ASSESSMENT OF THE TOXICITY OF STYRENE, STYRENE OXIDE, AND STYRENE GLYCOL IN PRIMARY CULTURES OF MOTOR AND SENSORY NEURONS

by

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Master of Science

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ABSTRACT

Chronic occupational exposure to styrene is associated with a number of adverse effects on the nervous system, including sensory neuropathy and neurophysiological alterations. In order to test styrene for neurotoxic potential and investigate its mechanism of action, primary co-cultures of murine spinal cord-dorsal root ganglia (DRG)-skeletal muscle were used in a simple *in vitro* neurotoxicity screen. The neurotoxicity of styrene and its major metabolites, styrene oxide and styrene glycol was evaluated after both short and long-term exposure. Endpoints used to characterize neurotoxicity were both morphological and neurophysiological, and included: (1) chromatolysis, (2) axonopathy, and (3) interference with action potential generation. The major findings of this study were: **[1]** styrene and styrene oxide were acutely cytotoxic to all cell types at concentrations in excess of 2.0 mM and 0.2 mM respectively. **[2]** There was no evidence of neurotoxicity attributable to styrene or its metabolites, with the exception of a slight chromatolytic effect induced by 0.2 mM styrene oxide in DRG neurons after long-term exposure. It is therefore possible that cytotoxic mechanisms rather than effects on neuron specific processes, underlie styrene's damage to cells of the nervous system. **[3]** Surprisingly, dimethyl sulfoxide (DMSO), a common *in vitro* solubility vehicle, was neurotoxic after long-term exposure, producing both chromatolysis and axonopathy. This study, therefore also established the upper limit of DMSO recommended for use as a solubility vehicle in long-term *in vitro* tests.

RÉSUMÉ

L'exposition à longue terme au styrène en milieu de travail est associée à un nombre d'effets neurotoxiques, tels que la neuropathie sensorielle et les déficits neurophysiologiques. Afin d'évaluer le potentiel neurotoxique du styrène et rechercher son mécanisme d'action, les cultures primaires de moelle épinière/ganglions rachidiens/muscle squelletique de souris embryonique peuvent être utilisés comme un indicateur *in vitro* de la neurotoxicité. La neurotoxicité du styrène et de ses métabolites, l'oxyde et le glycol de styrène, a été évaluée suite aux expositions courtes et longues. Les marqueurs de toxicité choisis afin de caractériser la neurotoxicité étaient tous deux morphologiques et neurophysiologiques. Ils incluaient: (1) la chromatolyse, (2) l'axonopathie, et (3) les intérferences avec les influx nerveux. Les résultats principaux indiquent que: **[1]** la styrène et l'oxyde de styrène étaient cytotoxiques, conséquemment à l'exposition à des concentrations qui excèdent 2.0 mM et 0.2 mM respectivement. **[2]** Il n'y a pas d'évidence d'effects neurotoxiques de styrène ou ses metabolites après une exposition de longue durée, à l'exception de la chromatolyse chez les neurones sensoriels par l'oxyde de styrène (0.2 mM). Il est donc possible que les mécanismes de la cytotoxicité plutôt que des effets sur des processus neuronaux sont à la base des atteintes aux cellules du système nerveux. **[3]** Un résultat imprévu a cependent été mis en évidence: le sulfoxide dimethyl (DMSO), un agent de solubilisation, a provoqué la neurotoxicité suite à l'exposition de longue durée. La neurotoxicité s'est manifestée sous forme d'axonopathie et de chromatolyse. Donc, cette étude à établi aussi le seuil de concentration de DMSO récommandé comme agent de solubilité chez les essaies *in vitro* à longue terme.

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1) **Kohn J**, Minotti S, Durham H (1994). Assessment of the toxicity of styrene, styrene oxide, and styrene glycol in primary cultures of motor and sensory neurons. ***Toxicology Letters*** (accepted for publication).

2) **Kohn J,** and Durham HD (1993). S9 liver fraction is cytotoxic to neurons in dissociated culture. ***NeuroToxicology*** 14(4): 381-386.

3) Robertson H, Renton K, **Kohn J**, White T (1992). Patterns of fos expression suggest similar mechanisms of action for the excitotoxins domoic and kainic acid. ***Annals of the New York Academy of Sciences*** 648: 330-331.

4) **Kohn J** and Durham H (1991). *In vitro* assessment of the neurotoxicity of styrene and its metabolites. ***Abstracts of the 24th Annual Symposium of the Society of Toxicology of Canada.***

5) Mount H, Quirion R, **Kohn-Alexander J,** Boksa P (1990). Subtypes of excitatory amino acid receptors involved in the stimulation of [^{3}H]dopamine release from cell cultures of rat ventral mesencephalon. ***Synapse*** 5: 271 - 280.

6) **Kohn J**, Howlett SE, Ferrier GR (1990). Induction of the arrhythmogenic transient inward current by the calcium paradox in isolated guinea pig ventricular myocytes.

Proceedings of the 33rd Annual Meeting of the Canadian Federation of Biological Sciences, Abstract 332.

7) Boksa P, Mount H, Chaudieu I, **Kohn J**, Quirion R (1989). Differential effects of NMDA, quisqualate, and kainate on [^{3}H]dopamine release from mesencephalic cell cultures. ***Society for Neuroscience Abstracts*** 15: 946.

PREFACE

This thesis follows a classical format, and is composed of 5 chapters and an Appendix. The background relating to occupational styrene exposure is introduced in Chapter 1, and the rationale for undertaking this study is presented. The biotransformation of styrene is then addressed, which is followed by an overview of its toxicology in humans and in animals. *In vitro* models that may be used for toxicity testing are described, and methods for characterizing neurotoxicity *in vitro* are discussed; the key experimental objectives of this study are also presented. Chapter 2 describes the methodologies used to evaluate the neurotoxicity of styrene and its metabolites *in vitro*, while Chapter 3 presents the results. In Chapter 4, the findings of this study are discussed in relation to styrene's possible mechanism(s) of action, along with some conclusions. A portion of this work has been presented in poster & abstract form at the 1991 Society of Toxicology of Canada Symposium, and some of it has been submitted to ***Toxicology Letters*** for publication (presently under revision). Chapter 5 describes the use of metabolic activation systems in neurotoxicity studies with cultured cells; problems associated with generating metabolites *in vitro* are discussed, and complementary experiments that used an S9 microsomal fraction to generate metabolites of styrene *in vitro* are introduced. The published account of these experiments can be found in the Appendix, entitled "S9 liver fraction is cytotoxic to neurons in dissociated culture" by J Kohn & HD Durham, which appeared in ***NeuroToxicology*** 14 (4): 381-386, 1993. This paper represents an original contribution to knowledge, and its main finding is that S9 microsomes can be used *in vitro* without inducing cytotoxicity in cultured neurons after several days of exposure.

