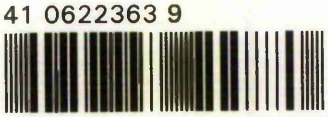


THE TOXICITY OF TRICRESYL PHOSPHATE TOWARDS CULTURED NERVE CELLS AND ISOLATED NERVE

Maxine Joanne Fowler

A thesis submitted in partial fulfillment of the requirements of The Nottingham Trent University for the degree of Doctor of Philosophy

May 2000

In loving memory of Ralph and Mary Jane

DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degree

We certify that the work submitted was carried out by the author. Due acknowledgements has been made of any assistance received.

Signed ..... (Candidate)

Signed ..... (Director of Studies)

"Standing on the shoulders of giants ... ..."

ACKNOWLEDGEMENTS

I would like to thank Alan Hargreaves for all his help during this time and also thanks to John Flaskos and Graham McLean for their words of wisdom.

Big thanks go to the giants, Alan, Cheryl, David, Debbie, Gino, Jean, Kevin, Lucy, Nadine, Nico and Shiva, who have all guided me through the rough times which have made me the scientist I am today.

Special thanks goes to my family, Mum, Dad and Paul who were always supportive, encouraging and listened to me throughout. Finally, I would like to thank Wayne for always believing in me and being the tower of strength I needed, even when it was hard for him.

ABSTRACT

The aim of this work was to study the effects of tricresyl phosphate (TCP; mixed isomers), tri*ortho*cresyl phosphate (T*O*CP), tri*para*cresyl phosphate (T*P*CP) and one of the metabolites of T*O*CP, cyclic phenyl saligenin phosphate (CPSP), on the outgrowth of neurites by differentiating mouse N2a neuroblastoma and rat C6 glioma cells.

At a concentration of 1 μg/ml (2.7 μM) TCP inhibited the outgrowth of axon-like processes in differentiating N2a cells. By contrast, TCP had no effect on the production of extensions by C6 glioma cells induced to differentiate by the addition of sodium butyrate suggesting a selective effect on neuronal cells under these conditions.

Further work showed that 1 μg/ml TCP, T*O*CP (2.7μM) and 1 μg/ml CPSP (3.8 μM) inhibit the outgrowth of axon-like processes by N2a for up to 48 hours, whilst 1 μg/ml T*P*CP (2.7μM) demonstrates a transient inhibition at 24 hours which diminishes after 48 hours. Inhibition of axon outgrowth was also seen with T*O*CP and CPSP after 4 hours exposure whereas no inhibitory effects were seen with TCP or T*P*CP at this time point. It was found that TCP and T*O*CP but not T*P*CP could be microsomally activated into a form that caused greater inhibition of axon outgrowth than seen in the corresponding control situations after 4 hours of exposure.

The Western blot data has showed that inhibited axon outgrowth was associated with cytoskeletal changes, consistently involving a reduction in neurofilament heavy chain (NF-H) phosphorylation and sometimes associated with a reduction in the levels of NF-H, tubulin and GAP-43.

The inhibitory effects of these OPs on differentiating N2a cells were shown to be attenuated in the presence of conditioned medium from rat C6 glioma cells, which are known to produce neurotrophic factors. This protective effect was observed at all time points. Preliminary studies showed that the effects of OPs on pre-differentiated cells mirrored the principal effect seen on the typical differentiating N2a cellular system, with a reduction in the number of pre-formed axons.

Taken together, the findings from this *in vitro* cellular system indicate that T*O*CP and its metabolite, which are both strongly neuropathic *in vivo*, cause selective inhibition of the outgrowth of axon-like processes by differentiating N2a cells, and that this effect is associated with cytoskeletal changes, notably in NFs. These *in vitro* patterns of toxicity agree with those observed *in vivo*, indicating that differentiating N2a cells represent a useful cellular system for studying the neurodegenerative effects of OPs and other similar compounds.

Contents

Page No.

List of Figures

List of Tables

ABBREVIATIONS

2,5HD	2,5-hexanedione
AChE	Acetylcholine esterase
AD	Alzheimer's disease
Al	Aluminum
ALS	Amyotrophic lateral sclerosis
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
C6	Rat glioma cell line
cAMP	Dibutyryl cyclic AMP
CM	Conditioned medium
CPSP	Cyclic phenyl saligenin phosphate
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DDT	Dichlorodiphenyltrichloroethane
DFP	Diisopropylfluorophosphate
DMEM	Dulbecco's Modified Eagles Medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ETCC	European Tissue Culture Collection
FBS	Foetal bovine serum
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HD	Huntington's Disease
IC_{50}	Half maximum inhibitory concentration
IDPN	β,β'-iminodipropionitrile
IF(s)	Intermediate filament(s)
IMS	Intermediate syndrome
kDa	kilo Dalton
M/S	Microsomes
MAPs	Microtubule associated proteins
MF(s)	Microfilament(s)
MPP+	1-methyl-4-phenyl-pyridine
MT(s)	Microtubule(s)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
N2a	Mouse neuroblastoma cell line
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NOAH	National Office of Animal Health
NBT	Nitro blue tetrazolium
NF(s)	Neurofilament(s)
NF-H	Neurofilament heavy chain
NF-L	Neurofilament light chain
NF-M	Neurofilament medium chain
NGF	Nerve growth factor
NTE	Neurotoxic esterase or neuropathy target esterase
OC(s)	Organochlorine(s)
OPIDN	Organophosphate induced delayed neurotoxicity
OP(s)	Organophosphate(s)
PBS	Phosphate buffered saline
PC12	Rat PC12 pheochromocytoma cell line

PD	Parkinson's Disease
PMSF	Phenylmethylsulfonyl fluoride
RMd09	Monoclonal antibody recognising non-phosphorylated NF-H
RPMI	Roswell Park Memorial Institute
SCa	Slow component a
SCb	Slow component b
SC*O*TP	Saligenin cyclic -*O*- tolyl phosphate
SDS	Sodium dodecyl sulphate
Ta51	Monoclonal antibody recognising phosphorylated NF-H
TBS	Tris buffered saline
TCP	Tricresyl phosphate
TCL	Trichlorphon
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TGF-β	Transforming growth factor -β
T*M*CP	Tri*meta*cresyl phosphate
T*O*CP	Tri*ortho*cresyl phosphate
T*P*CP	Tri*para*cresyl phosphate
Tween	Polyoxyethylene sorbiton monolaurate

Chapter One

Introduction

1.1 Organophosphates: A general introduction

Organophosphates (OPs) comprise one of the largest and most diverse family of chemicals. The majority of them have been used as agricultural pesticides since the 1980s. They have the advantage that they are degraded relatively quickly in the environment compared to organochlorine (OCs) compounds such as DDT and hexachloron, which are stable for years. However, one disadvantage is that they have a highly acute toxic effect, which has resulted in a large number of fatal intoxications (Kaloyanova and El Batawi, 1991).

Many studies conducted by independent research groups, such as Stephen *et al.*, (1995; 1996) and Stokes *et al.*, (1995), have shown that people working with OPs are experiencing symptoms associated with OP exposure. Stephens *et al.*, (1995), Mearns *et al.*, (1994) and Maizlish *et al.*, (1987) expressed concerns at the dangers of repeated low level exposure, which over months and years may result in chronic damage to the nervous system. These studies concluded that there was a need to reduce exposure to OPs as far as possible.

Although, severe acute poisonings are relatively uncommon in the UK and other Western countries, the potential still exists among agricultural workers, sheep dippers, farmers and pesticide sprayers (Jamal, 1995). Indeed, a farmer is suing a sheep dip manufacturer, Grampian Pharmaceuticals of Glasgow and Mallinckrodt Veterinary of Uxbridge, over allegations that chemicals in the sheep dip mix have caused his chronic heart condition. However, the UK Industry body that is representing the sheep dip manufacturers, the National Office of Animal Health (NOAH), denies any link between OP dip and the chronic heart condition (European Chemical News, 1995). Evidently, concern has emerged for humans and livestock due to the toxic effect of OPs.

The toxic effects of OPs can be subdivided into two types :-

(1) Acute toxicity

(2) Delayed toxicity

(1) **Acute toxicity**. These effects are seen with many pesticides and also with nerve agents such as isopropyl methyl phosphonofluoridate (sarin). These organophosphate esters are very powerful inhibitors of the enzyme acetylcholinesterase (AChE), preventing the enzyme from hydrolyzing acetylcholine to choline and acetate (Johnson, 1975a; Roth *et al.*, 1993). It has been suggested from work by Kiss *et al.*, (1983), Brill *et al.*, (1984), Rengstorff, (1985), and Roth *et al.*, (1993) that OPs also exert a direct toxic effect on the heart. The reasons for this are that the heart receives its nerve supply from both sympathetic and parasympathetic nerves and the action of these OPs are on both systems, thus acutely distorting the normal functional balance.

OPs directly inhibit both specific cholinesterase, which is found in neuromuscular structures and in erythrocytes, and non-specific cholinesterase in the plasma and liver, by phosphorylating the cholinesterase esteric binding site and thus preventing the enzyme from deactivating acetylcholine. As a result of this, acetylcholine levels build up at the nerve endings in the effector organs (such as the heart) causing overstimulation (Hayes, 1987; Kaloyanova and El Batawi, 1991; Koelle, 1994).

The inhibition of cholinesterase at post synaptic receptor sites causes hyperactivity due to the repetitive stimulation by excess acetylcholine. In skeletal muscle, it is seen as fasciculations, movement of muscle fibre groups and / or as twitching of large muscle groups. This is followed by fatigue, weakness and finally flaccid paralysis of the muscle. Another area affected is smooth muscle; this may involve constriction of airways and hyperactivity in the gastro-intestinal tract, contraction of pupillary constrictor muscles, ganglionic stimulation such as an increase in heart rate and blood pressure (Hayes, 1987; Koelle, 1994; Sidell, 1994).

Effects on the central nervous system include confusion, slurred speech, poor coordination and convulsions (Hayes, 1987). Other symptoms that can develop are excessive lacrimation, salivation, sweating, urination, defaecation and total weakness. Eventual death results from asphyxiation due to excessive broncho-constriction, bronchosecretion and paralysis of the respiratory muscles (Fedalei and Nardone, 1983).

There are antidotes available, which are cholinergic antagonists such as atropine and oxymes; these block the excessive stimulation, easing the symptoms and condition (Kaloyanova and El Batawi, 1991; Sidell, 1994).

(2) **Delayed toxicity**. This toxicity occurs following exposure to compounds such as tricresyl phosphate (TCP), which is a relatively weak inhibitor of acetylcholine esterase, such OPs are, currently, in use as agricultural and domestic pesticides e.g. mipafox, leptophos, trichlorphon etc.

This delayed neurodegenerative condition is known as organophosphate-induced delayed neurotoxicity (OPIDN) and is characterized by delayed manifestations after exposure to some OP esters (Hayes, 1987; Kaloyanova and El Batawi, 1991; Chambers and Levi, 1992; Huff and Abou-Donia, 1995). It has been suggested that OPIDN involves the dying back of long myelinated nerve axons, particularly in the sciatic nerve and the spinal cord. This damage to the axons is apparently irreversible, although recovery in mild poisonings may be due to compensation of lost nerve fibre.

It has been suggested by Senanayake and Karalliedde (1987) and De Bleecker *et al.*, (1994) that there exists an intermediate syndrome (IMS) between acute cholinergic crisis and the onset of OPIDN. IMS has been characterized by the weakness of cranial, respiratory and proximal limb muscles and is associated with the depression of tendon reflexes.

1.1.1 Delayed neurodegenerative condition: Organophosphate induced delayed neurotoxicity (OPIDN)

OPIDN has been defined as a neurodegenerative disorder characterized by sensory loss and ataxia associated with distal degeneration of sensory and motor axons in ascending and descending spinal cord tracts and peripheral nerves (Richardson, 1992). Initially, it was described as 'demyelination syndrome', as it was characterised by dying back of long myelinated nerve axons and damage to individual axons was apparently irreversible. Abou-Donia and Lapadula (1990) reported that this degeneration was

associated with axonal swellings containing cytoskeletal protein aggregates. However, the sequence of events which leads to axonal degeneration remains largely unknown (Abou-Donia, 1981; Zech and Chemnitius, 1987; Richardson, 1992).

Abou-Donia (1981) detailed the characteristics and features relating to OPIDN as follows:

(1) OPIDN is induced by delayed neuropathic OP esters; however, not all OP esters produce delayed neurotoxicity,
(2) there is an latent period of 6 - 14 days after administration of a single dose, before the onset of clinical symptoms,
(3) cellular damage is observed in sciatic, peroneal and tibial nerve, spinal cord and medulla but not in the higher brain. Itoh (1984) demonstrated that associated axonal lesions and demyelination occurred in both tractus spinocerebellaris and cerebellum in hens,
(4) lesions begin at the distal part of the long fibres and of large diameter peripheral nerves, and
(5) lesions are characterized by the degeneration of axons coupled with the subsequent secondary degeneration of the myelin sheath.

1.1.2 Reported cases of TCP poisoning resulting in OPIDN

Since 1899 there have been more than 40,000 reported episodes on TCP poisonings (e.g. Smith *et al.*, 1930; Abou-Donia, 1981; 1990). Table 1.1 shows the documented cases of TCP poisoning which subsequently led to OPIDN with the earliest case of TCP poisoning being in 1899 from the use of creosote oil as a chemotherapeutic treatment for pulmonary tuberculosis (Lapadula *et al.*, 1985).

The next outbreak occurred in the early 1930s, in Eastern and Southern states of America, when up to 10,000 to 20,000 people were affected by an alcoholic extract known as "Jamaican Ginger" (Ginger Jake or Jake) which contained Lindol. Lindol was used as a substitute solvent and was thought to be non-toxic and heat stable. However, it contained tri*ortho*cresyl phosphate, T*O*CP, which is thought to be the

neurotoxic isomer of TCP (Somkuti, 1990; Woolf, 1995) and this led to the development of clinical symptoms typical of OPIDN.

The symptoms suffered from ingesting this contaminated alcoholic extract depended upon the volume ingested but people were suffering from gastrointestinal effects such as nausea and possibly diarrhoea. After a few weeks, some people were suffering from tingling sensations in their extremities, general malaise and fatigue - the classical signs of OPIDN. Partial or full recovery was seen in some people, but others were left with residual limps, staggering gait and/or complete paralysis (Somkuti, 1990; Woolf, 1995).

Other epidemics that have been reported were mainly caused by the consumption of edible oils accidentally contaminated or adulterated with mineral oils containing the - *ortho* isomers of TCP (Woolf, 1995).

1.1.3 Symptomatology of OPIDN

The clinical symptoms of OPIDN after intake, usually by ingestion, of an OP compound were documented by Zech and Chemnitius (1987) as follows :

(1) After 48 hours, a range of gastrointestinal symptoms occur such as vomiting and diarrhoea (Travers, 1962),

(2) following a latency period of 6 - 14 days, the development of progressive weakness and ataxia occurs beginning distally in the lower limbs (Cherniack, 1988). This may possibly develop into flaccid paralysis of the lower limbs with a chance of it spreading to the muscles of the trunk, hands and arms. Knee and ankle jerks are impaired or totally absent. Muscle atrophy occurs due to motor nerve damage. There may be mild signs, if any, of impaired sensitivity or disturbance of cerebral functions (Smith and Spalding, 1959), and

(3) Weeks and months following the onset of OPIDN, there is the possibility that flaccid paralysis develops into spastic paralysis, which is known as upper motor neuron syndrome. Patients can suffer various degrees of chronic impairment with

Table 1.1 Documented cases of TCP poisoning and subsequently OPIDN as a result of exposure to triorthocresyl phosphate (TOCP) (adapted from Abou-Donia, 1990).

Year	Country	Cause	Number of Cases
1899	France	Therapy of tuberculosis with creosote containing 15 % TCP	59
1930	USA	Contaminated ginger extract	10,000-20,000
1295-1934	France, Germany, Switzerland	Parsley - extract 'Apiol' adulterated with TCP used for abortifacient	200-500
1937	South Africa	Contaminated cooking oil	60
1940	Switzerland	Contaminated cooking oil	80
1942	Britain	Contamination from manufacturing use	3
1945	Britain	Contaminated cottonseed oil	17
1943-1947	Germany	Used as cooking oil	10-20
1947	Switzerland	Contaminated food	73
1952	Switzerland	Contaminated olive oil	80
1955	South Africa	Contaminated water	11
1959	Morocco	Used as cooking oil	10,000
1960	India	Contaminated cooking oil	58
1966	Rumania	Contaminated alcohol	12
1967	Fiji Islands	Contaminated flour	?
1973	Morocco	Shoe glue exposure	40
1977 & 1978	Sri Lanka	Contaminated sesame oil	23
1988	India	Contaminated cooking oil	1,000

motor functions improving gradually during the first year of rehabilitation. However, long term recovery is limited and dependent upon intensive physiotherapy in severe cases.

Any muscular atrophy that occurs is the direct result of damage to the long motor neurons and is referred to as a 'dying back' process, as the degeneration of long motor neurons spreads from the periphery of the axon towards the nerve cell body (Abou-Donia, 1981; Zech and Chemnitius, 1987).

1.1.4 Protection against OPIDN

Considerable effort has been made in the search for prophylactic agents. If a non-specific serine esterase inhibitor such as phenylmethylsulfonyl fluoride (PMSF) is administered to hens before the neuropathic OP ester, the development of OPIDN is completely prevented. However, if PMSF is administered after the neuropathic OP ester, it aggravates both the clinical and morphological symptoms. This suggests that pre-treatment with PMSF prevents OPIDN by blocking OP-receptor interactions and therefore preventing the initial step of the disorder (Pope *et al.*, 1993).

1.1.5 Treatments for OPIDN

In an attempt to alleviate the symptoms of OPIDN, several compounds have been tried such as steroids, calcium channel blockers and gangliosides. Ehrich *et al.*, (1986) found that steroids did not alleviate the effects of T*O*CP on experimental roosters. El-Fawal (1989) and El-Fawal *et al.*, (1990) found that calcium channel blockers helped to alleviate the clinical response to phenyl saligenin phosphate without affecting the activity of the proposed target enzyme, neuropathy target esterase (NTE; section 1.3). Berry *et al.*, (1986) and Moretto and Lotti (1989) found that gangliosides alleviated the outcome of OPIDN, which had been induced by both T*O*CP and diisopropylfluorophosphate (DFP), without affecting NTE activity. It was concluded that the therapeutic effects were related to processes following initiation and preceding the expression of OPIDN.

1.2 Tricresyl phosphate (TCP) and related isomers

Organophosphate compounds such as tricresyl phosphate (TCP) are widely used as plasticizers, industrial hydraulic fluids and lubricant additives. These compounds have the technical advantages of being pliable and flame-, abrasion- and water-resistant (Bondy *et al.*, 1960; Cho *et al.*, 1994). However, they are too toxic to be used in connection with food packaging or in materials that come into contact with the body, as they are readily absorbed through intact skin (Hodge and Sterner, 1943).

One of the main uses of these compounds is in tri-aryl phosphate based hydraulic fluids which are used in hydraulic systems that generate very high temperatures and pressures (>5,000 psi). Contamination usually occurs due to the system developing leaks, resulting in the generation of aerosols in the work environment. The most likely route of exposure is through inhalation or dermal contact (Latendresse *et al.*, 1995).

TCP is produced in large quantities and finally ends up discharged into the aquatic environment and waste water treatment systems (Cho *et al.*, 1994; 1996). Presently this is a problem in Japan, where the Kurose River is being contaminated by TCP, the source being traced to agricultural plastic films, waste PVC pipes and used oils from cars (Cho *et al.*, 1996).

1.2.1 Composition of TCP

TCP is a commercially available product that is produced from a phenolic fraction containing *-ortho*, *-meta* and *-para* cresols and xylenols that are reacted with phosphorus oxychloride to form esters that contain symmetrical tri-esters and mixed esters. Therefore, the commercial product TCP consists of three isomers *-ortho*, *-meta* and *-para* (Bondy *et al.*, 1960; Glees and Janzik, 1965).

It was suggested by Bondy *et al.*, (1960) that the *-ortho* cresyl ester produces the delayed neuropathic effect, and that this was solely dependent upon the amount of *-ortho* cresyl ester present in the raw material. Previous work conducted by Henschler (1957) showed that the delayed neuropathic effect of TCP did not run parallel to the

proportion of -*ortho* isomer present but depended on the presence of mono -*ortho* cresyl ester, as only one -*ortho* cresyl group was needed for neurotoxic activity. Hence, it has been enforced that TCP should contain no more than 3 % of the -*ortho* isomer; however, this does not remove the toxic effect of TCP (Bondy *et al.*, 1960).

The other two cresols -*meta* and -*para* are thought to be non-neurotoxic *in vivo*. However, although the -*meta* and -*para* isomers have the same technical advantages as TCP, their isolation through intensive purification of these isomers would be very costly and hence commercially unrealistic (Bondy *et al.*, 1960).

1.2.2 Isomers of TCP

As previously mentioned the commercial product, TCP, consists of three isomers -*ortho*, -*para* and -*meta*, which are known as tri*ortho*cresyl phosphate (T*O*CP), tri*para*cresyl phosphate (T*P*CP) and tri*meta*cresyl phosphate (T*M*CP). However the distribution of these isomers has been suggested as totally random with different mixtures being formed such as -*O*-*M*-*M*-TCP, -*O*-*P*-*P*-TCP, -*O*-*M*-*P*-TCP as indicated by Glees and Janzik (1965).

Structurally the isomers only differ in the position of the methyl group around the benzene ring. Figure 1.1 shows the structural composition of the three isomers: T*O*CP, T*P*CP and T*M*CP.

1.2.3 Tri*ortho*cresyl phosphate (T*O*CP)

T*O*CP is an organophosphorus ester that is an undesirable impurity of TCP synthesis (Budavari *et al.*, 1996), chemically very inert (Bischoff *et al.*, 1967) with a molecular weight of 368.36 (Itoh *et al.*, 1981). This compound has many technical uses, such as a fire retardant in high speed brake lubricants, and as a component of air filter media, lacquer coatings, wood preservatives and methylating agents (Liepin and Pearce, 1976; Budavari *et al.*, 1996).

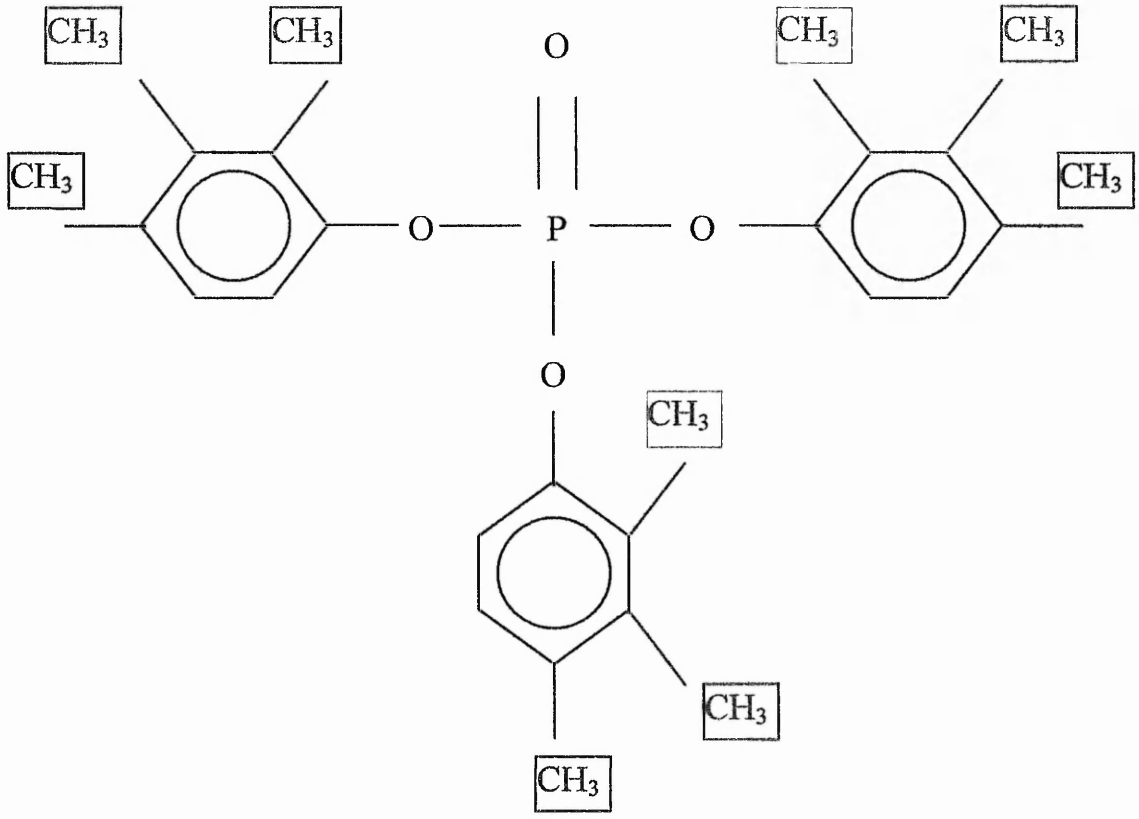


Figure 1.1 Structural composition of isomers of TCP with triorthocresyl phosphate (TOCP) ———— ; triparacresyl phosphate (TPCP) ———— ; trimetacresyl phosphate (TMCP) ———— (adapted from Glees and Janzik, 1965).

As previously mentioned, T*O*CP is able to induce a delayed neuropathy condition known as OPIDN, with symptoms such as ataxia, flaccid paresis and paralysis (Abou-Donia, 1981; Freudenthal *et al.*, 1993). It has been suggested by Freudenthal *et al.*, (1993) and others that T*O*CP causes axonal degeneration and subsequent demyelination of the axonal sheath of central and peripheral nerves.

The ability of TCP to induce OPIDN is dependent on the presence of an *-ortho* substituted methyl group in the aromatic series (Bischoff, 1967; Abou-Donia, 1990; 1992). *In vivo*, it is thought that the *-ortho* methyl phenol derivative of T*O*CP is microsomally metabolized into more potent neurotoxic metabolites such as 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one (CBDP or SC*O*TP), *O*-cresyl dihydrogen phosphate, hydroxymethyl T*O*CP (Casida *et al.*, 1961; Eto *et al.*, 1962; Taylor and Buttar, 1967; Sharma and Watanabe, 1974; Nomeir and Abou-Donia., 1986).

Eto *et al.*, (1962) proposed a pathway for the hydroxylation and cyclization reactions in the metabolism of T*O*CP. *In vivo* and *in vitro*, many of the *ortho*-cresyl phosphate esters are metabolized to form more powerful esterase inhibitors possibly involved in the hydroxylation and subsequent cyclization of *ortho*-hydroxymethyl derivatives (Casida *et al.*, 1961).

1.2.4 Tri*meta*cresyl phosphate (T*M*CP) and tri*para*cresyl phosphate (T*P*CP)

Work conducted by Bondy *et al.*, (1960) and Freudenthal *et al.*, (1993) has shown that both T*M*CP and T*P*CP have no measurable neurotoxic activity and are therefore unable to induce OPIDN (Patton *et al.*, 1986). Work conducted by Lapadula *et al.*, (1991) also agrees that T*P*CP can not induce OPIDN and consequently has no effect on protein phosphorylation.

1.3 Proposed molecular basis of OP toxicity

It has been proposed that there are two biochemical lesions that precede the onset of clinical OPIDN :

(1) organophosphorylation of the NTE, a membrane associated enzyme of unknown function in neurons (Zech and Chemnitius, 1987). The mechanism of NTE inhibition is discussed in detail in section 1.4.

(2) disruption of the axonal cytoskeleton through altered protein phosphorylation (Zech and Chemnitius, 1987; Abou-Donia and Lapadula, 1990).

It has been found that disruption of neuronal cytoskeletal components such as microtubules (MTs), neurofilaments (NFs) and microfilaments (MFs) may be involved in the pathogenesis of OPIDN (Abou-Donia *et al.*, 1988; Lotti, 1992). Suwita *et al.*, (1986) suggested that a neuropathic dose of TOCP in hens enhances phosphorylation of α- and β- tubulin and microtubule-associated protein 2 (MAP2) in the brain and spinal cord and of neurofilaments in the spinal cord. They concluded that the effects were related to altered calcium-calmodulin kinase II activity.

Abou-Donia (1993) hypothesized that OPs which cause OPIDN may interfere with protein kinases by phosphorylating their serine and / or threonine hydroxyl residues. He suggested that this action would adversely affect the regulation of normal neuronal proteins and result in axonal degeneration. This was supported by evidence from two observations that (1) early ultrastructural changes showed aggregation and accumulation of NFs and MTs (Prineas, 1969) and (2) the organisation of cytoskeletal proteins is significantly affected by protein-kinase mediated phosphorylation (Kenyon and Garcia, 1987).

Research conducted by El-Fawal and Ehrich (1993) suggested that the development of OPIDN may involve an increase in calcium influx with the subsequent activation of calpains (calcium activated neutral proteases) and precipitation of axonal cytoskeleton degeneration.

Abou-Donia (1993) found that hens given one oral dose of 750 mg/kg of T*O*CP suffered paralysis 21 days after treatment. This was accompanied by an increase in calcium / calmodulin-dependent kinase mediated phosphorylation of cytosolic proteins, α- and β-tubulin and the membrane protein, myelin basic protein. Lapadula *et al.*, (1992) have shown that chicken sciatic nerves treated with T*O*CP display increased levels of phosphorylation of tubulin, MAP2 and NFs.

It has been suggested that the hyperphosphorylation of cytoskeletal proteins and axonal swellings are causally linked and this decreases the rate of axonal transport leading to its accumulation, suggesting that the cytoskeleton is involved in the production of neuropathologic lesions (Abou-Donia, 1995). This confirms the close temporal relationship between increased phosphorylation of cytoskeletal proteins and the development of OPIDN (Abou-Donia, 1995).

1.4 Neurotoxic esterase or neuropathy target esterase (NTE)

Initial studies by Johnson *et al.*, (1969) over twenty years ago, provided possible evidence that the target site for initiation of OPIDN was a specific esterase known as neurotoxic esterase (NTE). Little is known regarding its physiological role or the pathophysiological processes which occur with extensive NTE inhibition but evidence suggests that modifications to NTE by a covalently bound neuropathic OP ester may form the initial biochemical lesions that leads to OPIDN (Glynn, 1999; 2000; Johnson, 1974; 1990).

1.4.1 Characteristics of NTE

NTE is an integral membrane protein in neurons, with a molecular weight of 155 to 178 kDa (Carrington *et al.*, 1985). NTE is believed to play an important role in neuronal development, possibly involved in signalling pathways controlling interactions between neurons and glial cells (Glynn, 1999; 2000; Johnson, 1974; 1990). However, it is believed not to be essential for the health of mature neurons (Lotti and Moretto, 1993).

NTE has serine esterase activity and efficiently catalyses the hydrolysis of phenyl valerate (PV), *in vitro*, but its physiological substrate is unknown (Abou-Donia, 1993; Glynn, 1999; 2000; Johnson, 1990). It has been estimated that 70 - 80 % inhibition of total NTE activity by a neuropathic OP ester is necessary for the development of OPIDN (Johnson *et al.*, 1980). This has been established by an assay designed by Johnson (1969; 1975b) that uses a hen brain homogenate exposed to neuropathic OP ester to determine the inhibited activity of NTE (adapted by Correll *et al.*, 1991).

1.4.2 Distribution of NTE

NTE is found in large concentrations in the brain cortex, spinal cord and sciatic nerve but is also present at lower concentrations in non-neuronal tissues such as human placenta, spleen, platelets, testes and lymphocytes (Gurba and Richardson, 1983; Williams, 1983; Bertoncin *et al.*, 1985; Carrington *et al.*, 1985; Lotti *et al.*, 1985). Experimental work conducted by Glynn *et al.*, (1997) on intracellular immunostaining using their NTE rabbit antiserum (R28), found that neuronal cell bodies and, in some cases, proximal axon segments from frozen chicken brain cortex sections positively stained for the presence of NTE. It was also noted that glial cells, sciatic nerves and motor end plates did not give a positive response to the NTE antiserum.

1.4.3 Suggested role of NTE inhibition in the initiation of OPIDN

It was proposed by Johnson (1974) that there is a two step process of initiation of OPIDN by neuropathic OP esters. The first step involves inhibition of NTE by organophosphorylation and a progressive decrease in its catalytic activity. This is followed by an alteration of the membrane micro-environment which is in contact with the phosphorylation site which, in turn, disturbs homeostasis of the protein leading to its degradation. However, OPIDN and its potential molecular mechanisms are still not fully understood. It has been suggested that the role of NTE in OPIDN may be due to either (1) the loss of an essential non-esterase function which is required by neurons and/or their axons, or (2) that having a negatively charged group covalently attached to the NTE active site (serine residue; a process known as 'aging') may convert NTE into a toxic form that may initiate neuropathy (Abou-Donia, 1990; Glynn, 1999; 2000;

Johnson, 1990). The suggested neuropathic events that may result from a gain of NTE function are (a) chemical transection of the axon (distal portion of axon from the initial vacuolar lesion which subsequently undergoes a Wallerian-type degeneration (Bouldin and Cavanagh, 1979)), (b) entry of calcium into the axon through intact axolemma, followed by (c) activation of calcium-dependent proteases (calpains) and proteolysis of the axonal cytoskeleton (George *et al.*, 1995).

However, it has been found that the stage two process of 'aging' does not occur when a non-neuropathic agent is present; such compounds appear to inhibit NTE but do not cause any delayed effects (Johnson *et al.*, 1987).

1.4.4 Chemical aspects of NTE inhibition

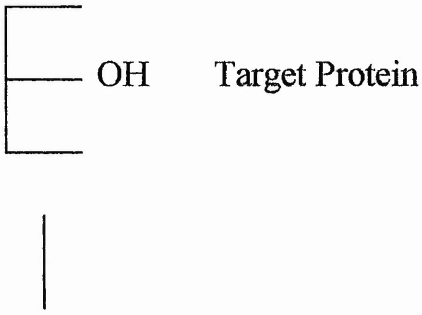
Johnson (1974) documented that inhibition of the enzyme depends entirely on the nature of the chemical group, which is covalently bound at its active site. Many NTE inhibitors have been classified into two major categories.

(1) Group A compounds inhibit NTE and are neurotoxic. Examples of these are phosphate, phosphoramidate and phosphonate compounds.

(2) Group B compounds inhibit NTE but have a protective effect against OPIDN. These include carbamates and sulphonyl fluorides (e.g. PMSF).

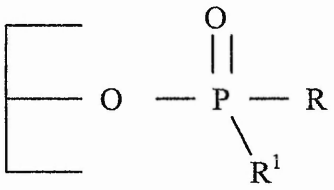
After inhibition of the enzyme by a Group A compound, hydrolysis may occur at one of the ester (R-O-P) or amide (R-N-P) bonds linking the phosphorous to the radicals R and R^1. This yields a stable ionized acidic group on the phosphorous, which is still attached to the protein. However, this stable acidic grouping can not be produced with Group B compounds indicating that they are chemically inactive (figure 1.2).

Reaction 1



In vivo organophosphorylation of active site of target inhibits NTE activity

Reaction 2



Modified Target Protein

Biological responses *in vivo* depend on the next chemical change

2a

If 1or 2, R - P bonds are C - O - P (Phosphate or Phosphonate)

THEN 'Aging' is possible

Formation of aged inhibited NTE that initiates neuropathy.

2b

If both R - P bonds are C - P (Phosphinates)

THEN 'Aging' is impossible

Inhibited NTE is protected against neuropathic OP esters, leading to protection against neuropathic effects.

Figure 1.2. Proposed two step process of initiation of OPIDN (adapted from Johnson, 1990).

1.4.5 *In vivo* studies relating NTE inhibition to OPIDN

As previously mentioned, Abou-Donia (1981) and Zech and Chemnitius (1987) claimed that several animal species were susceptible to the effects of neuropathic OP esters. It was from these studies that the importance of chickens/hens was recognised, leading to their subsequent use as the main test animal. The delay period observed with histopathological lesions and clinical signs is similar to that in humans and both sexes are susceptible. The clinical signs of delayed neuropathy in chickens can easily be observed, not interfering with the tests. Data banks on OPIDN in chickens facilitate testing of newly synthesized compounds.

There are a number of limitations with these animal experiments, such as the considerable time needed to perform the test, the large number of animals required (which has serious financial and ethical implications) and the fact that a lot of test material is needed to perform such work (Flaskos, 1995). However, while some of these limitations have been dealt with by the use of an *in vitro* assay of NTE (Johnson, 1990), such problems have also lead to an increase in the development of alternative systems, i.e. *in vitro* cellular system (Fedalei and Nardone, 1983; Henschler *et al.*, 1992; Nostrandt and Ehrich, 1992; Ehrich *et al.*, 1994; Flaskos *et al.*, 1994; Ehrich, 1995; Schmuck and Ahr, 1997).

1.4.6 *In vitro* studies relating NTE and OPIDN

Due to the limitations of *in vivo* experiments described above, work has begun to concentrate on the effects of OPs at the *in vitro* level. Presently, studies are being undertaken with regards to NTE activity which is found in both differentiated and undifferentiated cell lines of human and mouse origin (Fedalei and Nardone, 1983; Carrington et al., 1985). Although NTE is an initial target of neuropathic OPs, the nature of biochemical events following NTE inhibition and leading ultimately to OPIDN symptomatology is unknown and it is frustrating to know that NTE activity is not confined to neurons but it is also present in other cell types such as lymphocytes, which further complicates the issue. Therefore, if the mechanism of OPIDN

development is to be studied, the use of cell cultures is ethically superior and financially preferable (Flaskos, 1995).

1.5 The cytoskeleton as a potential toxicity target

As mentioned earlier, the disruption of the cytoskeleton has been suggested to be a primary response to exposure to toxic neuropathies and agents. The cytoskeleton is a fibrous network found in all types of eukaryotic cells and consists of 3 filamentous arrays, microtubules (MTs), intermediate filaments (IFs) and microfilaments (MFs). These arrays are structurally and biochemically different but jointly play a major role in the regulation of cellular processes such as cell morphogensis, cell movement and cell division (Lasek *et al.*, 1983). NFs, a class of IFs, and MTs are of particular interest due to their known importance in the control of axon growth and stability. Both of these networks represent potential toxicity targets and will therefore be discussed in detail.

1.6 Microtubules (MTs)

1.6.1 General properties of microtubules

Microtubules (MTs) have been implicated in many neuronal functions such as growth of axons and dendrites, maintenance of neuronal morphology and intracellular transport but the mechanisms involved are poorly understood (Heidemann *et al.*, 1981; Doering, 1993). MTs are important in developing axons and dendrites (Cambray-Deakin, 1991) in the elaboration and maintenance of neuronal processes (Ginzburg, 1991).

Within the nervous system MTs are involved in neurite extension and axoplasmic transport (Barra *et al.*, 1988), providing mechanical stability and channels along which membrane-bound organelles are transported (Jacobson, 1995). Okabe and co-workers confirmed that MTs are not static polymers but are dynamic structures that continue to assemble and elongate along the length of the nerve cell processes (Okabe and Hirokawa, 1988;1990).

1.6.2 Structure of MTs

MTs are cytoplasmic elements comprising tubulin heterodimers and non-tubulin associated proteins known as microtubule associated proteins (MAPs) (Cleveland, 1993). They are hollow cylindrical polymers composed of thirteen protofilaments (Okabe and Hirokawa, 1988; Hargreaves, 1997) with an external diameter of 25 nm and an internal diameter of 14 nm. The protofilaments are comprised of heterodimers of α- and β- tubulin subunits, with an estimated molecular weight of approximately 50 kDa each, arranged in a staggered fashion (Tilney *et al.*, 1973). They co-purify with MAPs after repeated cycles of tubulin assembly and disassembly *in vitro* (Shelanski *et al.*, 1973; Murphy and Borisy, 1975; Sloboda *et al.*, 1976) (figure 1.3).

MTs are inherently organised in a polar fashion with polymerisation and depolymerisation occurring at the ends of the structure (Heidemann *et al.*, 1981). The ends of the MTs have been defined depending upon their rate of growth, where the 'slow growing end' is known as the minus end and the 'fast growing end' is known as the plus end (Cleveland, 1993; Heidemann *et al.*, 1981). The minus ends are associated with microtubule organising centres (MTOCs) in non-neuronal cells such as basal bodies, centrioles and centromeres of chromosomes; it is from these regions that MTs assemble (Lodish *et al.*, 2000). Thus the polarity of the MTs becomes fixed in orientation with the plus end extending towards the extremities of the cell. In neuronal cells, the minus ends of the axonal MTs are oriented at the basal body of the axon with the plus end extending towards the tip of the axon (Lodish *et al.*, 2000; Bamburg *et al.*, 1986; Heidemann *et al.*, 1981). In dendrites, MTs often have mixed polarity with both plus and minus ends distal to the cell body. MTOCs are able to organise MTs by polymerisation of tubulin subunits at the plus ends, which leads to elongation of MTs (Lodish *et al.*, 2000). Recent work has suggested that MTs in both axons and dendrites are nucleated at the MTOC but are then released from the MTOC and delivered to the neurites (Yu *et al.*, 1993). The dynamics of MT assembly are controlled by the maintenance of constant polymer mass in a process called treadmilling (described by Margolis and Wilson, 1978). It involves a net gain of subunits at the plus end of MTs, which is balanced by a net loss at the minus end (Bergen and Borisy, 1980) and so the MT structure can be sustained.

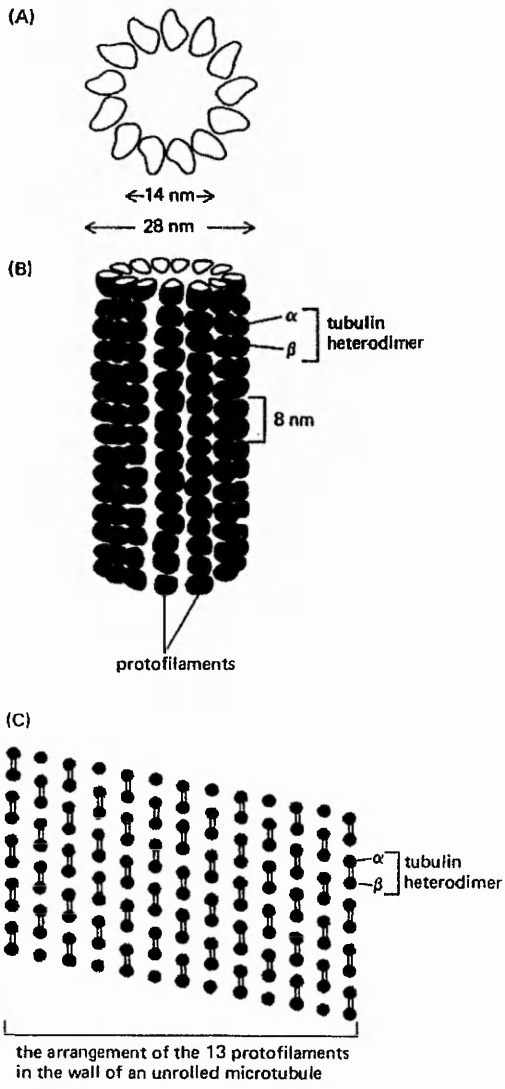


Figure 1.3. Schematic diagram of a microtubule showing how the tubulin polypeptides pack together to form the cylindrical wall. (A) shows the 13 polypeptides in a cross-section; (B) shows a side view of a short section of a microtubule with the tubulin polypeptides aligned into rows or protofilaments; (C) shows a portion of the microtubule wall "unrolled" to show the packing of the two kinds of tubulin polypeptides (adapted from Alberts et al., 1983).

In both instances, the growing end can be stabilized by several factors such as the presence of a GTP cap, binding of MAPs or interaction with membrane or other cytoskeletal elements (Kirschner and Mitchison, 1986; Gelfand and Bershadsky, 1991).

1.6.3 Biochemical properties of MTs

1.6.3.1 Tubulin

As explained above, α- and β- tubulin subunits are arranged as heterodimers in a thirteen protofilament structure with each subunit having a molecular weight of 50 kDa. Tubulin is a highly acidic, conserved protein and has the ability to bind a variety of MAPs (Cleveland, 1993; Hargreaves, 1997).

The carboxyl terminal domain within both the α- and β- subunits contains an area of 40 amino acids that is believed to possess phosphorylation sites for regulation (Hargreaves *et al.*, 1986; Wandosell *et al.*, 1987), binding sites for calcium (Serrano *et al.*, 1986) and sites for proteins that stabilize MTs such as MAPs (Serrano *et al.*, 1984a, 1984b; Macioni *et al.*, 1985).

The tubulin polymer undergoes several post-translational modifications, such as phosphorylation of Ser-444 of the β-tubulin subunit (Diaz-Nido *et al.*, 1990; Alexander *et al.*, 1991), acetylation of Lys-40 of α-tubulin (LeDizet and Piperno, 1987) and reversible removal of the carboxyl terminal tyrosine of α-tubulin (Barra *et al.*, 1988).

The synthesis of tubulin is auto regulated by a pool of unpolymerised tubulin and as this pool of tubulin free subunits is used up during MT assembly it, in turn, stimulates an increase in tubulin synthesis (Cleveland, 1993).

1.6.3.2 Microtubule-associated proteins (MAPs)

It has been suggested that MAPs may regulate the stability of MTs in axons and dendrites and be involved in determining balance between rigidity and plasticity in neuronal processes (Okabe and Hirokawa, 1988; Doering, 1994). MAPs are known to

be involved in MT assembly, stabilization of MTs and interactions of MTs with other cytoskeletal elements and organelles (Matus, 1988; Nixon *et al.*, 1990). For example, MAP1, MAP2 and tau are able to stimulate MT assembly (Murphy *et al.*, 1977, Cleveland *et al.*, 1993) and are implicated in the interaction with intermediate filaments (IFs) (Leterrier *et al.*, 1982) and actin filaments (Griffith and Pollard, 1978; Sattilaro *et al.*, 1981).

MAP2 comprises three closely related phosphoproteins: MAP2A and MAP2B, both of which have a molecular weight of approximately 280 kDa, and MAP2C with a molecular weight of 70 kDa, which is observed transiently during their development (Vallee, 1980; Matus *et al.*, 1981; Caceres *et al.*, 1984; De Camilli *et al.*, 1984). MAP2A and MAP2B are thermostable, elongated molecules with an estimated length of 185 nm (Olmsted, 1986). MAP2 projects radially from the MT surface (Vallee, 1980) and is abundant in the cell body and dendrites, where it forms cross-bridges between MTs and NFs (Leterrier *et al.*, 1982; Heimann *et al.*, 1985; Hirokawa *et al.*, 1988).

Tau protein is restricted to neurons, and axons in particular, and is known to be an exclusively axonal marker (Binder *et al.*, 1985). It consists of several polypeptides of molecular weights ranging approximately between 45 - 60 kDa (Nixon *et al.*, 1990). This phosphoprotein can be phosphorylated and has calmodulin binding sites (Olmsted, 1986). It plays an important role in promoting assembly of MTs (Weingarten *et al.*, 1975) and stabilizing MTs partly by inhibiting their disassembly (Black and Greene., 1982; Drubin and Kirschner, 1986; Kosik *et al.*, 1988). In particular, the work of Drubin and Kirschner (1986), who microinjected tau into fibroblasts, showed that tau could induce new MT assembly and stabilize existing MTs, consistent with its proposed role in the stability of axonal MTs.

MAPs that cross link MTs with other cytoskeletal elements and / or intracellular membranes include the MAP1 family. The MAP1 phosphoprotein consists of three unrelated proteins known as MAP1A, MAP1B and MAP1C with molecular weights of 350 kDa, 330 kDa and 320 kDa, respectively (Olmsted, 1986; Nixon *et al.*, 1990). These proteins are found in abundance within axons and dendrites.

Some members of the MAP1 group act as ATPase motors, which drive intracellular transport of macromolecules along the MT network. For example, dynein and kinesin are known to be MT associated ATPases performing the function of motors that move materials along MTs (Chauhan *et al.*, 1993).

Phosphorylation of both tubulin and MAPs has been shown to influence the organization, dynamics and assembly state of MTs (Avila *et al.*, 1987; 1988), the exact effect depending upon the target protein, protein kinase and phosphorylation site involved (Hargreaves *et al.*, 1986; Wandosell *et al.*, 1987; Serrano *et al.*, 1987). The phosphorylation state of MT proteins, therefore, plays an important role in the control and regulation of MT dynamics. Indeed, it has been found that the phosphorylation state of MAPs and tubulin may be altered in certain neurodegenerative conditions (e.g. Alzheimer's disease (Cork *et al.*, 1986; Lee *et al.*, 1988b)) and chemically induced peripheral neuropathies such as acrylamide (Berti-Mattera *et al.*, 1990; Chauhan *et al.*, 1993) and aluminum (Johnson and Jope, 1988).

1.6.4 Role of microtubules in axonal growth and development

As the developing neurite extends, behind the growth cone, microtubules begin to be assembled. With time, the axonal cytoskeleton becomes generally more stable and associated with this is an increase in post-translational modified tubulin and NF subunits (Cambray-Deakin, 1991). The organization of the developing neuron depends on elongation of MTs from a longitudinal structural element (Bamburg, 1986). There have been two suggestions regarding the mechanism of MT assembly within an axon: either (a) there is MT elongation at the proximal end (close to the cell body) which moves forward into the lengthening axon, or (b) tubulin subunits are transported to the tip of the axon and assemble on to the free ends of MTs (Bamburg, 1986). It is the first suggestion that has been supported by studies on slow axonal transport confirming that MTs do assemble at the neuronal cell body (Heriot *et al.*, 1985). However, Bamburg (1986) proposed that tubulin was transported in an unassembled form and that MTs within axons were stationary, as are NFs.

The organization of MTs has been found to be orientated in a particular fashion; within the axon, they are orientated with the plus end towards the axon tip (Heidemann *et al.*, 1981), and in mature dendrites the polarity orientation is mixed approximately half each way (Baas *et al.*, 1988; Mandell and Banker, 1995). The difference in orientation may be due to selective transport in the axon and dendrites (Doering, 1994).

It is clear from their observations that several MAPs are essential for the growth and development of the axon and dendrites but it is the overall structure and turnover of tubulin subunits and MAPs that help to maintain the structure, orientation and function of MTs (Heidemann et al., 1981; Bass et al., 1988; Doering, 1994; Mandell and Banker, 1995).

1.6.5 MTs as useful markers for neurodegenerative agents

It has been demonstrated that many neurodegenerative agents such as acrylamide, aluminium and taxol interfere with MTs in such a way to disrupt their normal function.

Acrylamide has been shown to produce neuronal degeneration in the central and peripheral nervous system of humans and experimental animals. It has been reported that acrylamide causes a depletion of MAPs in the hippocampus and cerebellum, the areas involving memory and motor functions, respectively (Chauhan *et al.*, 1993). They have found that this compound depletes MAP1 and MAP2 within the extrapyramidal systems of experimental rats and have suggested that it binds to MTs causing curling and induces neuronal degeneration (Chauhan *et al.*, 1993). Acrylamide administrated to rats induces phosphorylation of spinal cord NFs *in vitro* and *in vivo* (Howland and Alli, 1986; Gold *et al.*, 1988) and β-tubulin in sciatic nerve homogenates (Berti-Mattera *et al.*, 1990) prior to the onset of axon degeneration.

Diaz-Nido and Avila (1990) found that aluminum increased phosphorylation of NFs and MAP2, *in vivo*, whereas *in vitro* studies indicated a selective precipitation of highly phosphorylated NFs and MAPs to form insoluble aggregates.

Taxol compound, which can be extracted from the bark of Western Yew trees (*Taxus brevifolia*), has been found to have an effect on cell growth by stabilizing MTs and inducing mitotic arrest (Wani *et al.*, 1971). It binds to the tubulin dimer, decreasing the availability of tubulin for *in vitro* assembly and therefore stabilizing MTs. This is a potent neurotoxin due to its ability to cause MT bundling and inhibit slow axonal transport (Slichenmyer and Von Hoff, 1990).

Work conducted by Abou-Donia (1993) showed that when hens were exposed to the OP compound, T*O*CP, there was an increase in autophosphorylation of calcium/calmodulin kinase II and in the phosphorylation of cytoskeletal proteins such as MAPs and tubulin.

1.6.6 Axonal transport

Many MT disrupting agents exert their neurodegenerative effects via inhibition of axonal transport, a MT-dependent process responsible for the transport of materials required for growth and renewal from the cell body to the axon and dendrite (Jacobson, 1995). It also provides materials for the formation, maintenance and function of synapses, and transports materials to the cell body from the axon terminal allowing the re-circulation of axonal components. Finally, it facilitates movement of trophic molecules and other signals retrogradely from the extracellular environment at the tips of the axon and from the axon's post synaptic targets.

Transport kinetic studies have suggested that MTs, tubulin and NF proteins are transported along axons in the form of a structural complex of MTs and NFs (Hoffman and Lasek, 1975; Lasek and Hoffman, 1976; Lasek, 1980). They are components of two slow moving phases, which have been designated as Group IV or slow component b (SCb) and Group V or slow component a (SCa) (Hoffman and Lasek, 1975; Grafstein and Forman, 1980).

SCb has an advancement rate of 2 - 20 mm per day and it transports more than 100 different proteins including actin, fodrin, myosin-like protein, clathrin and many metabolic enzymes (Black and Lasek, 1979; Willard *et al.*, 1979; Garner and Lasek, 1981; Brady and Lasek, 1981).

SCa has a transport rate of 0.1 - 1.0 mm per day and is predominantly composed of NFs and tubulin with small amount of fodrin and actin (Hoffman and Lasek, 1975; Black and Lasek, 1980). It has been suggested by Nixon *et al.*, (1990) that subtypes of tau are transported at this rate, indicating that the polypeptide is attached to MTs undergoing transport (Tytell *et al.*, 1984).

It is believed that NF proteins may be transported as polymers (Nixon and Lewis, 1986; Hoffman and Lasek, 1975), whereas tubulin transport is more complex with evidence suggesting that some tubulin polymers move as soluble dimers which are then inserted into the plus end of pre-existing MTs (Bamburg *et al.*, 1986; Okabe and Hirokawa, 1990).

1.7 Intermediate Filaments (IFs)

Intermediate filament arrays include different proteins which are expressed in different cell types and these are classified as type I to V, based on similarities in gene sequences as shown in table 1.2 (Lewis and Cowan, 1986; Osborn and Weber, 1986; reviewed by Robson, 1989).

Table 1.2 Intermediate filament classification

Type	Name	Tissue/Cell
I	acidic cytokeratins	epithelial cells
II	basic cytokeratins	epithelial cells
III	vimentin	mesenchymal cells
	glial fibrillary acidic protein (GFAP)	glial cells
	desmin	muscle cells
	peripherin	neuronal cells
IV	neurofilaments	neuronal cells
V	nuclear lamins	all cell types

The structural subunit of IFs consists of a dimer of two parallel polypeptide chains joined by a core α-helical coil region which are highly homologous to each other. The dimers then associate laterally, in an anti-parallel order to form protofilament (tetramers) and then form highly assembled intermediates (octamers) which eventually give rise to filaments that are 10 - 12 nm wide (Hargreaves, 1997). Although the exact function of IFs has not yet been fully established, it is known that they are biochemically very stable and plays a critical structural role in many different cell types, including neurons.

1.8 Neurofilaments (NFs)

1.8.1 General properties of neurofilaments

NFs are unique to neurons of the central and peripheral nervous system (Dahl, 1983). They are found in axons, dendrites and the cell body, but are enriched in axons (Bershadsky and Vasiliev, 1988). NFs are implicated in the control of axon architecture, which involves the development of two basic dimensions, longitudinal axial and radial. NFs and MTs together maintain the cylindrical form of the axonal cytoskeleton and influence the radial aspect of the axon directly through inter-array cross-bridges that result in laterally spaced linear polymers.

These filaments also influence the diameter of the axon and it is believed that NFs carry out this function in the mature axon. Evidence to support this view has been documented by Lasek *et al.*, (1983) showing that (1) NFs are a predominant cytoskeletal polymer in most very large axons, and (2) the proportion of NFs in the axonal cytoskeleton increases as the axon increases in cross-sectional area. Muma *et al.*, (1993) described NFs as the key cytoskeletal element in large caliber myelinated axons and suggested that the number of NFs present correlates closely with the cross-sectional area of the axon. In the Aplysia giant neurons, it has been demonstrated that NFs are mainly concentrated in the axon. This distribution may result from NF proteins being selectively associated with the axonal cytoskeletal network after being synthesized and transported into the axon. Alternatively, NF proteins could be degraded more rapidly in the cell body than the axon (Lasek *et al.*, 1983).

Hirokawa *et al.*, (1984) demonstrated that NFs run parallel towards the growth cone within the axon and are extremely cross-bridged with other NF structures. By contrast, within the cell body NFs are present in a randomly orientated manner with a lesser degree of cross-bridging than in the axon. NFs in dendrites, again, run parallel to the axis but are dispersed and/or exist as small fasicles with fewer cross-bridges between other NFs than seen in the axon and cell body.

