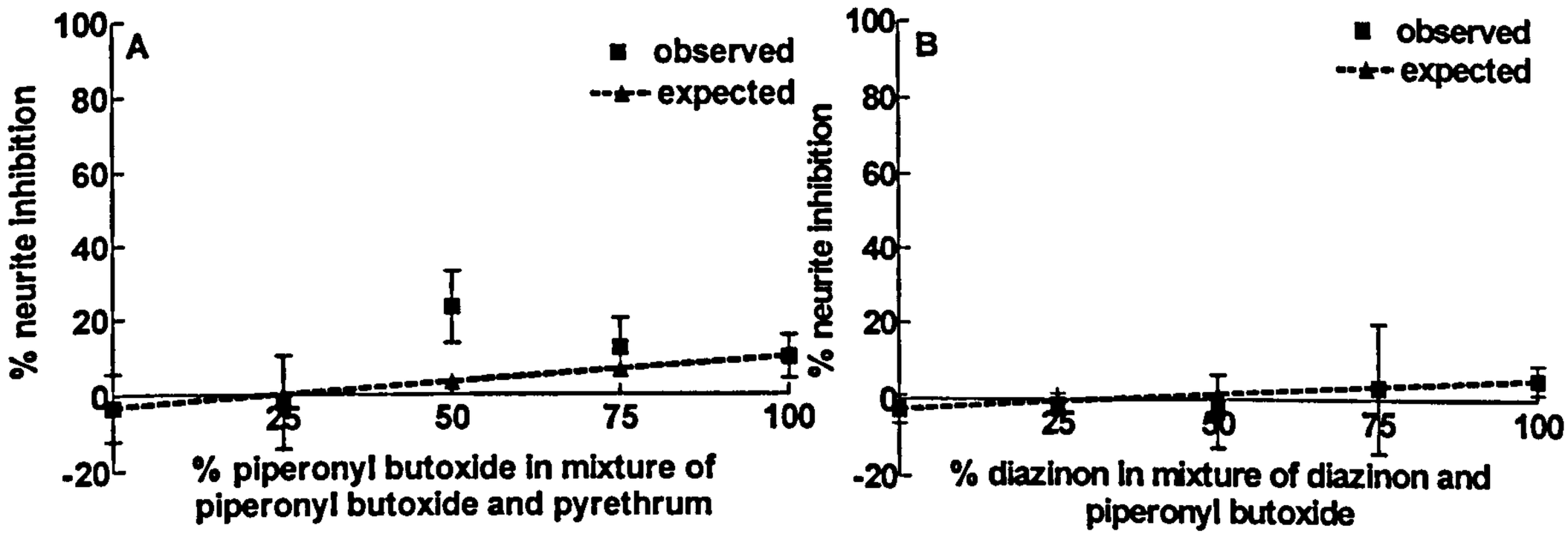


Figure 4.16: *Relationship between relative concentrations and the inhibition of neurite outgrowth with a mixture of A) pyrethrum with piperonyl butoxide and B) diazinon with piperonyl butoxide. Results of diazinon with pyrethrum were shown in figure 4.4. Pyrethrum was used at a maximum of 500 nM, diazinon at a maximum of 10 µM and piperonyl butoxide at a maximum of 10 nM. Error bars represent standard errors of at least 4 experiments. No significant synergy was seen with any combination.*

None of these combinations was found to produce statistically significant synergy. Thus the active ingredients of Commercial Formulation 1 were not synergistic within the mixture itself.

A slight *increase* in neurite length was observed at some proportions of different substances, as illustrated with pyrethrum and piperonyl butoxide. This increase represents greater neurite growth than the controls and is shown as a negative value for neurite inhibition, on this graph, and occasionally on others. Allowances are made for this effect in the statistical analysis, which sums the signed differences, between the observed and expected results at each point.

Each active ingredient from Commercial Formulation 1 was then combined with chlorpyrifos. The results are shown in Figure 4.17 and 4.18.

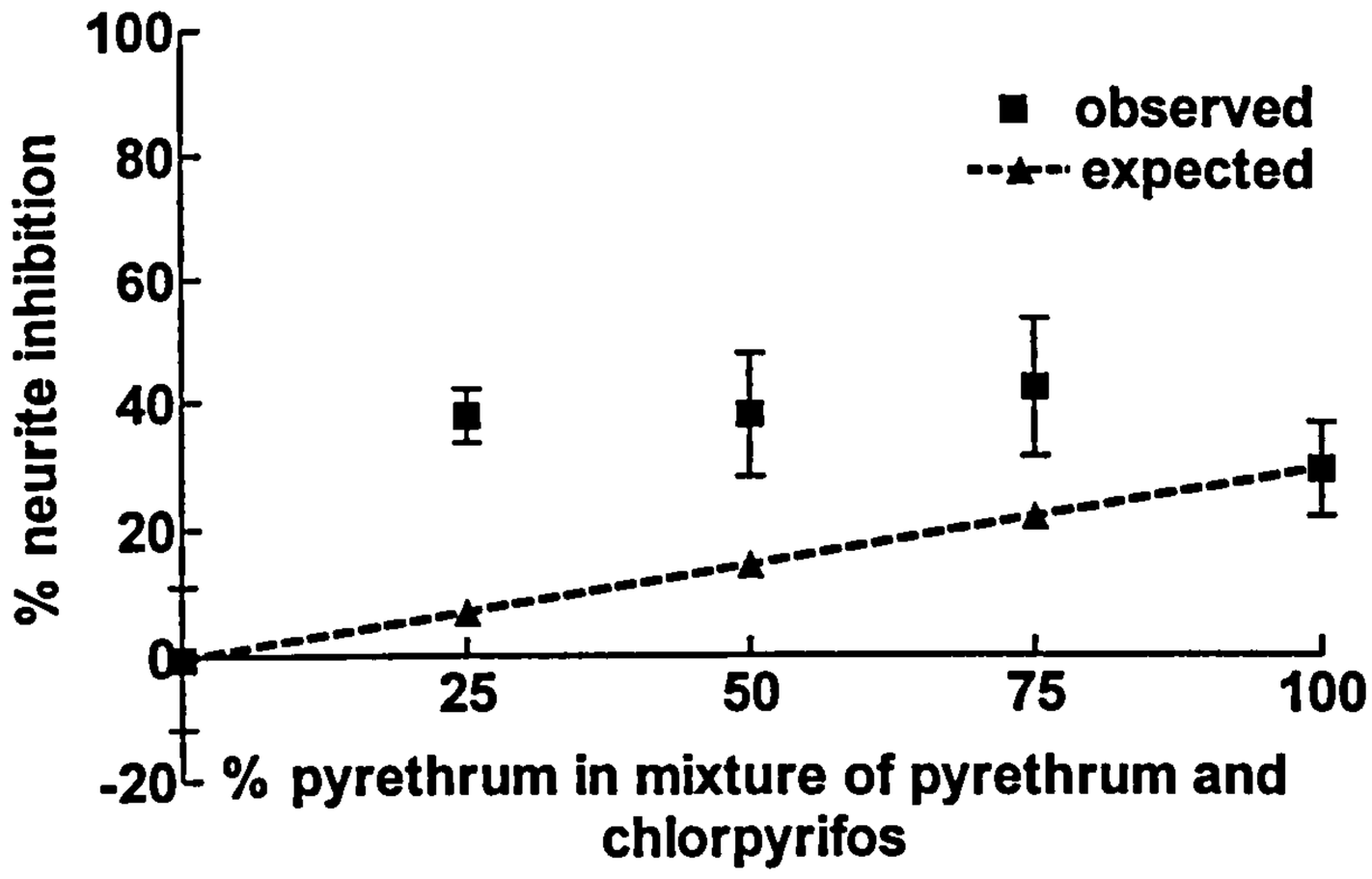


Figure 4.17: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of pyrethrum and chlorpyrifos. Chlorpyrifos was used at a maximum concentration of 10 µM and pyrethrum at 500 nM. Error bars represent standard errors of 4 separate experiments. Statistically significant synergy was observed (p<0.02).*

The combination of chlorpyrifos and pyrethrum produced statistically significant synergy (p<0.02).

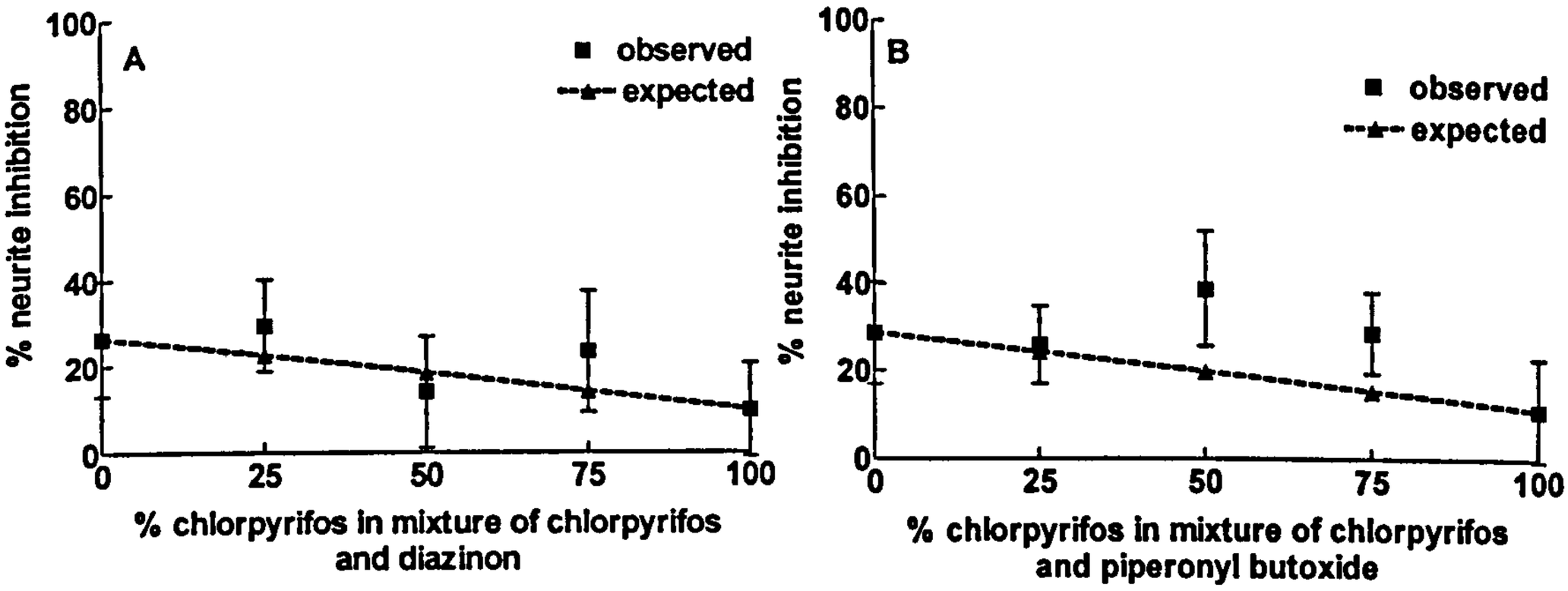


Figure 4.18: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of A) chlorpyrifos with diazinon and B) chlorpyrifos with piperonyl butoxide. Chlorpyrifos and diazinon were used at a maximum concentration of 10 µM, and piperonyl butoxide at a maximum of 10 nM. Error bars represent standard errors of 4 separate experiments. No statistically significant synergy was observed.*

No significant synergy was found with combinations of chlorpyrifos with diazinon or with piperonyl butoxide.

The active ingredients of Commercial Formulation 1, viz. diazinon, pyrethrum and piperonyl butoxide were formulated in DMSO. This mixture (at a maximum concentration of 10 ppb) was combined with chlorpyrifos as above and assessed for ability to inhibit neurite outgrowth. Commercial Formulation 1 was also combined with chlorpyrifos. The results are shown in Figure 4.19.

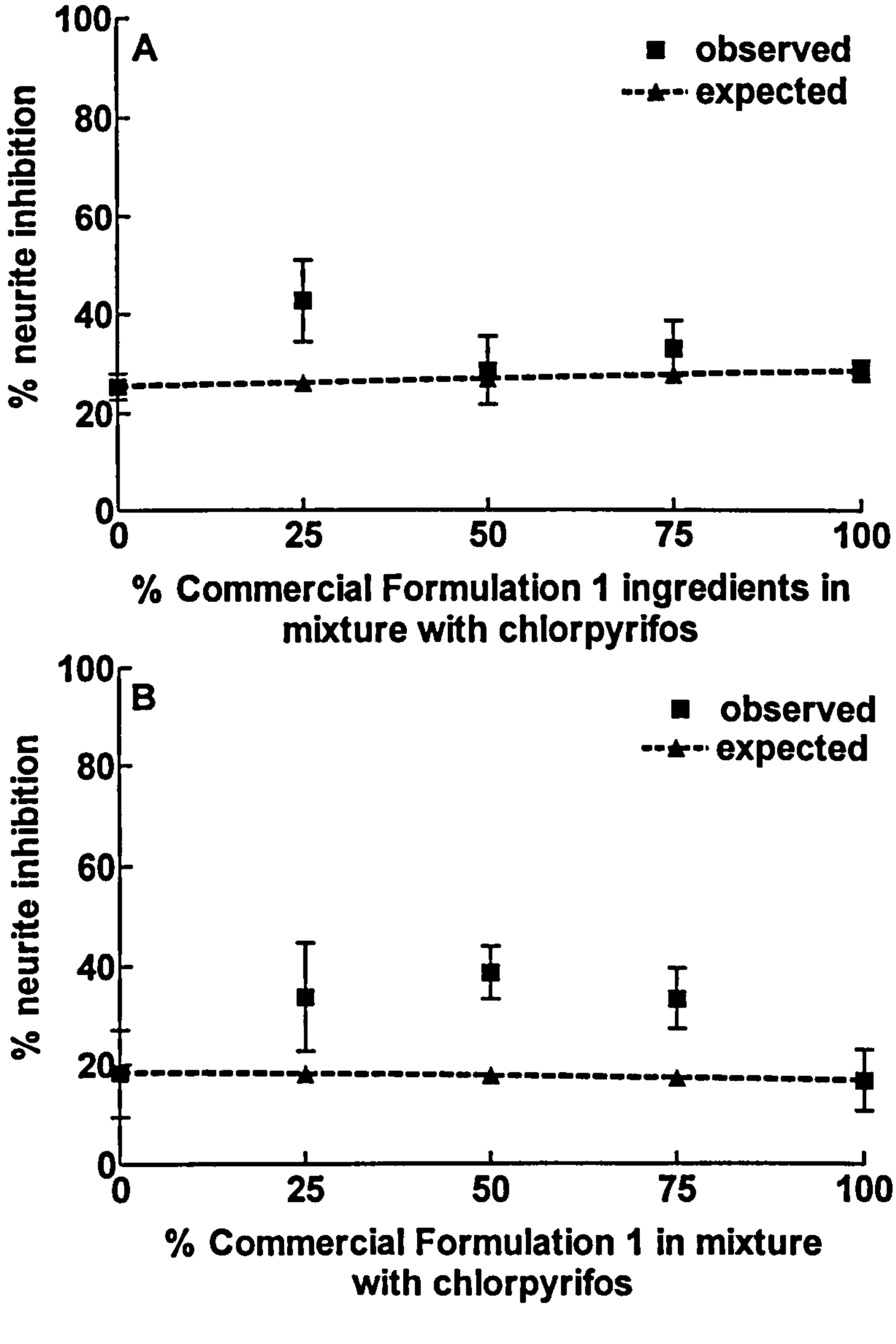
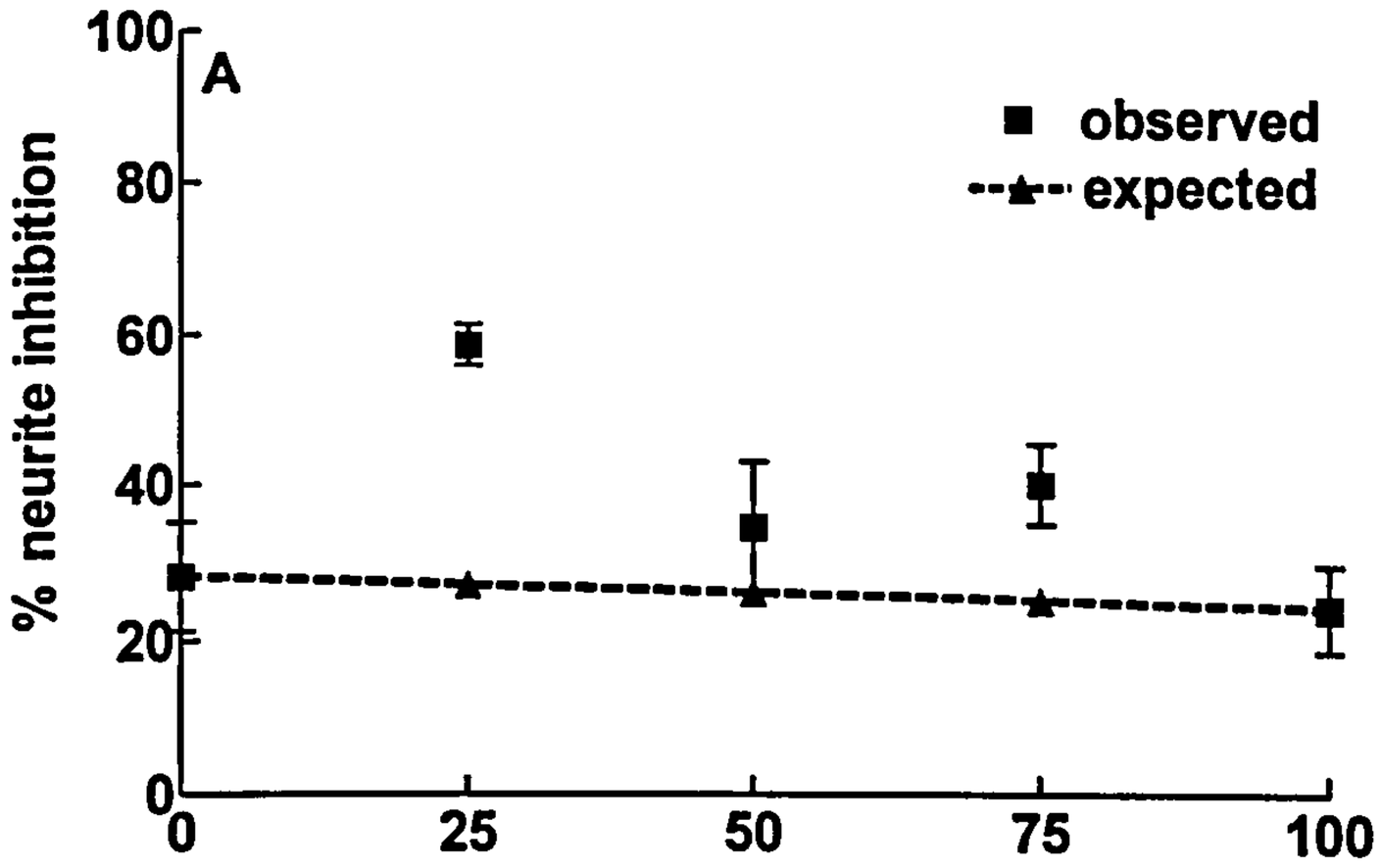


Figure 4.19: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of chlorpyrifos with A) ingredients of Commercial Formulation 1 and B) with Commercial Formulation 1. Chlorpyrifos was used at a maximum concentration of 10 μM, and the formulation and the mixture of ingredients of formulation 1 was used at a maximum concentration of 10 ppb. Error bars represent standard errors of 4-7 separate experiments. Statistically significant synergy was observed with combination of Commercial Formulation 1 with chlorpyrifos ($p<0.04$), but no significant synergy was seen with the ingredients of Commercial Formulation 1 and chlorpyrifos.*

Combination of chlorpyrifos with Commercial Formulation 1 produced statistically significant synergy ($p<0.04$).

This result contrasts with the fact that the active ingredients of Commercial Formulation 1 in the same proportions did not react synergistically with chlorpyrifos. It suggests that the vehicles of Commercial Formulation 1 or another ingredient in the formulation might have been responsible. To determine if this was the case, each of the vehicles was combined separately with chlorpyrifos; the results are shown in Figure 4.20.



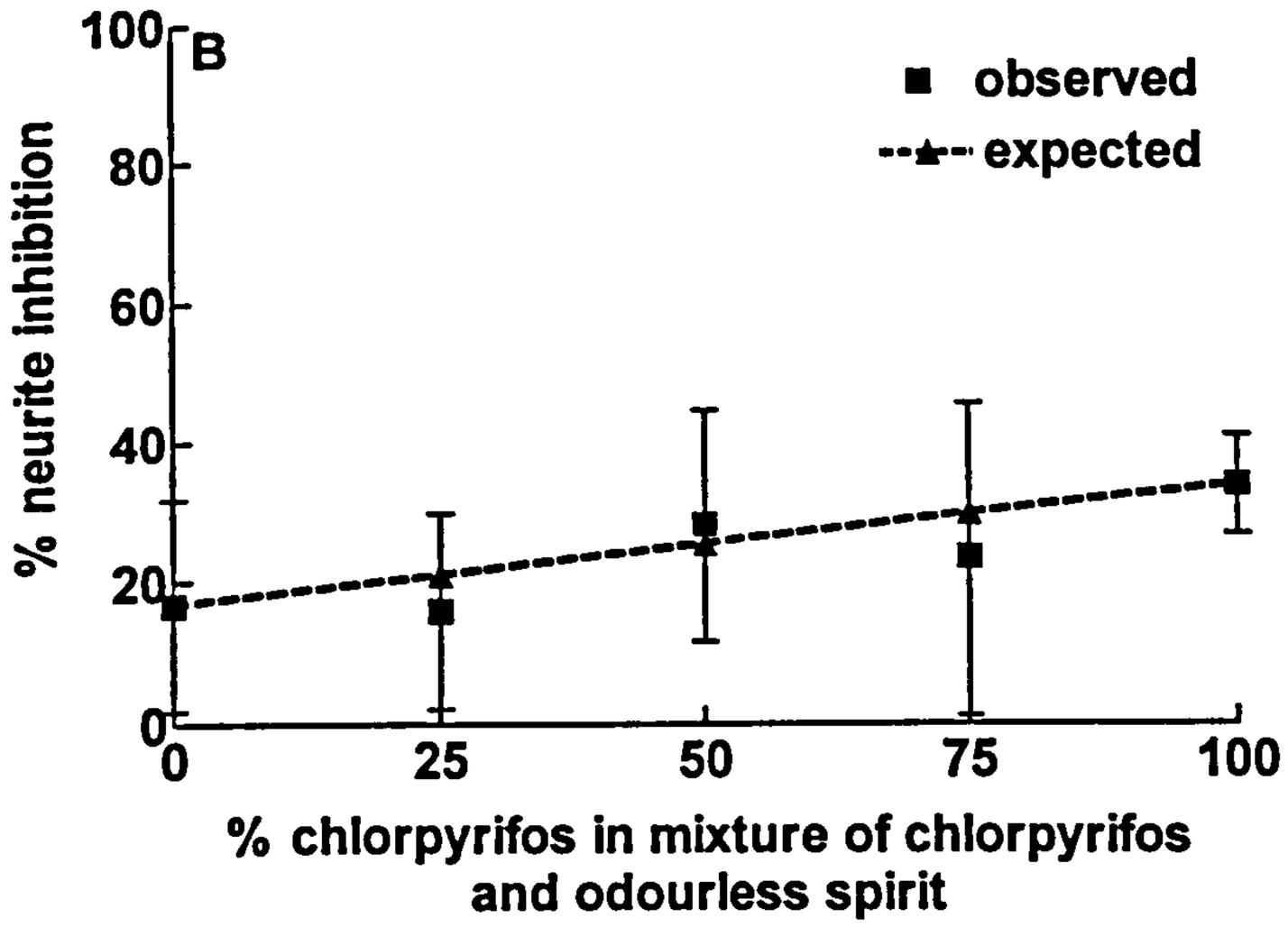


Figure 4.20: *Relationship between relative concentrations and the inhibition of neurite outgrowth of a mixture of chlorpyrifos with the spirits of Commercial Formulation 1. Chlorpyrifos was used at a maximum concentration of 10 μM, and the spirits were used at a maximum concentration of 100 ppb. Error bars represent standard errors of 4 separate experiments. Statistically significant synergy was observed with combination of chlorpyrifos with regular spirit ($p<0.02$) but not with odourless spirit.*

Statistically significant synergy was observed between chlorpyrifos and regular spirit ($p<0.02$), but not between chlorpyrifos and odourless spirit.