CHAPTER 1

GENERAL INTRODUCTION

RATIONALE

EXPERIMENTAL OBJECTIVES

1. INTRODUCTION

1.1. *Occupational styrene exposure: background & rationale for undertaking this study*

Styrene monomer is a commercially important organic solvent, widely used since the 1940s in the production of polymers, copolymers, synthetic rubber, and fibreglass reinforced polyester plastics. The most extensive occupational exposure to styrene occurs by inhalation, and to a lesser extent, by skin contact with the liquid form (IARC, 1984). In 1988, styrene production exceeded 9 billion pounds in the United States alone, which ranks it among the top ten synthetic organic chemicals produced in that country (USITC, 1988; Bond, 1989), and global consumption in 1990 was estimated to be 13.6 million metric tons (Barale, 1991). The United States National Institute of Occupational Health and Safety [NIOSH] has estimated that the number of workers potentially exposed to styrene on a full-time basis in the United States, is approximately 30,000, with a further 300,000 workers exposed on a part-time basis (NIOSH, 1983). Such widespread occupational use of styrene has prompted numerous investigations into its toxicology, and as a result, there is a large data base regarding styrene's metabolism and general toxicological properties (for reviews see Leibman, 1975; Bond, 1989).

Many toxicological studies of styrene in humans and animals have focused on its genotoxicity (Loprieno *et al.*, 1976; Vainio *et al.*, 1976, 1982; Byfält Nordqvist *et al.*, 1985; Barale, 1991 [review]; Dypbukt *et al.*, 1992; Zhang *et al.*, 1993) and carcinogenicity (Valic, 1983; Ponomarkov *et al.*, 1984; IARC, 1987). However, prior studies of occupationally exposed workers indicate that the nervous system may be a

particularly vulnerable target. Clinical and experimental evidence indicate that styrene is potentially neurotoxic and a number of neurological signs and symptoms are associated with occupational exposure. This can have implications for employees of the plastics industry, since the widespread use of plastics and expansion of the industry ensures that occupational exposure will continue into the future. Although long-term exposure to styrene is linked to a number of central and peripheral neuropathologies, previous investigations have not led to any conclusive determinations of its mechanism of action. This lack of conclusive mechanistic data warrants further investigation of its neurotoxicity. Since cultures of the central nervous system can be used in simple screens of suspected neurotoxicants, we have used primary co-cultures of murine spinal cord-dorsal root ganglia (DRG)-skeletal muscle to determine if styrene has neurotoxic potential, and to investigate the mechanism by which it can be toxic to motor and sensory neurons. Since styrene may require metabolic activation to be neurotoxic, its major metabolites, styrene oxide and styrene glycol, were also investigated for neurotoxic potential.

In this introductory chapter, the biotransformation of styrene will be reviewed, as well as the literature pertaining to its human and animal toxicology. Particular emphasis will be placed on the evidence for its neurotoxic potential. The experimental objectives will be defined, and the application of *in vitro* models to neurotoxicity testing will be introduced. The selection of endpoints for characterizing neurotoxicity *in vitro* will also be addressed.

1.2. *Overview of the biotransformation of styrene*

Frequently, the toxicity of organic solvents is mediated by the action of reactive metabolites, rather than by actions of the parent compounds themselves. The metabolism of solvents is generally catalyzed by the cytochrome P450 mixed function oxidase (MFO) superfamily of enzymes, which are localized mainly in the endoplasmic reticulum of the liver. As with other organic solvents, styrene is metabolized primarily by this enzymatic system, but some local metabolism has been demonstrated in extrahepatic tissues such as kidney, gastrointestinal mucosa, lung, skin, and nervous tissue (Leibman and Ortiz, 1968; Leibman, 1975; Ryan *et al.*, 1976; Ryan and Bend, 1977; Cantoni *et al.*, 1978; Wigaeus *et al.*, 1983; Ghersi-Egea *et al.*, 1988, 1993; Minn *et al.*, 1991), since drug-metabolizing enzymes are present in these tissues at low levels.

In both humans and laboratory animals, the first major metabolite generated in styrene's metabolic pathway (refer to Fig. 1, p. 3-7) is styrene-7,8-oxide (styrene oxide), a highly reactive epoxide (Leibman, 1975; Wigaeus *et al.*, 1983; IARC, 1987). Styrene oxide is subsequently hydrated to the next major metabolite, styrene glycol (1-phenyl-1,2-ethanediol), which is a more polar detoxification product (Leibman and Ortiz, 1969). This step is catalyzed by the action of epoxide hydrolase, an important hydrolytic enzyme located in close proximity to the cytochrome P450 MFOs (Ohtsuji and Ikeda, 1971). Alternatively, styrene oxide may be conjugated with glutathione by the glutathione S-transferase group of enzymes (Ryan and Bend, 1977; Watabe *et al.*, 1981). Since glutathione conjugation is a vital cellular defense mechanism against many reactive

electrophiles, this process presumably renders styrene oxide more polar and more readily excretable, and prevents it from binding covalently to vital macromolecular constituents such as lipids, proteins, and DNA. Styrene oxide is presumed to exert cellular damage by binding to these essential macromolecules, and has been shown to alkylate proteins and DNA both *in vitro* and *in vivo* (Marniemi *et al.*, 1977; Byfält Nordqvist *et al.*, 1985). Cytosolic glutathione-S-transferase and microsomal epoxide hydrolase both appear to play protective roles with regard to the toxicity of styrene oxide.

2. REVIEW OF THE HUMAN TOXICOLOGY OF STYRENE

2.1. *General toxicology*

There have been a number of investigations into the human toxicology of styrene, and many of these indicate that styrene's acute toxicity in both humans and animals is qualitatively similar. Styrene's acute toxicity is classified as low-to-moderate, and it may induce toxicity similar to other hydrocarbons that is not related to biotransformation (Leibman, 1975). If acute exposure concentrations are high enough, both functional and morphological changes can occur which may or may not be reversible upon termination of exposure (Bond, 1989). Acute exposure to high concentrations (600-800 ppm) has been reported to produce eye, nose, throat and respiratory system irritation (Wolf *et al.*, 1956; Leibman, 1975; Withey, 1976), and chemical pneumonitis can occur if aspiration occurs (Leibman, 1975). As a point of reference, the 8 hour time-weighted average permissible exposure limit is 50-100 ppm (Barale 1991; Arlien-Søborg, 1992). Skin

contact with liquid styrene monomer is highly irritating, producing erythema (Leibman, 1975; Withey, 1976; Bond, 1989), and long-term contact with the monomeric form will result in blistering and dermatitis (Withey, 1976; Bond, 1989).

2.2. *Systemic toxicology*

Organ toxicity due to occupational styrene exposure is not a common finding, although both hepatotoxicity and hematologic changes have both been described (Leibman, 1975; IARC, 1979; Hotz *et al.*, 1980; Härkönen *et al.*, 1984). Leibman (1975) has made reference to reports in the Russian literature of hepatic dysfunction in styrene exposed workers. However, findings such as liver enlargement and decrease in liver function are inconclusive, as these individuals may have been concurrently exposed to a number of other chemical agents.

Reported hematological disturbances in occupationally exposed workers include lymphocytosis, leucopenia, monocytosis, reduction in platelet number, lengthened coagulation time, and a lowered hemoglobin concentration (IARC, 1979). One *in vitro* study using cultured human blood cells found a dose-dependent inhibition of membrane-bound acetylcholinesterase, possibly due to changes in lipid-protein interactions as a result of changes in membrane fluidity (Korpela and Tähti, 1986).