1.8.2 The structure of neurofilaments

NFs consist of a polypeptide triplet divided into three chains with apparent molecular weights of 68 kDa (NF-L; light chain), 160 kDa (NF-M; medium chain) and 200 kDa (NF-H; heavy chain) subunits on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Hoffman and Lasek, 1975; Carden *et al.*, 1985). Each NF subunit is divided into 3 domains, which are a highly conserved central α-helical rod domain, an amino terminal head domain and a carboxyl terminal tail domain with the variations in molecular weight occurring due to changes in this tail domain (Geisler *et al.*, 1985).

Geisler and Weber (1981) and Liem and Hutchinson (1982) showed that purified NF-L self-assembles into smooth IF. It was initially thought that NF-M and NF-H acted as peripherally bound associated proteins on a backbone exclusively made from NF-L subunits, but it is now believed that NF-M and NF-H are IF proteins in their own right but co-assemble in the presence of NF-L to form the NF structure (Geisler and Weber, 1981; Liem and Hutchinson, 1982).

Electron microscopic observations of assembled NF decorated with antibodies to NFs suggest that NF-L is the central core unit (Willard and Simon, 1981; Sharp *et al.*, 1982; Hirokawa *et al.*, 1984). NF-M subunits appear to be peripherally situated throughout this central core unit, whereas NF-H forms a peripheral structure that projects radially, interconnecting NFs to other cytoskeletal elements.

Further immunoelectron microscopical studies have shown that the NF-H carboxyl terminal extension is responsible for cross-bridges between neighbouring NFs and other

cytoplasmic organelles, thus helping to stabilize the axonal cytoskeleton (Willard and Simon, 1981; Debus *et al.*, 1982; Sharp *et al.*, 1982; Hirokawa *et al.*, 1984) (figure 1.4).

1.8.3 Post translational modifications to neurofilaments

All three NF subunits undergo extensive post translational modifications involving phosphorylation of a carboxyl terminal domain (Julien and Mushynski, 1982; reviewed by Matus, 1988). NF-H and NF-M are much larger molecules than NF-L, due to the long sequence extensions on the carboxyl terminal tail (-COOH) of the conserved core domain. This -COOH domain contains multiples of the motif Lys-Ser-Pro and is known as the 'KSP region'. These regions are found in large numbers in both NF-M (10 repeats) and NF-H (>50 repeats) (Myers *et al.*, 1987; Dautigny *et al.*, 1988), whereas in NF-L there are only a few KSP regions.

These repeats are potential phosphorylation sites and the extent of phosphorylation depends on the number of KSP repeats. Thus NF-H is highly phosphorylated, whereas NF-L is phosphorylated the least. Nixon and Sihag (1991) documented that NF subunits can also be phosphorylated at the amino terminal head domain ($-NH_3$), but these sites are much less abundant than those on the -COOH domain. Nixon and Lewis (1987) documented that the NF subunit is regulated at steady-state by a dynamic balance between the process of phosphorylation and selective dephosphorylation.

The level and extent of NF phosphorylation in neurons appears to be a reflection of its relationship with different NF kinases and phosphatases. Toru-Delbauffe and Pierre (1983) suggested that phosphorylation of NF proteins, in particular NF-H, was able to mediate interactions between NFs and other cellular organelles. The NF protein subunits, *in vitro*, are phosphorylated by two groups of kinases.

The first of these groups is the second messenger dependent protein kinases, which phosphorylate sites on the amino terminal domain of NF-H. These kinases include protein kinase C, cyclic AMP-dependent protein kinase and calcium/calmodulin-

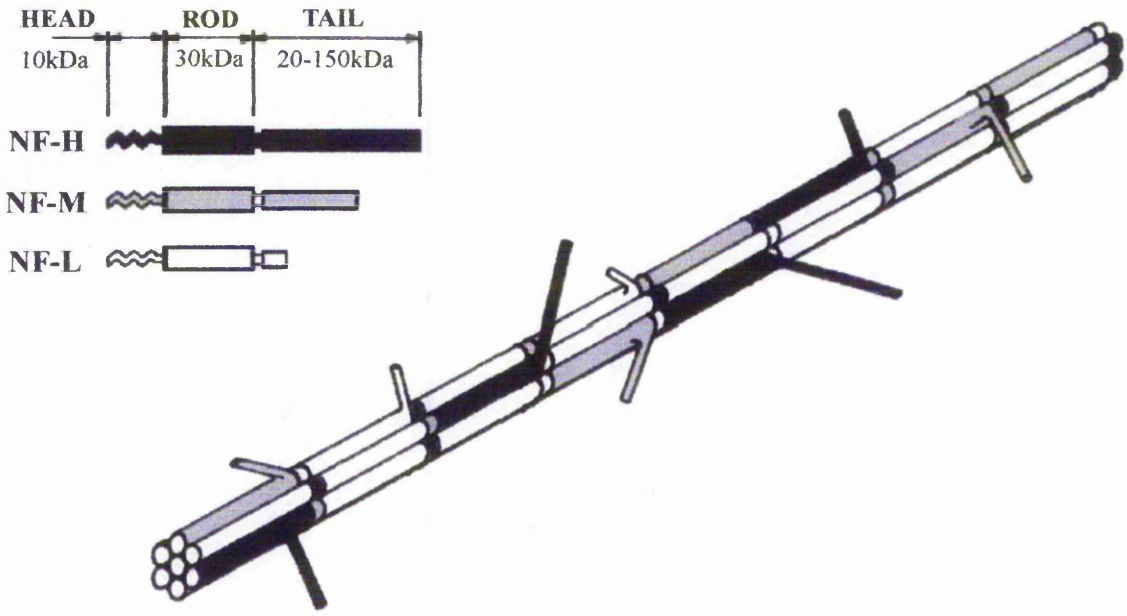


Figure 1.4. Schematic diagram of NFs in a mature axon. NFs are composed of three subunits with apparent molecular masses on SDS-PAGE of 70 kDa (NF-L), 140-160 kDa (NF-M) and 200 kDa (NF-H). The neurofilament subunits have a highly conserved central α-helical 'rod' domain, an amino-terminal 'head' domain and a carboxyl terminal 'tail' domain. The extreme length and unique structure of the tail domains, particularly of NF-H and NF-M, distinguish the NF subunits from the other intermediate filament proteins. When phosphorylated, the carboxyl terminal tails of NF-H and possibly of NF-M are believed to project radially from the filament core (adapted from Nixon and Sihag, 1991).

dependent protein kinases (Sihag and Nixon, 1990; Tokui *et al.*, 1990; Dosemeci and Pant, 1992). The second group comprises second messenger independent protein kinases, which phosphorylate the KSP repeats of the carboxyl terminal tail domain (Wible *et al.*, 1989; Roder and Ingram, 1991).

The turnover of phosphate groups on NF protein is maintained at a dynamic steady state (Nixon and Sihag, 1991). The current hypothesis states that NF-H phosphorylation plays an important role in the regulation of axonal diameter and of interactions between NFs and other cellular constituents (deWaegh *et al.*, 1992). It has been proposed that increased phosphorylation of the -COOH terminal domain of NF-H results in increased electrostatic repulsion leading to a decrease in NF density and consequently an increase in NF spacing and axonal diameter (Hirokawa *et al.*, 1984). Soussan *et al.*, (1994) found evidence to support the fact that increased axonal diameter is directly related to an increase in NF phosphorylation.

NF phosphorylation is thought to begin in the cell body soon after the synthesis of NFs and prior to their transfer into the axonal transport system. Carden *et al.*, (1985) and Nixon *et al.*, (1982) documented that phosphorylation of NF-H and NF-M occurs simultaneously with their transport into and along the axon, continuing during the anterograde intra-axonal movement towards the synaptic terminal (Nixon *et al.*, 1987; Lewis and Nixon, 1988).

Soussan *et al.*, (1994) conducted immunohistochemical studies which demonstrated that cell bodies and dendrites contain non-phosphorylated NF epitopes, whereas phosphorylated epitopes were localized mainly in axons. It was suggested by these workers that the level of NF phosphorylation increases as the distance from the cell body increases. However, Soussan *et al.*, (1994) found that variation in the distance from the cell body does not induce differences in NF-H phosphorylation.

1.8.4 Role of neurofilaments in axonal growth and development

Neuronal geometry is produced by a complex series of developmental interactions between the neuron and its local environment; for example, the direction of axonal growth and position of axonal branches are determined by the location of the environmental cues surrounding the axon (Lasek *et al.*, 1983).

The axonal cytoskeleton consists of NFs and MTs ordered into a continuous network by numerous cross-bridges. Renewal of the axonal network is by the addition of newly synthesized subunits in the cell body. Newly synthesized NF proteins are assembled into stable polymers within minutes after being synthesized in the cell body (reviewed by Grafstein and Forman, 1980).

These cytoskeletal polymers are transported in the form of assembled NF proteins and studies conducted on axonal transport of radiolabeled NF proteins suggest that the NF triplet protein moves coherently in a non-diffused form (Hoffman and Lasek, 1975; Black and Lasek, 1980). These NF proteins are rapidly incorporated into the cytoskeletal network of the axon. The basic axonal cytoskeletal pattern of synthesis, assembly and transport of NFs is established within the cell body and is conveyed into the axon by axonal transport, along the axon (Grafstein and Forman, 1980; Lasek *et al.*, 1983).

NFs play a more important role in the axon compared to the neuronal cell body. Axonal transport studies have shown that the cytoskeletal proteins do not degrade significantly whilst in transit between the cell body and the end of the axon. However, degradation will occur after the cytoskeletal elements have reached the axon terminal. Proteolytic degradation of NFs is an irreversible event that occurs selectively at the end of the axon and serves to halt the cytoskeleton's drive forward within the axon. Proteases that degrade NFs are calcium activated and are involved in mechanisms that control the length of the axon (Shea *et al.*, 1991).

degrade NFs are calcium activated and are involved in mechanisms that control the length of the axon (Shea *et al.*, 1991).

1.8.5 NFs as useful markers for neurodegenerative agents

There are a number of neurodegenerative agents which alter phosphorylation of neurofilaments; such agents include acrylamide, aluminum, β,β'-iminodipropionitrile (IDPN), 2,5-hexanedione (2,5-HD) and certain organophosphates (OPs) (reviewed by Hargreaves *et al.*, 1997). Generally, these agents can induce central and/or peripheral neuropathies with the early stages involving the aggregation of cytoskeletal proteins and the inhibition of axonal transport processes that are dependent on the integrity of neuronal cytoskeleton.

As described previously, the acute toxic effect of OPs involves the suppression of acetylcholinesterase, but with certain OPs such as tricresyl phosphate (TCP) these compounds are capable of inducing organophosphate delayed neurotoxicity (OPIDN) up to several weeks after exposure. However, the molecular basis of cytoskeletal disruption is not fully understood. *In vitro* studies have shown some changes; for example, Jensen *et al* (1992) found anomalous aggregation of phosphorylated NFs in central and peripheral axons which preceded axonal swelling and degeneration (Abou-Donia and Lapadula, 1990; Johnson 1990; Glynn, 1999; 2000).

1.9 The use of mammalian cell lines for *in vitro* studies

1.9.1 Major cell types in the nervous system

In the regulation of the mammalian central nervous system, the neuronal and glial cells play interactive roles. The neuronal cell's ultimate function is to communicate to other neighbouring neurons or effector cells through the transmission of impulses along axons. Glial cells do not participate in the active generation of impulses, nor do they produce axons or have a direct role to play in the information processing functions of the nervous system. However, some types of glial cells are influenced by neuronal activity and help regulate the ionic environment of the neurons and also remove some

neurotransmitters released by neurons. Therefore, neurons and glial cells are morphologically and functionally distinct cell types (Brown, 1991) either or both of which could be affected by neurotoxins *in vivo*.

1.9.2 Morphological features of nerve cells

Structurally, a differentiated neuronal cell consists of a cell body, an axon and a growth cone (figure 1.5). The cell body contains the nucleus and other organelles such as endoplasmic reticulum, ribosomes and Golgi apparatus. From this cell body extends a long projection known as the axon, which is extremely important for neurotransmission and axonal transport. The length of the axon varies greatly, being up to a thousand times longer than the cell body (Alberts *et al.*, 1983). These morphological features can be readily studied in a controlled environment using cultured neuronal cells (Flaskos *et al.*, 1994; 1998; Henschler *et al.*, 1992; Schmuck and Ahr, 1997).

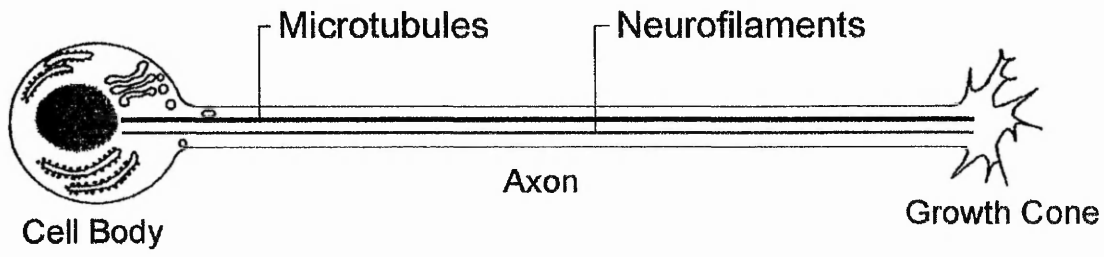


Figure 1.5. Schematic diagram of a neurone (adapted from Alberts et al., 1983).

1.9.3 The role of the cytoskeletal proteins in the growing axon

The role of the cytoskeleton in the development of the axon has been extensively studied through cellular systems and as described in sections 1.6, 1.7 and 1.8, cytoskeletal proteins such as MTs and NFs are essential for the growth, development and maintenance of the cell and its related neurites. The earliest events are believed to involve the reorganisation of the actin cytoskeleton at the cell membrane. From studies of PC12 cells and granule cells *in vitro*, actin appears to be important in the early stages

of outgrowth (Paves *et al.*, 1988; Cambray-Deakin *et al.*, 1987a). As the developing neurite extends, its growth cone (section 1.9.4) remains an actin-dominated zone high in calcium (Kater *et al.*, 1988). Behind the growth cone microtubules begin to be assembled, possibly due to a fall in calcium concentration. With time, the axonal cytoskeleton becomes generally more stable and associated with this stability is an increase in post-translationally modified tubulin and NF subunits (Cambray-Deakin, 1991). However, IFs in neurons are suggested to be involved in control of axonal diameter (Hoffman *et al.*, 1987). As the axon extends, its cytoskeleton behind the growth cone becomes more and more post-translationally modified by phosphorylation and acetylation. When synaptogenesis occurs there appears to be a reorganisation of the cytoskeleton (Burgoyne and Cambray-Deakin, 1988).

1.9.4 Role of the growth cone and growth-associated proteins in axon development

Studies using cultured cell lines have shown that the axon terminates within a structure known as the growth cone; this is a broad flat expansion with many long microspikes extending from it with the growth of the axon occurring via the growth cone (Gordon-Weeks, 1989). Goslin *et al.*, (1989) have found that a growth cone component interacts with microfilaments (MFs) and MTs both indirectly and directly, suggesting a link between axonal microtubules and elements of the growth cone cytoskeleton.

The growth cone determines the direction of growth by interpreting environmental cues found on cell surfaces (Dodd and Jessell, 1988) and its mobility is due to the presence of dynamic surface extensions such as microspikes or filopodia (in the case of nerve cells). Many cells extend thin, stiff protrusions which are about 0.1 μm wide and 5 to 10 μm long and contain a loose bundle of about 20 actin filaments (Diaz-Nido *et al.*, 1996; Alberts *et al.*, 1994). At the leading edge of the growth cone, actin filaments appear to organise themselves with the plus end attached to the membrane. It is at this edge that new microfilaments are nucleated and where monomers are subsequently added to the actin filament allowing them to elongate (Alberts *et al.*, 1994).

Research has shown that a protein known as growth associated protein-43 (GAP-43) is prominent within growth cone membranes and it has been hypothesized that this protein

regulates neurite growth (Skene, 1989; Pekiner *et al.*, 1996). GAP-43 occurs as a group of membrane-bound, rapidly transported (Group I) proteins with very acidic isoelectric points (4.3 – 4.5) and molecular weights ranging from 43 – 57 kDa (Skene, 1989). It is believed that GAP-43 is important for the initiation of the growth cone and also involved in the later stages of axonal development and maturity (Hall, 1982; Meiri *et al.*, 1986). Elevated GAP-43 expression continues throughout the period of axon elongation and synaptogenesis in all developing and regenerating systems studied (Skene, 1989; Pekiner *et al.*, 1996).

It has been documented that growth cones generate spontaneous transient elevations of intracellular calcium that regulate the rate of neurite outgrowth and that pulses of calcium inhibit neurite extension in Xenopus spinal neurons (Lautemilch and Spitzer, 2000). In neurons, it has been suggested that protein kinase C-regulated phosphorylation of GAP-43 may represent an important mechanism to transduce guiding signals into actin-cytoskeletal responses, mediating directed axonal growth (Rosner and Vacun, 1999). Also, it has been suggested by Meiri *et al.*, (1998) that stimulation of neurite outgrowth by neuronal cell adhesion molecules (NCAM) requires GAP-43 function.

1.9.5 Rationale for current *in vitro* studies of OP toxicity

As a direct consequence of the ethical and financial problems associated with animal experiments, together with the time consuming and laborious nature of such an approach for mechanistic studies, the introduction of cell culture systems was needed to allow the study of toxicity targeted at a specific cell type. Due to the relative inaccessibility and complexity of the nervous system, the use of cell cultures to study OPIDN is even more appropriate, as NTE activity is found in both differentiated and undifferentiated cell lines of human and mouse origin (Fedalei and Nardone, 1983; Carrington *et al.*, 1985). However, there have only been a few studies that have measured NTE inhibition in cultured neuronal cells (Nostrandt and Ehrich, 1992; El-Fawal and Ehrich, 1993).

OPIDN studies employing cell cultures and measuring parameters other than NTE have, so far, dealt with these systems more as a tool for predicting OPIDN rather than as a means for arriving rationally at an underlying mechanism of action (Veronesi, 1992). Such a system should provide a controlled environment for studying the sequence of events that occurs between phosphorylation, aging of NTE and the production of axonal degeneration as described *in vivo* by various research groups (Johnson *et al.*, 1980; 1990; Abou-Donia *et al.*, 1988; 1990; 1993). However, the problem with such cellular systems is that they lack the ability to simulate systemic effects as seen in animal models. For example, cellular systems are unable to mimic the function and action of the liver when toxic agents are bioactivated or degraded, bioeliminated and excreted from the body.

It is therefore important to use a relevant cell culture system which can be exploited towards the clarification of the biochemical mechanism responsible for the development of OPIDN. This, in turn, might lead to the identification of novel relevant endpoints that can be used for applied testing purposes (Flaskos, 1995).

1.9.6 Current *in vitro* mammalian cell line studies

Clearly, a major advantage of cellular systems is that they, permit evaluation of the direct effects of OPs on target cells as an alternative to animal studies, i.e. in primary screening of compounds. Not only is qualitative assessment of cytotoxic effects possible but the investigators can also measure intracellular changes at the molecular level. This has led to several publications by Fedalei and Nardone., (1983), Henschler *et al.*, (1992), Nostrandt and Ehrich, (1992), Ehrich *et al.*, (1994), Flaskos *et al.*, (1994), Ehrich, (1995) and Schmuck and Ahr, (1997) involving the use of *in vitro* cellular models to examine the neurotoxic effects of OPs.

In the majority of these studies, neuroblastoma cell culture lines have been used such as human neuroblastoma clones C-1300, SH-SY5Y, N-18, IMR32, rat PC12 pheochromocytoma, mouse N2a neuroblastoma, with work also being conducted on a rat C6 glioma cell line. Some of these cell lines have been used to evaluate levels of

NTE inhibition when exposed to neuropathic OP esters, as an alternative to hen brain homogenates from hens exposed to OP esters (Fedalei and Nardone., 1983).

However, Flaskos *et al.*, (1994) and Henschler *et al.*, (1992) have been interested in the effects of OPs on the cellular processes produced by both neuroblastoma and glioma cell lines. Experimental work conducted by Henschler *et al.*, (1992) and Schmuck and Ahr (1997) used human N-18 neuroblastoma and rat C6 glioma cell lines to screen a large number of OPs. In each case these cells were cultured, differentiated and treated with test compound for up to 3 weeks before the final assessment of the effects was made. This lengthy incubation led to high cytotoxicity levels, suggesting that the cultured cells were unhealthy. Furthermore, the conditions to which the cultured cells were exposed were not close to those seen when OPIDN is induced *in vivo* as the inhibition of NTE and of cytoskeletal proteins has been seen to occur within 24 hours of exposure to OPs (Johnson, 1977; 1987; Abou-Donia, 1993; 1995).

Work conducted by Flaskos *et al.*, (1994; 1998) has focused on the development of a more mechanistic approach, in which sublethal doses have been studied over short time intervals. Cultured cell lines were chosen with the purpose of the experiment in mind. For example, PC12 cells were used for studies of axon maintenance, as these cells can be effectively maintained in culture for several weeks before cells are lost through cell death and found that subcytotoixc levels of TCP (1 μg/ml) were found to inhibit the maintenance of neurites (Flaskos *et al.*, 1994). However, neuroblastoma and glioma cell lines were used to show the initial and early effects of test compounds, with no effect on cell viability after a 48 hour interval (Flaskos *et al.*, 1994; 1998). A disadvantage of the cultured cellular system is that it lacks the systemic effects of other organs such as the liver, however in the present work, attempts have been made to mimic the effects of the liver to bioactivate/inactivate toxin using microsomal activation (Sprague and Castle, 1985).

From this the neuronal and glial cell models have been established, allowing successful analysis of effects of test compounds on both proliferating and differentiated cells. The cell lines used for this study were N2a, a mouse neuroblastoma cell line, and C6, a rat glioma cell line, as mentioned above. These two cell lines are of particular interest as

they have been extensively used in research on neurite outgrowth (Hargreaves *et al.*, 1989; Shea *et al.*, 1991; 1995; Henschler *et al.*, 1992; Schmuck and Ahr, 1997; Flaskos *et al.*, 1998) and current work indicates that they demonstrate selective neurotoxic effects when exposed to neuropathic OP esters such as TCP, T*O*CP and TCL (Flaskos *et al.*, 1998; 1999).

1.10 Project Aim

As described in the introduction, it has been proposed that there are two biochemical lesions that precede the onset of OPIDN (1) organophosphorylation of NTE, and (2) the disruption of axonal cytoskeleton through altered protein phosphorylation. As the majority of research to date has concentrated on the effects of OPs *in vivo*, it was deemed important to determine whether these effects can also be seen in an *in vitro* situation, in order to provide a system that would, in due course, allow a further understanding of the molecular events involved. The aims of this work, therefore, were to investigate the neurotoxic effects of a known neuropathic OP, TCP (commercial product), two of its isomers, T*O*CP and T*P*CP and the metabolite CPSP on neurite outgrowth in cultured mouse N2a neuroblastoma cells and rat C6 glioma cells.

Experiments will be performed to :-

- investigate the morphological changes in axon outgrowth in N2a and C6 cell lines with TCP, T*O*CP, T*P*CP and CPSP over a range of time points.
- study neurotoxic markers in N2a cells when exposed to TCP, T*O*CP, T*P*CP and CPSP.
- investigate the *in vitro* effects of microsomal activation of TCP, T*O*CP and T*P*CP on the outgrowth of neurites by differentiating N2a cells.
- study the protective effect of conditioned medium (CM) produced by differentiated C6 cells, on differentiating N2a cells exposed to the compounds mentioned above.
- develop an *ex vivo* model to examine the effects of T*O*CP and T*P*CP on extracted rat vagus nerves.

It was expected that these experiments would help to establish the early cellular changes that occur when N2a cells are exposed to OPs, leading to a clearer understanding of the neurodegenerative effects of TCP, its isomers and one of its metabolites.

Chapter Two

Materials and Methods

2.1 Materials

All chemicals used for buffers and media were of the highest grade and purchased from Sigma-Aldrich Company Ltd (Poole, UK) and BDH (Leicester, UK). All tissue culture plasticware used was sterile and of the highest quality available from SLS, Nottingham.

2.1.1 Special Reagents

2.1.1.1 Test Compounds

Tricresyl phosphate (TCP; mixed isomers) was purchased from Aldrich Chemical Company, USA (reference number 0690UY). Tri*ortho*cresyl phosphate (T*O*CP; reference number 222017) and tri*para*cresyl phosphate (T*P*CP; reference number 221956) were purchased from ICN Biomedical Inc., Ohio, USA.

Cyclic phenyl saligenin phosphate (CPSP) was synthesized by Dr. I Coutts, Department of Chemistry and Physics, The Nottingham Trent University.

2.1.1.2 Western blotting materials

Optitran BA-S 83 reinforced nitrocellulose (membrane pore size 0.2 μm) was purchased from Schleicher & Schuell, Dassell, Germany. Whatman No.1 Filter Paper was purchased from Whatman, Kent, UK.

2.1.1.3 Primary monoclonal antibodies

RMd09 (anti-non-phosphorylated NF-H) and Ta51 (anti-phosphorylated NF-H) monoclonal antibodies were a gift from Dr. M. Carden, University of Kent, Canterbury. Anti-NF-H (phosphorylation independent; clone number N52), anti-MAP1 (clone number HM-1), anti-MAP1b (clone number AA6) and anti-GAP-43 (clone number GAP-7B10), anti-acetylated tubulin (clone number 6-11B-1) and anti-α-tubulin (B-5-1-2) were purchased from Sigma-Aldrich.

2.1.2 Animals

Male Wistar rats (150 - 200g) bred in house were used for the preparation of microsomes from liver (two used). Female Wistar rats (150-200g) bred at the University of Liverpool, Liverpool were used for vagus nerve and sciatic nerve experiments, *ex vivo* (24 used).

2.2 Methods

2.2.1 Tissue Culture Techniques

2.2.1.1 Cell lines

Mouse N2a neuroblastoma and rat C6 glioma cell lines were obtained from Flow Laboratories, Irvine, UK or the European Tissue Culture Collection (ETCC).

2.2.1.2 Maintenance and growth of cell lines

The cell lines were grown at 37 °C in a Jouan IG150 incubator with a humidified atmosphere of 5 % (v/v) CO_2 / 95 % (v/v) air. They were cultured in sterile growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing sodium bicarbonate and high glucose and supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, penicillin G (100 units/ml) and streptomycin (100 µg/ml).

The cells were maintained in the logarithmic phase of growth and passaged at 3 to 5 day intervals. The monolayer of cells was detached from the flask by using gentle jets of growth medium from a Pasteur pipette, centrifuged for 7 minutes at 1,000 rpm and resuspended in fresh growth medium in a sterile T25 cell culture flask. To ensure sterility of all procedures conducted on the used cell lines, a Class II (Gelaire BSB 4) laminar flow hood was used at all times.

2.2.1.3 Resuscitation N2a and C6 cells from liquid nitrogen store

A vial of cells was removed from the liquid nitrogen container and thawed quickly by incubation in a water bath set at 37 °C. The contents were diluted in 10 ml fresh growth medium and centrifuged for 7 minutes at 1,000 rpm. The cells were then resuspended and placed in a sterile T25 flask containing 10 ml fresh growth medium and left for 24 hours to allow the cells to form a monolayer, after which the growth medium was replaced. Cell growth was monitored until the cells were 60 - 80 % confluent, at which point they were deemed ready to be passaged for experimental use.

2.2.1.4 Cryopreservation of N2a and C6 cell lines in liquid nitrogen

The contents of a T25 flask of confluent cells were resuspended and centrifuged for 7 minutes at 1,000 rpm. The supernatant was discarded and the pellet gently resuspended in 1 ml of freezing medium [65 % (v/v) DMEM containing L-glutamine, penicillin G and streptomycin as mentioned above, 25 % (v/v) foetal bovine serum and 10 % (v/v) DMSO]. The cell suspension was placed into a cryo-vial lagged in tissue paper and incubated overnight at -70 °C. It was then transferred to a liquid nitrogen container for permanent storage.

2.2.1.5 Induction of cell differentiation in the presence or absence of test compounds

N2a and C6 cell lines were plated out at an initial density of 50,000 cells / ml in sterile 24 well culture dishes. The cells were grown for 24 hours in growth medium prior to the induction of cell differentiation in the presence or absence of various concentrations of test compounds.

N2a cell differentiation was induced by serum removal and the addition of 0.3 mM dibutyryl cyclic AMP in serum-free medium [DMEM containing L-glutamine, penicillin G and streptomycin at the concentrations mentioned above] (Shea *et al.*, 1991). The required test compound concentration was added freshly to the serum-free medium prior to its addition to the cells.

C6 cells were induced to differentiate by the addition of 2 mM sodium butyrate diluted in serum-free medium (Hargreaves *et al.*, 1989). Again various concentrations of test compound were added to the serum-free medium just prior to its addition to the cells.

Plates were then incubated for up to 48 hours, depending on the required exposure time.

2.2.1.6 Fixing and Staining of differentiated N2a and C6 cells

After incubating for the required length of time, the cells were fixed at -20 °C for 30 minutes in a fixing solution containing 10 % (v/v) methanol in 90 % (v/v) Tris buffered saline [TBS: 50 mM Tris, 200 mM NaCl; pH 7.4]. All solutions were adjusted to the correct pH at 25°C using a pH meter Model PW9409 purchased from Phillips (Pye-Unicom), Cambridge, UK unless otherwise stated.

The fixed cells were then stained for 1 minute at room temperature with Coomassie blue stain [1.25 g Coomassie blue-R250, 10 % (v/v) glacial acetic acid, 40 % methanol, 50 % (v/v) distilled water] washed with distilled water and allowed to air dry overnight. The stained cells were then viewed with the aid of an inverted light microscope.

2.2.1.7 Morphological measurements of fixed differentiated N2a and C6 cells

The total number of cells and the proportion of round versus flat cells were determined in each of five random fields per well. Neurites (cellular processes produced on cell differentiation) were also measured, as indicated below. The microscope used was an Olympus CK2 inverted light microscope (Olympus Optical Company Ltd., London, UK).

In the case of N2a cells, neurites were subdivided into :

(a) **extensions** : these are defined as processes of 0.5 - 2 cell body diameters in length, and

(b) **axon-like processes** : these are defined as processes that are greater than 2 cell body diameters in length with an extension foot (Keilbaugh *et al.*, 1991).

In the case of C6 cells, only one value was recorded. This was the number of cellular extensions, all of which were greater than 2 cell body diameters in length.

Cell shape has been divided into two different categories, round and flat cells. "Round" cells are typically round in shape and have a smooth rounded appearance, whereas "flat" cells have a more flattened and irregular appearance. This distinction helps to determine whether the test compound has a cytotoxic effect on the cells causing them to round up.

In some experiments, the lengths of N2a axon-like processes and C6 extensions were measured using an eyepiece graticule, which had been previously calibrated with a stage graticule.

2.2.1.8 Preparation of test compounds

The test compounds were diluted from a stock solution 200 µg/ml in absolute ethanol, which was present at a final concentration 0.05 % (v/v) in all samples. The concentrations of test compound were chosen on the basis that they were not considered to be cytotoxic towards proliferating cells, as determined by a number of cell viability assays such as the incorporation of ^{3}H-thymidine into DNA, Trypan blue exclusion and reduction of methyl blue tetrazolium (MTT) (Flaskos *et al.*, 1994). The control used was prepared in sterile DMEM with absolute ethanol, again giving a final concentration of 0.05 % (v/v).

2.2.1.9 Measurement of cell viability by the reduction of MTT

Cells were plated out at an initial density of 50,000 cells/ml in sterile 24 well culture dishes and induced to differentiate in the presence or absence of the test compound as described above. Thirty minutes prior to the end of the incubation, 50 µl MTT [5 mg MTT/ml phosphate buffered saline [PBS: 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4] were added to each well and the cells were incubated for the remainder of the incubation time in the designated incubator. The medium was removed from the wells and 1 ml of DMSO was added, the plates were agitated gently and the absorbance was read in a spectrophotometer at a wavelength of 570 nm (Mosmann, 1983; Sladowski *et al.*, 1993).

Time course experiments were initially undertaken to ensure that all subsequent experiments were assessed within the linear section of the reaction.

2.2.1.10 Trypan blue exclusion assay

Cells were plated out at an initial density of 50,000 cells / ml in sterile 24 well culture dishes and induced to differentiate in the presence or absence of the test compound as described previously. After the required incubation time, the cells were detached from the monolayer into suspension. Fifty µl of cell suspension were added to 50 µl of Trypan blue and mixed thoroughly. Ten µl of the cell suspension were applied on to a haemocytometer chamber for counting (Neubauer haemocytometer 1/400 mm^2 (B.S.748)). Cell viability was assessed by the cells' ability to exclude the dye and using the following equation :

$$\text{Cell viability} = \frac{\text{Live cells}}{\text{Total number of cells}} \times 100$$

2.2.1.11 Production of conditioned medium from differentiated rat C6 glioma cells

C6 cell line was plated out at an initial density of 50,000 cells / ml in sterile T25 culture flasks. The cells were grown for 24 hours in growth medium prior to the induction of cell differentiation. The cells were induced to differentiate by the addition of 2 mM sodium butyrate in serum-free medium (Hargreaves *et al.*, 1989). After a three day incubation the conditioned medium (CM; serum-free medium including secreted growth factors) was collected and stored at -70 °C until required.

2.2.1.12 Protective experiment using CM from differentiated rat C6 cells

When CM was used in protective experiments, the procedure was followed as described in section 2.2.1.5. However when the N2a cells were differentiated, the medium used was made from serum-free medium and CM in a ratio of 3 parts to 1 part (3 : 1), including 0.3 mM dibutyryl cyclic AMP as before.

2.2.2 One-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.2.1 Preparation of N2a and C6 cell extracts for gel electrophoresis and Western blotting

Cells were plated out at an initial density of 50,000 cells / ml into sterile T25 vented tissue culture flasks. The cells were grown for 24 hours and induced to differentiate in the presence or absence of the test compound as described previously.

After incubating for the required length of time, the cell monolayer was rinsed gently with ice cold DMEM with care being taken not to dislodge the cells. The cells were removed into 1 ml of 0.1 % (w/v) SDS in PBS, boiled for 1 minute and placed in an Eppendorf tube. The cell suspension was vortex mixed for approximately 1 minute and 50 µl aliquots were taken for protein estimation as described in section 2.2.4.

Once the protein content had been estimated, 100 µl of cell suspension were added to 100 µl of electrophoresis sample buffer [0.1M Tris-HCl, pH 6.8, 50 mM DTT, 3 % (w/v) sodium dodecyl sulphate (SDS), 20 % (w/v) glycerol, 0.1 % (w/v) bromophenol blue], denatured by boiling for 10 minutes and centrifuged for 1 minute at 10,000 g. Denatured proteins were then stored at -20°C until required.

2.2.2.2 Separation of proteins in cell extracts by gel electrophoresis

One-dimensional SDS-PAGE was performed in a 7.5 % (w/v) polyacrylamide resolving gel overlaid with a 4 % (w/v) stacking gel (Laemmli, 1970). A BIORAD MiniProtean® II electrophoresis cell kit was used and the gel plates were washed and cleaned with absolute ethanol prior to assembly. Once assembled and clamped on to the gel casting stand, the 7.5 % (w/v) resolving gel mixture was prepared as indicated below. It was then polymerised by the addition of 100 µl of 10 % (w/v) ammonium persulphate (APS) and 10 µl of N,N,N',N'-tetramethyl-ethylenediamine (TEMED). When the resolving gel had been poured between the glass plates, a layer of water-saturated butan-2-ol was added to the top to prevent any gel shrinkage.

Once the resolving gel had polymerized, the layer of butan-2-ol was rinsed off using SDS-PAGE buffer [25.6 mM Tris-base, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3]. The resolving gel was then overlaid with a 4 % (w/v) stacking gel and was polymerized in the presence of 50 µl of APS and 20 µl of TEMED.

7.5 % Resolving gel (10 ml) :

Reagent	Volume
40 % (w/v) acrylamide	1.88 ml
1.5 M Tris-HCl, pH 8.8	2.50 ml
10 % (w/v) SDS	100 µl
Distilled water	5.52 ml

4 % Stacking gel (10 ml) :

Reagent	Volume
40 % (w/v) acrylamide	1.00 ml
0.5 M Tris-HCl, pH 6.8	2.50 ml
10 % (w/v) SDS	100 µl
Distilled water	6.40 ml

Prior to the loading of cell extracts, the polymerised gels and clamp were removed from the casting stand and loaded into the inner cooling core of the electrophoresis tank and then into the buffer tank. The gels were totally submerged in SDS-PAGE buffer with care being taken to ensure all air bubbles were excluded.

Before separation by SDS-PAGE, the previously prepared cell extracts and molecular weight markers (SDS-PAGE pre-stained molecular weight standards ranging from 205,000 - 33,500 kDa) were boiled for 10 minutes, vortex mixed and loaded into the stacking gel. The gels were run at 40 mA per gel until the dye front had just reached the bottom of the gel, using the BIORAD Power Pac 300.

The gels were either stained with Coomassie brilliant blue, colloidal brilliant blue or silver staining reagent to visualize the protein bands and molecular weight markers. Alternatively, the proteins were electrophoretically transferred on to nitrocellulose membrane.

2.2.2.3 Staining polyacrylamide gels

2.2.2.3.1 Coomassie blue stain

The polyacrylamide gel was removed from the clamps and placed into a glass tray containing 1.25 % (w/v) Coomassie blue-R250 in a solution of 25 % (v/v) ethanol and 10 % (v/v) glacial acetic acid. The gel was placed on an orbital shaker allowing the uptake of Coomassie blue (Orbital shaker SO1 purchased from Stuart Scientific, Loughborough, UK). After up to 24 hours, the protein bands on the gel were viewed on a light box. In order to remove background colour, the gel was placed into a destaining solution [25 % (v/v) ethanol, 10 % (v/v) glacial acetic acid] on the orbital shaker and viewed after several hours.

2.2.2.3.2 Brilliant blue colloidal concentrate staining

As described above, after electrophoresis the gels were removed and placed in a glass tray containing a fixing solution containing 7 % (v/v) glacial acetic acid and 40 % (v/v) methanol. Prior to staining, 4 parts of the working concentrate was added to 1 part methanol and vortex mixed for 30 seconds. The staining suspension was added to the gel and left for 1 to 2 hours at room temperature and then viewed on a light box. If the gels required destaining, a two step procedure was followed. Firstly, a destain solution was added consisting of 10 % (v/v) glacial acetic acid and 25 % (v/v) methanol for 30

seconds and then the gels were rinsed twice in 25 % (v/v) methanol for 10 minutes (Neuhoff *et al.*, 1988).

2.2.2.3.3 BIORAD Silver stain

The electrophoresis polyacrylamide gels were placed in a fixative solution [40 % (v/v) methanol, 10 % (v/v) glacial acetic acid] overnight at room temperature. The gels were then incubated in the presence of the oxidizer [10 % (v/v) oxidizer in distilled water] for 10 minutes at room temperature on an orbital shaker. The oxidizer was removed and the gel was rinsed by three 10 minute washes with distilled water. The silver reagent [10 % (v/v) silver reagent in distilled water] was added to the gels after all the colour had been removed for a 30 minute incubation period. After this time the silver reagent was discarded and replaced with distilled water for 2 minutes. The developer [8 g developer in 250 ml distilled water] was then added for approximately 30 seconds or until the solution became cloudy, poured away and fresh developer added. This procedure was repeated until protein bands developed. To stop the reaction the gels were flooded with stop solution [5 % (v/v) glacial acetic acid in distilled water] (Merril *et al.*, 1981). The stained gels were viewed on a light box.

2.2.2.4 Western blotting of cell extracts separated by SDS-PAGE

The separated proteins were transferred electrophoretically on to nitrocellulose membrane filters in electroblotting buffer [39 mM glycine, 48 mM Tris-base, 0.0375 % (w/v) SDS, 20 % (v/v) methanol] (Towbin *et al.*, 1979). These proteins were transferred using the BIORAD Trans-Blot electrophoretic transfer cell at 30 V overnight at room temperature. The resultant Western blots were either dried between two pieces of clean filter paper and stored at room temperature or probed with relevant antibodies, as discussed in section 2.2.2.6.

2.2.2.5 Staining of Western blots using copper phthalocyanine 3,4',4",4"' tetrasulphonic acid (tetrasodium salt)

In order to assess the efficiency of the transfer of the protein bands, the Western blot was submerged in copper phthalocyanine 3,4',4",4"' tetrasulphonic acid (tetrasodium salt) staining solution [0.05 % (w/v) copper phthalocyanine 3,4',4",4"' tetrasulphonic acid (tetrasodium salt) in 12 mM hydrochloric acid in distilled water] for approximately 2 minutes, after which the protein bands on the nitrocellulose would have taken up the stain. The blot was destained by submerging it in a solution of 12mM sodium hydroxide (Bickar and Reid, 1992).

2.2.2.6 Immunoprobing of Western blots

The resultant Western blots were blocked by incubation at room temperature with 3 % (w/v) bovine serum albumin (BSA) in TBS (BSA/TBS) for at least 1 hour before being probed with primary antibodies overnight at 4°C.

The primary (monoclonal) antibodies used to probe the Western blots were :

1. RMd09 (which recognizes a non-phosphorylation dependent epitope on NF-H) and Ta51 (which recognizes a phosphorylation dependent epitope on NF-H), both of which were diluted 1/200 in BSA/TBS,
2. anti-NFH (phosphorylation independent), anti-MAP1, anti-MAP1b and anti-GAP-43, which were all diluted 1/500 in BSA/TBS, and
3. anti-acetylated tubulin and anti-α-tubulin, both diluted at 1/2000 in BSA/TBS.

The probed blot was washed for three 20 minute periods with TBS containing 0.1 % (v/v) Tween-20 (TBS/Tween) and then incubated for 2 hours at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG, diluted 1/1000 in 3 % (w/v) BSA/TBS (Dako Ltd, Cambridge, UK).

After extensive washings in TBS/Tween and a final wash in TBS, the blots were allowed to equilibrate in substrate buffer [0.75 M Tris-base, pH 9.5] for 5 minutes. The

developer was then added to the blots [33 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) [50 mg BCIP in dimethyl formamide (DMF)] and 44 µl nitro blue tetrazolium (NBT) [75 mg NBT in 70 % (v/v) DMF] added to 20 ml of substrate buffer]. Antigens were revealed by incubation of the probed blots in the developer, in the dark at room temperature. The reaction was stopped by extensive washing with distilled water, after which the blots were dried between 2 pieces of clean filter paper.

2.2.3 Protein estimation

Protein estimation was performed by the method described by Lowry *et al.*, (1951). To produce a calibration curve, a set of BSA standards were prepared ranging from 0 µg to 100 µg of protein from a 1 mg / ml stock solution. The aliquots of cell extracts and BSA standards were treated identically. To each tube, 1 ml of working Lowry reagent [100 ml of 2 % (w/v) sodium carbonate in 0.1 M sodium hydroxide containing 1 % (w/v) copper sulphate and 2.7 % (w/v) sodium potassium tartate] was added. The suspensions were vortex mixed and incubated at room temperature for 15 minutes. A volume of 100 µl of Folin & Ciocalteau phenol reagent (diluted 1 : 1 (v/v) with distilled water) was then added to each suspension and incubated for 30 minutes at room temperature. After this period of time, the absorbance was read at a wavelength of 690 nm.

2.2.4 Preparation of liver microsomes from male Wistar rats

Livers were extracted from two male Wistar rats under sterile conditions. The livers were washed several times in sterile DMEM and then with sterile 67 mM phosphate buffer, pH 7.4 (PO_4 buffer) on ice. The livers were minced finely with sterile scissors and forceps, before adding ice cold PO_4 buffer (40 ml per 10 g of tissue). The sample was transferred into an ice cold, sterile Dounce homogeniser (BDH) and homogenized by hand until all pieces of intact tissue had been homogenized.

After homogenizing the liver, the suspension was transferred into ice cold centrifuge tubes and centrifuged at 10,000 g for 23 minutes at 4°C using an MSE 24M centrifuge (MSE Scientific Ltd, UK). Following this centrifugation the supernatant was decanted

into Beckman centrifuge tubes and centrifuged using the Beckman L8-70 centrifuge at 105,000 g for 65 minutes at 4°C.

The supernatants were discarded and the pellets were washed with ice cold phosphate buffer. The pellets were re-homogenized with 5 ml of ice cold PO_4 buffer and transferred into fresh Beckman centrifuge tubes before being re-centrifuged at 105,000 g for 65 minutes at 4 °C.

After centrifugation, the supernatants were discarded and the pellets were re-homogenized in 4.5 ml PO_4/KCl [PO_4 buffer containing 1.15 % (w/v) potassium chloride]. Aliquots containing 100 µl of suspension were transferred to sterile Eppendorf tubes and stored at -70 °C until required. Five microsomal aliquots were kept for protein estimation as described previously in section 2.2.4.

2.2.5 Microsomal activation of OPs in cell culture using a NADPH generating system

Cells were plated out at an initial density of 50,000 cells / ml into sterile 24 well tissue culture plates. The cells were grown for 24 hours and then made to differentiate in the presence or absence of the test compound as described earlier (section 2.2.1.5).

In order to assess the effects of microsomal activation, a NADPH generating system was required. In each experiment, a set of controls was used to check the efficiency of the system. These included a 0.05 % ethanol control and 1.0 µg/ml test compounds (as described in section 2.2.1.5) and 0.05 % ethanol control and test compound in the presence or absence of microsomes and the NADPH generating system. These controls were set up to ensure that the effects seen on the N2a cells were due to activation of the test compound.

The NADPH generating system consists of 2 mM magnesium chloride, 4 mM glucose-6-phosphate and 0.4 mM NADP in 0.5 ml of serum-free medium containing 0.3 mM cAMP. To generate the system 2 units glucose-6-phosphate dehydrogenase and 2 µg of rat microsomes were added.

To allow this system to continually recycle, the generating system, enzyme, test compound and microsomes were introduced to the cells. This was done within a sterile insert which had a porous membrane, allowing the metabolite to reach the differentiating cells (inserts for cell culture 24 well dishes with pore size 0.4 μm from Fahrenheit, South Yorkshire, UK).

These experiments were carried out over periods from 4 up to 48 hours to assess the impact of microsomal activation on the toxicity of TCP, T*O*CP and T*P*CP.

2.2.6 The effects of tri*ortho*cresyl phosphate and tri*para*cresyl phosphate on rat vagus nerve regeneration *in vitro*

Prior to the extraction of vagus nerves from female Wistar rats, nitrocellulose discs measuring 3 cm x 3 cm were prepared and sterilized in 70 % (v/v) ethanol. All dissecting equipment was sterilized in 100 % (v/v) ethanol to ensure maximum sterility throughout the procedure.

Each rat was killed, bled and had its vagus nerve including the corresponding cell body removed. Each nerve was washed in sterile medium containing DMEM and antibiotics [penicillin G (100 units/ml) and streptomycin (100 μg/ml)] in the laminar flow hood.

After washing, the nerve was placed on to the nitrocellulose disc within a small Petri dish. The Petri dish contained 2 ml Ringer medium [Roswell Park Memorial Institute (RPMI) containing 2mM glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin] and 10 μl of the test compound. Care was taken to ensure that the nerve was stretched out fully. The nerves were incubated for 24 or 48 hours in a 37°C incubator with an atmosphere of 95 % (v/v) CO_2 / 5 % (v/v) air.

After the required incubation time, each vagus nerve was frozen rapidly on dry ice, wrapped in tin foil and sealed in a cryo-vial. The vagus nerves were stored at -70 °C until required.

2.2.7 Preparation of vagus nerve for SDS-PAGE analysis

Each vagus nerve was removed from storage at -70 °C separately and prepared for SDS-PAGE on ice, to ensure that no unnecessary degradation of the nerve proteins occurred. The length of the vagus nerve was measured in centimetres by stretching the nerve out on to a cold glass plate. This length determined the correct volume of electrophoresis sample buffer that was added (250 µl sample buffer per 1 cm length of vagus nerve). The nerve was homogenized in sample buffer using a hand operated Dounce homogeniser for 4 minutes, ensuring that the nerve was ground and only a small sheath was left. The suspension was placed in an Eppendorf tube, boiled for 5 minutes and centrifuged for 10 minutes at 10,000 g. The solubilised extract was stored at -20 °C until required.

2.2.8 Data manipulation

2.2.8.1 Statistical analysis

The statistical analysis performed on the morphological and molecular data was the Mann-Whitney U test where $p < 0.05$. This was used because it is non-parametric and tests for differences in two populations/samples.

2.2.8.2 Graphical representation

The IC_{50} data was plotted using the Microsoft Excel package. From these data, an exponential trendline was added with all other settings being custom settings. In order to determine the IC_{50} values for each experiment, the individual experimental data was plotted on graph paper by hand and from this the value was calculated. An example of this calculation is represented in the thesis.

2.2.8.3 Western blot quantification using QuantiScan package

Each Western blot was assessed using a densitometry package (QuantiScan), to give quantification to the visual blot data. Scanned readings were taken of whole bands ensuring minimal contamination with areas outside the band. As a standard, in all cases the background colour was subtracted before each band was scanned using a function within the package.

Chapter Three

Effects of the commercial product, tricresyl phosphate (TCP) on differentiating mouse N2a neuroblastoma and rat C6 glioma cells

3.1 Introduction

Tricresyl phosphate (TCP) is a commercially available OP which is widely used as a plasticizer, as a component of industrial hydraulic fluids and as a lubricant additive for applications such as in jet aircraft engines (Bondy *et al.*, 1960; Daughtrey *et al.*, 1996).

The commercial preparations of TCP used in this project are 95 % pure containing three isomers, *-ortho*, *-para* and *-meta,* with the percentage of the *-ortho* isomers varying between 25 and 40 %. Studies have shown that the *-ortho* isomer of TCP, known as T*O*CP, is capable of inducing a delayed neuropathy known as OP-induced delayed neuropathy (OPIDN) (Bondy *et al.*, 1960; Glees and Janzik, 1965). Consequently, the percentage of the *-ortho* isomer present within the industrial product has been controlled and set within strict limits of no more than 3 %. However, it has been documented that this level does not remove the toxic effect from the compound (Bondy *et al.*, 1960). TCP is, therefore, far too toxic to be used in connection with food packaging or in materials that come into contact with the body, as it is readily absorbed through intact skin (Hodge and Sterner, 1943).

The molecular basis of OPIDN has not yet been resolved, although animal studies have shown that the onset of clinical symptoms of OPIDN after exposure to TCP is preceded by a number of characteristic biochemical events (Johnson, 1990; Lapadula *et al.*, 1991; Suwita *et al.*, 1986). It has been suggested that the early disruption of the neuronal cytoskeleton plays a significant part in the onset of this disorder (Abou-Donia and Lapadula, 1990; Lapadula *et al.*, 1991; Suwita *et al.*, 1986).

Many animal studies have been conducted to study and evaluate the acute and delayed effects of OP neurotoxicity *in vivo*. However, such experiments and procedures have become very costly and time consuming (Abou-Donia, 1992). Therefore, this argues in favour of an *in vitro* cellular system to study the direct effects of OPs on cultured cells, establishing a simple and effective method for screening OP neurotoxicity.

Research has been conducted by Flaskos *et al.*, (1994; 1998) on an *in vitro* cellular system to help study effects of OPs. Consequently, they have shown that nerve growth

factor (NGF)-treated rat PC12 pheochromocytoma cells that have been exposed to non-cytotoxic levels of TCP resulted in the retraction of pre-formed extensions after 20 to 24 hour exposure (Flaskos *et al.*, 1994). Further work following on from this has shown that non-cytotoxic levels of TCP cause inhibition of outgrowth of axon-like processes in differentiating mouse N2a neuroblastoma as well as in rat PC12 pheochromocytoma cell lines (Flaskos *et al.*, 1998).

The aim of the present work was to investigate the effects of TCP exposure on differentiating mouse N2a neuroblastoma and rat C6 glioma cell lines, revealing any morphological and molecular changes that may occur. This was achieved by determination of :

(i) the non-cytotoxic levels of TCP in the cell cultures;

(ii) the ability of non-cytotoxic levels of TCP to inhibit the formation of axon-like processes or extensions in N2a and C6 cell lines after 24 and 48 hour exposure;

(iii) the IC_{50} values of TCP with respect to inhibition of neurite outgrowth for both cell lines at 24 and 48 hour time points;

(iv) disruption of cytoskeletal proteins at the same exposure times.

3.2 Morphological studies of N2a and C6 cell lines exposed to tricresyl phosphate (TCP)

In order to study the neurodegenerative effect of TCP, neurite outgrowth was measured in N2a and C6 cells induced to differentiate in the presence of TCP (sections 2.2.1.5 - 2.2.1.7)

3.2.1 Microscopic observations of N2a and C6 cells exposure to TCP

The following figures show the visual effects observed of N2a and C6 cell lines exposed to 1 μg/ml TCP (2.7μM) after 48 hours exposure.

Figure 3.1. *Differentiating N2a cells in the presence of 0.05% (v/v) ethanol (control) after a 48 hour incubation.* Projecting from each individual N2a cell are outgrowth processes defined as (a) axon-like processes (if greater than 2 cell body diameters in length) (arrows) or (b) extensions (shorter processes that are between 0.5 - 2 cell body diameters in length). This represents typical, healthy differentiating N2a cells with the appearance of large numbers of axon-like processes. All cells were fixed and stained with Coomassie blue stain.

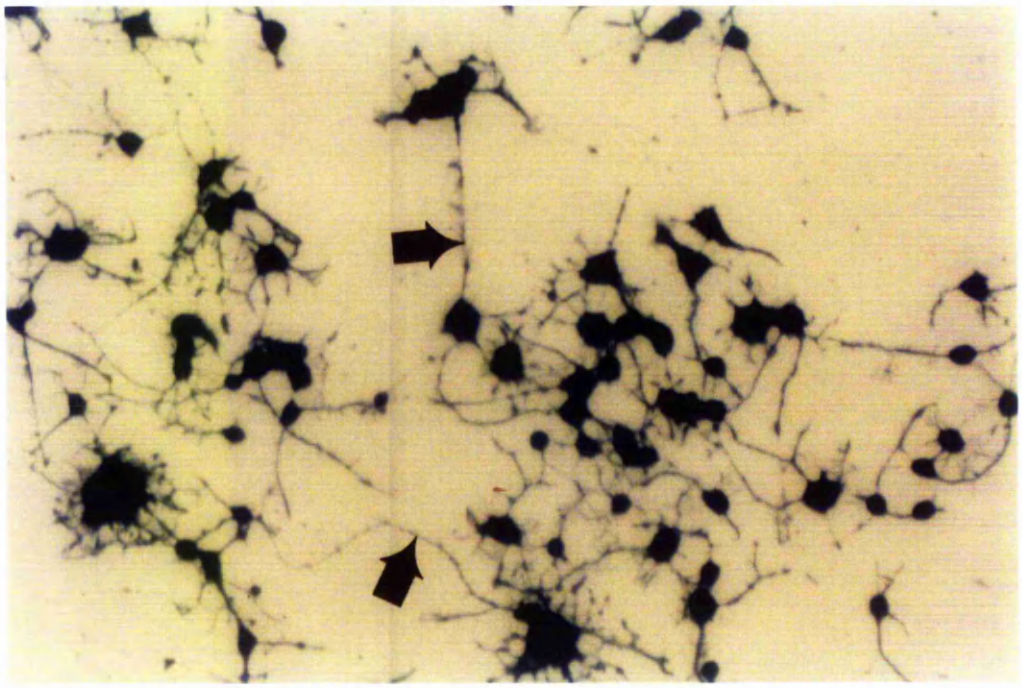


Figure 3.2. *Differentiating N2a cells incubated with 1 μg/ml TCP for 48 hours.* The absence of axon-like processes and extensions are characteristic symptoms that are seen after exposure to TCP.