As before, where synergy occurred, the approximate extent was calculated as previously described, to obtain the concentration of each substance that would have produced that extent of inhibition if it had been used independently. That concentration was then expressed as a fraction of the concentration when used in combination. This is shown in Table 4.21.

Substance	In the presence of:	Potentiation of toxicity (factor)
Regular spirit	*chlorpyrifos*	*1330 - 8550*
Chlorpyrifos	*regular spirit*	*1.4 - 3.6*
Commercial Formulation 1	chlorpyrifos	*710 - 2140*
Chlorpyrifos	Commercial Formulation 1	1.3 - 2.7
Pyrethrum	chlorpyrifos	*
Chlorpyrifos	pyrethrum	1.1 - 1.8

Table 4.21. *Potentiation of toxicity by combinations of pesticides or pesticide and solvent. The relationship between concentration and inhibition of neurite outgrowth from NB2a cells was calculated for each pesticide or formulation on its own. From that relationship, the concentration of each compound that would have produced the same inhibition of neurite outgrowth as that produced when it was used in combination was calculated. This was then expressed as a fraction of the concentration used in the combination. The range of values derives from the 3 different percentages (25:75; 50:50; 75:25) at which the products/formulations were combined. * indicates unable to assess value because of the unusual dose-response relationship of pyrethrum (see Figure 3.9A)*

4.7 Discussion

The hypothesis that combination of pairs of pesticides or their constituents leads to neurotoxicity that differs quantitatively from that expected from an additive effect, was supported by the results of this study. The assay developed here was able to demonstrate synergistic interactions with some, but not all combinations of pesticides and other substances, at concentrations that individually produced little or no detectable neurotoxicity.

Synergistic neurotoxicity of combinations of pesticide products commonly encountered by farmers or Gulf War veterans

Synergism of the direct effects on NB2a neuroblastoma cells was detected with various combinations of different pesticides encountered by farmers. Phosmet, both as a pure substance and in formulation (Nupor), when combined with pirimiphos methyl resulted in greater neurotoxicity than that expected from an additive effect. Likewise combination of diazinon with pure glyphosate or its formulation as Tough Weed Killer resulted in a synergistic increase in neurotoxicity, as did Tough Weed Killer in combination with pirimiphos methyl. Because there was no significant effect when pure glyphosate and pirimiphos methyl were tested in combination, a non-pesticide component of the Tough Weed Killer formulation is likely to be responsible. The surfactant used for another formulation of glyphosate, Roundup, was responsible for a series of poisonings associated with this product (Talbot *et al.*, 1991), and similar components may be responsible for the synergy observed here. Percutaneous absorption of pesticides depends on the vehicle of formulation (Gyrd-Hansen *et al.*, 1993), and it may be speculated that cell permeability may be similarly affected.

The mechanisms by which different substances react with each other were not determined. However the potentiation of neurotoxicity by up to 92 times that expected from additivity, by interaction of low concentrations of different products in combination may at least partly be responsible for the unexpected effects on individual health, especially in occupations such as farmers regularly exposed to combinations of pesticides and related products (Pilkington *et*

al., 1999; Bosma *et al.*, 2000; Committee on Toxicity of Chemicals in Food, 1999). Although much of the potentiation of toxicity with the pure pesticide combinations was small, this increased when the pesticide was present as a formulation; a situation more typical of the expected exposure to such products. From the *in vivo/in vitro* relationship suggested in Chapter 3, (*in vivo* LD_{50} (mg/kg) = 3-7 times *in vitro* IC_{50} (μM)) the equivalent *in vitro* toxicity of diazinon in the presence of Tough Weed Killer, which was increased synergistically from an IC_{50} of 68 μM to approximately 3 μM, would be equivalent to a LD_{50} range between 9 and 21mg/kg bodyweight. These results may explain to some degree the incidence of Gulf War Syndrome, as a result of multiple exposures to a variety of prophylactic components including various pesticides (Haley *et al.*, 1997; Chemical and Biological Defence Establishment, 1992; Abou-Donia *et al.*, 1996a) at concentrations which individually showed little or no ill effects.

Both pirimiphos methyl, a grain treatment, and glyphosate, a non-specific herbicide may enter the human food chain by normal consumption. Diazinon is used as an insecticide for sheep dips, and has been associated with cognitive impairment (Pilkington *et al.*, 1999; Bosma *et al.*, 2000). Pyrethroids have widespread use both in agriculture and in the domestic situation (Cremlyn, 1978) and phosmet has widespread use as an insecticide, especially against warble fly or tick infestation (Gyrd-Hansen *et al.*, 1993; Extoxnet, 2001). Low-level residues of these products may potentially present a significant hazard in combination.

Synergistic neurotoxicity of combinations of pesticide products commonly used in anti-head louse treatments

Synergism of neurotoxicity was observed with each combination of the active ingredients of anti-head louse treatments, carbaryl, malathion and pyrethrum, increasing the expected neurotoxicity by up to 75-fold. As a result of head louse resistance to one or more treatments (Ibarra and Hall, 1996) (Downs *et al.*, 1999) (Picollo *et al.*, 2000) (Bartels *et al.*, 2001), these preparations are often used consecutively. Head louse infestation is prevalent among children, who are therefore most at risk from the effects of combination of these pesticides applied directly and repeatedly to the scalp.

It could be anticipated that residual effects from one pesticide may increase the toxicity of subsequent treatments, with the lower infant bodyweight magnifying the risk. Application directly to the scalp of these lipophilic products together with associated solvents may promote passage through cell membranes or permit direct access to developing neurones. Malathion has previously been shown to produce a variety of abnormalities in developing chick embryos (Wyttenbach and Thompson, 1985). The effect of organophosphates and pyrethroids to alter the blood brain barrier (Gupta *et al.*, 1999a) may ease the passage to the brain. Pyrethroid-based insect repellents, a similar product, increase brain glutathione levels and show oxidative damage in brain, liver and kidneys in neonatal rats; an effect only recoverable over a long withdrawal period (Gupta *et al.*, 1999b).

Synergistic neurotoxicity of combinations with a commercial pesticide formulation

Synergism of the direct toxic effects on nerve cells was observed with the combination of chlorpyrifos and pyrethrum. It was also seen with chlorpyrifos and Commercial Formulation 1. At least part of this synergism was due to the regular spirit. As stated, organophosphates and pyrethroids are generally insoluble in water; so a range of different solvents is used in formulations of pesticide products (Cremlyn, 1978). The formulation of pesticides in solvent is taken into account when calculating safe exposure levels. However in the model used here, we have demonstrated enhanced toxicity of a pesticide (chlorpyrifos) in the presence of a solvent used in the formulation of a different pesticide. Thus the result is relevant to the situation of exposure to multiple agents; as seen from Table 4.21, the risk of neurotoxicity associated with exposure to chlorpyrifos may be amplified several hundred-fold in the presence of a second formulation or another pesticide.

No synergism was observed between chlorpyrifos and the active ingredients of Commercial Formulation 1 (which contained pyrethrum), yet there was synergism between chlorpyrifos and pyrethrum. However this difference is readily explained by the fact that the effective concentration of pyrethrum when used as a pure compound was more than 10^5 times greater

than the concentration of pyrethrum in the Commercial Formulation; so it is unlikely to represent a major hazard under normal circumstances.

The non-pesticide components of Commercial Formulation 1 are known to be primarily C9 to C13 branched chain alkenes, but little information is available about the neurotoxicity of these hydrocarbons. It is already known that chlorpyrifos is additive with xylene in its pro-convulsive activity (Wurpel *et al.*, 1993); here we have demonstrated synergism between chlorpyrifos and solvents in its effects on nerve cell differentiation. The solvents in the regular spirit may have acted in a number of ways to enhance the toxicity of chlorpyrifos. They may promote the passage of chlorpyrifos through the cell membrane, thus allowing higher effective intracellular concentrations. Alternatively, part of the enhanced toxicity in the presence of solvents may be due to an influence on some aspect of metabolism of the pesticides by the nerve cells, assuming a limited metabolic processing of pesticides by nervous tissue. Acetone, for example, increases the induction of some cytochrome P450 enzymes (Josephy, 1997a), while ethanol may be metabolised to acetone and then increase cytochrome P450 activity (Wickramasinghe, 1987). Similarly, although not detectable from these results, combinations of pesticides and/or solvents can result in reduced toxicity: co-exposure of toluene and n-hexane is known to decrease n-hexane toxicity *in vivo* (Takeuchi *et al.*, 1993). Other solvents are likely to produce similar effects.

This assay addresses many of the concerns and failings of previous models attempting to detect synergism. A major criticism of other assays is that the concentrations of two or more products in combination remain unchanged compared to those used individually, making it impossible to determine whether any effects result from synergism or are merely additive (Abou-Donia and Wilmarth, 1996; Marinovich *et al.*, 1996). By using proportional concentrations this problem is overcome; an additive response produces no increase in neurotoxic effect.

In addition, the use of low concentrations of the test substances, which result in minimal individual neurotoxicity, ensures that combination does not purely result in cell death. The

concentrations used may of course not be the minimum required to produce synergism. However although the synergy may be detected at much lower concentrations, it was necessary to ensure that such interactions resulted in a detectable end-point, even if extreme toxicity resulted from the combinations. Although concentrations equivalent to the approximate IC_{20} of the products was used in most of these studies, with some combinations very much lower concentrations were used (such as with Commercial Formulation 1), yet still resulted in significant detectable synergy. It was not possible therefore to predict the lowest concentrations that could still result in synergy with any combinations of products.

It is not clear how the concentrations used here relate to the *in vivo* situation. In many cases the degree of potentiation was small; so the resulting increase in neurotoxicity would still be unlikely to present a significant risk. In the case of combination with formulations however, which could increase the combined *in vitro* neurotoxicity many hundred times, this may represent a significantly increased risk in the *in vivo* situation. The potentiation of Commercial Formulation 1, for example, in the presence of chlorpyrifos was found to be between 710 and 2140 times greater toxicity. This is equivalent to an increase in the IC_{50} from 11 ppm (Chapter 3) to as low as 0.5 ppb, well below the permitted pesticide residue levels of most common products. One of the main active ingredients of Commercial Formulation 1 is diazinon, which has a United States Environmental Protection Agency maximum permitted residue level of 0.5 ppm, 1000 times higher than its effective IC_{50} in the presence of chlorpyrifos. The presence of chlorpyrifos could increase the expected toxicity at this maximum recommended intake to that expected to result from consumption of 500 ppm diazinon. In a 20 kg child, 500 ppm represents an intake of 25 ppm/kg, so the potential hazard is clear.

The mechanism by which organophosphates reduced neurite outgrowth, whether alone or in combination is not clear. Their ability to interact with acetylcholinesterase might play a role, as acetylcholinesterase has a trophic role influence in developing cells (Brimijoin and Koenigsberger, 1999; Bigbee *et al.*, 1999; Koenigsberger *et al.*, 1997). However, this role is unrelated to the inhibition of hydrolysis of acetylcholine and therefore the relative potencies of

the organophosphates to inhibit neurite outgrowth would not necessarily be related to their acute toxicities.

The compounds have other intracellular effects apart from those mentioned above. These include effects on Ca^{2+}-ATP-ase and calmodulin, known to occur with organophosphates (Abou-Donia *et al.*, 1993) and pyrethroids (Ferguson and Audesirk, 1990). It is unknown whether the induction of proliferation with low concentrations of organophosphates, noted in Chapter 3, would influence synergistic interaction in combination. However if the synergistic reduction in neurite outgrowth reflected an increase in proliferation of developing cells, this may have detrimental effects in developing animals, especially when laying down the cytoarchitecture of the brain. Further work would be necessary to determine if any of these mechanisms contribute to the synergism.

In conclusion, these data present evidence that, in addition to possible interactions between pesticides and the products in their formulations at the level of metabolism, there is a potential for interactions that lead to enhanced toxicity at the level of the nerve cell. The inhibition of neurite outgrowth is, of course, only one marker of neurotoxicity and, since it involves differentiating cells, may be of more relevance to exposure of the nervous system of the foetus or infant, rather than the mature nervous system. The synergism seen with combinations of anti-head louse treatments should, for this reason, raise the greatest concern. The results demonstrate the potential for a substantial increase in neurotoxicity accompanying various combinations of pesticides and their formulations. Increasing the number of different products to which an individual is exposed might be expected to increase the potential for synergism.

CHAPTER 5

THE EFFECT OF CHRONIC EXPOSURE TO ORGANOPHOSPHATES

5.1 Introduction

It has been postulated that exposure to chronic low levels of combinations of organophosphate pesticides can adversely affect the health of individuals, at concentrations below those demonstrated as hazardous from standard toxicity studies. The acute toxicity of organophosphate pesticides results from inhibition of acetylcholinesterase (Sultatos, 1994), while delayed toxicity can be associated either with organophosphate-induced delayed neuropathy (OPIND) (Johnson, 1990), which is related to inhibition of the enzyme neuropathy target esterase (NTE), or the myaesthenia-like Intermediate Syndrome (Senanayake and Karalliedde, 1987).

Exposure to chronic low level combinations has been suggested as playing a role in the ill health experienced by farmers exposed to sheep dip pesticides (Committee on Toxicity of Chemicals in Food, 1999; Jamal, 1997), where the organophosphate diazinon is most often implicated in the effects reported. Use of concentrated diazinon sheep dip has been found to contribute to cognitive impairment in some farmers, although chronic exposure to low levels has not been addressed (Pilkington *et al.*, 1999). Chronic exposure is also speculated as instrumental in symptoms of Gulf War veterans exposed to a cocktail of prophylactic treatments (Abou-Donia and Wilmarth, 1996; Haley *et al.*, 1999; Davies *et al.*, 1996).

Considerable controversy remains over whether prolonged exposure to one or more substance increases the observed neurotoxicity (Jamal, 1997; Rosenstock *et al.*, 1991; Stephens *et al.*, 1995). Neuropsychological effects of long term exposure and of chronic effects on both mature (Korsak and Sato, 1977; Stephens *et al.*, 1995; Stone *et al.*, 2000) and developing (Jena and Bhunya, 1992; Jarvinen *et al.*, 1983) nervous systems have been reported. However other attempts to demonstrate chronic neurotoxicity of individual compounds have proved negative (Chang *et al.*, 1992; Ivens *et al.*, 1998). Where chronic

toxic effects have been demonstrated there is evidence both for (Soliman *et al.*, 1983) and against (Prendergast *et al.*, 1997; Singh and Drewes, 1987; Socko *et al.*, 1999) the toxicity being related to inhibition of esterase activity.

Standard *in vivo* toxicity testing does not readily accommodate the phenomenon of acute exposure to pesticides in the presence of chronic low level pre-exposure. Synergy between pesticides has been shown to occur in some limited studies already performed on various combinations of products *in vivo* (Abou-Donia *et al.*, 1996a; McCain *et al.*, 1997; Audegond *et al.*, 1989) and *in vitro* (Marinovich *et al.*, 1996) as well as those investigated in this thesis (Chapter 4). These studies however demonstrate effects of simultaneous acute exposure to products, but do not address the effects resulting from acute exposure following chronic pre-exposure to these products at concentrations that produce neither acute nor delayed toxicity.

As mentioned in previous chapters, there is evidence that diverse organophosphates have sites of action that are unrelated to cholinesterase or NTE. Other groups of pesticides target similar or different neuronal mechanisms. The effects of carbamate pesticides are similar to organophosphates in terms of acute toxicity (Cremlyn, 1978), whereas pyrethroids act primarily to prolong opening of Na^+ channels to produce repetitive discharge (Aldridge, 1990). The toxic effects of pyrethroids manifest as paraesthesia, tremors and seizures.

Pesticides are seldom encountered in pure form, being formulated in a variety of different solvents, or in conjunction with potentiators. There is a potential for interactions not only between the pesticide compounds, but also between compounds, solvents and potentiators. Whole animal studies have demonstrated interactions between the effects of compounds which result from interference with liver metabolism such as the increased toxicity of organophosphates after induction of metabolising enzymes by solvents such as n-hexane (Pellin *et al.*, 1987; Abou-Donia *et al.*, 1985), methyl isobutyl ketone (Abou-Donia *et al.*, 1991), or increased cocaine metabolism after diazinon or alcohol exposure (Kump *et al.*, 1996).

Although data suggest that synergy may occur as a result of acute exposure to organophosphates in combination with other pesticides or solvents, evidence for toxic effects of chronic low doses of organophosphates remains equivocal. This is despite the likelihood that most pesticide exposure occurs over several years and involves a mixture of agents. It could be argued that the known ability of organophosphates to regulate expression of proteins related to cell growth and differentiation may alter cell function in such a way that response to further acute exposure to pesticides or other products may be modified.

The need for a model of chronic toxicity is therefore apparent, especially one which simulates the relationship to the exposure situation. Cell culture permits close control of drug concentration and exposure that is lacking in clinical or animal studies. This study again involves the use of an indicator of toxicity at the cellular level, viz. outgrowth of neurites from differentiating neuroblastoma cells (Flaskos *et al.*, 1998; Abdulla and Campbell, 1993), to assess differences in the response of neurones maintained with and without chronic low-level diazinon, to pesticides and associated compounds.

5.2 Research hypothesis investigated in this chapter

The neurotoxicity of pesticides to cells that have been chronically pre-exposed to the organophosphate diazinon differs quantitatively from that observed in cells that have not been pre-exposed to diazinon.

5.3 The assessment of the effects of pesticides to cells chronically pre-exposed to diazinon

In order to simulate the effects of chronic levels of organophosphate pesticides, NB2a neuroblastoma cells were cultured as usual but with the addition of 25 μM diazinon added to the culture medium, as described in 2.2.2. This concentration was chosen from preliminary studies, which demonstrated negligible effect on cell growth and neurite extension as shown in Figure 5.1.

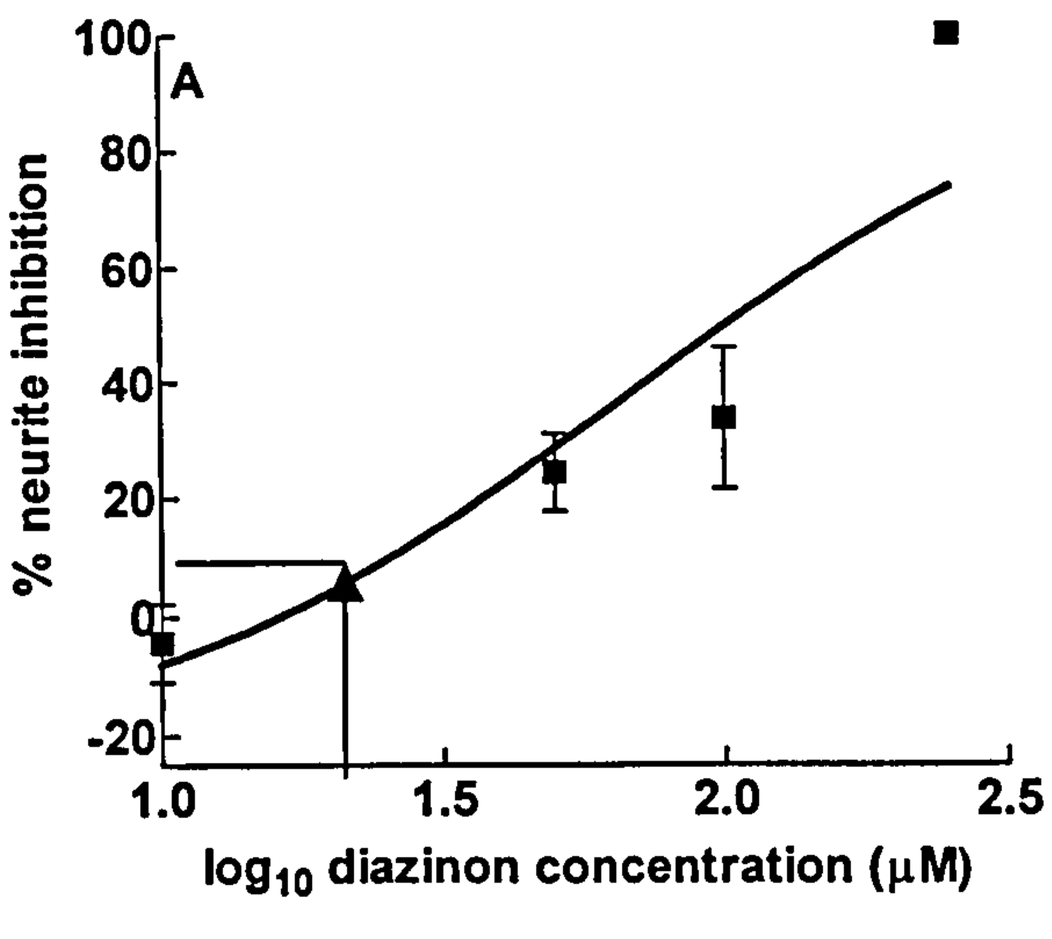


Figure 5.1: *Concentration response curve for the neurite inhibition produced by increasing concentrations of diazinon. Error bars are the standard errors of 6 separate experiments. The arrow marks the position of 25 μM diazinon and its resultant neurite inhibition.*

To ensure that there was no difference between the neurite outgrowth of cells chronically pre-exposed to diazinon and normal NB2a cells in the absence of other substances, the neurite outgrowth of all control groups chronically pre-exposed to diazinon from the individual experiments within this chapter were compared to that from control groups from cells that have not been pre-exposed, taken from the individual experiments in chapter 3. There was no significant difference between the two cell types with respect to neurite outgrowth in the absence of added test substance.

Effects of acute exposure to individual pesticides observed in 'normal' NB2a neuroblastoma cells were then compared to those in cells chronically pre-exposed to diazinon according to the methods in 2.2.3.

Diazinon

The effects on the inhibition of neurite outgrowth of cells pre-exposed to diazinon were compared to the inhibition in normal cells after differentiating for 24 h in diazinon. The results are shown in Figure 5.2.