2.3. *Genetic toxicology*

Occupational exposure to low concentrations of styrene, as well as to

concentrations above threshold limit values, are reported to be mutagenic, but the genotoxic properties of styrene are attributable to its major metabolite, styrene oxide (Barale, 1991). Styrene oxide has the properties of a direct-acting mutagen, and as a consequence of DNA base alkylations, DNA depurination has been observed (Barale, 1991). Occupational styrene exposure is reported to increase the frequency of sister chromatid exchanges, and has produced chromosomal aberrations, DNA single-strand breaks, and micronuclei in peripheral lymphocytes (Vainio *et al.*, 1981; Camurri *et al.*, 1983; Högstedt *et al.*, 1983; Nordensen and Beckman, 1984; IARC, 1987; Maki-Paakkanen 1987; Walles *et al.*, 1988; Bond, 1989; Yager *et al.*, 1993). Abnormal findings *in vitro* include chromosome breaks, aneuploidy, and micronuclei in human whole blood cultures and cultured human lymphocytes (Linnainmaa *et al.*, 1978*a*,*b*; Barale, 1991). In cultured human lymphocytes treated with styrene oxide, sister chromatid exchanges (Norppa *et al.*, 1980, 1981; Norppa and Vainio, 1983*a*; Bond, 1989; Zhang *et al.*, 1993), micronuclei and anaphase chromosome bridges (Turchi *et al.*, 1981), chromosomal aberrations (Fabry *et al.*, 1978), and alterations in cell-cycle progression (Zhang *et al.*, 1993) have all been detected.

2.4. *Neurotoxicology*

Many clinical, experimental, and epidemiological studies indicate that both acute and chronic occupational exposure to styrene is associated with central and peripheral nervous system dysfunction. Acute exposures to high concentrations (800 ppm) have

produced headache, nausea, and signs of CNS depression which include narcosis, drowsiness, stupor, and weakness, as well as impaired coordination and balance (Leibman, 1975; Withey, 1976; Härkönen, 1977). After low-level exposure, more subjective indications such as fatigue, mood changes, irritability, and concentration difficulties have also been reported (Härkönen, 1977).

Long-term exposure to concentrations within allowable limit values can also provoke many of these general symptoms, but more specific disturbances in neurological and neurophysiological functions have been observed after chronic exposure. After long-term exposure to high levels of styrene, neuroendocrine impairments have been described in female workers (Mutti *et al.*, 1984*a,c*; Arfini *et al.*, 1987). Neuropsychiatric effects have been observed (Flodin *et al.*, 1989), and long-term occupational exposure has been reported to affect complex neuropsychological functions, such as verbal learning skills and memory (Mutti *et al.*, 1984*a*). Neurobehavioural changes associated with chronic exposure include deficits in equilibrium, loss of coordinated movements and reduced psychomotor speed (Stewart *et al.*, 1968, Götell *et al.*, 1972; Gamberale and Hultengren 1974; Lindstrom *et al.*, 1976; Cherry *et al.*, 1980, 1981; Mutti *et al.*, 1984*a*). Disturbances of neurophysiological functions include increased reaction times, quantitative EEG abnormalities, vestibulo-oculomotor impairments and decreased visuomotor accuracy, acquired dyschromatopsia (acquired loss of colour vision and discrimination), slight alterations in motor and sensory nerve conduction velocities, and peripheral neuropathy with sensory involvement (Seppäläinen, 1976; Seppäläinen and Härkönen, 1976;

Härkönen, 1977; Lilis *et al.*, 1978; Rosen *et al.*, 1978; Seppäläinen, 1978; Ödkvist *et al.*, 1982; Treibig *et al.*, 1985; Behari *et al.*, 1986; Cherry and Gautrin, 1990; Gobba *et al.*, 1991; Matikainen *et al.*, 1993).

Although many studies have found evidence of neurological dysfunction linked to styrene exposure, other investigations report no adverse acute or chronic effects on the nervous system, and no correlation between peripheral neuropathy and styrene exposure (Seppäläinen and Härkönen, 1976; Triebig *et al.*, 1985, 1989). Due to such discrepancies, further mechanistic studies are required to more clearly define styrene's mode of action in the nervous system.

3. REVIEW OF STYRENE TOXICITY IN ANIMALS

3.1. *General Toxicity*

Animal studies have also addressed the toxicity of styrene. As in humans, acute toxicity is low-to moderate, with the LD_{50} (oral) in rats and mice equivalent to 5000 mg/kg and 320 mg/kg, respectively (Wolf *et al.*, 1956; Ohtsuji and Ikeda, 1971; Bond, 1989). In rats, the lowest reported lethal concentration after an 8-hour inhalation exposure is 5000 ppm (Bond, 1989). Acutely toxic effects in rats and guinea pigs after inhalation exposure to sublethal concentrations include eye, nose, and lung irritation, as well as pulmonary haemorrhage, congestion, edema, and exudation (Spencer *et al.*, 1942; IARC, 1979; Bond, 1989).

3.2. *Systemic toxicity*

The principal pathological findings in animals exposed to styrene are of respiratory system and liver toxicity, although renal congestion has also been reported. Damage to epithelial cells of the nose and trachea has been observed in rats, as well as degenerative changes in the respiratory mucosa (Ohashi *et al.*, 1985, 1986). In mice, repeated inhalation exposure to sublethal doses of styrene has produced hepatotoxicity, identifying the liver as a primary target organ in this species; males were significantly more susceptible than were females (Morgan *et al.*, 1993*a,b*). The liver toxicity was characterized by hepatic congestion, centrilobular necrosis, increased serum liver enzymes, and a dose-dependent decrease in hepatic glutathione levels (Morgan *et al.*, 1993*a,b*). Focal necrosis and altered enzyme levels have also been demonstrated in rats dosed orally with styrene (Srivastava *et al.*, 1982).

3.3. *Carcinogenicity and Genotoxicity*

There is only limited evidence for the carcinogenicity of styrene to experimental animals (IARC, 1987), with an increased incidence of lung tumours in male mice after oral administration (NCI, 1979). However, according to IARC (1987), there is sufficient evidence to classify styrene oxide as a carcinogen in experimental animals, and this has been demonstrated in rodents (Ponomarkov *et al.*, 1984; Lijinski, 1986; Conti *et al.*, 1988).

There are a number of reports in the literature concerning the mutagenic activity

of styrene and styrene oxide in both bacterial and mammalian systems, but in animals, as in humans, it is generally accepted that the genotoxic effects of styrene are attributable primarily to styrene oxide. In strains of *Salmonella typhimurium* which have been especially designed to detect mutagens, styrene oxide (but not styrene or styrene glycol) has induced mutagenic responses, such as base-pair substitutions (Milvy and Garro, 1976; Vainio *et al.*, 1976; DeMeester *et al.*, 1977). In the same bacterial strains, styrene will only induce mutagenesis after metabolic activation (Vainio *et al.*, 1976).

Styrene oxide is mutagenic to a number of mammalian cell types, including Chinese hamster cell lines, mouse cell lines, and rat hepatocytes, and has induced chromosomal aberrations, micronuclei and anaphase bridges in these systems (Vainio *et al.*, 1982). Rodents exposed to styrene or styrene oxide have also exhibited chromosomal damage (Norppa and Vainio, 1983*a,b*; Vainio *et al.*, 1982).