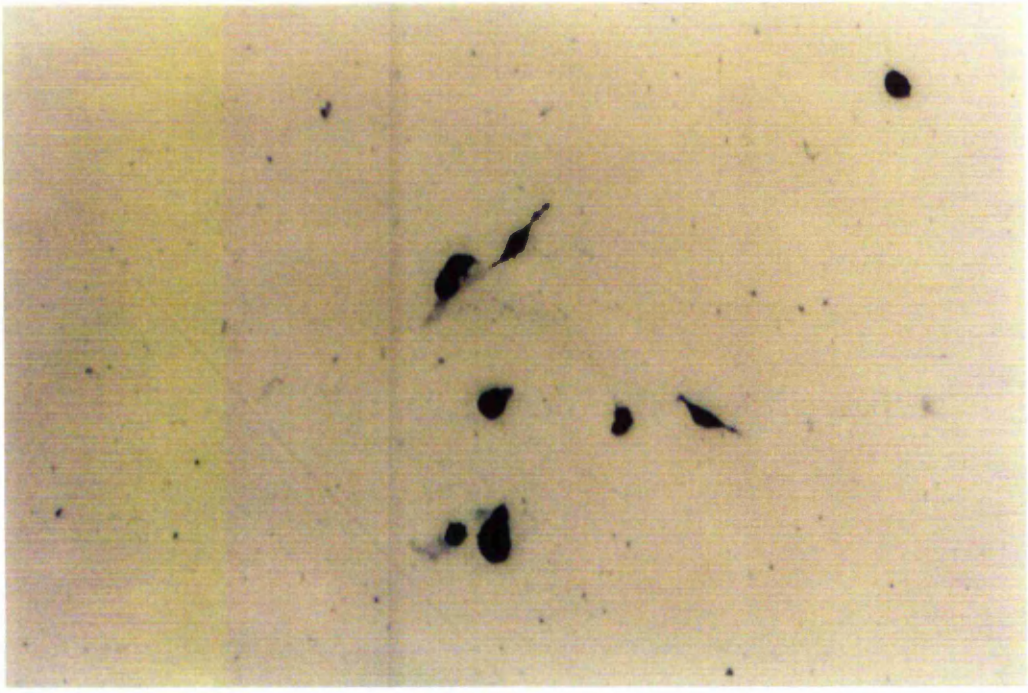


Figure 3.3. *C6 cells differentiating in the presence of 0.05 % (v/v) ethanol (control) after 48 hour exposure.* Note the presence of cellular projections which are defined as extensions (arrows).

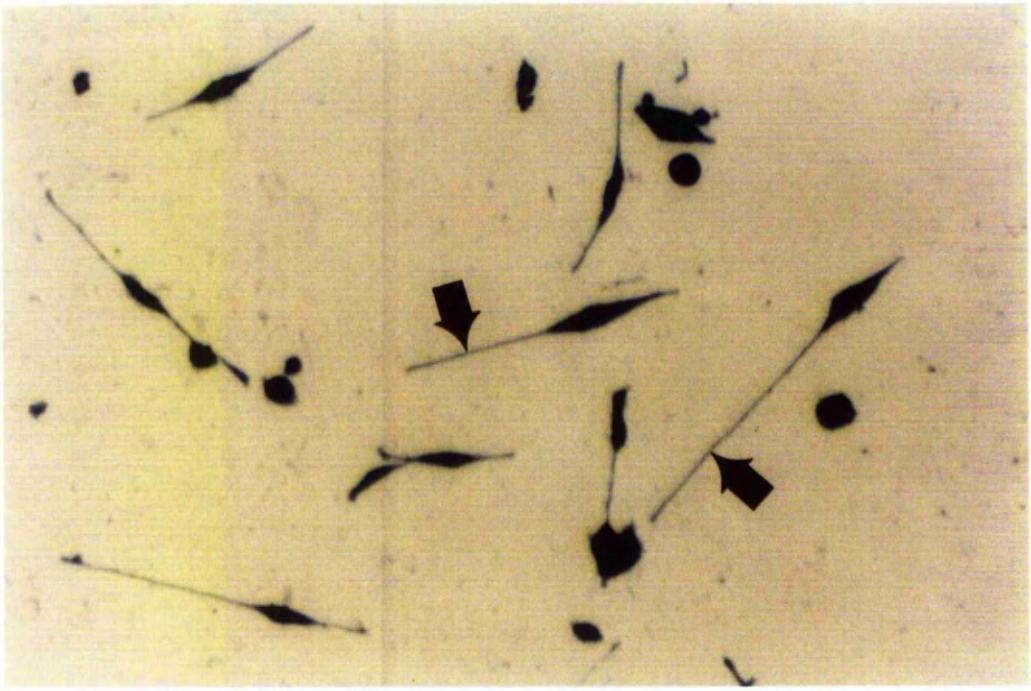
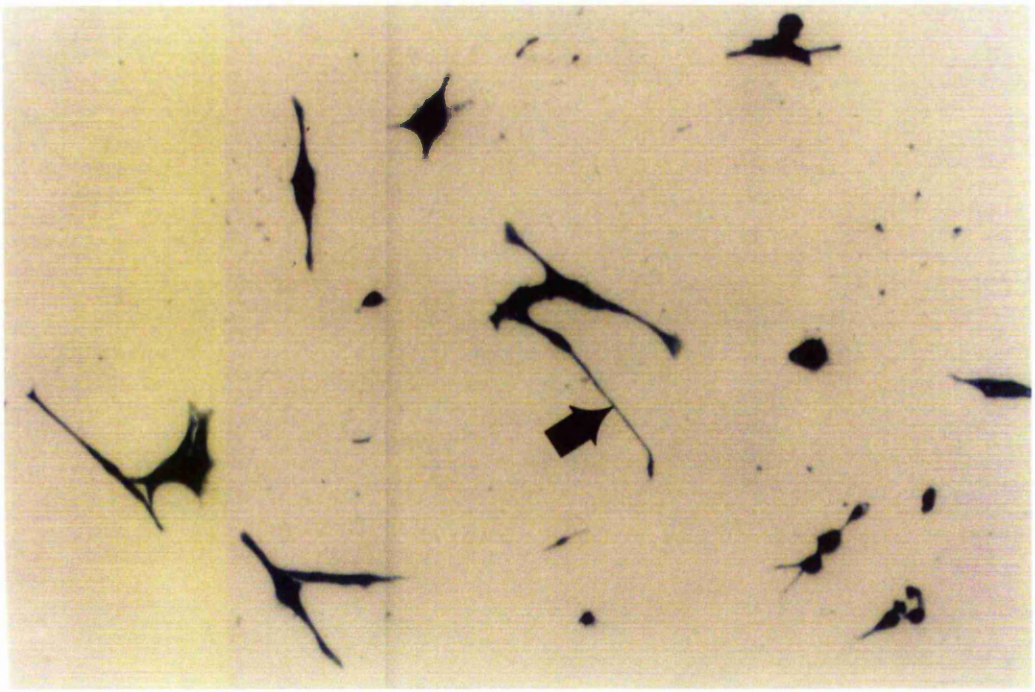


Figure 3.4. *Differentiating C6 cells incubated in the presence of 1 μg/ml TCP for 48 hours.* Note the presence of cellular extensions, suggesting glial cells are unaffected by the presence of TCP (arrow).



3.2.2 Determining non-cytotoxic levels of TCP using the MTT reduction assay

Previous work by Flaskos *et al.*, (1994) showed that the viability of proliferating N2a cells was unaffected by levels up to 10 μg/ml TCP. To establish the viability of differentiating N2a cells when exposed to levels of TCP, the MTT reduction assay was used; this assay determines cell growth at each concentration used.

Table 3.1 MTT reduction by differentiating N2a cells exposed to 1 μg/ml TCP

Differentiating N2a cells were incubated with 1 μg/ml TCP or with the 0.05 % (v/v) ethanol (control) for 24 and 48 hours and then incubated with MTT as described in section 2.2.1.9. The results presented below are measurements of absorbance values ± standard error for a total of 8 wells of cells cultured on 2 separate occasions.

	Control	**1 μg/ml TCP**
24 hours	**0.70** ± 0.12	**0.86** ± 0.13
48 hours	**0.82** ± 0.07	**0.80** ± 0.08

These results demonstrate that 1 μg/ml TCP does not cause a reduction in cell viability after a period of 24 or 48 hours exposure when compared to its corresponding control. The above results were not significantly different to the corresponding controls as determined by Mann-Whitney U test ($p > 0.05$), thus confirming that 1 μg/ml TCP is non-cytotoxic to the neuroblastoma (N2a) cell line at both time points. On the basis of this result, 1 μg/ml TCP was used as the highest concentration throughout this study.

3.2.3 Time course study of the effects of TCP on neurite outgrowth

The effects of TCP were assessed over a time range of 0 to 52 hours on differentiating N2a cells. These cells were incubated with either 0.05 % (v/v) ethanol (as the untreated control) or with 1 μg/ml TCP. The untreated control neuronal cells produced neurites of which 25 - 45 % were axon-like which was consistent throughout all experiments conducted as a requirement for 'normally behaving' N2a cells (Flaskos *et al.*, 1994; 1998).

As shown in table 3.2, the results obtained suggest that, while the number of small extensions remained constant, axon outgrowth was significantly reduced in the presence of TCP from the point of 24 hour exposure. Decreases in axon outgrowth were observed in both the control and TCP treated cells between 24 and 48 hours exposure, but relatively lower levels were maintained in the presence of TCP, as can be seen clearly in figure 3.5.

From previous work by Flaskos *et al.*, (1998) and the MTT results described in section 3.2., it has been shown that this concentration of TCP (1 μg/ml) is non-cytotoxic towards differentiating cells, as also determined by Trypan blue exclusion (data not shown). Furthermore, there was no overall change in cell morphology in relation to round and flat cells as defined in section 2.2.1.7, indicating a selective effect on the outgrowth of axon-like processes.

Table 3.2 Time course of the morphological effects of TCP on differentiating N2a cells.

N2a cells were induced to differentiate in the presence or absence of 1 μg/ml TCP. The cells were fixed and stained with Coomassie blue at different time points (section 2.2.1.6). Values are expressed as numbers observed per 100 cells ± standard errors from an average of 16 to 24 wells of cells cultured on 4 separate occasions.

		Extensions (%)	Axons (%)	Round cells (%)	Flat cells (%)
Zero Time		**16** ± 0.8	**7** ± 0.56	**17** ± 0.7	**83** ± 0.7
4 hours	**Control**	**42** ± 1.5	**24** ± 1.0	**24** ± 0.8	**76** ± 0.8
	TCP	**45** ± 1.7	**29** ± 1.4	**28** ± 1.3	**72** ± 1.3
8 hours	**Control**	**45** ± 1.9	**30** ± 1.4	**25** ± 0.9	**75** ± 0.9
	TCP	**49** ± 1.8	**44** ± 1.9	**29** ± 1.2	**71** ± 1.2
12 hours	**Control**	**44** ± 1.9	**45** ± 1.9	**25** ± 1.1	**75** ± 1.1
	TCP	**40** ± 1.3	**46** ± 0.9	**29** ± 1.2	**71** ± 1.2
24 hours	**Control**	**34** ± 1.2	**50** ± 1.4	**34** ± 1.1	**66** ± 1.1
	TCP	**41** ± 1.5	**32** ± 1.2	**35** ± 1.0	**65** ± 1.0
30 hours	**Control**	**26** ± 1.11	**32** ± 1.7	**27** ± 1.0	**73** ± 1.0
	TCP	**31** ± 2.0	**18** ± 1.5	**31** ± 1.9	**69** ± 1.9
48 hours	**Control**	**34** ± 1.4	**36** ± 1.5	**33** ± 1.0	**67** ± 1.0
	TCP	**39** ± 2.0	**19** ± 1.2	**42** ± 1.7	**58** ± 1.7
52 hours	**Control**	**33** ± 1.12	**42** ± 1.8	**35** ± 1.1	**65** ± 1.1
	TCP	**46** ± 2.38	**16** ± 0.9	**41** ± 1.4	**59** ± 1.4

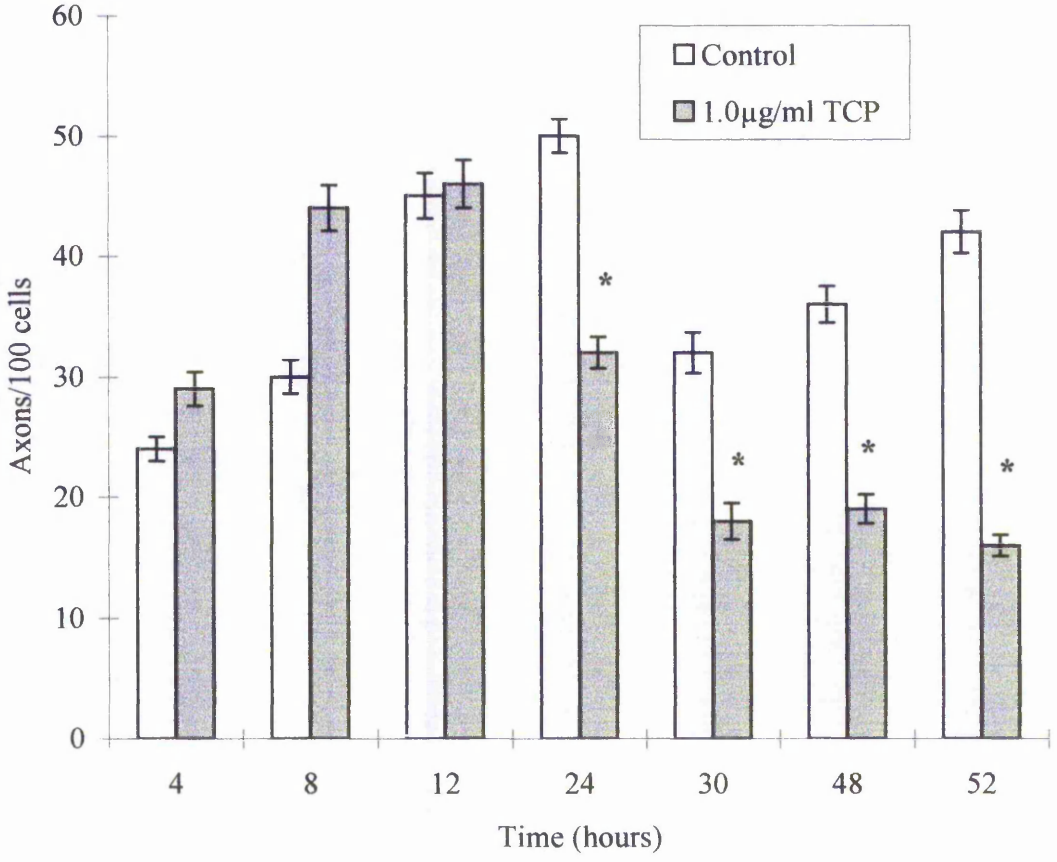


Figure 3.5. *The time course effects of TCP on axon outgrowth in differentiating N2a cells.* Shown are the numbers of axon-like processes per 100 cells counted at designated time points following exposure of cells to 1 µg/ml TCP. There was a significant decrease in axon outgrowth at each time point after and including 24 hours, as determined by the Mann-Whitney U test (* = $p < 0.0001$). Each data point represents an average of 16 to 24 wells of cells cultured on 4 separate occasions. Standard errors are shown as error bars.

3.2.4 Dose response study of the effects of TCP on differentiating N2a cells axonal processes

Dose response experiments were conducted to assess the impact of TCP on axon outgrowth and to establish the IC_{50} (the concentration required to inhibit axon outgrowth by 50 % of the control value) for two time points, 24 and 48 hours.

As can be seen for the 24 hour time point, axon outgrowth fell significantly in the presence of increasing concentrations of TCP, to approximately 30 % of the control levels, while there was no overall change in cell shape or in the number of short extensions (table 3.3).

After 48 hour exposure to TCP, axon outgrowth fell significantly in the presence of a range of TCP concentrations to approximately 30 % of control levels. However, the number of small extensions showed no significant effect overall, whilst the proportion of round to flat cells was found to be unaffected by different concentrations of TCP (table 3.3). In these dose response experiments, it was found that the reductions in axon number were statistically significant when compared to the corresponding controls after 24 and 48 hours as determined by Mann-Whitney U test ($p < 0.05$).

A graphical representation was then produced using the dose response data, allowing the IC_{50} value to be established at each time point. These were estimated to be 0.65 ± 0.09 μg/ml after 24 hour exposure to TCP and 0.64 ± 0.08 μg/ml after 48 hour incubation. These values suggest a slight decrease in toxicity towards axon outgrowth of differentiating N2a cells from 24 to 48 hours of exposure (figure 3.6 and 3.7) but statistical analysis using the Mann-Whitney U test showed no significant difference ($p > 0.5$).

Table 3.3 Dose response study of the morphological effects of TCP on differentiating N2a cells after 24 and 48 hour incubations.

N2a cells were induced to differentiate for 24 or 48 hours in the presence of a range of TCP concentrations. The cells were fixed and stained with Coomassie blue prior to morphological measurements. Data are expressed as the number recorded per hundred cells ± standard error from an average of 8 wells of cells cultured on 2 separate occasions for 24 hour exposure and an average of 8 wells of cells cultured on 2 separate occasions for 48 hour exposure.

Time (hours)	TCP (µg/ml)	Extensions (%)	Axons (%)	Round cells (%)	Flat cells (%)
24	0	40 ± 2.4	30 ± 2.2	30 ± 1.3	70 ± 1.3
	0.1	33 ± 3.0	26 ± 2.5	25 ± 1.3	75 ± 1.3
	0.25	41 ± 3.0	24 ± 2.1	30 ± 1.7	70 ± 1.7
	0.5	33 ± 2.7	15 ± 1.9	25 ± 1.6	85 ± 1.6
	0.75	44 ± 3.8	12 ± 1.2	32 ± 2.2	66 ± 2.2
	1.0	39 ± 2.8	12 ± 0.9	30 ± 1.7	70 ± 1.7
48	0	27 ± 2.6	32 ± 1.9	39 ± 1.5	61 ± 1.5
	0.1	31 ± 2.3	26 ± 1.8	37 ± 1.8	63 ± 1.8
	0.25	30 ± 4.1	19 ± 2.2	35 ± 3.5	65 ± 3.5
	0.5	38 ± 3.0	15 ± 2.0	40 ± 2.2	60 ± 2.2
	0.75	34 ± 3.3	13 ± 1.8	37 ± 1.9	63 ± 1.9
	1.0	40 ± 3.9	14 ± 1.8	36 ± 1.5	64 ± 1.5

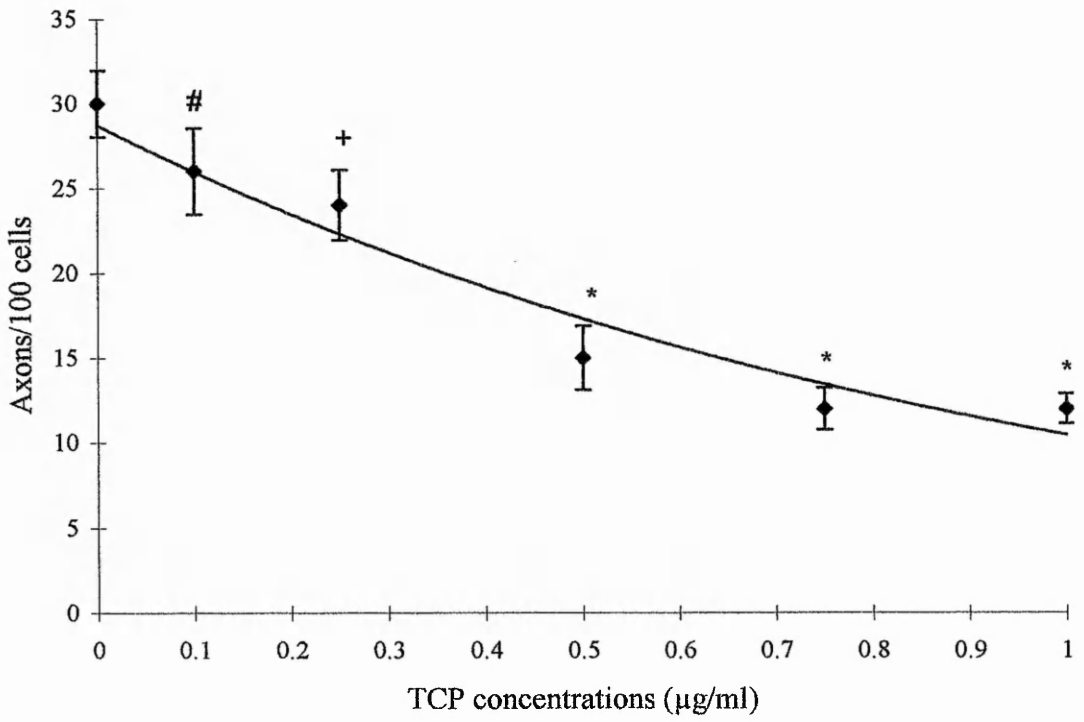


Figure 3.6. *Dose response study of the effects of TCP on axon outgrowth in differentiating N2a cells for 24 hours.* Presented are the numbers of axon-like processes per 100 cells following 24 hour incubation in the presence of increasing concentrations of TCP. All reductions in axon number were significant compared to controls, as determined by the Mann-Whitney U test (# = $p < 0.4$; + = $p < 0.03$; * = $p < 0.0001$). Each data point represents an average of 24 wells of cells from 6 dose response experiments. The IC_{50} value was calculated from an average of 24 wells of cells cultured on 6 separate occasions to be **0.65 ± 0.09 μg/ml**. Standard errors are shown as error bars.

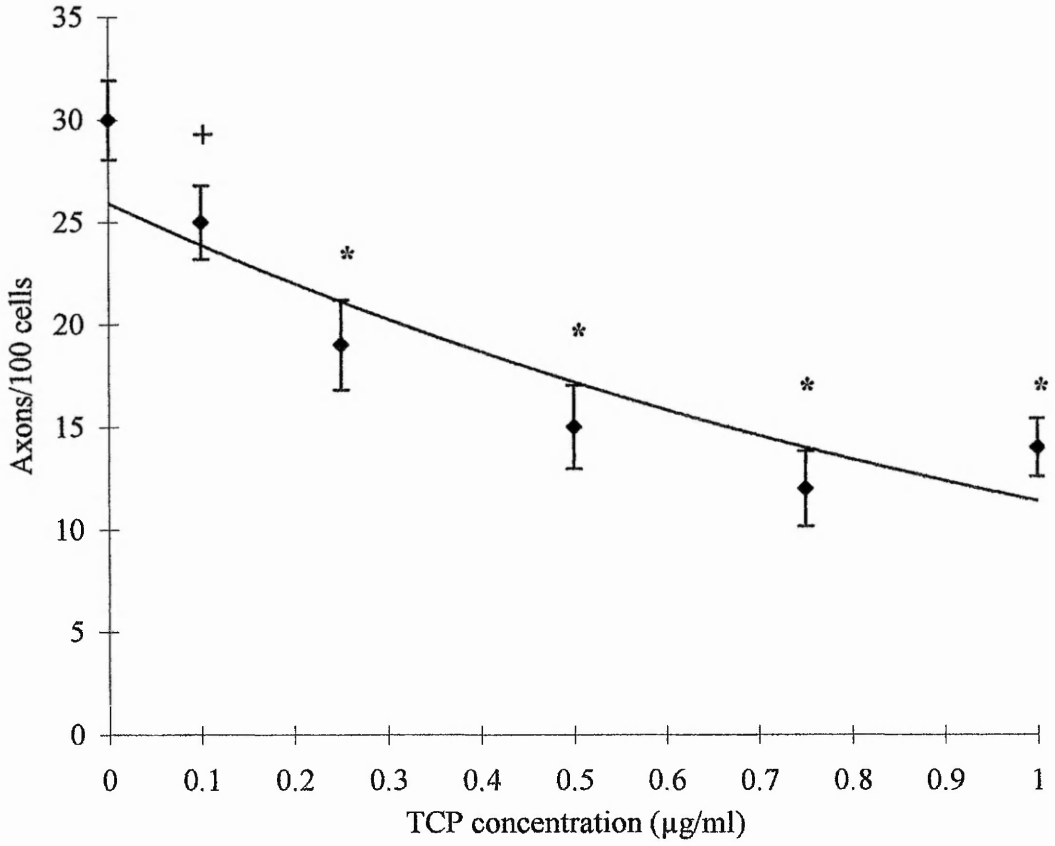


Figure 3.7. *Dose response study of the effects of TCP on axon outgrowth in differentiating N2a cells for 48 hours.* Presented are the numbers of axon-like processes per 100 cells following 48 hour incubation in the presence of increasing concentrations of TCP. All reductions in axon number were significant compared to controls, as determined by the Mann-Whitney U test (+ = $p < 0.004$; * = $p < 0.0001$). Each data point represents an average of 20 wells of cells from 5 dose response experiments. The IC_{50} value was calculated from an average of 20 wells of cells cultured on 5 separate occasions to be **0.64 ± 0.08 μg/ml**. Standard errors are shown as error bars.

3.2.5 Effects of TCP on differentiating rat C6 glial cells

Dose response experiments were also conducted on C6 cells induced to differentiate by the addition of 2 mM sodium butyrate. No significant changes were observed after 48 hours in the outgrowth of extensions, or in the proportion of round and flat cells, when C6 cells were treated with a concentration range of TCP up to 1 μg/ml (table 3.4 and figure 3.8). As described in section 2.2.1.7, C6 glial cells only form one type of outgrowth process when differentiating in the presence of sodium butyrate, which is defined as an extension.

Table 3.4. Dose response study of the morphological effects of TCP on differentiating C6 cells.

Differentiating C6 cells were exposed to a range of TCP concentrations for 48 hours. The cells were fixed and stained with Coomassie blue prior to morphological measurements. The results are an average of 8 wells of cells cultured on 2 separate occasions and are expressed as extensions per 100 cells (or the percentage of cells with rounded or flattened morphology) ± standard error.

TCP (μg/ml)	Extensions (%)	Round cells (%)	Flat cells (%)
0	**93** ± 4.89	**42** ± 2.16	**58** ± 2.16
0.1	**83** ± 4.30	**23** ± 1.94	**77** ± 1.94
0.5	**88** ± 4.30	**25** ± 1.65	**75** ± 1.65
1.0	**105** ± 4.34	**46** ± 2.52	**54** ± 2.52

It can be clearly seen from the data presented above that 1 μg/ml TCP appears to have no inhibitory effect on the number of extensions produced nor on the round to flat cell ratio, suggesting that TCP may have a selective effect on the type of cellular processes produced by different cell types.

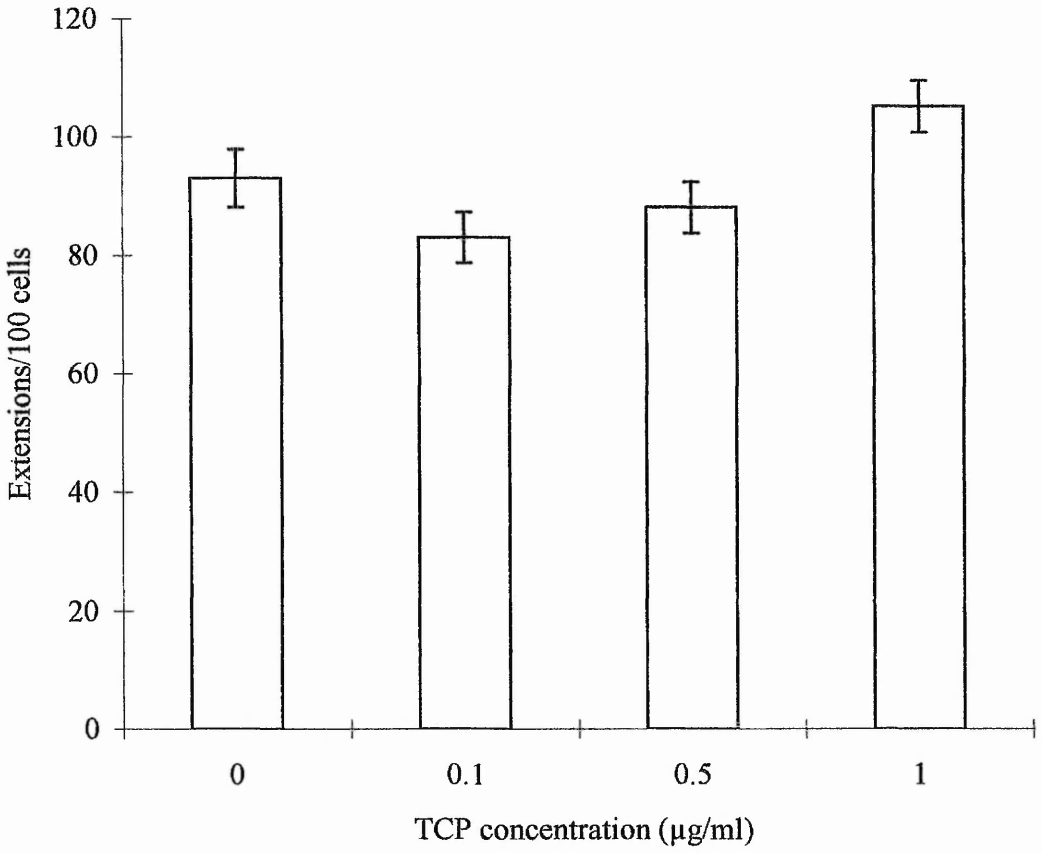


Figure 3.8. *Dose response study of the effects of TCP on differentiating C6 cells.* Shown are the numbers of extensions per 100 cells determined after 48 hours incubation in the presence of various concentrations of TCP. All concentrations showed no significant difference from the corresponding control, as determined by the Mann-Whitney U test ($p > 0.05$). Each data point represents an average of 8 wells of cells cultured on 2 separate occasions. Standard errors are shown as error bars.

3.2.6 Lengths of N2a and C6 processes as a visible marker of cell degradation

As seen in figures 3.1 to 3.4, there were visible differences between the axon lengths of control and TCP treated N2a cells and no visible difference in extension length in C6 cells. In order to further quantitate these effects, average lengths were taken of N2a axon-like processes and C6 extensions from plates of fixed and stained differentiating cells after exposure to 1 μg/ml TCP for 48 hours.

Table 3.5. Lengths of N2a and C6 processes following exposure to 1 μg/ml TCP for 48 hours

The lengths of axon-like processes or extensions were measured with the aid of an eye piece graticule for both N2a and C6 cells, respectively after a 48 hour incubation in the presence of 1 μg/ml TCP. The results are an average of 75 fields (from 2 separate experiments) for the N2a untreated cells and 40 fields (from 2 separate experiments) for N2a cells exposed to 1 μg/ml TCP. For differentiating C6 cells, 75 fields (from 2 separate experiments) were measured for both control and TCP treated cells.

	Control	1 μg/ml TCP
N2a cells : Axon length (μm)	**44.2** ± 4.73	**28.0** ± 2.56 $p < 0.0001$
C6 cells : Extension length (μm)	**76.5** ± 6.90	**72.2** ± 6.24 $p = 0.57$

The reduction seen in the length of axons in TCP treated N2a cells was statistically significant as determined by the Mann-Whitney U test ($p < 0.05$), whereas in C6 cells, no significant difference was observed in the length of extensions when comparing control cells and those treated with 1 μg/ml TCP. This, therefore, suggests that axon outgrowth in N2a cells is affected by TCP in a selective manner, whereas C6 cells are unaffected, as found in the dose response experiments described in table 3.4 and figure 3.8.

3.3 Molecular analysis of differentiating N2a cell extracts

3.3.1 Western blotting analysis of extracts from N2a cells exposed to 1 μg/ml TCP

The initial analysis was conducted on differentiating N2a cell extracts prepared from cells incubated for 24 and 48 hours in the presence or absence of 1 μg/ml TCP (section 2.2.2).

The resultant Western blots were probed with a series of monoclonal antibodies recognising different epitopes within neurofilament, microtubule structures and other cytoskeletal proteins. These are as follows :

- RMd09, which recognises a non-phosphorylation dependent epitope on NF-H;
- Ta51, which specifically binds to a phosphorylation dependent epitope on NF-H;
- N52, which recognises an NF-H epitope independent of its phosphorylation state;
- B-5-1-2, an antibody that recognises an α-tubulin epitope located at the C-terminal end of the α-tubulin isoform;
- GAP-7B10, which recognises GAP-43 in axons.

Probing of Western blots could reveal visible changes in antibody binding to cytoskeletal proteins caused by exposure to TCP. In some cases densitometric analysis was conducted in order to quantify the intensities of each band, providing extra information on the significance of each effect seen when compared to its corresponding controls (table 3.6).

As can be seen from figure 3.9, in the control cell extracts, there is (as expected) an increase in reactivity with RMd09, which recognises non-phosphorylated NF-H from 24 to 48 hours incubation, showing that the levels of expression increase as axons mature during cell differentiation. However, there was a reduction in reactivity with RMd09 (arrow) compared to the control in TCP treated cell extracts only after 48 hours.

In figure 3.10, a similar pattern of antibody binding can be seen with the antibody Ta51 (which recognises a phosphorylated epitope of NF-H). There is a decrease in antibody binding when comparing the corresponding control to the TCP treated cell extracts after 24 and 48. These effects seen with the anti-NF-H antibodies appear to be mirrored in the densitometry results obtained (table 3.6).

When the same cell extracts were probed with the N52 antibody, which recognises NF-H epitope independent of its phosphorylation state, once again there appeared to be a slight reduction in reactivity following both 24 and 48 hour exposure, when compared to its corresponding control (figure 3.11). Similarly, the densitometry package detected a subtle decrease in antibody intensity at both time points when compared to its corresponding control (table 3.6)

Figure 3.12 represents a Western blot probed with the anti-α-tubulin antibody, B-5-1-2. After 24 hours, a small decrease in antibody binding was observed and confirmed by densitometry (table 3.6). However, this loss in α-tubulin was not statistically significant and appears to be corrected after 48 hour exposure, with the antibody binding in TCP treated cell extracts being very similar to that of its corresponding control.

The Western blot seen in figure 3.13 represents the effects of TCP on levels of GAP-43 within differentiating N2a cells exposed to 1 μg/ml TCP. A slight reduction in antibody binding was seen in TCP treated cell extracts after 24 hours exposure; levels became significantly lower that the corresponding controls after 48 hours exposure. Densitometric analysis of two independent blots indicated no significant change in the levels of GAP-43 in TCP treated cell extracts compared to controls (table 3.6)

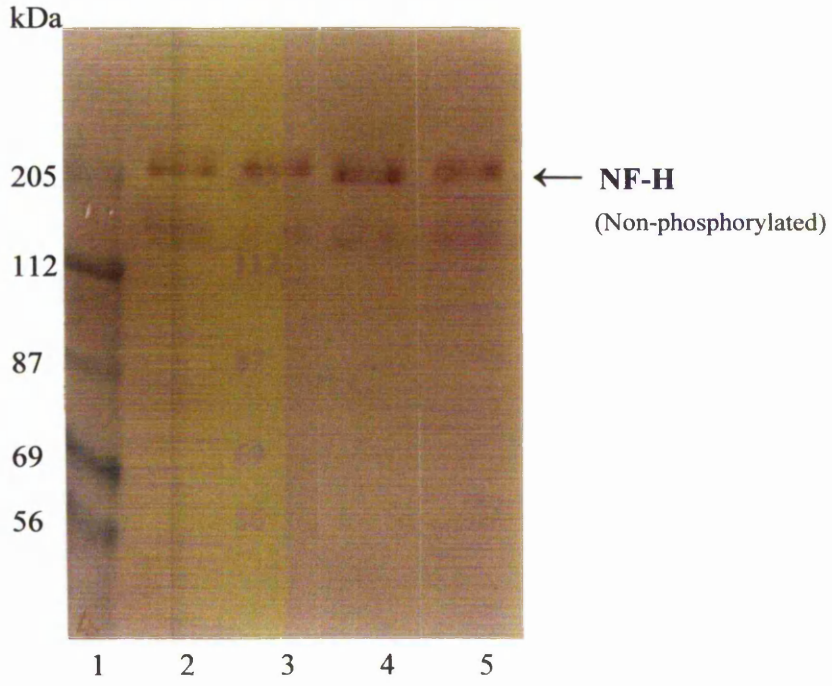


Figure 3.9. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml TCP, probed with neurofilament antibody, RMd09.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml TCP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml TCP for 48 hours. The blot was probed with antibody, RMd09 which recognises a non-phosphorylation dependent epitope on NF-H. The arrow indicates a reactive polypeptide with the apparent molecular weight of NF-H.

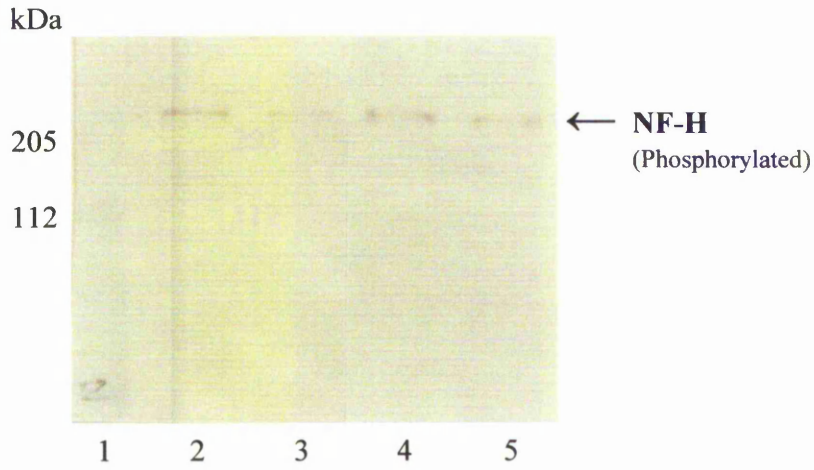


Figure 3.10. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml TCP, probed with neurofilament antibody, Ta51.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml TCP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml TCP for 48 hours. The blot was probed with antibody, Ta51 that recognises a phosphorylation dependent epitope on NF-H. The arrow indicates a reactive polypeptide with the apparent molecular weight of NF-H.

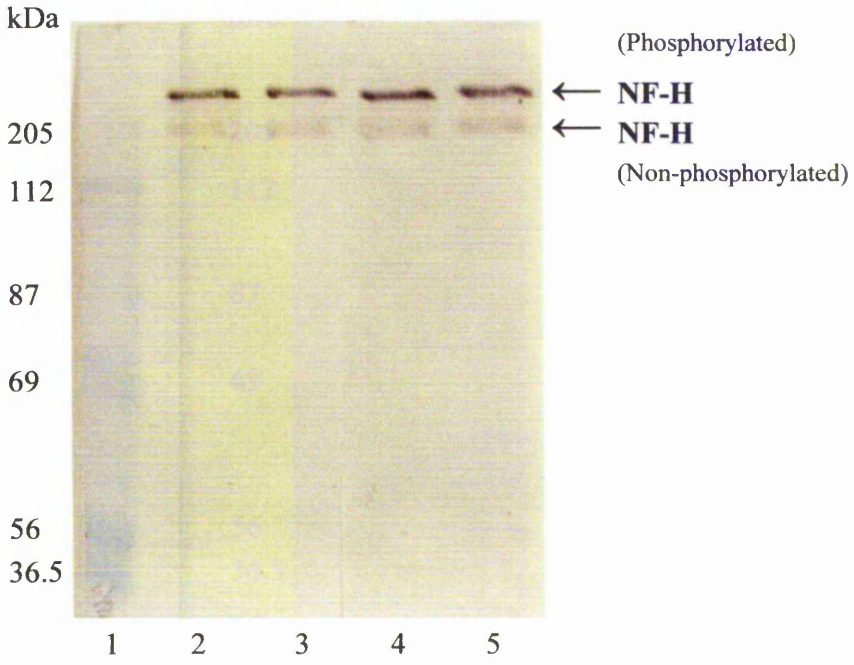


Figure 3.11. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml TCP, probed with neurofilament antibody, N52.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml TCP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml TCP for 48 hours. The blot was probed with antibody, N52 that recognises a NF-H epitope independent of the phosphorylation state. The arrows indicate a reactive polypeptide with the apparent molecular weight of NF-H.

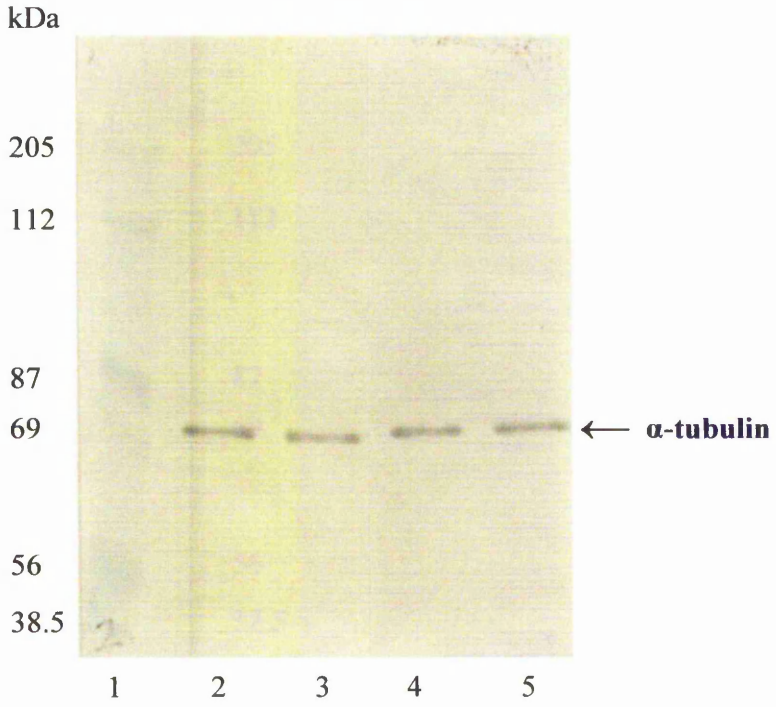


Figure 3.12. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml TCP, probed with anti-tubulin antibody, B-5-1-2.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml TCP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml TCP for 48 hours. The blot was probed with antibody, B-5-1-2 which recognises an epitope located at the C-terminal end of the α-tubulin isoform. The arrow indicates a reactive polypeptide with the apparent molecular weight of α-tubulin.

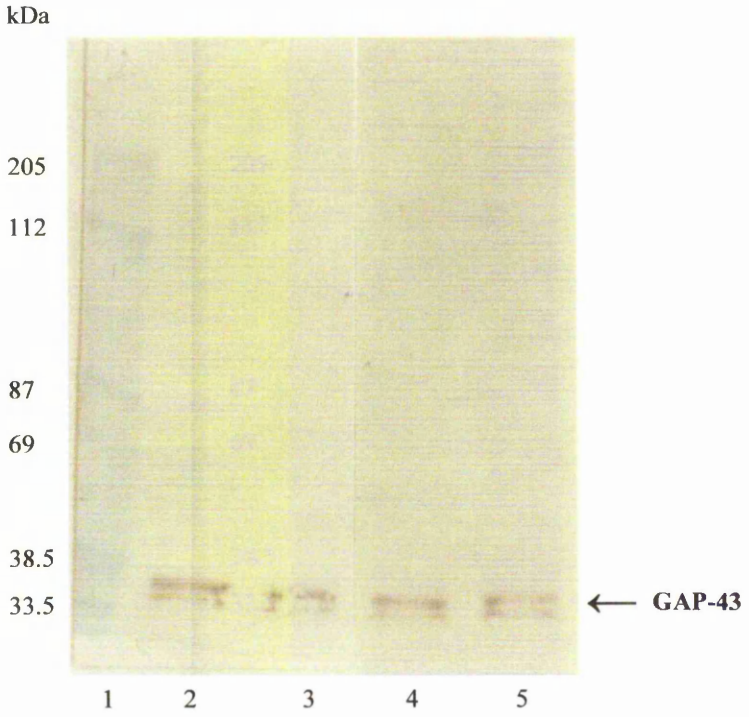


Figure 3.13. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml TCP, probed with GAP-7B10.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml TCP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml TCP for 48 hours. The blot was probed with antibody GAP-7B10, which recognises GAP-43 with in neuronal processes. The arrow indicates a reactive polypeptide with the apparent molecular weight of GAP-43

3.3.2 Quantitative analysis of Western blot data from extracts of differentiating N2a cells exposed to 1 µg/ml TCP

As the Western blot data was only assessed by visual comparisons, the intensities of antibody binding on each blot were then analysed quantitatively using a computer package known as QuantiScan. This package examines the intensities of each band from the Western blot giving it an arbitrary value that represents the band intensity. These arbitrary values are represented below as a percentage of their corresponding control. However, due to the limited supply of Western blots, statistical analyses of the observed trends were only conducted if there was 3 or more blots.

Table 3.6. Densitometric analysis of Western blots using QuantiScan

Data from the densitometry analysis has been tabulated below with values recorded representing the antibody intensities when compared to the corresponding control ± standard error. * represents statistical analysis performed using Mann-Whitney U test and the relevant p values are given.

	24 hour	48 hour	No. of experiments
Non-phosphorylation dependent epitope of NF-H (RMd09)	**98** ± 9.1	**58** ± 10.3	2
Phosphorylation dependent epitope of NF-H (Ta51)	**53** ± 4.5	**78** ± 1.0	2
Phosphorylation independent epitope of NF-H (N52)	**89** ± 3.3	**92** ± 18.0	2
α-tubulin (B-5-1-2)	**80** ± 2.1 * (p = 0.38)	**113** ± 4.0 * (p = 0.38)	3
GAP-43 (GAP-7B10)	**97** ± 5.0	**63** ± 17.0	2

3.4 Discussion

The time course experiment clearly shows that TCP is able to inhibit axon outgrowth as early as 24 hours following exposure. It is, therefore, not necessary to incubate the cells for the full 48 hours in following experiments, since the 24 hour incubation period with TCP displays similar morphological effects to later time points. From the MTT reduction assays (table 3.1), it can be seen that the range of TCP concentrations used had no inhibitory effect on cell growth after 24 and 48 hours. This suggests that the morphological effects seen (i.e. reduction in numbers of axon-like processes) are not due to the cytotoxic levels of TCP being used but are a direct response to the OP insult.

Morphologically, it has been shown that the commercial product TCP causes a significant reduction in the outgrowth of axon-like processes by differentiating N2a cells after both 24 and 48 hours but has no significant effect on the outgrowth of smaller extensions by N2a cells or processes by C6 cells. This indicates a selective effect of TCP towards differentiating neuroblastoma cells, inhibiting the ability of neuronal cells to produce axon-like processes. Evidence to support this can be seen in the dose response experiments, where 1 μg/ml TCP has a significant inhibitory effect on axon outgrowth in N2a cells but not on process outgrowth by C6 cells which have been subjected to the same experimental conditions. This data, coupled with the previously reported effects on PC12 cell differentiation (Flaskos *et al.*, 1994), suggests that non-cytotoxic levels of TCP exhibit selective inhibition of both the formation and maintenance of axon-like processes.

From the dose response experiments conducted, TCP was found to inhibit the outgrowth of axon-like processes of N2a cells in a dose-dependent manner after both 24 and 48 hours. From six separate dose response experiments, the IC_{50} values for axon outgrowth were estimated to be 0.65 ± 0.09 μg/ml and 0.64 ± 0.08 μg/ml after 24 and 48 hours, respectively. This suggests that the toxic effect of TCP on differentiating N2a cells was sustained over the whole incubation period.

The Western blot data and densitometric analysis gave an indication of the molecular changes in the axonal cytoskeleton of differentiating N2a cells following exposure to 1

μg/ml TCP. It is apparent from these data that TCP reduced antibody reactivity with a phosphorylation and non-phosphorylated - dependent epitope of NF-H and GAP-43 protein (only after 48 hours) but had little or no effect on the levels of phosphorylation - independent NF-H epitope or tubulin.

As mentioned earlier NFs are predominantly found in axons and, from the morphological changes observed, it is clear that TCP inhibits axon outgrowth in N2a cells and this, in turn, would suggest that the levels of NFs would be lower than the levels seen in the control extracts. However, this decrease is not evident (figure 3.11) suggesting that the retraction of axons is associated with re-packaging of NFs into the remaining length of the axon process or the perikaryon. The effects of TCP on NF-L and NF-M were not studied but, as they develop earlier than NF-H, it is likely that their levels are unaffected. However, further investigations, involving a study of NF-M and NF-L at earlier time points, are needed to complete the study.

In relation to the effects of TCP on NF-H's phosphorylation state (a post translational modification of NF-H), there appears to be a decrease in both phosphorylated and de-phosphorylated NF-H after 48 hours; this suggests that exposure to TCP reduces the phosphorylation state rather than the levels of NF-H. However, work by Suwita *et al.*, (1986) has suggested that there was an increase in phosphorylation of NF-H *in vitro*, in NFs crude chicken brain extract from T*O*CP-treated hens. Although no such increase was observed in the present work, the extent to which possible proteolysis might influence this result is not clear. Some of the differences between my results and these of Suwita *et al.*, (1986) could be related to the fact that total NF-H levels were assessed in the present work, whilst auto-phosphorylation of purified NFs was monitored by Suwita *et al.*, (1986). Degraded NFs would have been removed in the studies whilst their presence in the extracts could mask alterations in phosphorylation state of intact NFs.

The significant reduction in GAP-43 seen after 48 hours may influence the activity of the growth cones and lead to poor axonal development and maturation (Skene, 1989; Pekiner *et al.*, 1996).

Furthermore the study in this chapter uses a mixture of isomers which is only 95 % pure, whereas work by Lapadula *et al.*, (1985) used purified T*O*CP which was presumably metabolised at least partly to CPSP, *in vivo*. Therefore, it may be possible that the difference in chemical composition and metabolism could lead to these differences in results.

Further Western blot analysis was undertaken using other monoclonal antibodies that were raised against acetylated α-tubulin and microtubule associated proteins, MAP1 and MAP1B. The Western blots showed weak reactivity and multiple bands were revealed (data not shown). However, these blots showed no apparent overall decrease when compared to the corresponding control, implying that TCP had no effect on these proteins and providing further support for a selective effect of TCP on NF-H at these time points.

It can be concluded that sub-cytotoxic concentrations of TCP appear to selectively reduce the growth of axonal processes, associated with a reduction in non-phosphorylated and phosphorylated NF-H, in differentiating N2a cells after 48 hours exposure. As the commercial product has no inhibitory effects on the morphology of C6 cells but has a dramatic effect on the outgrowth of axon-like processes by N2a cells, the selective target for TCP would appear to be the outgrowth of axons by neuronal cells.

Chapter Four

Effects of tri*ortho*cresyl phosphate (T*O*CP) and tri*para*cresyl phosphate (T*P*CP) on differentiating mouse N2a neuroblastoma cells

4.1 Introduction

As previously mentioned, TCP consists of three different isomers known as tri*ortho*cresyl phosphate (T*O*CP), tri*para*cresyl phosphate (T*P*CP) and tri*meta*cresyl phosphate (T*M*CP) (Bondy *et al.*, 1960; Glees and Janzik, 1965). It has been documented that T*P*CP and T*M*CP contribute no neurotoxic effect to the commercial product TCP, but it is the presence of the *-ortho* isomer, T*O*CP, that gives the compound its neurotoxic effect (Bondy *et al.*, 1960). However, it is not known to what extent this toxicity is due to differences in uptake, bioelimination and bioavailability of the isomers following exposure (Bondy *et al.*, 1960; Glees and Janzik, 1965; Freudenthal *et al.*, 1993).

The two non-neurotoxic *-meta* and *-para* cresols, contribute very little, if any, toxic effect to the commercial product. Although they have the same properties and advantages as TCP, these compounds are not used in manufacture as they are impractical and very costly, due to the need for intense isolation and purification of these isomers before they are useful (Bondy *et al.*, 1960).

The *-ortho* isomer has been the cause of many world wide poisonings occurring after consumption of edible oils which have been accidentally contaminated or adulterated with mineral oils that contain the *-ortho* isomer (Nanda, 1995). Consequently, the *-ortho* isomer is interesting as it is known to produce a delayed neurotoxic effect in man and susceptible animal species on the central and peripheral nervous systems (Smith and Spalding, 1959; Abou-Donia, 1981; Biswas *et al.*, 1993; Nanda, 1995). The delayed neurotoxicity produced was recognised and is known as organophosphate induced delayed neurotoxicity (OPIDN), occurring after a latency period of 6 - 12 days (Abou-Donia, 1981; Zech and Chemnitius, 1987; Richardson, 1992).

Many research groups have been investigating the basis of this delayed neurotoxic effect by using the chicken animal model. Work has been conducted on the clinical and histopathological characteristics of OPIDN (Abou-Donia, 1981; Abou-Donia and Lapadula, 1990; Abou-Donia, 1993), the involvement of cytoskeletal proteins in the

mechanism of OPIDN (Abou-Donia, 1995), and the involvement of the neurotoxic esterase protein (NTE) in OPIDN onset (Johnson, 1969; 1974; 1990).

Alternatively, other research groups have been investigating the effects of OPs like T*O*CP on other biological systems. Such work has indicated that T*O*CP has a toxic effect upon rat testes, with exposed rats exhibiting decreases in sperm motility, vacuolations of the seminiferous epithelium and inhibition of neurotoxic and non-specific esterases (Somkuti *et al.*, 1987a; 1987b; 1991). Further experimental work confirmed that the *-para* isomer of TCP did not produce any of the toxic effects seen with the *-ortho* isomer (Somkuti *et al.*, 1987a; 1987b).

Ahmed *et al.*, (1993) documented that there was no difference between pregnant and non-pregnant rats in relation to the distribution of ^{14}C[T*O*CP] after 72 hours. However they concluded that organs such as lung, spleen, gall bladder and liver of mother and foetuses retained radioactive T*O*CP, suggesting that these may be additional target sites of T*O*CP toxicity.

Several other research groups have begun to study the toxic effects of OPs using cellular systems, with the main emphasis being on analysing levels of NTE inhibition following exposure to OPs such as T*O*CP, thus attempting to develop an *in vitro* test to evaluate and screen neurotoxicity compounds (Fedalei and Nardone, 1983; Ehrich, 1995). As mentioned in chapter one, work has also been conducted by Henschler *et al.*, (1992) and Flaskos *et al.*, (1994; 1998) on an *in vitro* cellular system to investigate the effects of OPs on neurite outgrowth.

It has been suggested that there are two biochemical lesions that precede the onset of OPIDN; these are (i) organophosphorylation of NTE (Zech and Chemnitius, 1987) and (ii) disruption of the axonal cytoskeleton through altered protein phosphorylation (Zech and Chemnitius, 1987; Abou-Donia and Lapadula, 1990). The majority of the mechanistic research has concentrated on the first lesion, although, *in vivo* experimental work has shown that the disruption of the neuronal cytoskeleton proteins such as MTs, NFs and MFs may be involved in the pathogenesis of OPIDN (Abou-Donia *et al.*, 1988; Lotti, 1992).

With *in vitro* experiments, the majority of the work has been conducted on tissues such as brain, spinal cord and sciatic nerve and it has been demonstrated that the cytoskeleton is affected following exposure to OPs (Abou-Donia *et al.*, 1988; Abou-Donia, 1995).

From the literature available, it is clear that very little work has been conducted on the early effects of OPs on brain, spinal cord and sciatic nerve proteins but even less work has been carried out with regard to the early effects of OP exposure in an *in vitro* cellular system. Previous experiments have shown that TCP has no effect on differentiated N2a cells after 4 and 8 hours (time course; section 3.2.3) but significantly inhibits axon outgrowth after 24 and 48 hour incubations. However, the contribution of T*O*CP and T*P*CP to this effect is not known. In order to have a better understanding of the effects of these isomers, they were studied separately on cells differentiating for various periods up to 48 hours.

This was achieved by :

(i) determining the non-cytotoxic levels of T*O*CP and T*P*CP by MTT reduction under all experimental conditions,

(ii) performing axonal outgrowth experiments on differentiated N2a cells at various time points ranging between 4 to 48 hours,

(iii) carrying out Western blot analysis of extracts prepared from N2a cells exposed to the isomers.

4.2 Morphological studies of differentiating N2a cells exposed to tri*ortho*cresyl phosphate (T*O*CP) and tri*para*cresyl phosphate (T*P*CP)

The results of the previous chapter established that differentiating N2a cells exposed to TCP showed a significant reduction in axon-like processes after 24 - 48 hours. However, it was not known which of the isomers was producing this effect. For the continuation of this work, pure *-ortho* and *-para* isomers were used, as they are known to be neurotoxic and non-neurotoxic *in vivo*, respectively. This should help to establish whether the isomers show different levels of toxicity when applied to the target cells.

4.2.1 Microscopic observation of N2a cells exposed to 1 µg/ml T*O*CP and T*P*CP for 48 hours

The following figures show the visual effects of 1 µg/ml T*O*CP and T*P*CP (2.7 µM) on differentiating N2a cells after 48 hours exposure.

Figure 4.1. *Differentiating N2a cells exposed to 0.05 % (v/v) ethanol (control) for 48 hours.* Note the abundance of axon-like processes (arrows) and small extensions, suggesting the cells are healthy. Cells were fixed and stained with Coomassie blue stain in all cases.