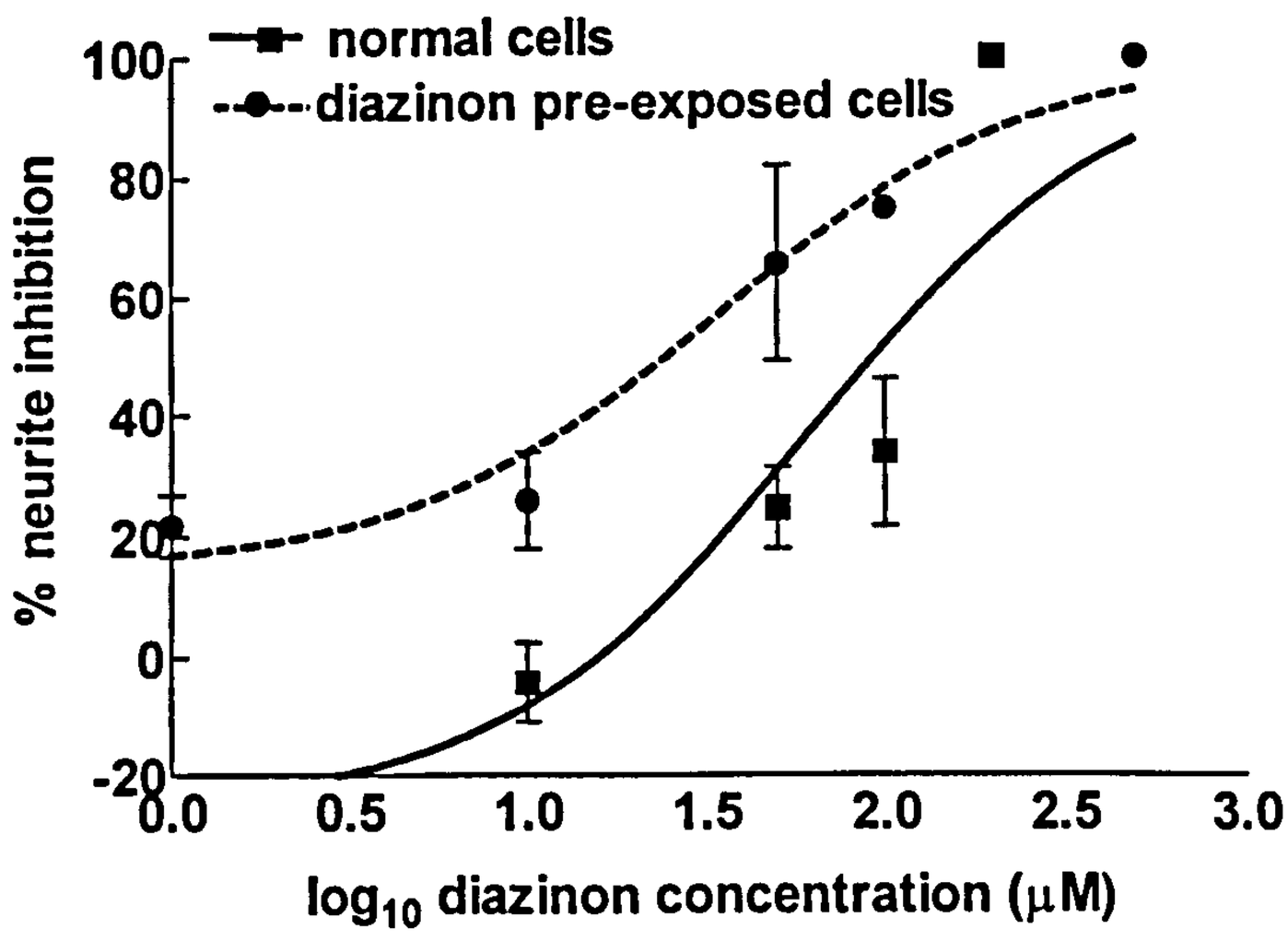


Figure 5.2: *Relationship between the concentration and the inhibition of neurite outgrowth observed in normal NB2a neuroblastoma cells and in cells pre-exposed to diazinon after differentiating in diazinon for 24 h. Error bars represent the standard error of 6 different experiments. The difference between the IC_{50} values was found to be statistically significant (p=0.001).*

The IC_{50} of cells pre-exposed to diazinon further exposed to diazinon was determined to be 35 μM (21-58 μM) compared to 68 μM (33-142 μM) in normal cells; a two-fold increase in neurotoxicity. The difference was found to be statistically significant (p=0.001). Statistical analysis of individual concentration points (one-way ANOVA including Bonferroni's correction for multiple comparisons) revealed a significant increase in inhibition of neurite outgrowth with 10, 50 and 100 μM diazinon in cells already pre-exposed to diazinon, compared to effects in normal cells ($p<0.01$, 0.05 and 0.001 respectively).

Because the cells had already been exposed chronically to diazinon, the results obtained here may have been due purely to a simple additive effect of the original 25 μM diazinon remaining unmetabolised in the cells, despite its removal from the culture medium 24 h before the acute exposure. The expected result, incorporating the expected toxicity from inclusion of this extra 25 μM diazinon, was determined from the original IC_{50} graph and is shown in Figure 5.3.

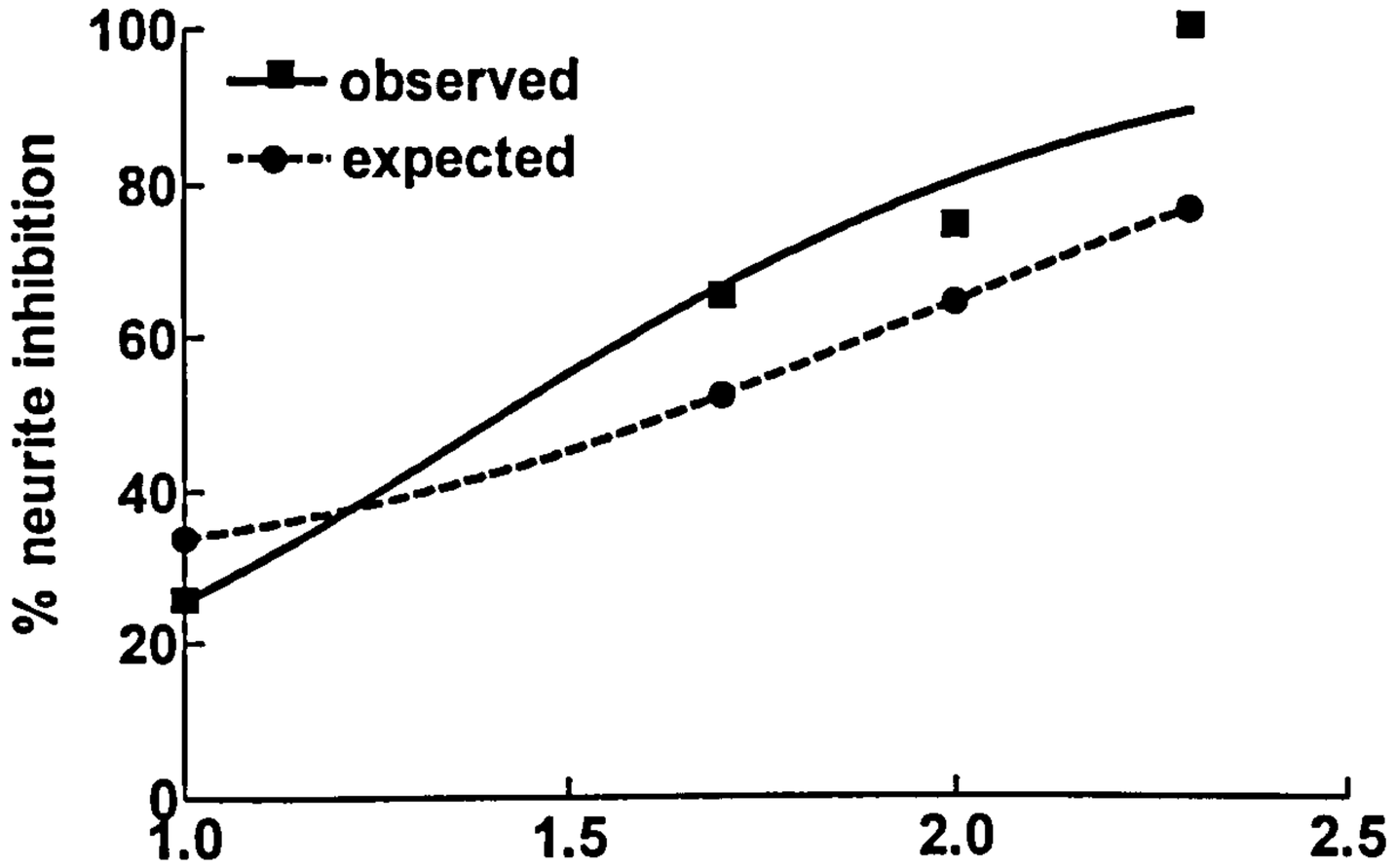


Figure 5.3: *Relationship between the concentration and the inhibition of neurite outgrowth observed in NB2a neuroblastoma cells pre-exposed to diazinon after differentiating in diazinon for 24 h, compared to the expected results calculated as a result of the chronic pre-exposure to 25 μM diazinon. The difference between the observed and expected results was statistically significant ($p<0.01$).*

The potentiation of effect above that anticipated as a result of an additive effect was statistically significant ($p<0.01$).

Glyphosate

The effects on the inhibition of neurite outgrowth of cells pre-exposed to diazinon were compared to the inhibition in normal cells after differentiating for 24 h in glyphosate. The results are shown in Figure 5.4.

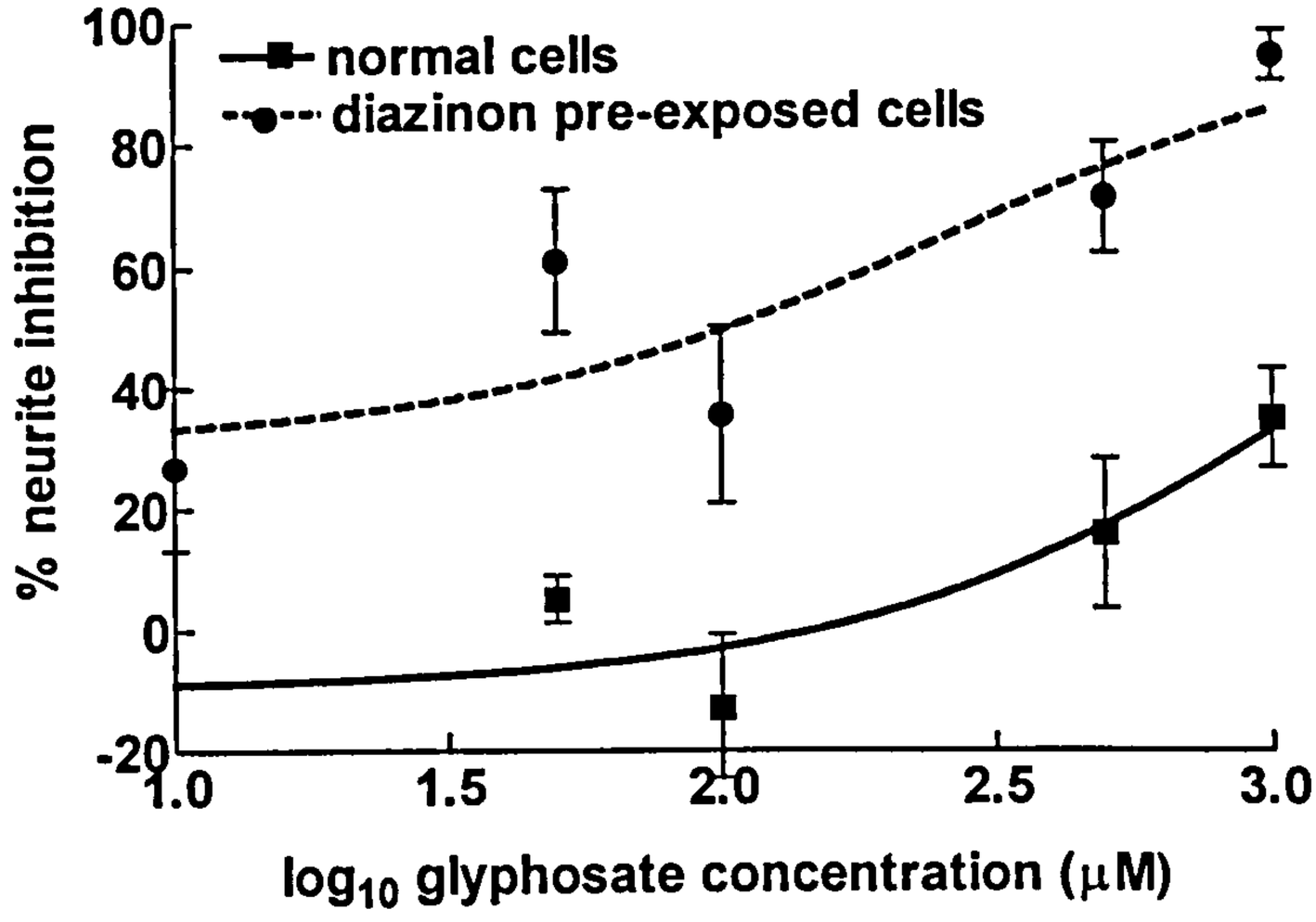


Figure 5.4: *Relationship between the concentration and the inhibition of neurite outgrowth observed in normal NB2a neuroblastoma cells and in cells pre-exposed to diazinon after differentiating in glyphosate for 24 h. Error bars represent the standard error of 5-6 different experiments. The difference between the two curves at 50, 100, 500 and 1000 μM was found to be statistically significant (p<0.01, 0.01, 0.01, 0.01).*

The IC_{50} of glyphosate in cells pre-exposed to diazinon was determined to be 273 μM (72-1041 μM), compared to 1587 μM (650-3878 μM) in normal cells. This represents an approximately 6-fold increase in neurotoxicity as a result of pre-exposure to diazinon. Due to the shape of individual response curves it was not possible to compare the IC_{50} values statistically. However statistical analysis of individual concentration points (one-way ANOVA including Bonferroni's correction for multiple comparisons) revealed a significant increase in inhibition of neurite outgrowth with glyphosate at 50, 100, 500 and 1000 μM in cells pre-exposed to diazinon compared to effects in normal cells ($p<0.01$ in all cases).

Verification that differences resulted from chronic pre-exposure to diazinon

Although the results demonstrated differences in pesticide toxicity in cells that had been chronically pre-exposed to diazinon, it was possible that the differences observed were purely the result of interaction of the pesticide with the residual diazinon remaining in the cells prior to treatment. To determine whether this was the case the methods given in 2.4.5 were used. Instead of the chronically pre-exposed cells, normal cells that had been exposed for 24 h to 25μM diazinon prior to plating were used. The cells were then exposed to a range of concentrations of glyphosate, shown to produce significant differences in response between normal and chronically pre-exposed cells (Figure 5.4). Combination of diazinon and glyphosate had also been observed to produce synergism in combination as a result of simultaneous exposure to the two substances (Chapter 4). The concentration response curve from these 'acutely pre-exposed' cells was compared to the curve for glyphosate in normal cells, and results are given in Figure 5.5.

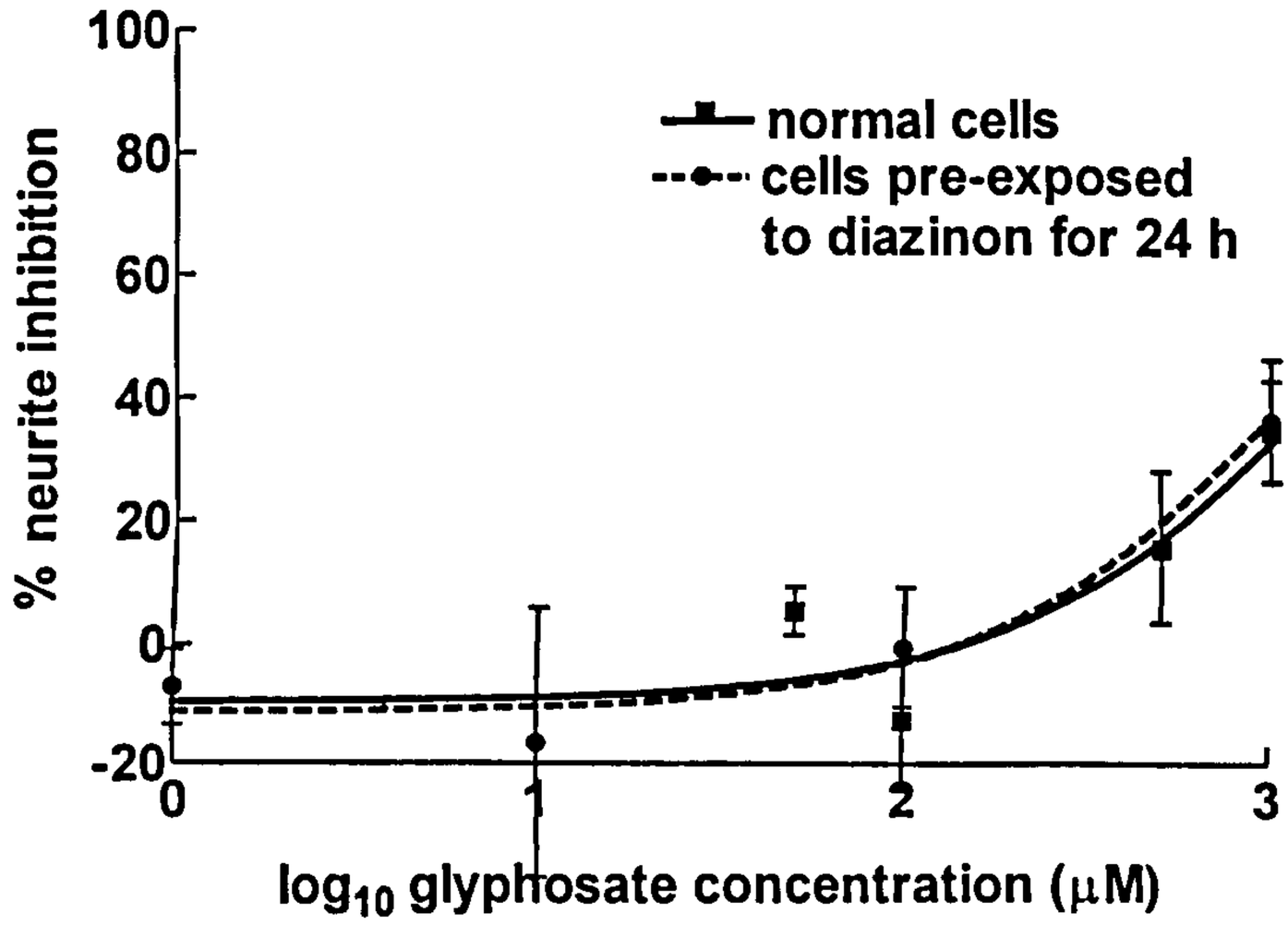


Figure 5.5: *Concentration response curves comparing the effects on neurite outgrowth inhibition of acute pre-treatment with diazinon for 24 h before exposure to glyphosate for 24 h. Error bars are the standard errors of 4-5 different experiments.*

The IC_{50} of glyphosate in cells that had been acutely pre-exposed to diazinon for 24 h was determined to be 1315 µM (444-3896 µM), compared to 1587 µM (650-3878 µM) in normal cells and 273 µM (72-1041 µM) in cells chronically pre-exposed to the same concentration of diazinon.

It is clear that acute pre-treatment with diazinon for 24 h prior to exposure to glyphosate for 24 h did not have any detectable effect on the response to the pesticide. Statistical analysis at individual points (one way ANOVA including Bonferroni's test for multiple comparisons) revealed no significant differences between acutely pre-exposed cells and normal cells. However a statistically significant difference was observed between cells acutely pre-exposed and chronically pre-exposed to diazinon at 1000 µM ($p<0.01$).

Phosmet

The effects on the inhibition of neurite outgrowth of cells pre-exposed to diazinon were compared to the inhibition in normal cells after differentiating for 24 h in phosmet. The results are shown in Figure 5.6.

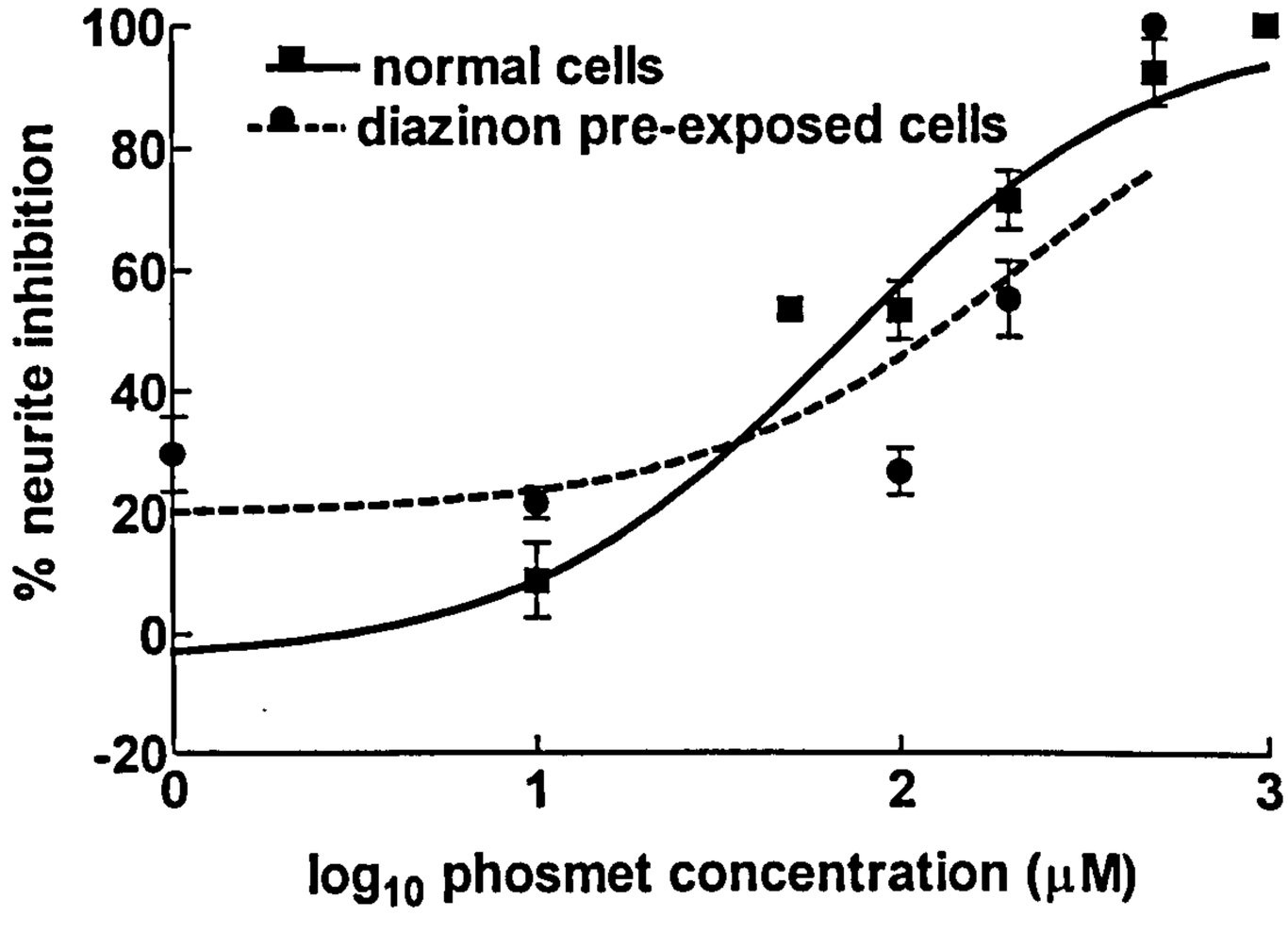


Figure 5.6: *Relationship between the concentration and the inhibition of neurite outgrowth observed in normal NB2a neuroblastoma cells and in cells pre-exposed to diazinon after differentiating in phosmet for 24 h. Error bars represent the standard error of 4-6 different experiments. The difference between the IC_{50} values was found to be statistically significant ($p<0.002$).*

The IC_{50} of phosmet in cells pre-exposed to diazinon was determined to be 213 μM (105-432 μM) compared to 72 μM (46-114 μM) in normal cells. The difference was found to be statistically significant ($p<0.002$). Statistical analysis of individual concentration points (one-way ANOVA including Bonferroni's correction for multiple comparisons) revealed a significant decrease in inhibition of neurite outgrowth (i.e. an increase in neurite growth) with 100 μM phosmet in cells pre-exposed to diazinon when exposed to phosmet compared to effects in normal cells ($p<0.01$).

Pirimiphos methyl

The effects on the inhibition of neurite outgrowth of cells pre-exposed to diazinon were compared to the inhibition in normal cells after differentiating for 24 h in pirimiphos methyl. The results are shown in Figure 5.7.