3.4. *Neurotoxicity*

As in humans, the nervous system of experimental animals is a major target of styrene. Following inhalation exposure to low levels, rats and guinea pigs have exhibited signs of CNS depression, weakness, and dyscoordination (Spencer *et al.*, 1942; Bond, 1989). Rats exposed to 300 ppm became somnolent (Savolainen and Pfäffli, 1977). Lethal concentrations have produced tremor, incoordination, clonic seizures, unconsciousness and coma, again indicating a primary effect on the CNS (Spencer *et al.*, 1942; Withey, 1976). In one study, the major metabolite, styrene glycol, was reported to be a more potent CNS

depressant than is styrene itself (Parkki MG *et al.*, 1976).

Besides acting as a general depressant, styrene also has effects on the nervous system that are more specific. Exposed primates have demonstrated vestibulo-ocular toxicity, expressed as alterations in the amplitude of electroretinogram c-waves, and styrene-treated rabbits have exhibited vestibular disturbances (Larsby *et al.*, 1978; Skoog and Nilsson, 1981). In rats, high concentrations of styrene are ototoxic, resulting in permanent high frequency hearing loss and an elevation of auditory thresholds (Pryor, 1987, Rebert *et al.*, 1993). In neurophysiological studies, no significant changes in nerve conduction velocity or spontaneous motor activity were detected in styrene-exposed rats (Seppäläinen, 1978; Husain *et al.*, 1985; Kulig, 1989; Jennings and Burden, 1993), but diminished performance in neurobehavioral tests (Husain *et al.*, 1985; Kulig, 1989), decreased grip-strength (Kulig, 1989), and changes in brain neurotransmitter levels have all been observed (Mutti *et al.*, 1984*b*; Husain *et al.*, 1980). Neuronal losses in sensory cortex, motor cortex, and hippocampus have been demonstrated in the rat (Rosengren and Haglid, 1989), and slight changes in protein composition of rat spinal cord axons have also been detected (Savolainen and Pfäffli, 1977); it has been suggested that these changes in axonal proteins in the spinal cord may be associated with the reductions in peripheral nerve conduction velocities observed in occupationally exposed workers (Savolainen and Pfäffli, 1977; Lilis *et al.*, 1978). Styrene exposure has also induced small but significant decreases in rat brain protein content (Savolainen and Pfäffli, 1977). These decreases are probably mediated by the covalent binding of styrene oxide, since styrene oxide has been

shown to bind to rat brain macromolecules (Savolainen and Vainio, 1977), probably at their sulfhydryl groups (Savolainen and Pfäffli, 1978), leading to organic degeneration of the CNS (Savolainen and Pfäffli, 1977; Savolainen et al., 1980). However, a reduction in protein synthesis is another possible explanation for the observed decreases in protein levels.

4. *IN VITRO* MODELS AND NEUROTOXICITY INVESTIGATIONS

4.1. *Advantages of in vitro models for neurotoxicity testing*

The intact mammalian nervous system is extremely complex, and results of animal studies are frequently difficult to interpret in relation to mechanism. For investigation of mechanisms of neurotoxicity, *in vitro* models can be very valuable tools. They have the advantage of permitting the examination of a neurotoxicant's effects on separate and discrete nervous system components. Nervous system cultures can therefore provide an opportunity to design readily interpretable mechanistic studies, and address specific questions about the action of neurotoxicants at the cellular and molecular levels.

In vitro, as *in vivo*, no one test can explore all possible targets of neurotoxicants. Several complementary *in vitro* tests need to be used, and ideally, such a test battery should include a system with metabolic capacity. Once a series of *in vitro* tests has been carried out, data obtained *in vitro* should be evaluated *in conjunction* with data from conventional animal studies to attain a meaningful neurotoxicity profile of a chemical. Once integrated, this combination of data should be used to assess morphological,

neurochemical, biochemical, or neurophysiological changes that are specifically targeted by neurotoxicants *in vivo*. Since the action of a xenobiotic that produces toxicity in whole animals will ultimately be exerted at the cellular level (Grisham and Smith, 1984), results obtained *in vitro* can be extrapolated to the whole organism if consideration is also given to factors such as absorption, distribution, and metabolism.

4.2. *The importance of in vitro models for neurotoxicity testing*

Occupational exposure to neurotoxic chemicals is presently a significant problem. NIOSH considers neurotoxic disorders as one of the 10 major occupational disorders in the United States (Gilioli, 1993), and neurotoxicity from chemical agents is considered a worldwide major public health concern. In order to evaluate the potential hazards of neurotoxic chemicals, *in vitro* screening models have gained popularity, and have become an important component of neurotoxicity testing, as toxicologists have recognized the need to develop sensitive, reliable, and reproducible alternatives to conventional whole animal neurotoxicity tests.

The development of *in vitro* neurotoxicity screens has also been stimulated by a significant increase in the number of potentially neurotoxic chemicals introduced into commerce and the environment, whose toxicology is largely unknown. There are between 60,000 and 100,000 chemicals in commerce today, but only about 750 of these have been properly evaluated for neurotoxic potential (Gilioli, 1993). With 2000 new chemicals introduced every year (Gilioli, 1993), they need to be properly identified and

characterized; however, the economics of conventional animal testing (cost and time constraints) make this a very expensive proposition. It is with this in mind that *in vitro models* have become important tools. With the recent improvements in cell culture methodology and the development of more sophisticated bioanalytical tools, *in vitro* models frequently play key roles in understanding the mechanisms of action of neurotoxicants at the cellular and molecular levels, rather than only at the organismal level.

4.3. *Neuronal culture systems for toxicity testing*

There are a number of different *in vitro* models that can be used for assessing the neurotoxic potential of drugs and chemicals. The choice of model, as well as the choice of endpoints to measure are selected based on observations made in animal studies. Source and age of tissue, as well as species, are other important considerations, since they can influence the expression of toxicity. The particular experimental model selected should also be determined by the nature of the problem that one wishes to investigate, and the questions one wishes to answer. The types of CNS cultures commonly used in toxicity tests and their applications, advantages and disadvantages, have been the subject of many reviews (see Schrier, 1982; Grisham and Smith, 1984; Laerum *et al.*, 1985; Walum *et al.*, 1990; Veronesi, 1992; Frazier, 1993; McFarlane Abdulla and Campbell, 1993). The cultures range from the very complex in which the tissue remains highly organized, to the very simple, in which very little cytoorganization is preserved. Generally, less disruption

of tissue means more preservation of the normal cytoarchitecture, but at the expense of a decreased ability to visualize and manipulate the cells in their living state. A few of these systems, in decreasing order of complexity, will be discussed below.

4.3.1. *Whole embryo or whole organ cultures*

Whole embryo or organ cultures are used primarily in developmental studies, rather than for those of a biochemical or morphological nature. Although the normal cytoarchitecture is maintained, a large number of animals are required, and these cultures require considerable manipulation for use in biochemical or morphological investigations. Since these tissues are cultured as a solid mass, it is very difficult to visualize morphological alterations unless histological processing of the tissue is carried out. Due to these methodological difficulties, these are not ideal models for neurotoxicity screening.