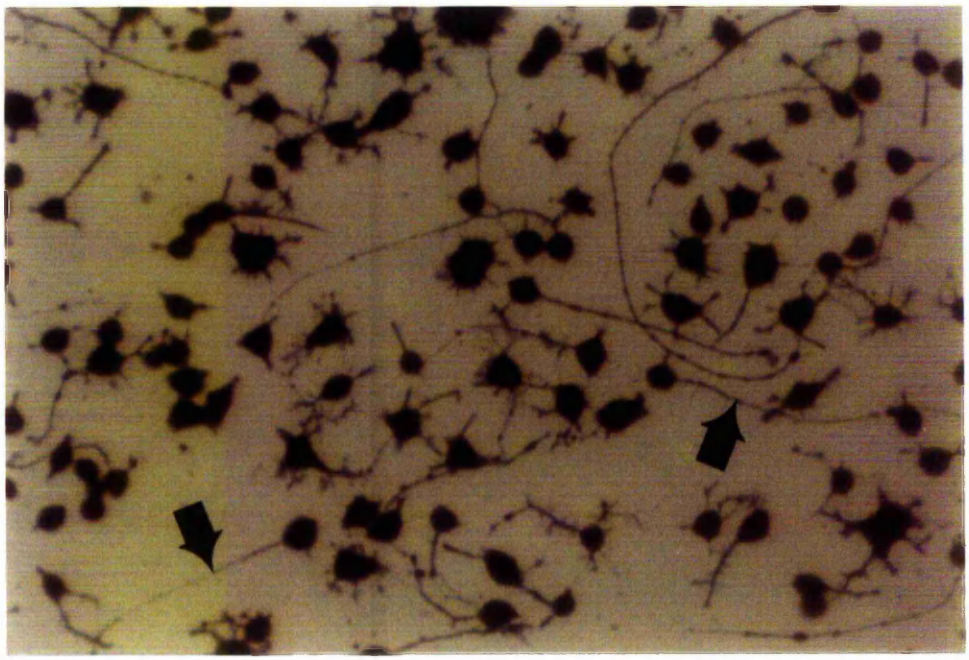


Figure 4.2. *Differentiating N2a cells incubated with 1 μg/ml TOCP for 48 hours.* Note the reduction in numbers of axon-like processes when compared to figure 4.1 which suggests that the effect of T*O*CP may be interfering with axon-like processes and not small extensions as they are present in high numbers.

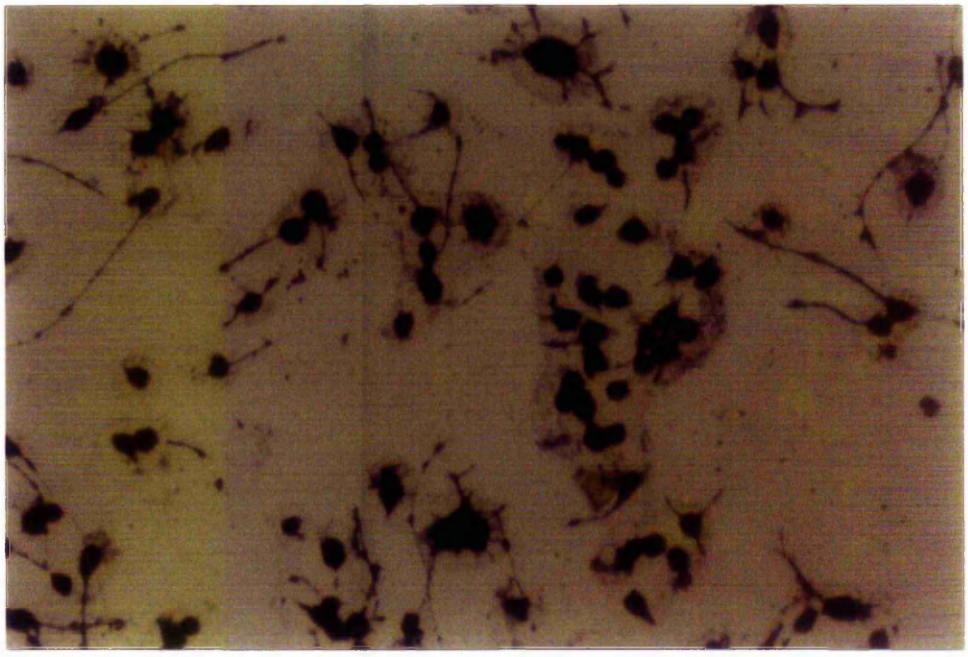
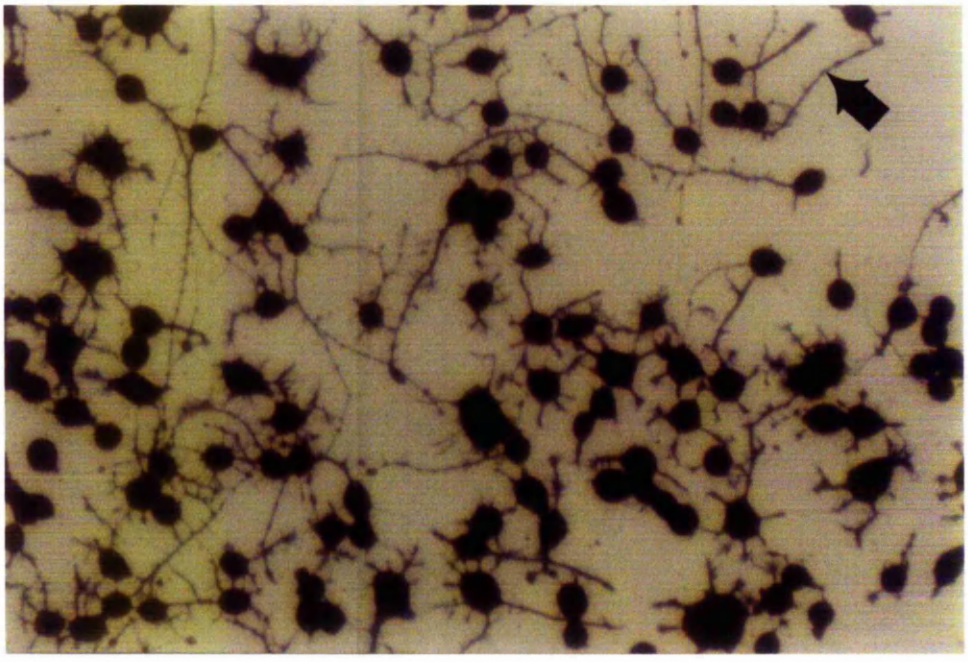


Figure 4.3. *Differentiating N2a cells exposed to 1 μg/ml TPCP for 48 hours.* Note the abundance of axon-like processes and small extensions when compared to the control; this suggests that T*P*CP has no inhibitory effect on these cells (arrow).



4.2.2 Determination of cell growth in the presence of T*O*CP and T*P*CP using MTT reduction

These experiments were performed to establish the viability of differentiating N2a cells when exposed to different levels of T*O*CP and T*P*CP. It was essential to establish the cytotoxic levels, if any, of the isomers before undertaking morphological studies described in chapter 3. This was achieved by using the MTT assay which measures levels of cell growth as an indicator to healthy, non-cytotoxic conditions.

Table 4.1. MTT reduction by differentiating N2a cells exposed to 1 µg/ml TOCP and TPCP

Differentiating N2a cells were incubated with 1 µg/ml T*O*CP/T*P*CP or 0.05 % (v/v) ethanol (control) for 24 and 48 hours and then subjected to the MTT reduction assay as described in section 2.2.1.9. The results are presented as mean absorbance (570nm) ± standard error for a total of 8 wells of cells cultured on 2 separate occasions.

	Control	**1 µg/ml T*O*CP**	**1 µg/ml T*P*CP**
24 hours	**0.70** ± 0.12	**0.79** ± 0.13	**0.75** ± 0.14
48 hours	**0.82** ± 0.07	**0.75** ± 0.06	**0.71** ± 0.07

The above results for T*O*CP and T*P*CP were not significantly different from the corresponding controls, as determined by Mann-Whitney U test ($p > 0.05$). These results demonstrate that 1 µg/ml T*O*CP and T*P*CP does not cause serious reductions in cell growth after a period of 24 or 48 hours, thus confirming that the concentrations used were non-cytotoxic to the N2a cell line.

4.2.3 Dose response study of the effects of T*O*CP on N2a axon outgrowth

Dose response experiments performed in the presence of T*O*CP show similar trends to those seen with TCP after 24 and 48 hour exposure (table 4.2, figure 4.4 and 4.5). This involved a decrease in axon outgrowth as the OP concentration increased; there was no significant change in cell shape or in the number of short extensions seen. A similar effect can also be seen after 48 hour exposure to a range of T*O*CP concentrations.

From the dose response graphs produced using this data (figure 4.4 and 4.5), the IC_{50} values for T*O*CP were established to be 0.74 ± 0.04 μg/ml after 24 hour exposure and 0.65 ± 0.08 μg/ml after 48 hours. This slight increase was found not to be significant, as determined by the Mann-Whitney U test. However, the toxic effect of T*O*CP after 24 hours mirrors the effect seen for 1 μg/ml TCP, suggesting that T*O*CP may be responsible for some, but not necessarily all of the neurotoxic effects.

4.2.4 Dose response study of the effects of T*P*CP on N2a axon outgrowth

When differentiating N2a cells were exposed to T*P*CP for 24 hours, axon outgrowth decreased in a dose dependent manner. All values were significantly lower than the corresponding control at all concentrations ($p < 0.05$), with the determined IC_{50} value being approximately 0.86 ± 0.04 μg/ml. However, there were no significant changes in the growth of small extensions or in cell shape, confirming the effect as not being cytotoxic but directed to axon outgrowth.

After 48 hour incubation, this trend was not observed and an IC_{50} could not be established. When comparing 1 μg/ml T*P*CP with its corresponding control, no overall inhibition of axon outgrowth was observed. Similarly, there was no significant inhibitory effect on the growth of extensions or on the round to flat cell ratio (table 4.3). Although inhibition of axon outgrowth was observed at some of the lower concentrations, an IC_{50} value was not reached (figure 4.6 and 4.7).

Table 4.2. Dose response effects of TOCP on differentiating N2a cells exposed for 24 and 48 hours

N2a cells were differentiated in the presence of a range of TOCP concentrations for 24 and 48 hours. The cells were fixed and stained with Coomassie blue at these time points. Data are expressed as the number recorded per hundred cells ± standard error from an average of 8 wells of cells cultured on 2 separate occasions.

Time (hours)	TOCP (µg/ml)	Extensions (%)	Axons (%)	Round cells (%)	Flat cells (%)
24	0	40 ± 2.3	34 ± 1.5	36 ± 1.9	64 ± 1.9
	0.1	34 ± 1.9	29 ± 2.2	33 ± 2.0	67 ± 2.0
	0.25	42 ± 2.1	24 ± 1.1	36 ± 1.7	64 ± 1.7
	0.5	43 ± 5.5	15 ± 1.6	25 ± 2.7	75 ± 2.7
	0.75	46 ± 5.0	16 ± 2.6	45 ± 2.1	54 ± 2.1
	1.0	45 ± 3.1	16 ± 1.2	40 ± 1.5	60 ± 1.5
48	0	38 ± 3.5	32 ± 1.6	34 ± 1.6	66 ± 1.6
	0.1	35 ± 3.6	20 ± 1.6	34 ± 1.5	66 ± 1.5
	0.25	28 ± 6.5	20 ± 4.4	17 ± 3.7	83 ± 3.7
	0.5	45 ± 5.7	22 ± 2.3	20 ± 3.2	80 ± 3.2
	0.75	29 ± 2.7	11 ± 1.4	12 ± 2.8	88 ± 2.8
	1.0	48 ± 4.7	14 ± 1.6	13 ± 2.0	87 ± 2.0

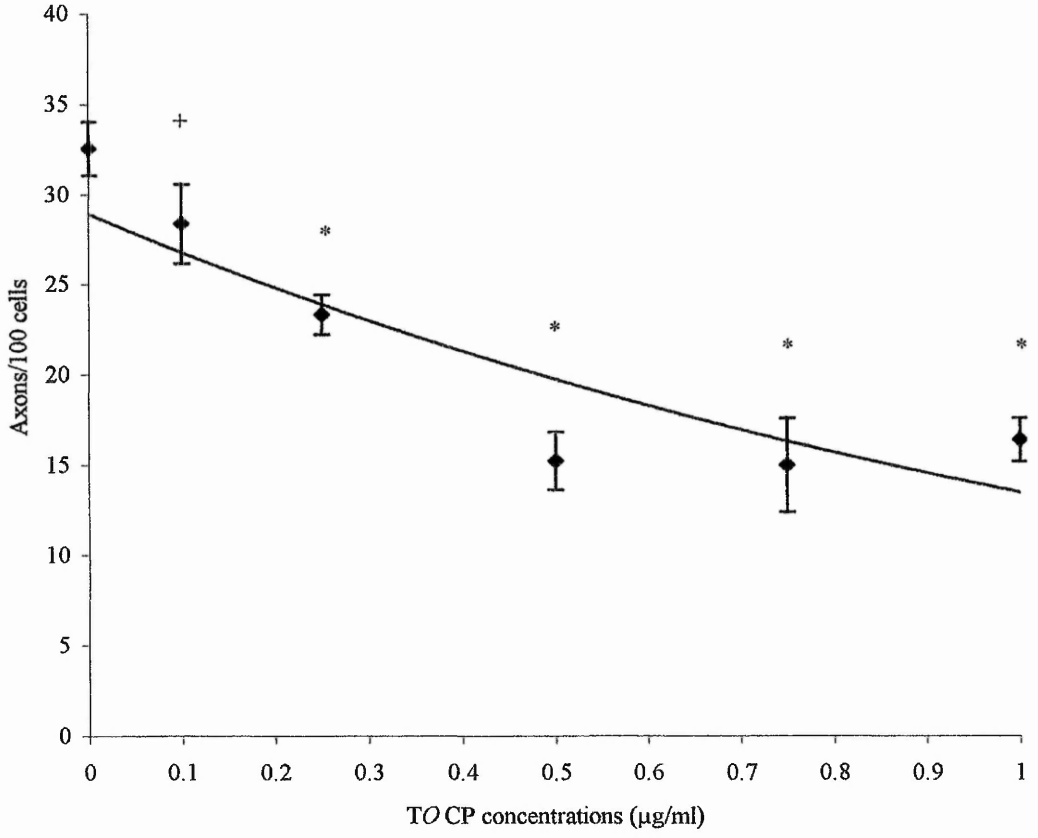


Figure 4.4. *IC_{50} determination for the effects of TOCP on axon outgrowth in N2a cells for 24 hour incubation.* Presented are the numbers of axon-like processes per 100 cells following 24 hours incubation in the presence of various concentrations of TOCP. All reductions are significant as determined by the Mann-Whitney U test (+ = $p < 0.2$; * = $p < 0.0001$). Each data point represents an average of 32 wells of cells from 8 separate dose response experiments. An IC_{50} value of **0.74 ± 0.04 µg/ml** was calculated from an average of 8 separate dose response experiments. Standard errors are shown as error bars.

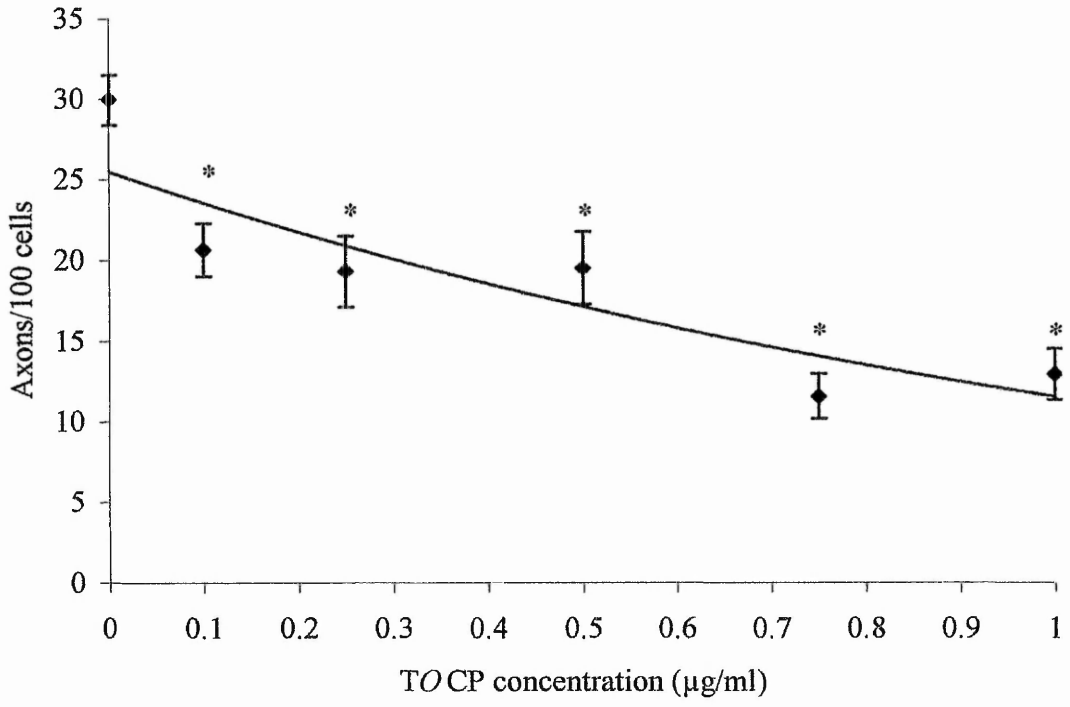


Figure 4.5. *IC_{50} determination for the effects of TOCP on axon outgrowth in N2a cells for 48 hour incubation.* Presented are the numbers of axon-like processes per 100 cells following 48 hours incubation in the presence of various concentrations of TOCP. All reductions are significant as determined by the Mann-Whitney U test (* = $p < 0.0005$). Each data point represents an average of 20 wells of cells from 5 separate dose response experiments. An IC_{50} value of **0.65 ± 0.08 µg/ml** was calculated from an average of 5 separate dose response experiments. Standard errors are shown as error bars.

Table 4.3. Dose response effects of TPCP on N2a cells exposed for 24 and 48 hours.

N2a cells were differentiated in the presence of a range of TPCP concentrations for 24 or 48 hours and subsequently fixed and stained with Coomassie blue. Data are expressed as the number recorded per hundred cells ± standard error from an average of 8 wells of cells cultured on 2 separate occasions.

Time (hours)	TPCP (µg/ml)	Extensions (%)	Axons (%)	Round cells (%)	Flat cells (%)
24	0	28 ± 1.6	29 ± 1.7	26 ± 1.4	74 ± 1.4
	0.1	32 ± 1.8	25 ± 1.9	27 ± 1.7	73 ± 1.7
	0.25	31 ± 2.1	20 ± 1.5	23 ± 1.6	77 ± 1.6
	0.5	36 ± 2.0	18 ± 1.5	25 ± 2.0	75 ± 2.0
	0.75	36 ± 2.1	16 ± 1.2	26 ± 1.8	74 ± 1.8
	1.0	36 ± 2.6	15 ± 1.1	24 ± 1.6	76 ± 1.6
48	0	31 ± 2.1	31 ± 3.1	32 ± 1.3	68 ± 1.3
	0.1	35 ± 2.8	18 ± 2.1	38 ± 2.1	62 ± 2.1
	0.25	34 ± 4.1	19 ± 1.3	35 ± 2.6	65 ± 2.6
	0.5	35 ± 2.2	22 ± 4.5	32 ± 1.6	68 ± 1.6
	0.75	39 ± 7.6	21 ± 3.6	39 ± 3.6	61 ± 3.6
	1.0	35 ± 2.0	28 ± 3.1	33 ± 2.0	67 ± 2.0

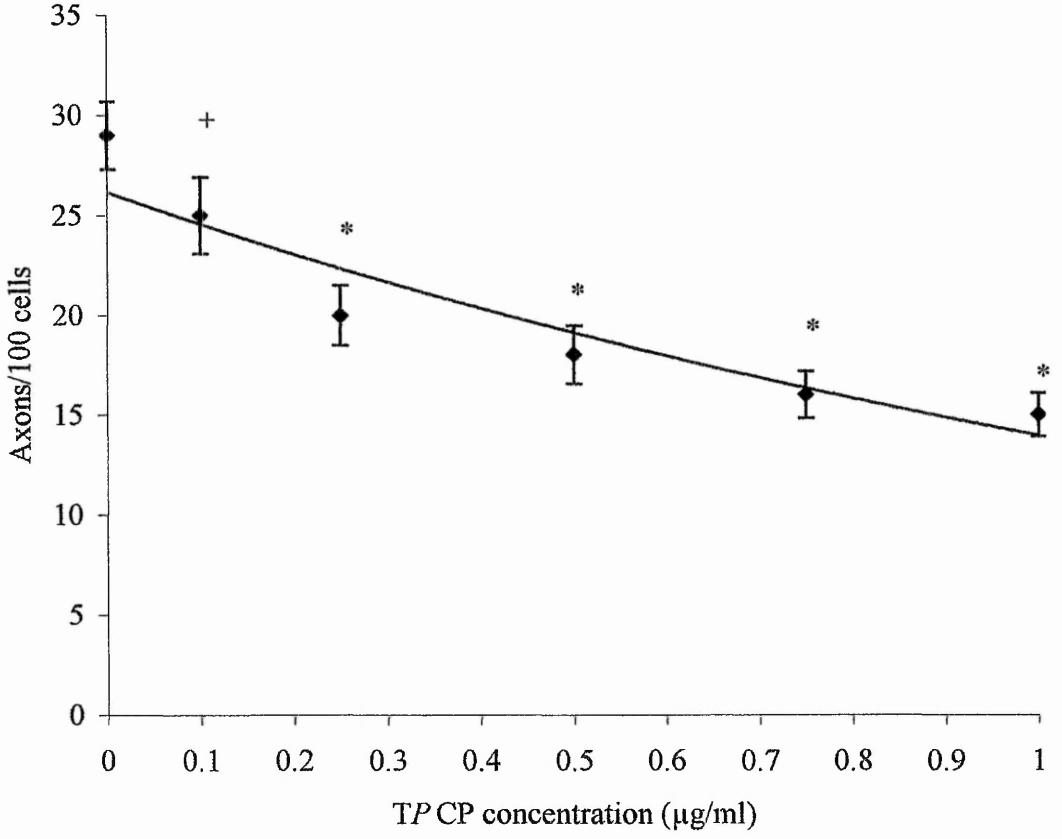


Figure 4.6. *IC_{50} determination for the effects of TPCP on axon outgrowth in N2a cells for 24 hour incubation.* Presented are the numbers of axon-like processes per 100 cells following 24 hours incubation in the presence of various concentrations of *TP*CP. All reductions were significant as determined by the Mann-Whitney U test (+ = $p < 0.2$; * = $p < 0.0002$). Each data point represents an average of 35 wells of cells from 7 separate dose response experiments. An IC_{50} value of **0.86 ± 0.04 µg/ml** was calculated from an average of 7 separate dose response experiments. Standard errors are shown as error bars.

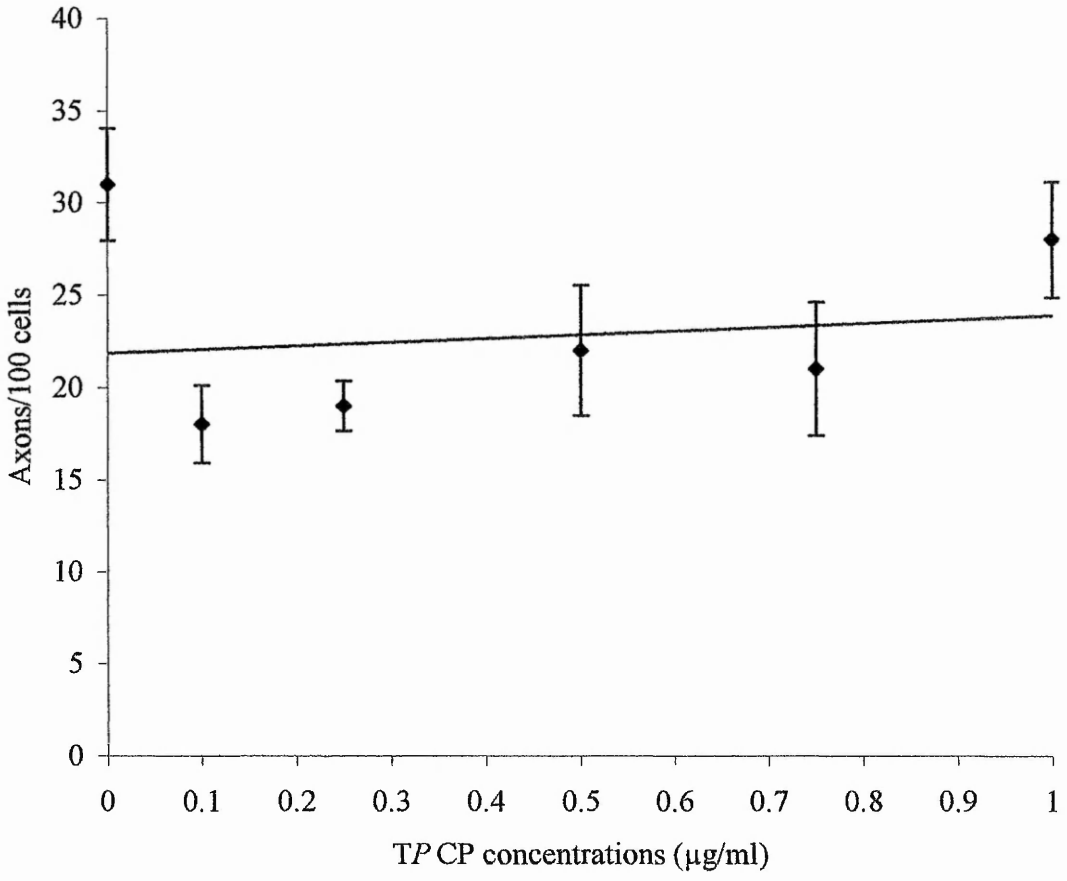


Figure 4.7. *IC_{50} determination for the effects of TPCP on axon outgrowth in N2a cells for 48 hour incubation.* Presented are the numbers of axon-like processes per 100 cells following 48 hours incubation in the presence of various concentrations of T*P*CP. Each data point represents an average of 20 wells of cells from 5 separate dose response experiments. The IC_{50} value could not be established from 5 separate dose response experiments. Standard errors are shown as error bars.

4.3 Molecular analysis of differentiating N2a cell extracts exposed to T*O*CP and T*P*CP

4.3.1 Western blotting analysis of N2a cells exposed to 1 μg/ml T*O*CP and T*P*CP for 24 and 48 hours

Western blots of cell extracts exposed to either control conditions (0.05 % (v/v) ethanol) or 1 μg/ml T*O*CP/T*P*CP at 24 and 48 hour time points, were probed with several monoclonal antibodies, in order to investigate the effects in more detail at the molecular level (section 2.2.2). This involved the use of a series of antibodies recognising different epitopes within NF and MT structures as described in section 3.3.1.

Each Western blot presented in the following pages looks at the visible changes in antibody binding when the cell extracts have been probed with different antibodies. Further work was conducted with the QuantiScan software package in order to quantify the intensities of each band, providing further information into the significance of each effect when compared to its corresponding control (table 4.4).

As can be seen in figure 4.8, a reduction in RMd09 antibody binding is reduced slightly in extracts incubated for 24 hours with 1 μg/ml T*O*CP compared with the control cell extract, and a further reduction is present after 48 hours. As with 1 μg/ml T*O*CP, a similar reduction in RMd09 antibody binding can seen in cells treated with 1 μg/ml T*P*CP after 24 hours when compared to the control cell extracts and after 48 hours exposure this reduction in antibody binding is still apparent. This Western blot, coupled with the denistometric analysis shown in table 4.4, suggests that both T*O*CP and T*P*CP reduce reactivity of the antibody towards the non-phosphorylation dependent epitope on NF-H at each time point.

Figure 4.9 shows a Western blot probed with Ta51 (which recognises phosphorylated NF-H) indicating that extracts from cells exposed to 1 μg/ml T*O*CP and T*P*CP show reduced reactivity with this epitope after 24 and 48 hours, with the level of reactivity being lower than the corresponding control at each time point. Densitometric analysis

using QuantiScan software confirmed this reduction in reactivity to a lower level than that observed with RMd09. Interestingly, figure 4.10 indicates that the binding of antibody N52 to NF-H is not reduced following 24 hours exposure to both isomers. However, after a further 24 hours exposure, the intensity of antibody binding appears to decrease in the T*O*CP and T*P*CP treated extracts, confirming earlier results that these isomers may cause a decrease in NF-H levels. However, it should be noted that the Western blot results using N52 were less reproducible than for the other two anti-NF-H antibodies.

From the Western blot probed with anti-α-tubulin antibody (figure 4.11), there is an apparent decrease in the levels reactivity with anti-α-tubulin in T*O*CP and T*P*CP - treated cell extracts after 24 hour incubations. However, after 48 hours the effect with 1 μg/ml T*O*CP appears to decrease further whereas with 1 μg/ml T*PCP* the decrease is similar to that seen at 24 hours, as confirmed by densitometric analysis.

Figure 4.12 represents a Western blot probed with the monoclonal antibody GAP-7B10, which recognises the axon growth associated protein GAP-43. The levels of antibody binding to T*O*CP treated cell extracts appear to be unchanged after 24 hours exposure whereas T*P*CP treated cell extracts appears to be slightly lower at this time point when compared to their corresponding controls. After 48 hours exposure these levels appear to be further reduced to extremely low levels with both isomers, as confirmed by densitometric analysis (table 4.4).

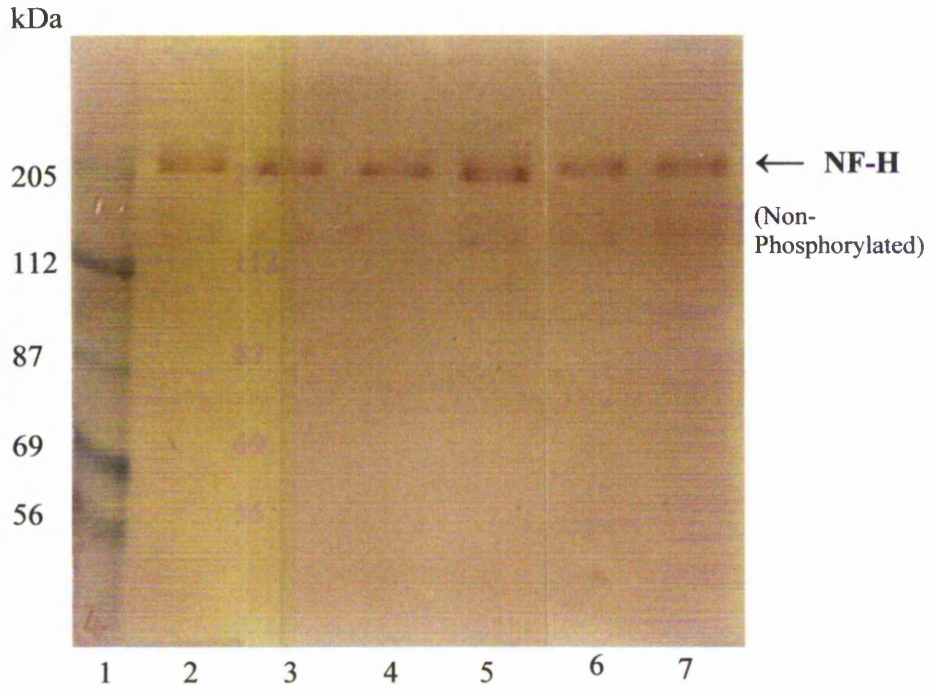


Figure 4.8. *Western blots of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with anti-neurofilament antibody, RMd09.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 24 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 24 hours; (5) control N2a cell extracts after 48 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 48 hours; (7) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 48 hours. The blot was probed with antibody, RMd09 that recognises non-phosphorylation dependent epitope on NF-H. The arrow indicates a reactive polypeptide with the apparent molecular weight of NF-H.

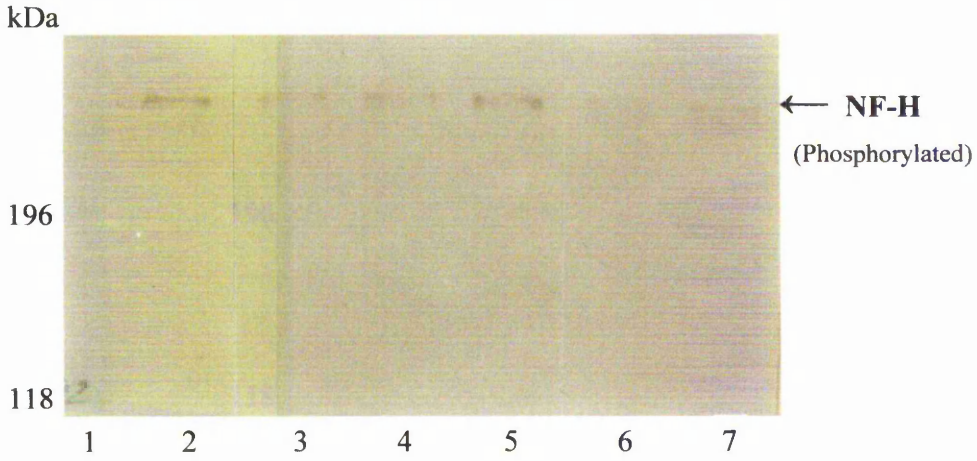


Figure 4.9. *Western blots of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with anti-neurofilament antibody, Ta51.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 24 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 24 hours; (5) control N2a cell extracts after 48 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 48 hours; (7) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 48 hours. The blot was probed with antibody Ta51, which recognises a phosphorylation dependent epitope on NF-H. The arrow indicates the polypeptide with the apparent molecular weight of NF-H.

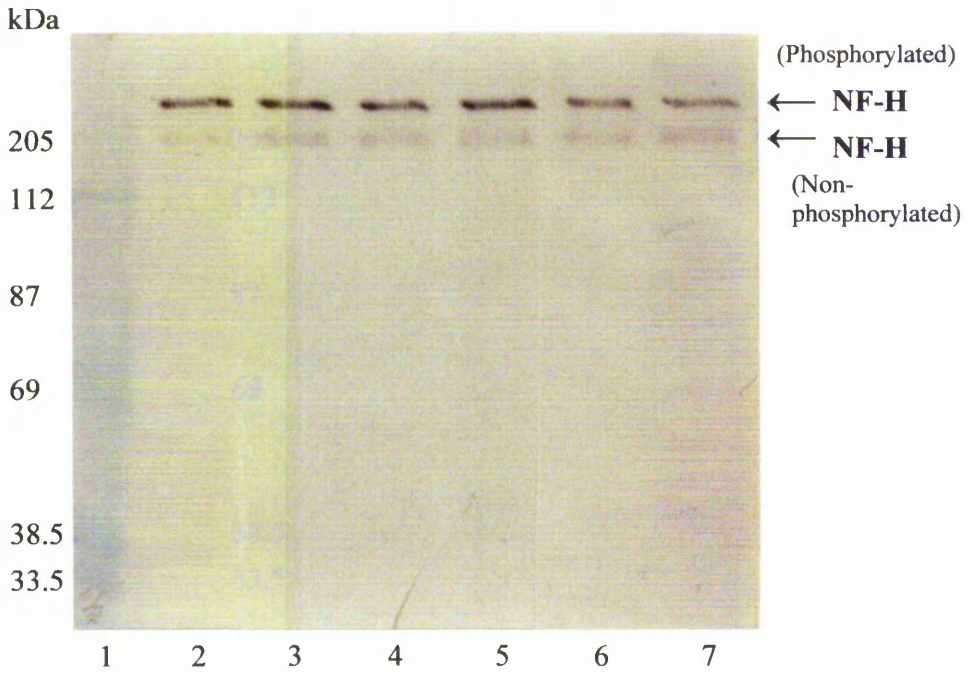


Figure 4.10. *Western blots of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with anti-neurofilament antibody, N52.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 24 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 24 hours; (5) control N2a cell extracts after 48 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 48 hours; (7) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 48 hours. The blot was probed with antibody, N52 that recognises NF-H epitope independent upon the phosphorylation state. The arrows indicate a reactive polypeptide with the apparent molecular weight of NF-H.

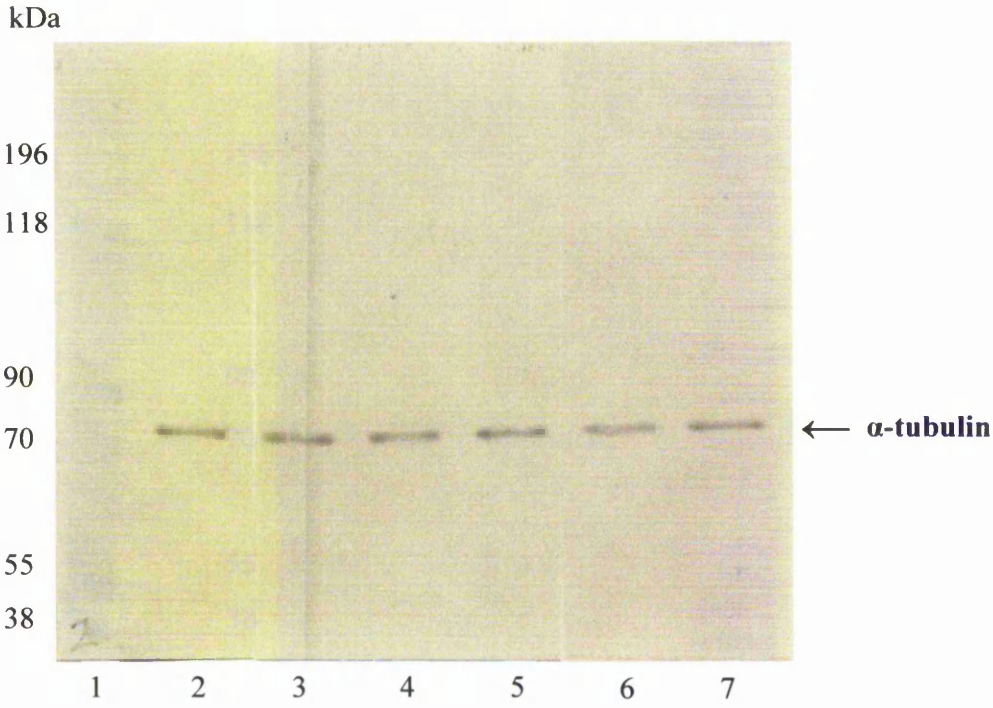


Figure 4.11. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml TOCP and TPCP, probed with anti-tubulin antibody, B-5-1-2.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 24 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 24 hours; (5) control N2a cell extracts after 48 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 48 hours; (7) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 48 hours. The blot was probed with antibody, B-5-1-2 that recognises an epitope located at the C-terminal end of the α-tubulin isoform. The arrow indicates a reactive polypeptide with the apparent molecular weight of α-tubulin.

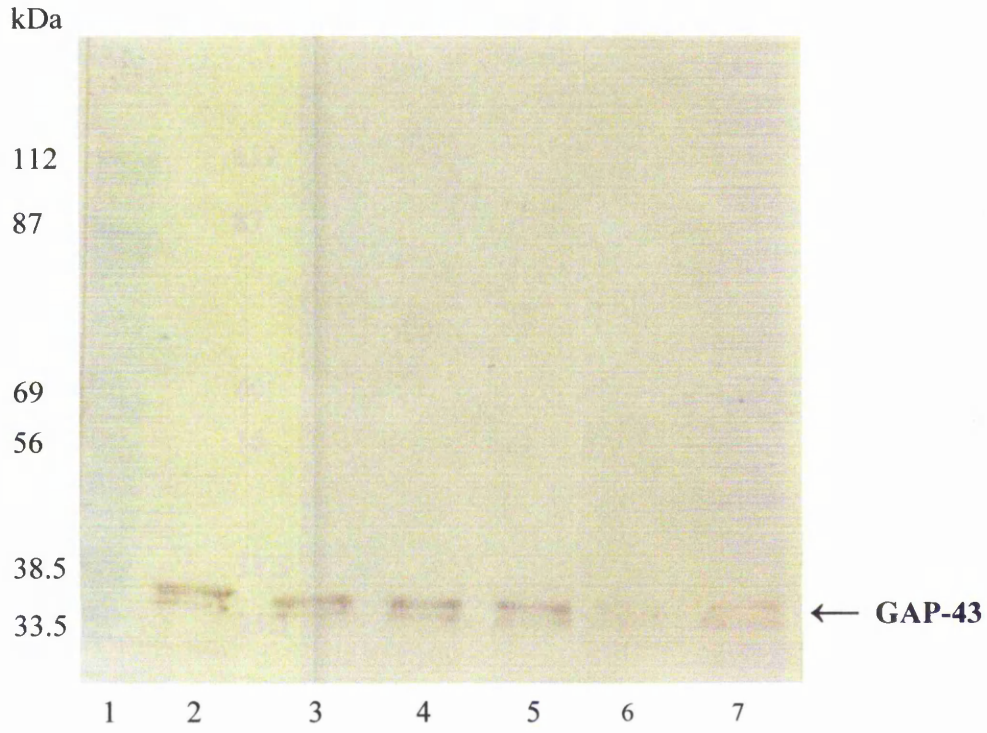


Figure 4.12. *Western blot analysis of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with GAP-7B10.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 24 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 24 hours; (5) control N2a cell extracts after 48 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 48 hours; (7) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 48 hours. The blot was probed with antibody GAP-7B10, which recognises GAP-43 with in neuronal processes. The arrow indicates a reactive polypeptide with the apparent molecular weight of GAP-43.

4.3.2 Quantitative analysis of Western blot data from extracts of differentiating N2a cells exposed to 1 µg/ml T*O*CP and T*P*CP

As the Western blot data was only assessed by visual comparisons, the intensities of antibody binding on each blot were then analysed quantitatively using a QuantiScan software package. This package examines the intensities of each band from the Western blot giving it an arbitrary value that represents the band intensity. These arbitrary values are represented below as a percentage of their corresponding control. However, due to the limited supply of Western blot data, statistical analysis was only assessed when the number of blots was three or more.

Table 4.4. Densitometric analysis of Western blots using QuantiScan

Data from the densitometry analysis has been tabulated below with values recorded representing the antibody intensities when compared to the corresponding control ± standard error from probed blots of two separate sets of cell extracts.

	1 µg/ml T*O*CP		1 µg/ml T*P*CP	
	24 hours	**48 hours**	**24 hours**	**48 hours**
Non-phosphorylation dependent epitope of NF-H (RMd09)	**86** ± 0.09	**61** ± 0.31	**86** ± 0.13	**86** ± 1.20
Phosphorylation dependent epitope of NF-H (Ta51)	**55** ± 1.50	**35** ± 2.00	**49** ± 2.00	**48** ± 1.00
Phosphorylation independent epitope of NF-H (N52)	**101** ± 21.1	**86** ± 23.7	**103** ± 13.4	**76** ± 18.1
α-tubulin (B-5-1-2)	**82** ± 4.70	**65** ± 5.40	**93** ± 1.50	**81** ± 1.20
GAP-43 (GAP-7B10)	**109** ± 9.00	**29** ± 3.20	**79** ± 6.00	**35** ± 15.0

4.4 Morphological studies of differentiating N2a cells exposed to 1 μg/ml T*O*CP and T*P*CP for 8 hours

Having established that differentiating N2a cells exposed to TCP and T*O*CP for 24 hours showed a significant reduction in axon-like processes, and that T*P*CP appears to be less neurotoxic, it was of further interest to determine whether isomer specific differences occur and were more distinguishable at earlier time points. For example, Enrich *et al.*, (1994) and Ehrich (1995) described inhibition of NTE in cultured N13 cells with in a few hours of exposure to OPDIN-inducing OPs. Experimental work was therefore conducted on differentiating N2a cells exposed to T*O*CP and T*P*CP after 4 and 8 hours.

4.4.1 Microscopic observation of N2a cells exposed to 1 μg/ml T*O*CP and T*P*CP after 8 hours

The following figures show the visual effects of the mentioned compounds after 8 hours exposure on the N2a cell line.

Figure 4.13. *Differentiating N2a cells exposed to 0.05 % (v/v) ethanol (control) for 8 hours.* Note the abundance of axon-like processes (arrows) and small extensions, suggesting the cells are healthy. Cells were fixed and stained with Coomassie blue stain, in all cases.

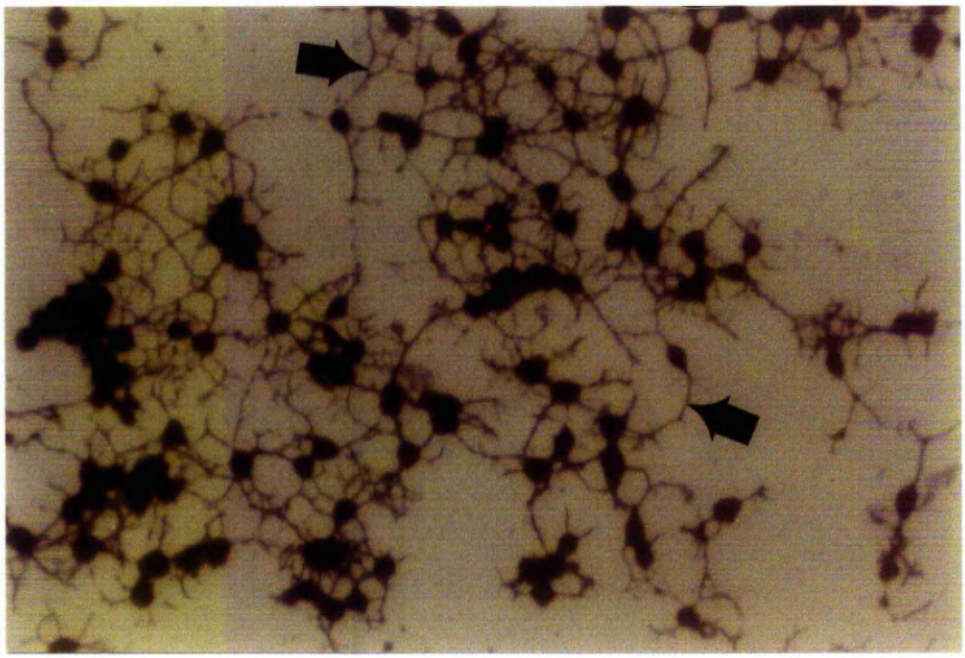


Figure 4.14. *Differentiating N2a cells exposed to 1 μg/ml TOCP for 8 hour* . Note the reduction in numbers of axon-like processes (arrow), which suggests that T*O*CP is inhibiting the production of axon-like processes at this concentration.

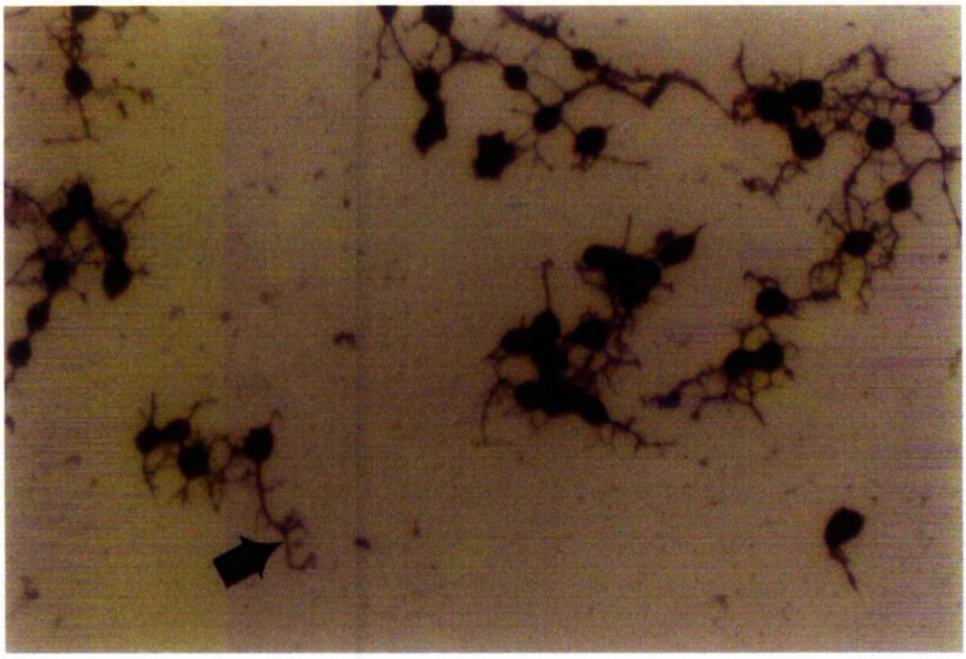
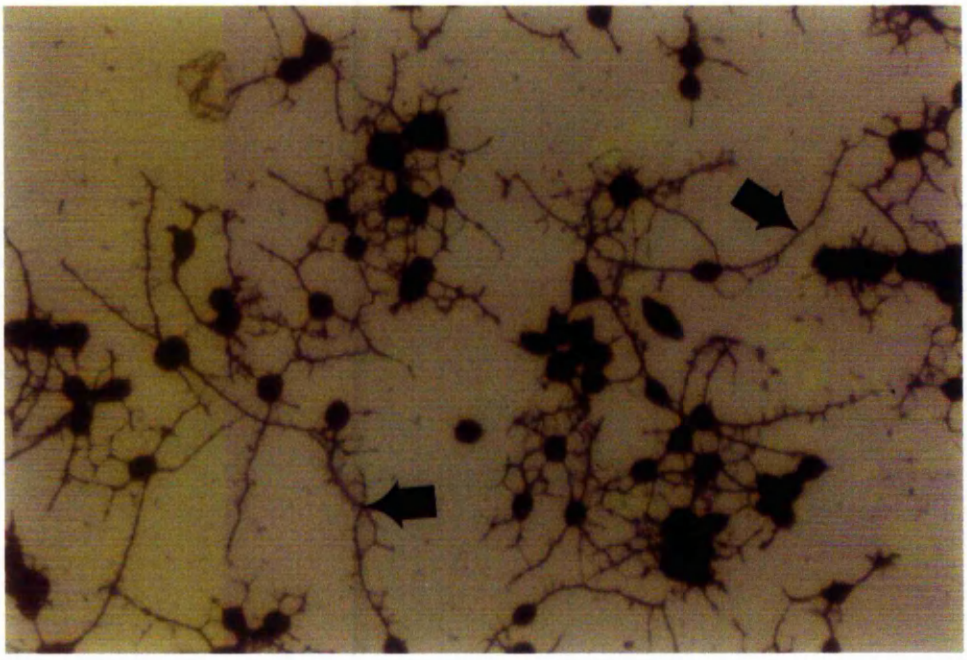


Figure 4.15. *Differentiating N2a cells incubated with 1 μg/ml TPCP for 8 hours.* Note the numbers of axon-like processes (arrows), suggesting that T*P*CP has no effect on these processes after 8 hour incubation (similar to figure 4.13).



4.5 Determination of cell growth in the presence of T*O*CP and T*P*CP after 4 and 8 hour incubations using MTT reduction

The growth of differentiating N2a cells when exposed to non-cytotoxic levels of T*O*CP and T*P*CP was established by MTT reduction as described earlier in this chapter.

Table 4.5. MTT reduction by differentiating N2a cells exposed to 1 µg/ml TOCP and TPCP

Differentiating N2a cells were incubated with 1 µg/ml OP compound or 0.05 % ethanol (control) for 4 and 8 hours and then subjected to the MTT reduction assay as described in section 2.2.1.9. The results are presented as mean absorbance (570nm) ± standard error for 8 wells of cells cultured on 2 separate occasions.

	Control	**1 µg/ml T*O*CP**	**1 µg/ml T*P*CP**
4 hours	**0.29** ± 0.01	**0.33** ± 0.01	**0.34** ± 0.01
8 hours	**0.29** ± 0.01	**0.32** ± 0.01	**0.33** ± 0.01

The presented results for T*O*CP and T*P*CP were not significantly different when compared to the corresponding controls, as determined by Mann-Whitney U test ($p > 0.05$). These results demonstrate that neither isomer, when used at concentrations no greater than 1 µg/ml, interferes with cell growth.

4.6 Morphological effects of *T*OCP and *T*PCP after 4 and 8 hours exposure

4.6.1 The effect 1 μg/ml *T*OCP and *T*PCP on differentiating N2a cells after 4 and 8 hour incubations

Table 4.6 shows that after 4 hours of exposure, axon outgrowth has been significantly inhibited by 1 μg/ml *T*OCP whereas *T*PCP appears to have no inhibitory effect on differentiating N2a cells. The number of small extensions and the round to flat cell ratio were not affected in any situation (figure 4.16).

After an eight hour incubation in the presence of the isomers, a similar pattern of inhibition can be seen with *T*OCP causing axon outgrowth to be inhibited to a level significantly lower than the corresponding control. By contrast, 1 μg/ml *T*PCP had no significant inhibitory effect on axon outgrowth in these cells at this time point (figure 4.16).

Table 4.6. The effect of 1 μg/ml TOCP and TPCP on differentiating N2a cells after 4 and 8 hours incubation

Differentiating N2a cells were exposed to 1 μg/ml *T*OCP and *T*PCP for 4 and 8 hours. The cells were then fixed and stained with Coomassie blue. Data are expressed as the number recorded per hundred cells ± standard error. These results are an average of 12 wells of cells cultured on 3 separate occasions.

Time (hours)	OP	Extensions (%)	Axons (%)	Round cells (%)	Flat cells (%)
4	Control	35 ± 1.2	35 ± 1.1	22 ± 0.8	78 ± 0.8
	*T*OCP	35 ± 1.4	19 ± 1.0	25 ± 1.4	75 ± 1.4
	*T*PCP	38 ± 1.3	29 ± 1.6	30 ± 1.5	70 ± 1.5
8	Control	38 ± 1.2	44 ± 1.2	22 ± 0.7	78 ± 0.7
	*T*OCP	38 ± 2.0	27 ± 1.8	31 ± 1.9	69 ± 1.9
	*T*PCP	41 ± 2.1	53 ± 2.2	35 ± 2.1	65 ± 2.1

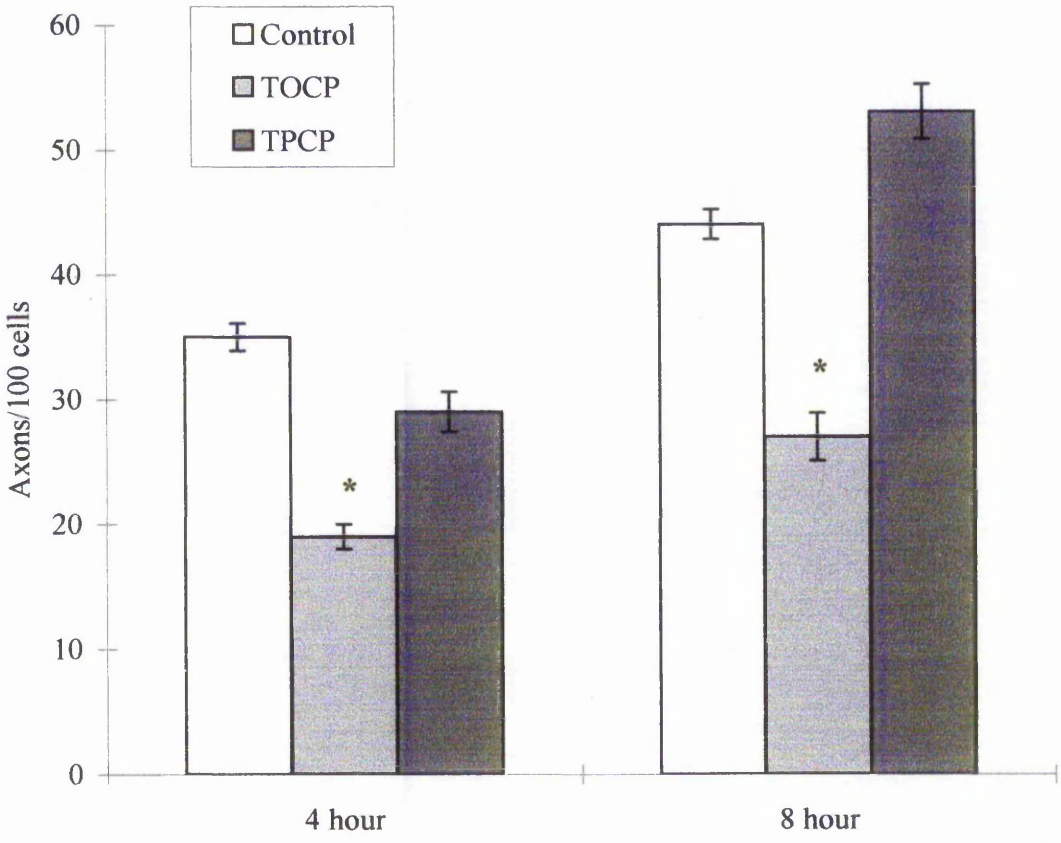


Figure 4.16. *The effects of 1 μg/ml TOCP and TPCP on axon outgrowth in N2a cells after 4 and 8 hour incubations.* Presented are the numbers of axon-like processes per 100 cells following 4 and 8 hour exposure in the presence of 1 μg/ml T*O*CP and T*P*CP. After 4 and 8 hour exposure, 1 μg/ml T*O*CP was found to cause a significant reduction in axon numbers compared to the controls, as determined by the Mann-Whitney U test (* = $p < 0.0004$). Each data point represents an average of 12 wells of cells cultured on 3 separate occasions. Standard errors are shown as error bars.