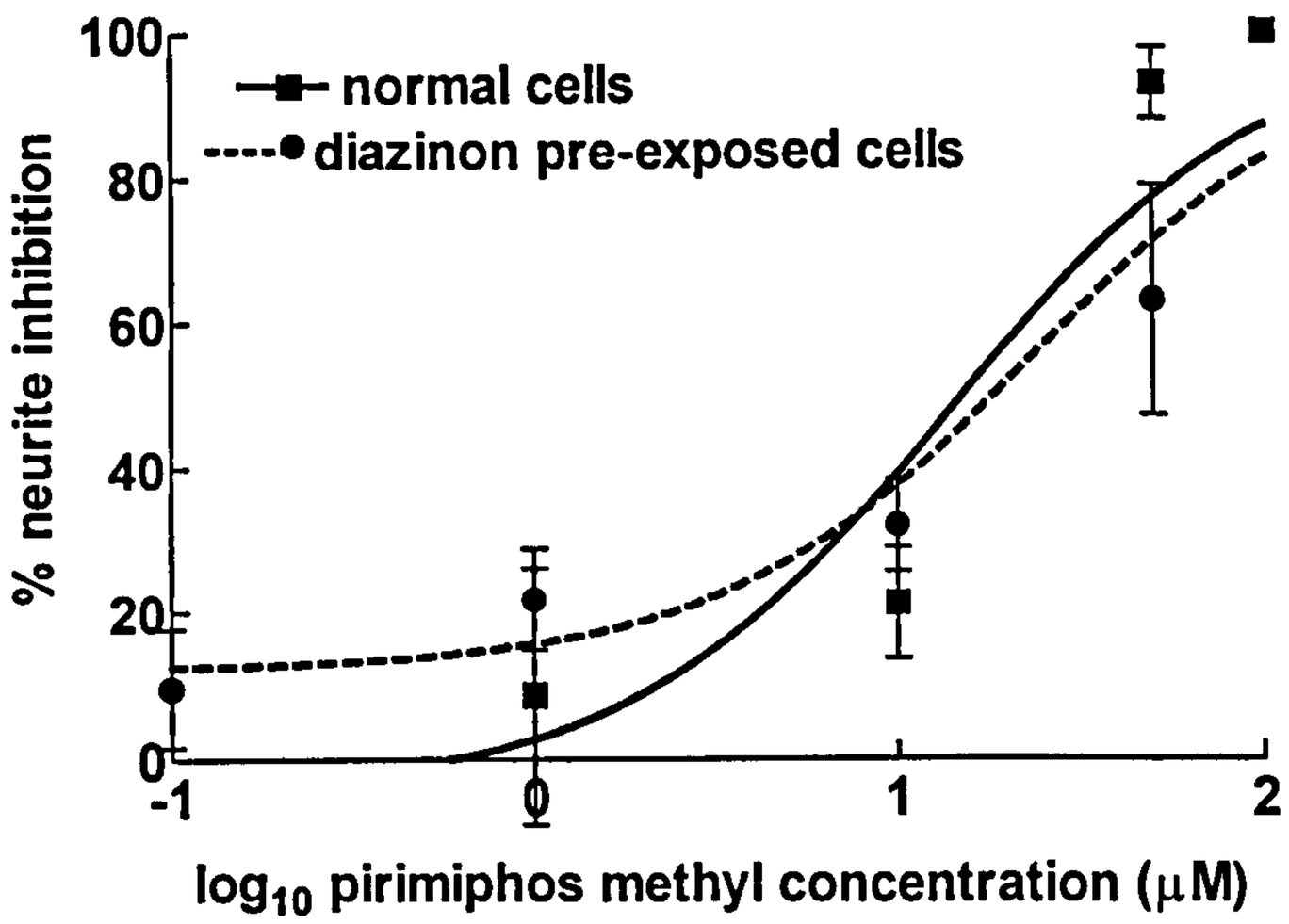


Figure 5.7: *Relationship between the concentration and the inhibition of neurite outgrowth observed in normal NB2a neuroblastoma cells and in cells pre-exposed to diazinon after differentiating in pirimiphos methyl for 24 h. Error bars represent the standard error of 6 different experiments.*

The IC_{50} of pirimiphos methyl in cells pre-exposed to diazinon was determined to be 24 μM (11-51 μM) compared to 14 μM (5-37 μM) for normal cells. The difference was not found to be statistically significant. Statistical analysis at individual concentrations revealed no significant differences in inhibition of neurite outgrowth in cells pre-exposed to diazinon compared to normal cells at any concentration of pirimiphos methyl.

Chlorpyrifos

The effects on the inhibition of neurite outgrowth of cells pre-exposed to diazinon were compared to the inhibition in normal cells after differentiating for 24 h in chlorpyrifos. The results are shown in Figure 5.8.

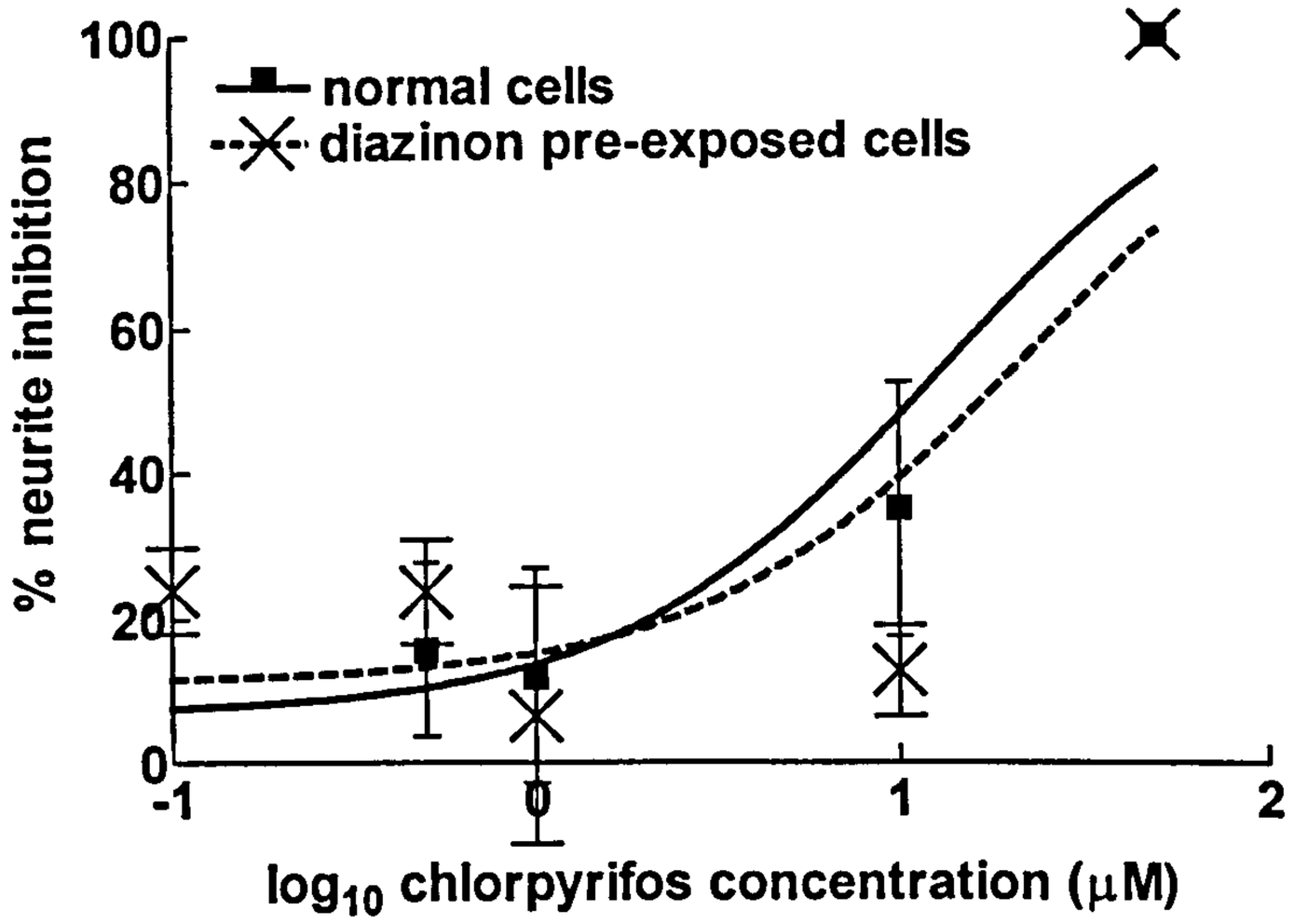


Figure 5.8: *Relationship between the concentration and the inhibition of neurite outgrowth observed in normal NB2a neuroblastoma cells and in cells pre-exposed to diazinon after differentiating in chlorpyrifos for 24 h. Error bars represent the standard error of 4-6 different experiments. The difference between the IC_{50} values was found to be statistically significant ($p<0.02$).*

The IC_{50} of chlorpyrifos in cells pre-exposed to diazinon was determined to be 22 μM (8-64 μM), compared to 13 μM (4-45 μM) in normal cells. The difference was found to be statistically significant ($p<0.02$).

At concentrations above 500 nM, little change in neurite length was measured in cells pre-exposed to diazinon rather than the expected decrease as observed in normal cells exposed to this concentration. Analysis based purely on the data presented therefore suggests that 1 or 10 μM chlorpyrifos had minimal inhibitory effect on neurite outgrowth in cells pre-exposed to diazinon.

However photomicrograph images of the effects reveal that these values may fail to demonstrate the true effects of chlorpyrifos in cells pre-exposed to diazinon. Normal cells exposed to chlorpyrifos at 500 nM and 10 µM are shown in Figure 5.9. A reduction in neurite outgrowth is seen with increasing concentration of chlorpyrifos, although even at 10 µM cells appear relatively healthy, with rounded cell bodies and numerous short neurites.

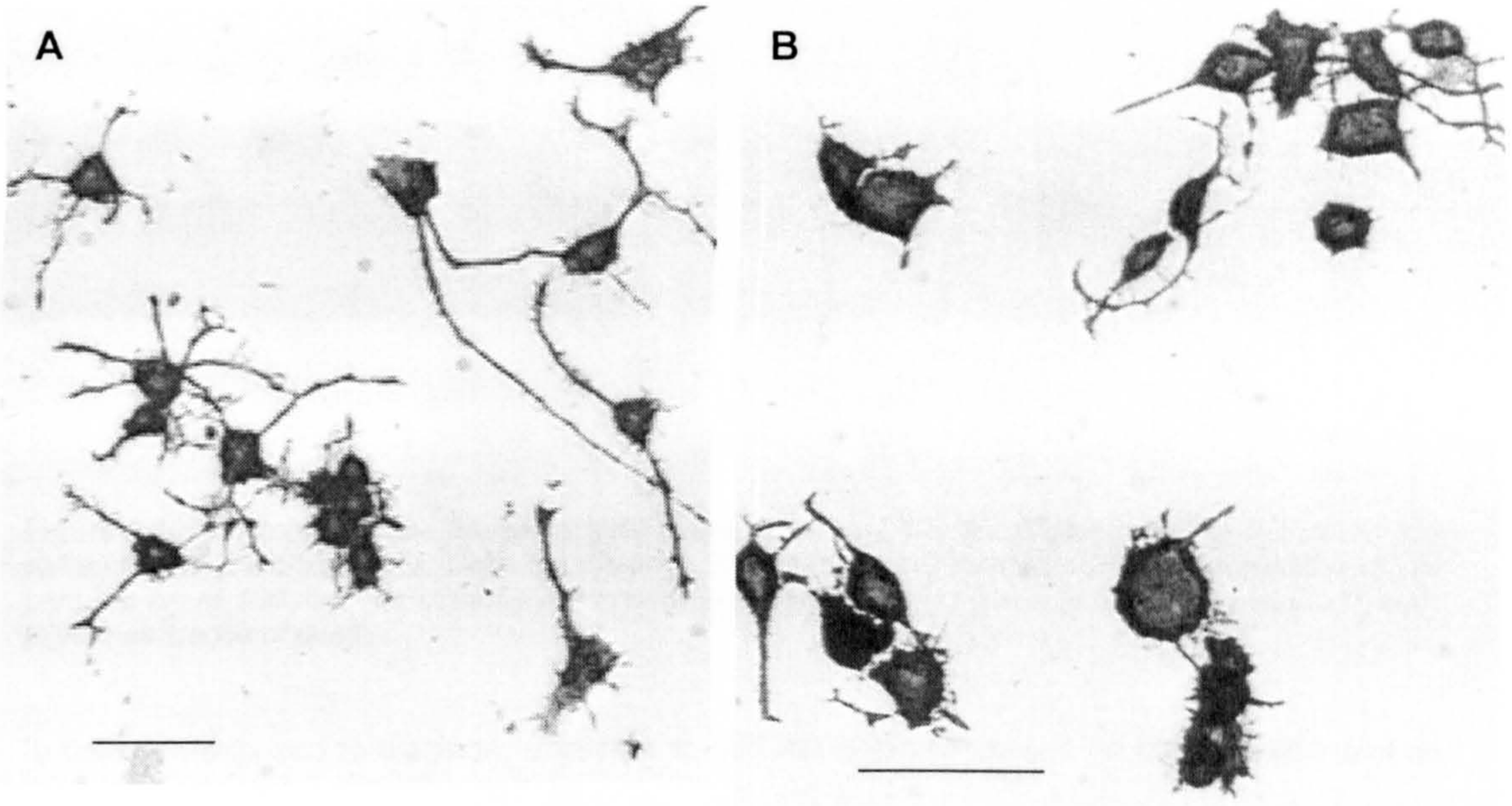


Figure 5.9: *Photomicrograph images of normal cells differentiating for 24 h in A) 500 nM and B) 10 µM chlorpyrifos. Cells were fixed in 10% formalin and stained with Coomassie Blue. Scale bars represent 100nm.*

Cells pre-exposed to diazinon exposed to the same concentrations of chlorpyrifos are strikingly different in appearance (Figure 5.10).

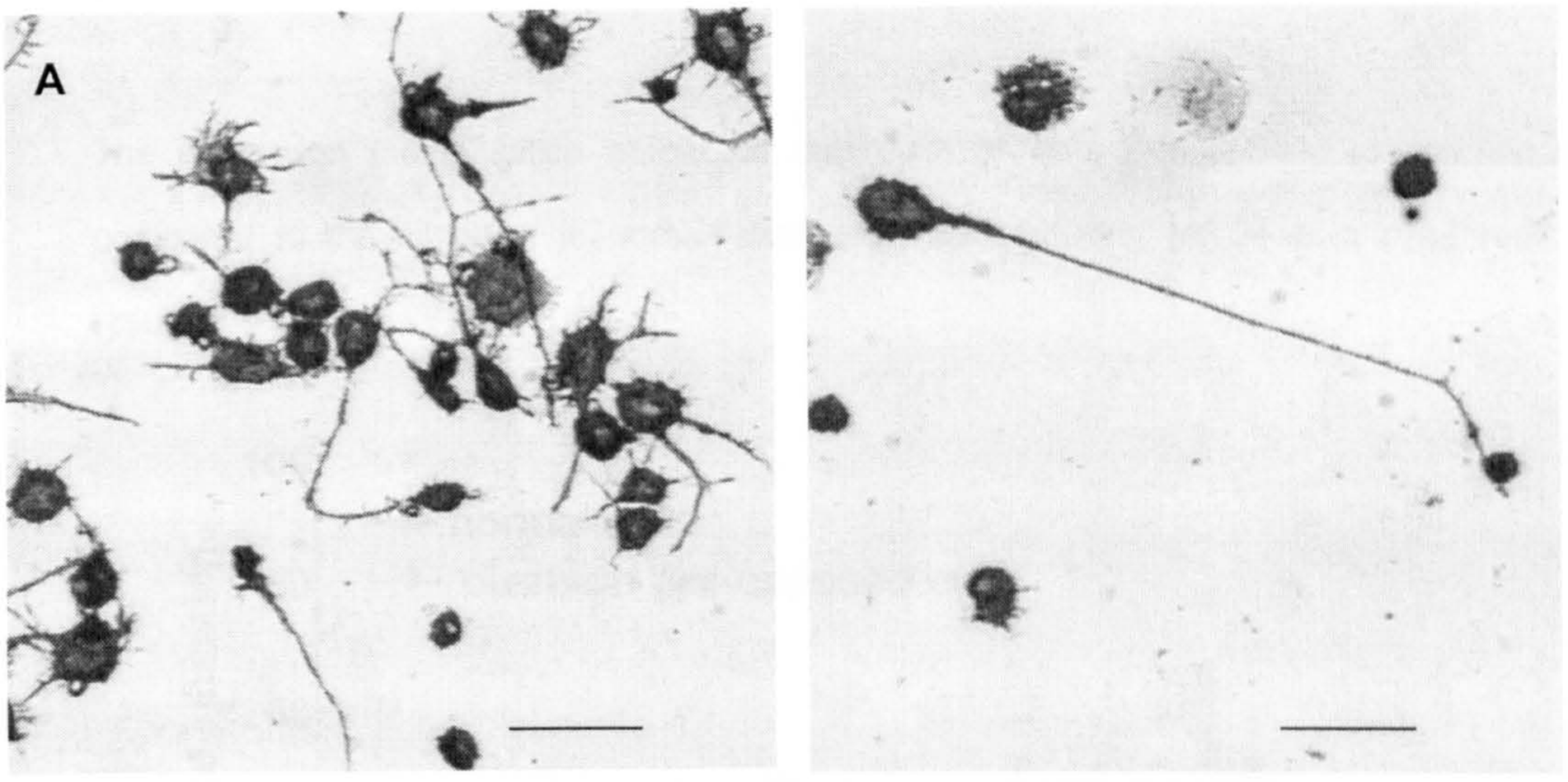


Figure 5.10: *Photomicrograph images of cells pre-exposed to diazinon differentiating for 24 h in A) 500 nM and B) 10 µM chlorpyrifos. Cells were fixed in 10% formalin and stained with Coomassie Blue. Scale bars represent 100 nm. The extremely long neurite extension can be seen in 5.10B, together with cells in various stages of death.*

In cells pre-exposed to diazinon, exposure to 500 nM chlorpyrifos had no observable effect on neurite length compared to the normal cells at the same concentration, and the cells appeared healthy with rounded bodies and numerous long and short neurites. At 10 µM chlorpyrifos however, considerable cell death was apparent, but some neurones exhibited an exceptional hyperextension of single, axon-like neurites, often connecting two or more neurones. A photomicrograph image of one such neurone exhibiting this phenomenon is shown in Figure 5.10B. There is an absence of branching although the single neurite is many times the length of the cell body. This hyperextension therefore confounded measurement of average neurite length.

Pyrethrum

The effects on the inhibition of neurite outgrowth of cells pre-exposed to diazinon were compared to the inhibition in normal cells after differentiating for 24 h in pyrethrum. The results are shown in Figure 5.11.

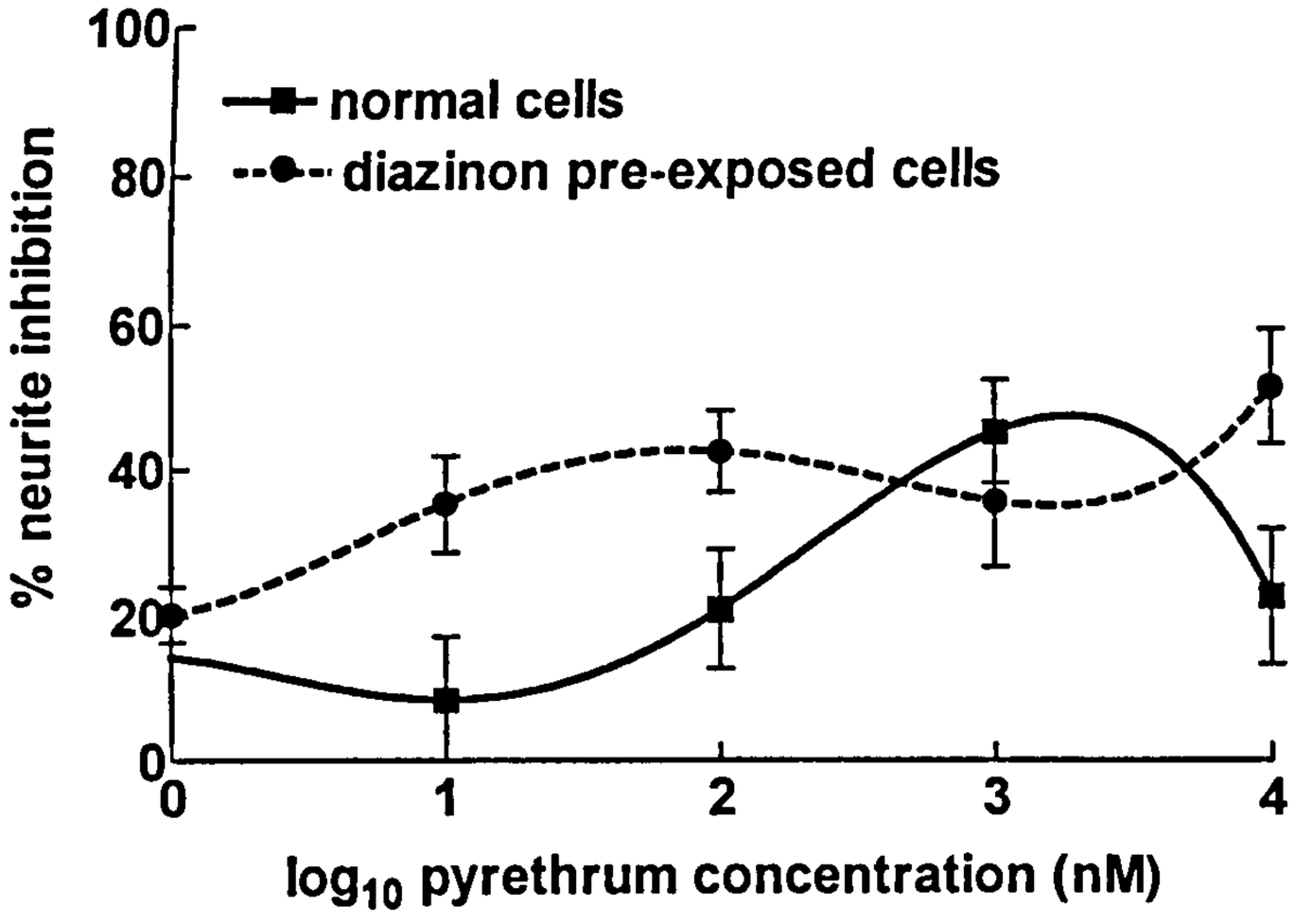


Figure 5.11: *Relationship between the concentration and the inhibition of neurite outgrowth observed in normal NB2a neuroblastoma cells and in cells pre-exposed to diazinon after differentiating in pyrethrum for 24 h. Error bars represent the standard error of 6-7 different experiments. The difference between the two curves at 10 μM was found to be statistically significant ($p<0.05$).*

As discussed in Chapter 3, an unusual response curve compared to that elicited by other pesticides was observed after exposure to pyrethrum in normal cells and this was reproduced in cells pre-exposed to diazinon. After an initial inhibition in neurite outgrowth with increasing concentrations of pyrethrum, further increase resulted in a reduction in this inhibition. A bell-shaped response curve was produced, preventing calculation of an accurate IC_{50} value, although in normal cells the maximum inhibition of outgrowth was seen at about 1 μM.

In cells pre-exposed to diazinon a similar pattern was observed. However the maximum neurotoxic effect in terms of neurite inhibition, was observed at much lower concentrations in these cells, reaching a maximum at about 0.1 μM rather than the 1 μM maximum seen in normal cells. Again no IC_{50} value could be calculated in cells pre-exposed to diazinon since a

50% inhibition level was not achieved. Statistical analysis of individual concentration points (one-way ANOVA including Bonferroni's correction for multiple comparisons) revealed a significant difference in response with 10 μM pyrethrum ($p<0.05$) in cells pre-exposed to diazinon, compared to effects in normal cells.

Formulations of glyphosate

Although glyphosate was not neurotoxic to cells under normal conditions, even at very high concentrations of this product, pre-exposure to chronic diazinon markedly increased the level of detectable neurotoxicity (Figure 5.4).

To investigate the effects of formulation of glyphosate in standard proprietary compounds on the toxicity observed in diazinon pre-treated cells, two such products were tested.

Roundup, 0.72% formulation of glyphosate

The effects on the inhibition of neurite outgrowth of cells pre-exposed to diazinon were compared to the inhibition in normal cells after differentiating for 24 h in Roundup. The results are shown in Figure 5.12.