4.3.2. *Organotypic cultures*

Organotypic models are either tissue slices or explants. Such preparations are maintained *in vitro* as part of a histologically complex tissue mass, similar to that from which it was derived. Intracellular relationships and neuronal circuitry that occur *in situ* (synaptic connections and spatial relationships) are preserved to some extent, and elongation of axons and dendrites, synaptogenesis, and myelination also occur. Although such tissues can be cultured over long periods of time, they are time consuming to maintain. These preparations are not ideal for long-term morphological studies that examine structural integrity, since microscopic examination of individual cells is more difficult than in monolayer preparations due to the thickness of the tissue explant or slice.

4.3.3. *Dissociated primary cultures*

A system widely used for neurotoxicity studies is the dissociated primary culture model. Primary cultures are composed of cells taken directly from an animal, usually, but not always, fetal. These monolayer cultures are more versatile than the previous models, and have a number of advantages. A good variety of cell types can be maintained, and different types can be co-cultured. One such co-cultured preparation is the spinal cord-DRG-skeletal muscle system. In this model, there is adequate heterogeneity of cell types (neurons, glia, fibroblasts, muscle). Cellular interactions that normally occur *in situ*, such as neuron-neuron, neuron-glia, and neuron-muscle can occur between cultured cells, and synapses and neuromuscular junctions can form. In nerve-muscle preparations, neurons and muscle develop at about the same rate, and neuronal processes will become myelinated. Myotubes form in bundles, become striated, and many will contract. Motor and sensory neurons in culture will differentiate and mature, to eventually resemble their counterparts *in situ*, and specific neuronal cell types can be identified by their morphology, neurophysiology and neurotransmitter profiles. Such cultures are stable over long periods of time which is important, particularly in long-term toxicity tests. Individual neurons can be readily visualized and identified, making the cultures suitable for studies requiring assessments of neuronal morphology. Dissociated primary cultures are also appropriate for electrophysiological studies, because action potentials may be generated spontaneously or in response to depolarizing stimulation.

As with any *in vitro* model, dissociated primary cultures have some disadvantages.

Although these cells are easy to manipulate in the living state, they need time (weeks) to develop, are technically demanding to culture, and they cannot be serially passaged (sub-cultured). Since the cells are dissociated, the normal cytoarchitecture is not preserved, and thus the accuracy of cellular interactions is compromised.

4.3.4. ***Cell lines***

The ability to be subcultured and undergo cell division defines a cell line. Most neural cell lines have been derived from nervous system tumour material. The first clonal line was established from a mouse neuroblastoma tumour (Augusti-Tocco and Sato, 1969; Schubert *et al.*, 1969), but continuous cell lines have also been established from hybrids of neuroblastoma-glioma and other cell types, or by fusion of neurons and glioma cells (Hamprecht, 1977; Walum *et al.*, 1990; Veronesi, 1992). Cell lines may be ideal for biochemical studies that require large quantities of single cells types with identical properties. They are relatively convenient to maintain, large numbers can be economically propagated, and don't require a large number of animals. However, the representation of cell types in the nervous system (and their interaction) is extremely limited, and the normal cytoarchitecture is lost. Although virtually immortal, cell lines don't always maintain their phenotypes with subsequent subcultures, thus their properties can vary with the number of serial passages. Also, these cells may not express a neurotoxicant's target molecules accurately since they differ substantially from intact neurons. If they cannot express the proper target molecules, they will not be useful for predicting neurotoxicity.

4.4 *Choice of model for this study*

The choice of an appropriate model for neurotoxicity testing is a compromise between the ease of manipulation and thoroughness of investigation. In this study, we have used primary co-cultures of dissociated spinal cord-DRG-skeletal muscle as a neurotoxicity screen. With this system, neurotoxicity can be assessed using biochemical, morphological, and electrophysiological techniques. This model is appropriate for the study that we have designed, as the extracellular environment can be readily controlled, and the sequence of pathological changes that may occur in living neurons over prolonged periods of time can be followed. Since the cells can be visualized by phase-contrast microscopy, the evolution of toxicity can also be analyzed in living cultures as a function of both dose and time. It is also likely that this model expresses the properties of the intact nervous system reliably enough to assess styrene's intrinsic potential for neurotoxicity.

Primary neuronal cultures have been used successfully in many other neurotoxicological investigations. A co-culture system similar to this one has been described by Shahar *et al.* (1987), and previous studies in our laboratory have successfully utilized cultures of murine spinal cord-DRG for studies of the known neurotoxicant 2,5-hexanedione (Durham, 1988; Durham *et al.*, 1989). Primary cultures of spinal cord neurons, cortical neurons, cerebellum, astrocytes, and DRG have been used to study the effects of a multitude of neurotoxic chemicals, including organophosphates, acrylamide, various heavy metals (such as lead acetate and aluminum), ethanol, solvents, and

neurotoxic amino acids, to name just a few (Seil and Lampert, 1969; Goldberg, 1980; Goldberg *et al.*, 1980; Hooisma *et al.*, 1980; Riopelle *et al.*, 1984; Veronesi *et al.*, 1984; Borenfreund and Babich, 1987; Holtzman *et al.*, 1987; Storey *et al.*, 1987; Davenport *et al.*, 1989; Sass *et al.*, 1993). Although these studies illustrate how neuronal cultures may be used to investigate different categories of chemicals, other studies using cultured neurons have shown that neurotoxicity observed *in vitro* can be representative of the neurotoxicity observed *in situ*. Support for such an *in vitro/in vivo* correlation is illustrated by a number of studies which have demonstrated that neurons in culture that are exposed to drugs or xenobiotics for a number of weeks may exhibit neuropathologies comparable to those induced by the same chemicals in the nervous system of intact animals (Veronesi *et al.*, 1983; Zagoren *et al.*, 1984; Spencer *et al.*, 1986, 1987; Holtzman *et al.*, 1987; Durham, 1988; Segal and Fedoroff, 1989*a,b*; Veronesi, 1992). The success of such prior *in vitro* studies therefore provided us with precedent for selecting our culture model as a neurotoxicity test system.

5. CHARACTERIZATION OF TOXICITY *IN VITRO*

5.1. *Endpoints and toxicity testing*

In any toxicological assessment, specific endpoints must be selected that will be used to define and characterize a cellular response to the toxicant. Endpoints can thus be defined as biochemical, morphological or physiological parameters that can be measured or observed after toxic insult, and can therefore be considered as "markers" of toxicity.

There are two types of endpoints that may be used to evaluate the toxic effects of chemicals on cultured cells: **[1]** those that define and characterize ***cytotoxicity*** (such as lethality or viability endpoints), and **[2]** those that define and characterize ***neurotoxicity*** (endpoints related to specific neuronal properties that are commonly affected by neurotoxicants).

In vitro, cytotoxic responses (affecting both neuronal and non-neuronal cells) generally predominate after acute (short-term) exposures to high concentrations of a toxicant. Under these conditions, specific non-lethal manifestations of neurotoxicity may be masked, but can become apparent after *longer* exposures to *lower* concentrations. With many neurotoxicants, the level of exposure (concentration and/or time) can determine whether cyto- or neurotoxicity is the principal indication.

5.2. *Cytotoxicity endpoints*

Endpoints of cytotoxicity usually measure properties that relate to basic functions required for viability of all cell types and are often assays of lethality. Such endpoints usually describe alterations in bioenergetics, macromolecular synthesis, or levels of intracellular free calcium, and are used to assess the degree or the progression of cellular damage, for example, by evaluation of membrane integrity, or loss of essential ions (Veronesi, 1992). Assays of membrane permeability are often used as a measure of cell injury that signifies impending cell death, since permeabilization of the membrane frequently occurs when basal level functions are severely compromised (Ekwall, 1983*a*;

Grisham and Smith, 1984).