4.7 Molecular effects of T*O*CP and T*P*CP after 4 and 8 hour incubations on differentiating N2a cells

4.7.1 Western blotting analysis of N2a cells exposed to 1 µg/ml T*O*CP and T*P*CP

Further analysis of the short term effects of T*O*CP and T*P*CP was conducted at a molecular level by probing cell extracts with the antibodies described in section 3.3.1. Scanning probed blots using QuantiScan software (table 4.7) again allowed quantitation of antibody reactivity.

The first point to mention is the fact that the intensity of antibody binding recognising non-phosphorylated NF-H increases in control cell extracts from 4 to 8 hours suggesting that the N2a cells were behaving normally with an increase in NF-H levels being detected (figure 4.17).

After a 4 hour incubation, T*O*CP and T*P*CP treated cell extracts probed with RMd09 appear to be unaffected, with antibody binding intensity mirroring that of the corresponding control. However after 8 hours exposure, it appears that both T*O*CP and T*P*CP cause a reduction in antibody binding (figure 4.17).

Figure 4.18 shows that the levels of phosphorylated NF-H are inhibited by the presence of T*O*CP and T*P*CP when compared to the corresponding control after 4 and 8 hour incubations. However after 8 hours, this reduction is still apparent in antibody binding with T*P*CP but the level of antibody binding with T*O*CP appears to be greatly reduced to substantially lower levels and similar trends have been confirmed by densitometry (table 4.7)

The Western blot in figure 4.19 details the effects of T*O*CP and T*P*CP on N52 reactivity, which recognises NF-H independent of its phosphorylation state. Reductions in the levels of NF-H were observed in cell extracts that were incubated with T*O*CP for 4 hours and these levels were further reduced after 8 hours, when compared to the corresponding control. However, T*P*CP appeared to inhibit N52 reactivity to a similar extent as T*O*CP after 8 hours but the inhibition seen at 4 hours was minimal.

The Western blot in figure 4.20, corresponds to extracts of N2a cells induced to differentiate in the presence or absence of 1 μg/ml T*O*CP and T*P*CP and then probed with anti-α-tubulin antibody. After 4 hours incubation, a reduction can be seen in antibody binding with T*O*CP treated cell extracts, whereas no effect can be seen with T*P*CP when compared to the corresponding controls. After 8 hours, a greater reduction in antibody binding can be seen with T*O*CP and T*P*CP.

The Western blot in figure 4.21 shows very weak reactivity with antibody GAP-7B10 with a possible slight reduction compared to controls in the presence of OPs. However, due to the weak reactivity observed and with the background noise being relatively high, meaningful densitometric analyses using QuantiScan software was not achieved at these time points.

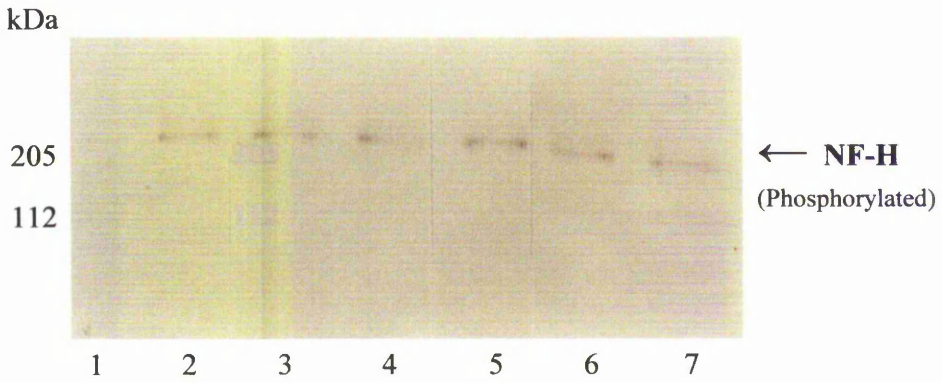


Figure 4.17. *Western blots of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with anti-neurofilament antibody, RMd09.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 4 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 4 hours; (5) control N2a cell extracts after 8 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 8 hours; (7) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 8 hours. The blot was probed with antibody, RMd09 that recognises a non-phosphorylation dependent epitope on NF-H. The arrow indicates a reactive polypeptide with the apparent molecular weight of NF-H.

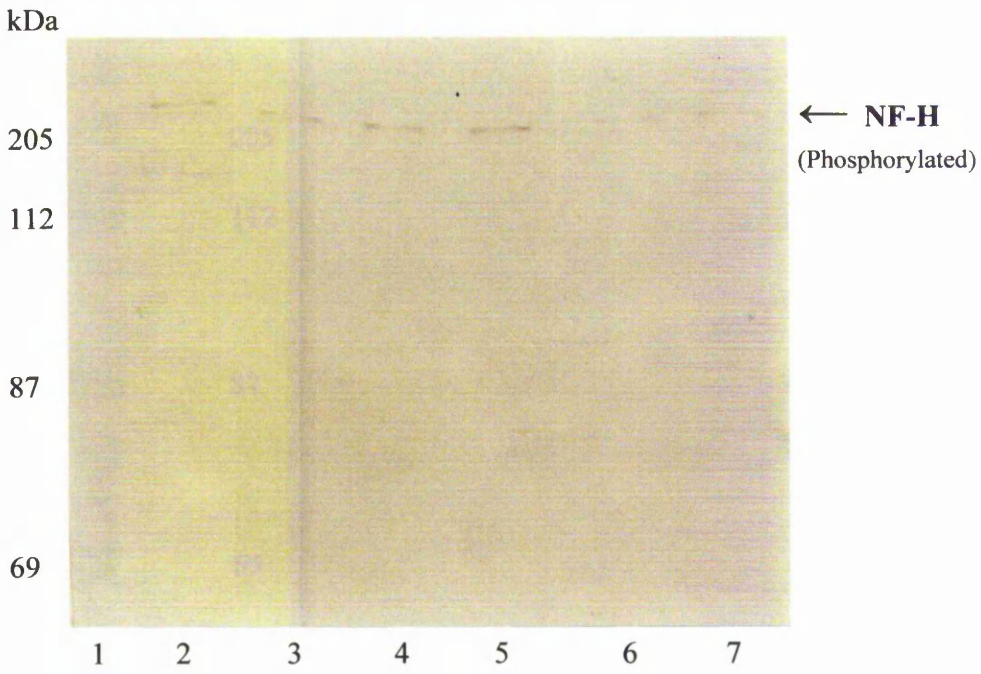


Figure 4.18. *Western blots of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with anti-neurofilament antibody, Ta51.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 4 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 4 hours; (5) control N2a cell extracts after 8 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 8 hours; (7) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 8 hours. The blot was probed with antibody, Ta51 that recognises a phosphorylation dependent epitope on NF-H. The arrow indicates a reactive polypeptide with the apparent molecular weight of NF-H.

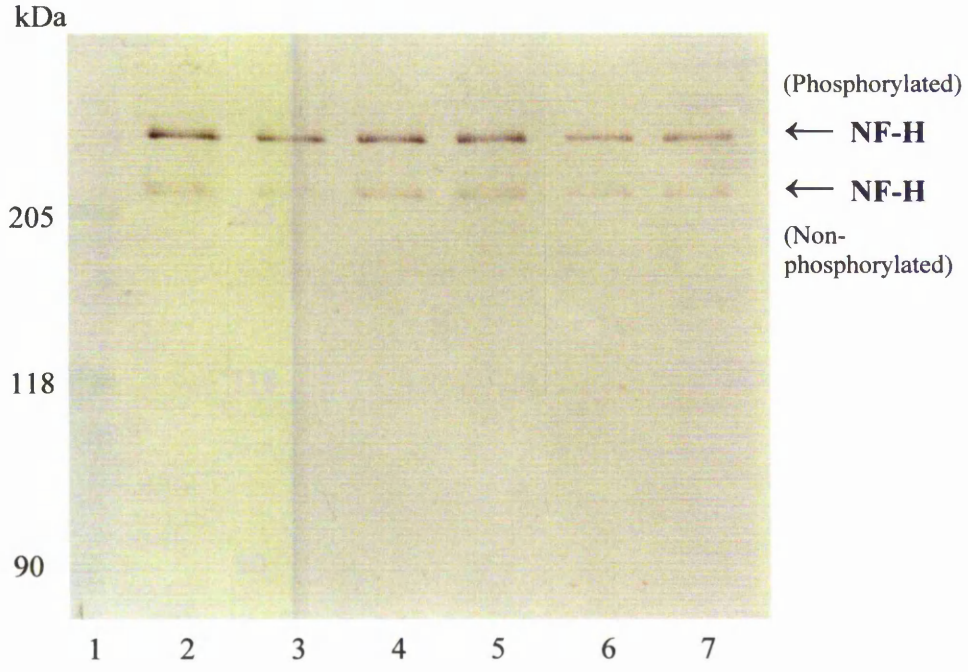


Figure 4.19. *Western blots of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with anti-neurofilament antibody, N52.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 4 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 4 hours; (5) control N2a cell extracts after 8 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 8 hours; (8) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 8 hours. The blot was probed with antibody, N52 that recognises a NF-H epitope independent upon the phosphorylation state. The arrows indicate a reactive polypeptide with the apparent molecular weight of NF-H.

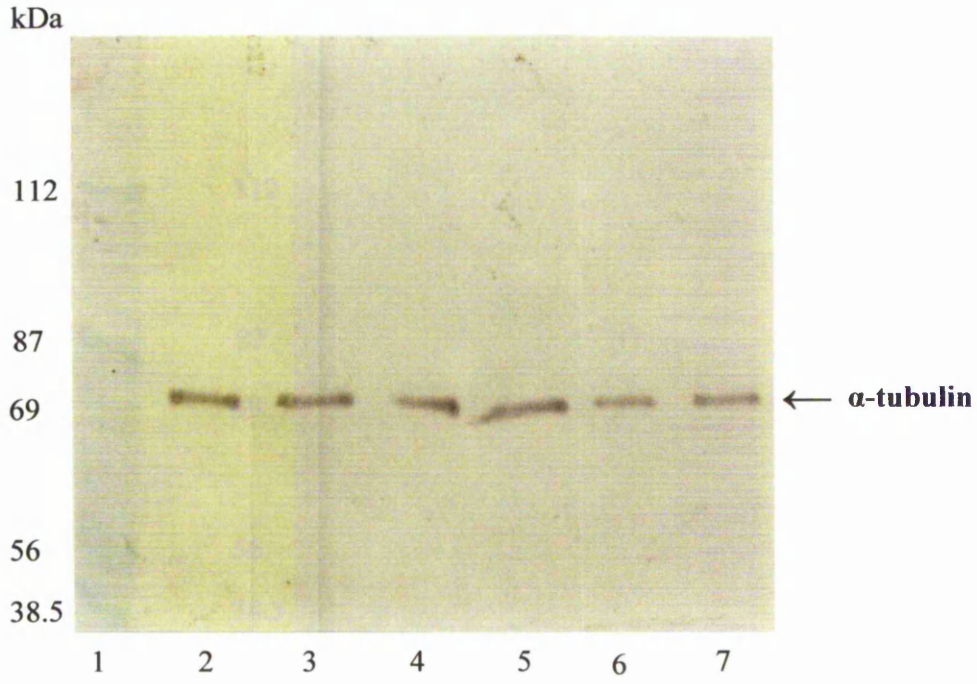


Figure 4.20. *Western blots of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with anti-tubulin antibody, B-5-1-2.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 4 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 4 hours; (5) control N2a cell extracts after 8 hour incubation; (7) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 8 hours; (8) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 8 hours. The blot was probed with antibody, B-5-1-2 that recognises an epitope located at the C-terminal end of the α-tubulin isoform. The arrow indicates a reactive polypeptide with the apparent molecular weight of α-tubulin.

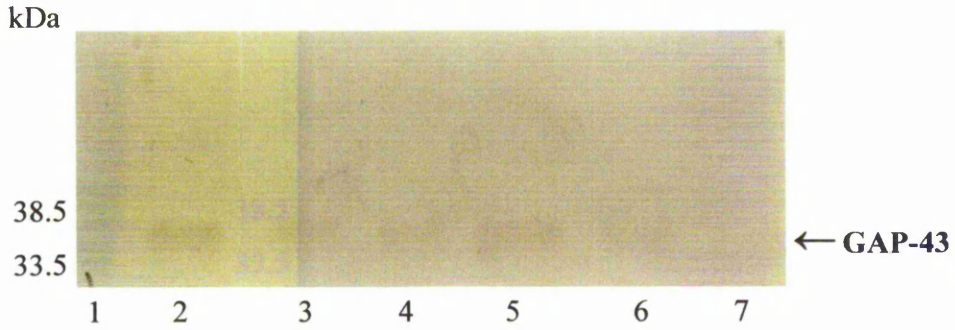


Figure 4.21. *Western blots of N2a cells induced to differentiate in the presence or absence of 1 μg/ml TOCP and TPCP, probed with GAP-7B10.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 4 hours; (4) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 4 hours; (5) control N2a cell extracts after 8 hour incubation; (6) extracts of N2a cells exposed to 1 μg/ml T*O*CP for 8 hours; (7) extracts of N2a cells exposed to 1 μg/ml T*P*CP for 8 hours. The blot was probed with antibody GAP-7B10, which recognises GAP-43 with in neuronal processes. The arrow indicates a reactive polypeptide with the apparent molecular weight of GAP-43.

4.7.2 Quantitative analysis of Western blot data from extracts of differentiating N2a cells exposed to 1 µg/ml *TOCP* and *TPCP* after 4 and 8 hours

As the Western blot data was only assessed by visual comparisons, the intensities of antibody binding on each blot were then analysed quantitatively using the QuantiScan software package as described in section 3.3.2 and 4.3.2. However, due to the limited supply of Western blot data, statistical analysis was only performed when the number of blots was three or more.

Table 4.7. Densitometric analysis of Western blots using QuantiScan

Data from the densitometric analysis has been tabulated below, with values recorded representing the antibody binding intensities when compared to the corresponding control ± standard error from probed blots of two separate sets of cell extracts.

	1 µg/ml *TOCP*		1 µg/ml *TPCP*	
	4 hours	**8 hours**	**4 hours**	**8 hours**
Non-phosphorylation dependent epitope of NF-H (RMd09)	**96** ± 1.50	**74** ± 6.00	**97** ± 5.50	**70** ± 8.00
Phosphorylation dependent epitope of NF-H (Ta51)	**66** ± 4.00	**38** ± 13.0	**87** ± 8.50	**68** ± 7.00
Phosphorylation independent epitope of NF-H (N52)	**65** ± 7.50	**52** ± 12.0	**90** ± 1.00	**55** ± 2.50
α-tubulin (B-5-1-2)	**80** ± 8.50	**58** ± 7.50	**104** ± 2.00	**69** ± 9.50

4.8 Discussion

MTT reduction assays confirmed that 1 μg/ml T*O*CP and T*P*CP had no cytotoxic effect on differentiating N2a cells from 4 to 48 hours of exposure. Thus, 1 μg/ml was used as the highest concentration of both isomers in cell differentiation experiments, in agreement with the non-cytotoxic dose for TCP found by Flaskos *et al.*, (1998) and as shown in chapter 3; section 3.2.2.

It is important to note that, when comparing outgrowth of axons in the control situations of 4 and 8 hours, a clear increase in axon numbers with time was observed which indicates that differentiating N2a cells are healthy and responding normally to the control environment (table 4.6). These values are also in broad agreement with the time course for axon outgrowth shown in figure 3.5.

The morphological data indicates that axon outgrowth by differentiating N2a cells exposed to T*O*CP for 4 and 8 hours is greatly decreased when compared to the corresponding control. In contrast to this isomer, T*P*CP appears to have little or no inhibitory effect on these cells at either time point (figures 4.13 to 4.15). This was confirmed by the morphological data that clearly show a large reduction in axon numbers when exposed to T*O*CP for both 4 and 8 hours and only for 4 hours when exposed to T*P*CP with total reversal seen after 8 hours to similar levels as the control (table 4.6).

To compare the later effects of T*O*CP and T*P*CP in more detail, dose response experiments were conducted for 24 and 48 hours exposure, which led to the determination of IC_{50} values for axon outgrowth at each time point. For differentiating N2a cells incubated with a range of T*O*CP concentrations up to 1 μg/ml, the morphological data showed that axon numbers decreased as the concentration of T*O*CP increased after 24 and 48 hour incubations, with all concentrations being significantly different from the control as determined by the Mann-Whitney U test ($p < 0.05$).

The IC_{50} values for 24 hour incubations were estimated at 0.74 ± 0.04 μg/ml (determined from eight separate dose response experiments) and for 48 hours

incubation to be 0.65 ± 0.08 μg/ml (determined from five separate dose response experiments). This suggests that T*O*CP becomes slightly more neurotoxic as time increases and that it has a sustained neurotoxic effect. However, these IC_{50} values appear to be similar to those found for TCP. As TCP is the commercial product, it is known to be 95 % pure and contain only 3 % of the *–ortho* isomer (due to strict limiting regulations, Bondy *et al.*, 1960). This leads to the conclusion that there is 30 times less T*O*CP present in TCP when compared to pure T*O*CP, suggesting that T*O*CP is not necessarily the sole contributor to the overall TCP toxic effect.

With the isomer T*P*CP, dose response experiments conducted over 24 and 48 hours showed a change in effects. After 24 hours exposure to a range of T*P*CP concentrations, the axon number produced by the differentiating N2a cells decreased in a dose-dependent manner as the concentration of T*P*CP increased. However, the fact that 1 μg/ml T*P*CP did not reduce axon outgrowth, after 48 hours, suggests that its effect on axon outgrowth is transient.

The IC_{50} value could only be estimated for 24 hour time point at 0.86 ± 0.04 μg/ml (determined from seven separate dose response experiments), as a value was unattainable after 48 hours (determined on five separate experimental occasions). Although T*P*CP has been noted in the literature as being non-neurotoxic (Bondy *et al.*, 1960; Patton *et al.*, 1986; Lapadula *et al.*, 1991; Freudenthal *et al.*, 1993), this compound does appear to exhibit neurotoxicity towards differentiating N2a cells after 24 hours exposure. However, this level of neurotoxicity is lower than that seen with TCP and T*O*CP and is not sustained after 48 hours, suggesting the effect of this isomer is short lived and the exposed cells have the ability to recover from the toxic effects experienced after 24 hours. The possible reasons for this are discussed later in chapter eight.

The Western blot data in figures 4.17 to 4.18 and their densitometric analysis (table 4.7) indicates that T*O*CP causes a greater reduction in phosphorylated NF-H but has no effect on non-phosphorylated NF-H after 4 hours when compared to T*P*CP. This may suggest that either proteolysis of phosphorylated NF-H or reduced phosphorylation of NF-H could be occurring with this isomer at this time point (Appendix II). It should be

that after 4 hours T*O*CP also significantly reduces the levels of NF-H, when no such effect could be seen with T*P*CP. Consequently, after 8 hours exposure, the presence of T*O*CP and T*P*CP significantly reduces both phosphorylated NF-H levels (RMd09) and the overall levels of NF-H (detected by N52). In the case of T*O*CP these changes are concomitant with the inhibition of axon outgrowth (figure 4.16 and table 4.6). However, this is not the case with T*P*CP, as axon outgrowth is not inhibited when compared to the corresponding control, suggesting that the molecular changes seen at 8 hours are temporal. Indeed partial recovery was detected with N52 and RMd09 and a sustained reduction of Ta51 reactivity was observed after 24 and 48 hours. This indicates that there may be a net reduction in the levels of phosphorylated NF-H at these later time points. This is discussed in more detail in the general discussion; chapter 8.

Visual and densitometric analysis of Western blots of cell extracts probed with anti-α-tubulin antibody indicates a reduction in levels from 4 and 8 hours incubation with T*O*CP and 8 hours, only, in the case of T*P*CP (figure 4.20 and table 4.7). In both cases, the reduced levels of α-tubulin were sustained at the later time points, being consistently lower in the case of T*O*CP (figure 4.11 and table 4.4). Other Western blots were probed with microtubule and MAPs antibodies but weak reactivity made it difficult to draw any firm conclusions, with no obvious differences between the isomers (data not shown).

Visual and densitometric analysis of Western blots probed with GAP-7B10 monoclonal antibody (which recognises GAP-43 in growth cones of axonal processes) showed very low reactivity with cell extracts after 4 – 8 hours exposure, making densitometric analysis difficult. Analysis of extracts following 24 – 48 hours exposure to either isomer indicates no considerable effect on GAP-43 levels at 24 hours but a significant reduction with both isomers at 48 hours (figure 4.21 and 4.12 and table 4.4). This inhibition in the expression of GAP-43 suggests that the growth cone processes are being either destroyed or damaged due to the isomers' presence but only at a later time point. Further work is required to increase blot replicates to yield sufficient data for statistical analysis.

From the data in table 4.6, it can be seen that T*O*CP but not T*P*CP has a significant inhibitory effect on axon outgrowth after 4 and 8 hours but no effect on the outgrowth of small processes. These early morphological effects could be related to the reductions in RMd09, Ta51 and N52 reactivity on Western blots, suggesting there could be an immediate dephosphorylation or proteolysis of phosphorylated NF-H. It can be concluded that T*O*CP has a sustained toxic effect on axon outgrowth at all time points. By contrast, 1 μg/ml T*P*CP appears to have a transient effect on axon outgrowth with toxicity being observed after 24 hours but not after 48 hours of incubation.

The Western blot data for each time points gives an insight in to the molecular effects of these isomers giving a clearer indication of the axonal targets of both isomers over time.

Chapter Five

Effects of microsomal activation of organophosphates and a synthetic metabolite of T*O*CP on axon outgrowth from mouse N2a neuroblastoma cells

5.1 Introduction

It was demonstrated in the 1960s that, when T*O*CP is metabolised in the intestine and livers of rats by microsomal hydroxylation and cyclisation (Eto *et al.*, 1962), several antiesterase metabolites were produced with the major esterase inhibitor isolated being 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one (CBDP or SC*O*TP). Other metabolites were also identified such as *O*-cresyl dihydrogen phosphate, hydroxymethyl T*O*CP, just to name a few (Casida *et al.*, 1961; Eto *et al.*, 1962; Taylor and Buttar, 1967; Sharma and Watanabe, 1974; Nomeir and Abou-Donia., 1986). Studies into the effects of these metabolites have concentrated, mainly, on CBDP which suggest that this metabolite mimics the effects of T*O*CP *in vivo* (Burka and Chapin, 1993; Biswas *et al.*, 1993).

It has been suggested that T*O*CP undergoes metabolic activation and biochemical detoxification. This metabolic activation takes place via two systems : (1) hepatic microsomal cytochrome P-450, which catalyses the hydroxylation of one of the three - *ortho* methyl groups to hydroxymethyl T*O*CP, and (2) plasma albumin which recycles this product to yield CBDP (Eto *et al.*, 1962; Sharma and Watanabe, 1974). It has also been suggested that the balance between activation and detoxification of this compound may influence the production of OPIDN (Abou-Donia, 1992; 1995).

5.1.1 Hepatic microsomal activation

The effects of microsomally activated TCP, T*O*CP and T*P*CP were analysed using the cellular model described in an attempt to mimic the effects of metabolism of OPs, *in vitro*. These experiments were conducted on the basis of a routine procedure used in the laboratory of Dr. W. G. McLean at Liverpool University, although it had to be adapted slightly to suit the experimental conditions used throughout this research.

The principle behind this technique is to stimulate prepared microsomes in the presence of a NADPH generating system that is continually recycled by the presence of ATP. Microsomes were isolated from rat liver as this organ is known to be a rich source of smooth endoplasmic reticulum (ER). The smooth ER of the hepatocyte contains

enzymes, such as cytochrome P-450, that detoxify drugs and harmful compounds (Alberts *et al.*, 2000).

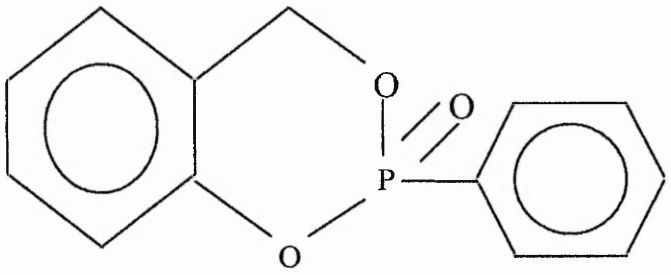
Several research groups have attempted to metabolically activate OPs. For example, Sprague and Castles (1985) used an *in vitro* neurotoxic esterase test with metabolic activation to assess the delayed neurotoxic potential of many different OPs such as T*O*CP, O,O-diphenyl-*o*-toyl phosphate (M*O*CP), etc. Ma and Chambers (1995) used rat hepatic microsomes to kinetically analyse cytochrome P-450 - mediated desulphuration (activation) and dearylation (detoxification). In the case of chlorpyrifos, they found that microsomes had a greater capacity to detoxify and a lower capacity to activate this compound when compared to parathion.

The aim of this section of work was to investigate the effects of microsomal activation on the toxicity of TCP, T*O*CP and T*P*CP towards differentiating N2a cells.

5.1.2 Characterisation of the cyclic phenyl saligenin phosphate (CPSP or PSP)

From the described pathway of T*O*CP degradation, one of the active metabolites was identified and Dr. I. Coutts at The Nottingham Trent University, Nottingham, prepared a structurally similar compound. The compound synthesised was identified as cyclic phenyl saligenin phosphate (CPSP or PSP), having the following chemical structure (figure 5.1). Structurally, this compound differs only slightly from CBDP by the absence of the methyl group and is known to have the same ability to inhibit NTE and cause OPIDN, *in vivo* (Nomeir and Abou-Donia, 1986).

Figure 5.1. Chemical structure of cyclic phenyl saligenin phosphate (CPSP or PSP)



This synthesised compound was then used in morphological studies on differentiated N2a cells in order to assess the level of toxicity produced compared to that seen with TCP and T*O*CP. Therefore, the aim was to assess the impact of CPSP on differentiated N2a cells by the following steps :

(i) determination of the cytotoxic effects of 1 μg/ml (3.8 μM) CPSP after 4, 8, 24 and 48 hour incubations,

(ii) dose response experiments for 4, 8, 24 and 48 hours exposure and estimation of IC_{50} values for 24 and 48 hours, and

(iii) molecular analysis of N2a cells exposed to 1 μg/ml CPSP after 4, 8, 24 and 48 hour incubations.

5.2 Effects of TCP, *TO*CP and *TP*CP on axon outgrowth by differentiating N2a cells in the presence and absence of a microsomal activation system

Investigations into the effects of microsomal activation of TCP and its isomers were carried out at different time points of 4, 24 and 48 hours, in order to assess the impact and the efficiency of the microsomal activation technique on the effects of OPs on differentiating N2a cells.

5.2.1 Analysis of the effects of microsomal activation of 1 µg/ml OPs on differentiating N2a cells after 4, 24 and 48 hours exposure

Initially, the effect of microsomes alone on axon outgrowth was assessed to determine if a neurotoxic effect was evident; no effect was observed.

As an internal control, a typical experiment was conducted as described in section 2.2.1.5 to 2.2.1.7, with axon outgrowth mirroring the effects seen in the earlier chapters (section 3.2.4, 4.2.3 and 4.6.1) when exposed to 1 µg/ml of test OP. However, when the cells are treated with the same concentration of OP compounds in the presence of the microsomal activation system (section 2.2.5), increased toxicity can be seen with both TCP and T*O*CP, whereas axon outgrowth in T*P*CP treated cells is unaffected.

For example, it is interesting to note that TCP normally has no toxic effect after 4 hours but after microsomal activation this compound is able to inhibit axon outgrowth in differentiating N2a cells. Similarly, an inhibitory effect is usually seen with T*O*CP after 4 hours but microsomal activation appears to enhance this effect even further (table 5.1).

After a 24 hour incubation period, it appears that the microsomal activation system increases the toxicity of TCP and T*O*CP slightly which is lower than that observed at 4 hours. In fact in the presence of T*P*CP it appeared to encourage axon outgrowth when compared to the 'NADPH system without microsomes' control (table 5.1).

In cells treated with TCP for 48 hours, there was a slight increase in the inhibition of axon outgrowth in the presence of activated microsomes compared to controls. However, the magnitude of this effect was lower than that seen at 4 or 24 hours. By contrast, activated microsomes caused no change in axon inhibitory effects of T*O*CP at this time point, whereas T*P*CP treated cells showed an increase in axon outgrowth under the same conditions (table 5.1).

Table 5.1. Microsomal activation of 1 μg/ml TCP, TOCP and TPCP after 4, 24 and 48 hour incubations.

Differentiating N2a cells were exposed to 1 μg/ml TCP, T*O*CP and T*P*CP in two separate experimental conditions - NADPH system without microsomes (M/S) or NADPH system with M/S in all cases incubated in the presence or absence of OPs for 3 time points. Cells were fixed and stained with Coomassie blue at these time points. Data expressed are axon numbers as a percentage of the corresponding control ± standard error. These results are an average of 6 wells cultured on three separate occasions.

		NADPH system without M/S	**NADPH system with M/S**
4 hour	**TCP**	**52** ± 1.9	**26** ± 0.9
	T*O*CP	**38** ± 2.2	**25** ± 1.3
	T*P*CP	**109** ± 3.8	**100** ± 1.9
24 hour	**TCP**	**59** ± 1.9	**41** ± 1.6
	T*O*CP	**60** ± 5.0	**52** ± 4.8
	T*P*CP	**78** ± 4.2	**98** ± 4.4
48 hour	**TCP**	**68** ± 2.8	**50** ± 2.5
	T*O*CP	**66** ± 3.6	**66** ± 3.5
	T*P*CP	**72** ± 2.9	**142** ± 2.9

5.2.2 Analysis of microsomal activation of differentiating N2a cells incubated in the presence of 0.1 μg/ml TCP, *TOCP* and *TPCP* for 4 hours

In order to determine whether non-neurotoxic concentrations of TCP, T*O*CP and T*P*CP could be metabolically activated, further experiments were performed at a concentration of 0.1 μg/ml on differentiating N2a cells.

Table 5.2. Microsomal activation of 0.1 μg/ml TCP and TOCP after 4 hour incubation of differentiating N2a cells

Differentiating N2a cells were incubated in the presence or absence of microsomally activated 0.1 μg/ml TCP and T*O*CP after 4 hours. Cells were fixed and stained with Coomassie blue at these time points. Data are expressed as axon numbers as a percentage of the corresponding control ± standard error. These results are an average of 8 wells of cells cultured on 2 separate occasions.

		NADPH system without M/S	**NADPH system with M/S**
4 hour	**TCP**	**55** ± 2.3	**59** ± 1.9
	T*O*CP	**69** ± 2.7	**39** ± 1.3

Although axon outgrowth was inhibited in the presence of 0.1 μg/ml for both compounds, microsomal activation of these compounds did not increase toxicity compared to the typical experiment (table 5.2). However, there was a decrease in axon numbers of T*O*CP activation compared to the NADPH control without microsomes.

5.3 Morphological studies on differentiating N2a cells exposed to cyclic phenyl saligenin phosphate (CPSP)

As the effects of microsomal activation had been assessed and presented earlier in this chapter, the emphasis was then placed on analysing the effects of the synthesised compound, cyclic phenyl saligenin phosphate (to be known as CPSP) on differentiating N2a cells. This was initially assessed over four time points, 4, 8, 24 and 48 hours, determining whether the metabolite was more neurotoxic than TCP and T*O*CP.

5.3.1 Microscopic observations of N2a cells exposed to 1 µg/ml CPSP

The following figures show the visual effects of 1 µg/ml CPSP after 8 and 48 hours exposure on the N2a cell line.

Figure 5.2a. *Effects of CPSP on N2a cell differentiation:* **(a)** *N2a cells differentiated for 4 hours in the presence of 0.05 % (v/v) ethanol (control).* Note the abundance of axon-like processes and small extensions, suggesting the cells are healthy (arrow); **(b)** *N2a cells differentiated in the presence of CPSP for 4 hours.* Note the reduction of axon-like processes. Cells were fixed and stained with Coomassie blue stain, in all cases.

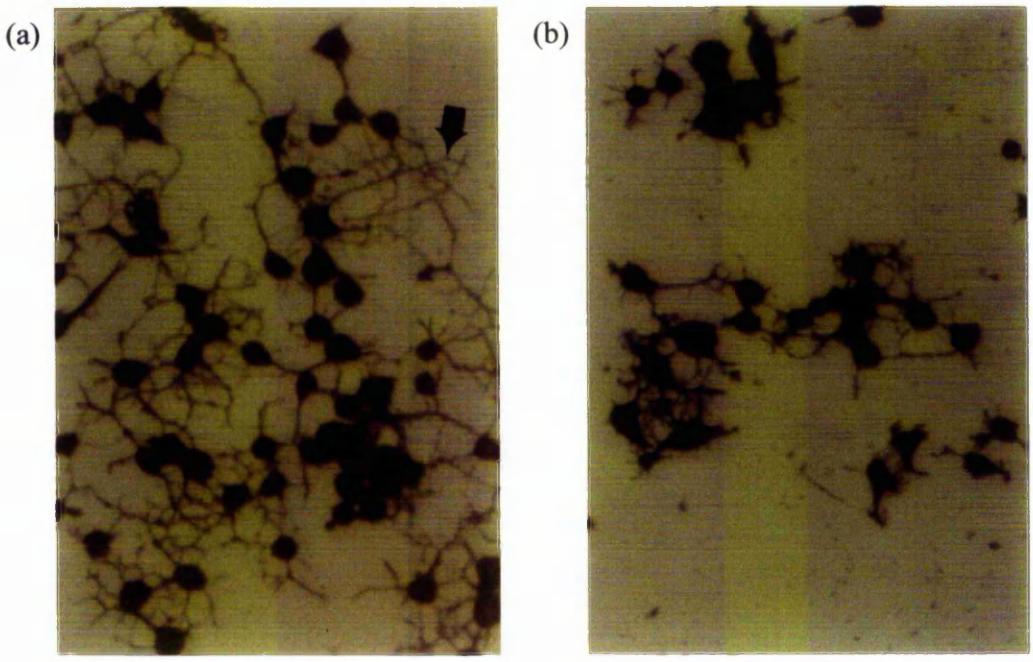
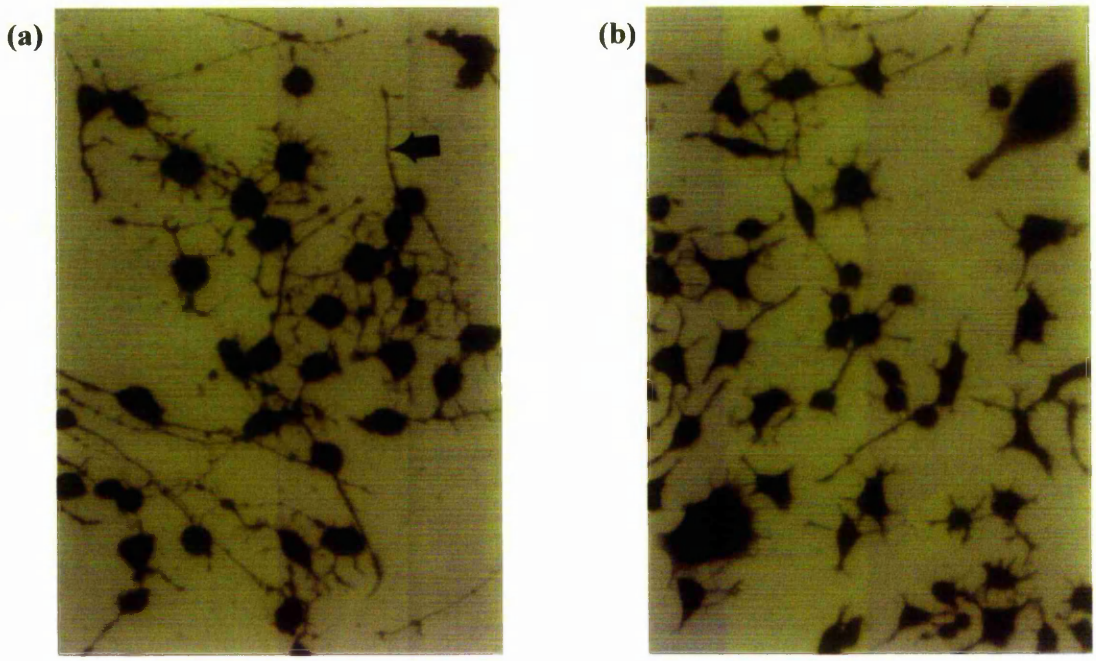


Figure 5.3. *Effects of CPSP on N2a cell differentiation:* **(a)** *N2a cells differentiated in the presence of 0.05 % (v/v) ethanol (control) for 48 hours.* Note the abundance of axon-like processes and small extensions, suggesting the cells are healthy (arrow); **(b)** *N2a cells differentiated in the presence of 1 μg/ml CPSP for 48 hours.* It can be clearly seen that the number of axon-like processes has decreased when compared to the control.



5.3.2 Determination of cell growth in the presence of CPSP using MTT reduction

The growth of differentiating N2a cells was determined in the presence of CPSP following 4, 8, 24 and 48 hours incubation using the MTT reduction assay described earlier to ensure that a non-cytotoxic concentration of this compound was being used.

Table 5.3. MTT reduction by N2a cells differentiating in the presence of 1 μg/ml CPSP

Differentiating N2a cells were incubated with 1 μg/ml CPSP or with 0.05 % (v/v) ethanol (control) for 4, 8, 24 and 48 hours and then subjected to the MTT reduction assay, as described in section 2.2.1.9. The results are presented as mean absorbance (570nm) ± standard error for a total of 8 wells of cells cultured on 2 separate experiments.

Time (hours)	Control	1 μg/ml CPSP
4	**0.29** ± 0.01	**0.27** ± 0.01
8	**0.29** ± 0.01	**0.28** ± 0.01
24	**0.39** ± 0.04	**0.38** ± 0.04
48	**0.53** ± 0.01	**0.57** ± 0.04

The results presented show that the viability of cells treated with 1 μg/ml CPSP after 4 and 8 hours were slightly lower than the control; however, these values were not significantly different, as determined by the Mann-Whitney U test ($p > 0.05$). The reduction of MTT by cells treated with CPSP for 24 and 48 hours was also not significantly different from the corresponding control, as determined by the Mann-Whitney U test ($p > 0.05$).

5.3.3 A dose response study of the effects of CPSP on N2a axon outgrowth

Having established that the highest CPSP concentration to be used was non-cytotoxic, differentiating N2a cells were incubated with a range of CPSP concentrations from 0 to 1 μg/ml over a 4 and 48 hour time period (figures 5.4a, 5.4b, 5.5a and 5.5b). Axon outgrowth decreased in a dose dependent manner with numbers of axons, at all concentrations of CPSP, being significantly lower than the corresponding control as determined by Mann-Whitney U ($p < 0.05$). There was no inhibitory changes in the growth of small extensions or in round to flat cell ratio (table 5.5). The IC_{50} values were determined for each time point and are represented as a concentration in μg/ml ± standard error (table 5.4).

Table 5.4. IC_{50} values for N2a cells exposed to for various times to CPSP

Time point (hours)	IC_{50} value
4	**0.66** ± 0.05 μg/ml
8	**0.57** ± 0.02 μg/ml
24	**0.73** ± 0.09 μg/ml
48	**0.78** ± 0.07 μg/ml

From the results presented in the table above, it clearly states that the IC_{50} values appear to inhibit axon outgrowth a similar extent after 4 and 8 hours but this value appears to get less as exposure times are increased to 24 and 48 hours. It was statistically determined by using Mann-Whitney U test ($p < 0.05$) that all of the IC_{50} values were not significantly different from one another irrespective of time.

Table 5.5. Dose response effects of 4 to 48 hours exposure to CPSP on differentiating N2a cells.

N2a cells were differentiated in the presence of a range of CPSP concentrations for 4, 8, 24 and 48 hours. The cells were fixed and stained with Coomassie blue at the specified time points. Data are expressed as the number recorded per hundred cells ± standard error. These results are an average 8 wells of cells cultured on two separate occasions.

Time (hours)	CPSP (µg/ml)	Extensions (%)	Axons (%)	Round cells (%)	Flat cells (%)
4	0	29 ± 1.1	36 ± 1.3	16 ± 0.5	84 ± 0.5
	0.1	35 ± 1.9	31 ± 1.4	17 ± 0.8	83 ± 1.3
	0.25	32 ± 1.4	24 ± 1.6	16 ± 0.6	84 ± 1.3
	0.5	30 ± 1.7	21 ± 1.8	26 ± 1.5	74 ± 1.3
	0.75	25 ± 1.6	14 ± 0.9	14 ± 0.9	86 ± 1.3
	1.0	29 ± 1.1	12 ± 0.8	15 ± 0.6	85 ± 0.6
8	0	33 ± 1.0	40 ± 1.2	18 ± 0.5	82 ± 0.5
	0.1	30 ± 1.6	30 ± 2.2	16 ± 0.8	84 ± 0.8
	0.25	32 ± 1.3	27 ± 1.7	16 ± 0.9	84 ± 0.9
	0.5	28 ± 1.4	19 ± 1.0	24 ± 1.3	76 ± 1.3
	0.75	28 ± 1.4	16 ± 1.0	19 ± 0.9	81 ± 0.9
	1.0	29 ± 1.1	14 ± 0.8	16 ± 0.8	84 ± 0.8
24	0	24 ± 0.8	47 ± 1.2	19 ± 0.8	81 ± 0.8
	0.1	22 ± 0.7	39 ± 1.1	19 ± 0.8	81 ± 0.8
	0.25	21 ± 0.6	30 ±1.1	21 ± 0.9	79 ± 0.9
	0.5	21 ± 0.6	26 ± 0.9	18 ± 0.7	82 ± 0.7
	0.75	21 ± 1.0	24 ± 1.0	21 ± 1.0	79 ± 1.0
	1.0	28 ± 1.5	20 ± 1.2	25 ± 2.0	75 ± 2.0
48	0	26 ± 0.9	46 ± 1.6	19 ± 0.6	81 ± 0.6
	0.1	24 ± 0.9	33 ± 1.5	20 ± 0.7	80 ± 0.7
	0.25	23 ± 0.7	27 ± 1.1	21 ± 0.8	79 ± 0.8
	0.5	23 ± 1.0	25 ± 1.5	20 ± 1.0	80 ± 1.0
	0.75	21 ± 1.0	20 ± 1.8	16 ± 1.0	84 ± 1.0
	1.0	22 ± 0.9	24 ± 1.5	18 ± 0.7	82 ± 0.7

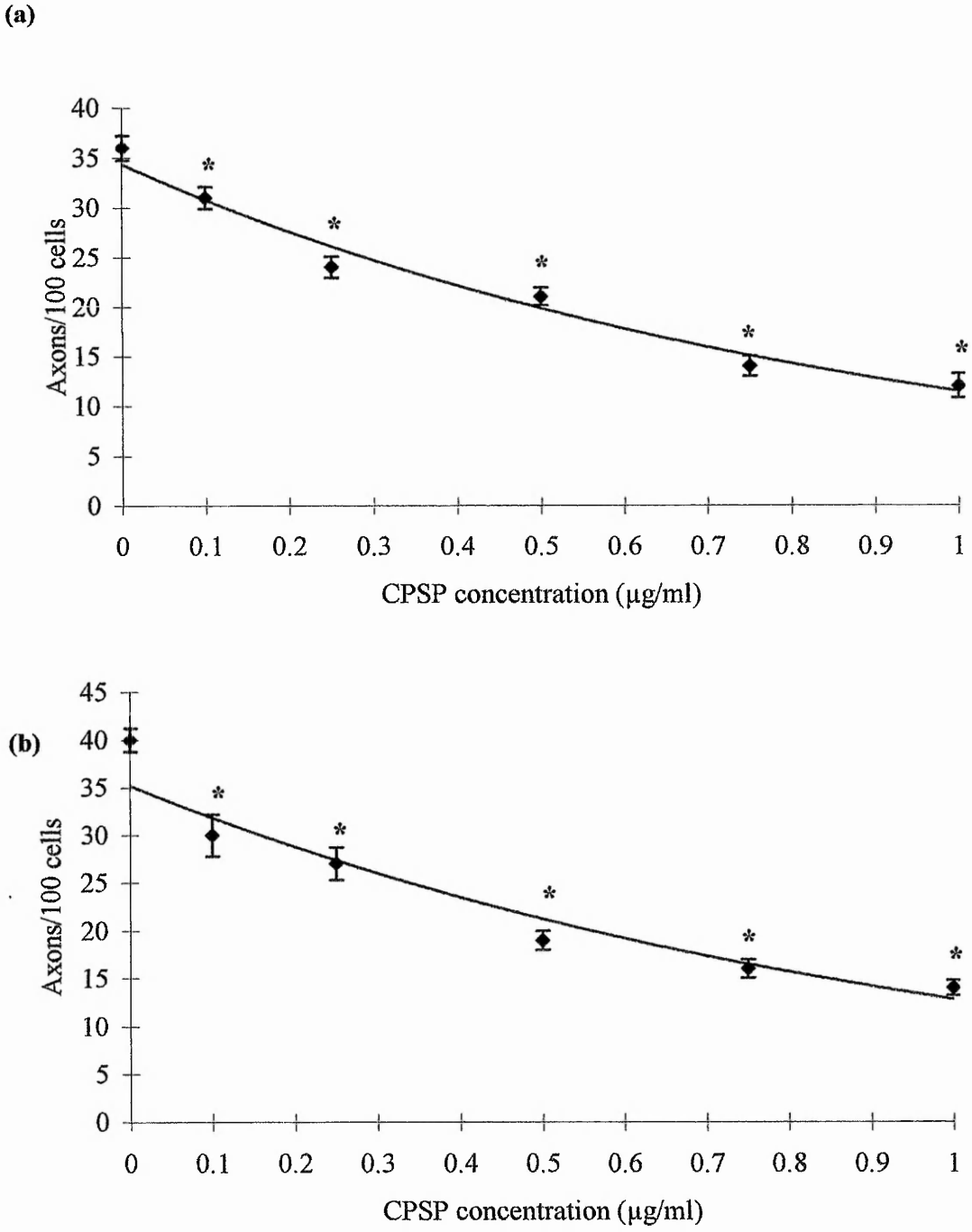


Figure 5.4. *IC_{50} determination for the effects of CPSP on axon outgrowth in differentiating N2a cells (a) after 4 hours incubation and (b) after 8 hours incubation.* Presented are the numbers of axon-like processes per 100 cells following incubation in the presence of differing concentrations of CPSP. All reductions are significant as determined by the Mann-Whitney U test (* = $p < 0.0001$). The IC_{50} value was determined to be (a) **0.66 ± 0.05 μg/ml**, and (b) **0.57 ± 0.02 μg/ml and** each data point represents an average of 8 wells of cells cultured on two separate occasions. Standard errors are shown as error bars.

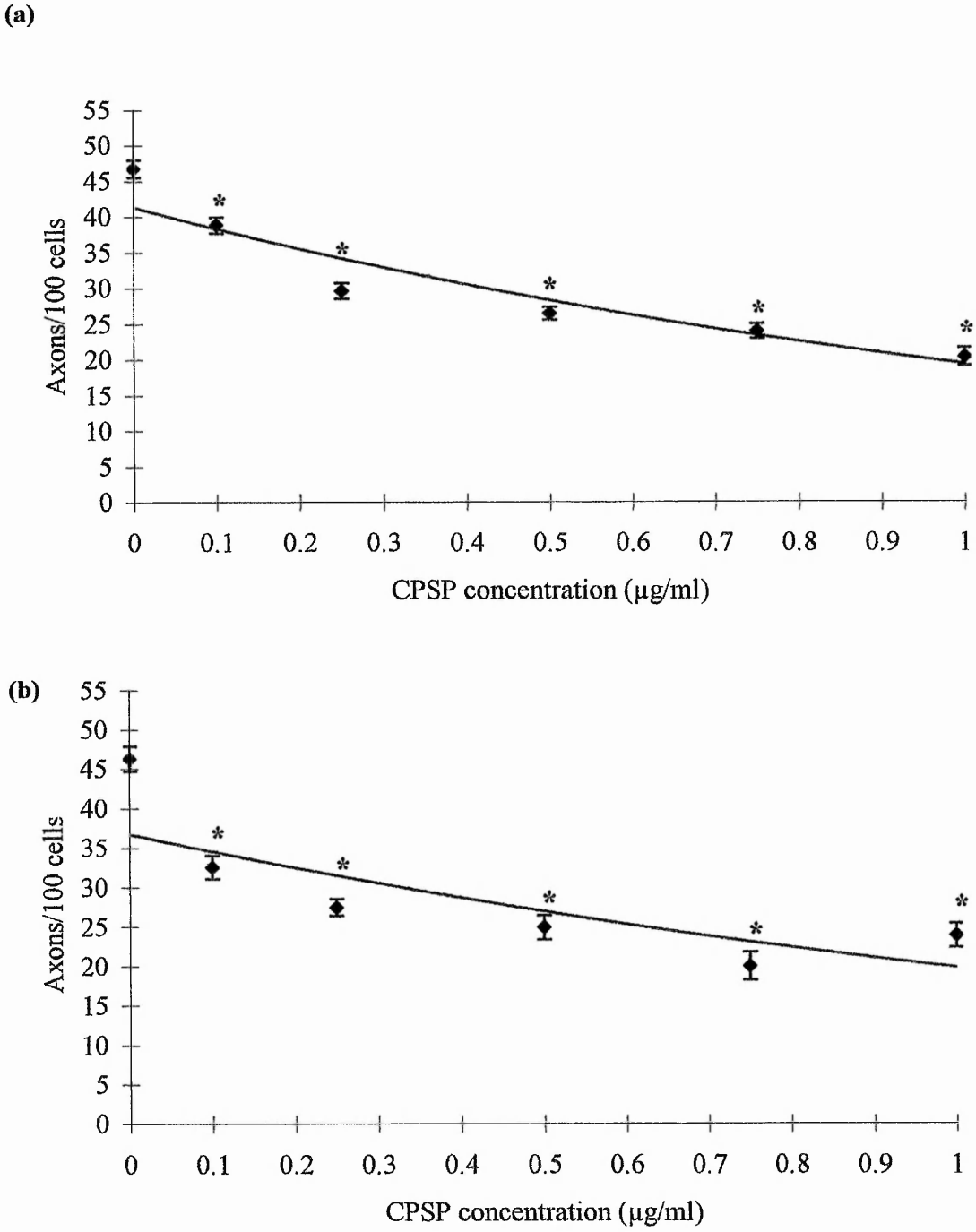


Figure 5.5. *IC_{50} determination for the effects of CPSP on axon outgrowth in differentiating N2a cells (a) after 24 hours incubation and (b) after 48 hours incubation.* Presented are the numbers of axon-like processes per 100 cells following incubation in the presence of differing concentrations of CPSP. All reductions are significant as determined by the Mann-Whitney U test (* = $p < 0.0001$). The IC_{50} value was determined to be (a) **0.73 ± 0.09 μg/ml**, and (b) **0.78 ± 0.07 μg/ml and** each data point represents an average of 8 wells of cells cultured on two separate occasions. Standard errors are shown as error bars.

5.4 Molecular analysis of the effects of CPSP on differentiating N2a cells

5.4.1 Western blotting analysis of extracts from differentiating N2a cells exposed to 1 μg/ml CPSP for 4, 8, 24 and 48 hours

The initial analysis was conducted on differentiating N2a cell extracts prepared following 4, 8, 24 and 48 hour incubations in the absence (i.e. control; 0.5% (v/v) ethanol) or presence of 1 μg/ml CPSP (section 2.2.2). The resultant Western blots were probed with a series of antibodies recognising different epitopes within NF and MT structures as described in section 3.3.1. Each Western blot indicates visually any changes in antibody binding when the cell extracts are probed with different antibodies. Further analysis was conducted with QuantiScan software in order to quantify densitometrically the intensities of each band, providing extra information as to the significance of each effect.

The fact that the levels of antibody reactivity with NF-H epitopes increase in intensity over time in control cell extracts, suggests that the differentiating N2a cells were behaving normally (figure 5.6 - 5.8). Figure 5.6a represents a Western blot of extracts of differentiating N2a cells exposed to 1 μg/ml CPSP after 4 and 8 hour incubations and then probed with RMd09 (which recognises a non-phosphorylated epitope on NF-H). The probed blot shows that reactivity with cell extracts exposed to CPSP is greatly reduced after 8 hours when compared to the corresponding control but no clear effect seen can be seen after 4 hours of incubation. The Western blot shown in figure 5.6b shows that CPSP has no significant inhibitory effect on antibody reactivity with the non-phosphorylation dependent epitope recognised by RMd09 after 24 and 48 hours.

Figure 5.7a displays a Western blot that represents cell extracts exposed to CPSP after 4 and 8 hours and probed with the antibody Ta51 (which recognises a phosphorylated epitope of NF-H). The effect of CPSP on differentiating N2a cells shows no reduction in antibody binding when compared to the corresponding control, after 4 to 48 hours (figure 5.7a and figure 5.7b).

In the case of differentiated N2a cells exposed to CPSP after 4 and 8 hours, it appears that the levels of N52 antibody binding (N52 recognises NF-H independent of its phosphorylation state) are lower than the controls (figure 5.8a). However, the Western blot of 24 and 48 hour cell extracts, probed with N52 showed similar levels of NF-H appearing over time, when compared to its corresponding control (figure 5.8b).

Figure 5.9a represents a Western blot that has been probed with B-5-1-2, an antibody that recognises the α-tubulin subunit. After 4 hours of exposure the level of antibody binding is significantly lower than that of the control but appears to recover slightly after a further 4 hour incubation. In figure 5.9b the Western blot represents differentiating N2a cell extracts exposed to 1 μg/ml CPSP for 24 and 48 hours and shows a slight reduction at 24 hours with a return to control levels by 48 hours.

The Western blot probed with GAP-7B10 (figure 5.10a) shows that CPSP treated cell extracts show a slightly higher reactivity than controls with this antibody after 4, 8 and 24 hours incubation. However, after 48 hours of exposure, the reactivity of GAP-7B10 shows a marked decrease compared to its corresponding control (figure 5.10b).

Densitometric analysis was conducted on the presented Western blots and the values obtained from the QuantiScan package show similar trends that have been described above, therefore confirming that such effects had occurred (table 5.6).

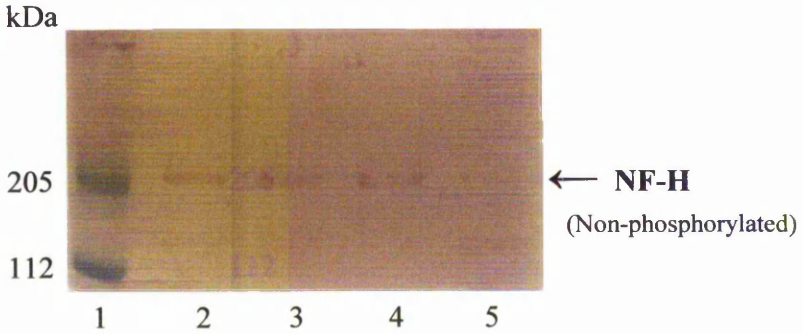


Figure 5.6a. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml CPSP, probed with neurofilament antibody, RMd09.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml CPSP for 4 hours; (4) control N2a cell extracts after 8 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml CPSP for 8 hours.

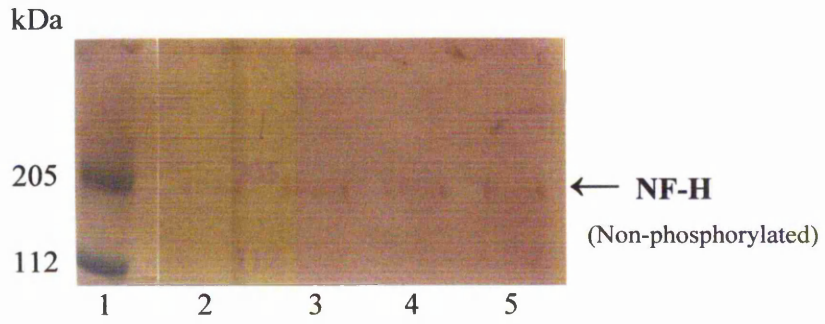


Figure 5.6b. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml CPSP, probed with neurofilament antibody, RMd09.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml CPSP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml CPSP for 48 hours.

These blots were probed with monoclonal antibody RMd09, which recognises a non-phosphorylation dependent epitope on NF-H. The arrow indicates a reactive polypeptide with the apparent molecular weight of NF-H.