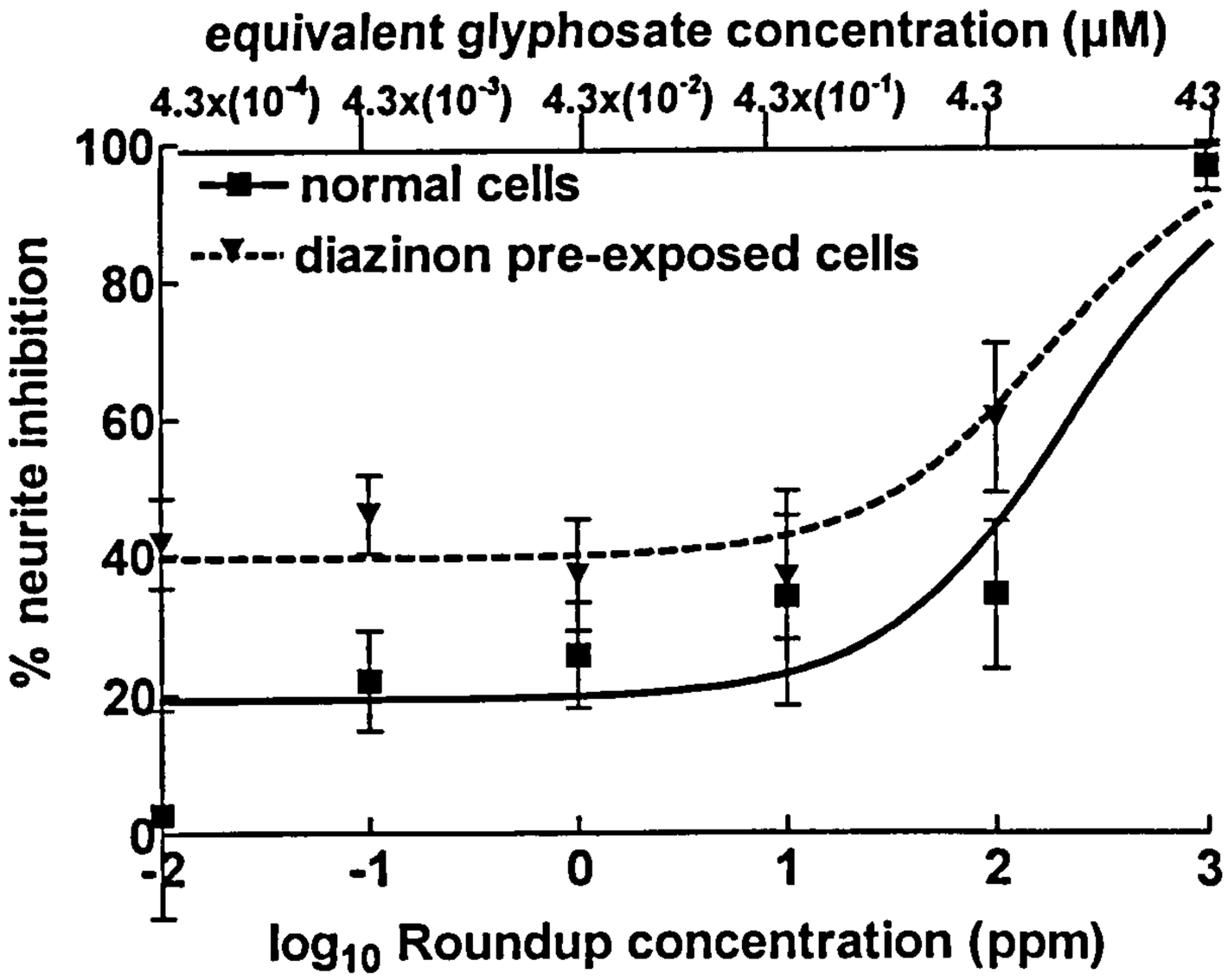


Figure 5.12: *Relationship between the concentration and the inhibition of neurite outgrowth observed in normal NB2a neuroblastoma cells and in cells pre-exposed to diazinon after differentiating in Roundup, 0.72% glyphosate for 24 h. Error bars represent the standard error of 5-7 different experiments. The equivalent concentration of glyphosate is indicated on a separate axis. The difference between the two curves at 0.01 and 0.1 ppm was found to be statistically significant ($p<0.05$ and 0.03).*

The IC_{50} of Roundup in cells pre-exposed to diazinon was determined to be 176 ppm (64-485 ppm) (equivalent to 8 µM glyphosate), compared to 223 ppm (74-679 ppm) in normal cells (equivalent to 10 µM glyphosate). Due to the shape of individual response curves it was not possible to analyse these results statistically although formulation of glyphosate in Roundup increased the toxicity of glyphosate (IC_{50} 1587 µM) by 165 fold in normal cells, and by over 200 fold in cells pre-exposed to diazinon.

Statistical analysis at individual concentration points however (one-way ANOVA including Bonferroni's correction for multiple comparisons), revealed a significant increase in inhibition of neurite outgrowth with 0.01 ppm Roundup in cells pre-exposed to diazinon compared to effects in normal cells ($p<0.05$).

Tough Weed Killer, 5% formulation of glyphosate trimesium

Glyphosate trimesium, a related formulation of glyphosate technical is often substituted for pure glyphosate in formulation. The effects on the inhibition of neurite outgrowth of cells pre-exposed to diazinon were compared to the inhibition in normal cells after differentiating for 24 h in Tough Weed Killer. The results are shown in Figure 5.13.

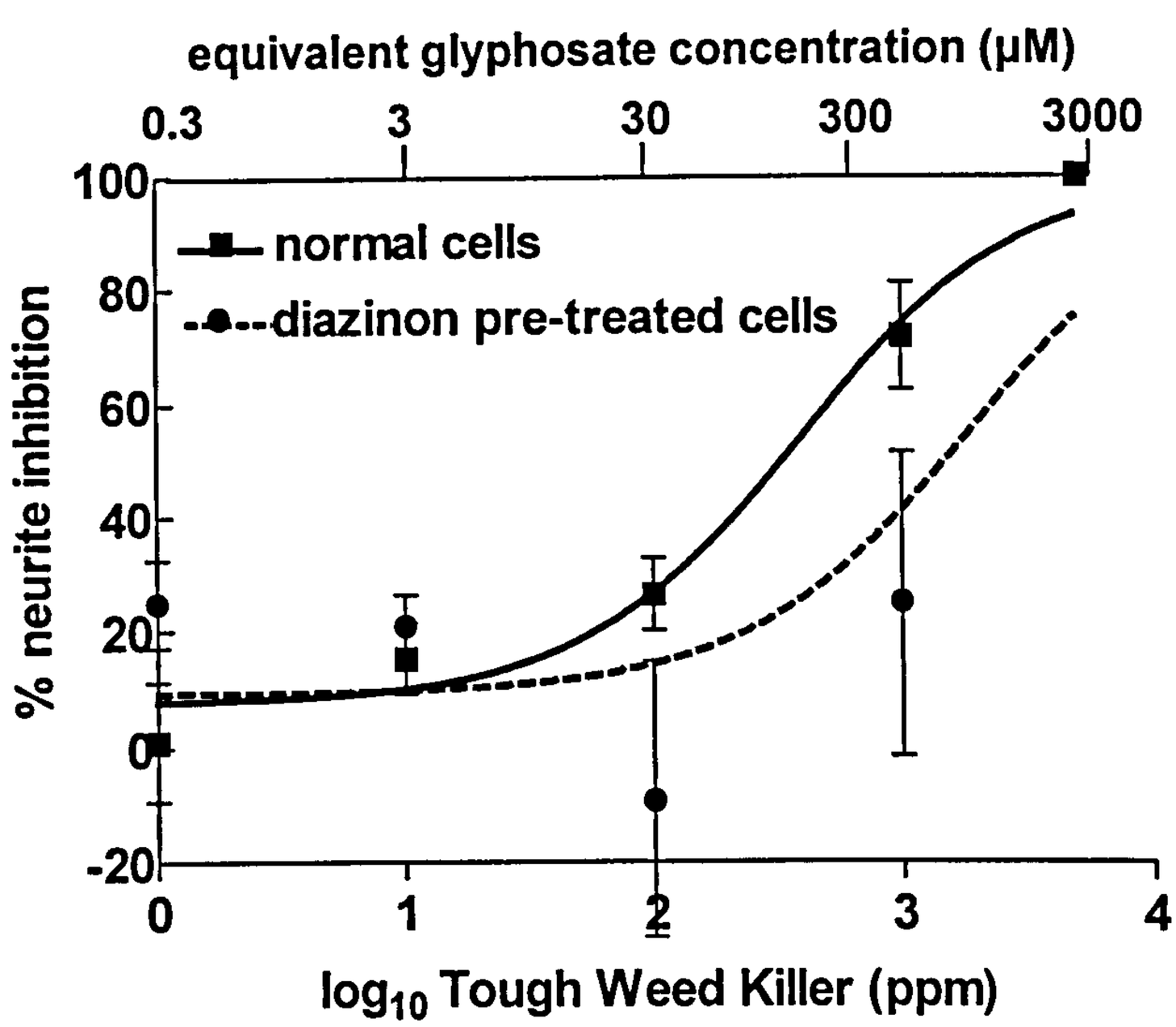


Figure 5.13: *Relationship between the concentration and the inhibition of neurite outgrowth observed in normal NB2a neuroblastoma cells and in cells pre-exposed to diazinon after differentiating in Tough Weed Killer, 5% glyphosate trimesium for 24 h. Error bars represent the standard error of 6 different experiments. The equivalent concentration of glyphosate is shown on a separate axis.*

The IC_{50} of Tough Weed Killer in cells pre-exposed to diazinon was determined to be 1843 ppm (431-7877) ppm (equivalent to 552 µM glyphosate), compared to 392 ppm (202-761 ppm) in normal cells (equivalent to 118 µM glyphosate). Formulation of glyphosate in Tough Weed Killer therefore increased the toxicity of glyphosate (IC_{50} 1587 µM) by 13 fold in normal cells, and by 3 fold in cells pre-exposed to diazinon. The unusual concentration response curves precluded statistical comparison of individual IC_{50} values.

Statistical analysis of individual concentration points (one-way ANOVA including Bonferroni's correction for multiple comparisons) did not reveal any significant difference in the inhibition of neurite outgrowth in cells pre-exposed to diazinon compared to the effects in normal cells when subsequently exposed to Tough Weed Killer. However, as discussed previously concerning the effects of chlorpyrifos, a dramatic increase in neurite length was seen in some cells. The wide variation in results depended on whether this phenomenon was noted in individual experiments. Some degree of hyperextension was observed in half of the six experiments performed, and in two of these the majority of the cells remaining in the wells showed this effect.

An example of cells pre-exposed to diazinon exposed to Tough Weed Killer at 100 ppm demonstrating an extreme example of hyperextension of neurites is shown in Figure 5.14, compared to normal cells exposed to the same concentration.

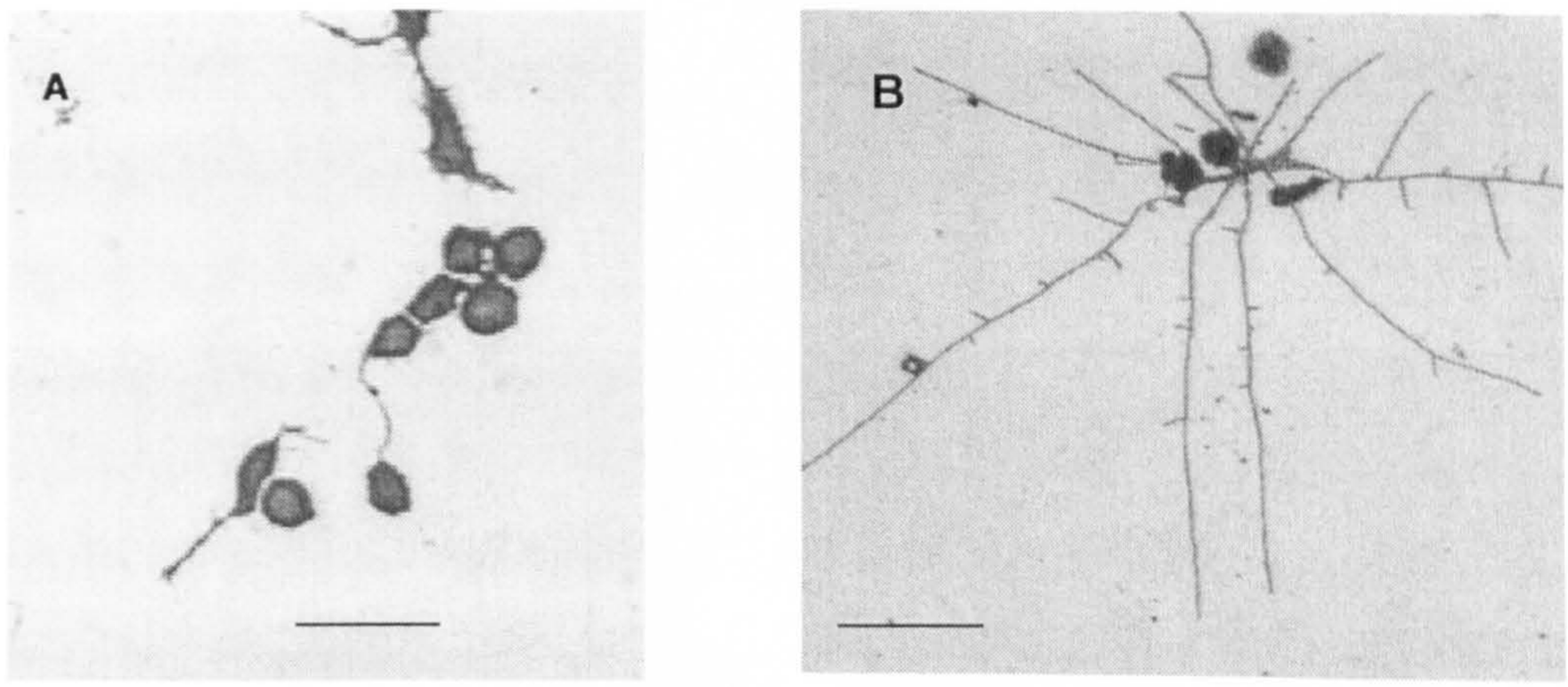


Figure 5.14: *Photomicrograph images of A) normal Nb2a cells and B) cells pre-exposed to diazinon differentiating for 24h in 100 ppm Tough Weed Killer (5% glyphosate trimesium). Cells were fixed in 10% formalin and stained with Coomassie Blue. The scale bars represent 100nm.*

Results for all the experiments performed here are summarized in Table 5.15.

test product	IC_{50} (μM) with 95% confidence limits	
	normal cells	pre-treated cells
diazinon	68 (33-142)	35 (21-58)
phosmet	72 (46-114)	213 (105-432)
pirimiphos methyl	14 (5-37)	24 (11-51)
chlorpyrifos	13 (4-45)	22 (8-64)
pyrethrum	1*	0.1*
glyphosate	1587 (650-3878)	273 (72-1041)
Roundup	10a	8a
Tough Weed Killer	118a	552a

Table 5.15: *Summary of the IC_{50} values for normal NB2a neuroblastoma cells and cells pre-exposed to diazinon differentiating for 24 h in the presence of various pesticides. * represents estimate of IC_{50} and a represents the IC_{50} of the equivalent glyphosate in the proprietary products.*

5.4 Discussion

The hypothesis that the neurotoxicity of pesticides to cells that have been chronically pre-exposed to the organophosphate diazinon differs quantitatively from that observed in cells that have not been pre-exposed to diazinon, was supported by the results of this study. The results demonstrated significant differences in response to pesticide exposure between cells that had been chronically pre-exposed (but not acutely pre-exposed) to diazinon compared to normal cells.

The neurotoxicity of cells chronically pre-exposed to diazinon

This is the first publication of results from a cellular model of *in vitro* toxicity investigating the effects on acute toxicity of chronic pre-exposure to minimally or non-toxic concentrations of organophosphates, in this case diazinon. There was no effect on neurite outgrowth in these cells as a result purely of acute pre-exposure to diazinon (Figure 5.5). When exposed to a variety of different pesticides however it was clear that the manifestation of response in NB2a neuroblastoma cells pre-exposed to diazinon depended on the product to which it is subsequently exposed.

Although it could be speculated that the results seen here would be observed with chronic pre-exposure to other organophosphates, this could only be confirmed by further investigation of cells pre-exposed to different products. Likewise it is unknown whether the effects would be observed if cells were exposed to lower concentrations or for a shorter period before subsequent challenge with different pesticides.

Earlier results (3.5) do not suggest any significant metabolism is occurring. Chronic diazinon exposure *in vivo* induces P450 enzymes (Kump *et al.*, 1996), resulting in more rapid metabolism of subsequent exposure to cocaine. P450 induction would increase the toxicity of those organophosphates which produced metabolites that were more toxic than the parent molecule such as chlorpyrifos oxon or pirimiphos methyl oxon, the principle metabolites of

chlorpyrifos and pirimiphos methyl. However no significant difference was seen in diazinon pre-treated cells exposed to pirimiphos methyl and a significant reduction was seen with chlorpyrifos, compared to normal cells. It is likely that effects were due mainly to factors other than induction of metabolism.

Another possible contribution to the observed effects could be from induction of other enzyme systems such as glutathione-S-transferase or the flavin monooxygenase system in cells pre-exposed to diazinon. Flavin monooxygenases are responsible for activation of organophosphates containing at least one C-P bond (Sultatos, 1994), so would apply only to activation of glyphosate in this study. Glutathione-S-transferase is known to inactivate malathion (Josephy, 1997b) but not parathion (Levine and Murphy, 1977), but Chambers *et al.* (Chambers *et al.*, 1994) found no differences in glutathione levels after induction with phenobarbital or β-naphthoflavone in rats exposed to parathion or paraoxon.

Effects on the cholinergic mechanisms in these cells may account more fully for the effects seen in this study. Acetylcholinesterase activity increases considerably in differentiating cells (Rieger *et al.*, 1976) and the increased expression is related to the adhesion properties and not to their acetylcholine hydrolysis activities (Koenigsberger *et al.*, 1997). Organophosphate tolerance is associated with increased acetylcholinesterase activity (Overstreet and Schiller, 1992), possibly in response to down-regulation of muscarinic receptors. Acetylcholinesterase itself is toxic to neuronal and glial cells (Calderon *et al.*, 1998); so any increase in expression may manifest as increased toxicity.

The subsensitivity (Overstreet and Schiller, 1992) associated with down-regulation or inhibition of muscarinic receptors (Sivam *et al.*, 1983; Dawson and Jarrott, 1981; Viana *et al.*, 1988) plays a role in tolerance resulting from chronic toxicity and may result from a direct effect on binding to the muscarinic or nicotinic receptors by the pesticides themselves, such as seen with chlorpyrifos oxon (Abou-Donia and Huff, 1995). Down-regulation of muscarinic receptors recovers rapidly after acetylcholinesterase inhibition by organophosphates, but the nicotinic receptors remain significantly depressed (Prendergast *et al.*, 1998). In contrast,

continuous infusion of physostigmine increases expression of nicotinic receptors (Overstreet and Schiller, 1992). Organophosphate-inactivated acetylcholinesterase increases the accumulation of acetylcholine, which may assume a toxic role by inhibition of neurite growth (Lipton *et al.*, 1988). The process is thought to involve second messengers such as cAMP (Stitcher *et al.*, 1977; Sevaljevic *et al.*, 1981).

Other mechanisms may play a role in these toxic effects including alterations in protease activity (Minara *et al.*, 1990; Leprince *et al.*, 1991), or other molecules such as somatostatin (Robbins *et al.*, 1982), choline or excitatory neurotransmitters (Overstreet and Schiller, 1992), GABA (Kar and Matin, 1972; Sivam *et al.*, 1983) or cGMP (Lundy and Magor, 1978). Chlorpyrifos has been shown to reduce binding activity of the transcription factor AP-1 (Crumpton *et al.*, 2000) suggesting non-cholinergic actions of some organophosphates on cell differentiation and phenotype.

Enhanced neuronal excitability, presumably by increasing acetylcholine at the synapse (Kaufer *et al.*, 1998b) has been shown to increase *cfos* gene expression and may activate long-term changes in expression of proteins mediating cholinergic neurotransmission and subsequently delay acetylcholine reduction, with potentially damaging long-term implications.

The increase in neurite length seen with increased concentrations of chlorpyrifos and Tough Weed Killer may reflect the secondary function of acetylcholinesterase as a cell adhesion molecule (Bigbee *et al.*, 1999), or even an increased production of NGF synergising with the effect of dibutyryl cyclic AMP on neurite outgrowth (Huang *et al.*, 1996).

The inhibition of neuropathy target esterase (NTE) is not considered to play a role here. The effects discussed are observed after an exposure period of 24 h whereas effects associated with inhibition of NTE are normally apparent after 10-14 days (Johnson, 1970) and involve dying back of long axons rather than inhibition of growth. The pesticides investigated have not been associated with this type of neuropathy apart from after repeated exposure to chlorpyrifos (Capodicasa *et al.*, 1991).

The biphasic response curve of pyrethrum was maintained after chronic pre-exposure to diazinon, although the toxicity increased 10-fold, which suggests that the mechanism of toxicity remained the same, but the sensitivity was increased after pre-exposure to organophosphates. The increase in toxicity may involve more complicated interactions, although an increase in acetylcholine synthesis (Eels *et al.*, 1992) and alterations in muscarinic and nicotinic receptors as a result of pyrethrum exposure (Eriksson and Nordberg, 1990) further highlights possible acetylcholine involvement in the increased toxicity of pyrethrum.

Effect of formulation on increased toxicity in chronically pre-exposed cells

The components of a formulation can increase *in vivo* and *in vitro* toxicity to a greater extent than can be explained by additivity. This was well illustrated in the model by glyphosate, both as the pure substance and in formulation. Even without pre-exposure to diazinon, formulation significantly increased the neurotoxicity (Figure 3.5). The toxicity of Roundup was significantly increased in cells pre-exposed to diazinon compared to normal cells, and this may be the case after chronic pre-exposure to other pesticides. The apparent reduction in toxicity to pre-exposed cells treated with another glyphosate formulation, Tough Weed Killer, compared to normal cells (Figure 5.14) was due to a hyperextension of neurites similar to that seen with chlorpyrifos (Figure 5.10B).

Correlation with illnesses suspected of being caused by pesticide exposure

The link between pesticide exposure and Gulf War Syndrome as well as the claims of ill-health suffered by farmers as a result of chronic exposure to pesticides has been studied to a limited degree *in vivo* (Abou-Donia *et al.*, 1996a; Haley *et al.*, 1999; Davies *et al.*, 1996). Where chronic toxic effects have been demonstrated there is evidence both for (Soliman *et al.*, 1983) and against (Prendergast *et al.*, 1997; Singh and Drewes, 1987; Socko *et al.*, 1999) the toxicity being related to inhibition of esterase activity, but clinical and epidemiological studies are difficult to interpret (Ray, 1998; Ray, 2000). Genetic polymorphisms of the PON-1

gene have been linked to increased toxicity due to maintenance of the more toxic oxon metabolites *in vivo* (Humbert *et al.*, 1993), and competition for xenobiotic metabolizing enzymes.

This study however, involves the simulation of chronic low-level exposure to a common organophosphate, diazinon, and shows synergism of effects both with other organophosphates and with other pesticides as well as highlighting the problems associated with formulation in solvents.

The prophylactic use of the reversible acetylcholinesterase inhibitor, pyridostigmine bromide, as a protection against subsequent organophosphate nerve gas exposure (Abou-Donia *et al.*, 1996a) in Gulf War Veterans may have increased the toxicity caused by subsequent exposure to pesticides, including organophosphates and pyrethroids, or by solvents.

Pyrethroid exposure may contribute significantly to the ill-health of Gulf War Veterans and farmers. Pyrethrum is the most neurotoxic *in vitro* of all the pure pesticides tested here; in contrast to the reported *in vivo* toxicity (Casida *et al.*, 1983), although a more direct route *in vivo* such as intravenous exposure greatly increases the neurotoxicity (Casida *et al.*, 1983). A breakdown in blood brain barrier can occur in times of stress (Friedman *et al.*, 1996), as a result of increased acetylcholine (also due to stress) (Kaufer *et al.*, 1998a) or as a consequence of pesticide exposure, including organophosphates and pyrethroids in young animals (Gupta *et al.*, 1999a; Gupta *et al.*, 1999b), and this may also increase neurotoxicity *in vivo*. Exposure to pyrethrum of cells already exposed to diazinon further increases the neurotoxicity seen in this model. Similar potentiation of neurotoxicity of the synthetic pyrethroid deltamethrin has been seen *in vivo* after exposure to organophosphates (Audegond *et al.*, 1989).