Even though cytotoxicity and viability endpoints can supply information about the intrinsic toxicity of a chemical, they have a limited ability to define a chemical's specifically neurotoxic mode of action. For many (but not all) chemicals, no acceptable correlation has been shown between cytotoxicity in culture assays, and neurotoxic potential in whole animals (Ekwall, 1983*b*). In other words, cytotoxicity *in vitro* is not an endpoint that correlates well with neurotoxicity in the whole animal. Cytotoxicity assays can, however, be useful for establishing the exposure regimen for neurotoxicity tests, by defining the threshold for lethality. They can also detect any non-specific effects of a chemical on cell attachment, which may be difficult to interpret.

5.3. *Cytotoxicity endpoints selected for this study*

In this study, the following endpoints were selected to characterize cytotoxicity: **[a]** blebbing of portions of the cell membrane (appearance of multiple surface protrusions); **[b]** vacuolation and granularity of the cell cytoplasm; **[c]** rounding of cellular shape; **[d]** detachment of cells from the substratum, and **[e]** cell death. Cytotoxicity tests were carried out to define the **L**owest **E**ffective **C**ytotoxic **C**oncentration, or **LECC**. The LECC is the lowest concentration of a chemical which can induce a cytotoxic response in cultured cells within 4 days of the initial exposure. This information (the LECC) is used to select sublethal concentrations for long-term tests, in order to reveal neuron-specific effects of toxicants, ie., specific effects on neuronal morphology, structure, or

function. When used in a preliminary screen, cytotoxicity tests also differentiate potential neurotoxicants from cytotoxicants.

5.4. *Neurotoxicity endpoints*

Neurotoxicity endpoints are more subtle than animal morbidity or lethality (Veronesi, 1992) and are related to the mechanism(s) of neurotoxicity. From a scientific viewpoint, the mechanistic approach is not just preferable, but is also necessary (Rowan and Goldberg, 1985). Neuron-specific endpoints used in mechanistic studies focus on neurochemical, morphological and neurophysiological properties that are common targets of neurotoxicants, and can provide clues about mechanism of action. Some examples of cellular targets for neurotoxicants include axonal transport mechanisms, synthesis of specific neuronal proteins, uptake and release of neurotransmitters, neuron-specific enzyme activity, and action potential generation and conduction. Neurotoxic endpoints can define either *reversible* effects of chemicals which do not induce conspicuous changes in neuronal morphology or physiology, or *irreversible* effects which have permanent morphological (or neurophysiological) consequences. Since no single neurotoxic endpoint can cover the entire range of potential targets, a variety of endpoints are generally chosen. These endpoints should represent a cross-section of the biological processes and cellular interactions normally present in intact nervous tissue. In any investigation of a potential neurotoxicant, a suitable combination of functional and morphological endpoints can greatly enhance the capability of a test battery to detect neurotoxicity (Mattsson *et al.*,

1990); in this manner, the most susceptible target of a neurotoxicant may be identified.

5.5. *Neurotoxicity endpoints selected for this study*

The particular neurotoxic endpoints chosen for this study were selected so that both morphological and physiological disturbances could be revealed. The selection of a neurophysiological endpoint was appropriate, because styrene is an organic solvent, and solvents can exert depressant or anaesthetic effects on neuronal membranes, decreasing the excitability of neuronal cells. Endpoints explored after long-term exposure to styrene and its metabolites were: **[1]** chromatolysis, a general indicator of neuronal stress, **[2]** modifications in neuronal morphology, and **[3]** alterations in gross neurophysiological properties. Since these endpoints were adapted from those used to assess neurotoxicity in whole animal studies, results could be interpreted with a reasonable degree of confidence. These endpoints will be discussed below in greater detail.

5.5.1. *Chromatolysis*

Chromatolysis (sometimes termed the cell body reaction) is a non-specific, characteristic response of the perikaryon to neuronal injury *in situ*, regardless of its cause. It has been described in both motor and DRG neurons (Nakano *et al.*, 1983; Sterman, 1984; Sterman and Delannoy, 1985; Goldstein *et al.*, 1987; Manetto *et al.*, 1988; Wakayama, 1992), and can occur in response to many types of neurological disturbances, such as toxic insult (Sterman, 1982, 1984; Sterman and Delannoy, 1985), axotomy (Sterman and Delannoy, 1985; Goldstein *et al.*, 1987), and neurological disease such as

amyotrophic lateral sclerosis (Nakano *et al.*, 1983; Wakayama, 1992) This indicates a common pathological reaction to various types of neuronal injury. Morphological indicators of chromatolysis include **[1]** peripheral nuclear displacement, **[2]** dispersal of Nissl substance, and **[3]** an abnormal accumulation of phosphorylated neurofilament (NF) proteins in the cell body (Lieberman, 1971; Goldstein *et al.*, 1987). In normal neurons, axons contain NFs that are more highly phosphorylated than those in the cell bodies and dendrites (Durham, 1990*b*). An increase in perikaryal NF phosphorylation can be visualized by immunocytochemical labelling with antibodies directed against phosphorylated NF proteins such as antibody SMI31, and an increase in perikaryal immunoreactivity can indicate neuronal stress. Since phosphorylation has been associated with slowing of NF transport, increased perikaryal immunoreactivity may be associated with either an increase in the degree of phosphorylation, or an increase in the number of neurofilaments (Goldstein *et al.*, 1987).

It is unclear whether chromatolytic changes are the direct result of injury to the cell body, or are secondary to changes in neuronal protein metabolism as part of a regenerative response, but a chromatolytic response signifies that neuronal injury has occurred. Two indicators of chromatolysis were used in this study to measure neuronal stress in response to treatment with styrene or its metabolites: (1) the percentage of motor and DRG neurons with eccentrically placed nuclei, and (2) the percentage of perikarya immunoreactive with SMI31.

5.5.2. *Alterations in neuronal morphology: axonopathy*

Axonopathy is manifested morphologically by axonal degeneration and/or the presence of focal axonal swellings. Axonal degeneration (also referred to as a "dying-back" neuropathy) is a pathological process that is characterized by retrograde degeneration of nerve fibres, and has been demonstrated in response to toxin-induced neuronal injury (Spencer and Schaumburg, 1977, Veronesi *et al.*, 1983; Gold *et al.*, 1992); there is usually a secondary loss of myelin. Since degenerating fibres of cultured neurons can be visualized by phase microscopy, axonopathy resulting from styrene (or metabolite) exposure can be detected in living cultures, or after fixation and immunocytochemical labelling.

The presence of focal axonal swellings is indicative of disturbances in axoplasmic transport. Abnormalities in the slow transport of cytoskeletal elements are manifested by the focal accumulation of neurofilaments (Spencer and Schaumburg, 1977) which are visible as swellings, especially in the distal regions of large diameter axons. If the defect is in fast axonal transport mechanisms, the swellings will be filled with membranous organelles (Sahenk and Mendell, 1979). In either case, axonal segments distal to swellings will degenerate when the supply of essential materials is interrupted. In cultures exposed to styrene or its metabolites, morphological indicators of axonal transport defects could be monitored after labelling with SMI31, since this antibody readily labels neuronal processes.