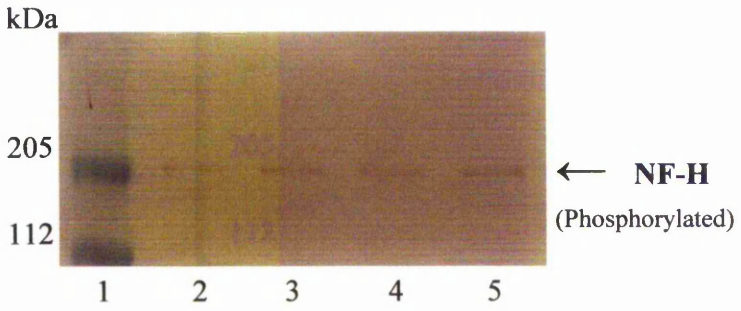


Figure 5.7a. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml CPSP, probed with neurofilament antibody, Ta51.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml CPSP for 4 hours; (4) control N2a cell extracts after 8 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml CPSP for 8 hours.

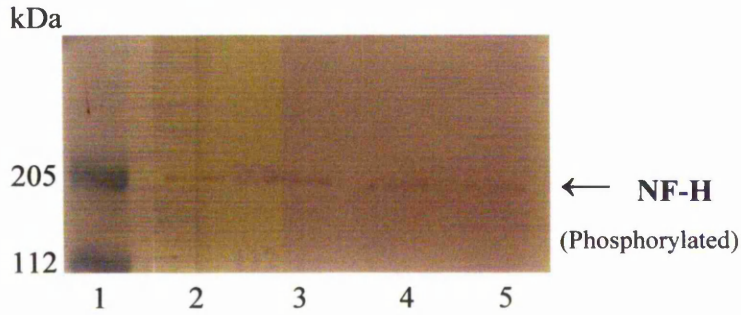


Figure 5.7b. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml CPSP, probed with neurofilament antibody, Ta51.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml CPSP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml CPSP for 48 hours.

These blots were probed with antibody, Ta51 which recognises a phosphorylation dependent epitope on NF-H. The arrow indicates a reactive polypeptide with the apparent molecular weight of NF-H.

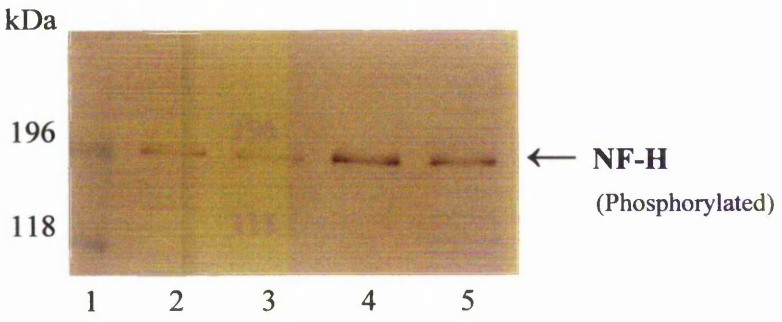


Figure 5.8a. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 µg/ml CPSP, probed with anti-neurofilament antibody N52.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hours incubation; (3) extracts of N2a cells exposed to 1 µg/ml CPSP for 4 hours; (4) control N2a cell extracts after 8 hours incubation; (5) extracts of N2a cells exposed to 1 µg/ml CPSP for 8 hours.

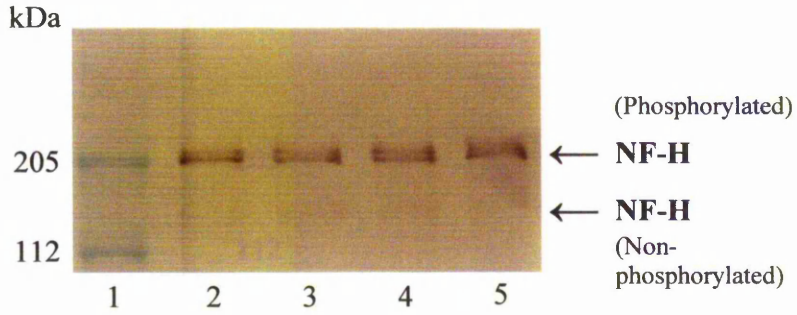


Figure 5.8b. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 µg/ml CPSP, probed with anti-neurofilament antibody, N52.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 µg/ml CPSP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 µg/ml CPSP for 48 hours.

These blots were probed with antibody, N52 which recognises a NF-H epitope independent of the phosphorylation state. The arrow(s) indicates a reactive polypeptide with the apparent molecular weight of NF-H.

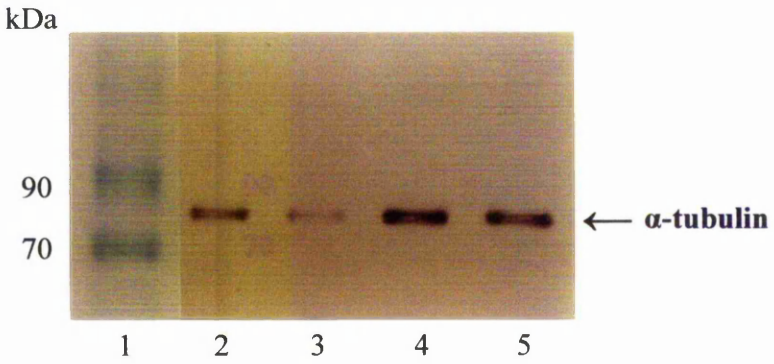


Figure 5.9a. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml CPSP, probed with anti-tubulin antibody, B-5-1-2.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml CPSP for 4 hours; (4) control N2a cell extracts after 8 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml CPSP for 8 hours.

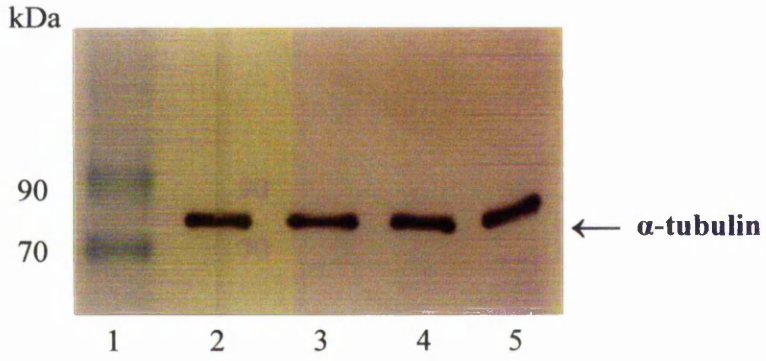


Figure 5.9b. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml CPSP, probed with anti-tubulin antibody, B-5-1-2.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml CPSP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml CPSP for 48 hours.

These blots were probed with antibody, B-5-1-2 which recognises an epitope located at the C-terminal end of the α-tubulin isoform. The arrow indicates a reactive polypeptide with the apparent molecular weight of α-tubulin.

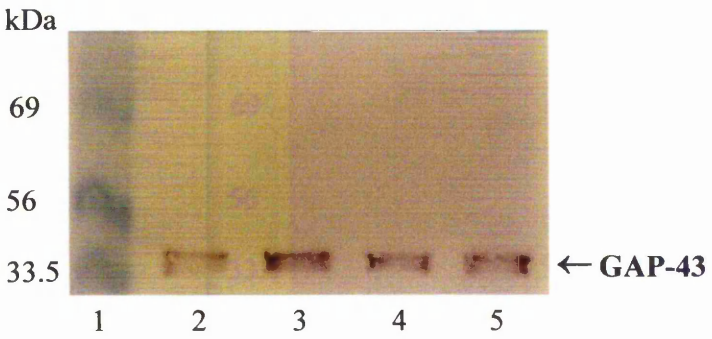


Figure 5.10a. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml CPSP, probed with GAP-7B10.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 4 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml CPSP for 4 hours; (4) control N2a cell extracts after 8 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml CPSP for 8 hours.

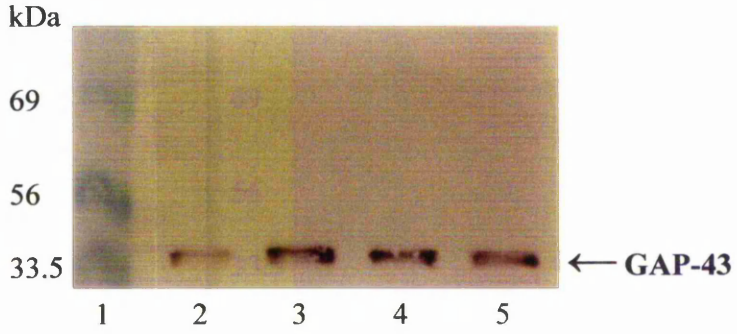


Figure 5.10b. *Western blot analysis of N2a cells induced to differentiate in the presence and absence of 1 μg/ml CPSP, probed with GAP-7B10.* Shown are : (1) prestained molecular weight markers; (2) control N2a cell extracts after 24 hour incubation; (3) extracts of N2a cells exposed to 1 μg/ml CPSP for 24 hours; (4) control N2a cell extracts after 48 hour incubation; (5) extracts of N2a cells exposed to 1 μg/ml CPSP for 48 hours.

These blots were probed with antibody GAP-7B10, which recognises GAP-43 within neuronal processes. The arrow indicates a reactive polypeptide with the apparent molecular weight of GAP-43.

5.4.2 Quantitative analysis of Western blot data from extracts of differentiating N2a cells exposed to 1 µg/ml CPSP after 4, 8, 24 and 48 hours

As the Western blot data was only assessed by visual comparisons, the intensities of antibody binding on each blot were then analysed quantitatively using the software, QuantiScan as previously described. Statistical analysis could not be performed on the presented Western blot data as the experiment was only performed once.

Table 5.6. Densitometric analysis of Western blots using QuantiScan

Data from the densitometric analysis has been tabulated below with values recorded representing the antibody intensities when compared to the corresponding control for a single blot.

	1 µg/ml CPSP			
	4 hours	**8 hours**	**24 hours**	**48 hours**
Non-phosphorylation dependent epitope of NF-H (RMd09)	**106**	**47**	**114**	**83**
Phosphorylation dependent epitope of NF-H (Ta51)	**130**	**210**	**107**	**95**
Phosphorylation independent epitope of NF-H (N52)	**54**	**73**	**83**	**114**
α-tubulin (B-5-1-2)	**59**	**74**	**89**	**100**
GAP-43 (GAP-7B10)	**213**	**130**	**211**	**71**

5.5 Discussion

Experiments were performed using the microsomal activation technique during 4, 24 and 48 hour incubations with N2a cells induced to differentiate in the presence of 1 μg/ml TCP, T*O*CP and T*P*CP. The experiments with TCP showed a greater reduction in axon outgrowth with microsomal activation when compared to the corresponding non-microsomally activated control after 4 to 24 hours. However, this effect was not as dramatic after a 48 hour incubation. With T*O*CP, increased toxicity could only be seen after 4 hours with no measurable effect after 24 or 48 hours and, characteristically, T*P*CP showed no increase in toxicity towards differentiating N2a cells at any time point.

This increased toxicity seen with both TCP and T*O*CP after 4 hours suggests that this was the optimum time point to detect metabolic activation, showing that these OPs can be metabolically activated into a more toxic compound such as the metabolite SC*O*TP (Casida *et al.*, 1961; Eto *et al.*, 1962; Taylor and Buttar, 1967; Sharma and Watanabe, 1974; Nomeir and Abou-Donia., 1986). However, T*P*CP appears to remain non-neurotoxic regardless of the presence of a metabolic activation system.

After 48 hours, microsomal activation had no effect on any of the OPs tested suggesting that either (1) the life span of the generating system had lapsed, or (2) the neurotoxic effect had already reached a peak, or (3) the concentration of OP present had been depleted and the cells had time to recover from these effects, or (4) that detoxification of the active metabolite(s) may be occurring.

To assess whether the effects of microsomal activation were OP concentration dependent, 0.1 μg/ml of each compound was used in a 4 hour incubation experiment. In this case with TCP no increased toxicity was observed, as the level of axon outgrowth was the same as that seen in the corresponding NADPH system without microsomes. However, a decrease in axon outgrowth was seen with 0.1 μg/ml T*O*CP suggesting microsomal activation may be occurring at this concentration but further work needs to be done to confirm these effects.

It can be concluded that 1 μg/ml TCP and T*O*CP can be metabolically activated to a compound with increased toxicity after 4 hours, agreeing with the suggestion that *in vivo* these compounds are rapidly activated, detoxified and bio-eliminated in the liver (Eto *et al.*, 1962). However, T*P*CP appears to be non-cytotoxic in all conditions, as it is unable to be metabolically activated into a more toxic compound. This is in agreement with the suggestion that the T*P*CP isomer contributes no neurotoxic effect *in vivo* (Bondy *et al.*, 1960). Further work should be undertaken to check whether the absence of microsomal activation after 48 hours was due to the expiry time of the components of the generating system.

Studies into the toxicity level of CPSP towards differentiating N2a cells were then instigated. From the MTT reduction studies, it was confirmed that 1 μg/ml CPSP was non-cytotoxic towards these cells under all experimental conditions employed. Consequently, this was used as the highest concentration for cell differentiation experiments.

To analyse the effects of CPSP, dose response experiments were conducted on differentiating N2a cells over a range of concentrations from 0.1 to 1 μg/ml. The results indicate that these cells respond in a dose dependent manner with axon numbers decreasing as the concentration of CPSP increases, at each time point.

At all time points, the estimated IC_{50} values determined show that CPSP has an axon inhibitory effect on differentiating N2a cells and that the values determined were very similar to those found with T*O*CP and TCP at 24 and 48 hour time points (section 4.2.3). Overall, these results indicate that CPSP has a sustained inhibitory effect on axon outgrowth, similar to that seen with T*O*CP and TCP. This indicates that although the compound synthesised does not present higher toxic effects than T*O*CP on differentiating N2a cells, it is still able to inhibit axon outgrowth to similar levels.

Differentiating N2a cell extracts exposed to 1 μg/ml CPSP at each time point were also analysed using the Western blot and immunoprobing technique, to detail the effects of this compound at a molecular level. Visual and densitometric analysis of Western blots probed with RMd09 suggest that CPSP has no effect after 4 hours on non-

phosphorylated NF-H but after 8 hours these levels are greatly reduced when compared to the corresponding control (figure 5.6a). After 24 – 48 hours, the Western blot data suggests that the levels of non-phosphorylated NF-H recover to a level similar to controls (figure 5.6b).

In the Western blot probed with Ta51 (which recognises phosphorylated NF-H), the level of antibody binding mimics that of the corresponding control after 4 hours but after 8 hours levels of phosphorylated NF-H reactivity appear to increase compared to the corresponding control and the same sample probed with RMd09, indicating that CPSP treatment results in increased phosphorylation of NF-H at this time point (figure 5.7a). The levels of phosphorylated NF-H after 24 and 48 hours appeared to mirror that of the control (figure 5.7b and table 5.6).

The Western blot data also indicated that 1 μg/ml CPSP treatment for 8 hours reduces non-phosphorylated NF-H (figure 5.6a) and increases phosphorylated NF-H (monitored with Ta51, figure 5.7a), suggesting an altered phosphorylation state at this time point and a possible target for this compound. This increased phosphorylation contrasts sharply with the reduction in Ta51 reactivity observed with both isomers at the same time point (chapter 4) but is in agreement with the observed increase in spinal cord NF-H phosphorylation in T*O*CP-treated hens (Suwita *et al.*, 1986). The switch in binding of antibodies dependent on phosphorylation state after 8 hours with CPSP suggests that it may cause a net increase in NF-H phosphorylation at this time point.

Western blot data probed with N52 (which recognises NF-H independent of its phosphorylation state) clearly shows levels of NF-H initially decrease, then steadily recovers (figure 5.8a and 5.8b) suggesting that CPSP has no effect on the levels of NF-H.

Interestingly, the effect of CPSP on levels of α-tubulin appear to be inhibitory at early time points (figure 5.9a) but a recovery in the levels is clearly observed after 48 hours (figure 5.9b), suggesting that this cytoskeletal protein is also able to recover to levels similar to those of the control.

Western blots probed with an antibody that recognises GAP-43, indicate that after 4, 8 and 24 hours, the levels of this protein remain constant or increase slightly but appear to decrease markedly after 48 hours. This suggests a lack of effect on the expression of this axon growth-associated protein until later stages of exposure, possibly requiring at least 48 hours with the GAP-43 before the expression is inhibited (figure 5.10a and figure 5.10b). This effect is similar to the basic trend observed for T*O*CP and T*P*CP treated cell extracts (table 4.4).

From the results presented within this chapter, it can be concluded that CPSP inhibits axon outgrowth, and has specific effects on the pattern of expression and/or phosphorylation state of distinct cytoskeletal and axon growth associated proteins at different time points.

Chapter Six

Protective effects of C6 conditioned medium on N2a cells exposed to TCP, T*O*CP, T*P*CP and CPSP

6.1 Introduction

Research into neurotrophic growth factors (for example glial cell line-derived neurotrophic factor (GDNF) see below) has centered around diseases such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and Alzheimer's disease (AD), with rigorous *in vivo* testing in animal models supporting the use of GDNF in a variety of neurodegenerative diseases (Lapchak *et al.*, 1997b).

It has been found that GDNF is a related member of the transforming growth factor-β (TGF-β) superfamily. It is a disulphide-bonded dimeric protein with an approximate molecular weight ranging between 40 - 45 kDa (Lapchak *et al.*, 1997b; Matheson *et al.*, 1997). However, unlike other TGF-β family members, GDNF signals through a complex of GDNF-α receptor and a cellular tyrosine kinase receptor molecule, c-ret (Lapchak *et al.*, 1997b).

Different research groups have found that GDNF influences dopaminergic neurons (Lin *et al.*, 1993; Beck *et al.*, 1995; Tomac *et al.*, 1995) and also motor neurons *in vivo* (Henderson *et al.*, 1994; Oppenheim *et al.*, 1995; Yan *et al.*, 1995). It has been found that, *in vitro*, GDNF selectively enhances the survival and development of dopaminergic neurons (Lapchak *et al.*, 1997a; Lin *et al.*, 1995; Oppenheim *et al.*, 1995). This has been demonstrated by an increase in number of surviving neurons in culture, an increase in dopamine (DA) uptake, cell size and extensions of neurites but no effect on non-dopaminergic neurons or glial cells. Work by Lapchak *et al.*, (1997a; 1997b) concluded that GDNF improves the survival of cells, preventing further cell death by stimulating them to regrowth.

Matheson *et al.*, (1997) concluded that GDNF and NGF rescued nearly 100 percent of sensory neurons in a neonatal, sciatic nerve axotomy-induced cell death model, whilst determining whether growth factors could influence dorsal root ganglion (DRG) neuron survival, *in vivo*. They proposed that the biological responsiveness of DRG neurons to GDNF both *in vivo* and *in vitro* suggest that this factor may play a role in the development and maintenance of sensory neurons.

Work by Henderson *et al.*, (1994) found GDNF to be 75 times more potent than any other neurotrophin in supporting the survival of purified embryonic rat motor neurons in culture. *In vivo*, GDNF rescues and prevents atrophy of facial motor neurons that have been deprived of target-derived survival factors by axotomy, suggesting that GDNF may be a physiological trophic factor for spinal motor neurons. It is believed that GDNF is a valuable neurotrophic factor which is able to increase the survival of motor neurons and dopamine neurons when injured due to recent toxic insult (Henderson *et al.*, 1994). Treatments with GDNF in vivo also prevents induced death and atrophy of both avian and mouse spinal motor neurons following peripheral axotomy (Oppenheim *et al.*, 1995).

Furthermore, it has been documented that GDNF has been identified in peripheral tissues such as intestine, aorta, bladder, etc (Peters *et al.*, 1998). Work by Trupp *et al.*, (1995) has shown that the expression of GDNF mRNA was significantly higher in peripheral organs than in neuronal tissues in developing rats (Trupp *et al.*, 1995). However, studies have shown that with the administration of exogenous neurotrophic factors after nerve injury mimics those effects seen of target organ-derived trophic factors on neuronal cells (Terenghi, 1999). These studies suggest that the therapeutic potential of neurotrophic factors for the treatment of peripheral nerve injury and for neuropathies (Terenghi, 1999).

As mentioned earlier conditioned medium (CM) cultured from rat glial cells is a good source of GDNF (Suter-Crazolora and Unsicker, 1996). In the present work, CM was collected from differentiating C6 cells (section 2.2.1.12) and used in preliminary experiments, to analyse the possible protective effects against any insult produced by TCP, T*O*CP, T*P*CP and CPSP.

The aim of the present work was to determine whether neurotrophic factors produced by differentiating C6 glial cells had a protective effect on differentiating N2a cells exposed to TCP, T*O*CP, T*P*CP and CPSP. The effects were analysed by morphological studies over 4, 8, 24 and 48 hour time points.

6.2 Morphological studies on the protective effects of CM on differentiating N2a cells exposed to TCP, T*O*CP, T*P*CP and CPSP

As mentioned in section 6.1, neurotrophic proteins such as GDNF have a protective and reparative effect on motor neurons. (Henderson *et al.*, 1994; Oppenheim *et al.*, 1995; Yan *et al.*, 1995). It is hypothesized that CM from differentiating C6 cells could have a protective effect on N2a cells exposed to neurodegenerative agents such as TCP, T*O*CP, T*P*CP and CPSP over a period of 4, 8, 24 and 48 hours.

6.2.1 Studies on N2a cells exposed to 1 µg/ml TCP, T*O*CP, T*P*CP and CPSP for 48 hours

The following figures show the visual effects on N2a cells of exposure to OPs at a concentration of 1 µg/ml for 48 hours.

Figure 6.1. *Effects of CM on N2a cell differentiation*: **(a)** *N2a cells differentiating for 48 hours in the presence of 0.05 % (v/v) ethanol (control) known as a "typical experiment"*; **(b)** *control N2a cells differentiating for 48 hours in the presence of CM.* In both cases, note the abundance of axon-like processes (arrows) and small extensions, suggesting the cells are healthy. Cells were fixed and stained with Coomassie blue stain.

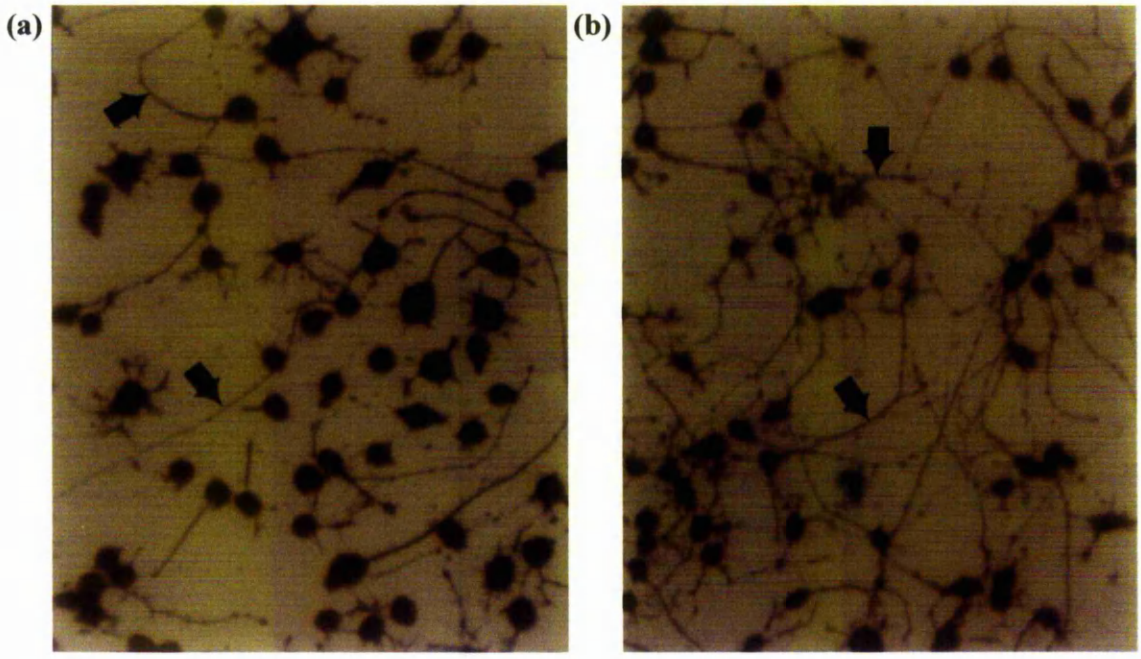


Figure 6.2. *Effects of CM on N2a cell differentiating in the presence of TCP* **(a)** *N2a cells differentiating in the presence of 1 μg/ml TCP for 48 hours*; **(b)** *differentiating N2a cells incubated with 1 μg/ml TCP for 48 hours in the presence of CM.* Clearly the number of axonal processes (arrows) has increased in (b) suggesting that the effects of TCP are attenuated in the presence of CM

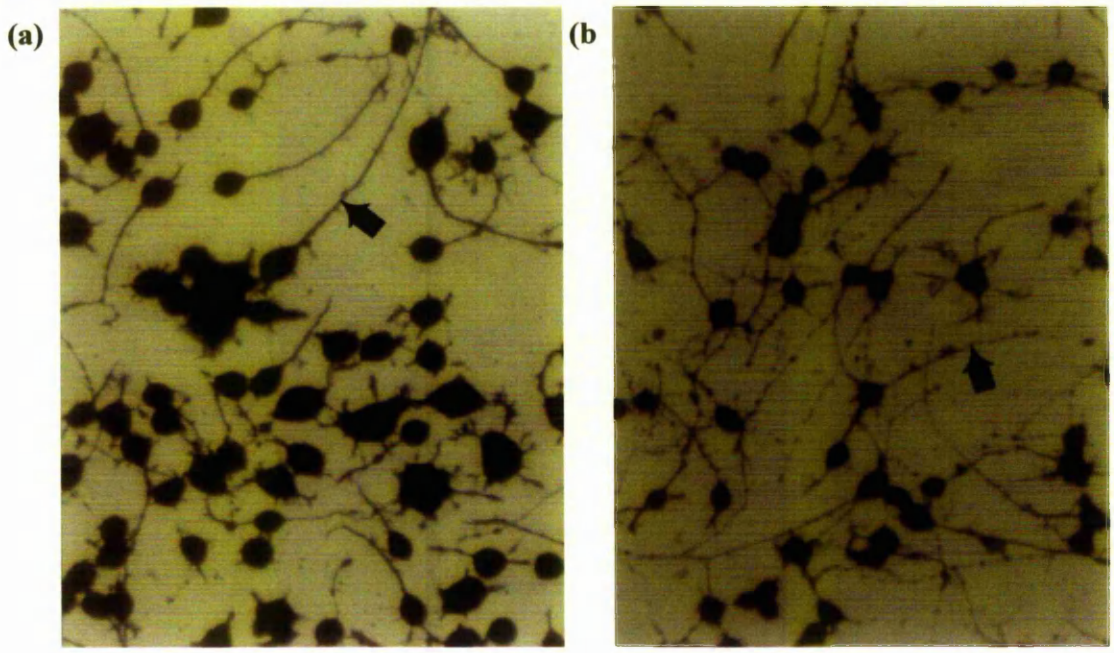


Figure 6.3. *Effects of CM on N2a cell differentiating in the presence of TOCP* **(a)** *differentiating N2a cells exposed to 1 μg/ml TOCP after 48 hours*; **(b)** *differentiating N2a cells exposed to 1 μg/ml TOCP in the presence of CM after 48 hour incubation.* Note the abundance of axonal processes (arrows) in (b) suggesting CM has reversed the inhibitory effect of T*O*CP.

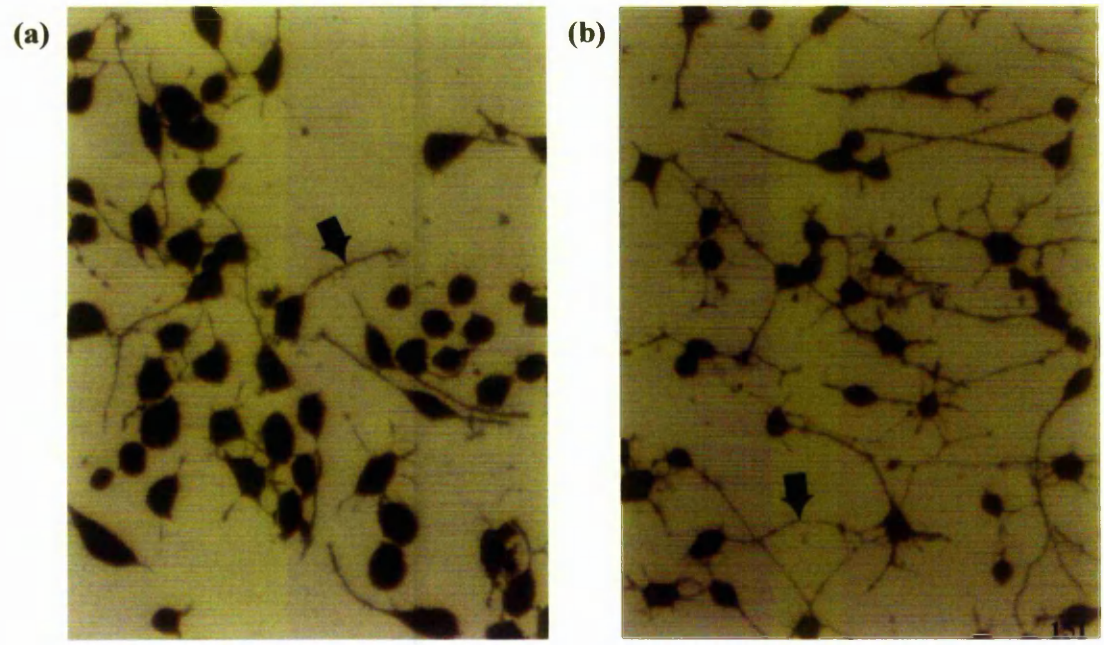


Figure 6.4. *Effects of CM on N2a cell differentiating in the presence of TPCP* (a) *N2a cells differentiated in the presence of 1 μg/ml TPCP for 48 hours*; (b) *differentiating N2a cells incubated with 1 μg/ml TPCP for 48 hours in the presence of CM.* Note the increase in the number of axonal processes (arrows) in (b) suggesting the presence of CM encourages the production of these processes.

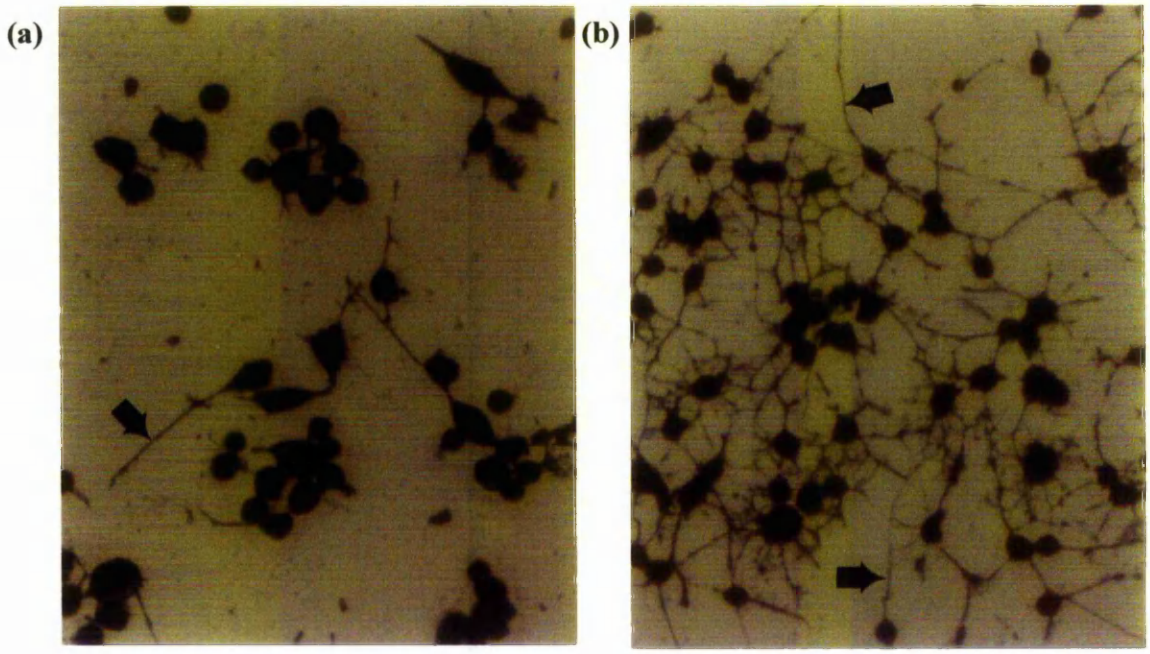
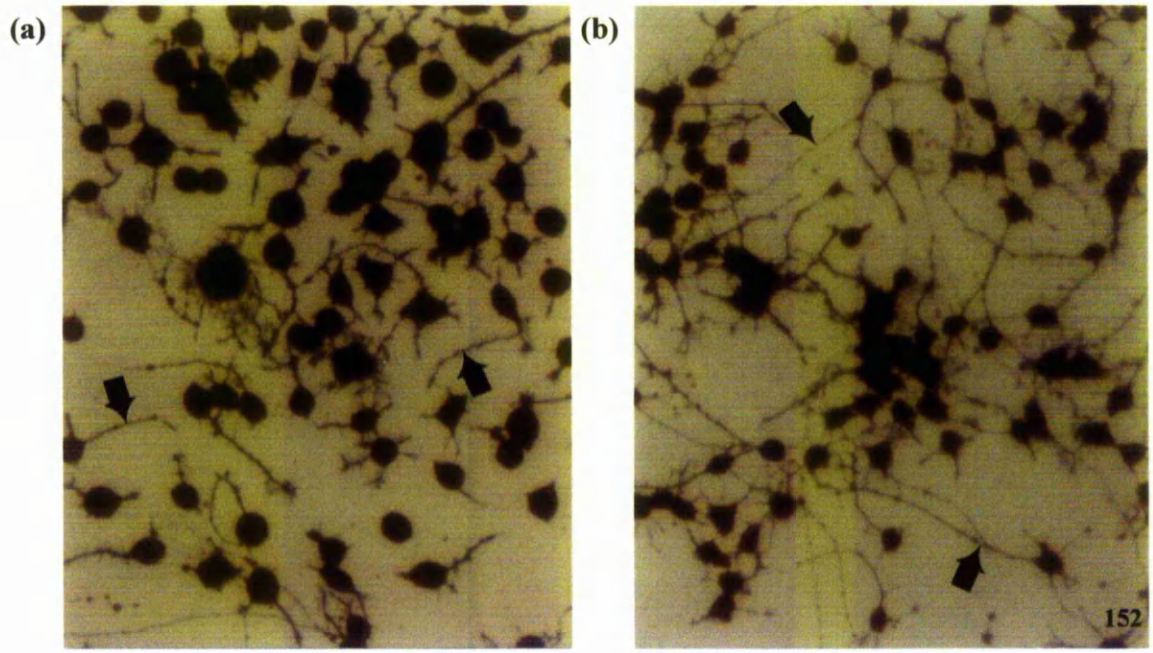


Figure 6.5. *Effects of CM on N2a cell differentiating in the presence of CPSP* (a) *differentiating N2a cells exposed to 1 μg/ml CPSP after a 48 hour incubation;* (b) *differentiating N2a cells exposed to 1 μg/ml CPSP in the presence of CM after 48 hour incubation.* Note the abundance of axonal processes (arrows) in (b) when compared to the typical experiment



6.3 Time course study of the effects of C6 CM on differentiating N2a cells exposed to TCP, *TOCP*, *TPCP* and CPSP.

The effects of CM were assessed on differentiating N2a cells to determine whether or not the axon inhibitory effects of the OPs could be prevented. Four time points (4, 8, 24 and 48 hours) were assessed in experiments conducted in the presence and absence of CM to ensure that the OPs being tested were working correctly and that any apparent protective effects were due to the presence of CM. Experiments performed in the absence of CM will be referred to as typical experiments (section 2.2.1.5)

After 4 hours incubation in the presence of CM but without OP the number of axons was significantly increased compared to the corresponding typical experiment, with the effects of the OPs used being blocked. The number of extensions and the ratio of round to flat cells remained constant under all conditions, mimicking the effects seen in the typical experiment, thus suggesting no additional morphological effects (table 6.1 and figure 6.6).

An 8 hour incubation with CM showed a similar pattern to the 4 hour exposure time with increased numbers of axons compared to the corresponding typical experiment. Again, it appears that the number of extensions and the ratio of round to flat cells was not affected nor altered in any way (table 6.2 and figure 6.7). However, it is important to note that after 8 hour incubation with TCP and T*O*CP, the number of axons compared to their corresponding controls appears to be slightly reduced in the presence of CM.

After both 24 and 48 hours, the number of axons per 100 cells was still higher in the presence of CM than the corresponding typical experiment (table 6.3, figure 6.8, table 6.4 and figure 6.9). The inhibitory effect normally seen with TCP and T*O*CP appeared to be completely blocked after 24 and 48 hours exposure in the presence of CM.

Table 6.1. Protective effects of CM on differentiating N2a cells exposed to TCP, TOCP, TPCP and CPSP for 4 hours.

N2a cells were differentiated for 4 hours in the absence (typical) and presence of CM and 1 μg/ml TCP, T*O*CP, T*P*CP and CPSP and then fixed and stained with Coomassie blue. Data are expressed as the number recorded per hundred cells ± standard error. These results are an average of 8 wells of cells cultured on two separate occasions.

		Extensions (%)	**Axons (%)**	**Round cells (%)**	**Flat cells (%)**
Typical	**Control**	**27** ± 2.1	**33** ± 1.6	**15** ± 0.8	**85** ± 0.8
	1 μg/ml TCP	**30** ± 2.2	**32** ± 2.0	**14** ± 0.9	**86** ± 0.9
	1 μg/ml T*O*CP	**21** ± 2.2	**18** ± 1.6	**12** ± 1.5	**88** ± 1.5
	1 μg/ml T*P*CP	**28** ± 1.2	**33** ± 3.2	**17** ± 1.3	**83** ± 1.3
	1 μg/ml CPSP	**28** ± 1.1	**19** ± 1.1	**16** ± 1.3	**84** ± 1.3
With CM	**Control**	**33** ± 1.5	**55** ± 2.8	**20** ± 1.0	**80** ± 1.0
	1 μg/ml TCP	**29** ± 1.4	**47** ± 2.4	**20** ± 1.2	**80** ± 1.2
	1 μg/ml T*O*CP	**30** ± 1.5	**53** ± 2.2	**20** ± 1.0	**80** ± 1.0
	1 μg/ml T*P*CP	**27** ± 1.4	**52** ± 2.3	**17** ± 0.7	**83** ± 0.7
	1 μg/ml CPSP	**31** ± 1.1	**52** ± 1.5	**19** ± 0.8	**81** ± 0.8

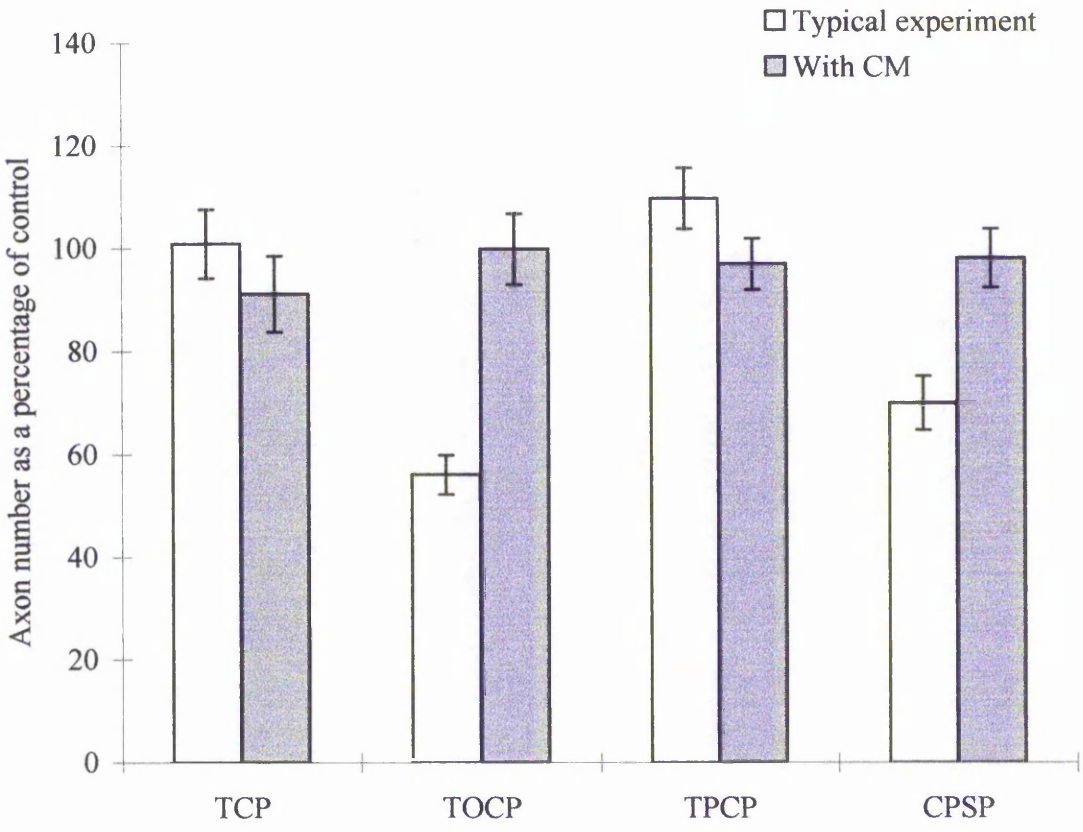


Figure 6.6. *Protective effects of CM on differentiating N2a cells exposed to TCP, TOCP, TPCP and CPSP for 4 hours.* Shown are the numbers of axon-like processes per 100 cells as a percentage of the corresponding control following 4 hours incubation in the presence of each OP at 1 μg/ml. All increases in the presence of CM were found to be significant when compared to the corresponding typical experiment performed in the absence of CM, as determined by the Mann-Whitney U test ($p < 0.05$). Each data point represents an average of 8 wells of cells cultured on two separate occasions. Standard errors are shown as error bars.

Table 6.2. Protective effects of CM on differentiating N2a cells exposed to TCP, TOCP, TPCP and CPSP for 8 hours.

The N2a cells were differentiated for 8 hours in the absence (typical) and presence of CM and 1 µg/ml TCP, T*O*CP, T*P*CP or CPSP and then fixed and stained with Coomassie blue. Data are expressed as the number recorded per hundred cells ± standard error. These results are an average of 8 wells of cells cultured on two separate occasions.

		Extensions (%)	**Axons (%)**	**Round cells (%)**	**Flat cells (%)**
Typical	**Control**	**33** ± 2.8	**45** ± 3.7	**18** ± 1.5	**82** ± 1.5
	1 µg/ml TCP	**27** ± 1.3	**41** ± 1.8	**18** ± 1.2	**82** ± 1.2
	1 µg/ml T*O*CP	**24** ± 1.4	**14** ± 2.4	**15** ± 1.0	**85** ± 1.0
	1 µg/ml T*P*CP	**30** ± 2.5	**27** ± 1.8	**18** ± 1.2	**82** ± 1.2
	1 µg/ml CPSP	**33** ± 2.1	**14** ± 1.2	**16** ± 0.8	**84** ± 0.8
With CM	**Control**	**38** ± 2.2	**74** ± 3.0	**22** ± 1.4	**78** ± 1.4
	1 µg/ml TCP	**30** ± 1.9	**60** ± 3.9	**18** ± 0.9	**82** ± 0.9
	1 µg/ml T*O*CP	**34** ± 1.4	**62** ± 3.0	**22** ± 1.1	**78** ± 1.1
	1 µg/ml T*P*CP	**38** ± 2.5	**74** ± 4.1	**24** ± 1.6	**76** ± 1.6
	1 µg/ml CPSP	**39** ± 1.4	**70** ± 2.6	**22** ± 0.8	**78** ± 0.8

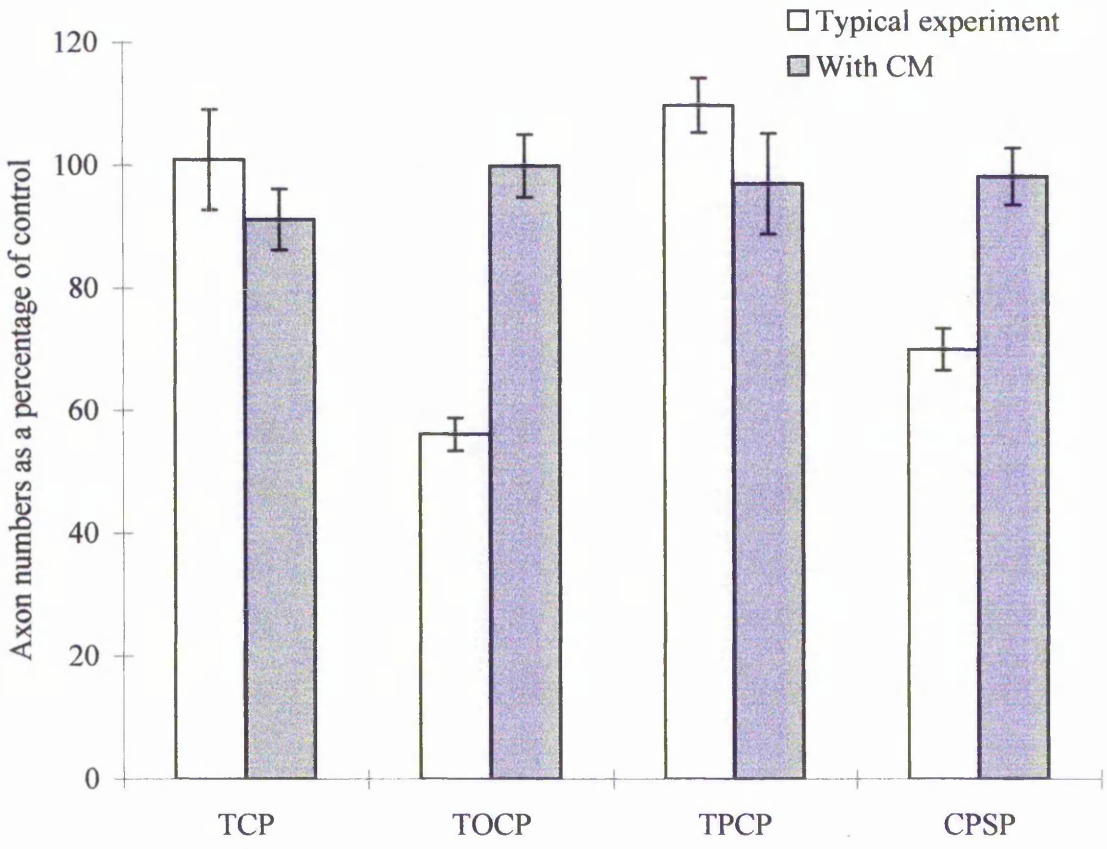


Figure 6.7. *Protective effects of CM on differentiating N2a cells exposed to TCP, TOCP, TPCP or CPSP for 8 hours.* Shown are the numbers of axon-like processes per 100 cells as a percentage of the corresponding control following 8 hours incubation in the presence of 1 μg/ml of each OP. All increases were found to be significant when compared to the corresponding experiment performed in the absence of CM, as determined by the Mann-Whitney U test ($p < 0.05$). Each data point represents an average of 8 wells of cells cultured on two separate occasions. Standard errors are shown as error bars.

Table 6.3. Protective effects of CM on differentiating N2a cells exposed to TCP, TOCP, TPCP and CPSP for 24 hours.

N2a cells were differentiated for 24 hours in the absence (typical) and presence of CM and 1 μg/ml TCP, T*O*CP, T*P*CP and CPSP and then fixed and stained with Coomassie blue. Data are expressed as the number recorded per hundred cells ± standard error. These results are an average of 12 wells of cells cultured on three separate occasions.

		Extensions (%)	**Axons (%)**	**Round cells (%)**	**Flat cells (%)**
Typical	**Control**	**30** ± 1.3	**49** ± 2.1	**20** ± 0.9	**80** ± 0.9
	1 μg/ml TCP	**24** ± 1.1	**24** ± 1.3	**19** ± 1.1	**81** ± 1.1
	1 μg/ml T*O*CP	**27** ± 1.6	**20** ± 0.8	**18** ± 0.9	**82** ± 0.9
	1 μg/ml T*P*CP	**27** ± 1.4	**21** ± 1.4	**19** ± 0.8	**81** ± 0.8
	1 μg/ml CPSP	**22** ± 1.1	**24** ± 1.8	**17** ± 0.8	**83** ± 0.8
With CM	**Control**	**31** ± 0.8	**83** ± 8.4	**21** ± 0.6	**79** ± 0.6
	1 μg/ml TCP	**30** ± 1.0	**72** ± 1.9	**22** ± 0.6	**78** ± 0.6
	1 μg/ml T*O*CP	**31** ± 1.0	**70** ± 2.3	**22** ± 0.6	**78** ± 0.6
	1 μg/ml T*P*CP	**30** ± 0.9	**73** ± 2.0	**24** ± 0.7	**76** ± 0.7
	1 μg/ml CPSP	**30** ± 1.3	**75** ± 2.8	**23** ± 0.9	**77** ± 0.9

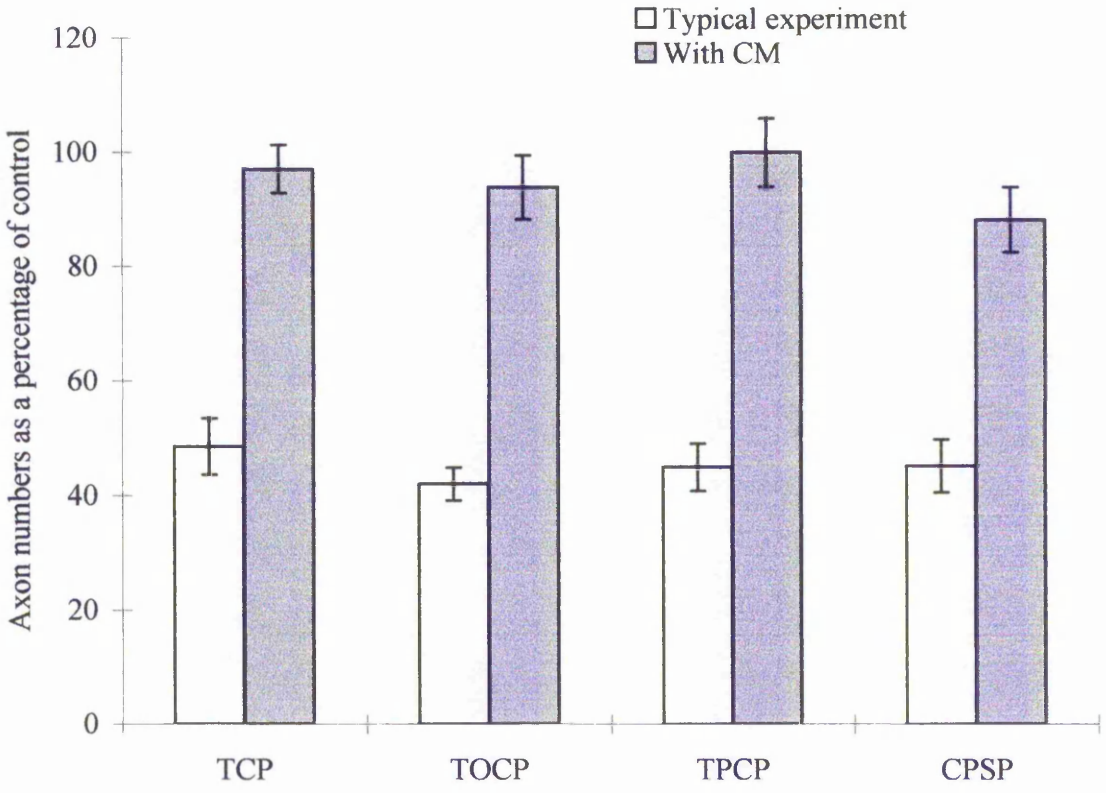


Figure 6.8. *Protective effects of CM on differentiating N2a cells exposed to TCP, TOCP, TPCP or CPSP for 24 hours.* Shown are the numbers of axon-like processes per 100 cells as a percentage of the corresponding control following 24 hours incubation in the presence of 1 μg/ml of each OP. All increases were found to be significant when compared to the corresponding experiment performed in the absence of CM, as determined by the Mann-Whitney U test ($p < 0.05$). Each data point represents an average of 12 wells of cells cultured on three separate occasions. Standard errors are shown as error bars.

Table 6.4. Protective effects of CM on differentiating N2a cells exposed to TCP, TOCP, TPCP and CPSP for 48 hours.

N2a cells were differentiated for 48 hours in the absence (typical) and presence of CM and 1 μg/ml TCP, T*O*CP, T*P*CP or CPSP and then fixed and stained with Coomassie blue. Data are expressed as the number recorded per hundred cells ± standard error. These results are an average of 8 wells of cells cultured on two separate occasions.

		Extensions (%)	**Axons (%)**	**Round cells (%)**	**Flat cells (%)**
Typical	**Control**	**26** ± 0.8	**45** ± 2.7	**21** ± 1.5	**79** ± 1.5
	1 μg/ml TCP	**25** ± 1.1	**26** ± 1.5	**19** ± 1.6	**81** ± 1.6
	1 μg/ml T*O*CP	**21** ± 1.5	**22** ± 1.5	**18** ± 1.8	**82** ± 1.8
	1 μg/ml T*P*CP	**24** ± 0.8	**39** ± 3.1	**20** ± 1.0	**80** ± 1.0
	1 μg/ml CPSP	**25** ± 1.4	**32** ± 2.4	**18** ± 0.8	**82** ± 0.8
With CM	**Control**	**26** ± 0.9	**64** ± 3.0	**22** ± 1.2	**78** ± 1.2
	1 μg/ml TCP	**24** ± 0.6	**66** ± 2.8	**22** ± 1.2	**78** ± 1.2
	1 μg/ml T*O*CP	**27** ± 0.8	**74** ± 3.6	**24** ± 1.3	**76** ± 1.3
	1 μg/ml T*P*CP	**28** ± 1.0	**67** ± 2.5	**26** ± 1.8	**74** ± 1.8
	1 μg/ml CPSP	**27** ± 1.5	**79** ± 3.8	**22** ± 1.3	**78** ± 1.3

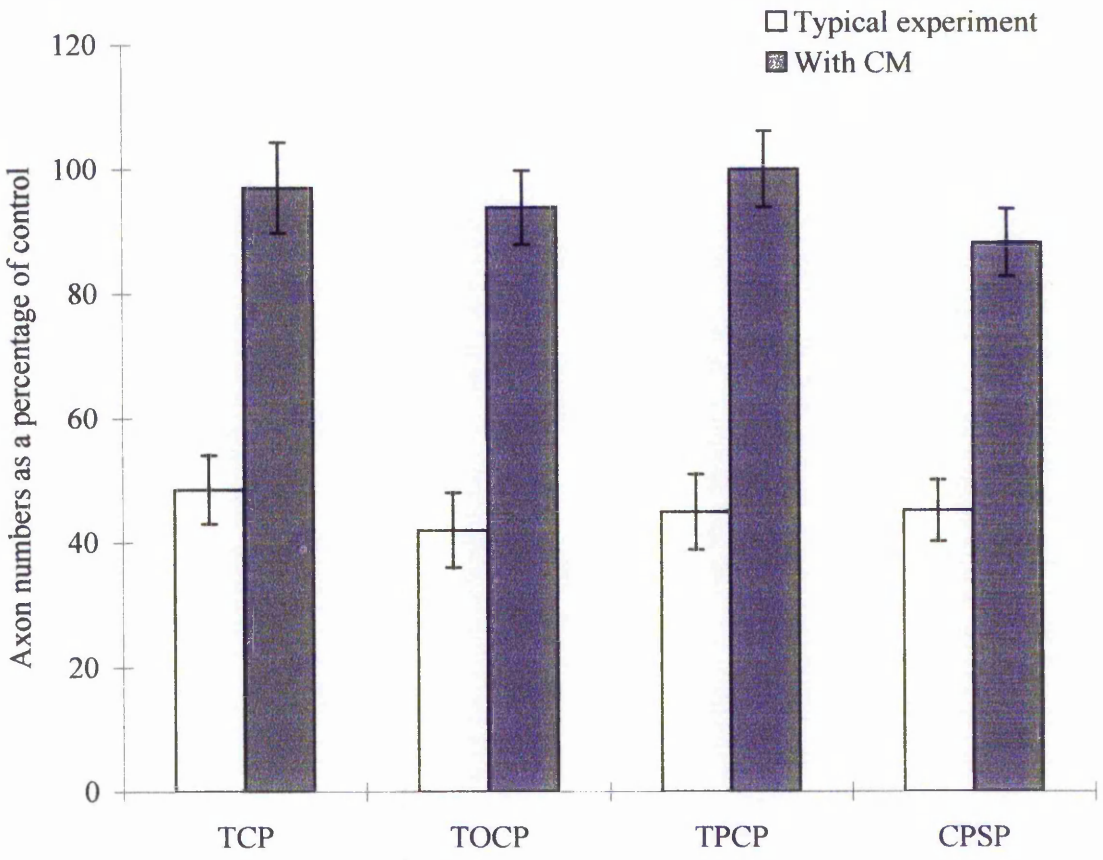


Figure 6.9. *Protective effects of CM on differentiating N2a cells exposed to TCP, TOCP, TPCP or CPSP for 48 hours.* Shown are the numbers of axon-like processes per 100 cells as a percentage of its corresponding control following 48 hours incubation in the presence of 1 μg/ml TCP, T*O*CP, T*P*CP and CPSP. All increases were found to be significant when compared to the corresponding experiment performed in the absence of CM, as determined by the Mann-Whitney U test ($p < 0.05$). Each data point represents an average of 16 wells of cells from 4 separate experiments. Standard errors are shown as error bars.