CHAPTER 6

DETERMINATION OF POSSIBLE MECHANISMS OF PESTICIDE SYNERGY

6.1 Introduction

The results observed in Chapter 4 indicated that some, although not all combinations of pesticides and their formulation products can produce synergistic reactions, manifesting as a reduction in neurite outgrowth in NB2a neuroblastoma cells. The results from Chapter 5 show that these effects can also result from chronic pre-exposure of cells to the organophosphate diazinon. However none of the results gave any indication of the mechanisms involved in the synergistic reactions observed.

Organophosphates can affect various different endpoints at concentrations well below those that affect cell viability (Rowles *et al.*, 1995). The primary toxic effect of organophosphates involves the irreversible inhibition of the enzyme acetylcholinesterase by binding at the active site and preventing the enzyme from participating in the reaction to hydrolyse acetylcholine to choline and acetyl CoA (Sultatos, 1994). At neuronal synapses the resulting accumulation of acetylcholine maintains continuous stimulation of receptors. Whether this esterase inhibition, despite the lack of correlation with acute neurotoxicity (Marinovich *et al.*, 1996), can translate into the toxicity observed in this assay, which lacks synapses, is not known. Inhibition of acetylcholinesterase activity does correlate with the presence of abnormal neurofilament inclusions in dorsal root ganglia neurones (Dupree *et al.*, 1995; Bigbee *et al.*, 1999), and so could disrupt cellular transport functions.

Acetylcholinesterase has various other roles in cells, dependent on the stage of cell development (Coleman and Taylor, 1996). Apart from its function at cholinergic nerve synapses, it has been found to be toxic to cells (Calderon *et al.*, 1998), in a way that is not related to inhibition of its catalytic site. Acetylcholinesterase is not confined to cholinergic cells (Grisaru *et al.*, 1999) so may potentially play a role in the synergistic increase in toxicity observed in previous chapters. It is expressed by neurones during periods of neuritogenesis

(Sharma and Bigbee, 1998) and is associated with cell adhesion, by means of a second active site on the molecule at the peripheral anionic site (Johnson and Moore, 1999), which is not apparently influenced by inhibition of the catalytic site. The process of cell adhesion is involved in cell plasticity and may influence the extension of neurites from developing neurones (Koenigsberger *et al.*, 1997). Organophosphate pesticides may interact with the adhesion role of acetylcholinesterase, and potentially produce synergistic effects by this mechanism.

Alternatively the synergistic effects on neurite outgrowth may be due to a change in cell viability resulting from a combination of pesticides. A reduction or even an increase in cell density, due to cell death or proliferation, could affect the ability of the cells to produce neurites. Likewise an effect on axonal transport by interference with cytoskeletal proteins such as neurofilaments (Flaskos *et al.*, 1998) may result in a decrease in viability.

The purpose of this part of the investigation therefore was to determine whether effects on acetylcholinesterase activity, cell viability or adhesion could be detected in combinations of pesticides, which could explain the synergistic reactions in Chapter 5.

6.2 Research hypothesis investigated in this chapter

The synergistic effect of combinations of pesticides is related to a synergistic action as inhibitors of acetylcholinesterase, synergistic effects on cell viability or synergistic impairment of cell adhesion.

6.3 Detection of changes in acetylcholinesterase activity

The methods used to investigate the possible mechanisms resulting in the synergistic reactions observed with some combinations of *pesticides and formulations* as seen in Chapter 4 are detailed in 2.6. Various pairs of pesticides that had produced significant synergy were investigated for changes in the rate of acetylcholinesterase activity, and these were compared with a combination (diazinon with chlorpyrifos) that did not produce any synergistic effect on inhibition of neurite outgrowth.

Combination of phosmet and pirimiphos methyl

Changes in the rate of acetylcholinesterase activity produced by exposure to a combination of phosmet and pirimiphos methyl for 1 h and 24 h are shown in Figure 6.1.

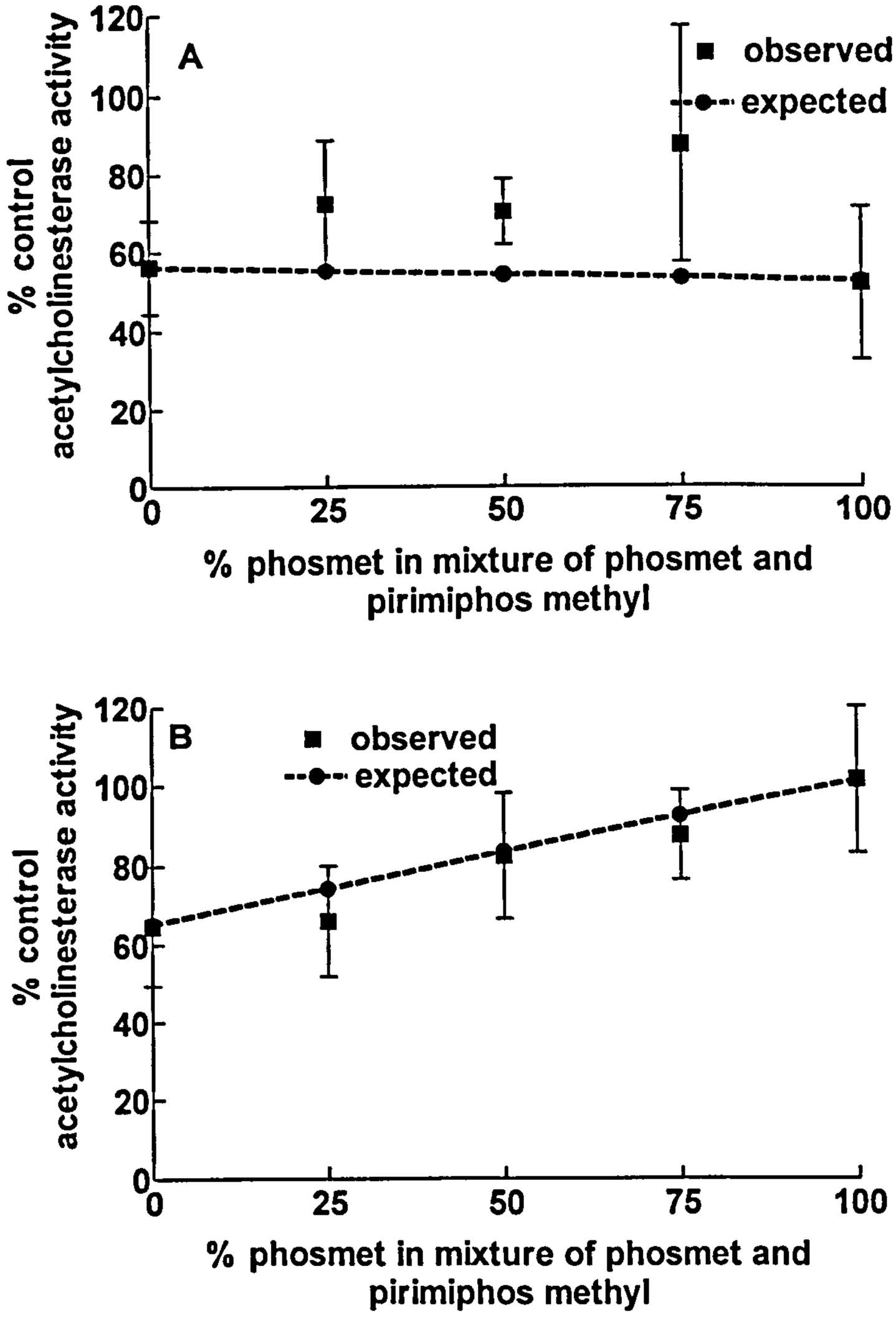


Figure 6.1: *Relationship between the relative concentrations and acetylcholinesterase activity observed in NB2a neuroblastoma cells after exposure to a mixture of phosmet and pirimiphos methyl for A) 1 h and B) 24 h. Each pesticide was used at a maximum concentration of 6 µM. Error bars represent standard errors of 4 separate experiments. The combination produced statistically significantly less inhibition of acetylcholinesterase activity than would be expected from an additive effect, after 1 h of exposure ($p < 0.05$). No significant increase was seen after 24 h.*

The difference between the observed and expected rate of acetylcholinesterase activity was found to be statistically significant ($p<0.05$) after 1 h exposure to a mixture of phosmet and pirimiphos methyl. The rate of acetylcholinesterase activity as a result of exposure to the combination of the two pesticides was actually *greater* than would be expected purely from an additive effect on activity, i.e. the substances interacted in an inhibitory, not a synergistic manner. No significant difference was seen between the observed and expected rate of acetylcholinesterase activity after 24 h exposure to a mixture of phosmet and pirimiphos methyl.

Combination of diazinon and glyphosate

Changes in the rate of acetylcholinesterase activity produced by exposure to a combination of diazinon and glyphosate for 1 h and 24 h are shown in Figure 6.2.

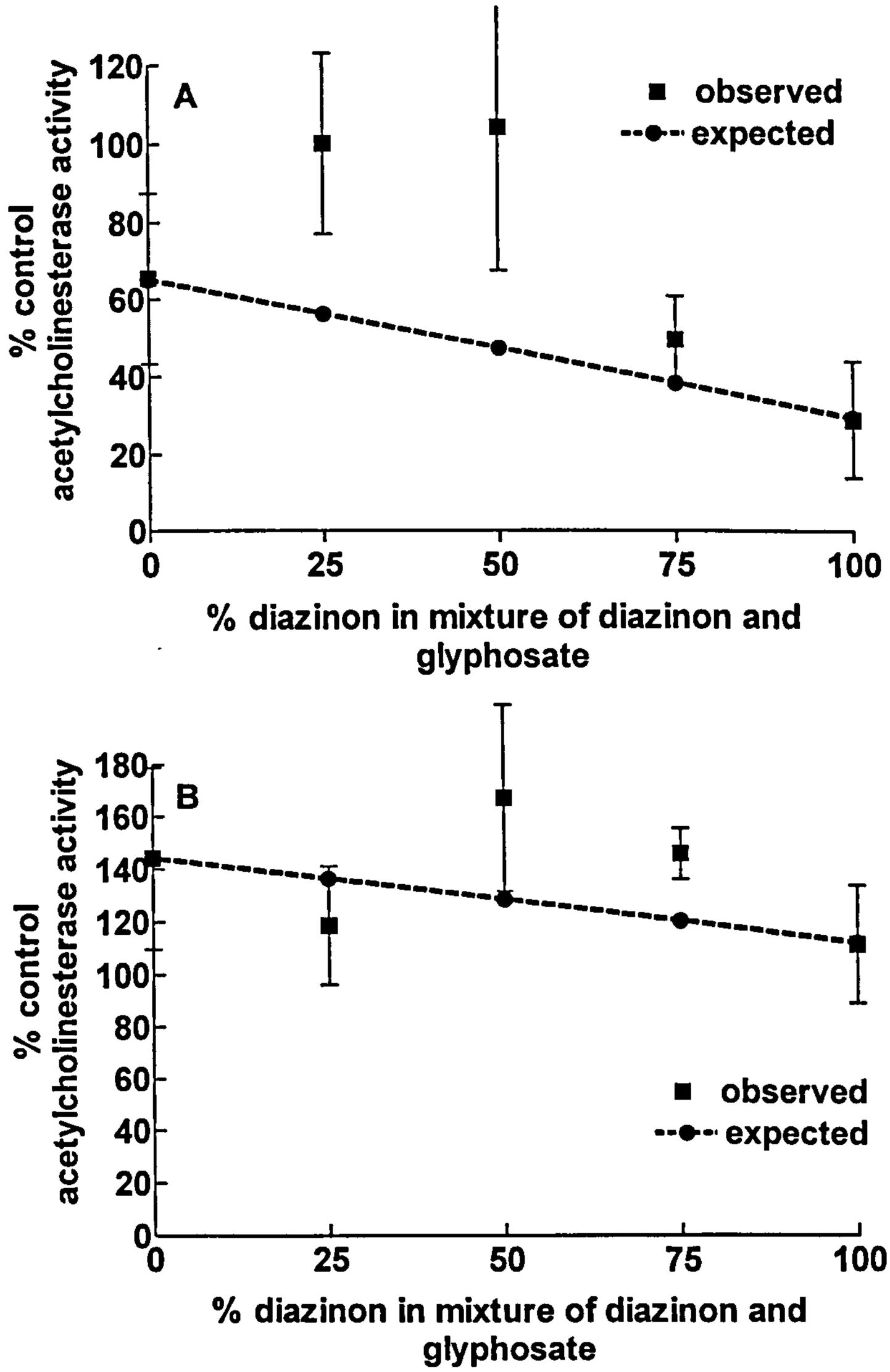


Figure 6.2: *Relationship between the relative concentrations and acetylcholinesterase activity observed in NB2a neuroblastoma cells after exposure to a mixture of diazinon and glyphosate for A) 1 h and B) 24 h. Diazinon was used at a maximum concentration of 10 µM and glyphosate at a maximum concentration of 500 nM. Error bars represent standard errors of 4-5 separate experiments. The reduction in inhibition of enzyme activity after 1 h or 24 h exposure was not statistically significant.*

No significant difference was seen between the observed and expected rate of acetylcholinesterase activity after 1 h exposure to a mixture of glyphosate and diazinon, despite the appearance of the curve. The results were biased by large changes in some of the individual experiments.

The difference between the observed and expected rate of acetylcholinesterase activity after 24 h exposure to a mixture of diazinon and glyphosate was found to be almost statistically significant (p=0.07).

Combination of malathion and carbaryl

Changes in the rate of acetylcholinesterase activity produced by exposure to a combination of malathion and carbaryl for 1 h and 24 h are shown in Figure 6.3.

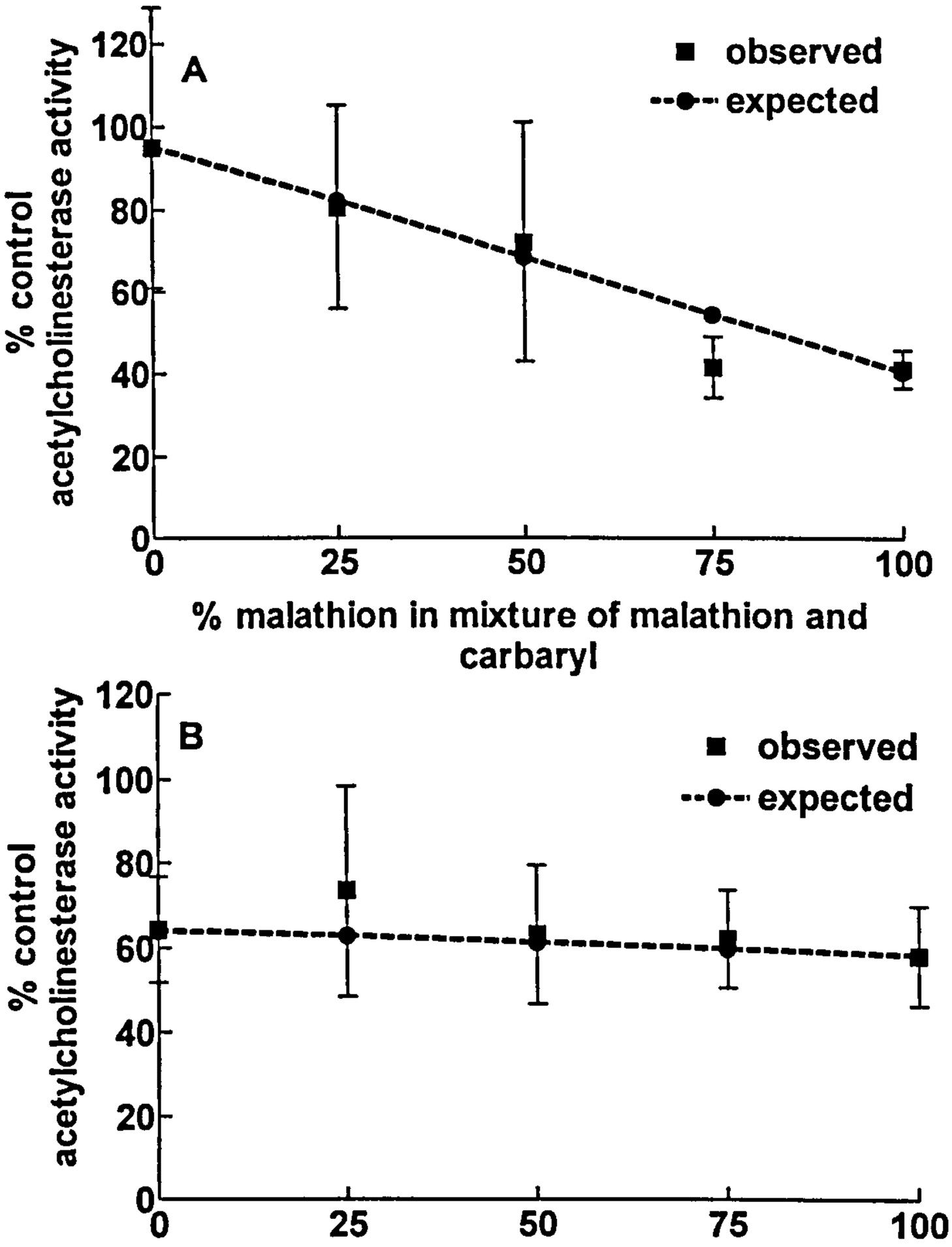


Figure 6.3: *Relationship between the relative concentrations and acetylcholinesterase activity observed in NB2a neuroblastoma cells after exposure to a mixture of malathion and carbaryl for A) 1 h and B) 24 h. Malathion was used at a maximum concentration of 10 µM and carbaryl at a maximum concentration of 1 µM Error bars represent standard errors of 4 separate experiments. No statistically significant increase in enzyme activity was observed after 1 h or 24 h.*

No significant difference was seen between the observed and expected rate of acetylcholinesterase activity after 1 h or 24 h exposure to a mixture of malathion and carbaryl.

Combination of diazinon and chlorpyrifos

Changes in the rate of acetylcholinesterase activity produced by exposure to a combination of diazinon and chlorpyrifos for 1 h and 24 h are shown in Figure 6.4.

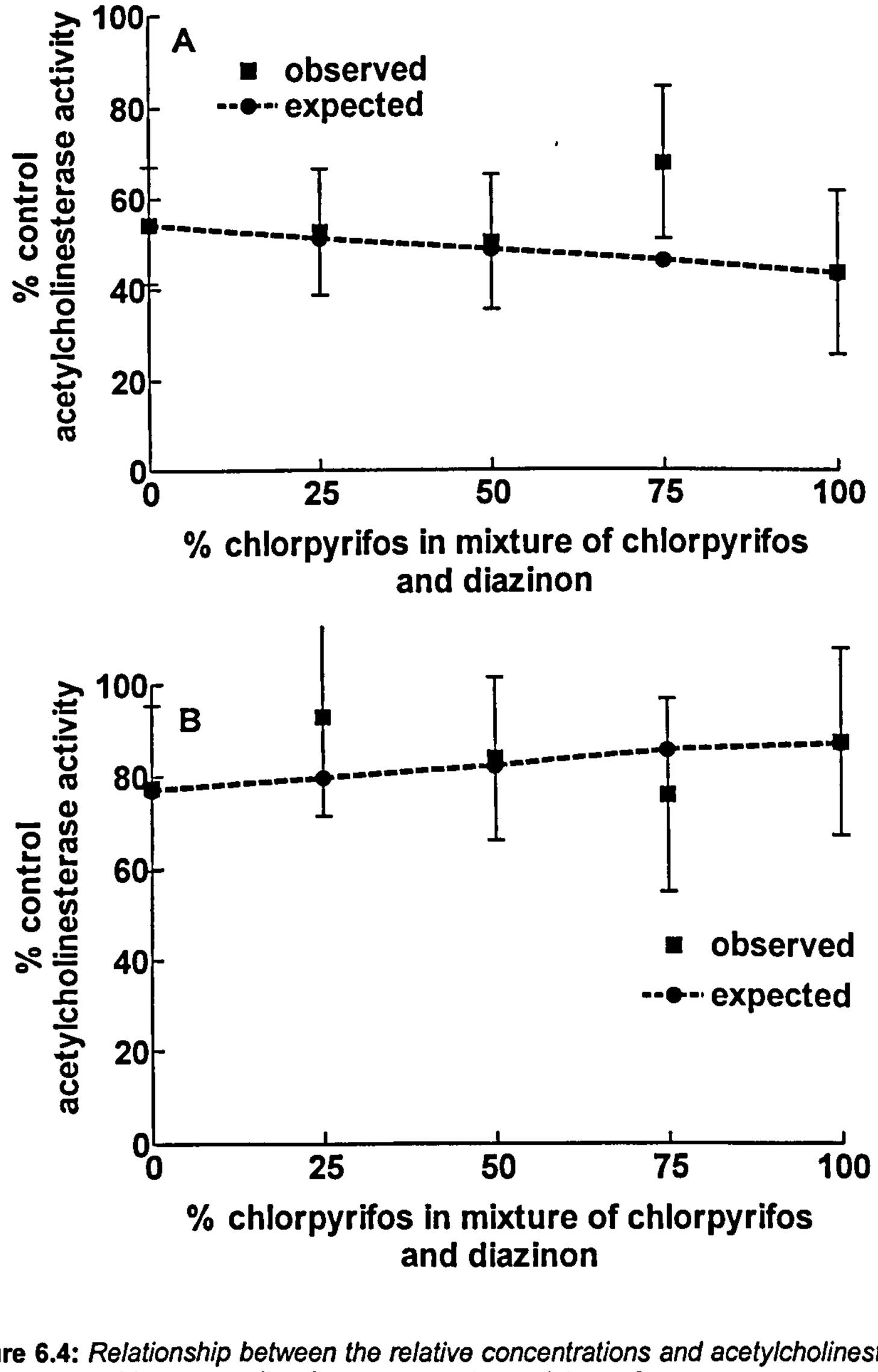


Figure 6.4: *Relationship between the relative concentrations and acetylcholinesterase activity observed in NB2a neuroblastoma cells after exposure to a mixture of chlorpyrifos and diazinon for A) 1 h and B) 24 h. Chlorpyrifos was used at a maximum concentration of 1 μM and diazinon at a maximum concentration of 10 μM Error bars represent standard errors of 5-6 separate experiments. No statistically significant increase in enzyme activity was observed after 1 h or 24 h.*

No significant difference was seen between the observed and expected rate of acetylcholinesterase activity after 1 h or 24 h exposure to a mixture of chlorpyrifos and diazinon.

In summary, the ability to inhibit the rate of acetylcholinesterase activity was significantly reduced 1 h after exposure to the synergistic mixture of phosmet and pirimiphos methyl compared to that predicted from an additive effect of the two substances, but no such difference was observed 24 h after exposure to this mixture. A similar reduction in ability to inhibit enzyme activity was also seen 24 h after exposure to a mixture of diazinon and glyphosate, and this was almost statistically significant. No other differences were observed between the expected and observed rates of enzyme activity in the remaining synergistic combinations or the non-synergistic combination of chlorpyrifos and diazinon.

Summary of effects on acetylcholinesterase inhibition by pesticides

NB2a neuroblastoma cells

Mean acetylcholinesterase activity rates in NB2a neuroblastoma cells after 1 h and 24 h exposure to each of the individual pesticides used here are summarized in Figure 6.5. The concentrations are the maximum at which synergy was investigated.