5.5.3. *Alterations in membrane electrical properties*

Organic solvents can exert a depressant effect on the nervous system by acting directly on neuronal membranes. This may induce non-specific, reversible changes in membrane permeability, and modified lipid-lipid and lipid-protein relationships (Bridges *et al*, 1983). Neuronal excitability will be decreased due to altered membrane fluidity, leading to inhibition of action potential (AP) production and propagation. Neurotoxicity can be therefore be manifested neurophysiologically as gross alterations in membrane electrical properties. In dissociated cultures, spinal motor neurons (but not DRG neurons) can spontaneously generate bursts of APs. Both types of neurons will generate APs in response to depolarizing electrical stimulation. Measurements of resting membrane potential (RMP), spontaneous electrical activity, as well as AP amplitude and duration were used as a simple screen to detect general effects of styrene and its metabolites on the ability of neurons to conduct electrical signals, since interference with neuronal excitability may be manifested as gross changes in general neurophysiological properties.

6. EXPERIMENTAL OBJECTIVES

The specific aim of this study was to investigate the mechanism of action and assess the neurotoxic potential of styrene and its major metabolites *in vitro*, using morphological and neurophysiological endpoints. The experimental strategy employed a "tiered approach", meaning that the order of the test protocols proceeded from the more general (cytotoxicity tests) to the more specific (neurotoxicity tests). In summary, the

ultimate objectives of this study were:

[1] To separate the cytotoxic and the neurotoxic effects of styrene, styrene oxide, and styrene glycol using short-term tests, and to determine the LECC for each chemical.

[2] To see if sublethal concentrations of styrene (or its metabolites) altered the morphology of motor and sensory neurons after long-term exposure. Eccentric nuclei, SMI31-positive somata, axonal degeneration, or axonal swellings were used as morphological endpoints.

[3] To detect decreases in neuronal excitability after short and long-term exposure to styrene, by screening for alterations in resting membrane potential, action potential amplitude, and action potential duration.

CHAPTER 2

MATERIALS

AND

METHODS

1. MATERIALS AND METHODS

1.1. *Preparation of Cultures*

Primary co-cultures of dissociated spinal cord-DRG-skeletal muscle were prepared from day 13, CD1-mouse embryos. Spinal cords with DRG attached were excised, finely minced, and enzymatically dissociated using 0.25% trypsin (Gibco Canada Inc., Burlington, ONT). At this stage of development, the spinal column has not yet fused around the spinal cord, and is relatively easy to remove. Hindlimb muscle was taken from the same embryos. The tissues were further dissociated mechanically by gentle trituration with a Pasteur pipette. Spinal cord-DRG and muscle were seeded at a density of 175,000 and 8000 cells per well respectively, in Nunclon 4-well culture plates (Gibco) containing 13 mm diameter round glass coverslips (J.B.EM Services, St. Laurent, QUE., Canada). To promote adhesion of dissociated cells, coverslips were coated with poly-D-lysine, and either MATRIGEL^{IM} basement membrane matrix or Laminin (all from Collaborative Research Inc., Bedford, MA). On day 4, cultures were treated for 24 hours with cytosine-β-D-arabino-furanoside (Calbiochem-Behring Corp., La Jolla, CA), to reduce the proliferation of non-neuronal cells. Cultures were maintained in hormonally supplemented N3 medium plus 2% horse serum as previously described (Durham, 1988) and were used for experiments 5-8 weeks post-plating, to allow for maturation.

1.2. *Short-term cytotoxicity testing*

Cytotoxicity assays were carried out with styrene, styrene oxide and styrene glycol

([±]phenyl 1,2-ethanediol) (Aldrich Chemical Co., Milwaukee, WI), in order to determine the lowest effective cytotoxic concentration (LECC) for each chemical. Since styrene and styrene oxide are insoluble in aqueous media, dimethyl sulfoxide [DMSO] (BDH, Montreal, QUE) was used as a solubility vehicle. Test concentrations were prepared from 30% styrene or styrene oxide stock solutions, dissolved in DMSO. Control cultures received volumes of DMSO equivalent to those in treated cultures. Due to the volatile nature of these chemicals, experiments were carried out in 35 mm glass culture dishes (Bellco Glass Inc., Vineland, NJ) which were sealed with Dow Corning high-vacuum silicone grease (Fisher Scientific Ltd, Montreal, QUE) and clamped with Thomas pinch clamps (Arthur H. Thomas Co., Philadelphia, PA). Although the physicochemical properties of styrene glycol indicated that it was hydrophilic, and not volatile at 37°C, it was not fully soluble at room temperature. DMSO was therefore used to facilitate its dissolution into aqueous medium. Cytotoxicity tests with styrene glycol were carried out in sealed culture dishes, until it was ascertained that no cytotoxicity was attributable to it. Subsequent experiments with styrene glycol were then performed in an unsealed culture system.

1.3. *Determination of the lowest effective cytotoxic concentrations*

For determining the LECCs, cultures were exposed to various concentrations of either styrene, styrene oxide, or styrene glycol (in separate experiments) for four days, until the threshold of lethality for each chemical was determined. Treated cultures were

examined daily by phase contrast microscopy for cytotoxic manifestations in both neuronal and non-neuronal cells. Morphological endpoints of cytotoxicity included cell membrane blebbing; vacuolation and granularity of cytoplasm; rounding or detachment of cells from the substratum, axonal degeneration, and cell death. After four days of exposure, the degree of cytotoxicity induced by each concentration of chemical was graded on a scale of 0 to 4. This scale was termed the *cytotoxicity index*, and was defined as follows:

Grade 0 = indistinguishable from untreated control cultures

Grade 1 = slight granularity of the cytoplasm

Grade 2 = cell membrane blebbing and vacuolation of the cytoplasm

Grade 3 = rounding of cellular shape and detachment from the substratum

Grade 4 = cell death

On this scale, the LECC was defined as a Grade 2 cytotoxic response. Once the LECCs were determined, cytotoxicity tests for each chemical were repeated three times (at the LECC), using two coverslips per test.

1.4. *Cell viability assays*

Loss of cell viability was confirmed on day four by the ethidium uptake assay. This assay employs a nucleic acid stain, the ethidium homodimer, to label nuclei of dead and dying cells. Neurons are considered irreversibly damaged and therefore no longer viable when their plasma membranes lose integrity and become permeable to exogenous

molecules, such as the ethidium homodimer. When the ethidium reagent is taken up into a cell, it is translocated to the nucleus where it intercalates with DNA due to its high affinity for nucleic acids. Nuclei of non-viable cells will exhibit red fluorescence when examined by epifluorescence optics, and loss of cell viability can be therefore be verified. The assay was carried out as follows: 4.0 mM ethidium homodimer (Molecular Probes Inc., Eugene, OR) was dissolved in minimal essential medium (MEM) without sodium bicarbonate, enriched with 5g/litre glucose, and adjusted to pH 7.0 with 1M NaOH. Cultures were exposed to this solution for 15 minutes, and examined by epifluorescence microscopy (using a conventional fluorescein filter set) for nuclear uptake of the ethidium reagent.