6.4 Discussion

The data presented in this chapter indicate that CM has a protective effect on differentiating N2a cells exposed to OPs, encouraging axon outgrowth. CM has the ability to significantly increase the outgrowth of axon-like processes in control cells (non-OP treated) after 4, 8, 24 and 48 hour incubations. Under typical experimental conditions (i.e. in the absence of CM) the OP compounds being tested were clearly affecting the outgrowth of these processes, as previously observed and discussed in earlier chapters.

The results of cell differentiation experiments at all time points suggest that CM protects against the axon inhibitory effects of TCP, T*O*CP, T*P*CP and CPSP. However, experiments particularly at the 8 hour time point indicated that protection was not always complete (table 6.1 - 6.4 and figure 6.6 - 6.9). The fact that at all time points the ratio of round to flat cells remained constant as can be seen in the typical control experiment, suggests that the effect of the CM is directed specifically towards the recovery of axon-like processes of OP-treated differentiating N2a cells.

Given that C6 cells are known to produce neurotrophic factors including GDNF (Suter-Crazolora and Unsicker, 1996), it is likely that a GDNF-like growth factor produced by C6 cells has a positive effect on N2a axon outgrowth in these experiments. This model, therefore, suggests that in agreement with Matheson *et al.*, (1997) and Oppenheim *et al.*, (1995) a glial derived neurotrophic factor may play a significant part in the development and maintenance of axons in these cells after a recent toxic insult. Such a growth factor could reverse or block a toxin-induced reduction in axon outgrowth and consequently maintain the neuronal cells in a more healthy state.

In order to further characterise the morphological data produced, Western blot analysis would have been desirable. However on preparing the cell extracts for these experiments, a set of cell extracts which had undergone the typical experimental conditions were not included, thus rendering the analysis of the cell extracts in the presence of CM useless. Ideally, this analysis should be completed to give a clearer understanding of the protective effects of CM on axonal cytoskeletal proteins.

In summary, the protective effect of CM on OP treated differentiating N2a cells is clear, with reversal of axonal inhibition after 4, 8, 24 and 48 hour time points. Further work is now required to establish the molecular basis of this effect.

Chapter Seven

Preliminary studies on the effects of OPs on pre-differentiated systems

7.1 Introduction

Although the work presented in this thesis has concentrated mainly on the effects of OPs on a differentiating cell culture system, it was also of interest to study the effects of these compounds on pre-differentiated cells. As previously mentioned, the majority of *in vivo* studies have been conducted on hens as it has been confirmed that their symptoms mirror those seen in humans (El-Fawal *et al.*, 1990). As it is known that OPIDN induced by TCP and T*O*CP involves axonal degradation with secondary breakdown of myelin and degeneration of muscle, investigations into these effects have been studied in other systems (Cavanagh, 1964; Prentice and Roberts, 1984).

Research into the mechanism(s) of other peripheral neuropathies has involved the use of parasympathetic nerves such as vagus and sciatic nerves. These nerves have been used to study the effects of neurotoxins on (1) fast and slow axonal transport (Archer and McLean, 1988; McLean, 1988; McLean *et al.*, 1993; Archer *et al.*, 1994), (2) cytoskeletal proteins such as NFs and MTs in regenerating nerves (Pekiner and McLean, 1991; Mullins *et al.*, 1991; McLean *et al.*, 1994) and (3) nerve recovery after nerve crush (Bresjanac and Sketelj, 1989).

Another type of pre-differentiated system is one in which neuronal cell lines are encouraged to differentiate *in vitro* and are then maintained for longer periods of time in the presence of toxin leading to studies of their effects on the maintenance and stabilisation of such cells and processes. An example of such a cell line is the rat PC12 pheochromocytoma cells which can be maintained with pre-formed axons, in the presence of nerve growth factor (NGF) for up to 5 days (Flaskos *et al.*, 1994). Work by Flaskos *et al.*, (1994) showed that 1 μg/ml TCP caused neurite retraction in PC12 cells induced to differentiate in the presence of NGF. It was therefore of interest to know if pre-differentiated N2a cells were affected in a similar manner

As part of this thesis, preliminary studies were made on two pre-differentiated systems which were :

(1) mouse N2a neuroblastoma cells that were pre-differentiated for 16 hours and then exposed to TCP, T*O*CP, T*P*CP and CPSP for 24 hours, and

(2) peripheral vagus nerves excised from Wistar rats (section 2.2.6 and 2.2.7). The aim of the work was to analyse the effects of 24 and 48 hours exposure to T*O*CP and T*P*CP on peripheral vagus nerve *in vitro*.

7.2 Effects of 24 hours exposure to TCP, T*O*CP, T*P*CP and CPSP on axon maintenance in pre-differentiated N2a cells

Experiments conducted by Flaskos *et al.*, (1994) on PC12 cells demonstrated that TCP caused neurite retraction. A further study indicated that TCP also inhibited axon outgrowth by both PC12 and N2a cells (Flaskos *et al.*, 1998). Since only outgrowth studies had been carried out on N2a cells, it was important to assess the effects of TCP, T*O*CP, T*P*CP and CPSP on differentiated N2a cells with pre-formed axons. The procedure taken was to plate out the N2a cells and induce them to differentiate in the presence of 0.3 mM cAMP for 16 hours before exposing the cells and axonal processes to 1 μg/ml OP compound for a further 24 hours as described in section 2.2.1.5.

7.2.1 The effects of TCP, T*O*CP, T*P*CP and CPSP on N2a cells with pre-formed axons

The effects of these OP compounds are summarised in table 7.1 and figure 7.1. The data clearly show similar inhibitory trends with respect to axon outgrowth for all OP compounds tested, with these values being significantly lower than the corresponding control, as determined by the Mann-Whitney U test ($p < 0.05$). However the number of smaller extensions present was also reduced showing, significantly lower values than the corresponding control. The one exception was CPSP which appeared to cause a reduction in the outgrowth of axon-like extensions only. When other aspects of cell morphology were examined a greater degree of cell rounding was observed with TCP, T*O*CP and T*P*CP than in control or CPSP treated cells.

Table 7.1. The effects of TCP, TOCP, TPCP and CPSP on N2a cells with pre-formed axons

N2a cells were differentiated for 16 hours then incubated in the absence or presence of OPs for a further 24 hours. The cells were then fixed and stained with Coomassie blue. Data are expressed as the number observed per hundred cells ± standard error from an average of 8 wells of cells cultured on two separate occasions.

	Extensions (%)	**Axons (%)**	**Round cells (%)**	**Flat cells (%)**
Control	**24** ± 1.3	**45** ± 1.6	**24** ± 0.9	**76** ± 0.9
1 µg/ml TCP	**14** ± 1.1	**15** ± 1.6	**37** ± 1.5	**63** ± 1.5
1 µg/ml TOCP	**13** ± 1.1	**11** ± 1.1	**44** ± 1.9	**56** ± 1.9
1 µg/ml TPCP	**17** ± 1.2	**20** ± 1.8	**33** ± 1.9	**67** ± 1.9
1 µg/ml CPSP	**30** ± 2.3	**28** ± 2.4	**20** ± 1.5	**80** ± 1.5

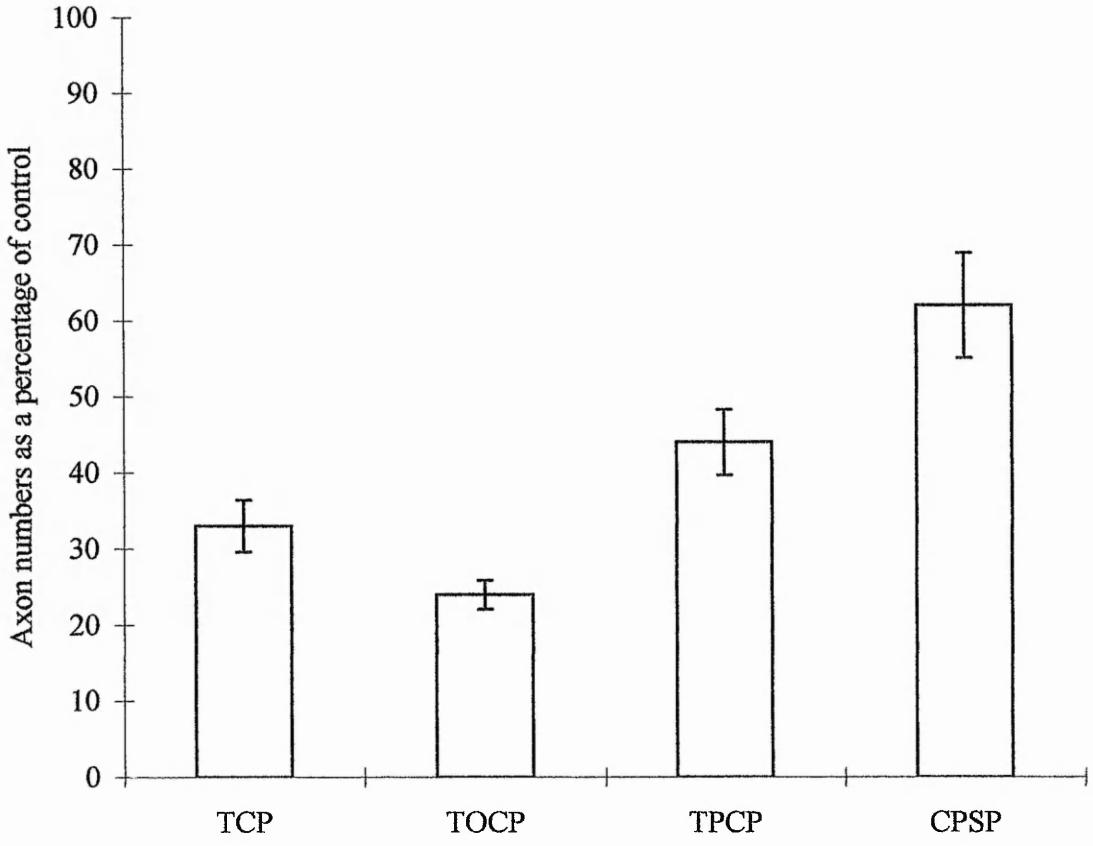


Figure 7.1. *The effects of 1 μg/ml TCP, TOCP, TPCP and CPSP on pre-differentiated N2a cells.* Shown are the numbers of axon-like processes per 100 cells as a percentage of the corresponding control, following 24 hour incubation in the presence of 1 μg/ml TCP, T*O*CP, T*P*CP and CPSP. All of these OP compounds were found to cause significant reductions in axon outgrowth when compared to controls, as determined by the Mann-Whitney U test ($P < 0.05$). Each data point represents an average of 8 wells of cells cultured on two separate occasions. Standard errors are shown as error bars.

7.3 The effects of T*O*CP and T*P*CP on extracted rat vagus nerves

To investigate the effects of 1 μg/ml T*O*CP and T*P*CP on pre-differentiated tissue systems, intact vagus nerves were excised from Wistar rats and exposed to the above compounds for 24 or 48 hours, as described in section 2.2.6. The resultant nerves were then homogenised in SDS-PAGE sample buffer ready for electrophoretic analysis. For each condition, OP treated or control, four separate nerves were prepared from different animals for each time point.

Initially each vagus nerve preparation was electrophoretically separated and stained using BIORAD silver stain (section 2.2.2.3.3) to check the protein profile and the reproducibility between each nerve (figure 7.2). Nerve preparations were then blotted on to nitrocellulose and probed with anti-NF-H antibodies (section 2.2.2). However, the results from these Western blots did not give a consistent pattern of binding for replicate nerves, indicating that the reproducibility and/or survival between the nerves was not consistent. To minimise variation between like samples, a pooled sample was analysed and assumed to be representative of the individual samples previously investigated.

The Western blot in figure 7.3, which was probed with monoclonal antibody N52 (which recognises total NF-H levels independent of its phosphorylation state), shows that after 24 hours exposure the antibody binding decreased slightly in extracts exposed to T*O*CP, whereas no reduction was seen with T*P*CP. However, after 48 hours exposure the levels of antibody binding were equal to the corresponding control. This result suggests that there may be a reduction in NF-H levels after 24 hours exposure to T*O*CP but no effect at 48 hours. Densitometric analysis was used to confirm the visual differences with T*O*CP causing a 10 % reduction after 24 hours but was seen to recover to levels of 103 % with respect to the corresponding control at 48 hours. Antibody binding with T*P*CP treated extracts was higher than the control after 24 hours (at 113 %) and decreased slightly to 98 % of the control after 48 hours.

The pooled extracts were then probed with the GAP-7B10 antibody, but no changes in antibody binding were observed when compared to the corresponding control. This indicates that levels of GAP-43 were not significantly reduced by the presence of T*O*CP and T*P*CP (figure 7.4).

Other Western blots were undertaken using previously described antibodies (RMd09, Ta51 and B-5-1-2) but the resultant blots were not very clear and therefore the results were inconclusive. However, further work investigating the effects on other cytoskeletal proteins would be extremely beneficial, although these Western blots need to be limited due to the insufficient material.

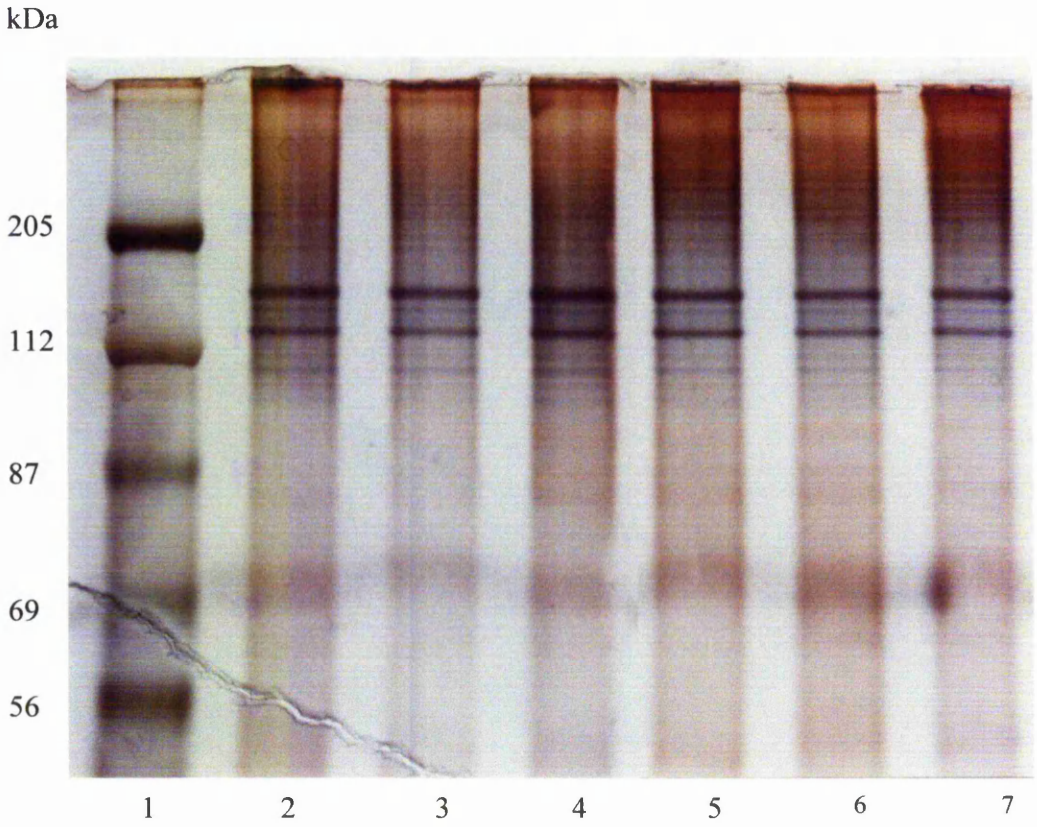


Figure 7.2. *BIORAD silver stained SDS-PAGE of pooled vagus nerves.* Shown are : (1) prestained molecular weight markers; (2) pooled control vagus nerve extracts after 24 hour incubation; (3) pooled vagus nerve extracts exposed to 1 µg/ml T*O*CP for 24 hours; (4) pooled vagus nerve extracts exposed to 1 µg/ml T*P*CP for 24 hours; (5) pooled control vagus nerve extracts after 48 hour incubation; (6) pooled vagus nerve extracts exposed to 1 µg/ml T*O*CP for 48 hours; (7) pooled vagus nerve extracts exposed to 1 µg/ml T*P*CP for 48 hours.

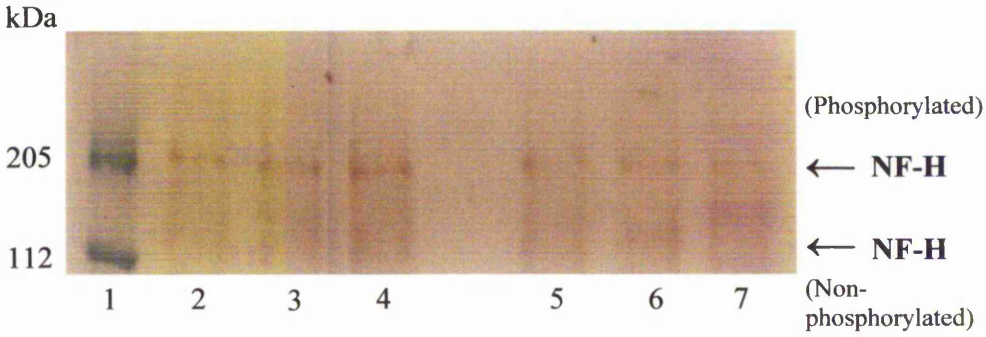


Figure 7.3. *Western blots of pooled vagus nerve preparations exposed to 1 μg/ml TOCP and TPCP, probed with anti-neurofilament antibody, N52.* Shown are : (1) prestained molecular weight markers; (2) pooled control vagus nerve extracts after 24 hour incubation; (3) pooled vagus nerve extracts exposed to 1 μg/ml T*O*CP for 24 hours; (4) pooled vagus nerve extracts exposed to 1 μg/ml T*P*CP for 24 hours; (5) pooled control vagus nerve extracts after 48 hour incubation; (6) pooled vagus nerve extracts exposed to 1 μg/ml T*O*CP for 48 hours; (7) pooled vagus nerve extracts exposed to 1 μg/ml T*P*CP for 48 hours. The blot was probed with antibody, N52 which recognises NF-H independent of its phosphorylation state. The arrows indicate a reactive polypeptide with the apparent molecular weight of NF-H.

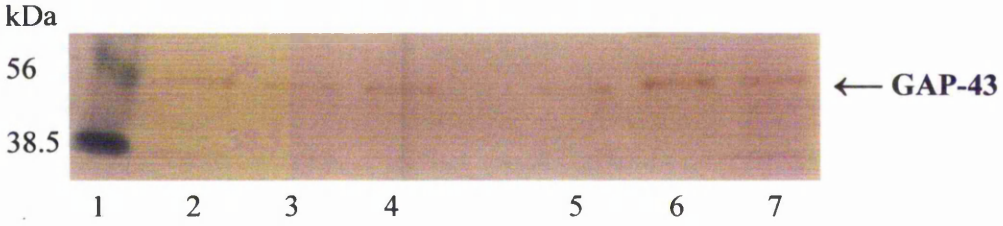


Figure 7.4. *Western blots of pooled vagus nerve preparations exposed to 1 µg/ml TOCP and TPCP, probed with GAP-7B10.* Shown are : (1) prestained molecular weight markers; (2) pooled control vagus nerve extracts after 24 hour incubation; (3) pooled vagus nerve extracts exposed to 1 µg/ml T*O*CP for 24 hours; (4) pooled vagus nerve extracts exposed to 1 µg/ml T*P*CP for 24 hours; (5) pooled control vagus nerve extracts after 48 hour incubation; (6) pooled vagus nerve extracts exposed to 1 µg/ml T*O*CP for 48 hours; (7) pooled vagus nerve extracts exposed to 1 µg/ml T*P*CP for 48 hours. The blot was probed with antibody, GAP-7B10 which recognises GAP-43 with in neuronal processes. The arrow indicates a reactive polypeptide with the apparent molecular weight of GAP-43.

7.4 Discussion

From the experiments with pre-differentiated N2a cells, it can be concluded that all of the compounds tested appear to have inhibitory effects on differentiating N2a cells with formed or pre-formed axons, with T*O*CP exhibiting the greatest inhibition. This, coupled with results from earlier chapters, suggests that irrespective of whether the axons were forming or pre-formed, there still appears to be an inhibitory effect with these compounds. This is consistent with the reduction in axon outgrowth observed in previously (chapter 4) and agrees with the previously observed retraction of neurite in TCP-treated PC12 cells (Flaskos *et al.*, 1998). This gives further support to the validity of this cell culture system for studying the toxicity of OPs.

The observed reduction in numbers of pre-formed extensions in cells treated with TCP, T*O*CP and T*P*CP indicates that in pre-differentiated systems these OPs may also be interfering with the maintenance of extensions, indicating that additional molecular targets may be affected. Alternatively, the cells may be at the limits of survival due to the length of time being in a pre-differentiated state without the presence of essential growth factors. Although CPSP is not the most potent inhibitor of axon outgrowth, it does appear to have a selective effect on these processes in both differentiating and pre-differentiated cells.

Therefore, the length of experimental time required to assess the impact of these compounds could have been a contributing factor towards the stability and morphology of the cells. This highlights the relatively fragile nature of differentiated N2a cells compared to the PC12 cell line, which successfully survives tissue culture treatments for up to 5 days after differentiation. Differentiated N2a cells are useful for assessing effects for up to 24 hours but for longer time points careful consideration must be taken to ensure a healthy and stimulating environment for the N2a cells to grow and/or differentiate in.

Preliminary experiments were carried out with isolated nerve to take into account the complexities of cell : cell interactions in nerve tissue. However, as a result of the delicate nature of the vagus nerves once removed from the animal, reproducibility

between nerve extracts was poor. There was evidence that several of the treated nerves were damaged and regeneration was unclear. This was not helped by the fact that several Western blots gave smeared patterns and low reactivity of the same antibodies with individual control nerve extracts. To reduce this variation between individually treated nerves, a pooled sample containing extracts from each nerve was then analysed. Therefore, this sample was used as a representative of each nerve extracted from each animal (where n = 4).

Due to the limited supply of samples, only a few Western blots were produced. However, from the Western blot data shown in figure 7.2 it can be seen that in vagus nerves exposed to 1 μg/ml T*O*CP, the level of total NF-H within the nerve was reduced after 24 hours but not after 48 hours when compared to its corresponding control. This may be suggesting that after 48 hours the nerve is regenerating and therefore less affected by the presence of T*O*CP at this time point. By contrast, the levels of NF-H in T*P*CP treated nerve extracts appear to mirror those of the corresponding control, indicating that the nerve is unaffected by T*P*CP and is also able to regenerate normally. Interestingly, the Western blot probed with the GAP-43 antibody showed that these compounds were not inhibiting but slightly increasing its expression and leading us to believe that the nerve may be regenerating over the 24 to 48 hour time period. This effect mirrors that seen with CPSP on differentiating N2a cells up to 24 hours (figure 5.10b).

Although the data obtained from the Western blots of whole nerve are interesting, there is a definite need to clarify these results by further work on these and other extracted vagus nerves. Since the results presented within this chapter are preliminary studies on the effects of the OP compounds on pre-differentiated systems, further work is essential to confirm these findings and help to establish further markers of toxicity.

8.0 General Discussion

As research and governmental committees have agreed on the need to reduce the use of animals in chemical testing, research programmes to develop alternative methods have been instigated, with the *in vitro* cellular system being just one example (Veronesi, 1992). Such a cellular system provides a controlled environment for studying the sequence of events that occur after any type of chemical insult on a specific cell type. The problem with such systems is that they lack the ability to promote systemic effects (like the action of the liver) as seen in animal models. However, these systems do permit the evaluation of the direct toxic effects on cultured cells; not only is quantitative assessment of cytotoxic effects possible but investigators can also examine the extent of intracellular changes at the molecular level. Such an approach also overcomes the ethical issues associated with the use of large numbers of animals.

The work presented within this thesis has given some insight into the neurotoxic effects of TCP, two of its related isomers and a structural analogue of its active metabolite on differentiating cell lines. These compounds show a selective effect on outgrowth of axon-like processes produced by cells of neuronal origin, an effect which correlates with altered NF-H levels (Flaskos *et al.*, 1998). Also, isomer specific toxicity was observed at earlier time points, which corresponds to the toxicity pattern seen *in vivo* (Fowler *et al.*, 1997; 2000).

It is important to mention that the effect seen in differentiating N2a cells exposed to the *-para* isomer at 1 μg/ml is a transient one, in which inhibition of axon outgrowth is observed after 24 hours but usually not after 48 hours, suggesting that the inhibitory effect is not sustained. There are several possible reasons for this transient effect and these are as follows:

- the initial neurotoxic action may be short lived and with time the cells are able to overcome the toxic effects, making it a less effective compound, or

- the half life or solubility of the isomer, in an aqueous solution, is relatively poor (Chambers, 1992) and the toxic levels in the culture conditions are limited, giving the cells enough time to recover and attenuate the inhibitory effect over time, or

- the steric effect i.e. the different position of the methyl group may result in a reversible or distinct binding to target proteins.

It is important to mention that an OP concentration of up to 1 μg/ml was used throughout this work for a number of reasons. Earlier studies by Flaskos *et al.*, (1994) showed that this concentration of TCP had an inhibitory effect on the maintenance of neurites in cultured rat PC12 pheochromocytoma cells with no overall effect on cell viability as determined by Trypan blue exclusion and [^{3}H]thymidine incorporation. Moreover, this level of OP causes no significant cell death under any of the conditions tested in the current work. Furthermore, the concentration of OPs (1 μg/ml) used is several hundred fold lower than the dose used to induce OPIDN *in vivo* (Abou-Donia and Lapadula, 1990) and is comparable to the levels found in nerve tissue of animal models of OPIDN (Ahmed *et al.*, 1993; Somkuti and Abou-Donia, 1990).

This system has clearly demonstrated the selective effect of TCP on the outgrowth of axon-like processes in differentiating cells of a neuronal nature, with a lack of effect seen on short extensions produced by the same cells and processes produced by differentiating C6 glioma cells (chapter 3 and Flaskos *et al.*, 1998). These data are in agreement with numerous studies stating that the longer neuronal processes are first affected and damaged (Abou-Donia, 1981; 1992; 1995; Abou-Donia and Lapadula, 1990; Johnson, 1974; 1975a; 1975b). In order to further quantify the effects of OPs on the different types of processes produced by differentiating neuronal and glial cells, an image analysis package was used to measure neurite length as described by McLean *et al.*, (1998). These results mirrored previously observed trends, giving an excellent correlation between the two different approaches (data presented in Appendix I), and were also in agreement with measurements made using an eyepiece graticule (table 3.5).

However, work by Henschler *et al.*, (1992) suggested that a range of OPs caused the inhibition of neurite outgrowth in both differentiating N-18 neuroblastoma and C6 glioma cell lines. It is important to note that this research group does not clearly distinguish, in terms of neurite length, between the two different kinds of processes in differentiating neuronal cells described by Flaskos *et al.*, (1994; 1998). As a consequence, subtle but important changes could be missed or sensitivity could be lost through this generalisation. There are other possible reasons to explain the glial toxicity obtained by Henschler's research group, one of which is the doubtful viability of the cells in culture due to the extended length of incubation times. They exposed N-18 and C6 cells to different concentrations of OPs for 14 days and then a further 6 days in the presence of cAMP to induce neurite outgrowth before analysing the cells for the effects of OPs. As a result, high levels of cytotoxicity were observed. In my opinion, the length of time these cells were exposed to the OPs and then allowed to differentiate was far too long and consequently could contribute to the effects seen being cytotoxic rather than selectively neurotoxic. Incubation and exposure times used routinely throughout my work did not exceed 48 hours, as *in vivo* as molecular changes have been observed within this time scale by other researchers such as Abou-Donia *et al.*, (1981; 1990; 1995), Suwita *et al.*, (1986) and Zech and Chemnitius (1987).

Since the exposure time of any compound *in vivo* may only last for a few minutes/hours, using short incubation times is mechanistically more relevant (with respect to OPDIN), since we can study the early biochemical effects leading to clinical OPIDN manifestations. Therefore, the studies conducted in this thesis at the later time points 24 and 48 hours gave useful information but may be too long for detection of early toxicity markers, which encouraged us to carry out further experiments at earlier time points. Indeed, it can be concluded from studies undertaken at 4 and 8 hours that isomer specific differences were identified (chapter 4).

As previously mentioned, an important feature lacking from such a cellular system is the metabolic action of the liver. In an attempt to overcome this problem microsomal activation was used to mimic the function of the liver. It is known that TOCP is bio-activated by the cytochrome P-450 system in the liver into a more neurotoxic compound(s) before being detoxified and eliminated (Bischoff, 1967; Sprague and

Castle, 1985; Burka and Chapin, 1993). It was essential that measures were taken to ensure the effects seen throughout these experiments were due to microsomal activation only, so care was taken to include the correct controls. Initially, the effects of the microsomes were assessed to determine if they would change the outcome of the experiment, while routine controls included the activation system without microsomes.

It can be concluded that an increase in toxicity was seen with TCP and T*O*CP after microsomal activation, as determined by a greater inhibition of axon outgrowth than seen in previous experiments, after 4 hours of exposure (section 4.6.1). This confirms that TCP (which contains T*O*CP) and T*O*CP can be microsomally activated into a more neurotoxic compound(s) as is thought to occur *in vivo* (Taylor and Buttar., 1967; Eto *et al.*, 1962; Casida *et al.*, 1961) but only for short periods of time i.e. 4 hours or less, whereas T*P*CP shows no increase in neurotoxic effect at this time point with or without microsomal activation. However, to ensure the workings of such a microsomal system, a positive control with a known metabolite or metabolic inhibitors of P-450, could be useful to confirm the efficiency of the system at later time points.

It would be beneficial for further work to be carried out to analyse the effects of this system on TCP, T*O*CP and T*P*CP at earlier time points than 4 hours, such as ten minutes or one hour maximum, as work by Ehrich *et al.*, (1997) has shown that NTE can be inhibited within 20 minutes of exposure to OPs, *in vitro*. Indeed preliminary work by Ahmed (1998) indicates the 10 minutes exposure to T*O*CP is sufficient to induce later changes (at 8 hours incubations) in the outgrowth of axon-like processes. Therefore it would be useful to extend this work to establish whether short exposure to OPs can inhibit the outgrowth of axon-like processes and to identify the molecular changes involved.

Several major conclusions can be drawn from the molecular analyses conducted on the differentiated N2a cell extracts, which have been incubated in the presence and absence of OPs. The first being that there is a clear reduction in phosphorylated NF-H and non-phosphorylated NF-H when compared to the corresponding control under most conditions with TCP, T*O*CP and T*P*CP. This effect could be due to a reduction in NF synthesis or to increased degradation, which could possibly involve calpain-activated

proteolysis. For example, work by El-Fawal and Ehrich (1993) suggested that the degeneration of the cytoskeleton, known to be involved in the development of OPIDN, may involve the activation of calpain due to a calcium influx (El-Fawal and Ehrich, 1993; El-Fawal *et al.*, 1990; Abou-Donia, 1990; 1995).

The levels of reactivity with all three antibodies seem to decrease from 4 to 8 hour incubation with some recovery with RMd09 and N52 at later time points, whereas Ta51 reactivity remains relatively low. This may indicate this NF-H levels are reduced at early time points but recover later, while the phosphorylation state is reduced early on and remains low throughout. It is also possible that TCP and/or its isomers may bind to the KSP regions (serine group) of NF-H in neuronal cells. In order to establish whether this can occur, further work should be undertaken to assess the effects of these OPs on the reactivity of purified NFs with phosphorylation state dependent antibodies.

The levels of other cytoskeletal proteins such as tubulin and GAP-43, were also investigated in the same cell extracts after 4 to 48 hours exposure. Similar trends were observed with both isomers showing an inhibitory effect after 8 hours (in the case of T*O*CP and T*P*CP) and 48 hours (for TCP, T*O*CP and T*P*CP) (figure 4.11, 4.12, 4.20 and 4.21; table 4.4 and 4.7). For tubulin, similar inhibitory effects were seen at 8 hours and 48 hours (although in the latter, to a greater degree), which suggests that reduced tubulin levels at this time point could be an early toxicity marker for both T*O*CP and T*P*CP as the detection of the insult is clearly visible.

With CPSP, it appears that after 8 hours only the level antibody reactivity of phosphorylated NF-H is increased with a concomitant reduction in non-phosphorylated NF-H (figure 5.6a). Such effects appear to be in agreement with the findings of Suwita *et al.*, (1986) who indicated an increase in calcium/calmodulin protein kinase mediated phosphorylation of spinal cord NF-H from OP treated hens. However, it is important to note that this blot was only completed on one set of cell extracts and should be repeated before any firm conclusions can be drawn from this apparent shift in phosphorylation state.

However, although the predominant effect of these compounds appears to be selective towards the NF structures within the cell body and axonal processes, effects can be seen with other cytoskeletal proteins. The level of α-tubulin antibody reactivity was inhibited in CPSP-treated cells after 4 hours but then steadily increased with time. This suggest that tubulin is more vulnerable at earlier time points, when the axonal cytoskeletal framework is being built but is able to recover to levels similar to the corresponding controls (figure 5.9a and 5.9b). With the antibody GAP-7B10, a significant decrease in antibody binding was observed only after 48 hours, suggesting that only prolonged exposure to CPSP cause inhibition of GAP-43 protein expression (figure 5.10a and 5.10b).

One of the most dramatic effects seen was with CPSP-treated cell extracts that were probed with NF-H monoclonal antibodies, with a significant increase in phosphorylated NF-H and decrease in non-phosphorylated NF-H being observed after 8 hours (graphical data presented in Appendix II). This, however, was not seen in T*O*CP and T*P*CP-treated cell extracts after 8 hours, suggesting some differences in the target(s) of these OPs. The same graphs also reveal that compounds able to inhibit axon outgrowth within 4 hours of exposure also reduce reactivity with N52. This suggests that early reduction in axon outgrowth is associated with a decrease in the levels of NF-H. In the case of T*O*CP, the concomitant decrease in Ta51 but not RMd09 reactivity shows a selective reduction in the levels of phosphorylated NF-H.

Using short incubation times is mechanistically more relevant to study the earlier events following OP exposure and reduces the practical disadvantages of using extended incubation times, which risk being influenced by cytotoxic effects. For example, with the neurite outgrowth and Western blot data similar changes can be seen after 24 and 48 hours incubation with both the neurotoxic T*O*CP and the non-neurotoxic T*P*CP (table 4.2 and figure 4.3; figure 4.4 - 4.7). However, the use of the shorter exposure times, in particular 4 hours, allows us to see the clearer distinction between the neurotoxic and non-neurotoxic isomer at both the morphological and molecular level (table 4.6 and figure 4.16). To further support this statement, 4 hours proved to be the optimum time point to detect metabolic activation and further distinguish between the two isomers (table 5.1).

As OPIDN is known to be a debilitating condition, the search for a potential treatment is important. Of particular interest to the present work was recent literature on the protective effects of neurotrophic factors produced by differentiating glioma cells (Suter-Crazzolara and Unsicker, 1996). These workers documented that expression of neurotrophic factors such as GDNF was clearly evident within primary cultured astrocytes, B49 glioma and C6 glioma cells and that the growth factor was secreted into the cell culture medium.

My own experimental work using CM from C6 cells showed that it had the ability to block the inhibitory effects of TCP, T*O*CP, T*P*CP and CPSP on the outgrowth of axon-like processes. This suggests that neurotrophic factors in CM prevent the neurodegenerative effects of the OPs, making neurite outgrowth resistant to their toxic effects. However, to further confirm these findings, it would be useful to titrate the CM, selecting a concentration that did not have any effect in itself on neurite outgrowth. Further work on neurotrophic factors purified from CM would also help to establish the molecular basis of this protective effect.

In order to assess the effects of these OPs even further, preliminary work was conducted on two pre-differentiated systems. The effects seen with the pre-differentiated N2a cells appear to mirror the effects seen with differentiating cells, as described in earlier result chapters in that all OPs reduce axon numbers following 24 hour exposure. This, in agreement with previous data, using differentiated PC12 cells (Flaskos *et al.*, 1994), confirms that the OPs tested inhibit both the outgrowth and maintenance of axon-like processes in differentiating N2a cells. However, unlike the response of differentiating cells, inhibition of the outgrowth of smaller processes was also observed in the case of TCP, T*O*CP and T*P*CP. This could indicate that pre-formed neurites are more sensitive to OP exposure and/or that there are some additional molecular targets in pre-differentiated cells. This was not the case for CPSP, which affected only axon-like processes in both differentiating and pre-differentiated cells. Due to time constraints, it was not possible to detect molecular markers and to further evaluate this N2a pre-differentiated cellular system. Further experimental work is required at this and also at shorter exposure times (i.e. 4 or 8 hours) to investigate whether similar isomer and axon specific effects can be observed.

Preliminary work was carried out on *ex vivo* rat vagus nerve extracts in order to study effects of OPs on whole tissue. The excised vagus nerves were initially analysed separately; however, the reproducibility between the controls was inconsistent. Therefore to give an overall response, extracts from like-treated samples were pooled. Immunoprobing of the resultant Western blots was not conclusive, although it does show that T*O*CP but not T*P*CP has an inhibitory effect on the levels of NF-H after 24 hours of incubation. The recovery of NF-H levels at 48 hours, also confirmed by densitometric analysis, may indicate the process of regeneration is well underway by this stage.

However, to give a clearer understanding into the *in vivo* effects of these OPs, further work would be useful to assess the axonal transport of proteins along excised nerves. This would involve radiolabelling newly synthesised proteins in the vicinity of the cell bodies coupled with further electrophoretic and autoradiographic analysis of nerve pieces at different time points and various distances from the cell bodies (McLean *et al.*, 1993). This will help to detect temporal changes in the stability and transport of specific cytoskeletal proteins following exposure to different concentrations of OPs.

One problem endured during this work was the weak reactivity of several of the monoclonal antibodies used which led to difficulty in drawing firm conclusions, particularly at early time points. Further work with a more sensitive detection method such as enhanced chemiluminescence (ECL) might help to improve the quality of the blots.

As the current understanding of NTE in OPIDN is incomplete, it has been suggested by Glynn (1999; 2000) that it may be due to either (1) the loss of an essential non-esterase function which is required by neurons and/or their axons, or (2) that having a negative charge at the active site of NTE induces a toxic effect which may lead to neuropathy (Abou-Donia, 1990; Glynn, 1999; 2000; Johnson, 1990; Johnson and Read, 1987). Hence, it was of interest to the project to assess the potential role of neuropathy esterase (NTE) in the observed effects of OPs *in vitro*. Therefore, preliminary studies were undertaken to establish the presence of NTE in N2a cells and determine the effects of OPs on the levels of NTE found within differentiating N2a cells, using anti-NTE

antiserum, which was kindly provided by colleagues at the MRC Toxicology Unit, University of Leicester. Western blots probed with this anti-serum have shown that NTE can be detected in these cells and that reduced levels of NTE are observed after exposure to TCP, T*O*CP and T*P*CP for 24 hours (data not shown).

To date, further collaborative work has been undertaken (Sachana, 1999) to study the effects of OPs on NTE in N2a cells. It has been shown that our preparation of CPSP reduces NTE to less than 2 % of controls at 4 and 8 hour time points, indicating that NTE is blocked at a very early stage following exposure to CPSP. It has been shown that phenyl saligenin phosphate (PSP) caused an increase in calpain activity in chicken brains within 4 days in sciatic nerves within 2 days. This increase in activity did not occur if the L-type calcium channel blocker, nifedipine was administered to the birds (George *et al.*, 1995; El-Fawal *et al.*, 1990). Further work on the effects of blocking calcium channels on calpain activity following OP administration would be of interest.

To establish the crucial link between NTE inhibition and cytoskeletal changes observed in this thesis, it is important to study the changes in signalling pathways and cascades. To determine whether these pathways are blocked and/or inhibited by the presence of OPs, investigations on MAP kinases would be very useful.

In summary, the results from this project have helped to establish the pattern of toxicity of these OPs towards differentiating N2a cells. Of particular importance is the fact that the most neurotoxic isomer, *in vivo*, and its metabolite are the most effective at inhibiting outgrowth of axon-like processes at early time points *in vitro* and only OP preparations containing the *-ortho* isomer can be metabolically activated by microsomes. This suggests that we have a mechanistic system, which reflects *in vivo* toxicity patterns at least for T*O*CP.

These findings support the view that differentiating and pre-differentiated N2a cells represent a useful cellular system for studying the neurodegenerative effects of TCP, T*O*CP, T*P*CP and CPSP. This system has the potential to be used in screening other neurotoxic and cytotoxic compounds (Flaskos *et al.*, 1999) and is particularly useful for studying the sequence of molecular events that follow OP exposure.

References

Abou-Donia, M. B. (1981) Organophosphorus ester-induced delayed neurotoxicity. Annual Review of Pharmacology and Toxicology 21, 511-548.

Abou-Donia, M. B. (1992) Triphenyl phosphite : A Type II organophosphorus compound-induced delayed neurotoxic agent in Organophosphates. *Chemistry, Fate and Effects* (Edited by Chambers, J. E and Levi, P. E.) pp. 327-349, Academic Press, New York.

Abou-Donia, M. B. (1993) The cytoskeleton as a target for organophosphorus ester-induced delayed neurotoxicity (OPIDN). Chemical-Biological Interactions 87, 383-393.

Abou-Donia, M. B. (1995) Involvement of cytoskeletal proteins in the mechanism of organophosphorus ester-induced delayed neurotoxicity. Clinical and Experimental Pharmacology and Physiology 22, 358-359.

Abou-Donia, M. B. and Lapadula, D. M. (1990) Mechanism of organophosphorus ester-induced delayed neurotoxicity: Type I and Type II. Annual Review of Pharmacology and Toxicology 30, 405-440.

Abou-Donia, M. B., Lapadula, D. M. and Suwita, E. (1988) Cytoskeletal proteins as targets for organophosphorus compound and aliphatic hexacarbon-induced neurotoxicity. Toxicology 49, 469-477.

Ahmed, A. E., Jacob, S., Soliman, S., Ahmed, N., Osman, K., Loth, J-P. and Romero, N. (1993) Whole-body autoradiographic disposition, elimination and placental transport of [^{14}C]tri-*ortho*cresyl phosphate in mice. Journal of Applied Toxicology 13 (4), 257-267.

Ahmed, I. (1998) Neurodegenerative effects of organophosphate compounds on cultured N2a cells mouse neuroblastoma cells. M.Sc Thesis.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K and Watson, J. D. (1983) *Molecular Biology of the Cell* (2nd Edition), Garland Publishing Inc., New York.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K and Watson, J. D. (1994) *Molecular Biology of the Cell* (3rd Edition), Garland Publishing Inc., New York.

Alexander, J. E., Hunt, D. F., Lee, M. K., Shabanowitz, J., Michel, H., Berlin, S. C., MacDonald, T. L., Sundberg, R. J., Rebhum, L. I. and Frankfurter, A. (1991) Characterization of post translational modifications in neuron-specific class III beta-tubulin by mass spectrometry. Proceedings of the National Academy of Science of the United States of America 88 (11), 4685-4689.

Archer, D. R. and McLean, W. G. (1988) Changes in fast axonally transported proteins in the regenerating rabbit vagus nerve. Neuroscience Letters 87, 151-156.

Archer, D. R., Dahlin, L. B. and McLean, W. G. (1994) Changes in slow axonal transport of tubulin induced by local application of colchicine to rabbit vagus nerve. Acta Physiologica Scandinavica 150, 57-65.

Avila, J., Diaz-Nido, J., Wandosell, F., Hargreaves, A. J., Hernandez, M. A. and Serrano, L. (1988) Phosphorylation of tubulin by different protein kinases in *Structure and Functions of the Cytoskeleton* (Edited by Rousset, B. A. F.) pp. 439-445, John Libbey Eurotext, Paris-London.

Avila, J., Serrano, L., Hernandez, M. A., Wandosell, F., Montejo, E., Diaz-Nido, J. and Hargreaves, A. J. (1987) Regulation of the polymerization of microtubule protein in *The Cytoskeleton in Cell Differentiation and Development* (Edited by R. B. Maccioni and J. Archega) pp. 95-103, ICSU Press, Oxford.

Baas, P. W., Deitch, J. S., Black, M. M. and Banker, G. A. (1988) Polarity orientation of microtubules in hippocampal neurons: Uniformity in the axon and non-uniformity in the dendrite. Proceedings of the National Academy of Science of the United States of America 85, 8335-8339.

Bamburg, J. R., Bray, D. and Chapman, K. (1986) Assembly of microtubules at the tip of growing axons. Nature 321, 788-790.

Barra, H. S., Arce, C. A. and Argarana, C. E. (1988) Posttranslational tyrosination / detyrosination of tubulin. Molecular Neurobiology 2, 133-153.

Beck, K. D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R. A., Rosenthal, A. and Hefti, F. (1995) Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. Nature 373, 339-341.

Bergen, L. G. and Borisy, G. G. (1980) Head to tail polymerization of microtubules *in vitro*. Journal of Cell Biology 84, 141-150.

Berry, M. M., Trosko, B. K., Tanaka, D. Jr. and Bursian, S. J. (1986) The effect of a ganglioside mixture on the development of tri-*o*-tolyl phosphate-induced delayed neurotoxicity. Toxicologists 6, 219.

Bershadsky, A. D and Vasiliev, J. M. (1988) Systems of Intermediate filaments in *Cytoskeleton* pp. 133-150, Plenum Press, New York.

Berti-Mattera, L. N., Eichberg, J., Schrama, L. and LoPachin, R. M. (1990) Acrylamide administration alters protein phosphorylation and phospholipid metabolism in rat sciatic nerve. Toxicology and Applied Pharmacology 103 (3), 502-511.

Bertoncin, D., Russolo, A., Caroldi, S., and Lotti, M. (1985) Neuropathy target esterase in human lymphocytes. Archives of Environmental Health 40 (3), 139-144.

Bickar, D. and Reid, P. D. (1992) A high-affinity protein stain for Western blots, tissue prints and electrophoretic gels. Analytical Biochemistry 203, 109-115.

Binder, L. I., Frankfurter, A. and Rebhun, L. I. (1985) The distribution of tau polypeptides in the mammalian central nervous tissue. Journal of Cell Biology 101, 1371-1378.

Bischoff, A. (1967) The ultra-structure of tri-*ortho*-cresyl phosphate poisoning. I. Studies on myelin and axonal alternations in the sciatic nerve. Acta Neuropathologica 9, 158-174.

Biswas, S., Biswas, S. K. and Bhattacharyya, A. K. (1993) Effect of tri-*ortho*cresyl phosphate on hen nervous system. Indian Journal of Medical Research 98, 96-101.

Black, M. M. and Greene, L. A. (1982) Changes in the Colchicine susceptibility of microtubules associated with neurite outgrowth: Studies with nerve growth factor-responsive PC12 pheochromocytoma cells. Journal of Cell Biology 95, 379-386.

Black, M. M. and Lasek, R. L. (1979) Axonal transport of actin : slow component b is the principal source of actin for the axon. Brain Research 171, 401-413.

Black, M. M. and Lasek, R. L. (1980) Slow components of axonal transport: two cytoskeletal networks. Journal of Cell Biology 86, 616-623.

Bondy, H. F., Field, E. J., Worden, A. N. and Hughes, J. P. W. (1960) A study on the acute toxicity of the tri-aryl phosphates used as plasticizers. British Journal of Industrial Medicine 17, 190-200.

Brady, S. T. and Lasek, R. J. (1981) Nerve specific enolase and creatine phosphokinase in axonal transport soluble proteins and the axoplasm matrix. Cell 2, 515-523.

Bresjanac, M. and Sketelj, J. (1989) Neurite-promoting influences of proliferating Schwann cells and target-tissues are not prerequisite for rapid axonal elongation after nerve crush. Journal of Neuroscience Research 24, 501-507.

Brill, D. M., Maisel, A. S. and Prabhu, R. (1984) Polymorphic ventricular tachycardia and other complex arrhythmias in organophosphate insecticide poisoning. Journal of Electrocardiology 17, 97-102.

Brown., A. G. (1991) An introduction to neuroscience in nerve cells and the nervous system, pp. 117-125, Springer-Verlag, London.

Budavari, S., O'Neil, M. J., Smith, A., Heckleman, P. E. and Kinneray, J. F. (Editors) (1996) The Merck Index. An Encyclopedia of Chemicals, Drugs and Biologicals, pp. 1663, Merck & Co., Inc., Whitehouse Station, NJ. (12^{th} edition).

Burgoyne, R. D. and Cambray-Deakin, M. A. (1988) The cellular neurobiology of neuronal development: the cerebellar granule cells. Brain Research 472 (1), 77-101.

Burka, L. T. and Chapin, R. E. (1993) Toxicokinetics of [^{14}C]-saligenin cyclic-*o*-tolyl phosphate in anesthetized male F-344 rats. Reproductive Toxicology 7, 81-86.

Caceres, A., Banker. G., Steward, O., Binder, L. and Payne, M. (1984) MAP2 is localized to the dendrites of hippocampal neurons which develop in culture. Developmental Brain Research 13, 314-318.

Cambray-Deakin, M. A. (1991) Cytoskeleton of the growing axon in *The Neuronal Cytoskeleton*, pp. 233-255, Wiley Liss, New York.

Cambray-Deakin, M. A., Morgan, A. and Burgoyne, R. D. (1987) Sequential appearance of cytoskeletal components during the early stages of neurite outgrowth from cerebellar granule cells *in vitro*. Brain Research 465 (1-2), 197-207.

Carden, M. J., Schlaepfer, W. W. and Lee, V. M.-Y. (1985) The structure, biochemical properties, and immunogenicity of neurofilament peripheral regions are determined by phosphorylation state. Journal of Biological Chemistry 260 (17), 9805-9817.

Carden, M. J., Trojanowski, J. Q., Schlaepfer, W. W. and Lee V. W. (1987) Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. Journal of Neuroscience 7, 3489-3504.

Carrington, C. D. and Abou-Donia, M. B. (1984) The correlation between the rate of neurotoxic esterase activity and sensitivity to organophosphorus-induced delayed neurotoxicity. Toxicology and Applied Pharmacology 75, 350-357.

Carrington, C. D. and Abou-Donia, M. B. (1985) Characterization of [^{3}H]diisopropyl phosphorofluoridate-binding proteins in hen brain. Rates of phosphorylation and sensitivity to neurotoxic and non-neurotoxic organophosphorus compounds. Biochemical Journal 228, 537-544.

Carrington, C. D., Carrington, M. N. and Abou-Donia, M. B. (1985) Neurotoxic esterase in cultured cells : an *in vitro* alternative for the study of organophosphorus compound-induced delayed neurotoxicity in *Alternative methods in toxicology. Volume 3 : In vitro Toxicology*, pp. 457-465, Mary Ann Liebert Inc., Publishers, New York.

Casida, J. E., Eto, M. and Baron, B. L. (1961) Biological activity of tri*ortho*cresyl phosphate metabolite. Nature 191, 1396.

Cavanagh, J. B. (1964) Peripheral nerves changes in *ortho* cresol phosphate poisoning in the cat. Journal of Pathology and Bacteriology 87, 365-383.

Chambers, H. W. (1992) Organophosphorus Compounds: An Overview in *Organophosphates. Chemistry, Fate and Effects* (Edited by Chambers, J. E. and Levi, P. E.) pp. 3-17, Academic Press, New York.

Chauhan, N. B., Spencer, P. S. and Sabri, M. I. (1993) Acrylamide-induced depletion of microtubule-associated proteins (MAP1 and MAP2) in the rat extrapyramidal system. Brain Research 602, 111-118.

Cherniack, M. G. (1988) Toxicological screening of organophosphorus-induced delayed neurotoxicity : complications in toxicity testing. NeuroToxicology 9 (2), 249-272.

Cho, K. J., Hirakawa, T., Mukai, T., Takimoto, K. and Okada, M. (1996) Origin and stormwater runoff of TCP (tricresyl phosphate) isomers. Water Research 30 (6), 1431-1438.

Cho, K. J., Takimoto, K. and Okada, M. (1994) Fate of tricresyl phosphate isomers in Kurose River (Japan). Water Science Technology 30 (10), 189-197.

Cleveland, D. W. (1993) Tubulin and associated proteins in *Guidebook to the Cytoskeletal and Motor Proteins* (Edited by Kreis, T. and Vale, R.), pp. 101-105, Sambrook and Toole Publication, Oxford University Press.

Cork, L. C., Sternberger, N. H., Sternberger, L. A., Casanova, M. F., Struble, R. G. and Price, D. L. (1986) Phosphorylated neurofilament antigens in neurofibrillary tangles in Alzheimer's disease. Journal of Neuropathological and Experimental Neurology 45, 56-64.

Correll, L. and Ehrich, M. (1991) A microassay method for neurotoxic esterase determinations. Fundamental and Applied Toxicology 16, 110-116.

Dahl, D. (1983) Immunohistochemical differences between neurofilaments in perikarya, dendrites and axons. Experimental Cell Research 149, 397-408.

Daughtrey, W., Biles, R., Jortner, B. and Ehrich, M. (1996) Subchronic delayed neurotoxicity evaluation of jet engine lubricants containing phosphorus additives. Fundamental and Applied Toxicology 32, 244-249.

Dautigny, A., Pham-Dinh, D., Roussel, C., Felix, J. M., Nussbaum, J. L. and Jolles, P. (1988) The large neurofilament subunit (NF-H) of the rat: cDNA cloning and *in situ* detection. Biochemical and Biophysical Research Communications 154 (3), 1099-1106.

De Bleecker, J., Lison, D., Van Den Abeele, K., Willems, J. and De Reuck, J. (1994) Acute and subacute organophosphate poisoning in the rat. NeuroToxicology 15 (2), 341-348.

De Camilli, P., Miller, P. E., Navone, F., Theurkauf, W. E. and Vallee. R. B. (1984) Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. Neuroscience 11, 817-846.

Debus, E., Flugge, G., Weber, K. and Osborn, M. (1982) A monoclonal antibody specific for the 200 K polypeptide of the neurofilament triplet. EMBO Journal 1, 41-45.

deWaegh, S. M., Lee, V. M.-Y. and Brady, S. T. (1992) Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating Schwann cells. Cell 68, 451-463.

Diaz-Nido, J., Serrano, L., Lopez-Otin, C., Vandekerckhove, J. and Avila, J. (1990) Phosphorylation of a neuronal specific beta-tubulin isotype. Journal of Biological Chemistry 265 (23), 13949-13954.

Diaz-Nido, J., Ulloa, L., Sanchez, C. and Avila, J. (1996) The role of the cytoskeleton in the morphological changes occurring during neuronal differentiation. Cell and Developmental Biology, 7, 733-739.

Dodd, J. and Jessell, T. M. (1988) Axon guidance and patterning of neuronal projections in vertebrates. Science 242 (4879), 692-699.

Doering, L. C. (1993) Probing modifications of the neuronal cytoskeleton. Molecular Neurobiology 7, 265-291.

Dosemeci, A. and Pont, H. C. (1992) Association of cyclic AMP dependent protein kinase with neurofilaments. Biochemical Journal 282, 477-481.

Drubin, D. G and Kirschner, M. W. (1986) Tau protein in living cells. Journal of Cell Biology 103 (6,2), 2739-2746.

Ehrich, M. (1995) Using neuroblastoma cell lines to address differential specificity to organophosphates. Clinical and Experimental Pharmacology and Physiology 22, 291-292.

Ehrich, M., Correll, L. and Veronesi, B. (1994) Neuropathy target esterase inhibition by organophosphorus esters in human neuroblastoma cells. NeuroToxicology 15 (2), 309-314.

Ehrich, M., Correll, L. and Veronesi, B. (1997) Acetylcholinesterase and neuropathy target esterase inhibitions in neuroblastoma cells to distinguish organophosphorus compounds causing acute and delayed neurotoxicity. Fundamental and Applied Toxiciology 38, 55-63.

Ehrich, M., Jortner, B. S. and Gross, W. B. (1986) Dose-related beneficial and adverse effects of dietary corticosterone on organophosphorus-induced delayed neuropathy in chickens. Toxicology and Applied Pharmacology 82 (2), 250-260.