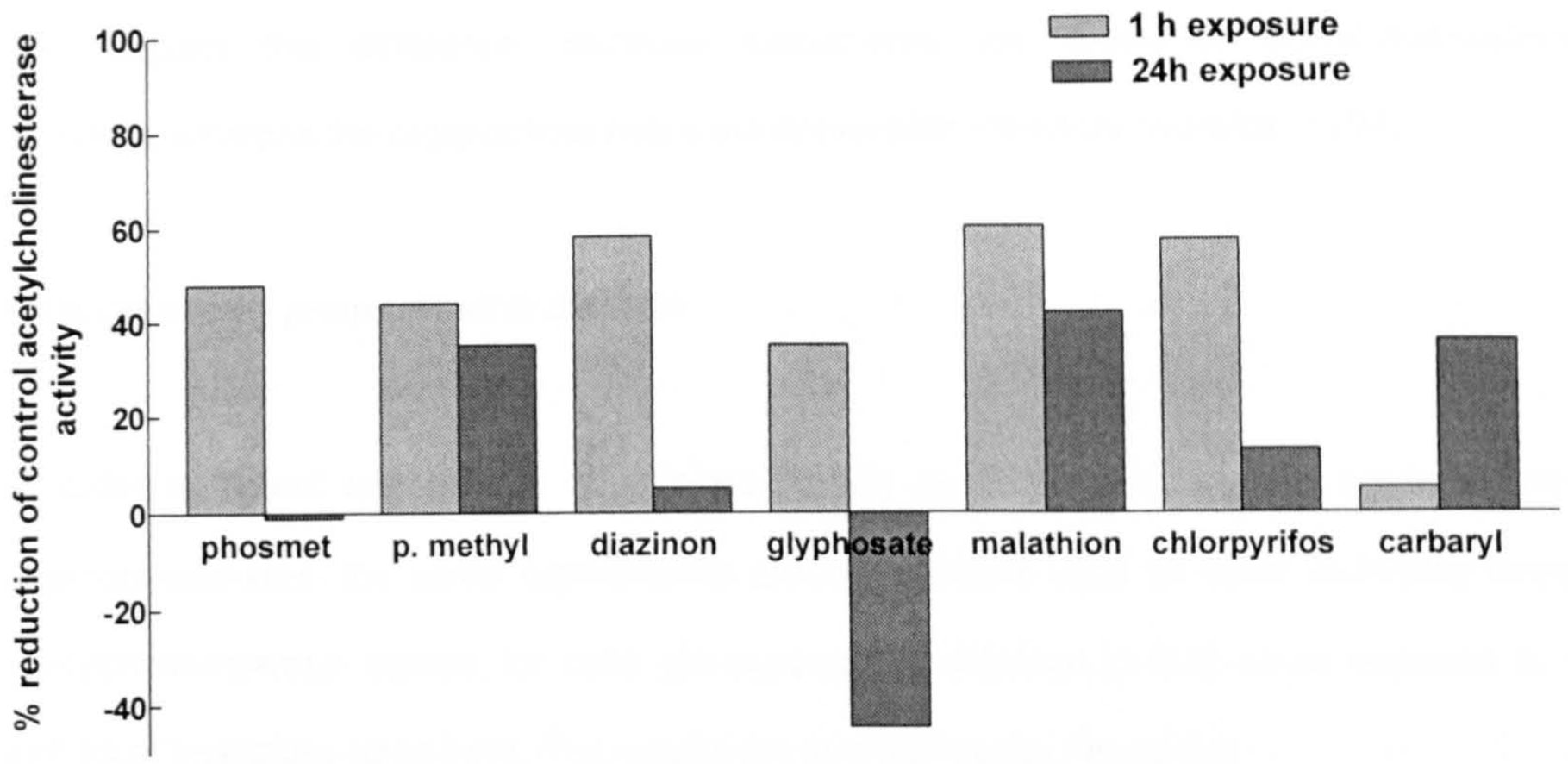


Figure 6.5: *Summary of the mean changes in rate of acetylcholinesterase activity after exposure of NB2a neuroblastoma cells for 1 h and 24 h to various pesticides. Phosmet and pirimiphos methyl were assessed at 6 µM, diazinon and malathion at 10 µM, chlorpyrifos and carbaryl at 1 µM and glyphosate at 500 µM. Results are the means of a minimum of 4 separate experiments.*

A reduction in rate of acetylcholinesterase activity compared to untreated controls was observed in NB2a neuroblastoma cells 1 h after exposure to all the organophosphate pesticides tested here, including glyphosate. In most cases the rates of activity fell to approximately 50% of the control values, despite the concentrations being chosen to have minimal effect on neurotoxicity. The enzyme activity after exposure to phosmet, diazinon and chlorpyrifos had almost or fully returned to the rate of untreated control cells after 24 h.

The rate of acetylcholinesterase activity was considerably lower than control activity 1 h after exposure to glyphosate although this depression in rate of activity was replaced by an apparent increase in activity after 24 h, compared to untreated controls. Until recently glyphosate was not considered to be an acetylcholinesterase inhibitor (Marrs, 1993). These results however demonstrate considerable inhibition 1 h after exposure, and this is confirmed by recent studies with serum proteins, also demonstrating considerable acetylcholinesterase inhibition with glyphosate (El-Demerdash *et al.*, 2001) *in vitro*.

In contrast with the organophosphates, the enzyme activity 1 h after exposure to the carbamate carbaryl was comparable to the controls, although a reduction in activity was seen

after 24 h exposure. Differences in metabolism of carbaryl compared to organophosphates may explain this difference, because *carbamates* are reversible acetylcholinesterase inhibitors, whereas the organophosphates are irreversible inhibitors (Sultatos, 1994).

Cells chronically pre-exposed to diazinon

In order to detect any change in enzyme activity as a result of chronic pre-exposure to organophosphates, the same experimental techniques were used to obtain individual rates of acetylcholinesterase activity for cells pre-exposed to diazinon (2.2.2) when exposed to the individual pesticides used here. The results are summarised in Figure 6.6.

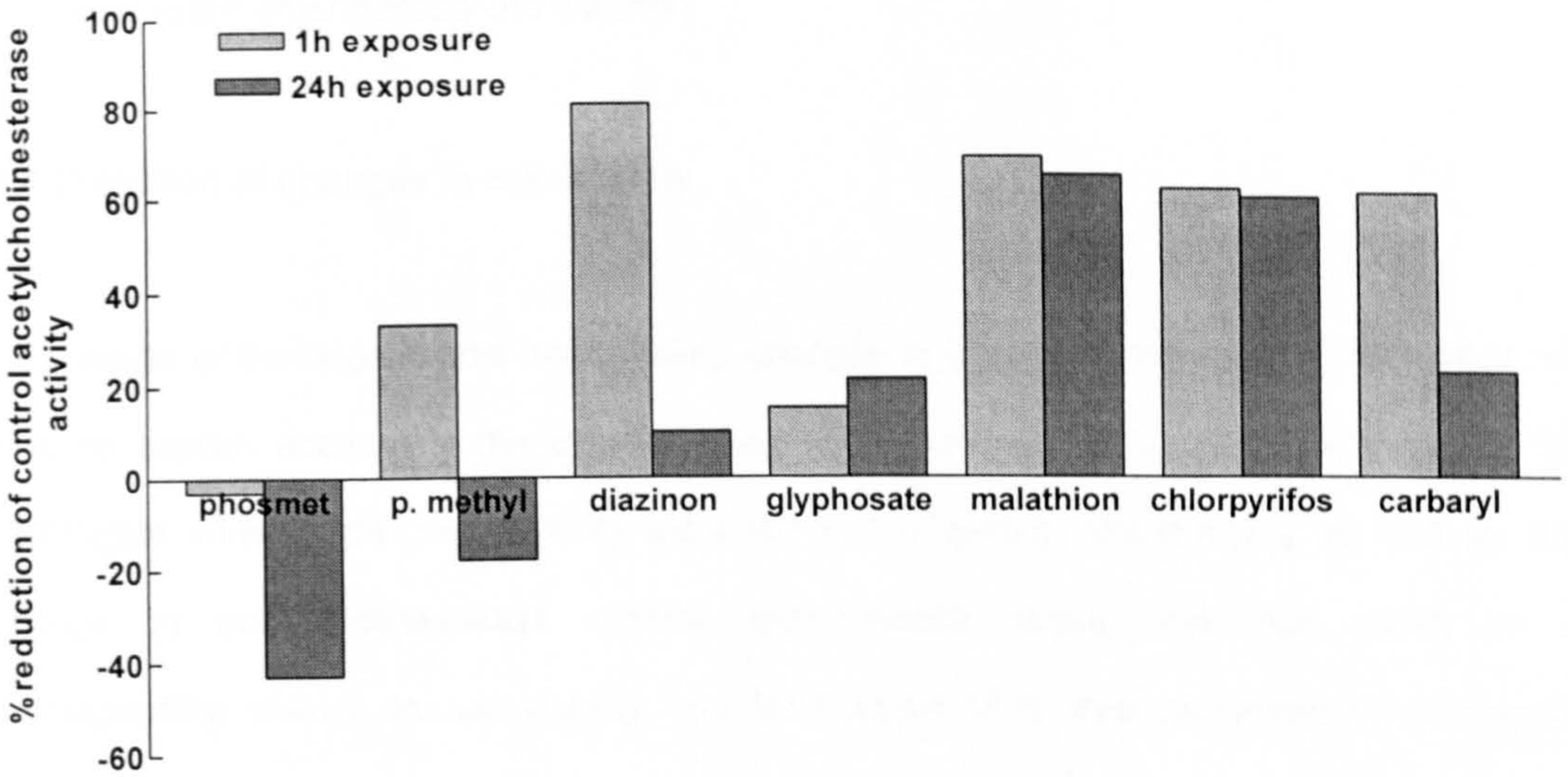


Figure 6.6*: Summary of the mean changes in rate of acetylcholinesterase activity after exposure of NB2a neuroblastoma cells chronically pre-exposed to diazinon, for 1 h and 24 h to various pesticides. Phosmet and pirimiphos methyl were assessed at 6 µM, diazinon and malathion at 10 µM, chlorpyrifos and carbaryl at 1 µM and glyphosate at 500 µM. Results are the means of a minimum of 4 separate experiments.*

Exposure to pesticides of cells chronically pre-exposed to diazinon resulted in apparent differences in their effects on acetylcholinesterase activity compared to their effects in normal cells (Figure 6.5). As in normal cells, a reduction in the rate of acetylcholinesterase activity was seen 1 h after exposure to all the pesticides, with the exception of phosmet where no inhibitory effect was observed. With malathion and chlorpyrifos similar reductions were maintained after 24 h suggesting that not only was the acetylcholinesterase inhibition irreversible (Sultatos, 1994) but that little *de novo* production of enzyme had occurred. An

increase in rate of activity was seen with phosmet and pirimiphos methyl, suggesting an increased expression of the enzyme.

Comparison of the mean untreated control rates of acetylcholinesterase activity from all the experiments in normal cells and cells pre-exposed to diazinon revealed that even in the absence of acute exposure to pesticides, the rate of enzyme activity was statistically significantly reduced (Student's t-test) in pre-exposed cells ($p<0.00002$) compared to NB2a cells that had not been chronically pre-exposed to diazinon. The difference in acetylcholinesterase activity represented a 2.6 times greater rate in the normal cells than in pre-exposed cells. This presumably reflected the inhibition of activity resulting from constant exposure to the organophosphate diazinon.

6.4 Detection of changes in cell viability

The results of the experiments investigating changes in acetylcholinesterase activity were not able to explain adequately the synergy observed in normal NB2a cells in Chapter 4. To investigate whether the neurotoxicity exhibited in the synergy experiments as well as the change in acetylcholinesterase activity demonstrated above was due purely to a corresponding change in cell viability, the MTT assay (2.7) was performed on the same combinations of pesticides at the same concentrations used in the above acetylcholinesterase activity assays.

The MTT assay determines the metabolism by the cells of the product MTT and is therefore a representation of the number of viable cells in a culture. Reduction in cell number, either by necrosis or apoptosis would be detected as a reduction in metabolism. The induction of neurite outgrowth could be affected by a reduction in cell number, either by a direct toxic effect on the cells or by the change in density of the cells affecting the neurite length of the remaining cells in the culture.

Combination of phosmet and pirimiphos methyl

The mean results of MTT assays to demonstrate the viability of cells exposed to a mixture of phosmet and pirimiphos methyl are shown in Figure 6.7.

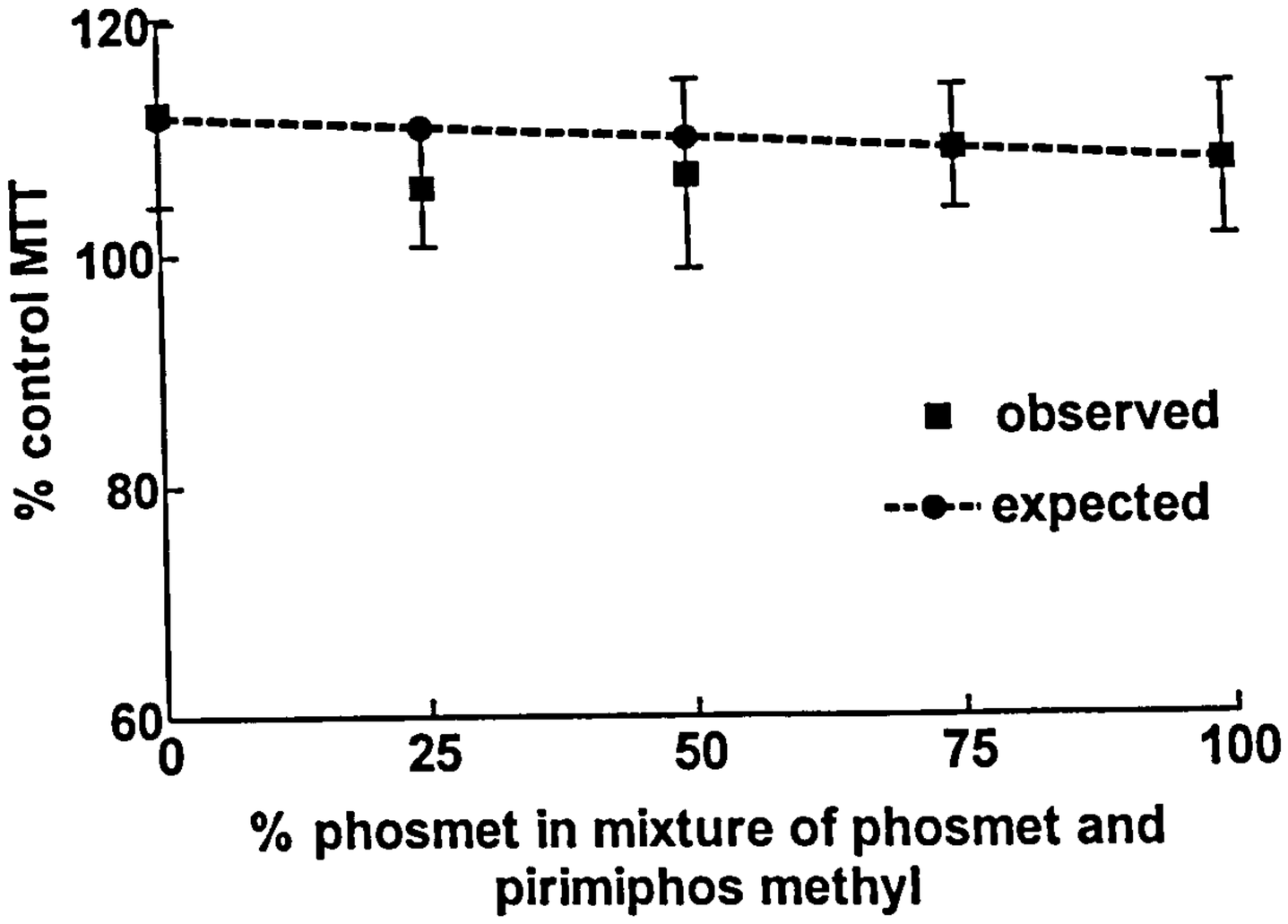


Figure 6.7: *Relationship between the relative concentrations and MTT metabolism observed in NB2a neuroblastoma cells after exposure to a mixture of phosmet and pirimiphos methyl for 24 h. Phosmet was used at a maximum concentration of 6 μM and pirimiphos methyl at a maximum concentration of 6 μM. Error bars represent the standard errors of 6 separate experiments.*

No significant difference was seen between the observed and expected viability of cells exposed to a combination of phosmet and pirimiphos methyl for 24 h.

Combination of diazinon and glyphosate

The mean results of MTT assays to demonstrate the viability of cells exposed to a mixture of diazinon and glyphosate are shown in Figure 6.8.

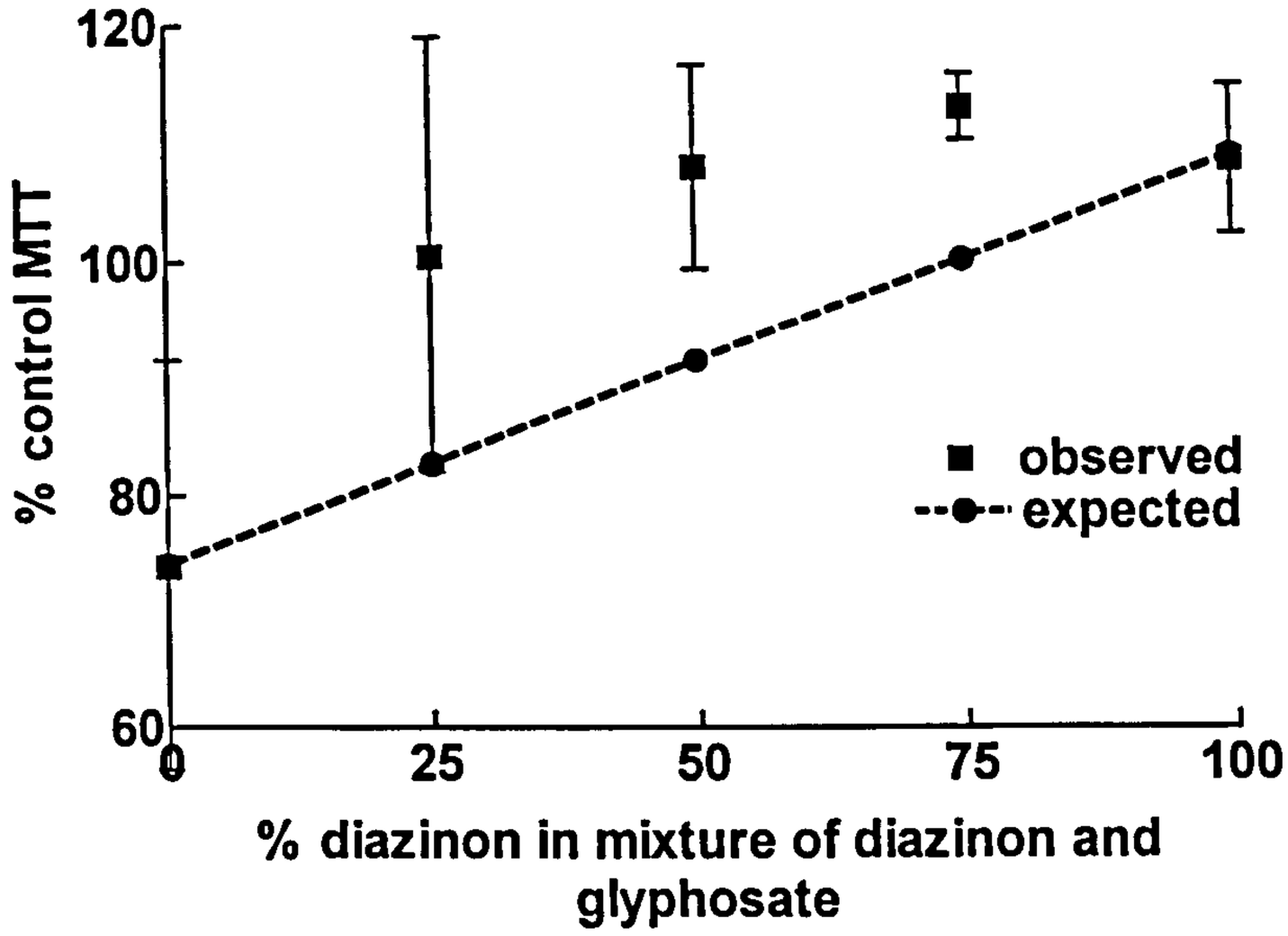


Figure 6.8: *Relationship between the relative concentrations and MTT metabolism observed in NB2a neuroblastoma cells after exposure to a mixture of diazinon and glyphosate for 24 h. Diazinon was used at a maximum concentration of 10 µM glyphosate at a maximum concentration of 500 µM. Error bars represent the standard errors of 4 separate experiments. A statistically significant difference was observed (p=0.022).*

The difference between the observed and expected cell viability was found to be statistically significant (p=0.022) after 24 h exposure to a mixture of diazinon and glyphosate. Significantly less inhibition of viability was observed in cells exposed to a mixture of diazinon and glyphosate compared to that expected from a purely additive effect on cell viability.

Combination of malathion and carbaryl

The mean results of MTT assays to demonstrate the viability of cells exposed to a mixture of malathion and carbaryl are shown in Figure 6.9.

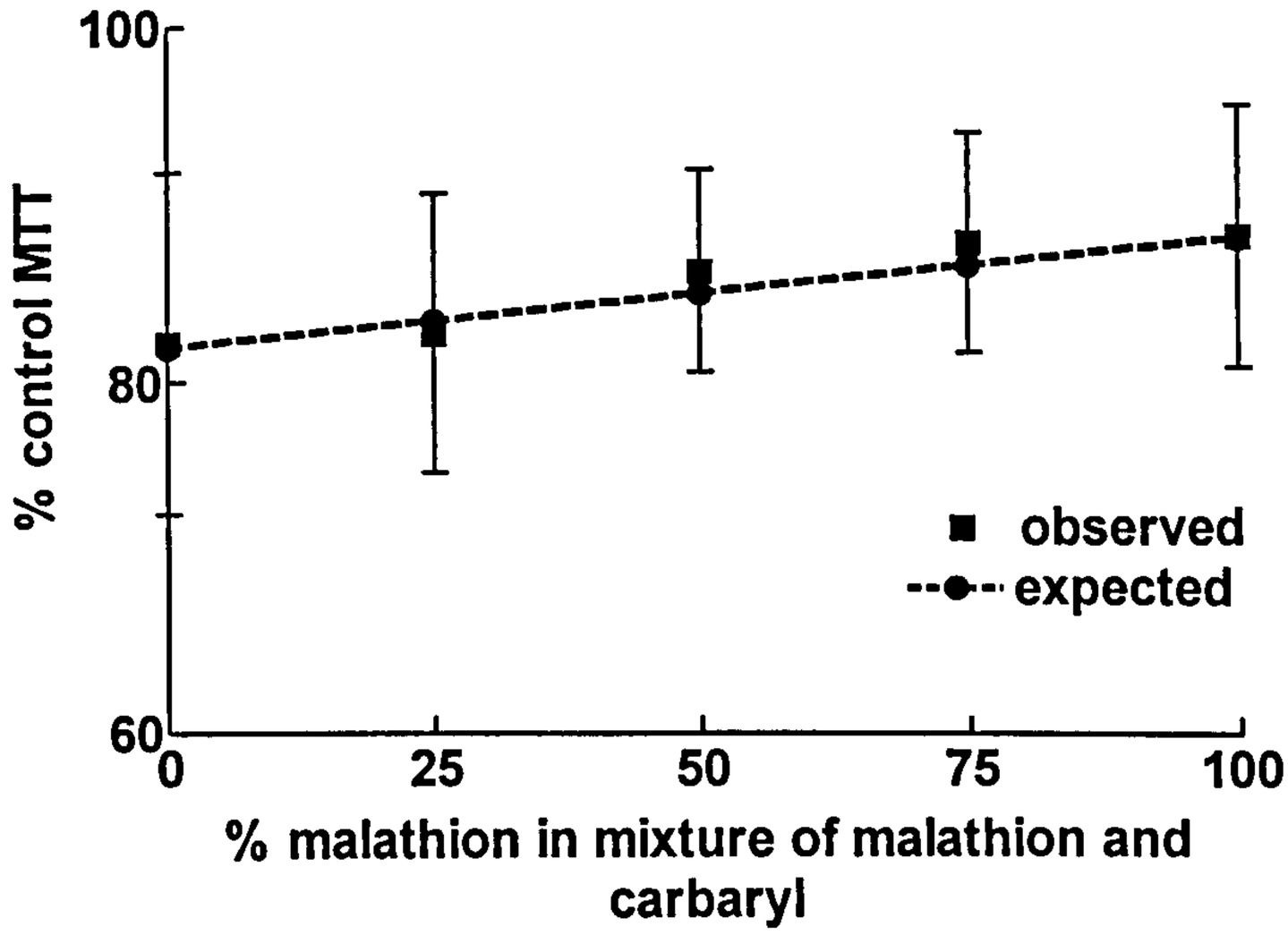


Figure 6.9: *Relationship between the relative concentrations and MTT metabolism observed in NB2a neuroblastoma cells after exposure to a mixture of malathion and carbaryl for 24 h. Malathion was used at a maximum concentration of 10 µM and carbaryl at a maximum concentration of 1 µM. Error bars represent the standard errors of 4 separate experiments.*

No significant difference was seen between the observed and expected viability of cells exposed to a combination of malathion and carbaryl for 24 h.

Combination of chlorpyrifos and diazinon

The mean results of MTT assays to demonstrate the viability of cells exposed to the non-synergistic mixture of chlorpyrifos and diazinon are shown in Figure 6.10.

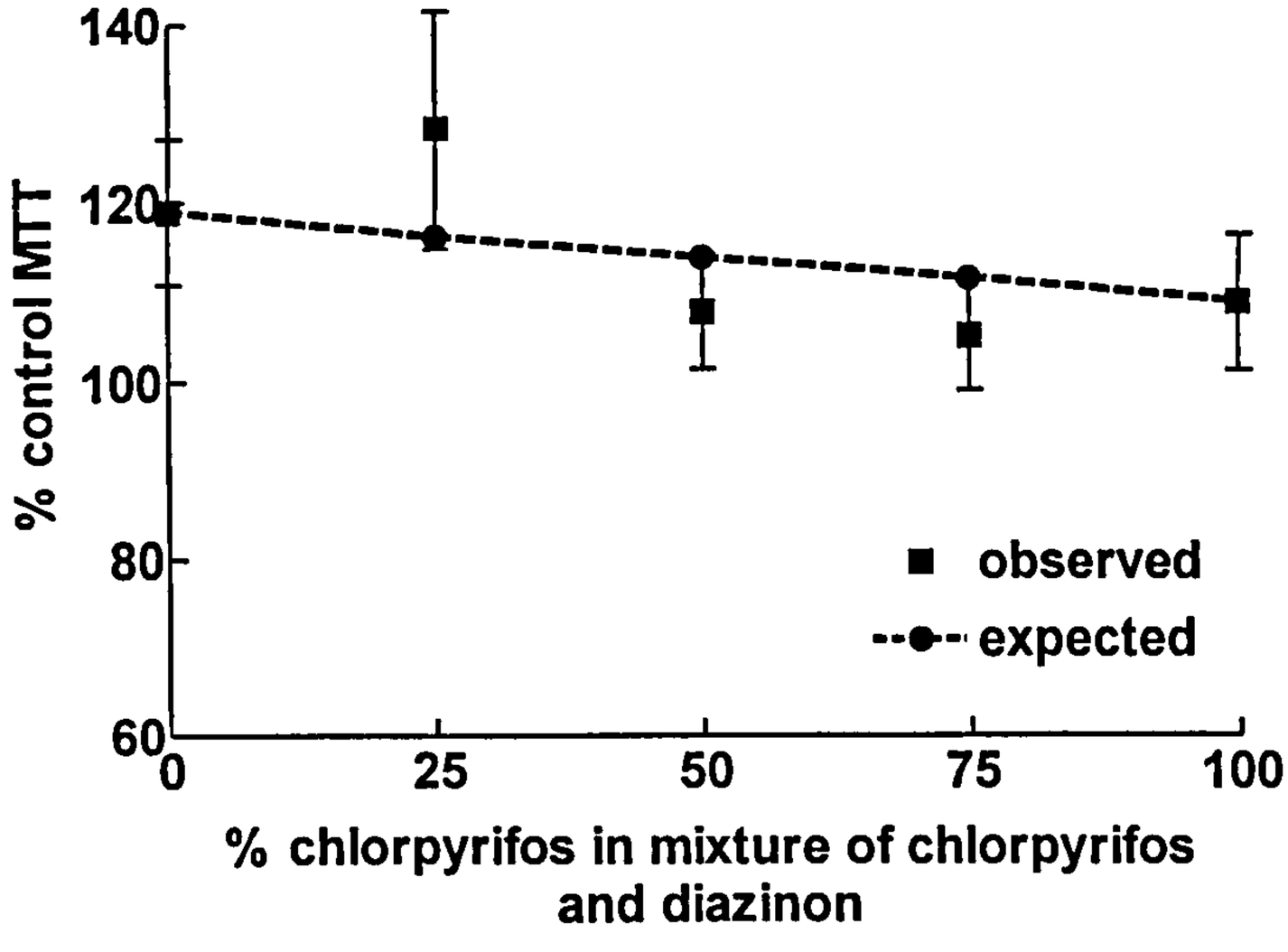


Figure 6.10: *Relationship between the relative concentrations and MTT metabolism observed in NB2a neuroblastoma cells after exposure to a mixture of diazinon and chlorpyrifos for 24 h. Diazinon was used at a maximum concentration of 10 μM and chlorpyrifos at a maximum concentration of 1 μM. Error bars represent the standard errors of 4 separate experiments.*

No significant difference was seen between the observed and expected viability of cells exposed to a combination of chlorpyrifos and diazinon for 24 h.

Summary of effects on cell viability by pesticides

The MTT values for each individual pesticide at the maximum concentration used, are summarized in Figure 6.11. These are compared to values in cells chronically pre-exposed to diazinon, exposed to the same concentrations of the pesticides, in order to detect any differences in response after chronic pre-exposure to organophosphates.

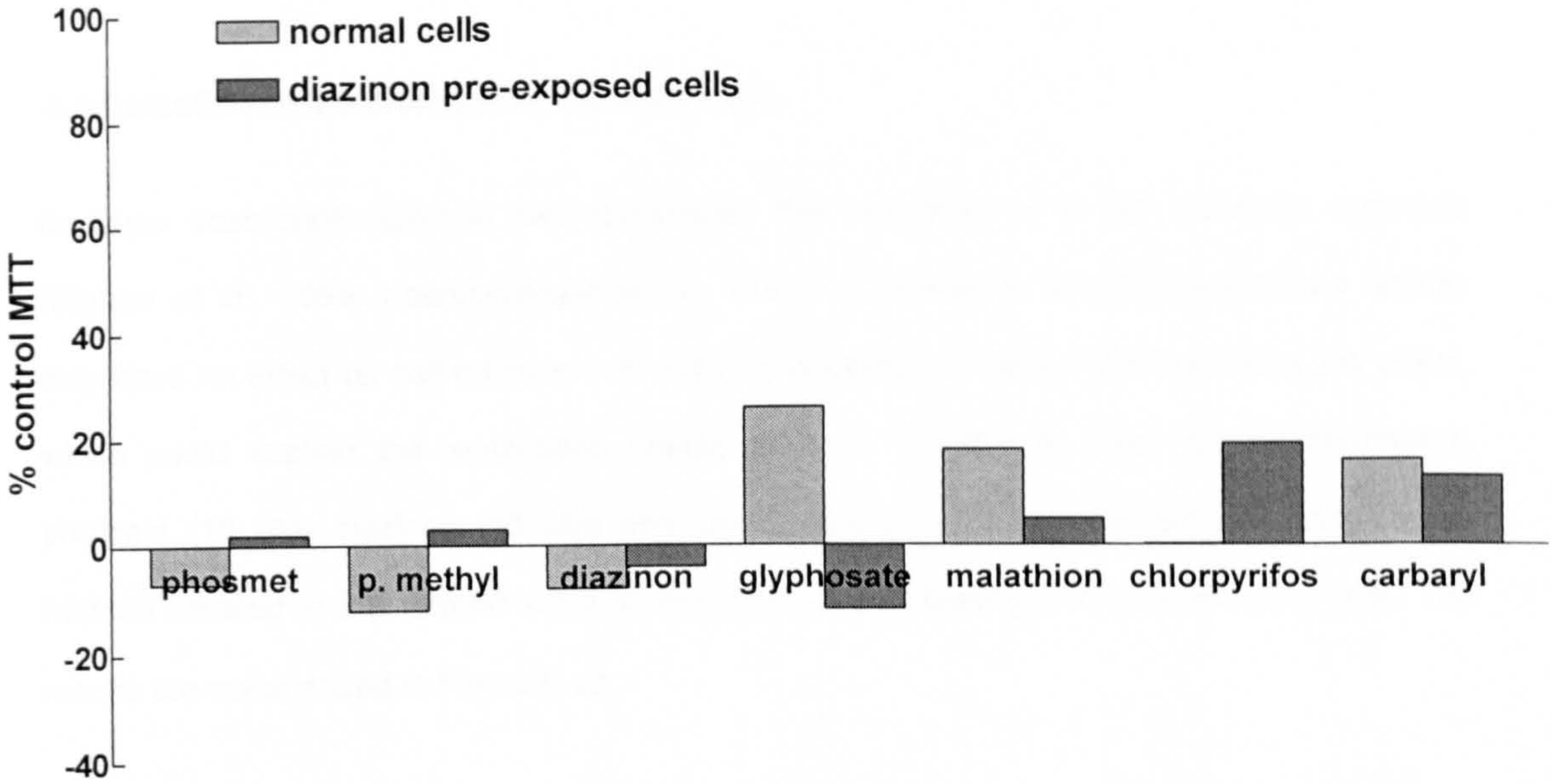


Figure 6.11: *Summary of results of the mean changes of MTT metabolism after treatment with pesticides at low concentrations for 24 h in normal NB2a neuroblastoma cells and in those chronically pre-exposed to diazinon. Phosmet and pirimiphos methyl were assessed at 6 µM, diazinon and malathion at 10 µM, chlorpyrifos and carbaryl at 1µM and glyphosate at 500 µM. Results are the means of a minimum of 4 separate experiments.*

Although individual differences were observed in the MTT metabolism of NB2a cells exposed to the pesticides compared to controls, they were not found to be statistically significant. Some differences in MTT metabolism were also observed between normal cells and those chronically pre-exposed to diazinon, although none of the differences was statistically significant at the 95% confidence limits. Effects on cell viability were not considered to be responsible for all the synergistic effects on neurotoxicity observed in Chapter 4 and 5, although may be involved in some individual cases.

6.5 Detection of the changes in cell adhesion.

Because acetylcholinesterase also possesses the properties of a cell adhesion molecule (Bigbee *et al.*, 1999; Koenigsberger *et al.*, 1997), a change in acetylcholinesterase activity may have an effect on cell adhesion. In order to investigate whether this may have any effect, which could explain the synergistic effects seen in Chapter 4, three organophosphates, phosmet (10 μM) diazinon (10 μM) and chlorpyrifos (1 μM) were tested according to the method detailed in 2.8. Effects were studied both in proliferating and differentiating cells. The results are summarized in Figure 6.12.

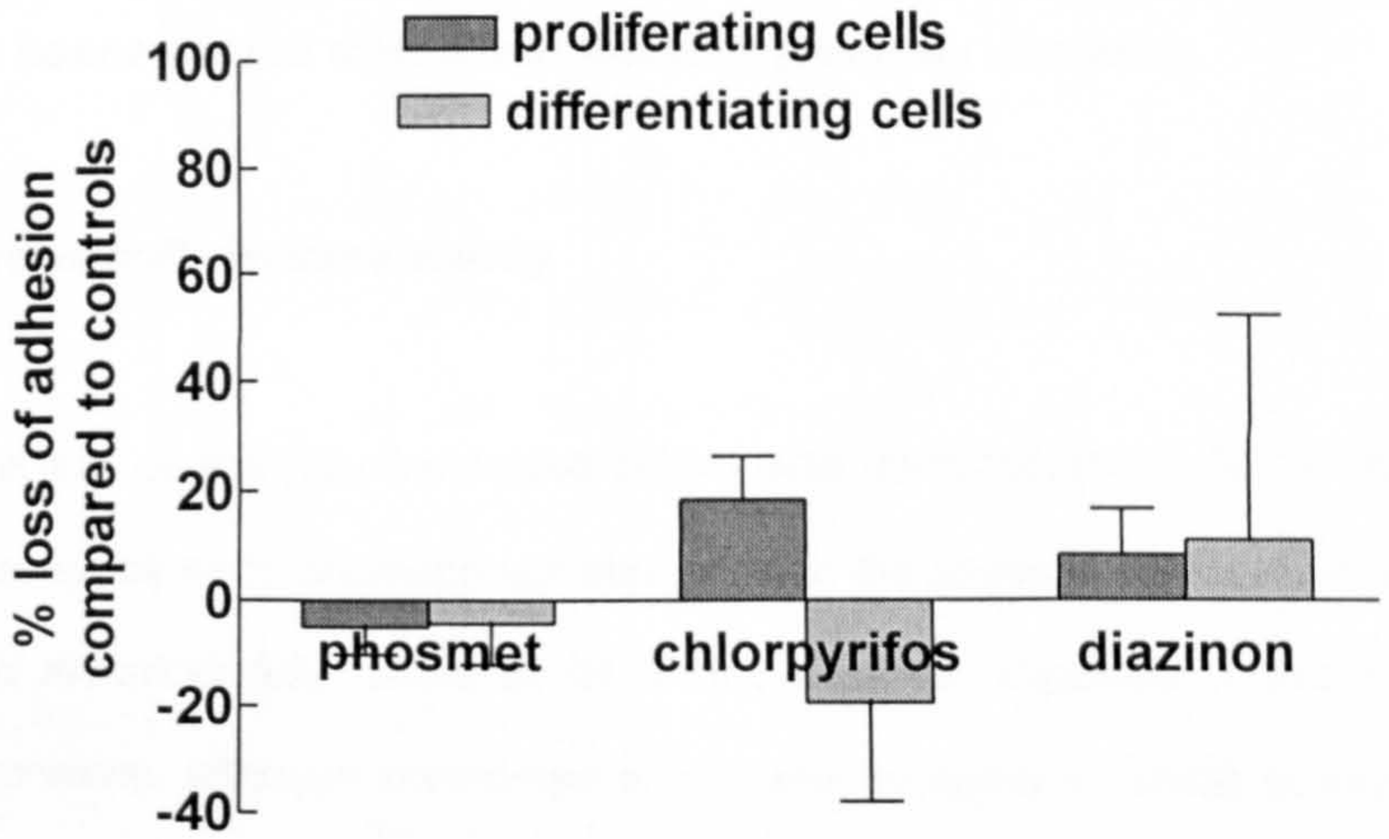


Figure 6.12: *Changes in the adhesion of NB2a neuroblastoma cells differentiating or proliferating for 24 h in the presence of phosmet (10 μM), chlorpyrifos (1 μM) and diazinon (10 μM). Error bars represent the standard errors of 4 separate experiments.*

None of the treatments showed any significant difference in adhesion compared to controls in proliferating or differentiating cells. Because of the difficulties involved in this method and the wide variation in results, it was not used to detect differences with combinations of products, and no further attempt was made to determine if adhesion played a role in the synergistic effects on neurite outgrowth observed in the studies previously described.

6.6 Discussion

The hypothesis that the synergistic effect of combinations of pesticides is related to their synergistic action as inhibitors of acetylcholinesterase and/or synergistic effects on cell viability was not adequately supported by the results of this study.

Changes in acetylcholinesterase activity or cell viability were not able to explain the synergism of effect on inhibition of neurite outgrowth observed with some pesticide combinations. The variations produced by effects on cell adhesion meant it was not possible to demonstrate whether changes in adhesion played a role in the synergy observed but it was also considered unlikely. The experiments investigating these potential areas of involvement did however reveal other effects that were previously unreported.

Acetylcholinesterase activity

Inhibition of acetylcholinesterase activity was demonstrated in NB2a neuroblastoma cells 1 h after exposure to organophosphates, even in the absence of metabolic activation. This activity had almost or fully recovered 24 h after the initial exposure. It was interesting to note that glyphosate, although considered to possess no ability to inhibit acetylcholinesterase (Marrs, 1993), inhibited enzyme activity after 1 h of exposure. More recent investigations have also reported this effect on acetylcholinesterase in serum by glyphosate (El-Demerdash *et al.*, 2001).

The reduction in activity contrasted with findings of Barber (Barber *et al.*, 1999b) who found no acetylcholinesterase inhibition with chlorpyrifos in the absence of activation in human SY5Y neuroblastoma cells. However others have found similar levels of inhibition to those obtained here in SY5Y cells with unactivated protoxicants such as diazinon and pirimiphos methyl (Veronesi and Ehrich, 1993). Differences in techniques are assumed to explain the lack of inhibition observed, not least the fact that much higher concentrations (10^{-4}M) were used (Barber *et al.*, 1999b) which were toxic to NB2a cells in these studies.

The concentrations of all the pesticides used were chosen to produce minimal toxic effect on neurite outgrowth. Despite this, the esterase activity after 1 h exposure fell to approximately half of the control activity and confirmed investigations by other laboratories (Koenigsberger *et al.*, 1997; Layer *et al.*, 1993) demonstrating the absence of any relationship between acetylcholinesterase inhibition and the inhibition of neurite outgrowth. The mean activity in normal control cells was found to be 3.67×10^{-11} moles substrate/l hydrolysed per cell/min; slightly higher than the equivalent reported rate in human SY5Y neuroblastoma cells which has been calculated to be 4×10^{-12} moles substrate/l hydrolysed per cell/min (Rowles *et al.*, 1995), and reflecting the observation that NB2a cells have 10 times more acetylcholinesterase than human neuroblastoma *cells* (Stieger *et al.*, 1989). The activity in brain is higher than other tissues such as muscle, where 10 fold less activity is detected (Ellman *et al.*, 1961). In the cells pre-exposed to diazinon the mean rate of activity fell to 1.03×10^{-11} moles substrate/l hydrolysed per cell/min. It was not possible to say whether this reduction in enzyme activity reflected inhibition by the chronic exposure to diazinon or a down-regulation in enzyme expression in response to a reduction in muscarinic receptors (Sivam *et al.*, 1983; Costa *et al.*, 1982b) or nicotinic receptors (Lim *et al.*, 1987).

The recovery of esterase activity in normal cells within 24 h suggested one of three possible mechanisms. The most likely explanation was that *de novo* synthesis of enzyme had occurred, and that the increase was in excess of the residual organophosphate, since the *de novo* enzyme synthesized was not itself inhibited. The recovery by *de novo* synthesis has previously been reported (Rieger *et al.*, 1976). Alternatively, although as described in previous chapters, the extent of metabolism by P450 enzymes in NB2a neuroblastoma cells is not considered to be significant, it is possible that these cells metabolised the organophosphates to a non-toxic metabolite by a metabolic pathway. However, even if the organophosphates were metabolized, it is well known that they are irreversible acetylcholinesterase inhibitors (Sultatos, 1994), so a recovery of activity in that 24 h period is unlikely.

Finally it is also possible that the apparent recovery in acetylcholinesterase activity after 24 h actually reflected a reduction in the number of cells differentiating after 1 h exposure compared to controls. This could be the result of an initial increase in cell proliferation at the expense of differentiation. Acetylcholinesterase is up regulated in differentiating cells (Rieger *et al.*, 1976) including NB2a neuroblastoma cells. In our preliminary studies acetylcholinesterase activity was undetectable in proliferating cells using these methods. Low concentrations of individual pesticides however, have been shown by other laboratories, to induce cell proliferation (Sharma and Obersteiner, 1981; Obersteimer and Sharma, 1978; Watanabe and Sharma, 1975), and indeed many of the organophosphates tested produced a small increase in the cell viability within 24 h (Figure 6.11), suggesting a delay in differentiation at the expense of proliferation. In situations where proliferation occurred at the expense of differentiation, the detectable rate of enzyme activity would therefore be less than expected.

Although the effects observed are interesting insofar as they reveal possible effects of exposure to organophosphates that have so far been uninvestigated in relation to mechanisms of pesticide toxicity, the observations are not able to explain the increased synergistic neurotoxicity of all combinations of the pesticides observed in Chapter 4. No significant effects on acetylcholinesterase were observed with the combination of malathion and carbaryl, despite this combination producing a significant inhibition of neurite outgrowth. It is unlikely that the synergy resulted from an effect on inhibition of acetylcholinesterase in the cells; this is consistent with other studies showing no correlation between cytotoxic effects and acetylcholinesterase inhibition (Sharma and Obersteiner, 1981; Marinovich *et al.*, 1996).

The depressed rates of acetylcholinesterase activity in cells chronically exposed to diazinon may suggest at least a partial explanation for the ill health claimed by some individuals. Further exposure to pesticides resulted in further depression of enzyme activity and even after 24h little recovery was apparent with many of the test pesticides, as was generally seen in normal cells. However it is not possible to discount a promotion of proliferation in these cells reducing the effective concentration of the pesticide in individual cells.

Changes in cell viability

The only significant effects observed with the combinations of synergistic pesticides investigated, resulted from an apparent increase in cell viability resulting from combination of glyphosate with diazinon. If a decrease in cell numbers, for example as a result of cell death, were to explain the increased synergistic neurotoxicity, a decrease in viability would have been expected with combination of pesticides.

A considerable reduction in viability however was observed in cells exposed to 100% (500 μM) glyphosate. This was not seen in cells exposed to any of the other organophosphates, and may result from its property as an amino acid inhibitor (Extoxnet, 2001). The apparent increase in viability with the combination of glyphosate and diazinon may therefore have reflected the reduction in cell viability of cells in the presence of glyphosate alone, the inclusion of diazinon providing a protective role against the cytotoxic effects of glyphosate. The unexpected reduction in acetylcholinesterase activity after exposure to glyphosate discussed above could similarly be explained by this reduction in cell viability.

No other effects on viability were seen that could adequately explain the results from the synergy investigations. This was not unexpected since Rowles *et al.* (1995) demonstrated the lack of correlation between cell viability and various endpoints such as esterase inhibition or markers of membrane integrity. These were affected at concentrations of organophosphate exposure 100-1000 times lower than those affecting cell viability, and which were similar (10^{-4} – 10^{-6}M) to those used in our studies.

Other possible explanations of synergy

The acute toxic actions of organophosphates *in vivo* mainly derive from the accumulation of acetylcholine resulting from the inhibition of acetylcholinesterase. Acetylcholine interacts with muscarinic receptors resulting in various effects on second messengers such as increased metabolism of cyclic AMP (Liu *et al.*, 1986) or GMP (Lundy and Magor, 1978). This may alter

GABA or dopamine turnover (Sivam *et al.*, 1983) or produce changes in the metabolism of phosphoinositides (Costa, 1990), which themselves play a role in the control of proliferation (Vicentini and Villereal, 1986; Ashkenazi *et al.*, 1989). These effects may in turn lead to abnormal phosphorylation of a variety of substrates culminating in toxic effects. Organophosphates can directly affect second messengers through interaction with muscarinic and other receptors (Volpe *et al.*, 1985; Katz and Marquis, 1989; Katz and Marquis, 1992) and have been shown to have effects on adenylate cyclase (Abou-Donia and Huff, 1995), phosphorylase a (Kauffman *et al.*, 1990), and protein kinase C activity (Pakaski *et al.*, 2000).

Abnormal phosphorylation may play a role in effects observed in this study. Organophosphates activate CaM kinase resulting in hyperphosphorylation of essential cytoskeletal components such as the neurofilament triplet, MAP-2, tubulin (Abou-Donia *et al.*, 1988) or tau (Gupta and Abou-Donia, 1999). Continuous exposure to pesticides may prevent binding of components such as microtubules, thereby disrupting axonal transport. A correlation of increased abnormal perikaryal neurofilament inclusion of cells with low level acetylcholinesterase activity is seen after treatment with the compound BW284c51, consistent with the idea that the regulation of cell adhesion and neurite outgrowth is via the cytoskeleton (Dupree *et al.*, 1995). Increases in trans-membrane potential or production of mitochondrial enzymes (Carlson and Ehrich, 1999) may also influence subsequent toxicity of exposure to a second pesticide. Neurite outgrowth is partly regulated by calcium influx through voltage dependent Ca^{2+} channels (Ferguson and Audesirk, 1990).

Combinations of different effects may explain the synergy observed with various pesticide combinations, but further analyses of these effects were beyond the scope of this thesis.