El-Fawal, H. A. N. and Ehrich, M. (1993) Calpain activity in organophosphorus-induced delayed neuropathy (OPIDN) : Effects of a phenylalkyalmine calcium channel blocker. Annals of the New York Academy of Sciences 679, 325-329.

El-Fawal, H. A. N., Cornell, L., Gay, L. and Ehrich, M. (1990) Protease activity in brain, nerve, and muscle of hens given neuropathy-inducing organophosphates and a calcium channel blocker. Toxicology and Applied Pharmacology 103, 113-142.

El-Fawal, H. A. N., Jornter, B. S. and Ehrich, M. (1989) Effect of verapamil on organophosphorus-induced delayed neuropathy in hens. Toxicology and Applied Pharmacology 97, 500-511.

Eto, M., Casida, J. E. and Eto, T. (1962) Hydroxylation and cyclization reactions involved in the metabolism of tri-*o*-cresyl phosphate. Biochemical Pharmacology 11, 337-352.

European Chemical News (1995) Farmer sues sheep dip manufacturers. European Chemical News 11-17, 22.

Fedalei, A. and Nardone, R. M. (1983) An *in vitro* alternative for testing the effect of organophosphates on neurotoxic esterase activity in *Alternative Methods in Toxicology. Volume 1 : Product safety evaluation*, pp. 254-269, Mary Ann Liebert Inc., Publishers, New York.

Flaskos, J. (1995) The use of neuronal cell cultures in the study of delayed organophosphate action in 33rd International Congress on Forensic and first on Environmental Toxicology, 27-31.

Flaskos, J., Fowler, M. J., Teurtrie, C. and Hargreaves, A. J. (1999) The effects of carbaryl and trichlorphon on differentiating mouse N2a neuroblastoma cells. Toxicology Letters 110, 79-84.

Flaskos, J., McLean, W. G. and Hargreaves, A. J. (1994) The toxicity of organophosphate compounds towards cultured PC12 cells. Toxicology Letters 70, 71-76.

Flaskos, J., McLean, W. G., Fowler, M. J. and Hargreaves, A. J. (1998) Tricresyl phosphate inhibits the formation of axon-like processes and disrupts neurofilaments in cultured mouse N2a and rat PC12 cells. Neuroscience Letters 242, 101-104.

Fowler, M. J., Flaskos, J., McLean, W. G. and Hargreaves, A. J. (1997) The effects of tricresyl phosphate on axon outgrowth on mouse neuroblastoma cells. Biochemical Society Transactions 25, S574.

Fowler, M. J., Flaskos, J., McLean, W. G. and Hargreaves, A. J. (2000) Effects of neuropathic and non-neuropathic isomers of tricresyl phosphate and their microsomal activation on the production of axon-like processes by differentiating mouse N2a neuroblastoma cells. Journal of Neurochemistry (In press; April 2000).

Freudenthal, R. I., Rausch, L., Gerhart, J. M., Barth, M. L., Mackerer, C. R. and Bisinger, E. C. (1993) Subchronic neurotoxicity of oil formulations containing either tricresyl phosphate or tri-*ortho*cresyl phosphate. Journal of the American College of Toxicology 12 (4), 409-416.

Garner, J. and Lasek, R. J. (1981) Clathrin is axonally transported as part of slow component b: The microfilament complex. Journal of Cell Biology 88, 172-178.

Geisler, N. and Weber, K. (1981) Self-assembly *in vitro* of the 68,000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate-size filaments. Journal of Molecular Biology 151, 565-571.

Geisler, N., Fischer, S., Vandekerckhove, J., Van Damme, J., Plessmann, U. and Weber, W. (1985) Protein-chemical characterization of NF-H, the largest mammalian neurofilament component; intermediate filament-type sequences followed by a unique carboxy-terminal extension. EMBO Journal 4 (1), 57-63.

Gelfand, V. I. and Bershadsky, A. D. (1991) Microtubule dynamics - mechanism, regulation and function. Annual Review of Cell Biology 7, 93-116.

George, E. B., Glass, J. W. and Griffin, J. W. (1995) Axotomy-induced axonal degeneration is mediated by calcium influx through ion-specific channels. Journal of Neurochemistry 15, 6445-6452.

Ginzburg, I. (1991) Neuronal polarity: targeting of microtubule components into axons and dendrites. TIBS 16, 257-261.

Glees, P. and Janzik, H. (1965) Chemically (TCP) induced fibre degeneration in the central nervous system, with reference to clinical and neurophamacological aspects. Progress in Brain Research 14, 97-121.

Glynn, P. (2000) Neural development and neurodegeneration: two faces of Neuropathy Target Esterase. Progress in Neurobiology 61, 61-74.

Glynn, P. (1999) Neuropathy Target Esterase. Biochemical Journal 61, 61-74.

Glynn, P., Holton, J. L., Nolan, C. C., Read, D. J., Brown, L., Hubbard, A. and Cavanagh, J. B. (1998) Neuropathy target esterase: immunolocalization to neuronal cell bodies and axons. Neuroscience 83 (1), 295-302.

Gold, B. G., Price, D. L., Griffin, J. W., Rosenfeld, J., Hoffman, P. N., Sternberger, N. H. and Sternberger, L. A. (1988) Neurofilament antigens in acrylamide neuropathy. Journal of Neuropathological Experimental Neurology 47 (2), 145-157.

Gordon-Weeks, P. R. (1989) Growth at the growth cone. TINS 12 (7), 238-239.

Goslin, K., Birgbauer, E., Banker, G. and Solomon, F. (1989) The role of cytoskeleton in organizing growth cones : a microfilament-associated growth cone component depends upon microtubules for its localization. Journal of Cell Biology 109, 1621-1631.

Grafstein, B. and Forman, D. S (1980) Intracellular transport in neurons. Physiological Reviews 60, 1167-1283.

Griffith, L. and Pollard, T. D. (1978) Evidence for actin filaments-microtubule interaction mediated by microtubule-associated proteins. Journal of Cell Biology 78, 958-965.

Gurba, P. E. and Richardson, R. J. (1983) Partial characterization of neurotoxic esterase of human placenta. Toxicology Letters 15, 13-17.

Hall, M. E. (1982) Changes in synthesis of specific proteins in axotomised dorsal root ganglia. Experimental Neurology 76, 83-93.

Hargreaves, A. J. (1997) The cytoskeleton as a target in cell toxicity. Advances in Molecular and Cell Biology. JAI Press Inc. 20, 119-144.

Hargreaves, A. J., Wandosell, F. and Avila, J. (1986) Phosphorylation of tubulin enhances its interaction with membranes. Nature 323, 827-828.

Hargreaves, A. J., Yusta, B., Avila, J., Hesketh, J. E., Aranda, A. and Pascual, A. (1989) Sodium butyrate induces major morphological changes in C6 glioma cells that are correlated with increased synthesis of a spectrin-like protein. Developmental Brain Research 45, 291-295.

Hayes, A. W. (1987) Chapter 5. Organophosphorus insecticides in *Principles and methods of Toxicology.* 2nd Edition, pp. 140-141, Raven Press, New York.

Heidemann, S. R., Landers, J. M and Hamborg, M. A. (1981) Polarity orientation of axonal microtubules. Journal of Cell Biology 91, 661-665.

Heimann, R., Shelanski, M. I. And Liem, R. K. H. (1985) Microtubule-associated proteins bind specifically to the 70 kDa neurofilament protein. Journal of Biological Chemistry 260 (22), 12160-12166.

Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simpson, L. C., Moffet, B., Vandlen, R. A., Koliatsos, V. E. and Rosenthal, A. (1994) GDNF : a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266, 1062-1064.

Henschler, D., Schmuck, G., Van Aerssen, M. and Schiffmann, D. (1992) The inhibitory effect of neuropathic organophosphate esters on neurite outgrowth in cell cultures : a basis for screening for delayed neurotoxicity. Toxicology *in vitro* 6 (4), 327-335.

Heriot, K., Gambetti, P. and Lasek, R. J. (1985) Proteins transported in slow component A and component B of axonal transport are distributed differently in the transverse plane of the axon. Journal of Cell Biology 100, 1167-1172.

Hirokawa, N., Glicksman, M. A. and Willard, M. B. (1984) Organization of mammalian neurofilament polypeptides within the neuronal cytoskeleton. Journal of Cell Biology 98, 1523-1536.

Hirokawa, N., Hisanaga, S. and Shiomura, Y. (1988) MAP2 is a component of cross-bridges between microtubules and neurofilaments in the neuronal cytoskeleton - quick-freeze, deep-etch immunoelectron microscopy and reconstitution studies. Journal of Neuroscience 8 (8), 2769-2779.

Hodge, H. C. and Sterner, J. H. (1943) The skin absorption of triorthocresyl phosphate as shown by radioactive phosphorus. International bibliography, information, documentation 79, 225-234.

Hoffman, B. J., Scheffel, U., Lever, R. A., Karpa, M. D. and Hartig, P. R. (1987) N1-methyl-2-125I-lysergic acid diethylamide, a preferred ligand for *in vitro* and *in vivo* characterisation of serotonin receptors. Journal of Neurochemistry 48 (1), 115-124.

Hoffman, P. N. and Lasek, R. J. (1975) The slow component of axonal transport: Identification of major structural polypeptides of the axon and their generality among mammalian neurons. Journal of Cell Biology 66, 351-366.

Howland, R. D. and Alli, P. (1986) Altered phosphorylation of rat neuronal cytoskeletal proteins in acrylamide induced neuropathy. Brain Research 363 (2), 3333-3339.

Huff, R. A. and Abou-Donia, M. B. (1995) *In vitro* effect of chlorpyrifos oxon on muscarinic receptors and adenylate cyclase. NeuroToxicology 16 (2), 281-290.

Itoh, H., Kishida, H., Tadokoro, M. and Oikawa, K. (1984) Studies on the delayed neurotoxicity of OPs compounds-II. Journal of Toxicological Sciences 9, 37-50.

Itoh, H., Tadokoro, M. and Oikawa, K. (1981) Studies on the delayed neurotoxicity of organophosphorus compounds-I. Journal of Toxicological Sciences 6, 297-300.

Jacobson, M. (1995) Axonal development in *Developmental Neurobiology.* 3rd Edition, pp. 163-215, Plenum Press, New York.

Jamal, G. A. (1995) Long term neurotoxic effects of organophosphate compounds. Adverse Drug Reaction Toxicological Review 14 (2), 85-99.

Jensen, K. F., Lapadula, D. M., Knoth Anderson, J., Haykal-Coates, N. and Abou-Donia, M. B. (1992) Anomalous phosphorylated neurofilament aggregations in central and peripheral axons of hens treated with tri-*ortho*-cresyl phosphate (T*O*CP). Journal of Neuroscience Research 33, 455-460.

Johnson, G. V. W. and Jope, R. S. (1988) Phosphorylation of rat brain cytoskeletal proteins is increased after orally administered aluminum. Brain Research 456 (1), 95-103.

Johnson, M. K. (1990) Organophosphates and delayed neuropathy - Is NTE alive and well ? Toxicology and Applied Pharmacology 102, 385-399.

Johnson, M. K. (1974) The primary biochemical lesion leading to the delayed neurotoxic effects of some organophosphorus esters. Journal of Neurochemistry 23, 785-789.

Johnson, M. K. (1969) The delayed neurotoxic effect of some organophosphorus compounds. Identification of the phosphorylation site as an esterase. Biochemical Journal 114, 711-717.

Johnson, M. K. (1975a) The delayed neuropathy caused by some organophosphorus esters : mechanism and challenge. Critical Reviews in Toxicology 3, 289-316.

Johnson, M. K. (1975b) Organophosphate esters causing delayed neurotoxic effects. Mechanism of action and structure / activity studies. Archives of Toxicology 34, 259-288.

Johnson, M. K. (1982) The target for initiation of delayed neurotoxicity by organophosphorus esters: Biochemical studies and toxicological applications *in Reviews in Biochemical Toxicology* (Edited by Hodgson, E., Bend, J. R. and Philpot,, R. M.) 4, 141-212.

Johnson, M. K. and Lotti, M. (1980) Delayed neurotoxicity caused by chronic feeding of organophosphates requires a high-point of inhibition of neurotoxic esterase. Toxicology Letters 5, 99-102.

Johnson, M. K. and Read, D. J. (1987) The influence of chirality on the delayed neuropathic potential of some organophosphorus esters : neuropathic and prophylactic effects of stereoisomeric esters of ethyl phenylphosphonic acid (EPN oxon and EPN) correlate with quantities of aged and unaged neuropathy target esterase *in vivo*. Toxicology and Applied Pharmacology 90, 103-115.

Julien, J. P and Mushynski, W. E. (1982) Multiple phosphorylation sites in mammalian neurofilament polypeptides. Journal of Biological Chemistry 257, 10467-10470.

Kaloyanova, F. P. and El Batawi, M. A. (1991) Chapter 2. Organophosphorus Compounds in *Human toxicology of pesticides*, pp. 3-33, CRC Press, USA.

Kater, S. B., Mattson, M. P., Cohan, C. and Connor, J. (1988) Calcium regulation of the neuronal growth cone. Trends in Neurosciences 11 (7), 315-321.

Keilbaugh, S. A., Prusoff, W. H. and Simpson, M. V. (1991) The PC12 as a model for studies of the mechanism of induction of peripheral neuropathy by anti-HIV-1 dideoxynucleoside analogs. Biochemical Pharmacology 42 (1), R5-R8.

Kenyon, G. L. and Garcia, G. A. (1987) Design of kinase inhibitors. Medical Research Reviews 7 (4), 389-416.

Kirschner, M. W. and Mitchison, T. (1986) Microtubule dynamics. Nature 324, 621.

Kiss, Z. and Fazekas, T. (1983) Organophosphate poisoning and complete heart block. Journal of the Royal Society of Medicine 76, 85.

Koelle, G. B. (1994) Pharmacology of organophosphates. Journal of Applied Toxicology 14 (2), 105-109.

Kosik, K. S., Orecchio, L. D., Bakalis, S., Duffy, L. and Neve, R. L. (1988) Partial sequence of MAP2 in the region of a shared epitope with Alzheimer neurofibrillary tangles. Journal of Neurochemistry 51 (2), 587-598.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680-685.

Lapadula, D. M., Patton, S. E., Campbell, G. M. and Abou-Donia, M. B. (1985) Characterization of delayed neurotoxicity in the mouse following chronic oral administration of tri-*ortho*-cresyl phosphate. Toxicology and Applied Pharmacology 79, 83-90.

Lapadula, E. S., Lapadula, D. M. and Abou-Donia, M. B. (1991) Persistent alternations of calmodulin kinase II activity in chickens after an oral dose of tri-*ortho*-cresyl phosphate. Biochemical Pharmacology 42 (1), 171-180.

Lapadula, E. S., Lapadula, D. M. and Abou-Donia, M. B. (1992) Biochemical changes in sciatic nerve of hens treated with tri-*o*micron-cresyl phosphate - increased

phosphorylation of cytoskeletal proteins. Neurochemistry International 20 (2), 247-255.

Lapchak, P. A. (1996) Therapeutic potentials for glial cell line-derived neurotrophic factor (GDNF) based upon pharmacological activities in the CNS. Reviews in the Neuroscience 7, 165-176.

Lapchak, P. A., Gash, D. M., Jiao, S., Miller, P. J. and Hilt, D. (1997a) Glial cell line-derived neurotrophic factor: a novel therapeutic approach to treat motor dysfunction in Parkinson's disease. Experimental Neurobiology 144, 29-34.

Lapchak, P. A., Hilt, D. and Araujo, D. M. (1997b) Glial cell line-derived neurotrophic factor: drug development. Drug News and Perspectives 10 (6), 347-353.

Lasek, R. J (1980) The dynamics of neuronal structures. Trends in Neuroscience 3, 87-91.

Lasek, R. J. (1982) Translocation of the neuronal cytoskeleton and axonal locomotion. Philosophical Transactions of the Royal Society of London Series b Biological Sciences Journal 299, 313-327.

Lasek, R. J., Oblinger, M. M. and Drake, P. F. (1983) Molecular biology of neuronal geometry : expression of neurofilament genes influences axonal diameter. Cold Spring Harbor Symposia on Quantitative Biology 48, 731-744.

Latendresse, J. R., Brooks, C. L. and Capen, C. C. (1995) Toxic effects of butylated triphenyl phosphate-based hydraulic fluid and tricresyl phosphate in female F344 rats. Veterinary Pathology 32, 394-402.

Lautermilch, N. J. and Spitzer, N. C. (2000) Regulation of calcineurin by growth cone calcium waves controls neurite extension. Journal of Neuroscience 20 (1), 315-325.

LeDizet, M. and Piperno, G. (1987) Identification of an acetylation site of *Chlamydomonas* α-tubulin. Proceedings of the National Academy of Science of the United States of America 84, 5720-5724.

Lee, V. M-Y., Otvos, L. Jr., Carden, M. J., Hollosi, M., Dietzschold, B. and Lazzarini, R. A. (1988) Identification of the major phosphorylation site in mammalian neurofilaments. Proceedings of the National Academy of Science of the United States of America 85, 1998-2002.

Leterrier, J-F., Liem, R. K. H. and Shelanski, M. L. (1982) Interactions between neurofilaments and microtubule-associated proteins: a possible mechanism for intraorganellar bridging. Journal of Cell Biology 95, 982-986.

Lewis, S. A. and Cowan, N. J. (1986) Anomalous placement of introns in a member of the intermediate filament multigene family : an evolutionary conundrum. Molecular and Cellular Biology 6 (5), 1529-1534.

Lewis, S. E. and Nixon, R. A. (1988) Multiple phosphorylated variants of the high molecular weight subunit of neurofilaments in axons of retinal cell neurons: characterization and evidence for their differential association with stationary and moving neurofilaments. Journal of Cell Biology 107 (6), 2689-2701.

Liem, R. K. and Hutchinson, S. B. (1982) Purification of individual components of the neurofilament triplet: filament assembly from the 70,000 dalton subunit. Biochemistry 21, 3221-3226.

Liepins, R. and Pearce, E. (1976) Chemistry and toxicity of flame retardant of plastics. Environmental Health Perspectives 17, 55-63.

Lin, L-F. H., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993) GDNF : a glial cell line-derived neurotrophic factor for mid-brain dopaminergic neurons. Science 260, 1130-1132.

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D. and Darnell, J. (2000) *Molecular cell biology* (Fourth Edition), W. H. Freeman and Company.

Lotti, M. (1992) The pathogenesis of organophosphate polyneuropathy. Critical Reviews in Toxicology 21 (6), 465-487.

Lotti, M. and Moretto, A. (1993) The search for the physiological functions of NTE : is NTE a receptor? Chemical-Biological Interactions 87, 407-416.

Lotti, M., Wei, E. T., Spear, R. C. and Becker, C. E. (1985) Neurotoxic esterase in Rooster testis. Toxicology and Applied Pharmacology 77, 175-180.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

Ma, T. and Chamber, J. E. (1995) A kinetic analysis of hepatic microsomal activation of parathion and chlorpyrifos in control and Phenobarbital-treated rats. Journal of Biochemical Toxicology 10 (2), 63-68.

Maccioni, R. B., Serrano, L. and Avila, J. (1985) Structural and functional domains of tubulin. BioEssays 2 (4), 165-169.

Maizlish, N., Schenker, M., Weisskopf, C., Sieber, J. and Samuels, S. (1987) A behavioural evaluation of pest control workers with short-term, low level exposure to the organophosphate diazinon. American Journal of Indian Medicine 12, 153-172.

Mandell, J. W. and Banker, G. A. (1995) The microtubule cytoskeleton and the development of neuronal polarity. Neurobiology of Aging 16 (3), 229-238.

Margolis, R. and Wilson, L. (1978) Opposite end assembly and disassembly of microtubules at steady state *in vitro*. Cell 13, 1-8.

Matheson, C. R., Carnahan, J., Urich, J. L., Bocangel, T. J. and Qiao Yan, Z. (1997) Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor for sensory neurons: comparison with the effects of the neurotrophins. Journal of Neurobiology 32 (1), 22-32.

Matus, A. (1988) Neurofilament protein phosphorylation - where, when and why. TINS 11 (7), 291-292.

Matus, A., Bernhardt, R. and Hugh-Jones, T. (1981) High molecular weight microtubules-associated proteins are preferentially associated with dendritic microtubules in brain. Proceedings of the National Academy of Science of the United States of America 78, 3010-3014.

McLean, W. G. (1988) Pressure-induced inhibition of fast axonal transport of proteins in the rabbit vagus nerve in galactose neuropathy: prevention by an aldose reductase inhibitor. Diabetologia 31, 443-448.

McLean, W. G., Holme, A. D., Janneh, O., Southgate, A., Howard, C. V. and Reed, M. G. (1998) The effect of benomyl on neurite outgrowth in mouse NB2A and human SH-SY-5Y neuroblastoma cells in vitro. NeuroToxicology 19 (4-5), 629-639.

McLean, W. G., Kanje, M. and Remgard, P. (1993) An *in vitro* system for the study of slow axonal transport. Brain Research 613, 295-299.

McLean, W. G., Roberts, R. E. and Mullins, F. H. (1994) Post-translational modifications of microtubule- and growth-associated proteins in nerve regeneration and neuropathy. Biochemical Society Transactions 23, 76-81.

Mearns, J., Dunn, J. and Lees-Haley, P. R. (1994) Psychological effects of organophosphate pesticides: a review and call for research by psychologists. Journal of Clinical Psychology 50, 286-294.

Meiri, K. F., Saffell, J. L., Walsh, F. S. and Doherty, P. (1998) Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. Journal of Neuroscience 18 (24), 10429-10437.

Meiri, K. F., Johnson, M. I. and Willard, M. B. (1988) Distribution and phosphorylation of growth associated protein GAP-43 in regenerating sympathetic neurons in culture. Journal of Neuroscience 8, 2571-2581.

Meiri, K. F., Pfenninger, K. H. and Willard, M. B. (1986) Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. Proceedings of the National Academy of Science of the United States of America 83, 3537-3541.

Merril, C. R., Goldman, D., Sedman, S. A. and Ebert, M. H. (1981) Ultra sensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211, 1437-1438.

Mitchison, T. and Kirschner, M. (1984) Dynamic instability of microtubule growth. Nature 312, 237-242.

Moretto, A. and Lotti, M. (1989) Effects of gangliosides on neuropathy target esterase. Toxicologist 9, 74.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival : application to proliferation and cytotoxicity assay. Journal of Immunological Methods 65, 55-63.

Mullins, F. H., Hargreaves, A. J. and McLean, W. G. (1991) Tubulin : tyrosine ligase activity in regenerating peripheral nerve. Biochemical Society Transactions 19, 1143-1146.

Muma, N. A. and Hoffman, P. N. (1993) Neurofilaments are intrinsic determinants of axonal caliber. Micron 24 (6), 677-683.

Murphy, D. B. and Borisy, G. G. (1975) Association of high molecular weight proteins with microtubules and their role in microtubule assembly *in vitro*. Proceedings of the National Academy of Science of the United States of America 72 (7), 2696-2700.

Murphy, D. B., Vallee, R. B. and Borisy, G. G. (1977) Identity and polymerization stimulatory activity of the non-tubulin proteins associated with microtubules. Biochemistry 16 (12), 2598-2605.

Myers, M. W., Lazzarini, R. A., Lee, V. M. Y., Schlaepfer, W. W. and Nelson, D. L. (1987) The human mid-size neurofilament subunit: a repeated protein sequence and the relationship of its gene to the intermediate filament gene family. EMBO Journal 6 (6), 1617-1626.

Nanda, S. (1995) Biochemical, neuropathological and behavioural studies in hens induced by acute exposure to tri-*ortho*cresyl phosphate. International Journal of Neuroscience 82, 243-254.

Neuhoff, V., Arold, N., Taube, D. and Ehrhardt, W. (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9, 255-262.

Nixon, R. A. and Lewis, S. E. (1986) Differential turnover of phosphate groups on neurofilament subunits in mammalian neurons *in vivo*. Journal of Biological Chemistry 261 (35), 16298-16301.

Nixon, R. A. and Lewis, S. E. (1987) Phosphorylation and dephosphorylation of neurofilament proteins in retinal ganglion cell neurons *in vivo*. Advances in Experimental Medical Biology 221, 167-186.

Nixon, R. A. and Sihag, R. K. (1991) Neurofilament phosphorylation : a new look at regulation and function. TINS 14 (11), 501-506.

Nixon, R. A., Brown, B. A. and Marotta, C. A. (1982) Posttranslational modification of a neurofilament protein during axoplasmic transport : Implications for regional specialization of CNS axons. Journal of Cell Biology 94, 150-158.

Nixon, R. A., Fischer, I. and Lewis, S. E. (1990) Synthesis, axonal transport, and turnover of the high molecular weight microtubule-associated protein MAP1A in mouse retinal ganglion cells : Tubulin and MAP1A display distinct transport kinetics. Journal of Cell Biology 110, 437-448.

Nixon, R. A., Lewis, S. E. and Marotta, C. A. (1987) Post-translational modification of neurofilament proteins by phosphate during axoplasmic transport in retinal ganglion cell neurons. Journal of Neuroscience 7 (4), 1145-1158.

Nomeir, A. A. and Abou-Donia, M. B. (1986) Studies on the metabolism of the neurotoxic tri-*o*-cresyl phosphate. Distribution, excretion and metabolism in male cats after a single, dermal application. Toxicology 38, 15-33.

Nostrandt, A. C. and Ehrich, M. (1992) Development of a model cell culture system in which to study early effects of neuropathy-inducing organophosphorus esters. Toxicology Letters 60, 107-114.

Okabe, S. and Hirokawa, N. (1988) Microtubule dynamics in nerve cells : analysis using microinjection of biotinylated tubulin in to PC12 cells. Journal of Cell Biology 107, 651-664.

Okabe, S. and Hirokawa, N. (1990) Turnover of fluorescently labelled tubulin and actin in the axon. Nature 343, 479-482.

Olmsted, J. B. (1986) Microtubule-associated proteins. Annual Review of Cell Biology 2, 421-457.

Oppenheim, R. W., Houenou, L. J., Johnson, J. E., Lin, l-F. H., Li, L., Lo, A. C., Newsome, A. L., Prevette, D. M. and Wang, S. (1995) Developing motor neurons rescued from programmed and axotomy cell death by GDNF. Nature 373, 344-346.

Osborn, M. and Weber, K. (1986) Intermediate filament proteins : a multigene family distinguishing major cell lineage. TIBS 11, 469-472.

Patton, S. E., Lapadula, D. M. and Abou-Donia, M. A. (1986) Relationship of tri-*o*-cresyl phosphate-induced delayed neurotoxicity to enhancement if *in vitro* phosphorylation of hen brain and spinal cord proteins. Journal of Pharmacology and Experimental Therapeutics 239 (2), 597- 605.

Paves, H., Neuman, T., Metsis, M. and Saarma, M. (1988) Nerve growth factor induces rapid redistribution of F-actin in PC12 cells. Federation of European Biochemical Societies (FEBS) Letters 235 (1-2), 141-143.

Pekiner, C. and McLean, W. G. (1991) Neurofilament protein phosphorylation in spinal cord of experimentally diabetic rats. Journal of Neurochemistry 56 (4), 1362-1367.

Pekiners, C., Dent, E. W., Roberts, R. E., Meiri, K. F. and McLean, W. G. (1996) Altered GAP-43 immunoreactivity in regenerating sciatic nerve of diabetic rats. Diabetes 45, 199-204.

Peters, R. J., Osinski, M. A., Hongo, J. A., Bennett, G. L., Okragly, A. J., HaakFrendscho, M. and Epstein, M. L. (1998) GDNF is abundant in adult rat gut. Journal of the autonomic nervous system 70 (1-2), 115-122.

Pope, C. N., diLorenzo, K. and Ehrich, M. (1995) Possible involvement of a neurotrophic factor during the early stages of organophosphate-induced delayed neurotoxicity. Toxicology Letters 75, 111-117.

Pope, C. N., Tanaka, D. and Padilla, S. (1993) The role of neurotoxic esterase (NTE) in the prevention and potentiation of organophosphorus-induced delayed neurotoxicity (OPIDN). Chemical-Biological Interactions 87, 395-406.

Prentice, D. E. and Roberts, N. L. (1984) Acute delayed neurotoxicity in hens dosed with tri-*ortho*-cresyl phosphate (T*O*CP): Correlation between clinical ataxia and neuropathological findings in *Delayed Neurotoxicity* (Edited by J. M. Cranmer and E. J. Hixson), pp. 139-144, Intox Press, AK.

Prineas, J. (1969) The pathogenesis of dying-back polyneuropathies Part I. An ultrastructural study of experimental tri-*ortho*-cresyl phosphate intoxication in the cat. Journal of Neuropathological and Experimental Neurology 28, 571-597.

Rengstorff, R. H. (1985) Accidental exposure to sarin: vision effects. Archives of Toxicology 56, 201-203.

Richardson, R. J. (1992) Chapter 16. Interaction of organophosphorus compounds with neurotoxic esterase in *Organophosphates. Chemistry, Fate and Effects* (Edited Chambers, J. E and Levi, P. E.) pp. 299-320, Academic Press, New York.

Richardson, R. J., Davis, C. S. and Johnson, M. K. (1979) Subcellular distribution of marker enzymes and of neurotoxic esterase in adult hen brain. Journal of Neurochemistry 32, 607-615.

Robson, R. M. (1989) Intermediate filaments. Current Opinion in Cell Biology 1, 36-43.

Roder, H. M. and Ingram, V. M. (1991) Two novel kinases phosphorylate tau and the KSP site of heavy neurofilament subunits in high stoichiometric ratios. Journal of Neuroscience 11 (11), 3325-3343.

Roth, A., Zellinger, M. D., Arad, M. and Atsmon, J. (1993) Organophosphates and the Heart. Chest 103, 576-582.

Sachana, M. (1999) The use of cultured neuronal cells to study the neurodegenerative effects of OPs. M.Sc Thesis.

Sattilaro, R. F., Dentler, W. L. and LeCluyse, B. L. (1981) Microtubule associated proteins (MAPs) and the organization of actin filaments *in vitro*. Journal of Cell Biology 90, 467-473.

Schmuck, G. and Ahr, H. J. (1997) Improved *in vitro* method for screening organophosphate-induced delayed polyneuropathy. Toxicology *in vitro* 11, 263-270.

Senanayake, N. and Karalliedde, L. (1987) Neurotoxic effects of organophosphorus insecticides. An intermediate syndrome. The New England Journal of Medicine 316, 761-763.

Serrano, L., Avila, J. and Maccioni, R. B. (1984a) Controlled proteolysis of tubulin by subtilism localization of the site for MAP2 interaction. Biochemistry 23 (20), 4675-4681.

Serrano, L., De La Torre, J., Maccioni, R. B. and Avila, J. (1984b) Involvement of the carboxyl terminal domain of tubulin in the regulation of microtubule assembly. Proceedings of the National Academy of Science of the United States of America 81 (19), 5989-5993.

Serrano, L., Diaz-Nido, J., Wandosell, F. and Avila, J. (1987) Tubulin phosphorylation by casein kinase II is similar to that found *in vivo*. Journal of Cell Biology 105 (4), 1731-1739.

Serrano, L., Valencia, A., Caballero, R. and Avila, J. (1986) Localization of the high affinity calcium binding site on tubulin molecule. Journal of Biological Chemistry 261 (15), 7076-7081.

Sharma, R. P. and Watanabe, P. G. (1974) Time related disposition of tri*ortho*tolyl phosphate (T*O*TP) and metabolites in chickens. Pharmacology Research Communications 6, 475-484.

Sharp, G. A., Shaw, G. and Weber, K. (1982) Immunoelectron microscopical localization of the three neurofilament triplet proteins along neurofilaments of cultured dorsal root ganglion neurons. Experimental Cell Research 137, 403-413.

Shea, T. B. (1995) Differential synthesis and cytoskeletal deposition of neurofilament subunits before and during axonal outgrowth in NB2a/dl cells: Evidence that segregation of phosphorylated subunits within the axonal cytoskeleton involves selective deposition. Journal of Neuroscience Research 40, 225-232.

Shea, T. B., Beermann, M. L. and Nixon, R. A. (1991) Multiple proteases regulate neurite outgrowth in NB2a/dl neuroblastoma cells. Journal of Neurochemistry 56, 842-850.

Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Microtubule assembly in the absence of added nucleotides. Proceedings of the National Academy of Science of the United States of America 70 (3), 765-768.

Sidell, F. R. (1994) Clinical effects of Organophosphorus cholinesterase inhibitors. Journal of Applied Toxicology 14 (2), 111-113.

Sihag, R. K. and Nixon, R. A. (1990) Phosphorylation of the amino-terminal head domain of the middle molecular mass 145 kDa subunit of neurofilaments. Journal of Biological Chemistry 265 (7), 4166-4171.

Skene, P. J. H. (1989) Axonal growth-associated proteins. Annual Review of Neuroscience 12, 127-156.

Sladowski, D., Steer, S. J., Clothier, R. H. and Balls, M. (1993) An improved MTT assay. Journal of Immunological Methods 157, 203-207.

Slichenmyer, W. J. and Von Hoff, D. D. (1990) New natural products in cancer chemotherapy. Journal of Clinical Pharmacology 30 (9), 770-778.

Sloboda, R. D., Dentler, W. L. and Rosenbaum, J. L. (1976) Microtubule-associated proteins and the stimulation of tubulin assembly *in vitro*. Biochemistry 15 (20), 4497-4505.

Smith, H. V. and Spalding, J. M. K. (1959) Outbreak of paralysis in Morocco due to *ortho*-cresyl phosphate poisoning. The Lancet 277, 1019-1021.

Smith, M. I., Elvove, E., Valer, P. J., Frazier, W. H. and Mallory, G. E. (1930) Pharmacological and chemical studies of the cause of so-called ginger paralysis. United States Public Health Reports 45, 1703-1716.

Somkuti, S. G. and Abou-Donia, M. B. (1990) Disposition, elimination, and metabolism of tri-*o*-cresyl phosphate following daily oral administration in Fischer 344 male rats. Archives of Toxicology 64, 572-579.

Somkuti, S. G., Lapadula, D. M., Chapin, R. E. and Abou-Donia, M. B. (1991) Light and electron microscopic evidence of tri-*ortho*-cresyl phosphate (T*O*CP)-mediated testicular toxicity in Fischer 344 rats. Toxicology and Applied Pharmacology 107, 35-46.

Somkuti, S. G., Lapadula, D. M., Chapin, R. E., Lamb IV, J. C. and Abou-Donia, M. B. (1987a) Reproductive tract lesions resulting from subchronic administration (63 days) of tri-*ortho*-cresyl phosphate in male rats. Toxicology and Applied Pharmacology 89, 49-63.

Somkuti, S. G., Lapadula, D. M., Chapin, R. E., Lamb IV, J. C. and Abou-Donia, M. B. (1987b) Time course of the tri-*ortho*-cresyl phosphate induced testicular lesion in F-344 rats: enzymatic, hormonal and sperm parameter studies. Toxicology and Applied Pharmacology 89, 64-72.

Soussan, L., Barzilai, A. and Michaelson, D. M. (1994) Distinctly phosphorylated neurofilaments in different classes of neurons. Journal of Neurochemistry 62, 770-776.

Sprague, G. L. and Castles, T. R. (1985) Estimation of the delayed neurotoxic potential and potency for a series of triaryl phosphates using an *in vitro* test with metabolic activation. NeuroToxicology 6 (1), 79-86.

Stephens, R., Spurgeon, A. and Berry, H. (1996) Organophosphates: The relationship between chronic and acute exposure effects. NeuroToxicology and Teratology 18 (4), 449-453.

Stephens, R., Spurgeon, A., Calvert, I. A., Beach, J., Levy, L. S., Berry, H. and Harrington, J. M. (1995) Neuropsychological effects of long-term exposure to organophosphates in sheep dip. The Lancet 345, 1135-1139.

Stokes, L., Stark, A., Marshall, E. and Narang, A. (1995) Neurotoxicity among pesticide applicators exposed to organophosphates. Occupational and Environmental Medicine 52, 648-653.

Suter-Crazzolara, C. and Unsicker, K. (1996) GDNF mRNA levels are induced by FGF-2 in rat C6 glioblastoma cells. Molecular Brain Research 14, 175-182.

Suwita, E., Lapadula, D. M. and Abou-Donia, M. B. (1986) Calcium and calmodulin-enhanced *in vitro* phosphorylation of hen brain cold-stable microtubules and spinal cord neurofilament triplet proteins after a single does of tri-*ortho*-cresyl phosphate. Proceedings of the National Academy of Sciences of the United States of America 83, 6174-6178.

Taylor, J. D. and Buttar, H. S. (1967) Evidence for the presence of 2-(*o*-cresyl)-4H-1:3:2-benodioxaphosphorane-2-one in cat intestine following tri*ortho*cresyl phosphate administration. Toxicology and Applied Pharmacology 11, 529-327.

Terenghi, C. (1999) Peripheral nerve regeneration and neurotrophic factors. Journal of anatomy 194 (1), 1-14.

Tilney, L. G., Bryan, J., Bush, D. J., Fujiwara, K. and Mooseker, M. (1973) Microtubules: Evidence for 13 protofilaments. Journal of Cell Biology 59, 267-275.

Tokui, T., Yamauchi, T., Yano, T., Knish, Y., Kusagawa, M., Yatani, R. and Inagaki, M. (1990) Calcium-calmodulin-dependent protein kinase II phosphorylates various types of non-epithelial intermediate filament proteins. Biochemical and Biophysical Research Communications 169 (3), 896-904.

Tomac, A., Lindqvist, E., Lin, L-F. H., Ogren, S. O., Young, D., Hoffer, B. J. and Olson, L. (1995) Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. Nature 373, 335-339.

Toru-Delbauffe, D. and Pierre, M. (1983) A rat brain protein kinases phosphorylating specifically neurofilaments. Federation of European Biochemical Societies (FEBS Letters) 162 (2), 230-234.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets : Procedure and some applications. Proceeding of the National Academy of Science of the United States of America 76, 4350-4354.

Travers, P. R. (1962) The results of intoxications with *ortho*-cresyl phosphate absorbed from contaminated cooking oil, as seen in 4,029 patient in Morocco. Proceedings of the Royal Society of Medicine 55, 57-60.

Trupp, M., Ryden, M., Jornvall, H., Funakoshi, H., Timmusk, T., Aarenas, E. and Ibanez, C. F. (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. Journal of cell biology 130 (1), 137-148.

Tytell, M., Brady, S. T. and Lasek, R. J. (1984) Axonal transport of a subclass of tau proteins : Evidence for the regional differentiation of microtubules in neurons. Proceedings of the National Academy of Science of the United States of America 81, 1570-1574.

Vallee, R. B. (1980) Structure and phosphorylation of microtubule-associated protein 2 (MAP 2). Proceedings of the National Academy of Science of the United States of America 77 (6), 3206-3210.

Veronesi, B., Ehrich, M., Blusztajn, J. K., Oortgiesen, M. and Durham, H. (1997) Cell culture models of interspecies selectivity to organophosphorus insecticides. NeuroToxicolgy 18 (1), 283-298.

Wandosell, F., Serrano, L. and Avila, J. (1987) Phosphorylation of α-tubulin carboxyl-terminal tyrosine prevents its incorporation in to microtubules. Journal of Biological Chemistry 262 (17), 8268-8273.

Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P. and McPhail, A. T. (1971) Plant antitumor agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. Journal of American Chemical Society 93, 2325-2327.

Weingarten, M. D., Lockwood, A. H., Hwo, S-Y. and Kirschner, M. W. (1975) A protein factor essential for microtubule assembly. Proceedings of the National Academy of Science of the United States of America 72 (5), 1858-1862.

Wible, B. A., Smith, K. E. and Angelides, K. J. (1989) Resolution and purification of a neurofilament-specific kinase. Proceedings of the National Academy of Science of the United States of America 86, 720-724.

Willard, M. B. and Hulebak, K. L. (1977) The intra-axonal transport of polypeptide H: evidence for a fifth (very slow) group of transported proteins in the retinal ganglion cells of the rabbit. Brain Research 136, 289-306.

Willard, M. B. and Simon, C. (1981) Antibody decoration of neurofilaments. Journal of Cell Biology 89, 198-205.

Williams, D. G. (1983) Intramolecular group transfer is a characteristic of neurotoxic esterase and is independent of the tissue source of the enzyme. Journal of Biochemistry 209, 817-829.

Woolf, A. D. (1995) Ginger Jake and the Blues : A tragic song of poisoning. Veterinary and Human Toxicology 37 (3), 252-254.

Yan, Q., Matheson, C. and Lopez, O. T. (1995) In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature 373, 341-344.

Yu, W., Centonze, V. E., Ahmad, F. J., *et al.* (1993) Microtubule nucleation and release from the neuronal centrosome. Journal of Cell Biology 122, 349-359.

Zech, R. and Chemnitius, J. M. (1987) Neurotoxicant sensitive esterase. Enzymology and pathophysology of organophosphorus ester-induced delayed neuropathy. Progress in Neurobiology 29, 193-218.

Appendix I

Raw data from image package (as described by McLean *et al.*, 1998)

As previously described in chapters 3 and 4, mouse N2a cells were differentiated in the absence and presence of OPs (TCP, T*O*CP and T*P*CP) and then fixed and stained with Coomassie blue (2.2.1.6 and 2.2.1.7). The package determined average neurite length and average number of extensions per cell ± standard deviation from an average of cells cultured on 4 separate occasions. Statistical analysis was determined by Mann-Whitney U test (where $p < 0.05$) and is indicated in parentheses.

TCP (µg/ml)	**Time (hours)**	**Average neurite length / cell (arbitrary values)**	**Average neurite extensions / cell (arbitrary values)**
0	24	204.6 ± 85.0	6.11 ± 1.4
1.0		114.5 ± 49.8 ($p < 0.013$)	5.00 ± 1.5
0	48	56.3 ± 22.0	2.09 ± 1.0
1.0		27.9 ± 12.6 ($p < 0.003$)	1.08 ± 1.2

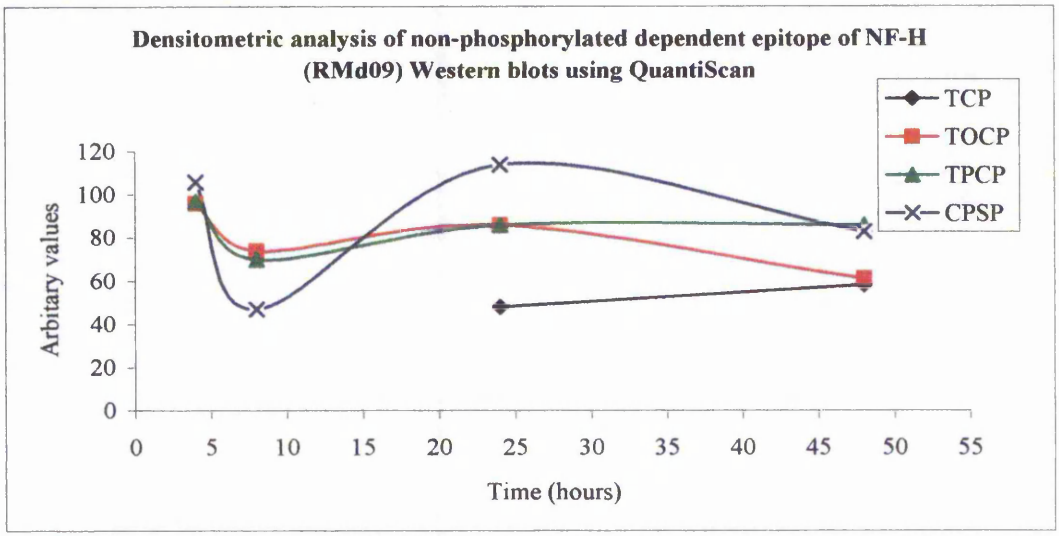
T*O*CP (µg/ml)	**Time (hours)**	**Average neurite length / cell (arbitrary values)**	**Average neurite extensions / cell (arbitrary values)**
0	24	107.6 ± 37.6	4.00 ± 0.9
1.0		78.3 ± 3.28 ($p < 0.06$)	1.67 ± 1.2
0	48	75.0 ± 45.5	1.57 ± 0.5
1.0		39.6 ± 33.3 ($p < 0.03$)	0.89 ± 0.6

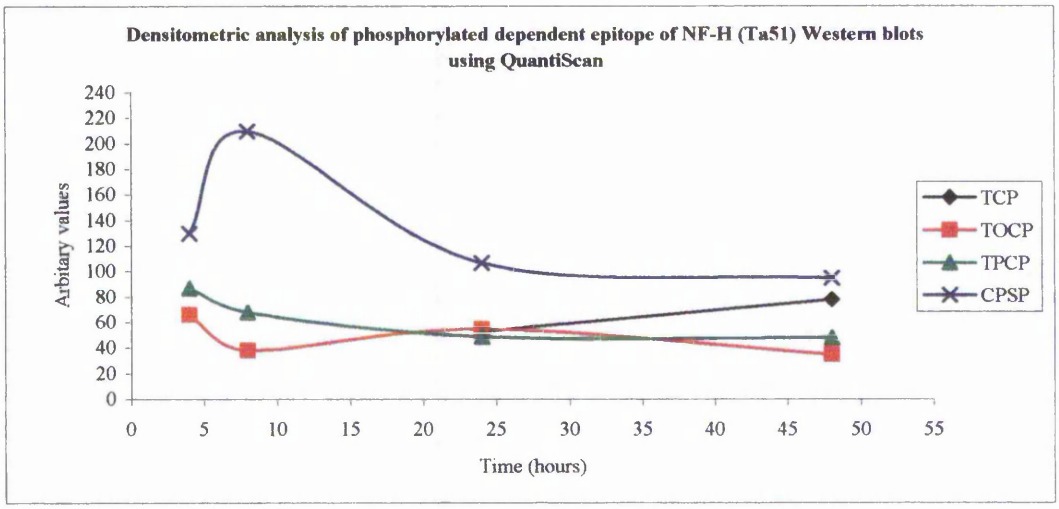
TPCP (μg/ml)	**Time (hours)**	**Average neurite length / cell (arbitrary values)**	**Average neurite extensions / cell (arbitrary values)**
0	24	111.7 ± 48.6	1.50 ± 0.5
1.0		61.3 ± 17.7 ($p < 0.005$)	1.33 ± 0.2
0	48	82.2 ± 19.6	1.00 ± 0.5
1.0		76.9 ± 26.6 ($p = 0.5$)	1.20 ± 0.4

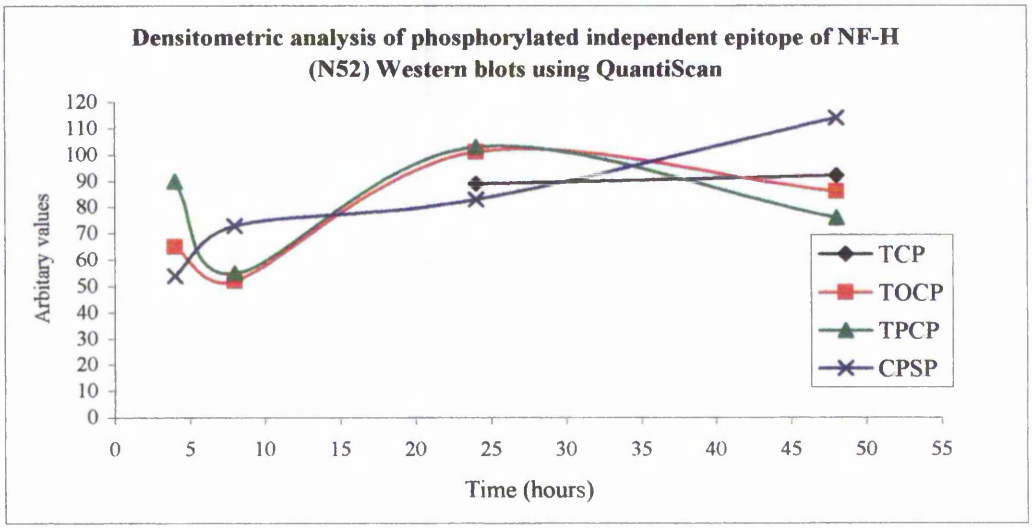
Appendix II

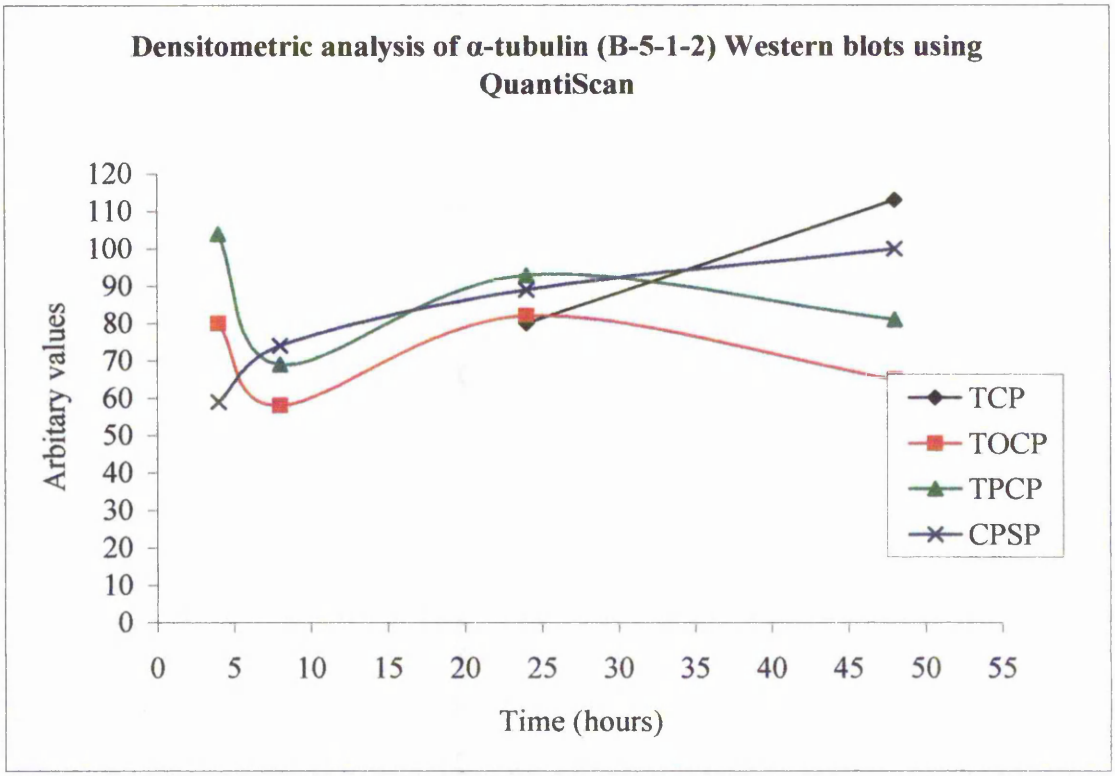
Graphical representation of QuantiScan data

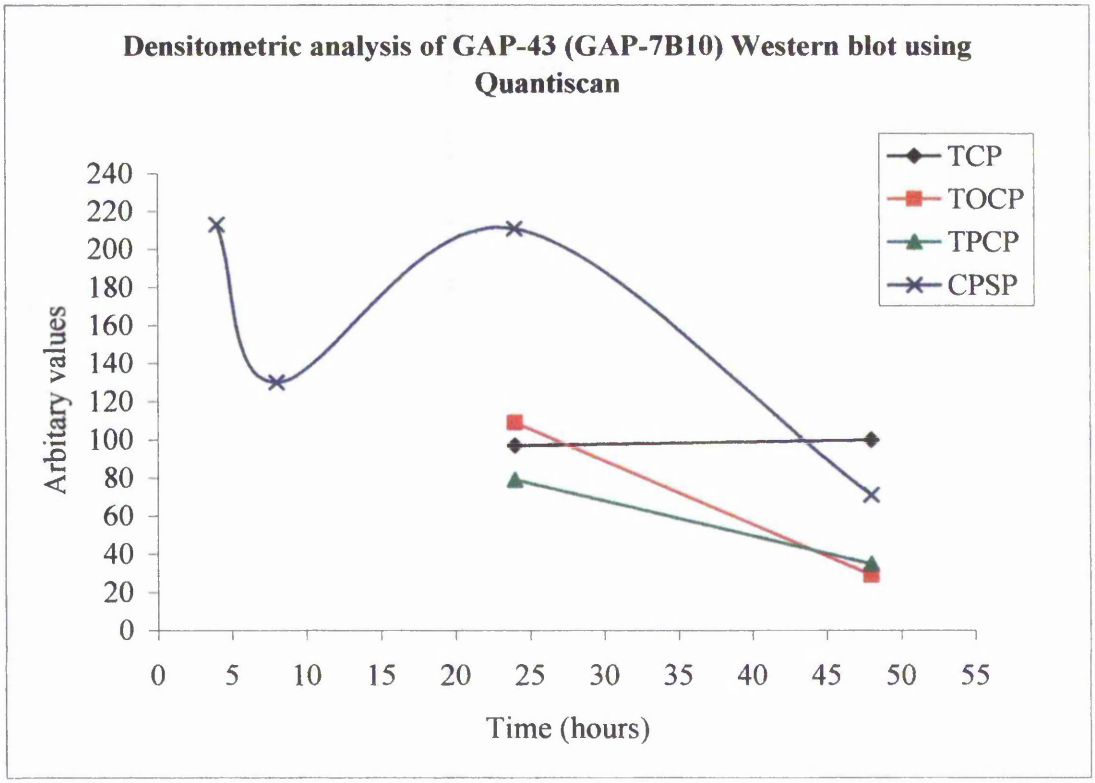
The following graphs represents the arbitrary values (from the QuantiScan package detailed in table 3.6, 4.4, 4.7 and 5.6) plotted against time of exposure (i.e. 4, 8, 24 and 48 hours) towards each OP tested. The graphs represent the effects of OP exposure on reactivity of antibodies against non-phosphorylated NF-H (RMd09), phosphorylated NF-H (Ta51), phosphorylation independent NF-H (N52), α-tubulin (B-5-1-2) and GAP-43 (GAP-7B10).


Densitometric analysis of non-phosphorylated dependent epitope of NF-H (RMd09) Western blots using QuantiScan
TCP
TOCP
TPCP
CPSP
Arbitary values
Time (hours)


Densitometric analysis of phosphorylated dependent epitope of NF-H (Ta51) Western blots using QuantiScan
TCP
TOCP
TPCP
CPSP
Arbitary values
Time (hours)


Densitometric analysis of phosphorylated independent epitope of NF-H (N52) Western blots using QuantiScan
TCP
TOCP
TPCP
CPSP
Arbitary values
Time (hours)


Densitometric analysis of α-tubulin (B-5-1-2) Western blots using QuantiScan
Arbitary values
120
110
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55
Time (hours)
TCP
TOCP
TPCP
CPSP


Densitometric analysis of GAP-43 (GAP-7B10) Western blot using Quantiscan
Arbitary values
240
220
200
180
160
140
120
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45 50 55
Time (hours)
TCP
TOCP
TPCP
CPSP

Appendix III

Publications

1. Flaskos, J., McLean, W. G., Fowler, M. J. and Hargreaves, A. J. (1998) Tricresyl phosphate inhibits the formation of axon-like processes and disrupts neurofilaments in cultured mouse N2a and rat PC12 cells. Neuroscience Letters 242, 101-104.

2. Flaskos, J., Fowler, M. J., Teurtrie, C. and Hargreaves, A. J. (1999) The effects of carbaryl and trichlorphon on differentiating mouse N2a neuroblastoma cells. Toxicology Letters 110, 79-84.

3. Fowler, M. J., Flaskos, J., McLean, W. G. and Hargreaves, A. J. (1997) The effects of tricresyl phosphate on axon outgrowth on mouse neuroblastoma cells. Biochemical Society Transaction 25 : S574-S574.

4. Hargreaves, A. J., McLean, W. G., Fowler, M. J. and Flaskos, J. (1999) Selective toxicity of tricresyl phosphate towards cultured cells of neuronal origin. 33rd International Congress on Forsenic (TIAFT) and 1st on Environmental Toxicology (GRETOX 1995), 143.

5. Fowler, M. J., Flaskos, J., McLean, W. G. and Hargreaves, A. J. (1999) Inhibition of axon outgrowth in mouse N2a neuroblastoma cells by triorthocresyl phosphate. NeuroToxicology, in press.

Accepted by Journal of Neurochemistry :

Fowler, M. J., Flaskos, J., McLean, W. G. and Hargreaves, A. J. (2000) Effects of neuropathic and non-neuropathic isomers of tricresyl phosphate and their microsomal activation on the production of axon-like processes by differentiating mouse N2a neuroblastoma cells.

In preparation :

Fowler, M. J and Hargreaves, A. J. (2000) Effects of β-ODAP on cell growth and neurite outgrowth in cultured N2a and C6 cells.