1.5. *Long-term neurotoxicity testing: morphological examination*

In long-term tests, cultures were treated with sublethal concentrations of each chemical over extended periods of time, to mimic chronic exposure conditions. In separate experiments, cultures were exposed to a concentration of each chemical that was just below the LECC for up to 8 weeks. These concentrations were: 2 mM styrene; 0.2 mM styrene oxide; 10 mM styrene glycol. Control cultures received the appropriate volumes of DMSO, which were 0.006% (styrene oxide), 0.04% (styrene glycol), and 0.05% (styrene). Experiments were carried out in sealed dishes, with the exception of some involving styrene glycol. Control and treated cultures were monitored daily by phase microscopy for morphological indications of neuronal stress or cytotoxicity. At weekly

intervals, both and control and treated cultures were fixed, immunocytochemically processed, and labelled with antibody SMI31. SMI31-labelled neuronal processes were examined for signs of focal axonal swellings or axonal degeneration. The number of motor and DRG neurons exhibiting peripherally displaced (eccentric) nuclei or SMI31-reactive perikarya were counted separately, and expressed as a percentage of the total number of motor or DRG neurons enumerated (75-200 DRG and motor neurons were counted per coverslip). Immunoreactive cells were identified as those with perikaryal staining that was clearly above background. Motor neurons were identified on the basis of perikaryal size (greater than 20 μm), and the presence of large, tapering, and branching dendrites. DRG neurons exhibit a rounded shape, a characteristic nucleus, and an absence of dendrites. Each time point and exposure concentration was examined in duplicate cultures in four or five separate experiments. Mean cell counts for each experiment were pooled, and expressed as mean values ± SEM. Means were compared using the Student's t-Test, (unpaired, two-tailed, $p = \leq 0.05$). Different culture batches were used throughout these experiments, in order to simulate any natural variability in sensitivity to neurotoxicants that can occur among cell populations.

1.6. *Immunocytochemistry*

Immunocytochemical processing of cultures was carried out at weekly intervals, and immunostaining was visualized by using a commercially available horseradish peroxidase detection system (Vectastain® ABC Kit, Vector Laboratories, Burlingame,

CA). This method uses an avidin-biotin complex to visualize an antigen-antibody complex. Cells on coverslips were fixed for 4 minutes in methanol and 2 minutes in acetone (both @ -20° C), followed by 20 minutes in 3% skim milk (dissolved in 0.05 M TRIS buffer, pH 7.6) to block non-specific binding sites. Coverslips were then processed as follows: **[1]** 30 minute incubation with the primary antibody SMI31 (diluted 1:2000 [Sternberger Monoclonals Inc., Baltimore, MD]); **[2]** 30 minutes with the secondary antibody (biotinylated anti-mouse IgG 1:100 [Dimension Laboratories Inc., Mississauga, ONT]); **[3]** 20 minutes in 0.3% H_2O_2 to block endogenous peroxidase activity; **[4]** 30 minutes in Vectastain® (Vector Laboratories), and finally, **[5]** 10 minutes in the chromogen, 3',3'-diaminobenzidine tetrahydrochloride [5%] (Gibco), plus 0.03% H_2O_2. This yielded a coloured reaction product. Incubations in antibody or Vectastain® were always followed by three rinses in TRIS buffer. Coverslips were rinsed in distilled water, mounted in glycerol/phosphate-buffered saline (1:1, v/v), sealed with paraffin wax, and examined by phase microscopy.

1.7. *Intracellular recordings of membrane electrical properties*

Resting membrane potential, and amplitude and duration of spontaneous and evoked APs were recorded from motor and DRG neurons in cultures exposed to toxicants for 30 min, 4 days, and 8 weeks. In short-term experiments designed to mimic acute exposure conditions, baseline intracellular recordings were obtained from neurons prior to styrene (or DMSO) addition, and then again after 30 minutes of exposure. In cultures

exposed to toxicants (or DMSO controls) for 4 days or 8 weeks, intracellular recordings were obtained from separate DMSO control and toxicant-treated cultures. Recordings were made at room temperature, and cells were bathed in bicarbonate-free MEM (Gibco) enriched with 5g/litre glucose, adjusted to pH 7.0 with 1M NaOH. Neurons were impaled with glass microelectrodes filled with 3 M KCl or 4 M potassium acetate (30-60 MΩ resistance). Intracellular injection of current and recording of membrane potentials was accomplished through the same electrode using a WPI model KS-700 dual microprobe system. Neurons were stimulated with a 5 μS depolarizing pulse (Model A310 Accupulser, World Precision Instruments Inc.). The APs generated were monitored on a Tektronix 5110 oscilloscope (Tektronix Inc., Beaverton, OR), and simultaneously photographed with Kodak RAR oscillograph film 2495 (Grass Instruments, Quincy, MA), using a Nihon-Kohden continuous recording oscilloscope camera (Model RLG 6201, Nihon-Kohden [America] Inc., Irvine, CA). Intracellular recordings of RMP, AP amplitude, and AP duration were measured using JAVA Video Analysis Software (Jandel Scientific, Corte Madera, CA). Measurements were expressed as mean values ± SD.

CHAPTER 3

RESULTS

FIGURES AND TABLES

1. RESULTS

1.1. *Short term cytotoxicity tests*

Fig. 2 illustrates the dose-response curves obtained for styrene, styrene oxide, and styrene glycol. Concentrations of each chemical tested when determining the LECCs are plotted against the degree of cytotoxicity they induced (expressed as a value between 0 and 4, as defined by the cytotoxicity index). The LECCs for each chemical were defined as those concentrations inducing a grade 2 cytotoxic response within 4 days of initial exposure, and are indicated above the curve.

Exposure of the cultures to styrene or styrene oxide induced a dose-related cytotoxic response in all cell types in the cultures (neurons, glia, fibroblasts). The LECCs for these chemicals were 2.8 mM and 0.4 mM respectively. Although parallel dose-response curves were generated for styrene and styrene oxide, styrene oxide was more potent at inducing a cytotoxic response by almost one order of magnitude. As is evident in Fig. 2, styrene glycol did not elicit cytotoxicity, even after 10 days of exposure to concentrations as high as 10 mM, in either sealed or unsealed culture dishes.

Morphological manifestations of cytotoxicity are illustrated in Figure 3. DMSO-treated control cultures (Fig. 3A) exhibited no morphological signs of cytotoxicity or significant nuclear uptake of ethidium homodimer (Fig. 3B). The grade 3 to 4 cytotoxic response induced by 4.0 mM styrene in both neuronal and non-neuronal cells after four days is illustrated in Figure 3C. Cytotoxicity was manifested as blebbing, vacuolation, granularity of processes with their subsequent degeneration, detachment from the

substratum, and the ultimate manifestation of cytotoxicity was cell death. Loss of viability was confirmed by a significant uptake of ethidium homodimer into nuclei of the same cells (Fig. 3D). Although styrene oxide was more potent than styrene, by almost an order of magnitude, it induced qualitatively similar cytotoxic effects (Fig. 3E, F). Cytotoxicity was observed in all types of neuronal cells (motor neurons, DRG neurons, and interneurons) and non-neuronal cells at the same exposure levels.

1.2. *Long-Term Exposure*

1.2.1. *Chromatolysis*

Immunocytochemical labelling with SMI31 was used to visualize chromatolytic neurons, since one of the hallmarks of chromatolysis is an accumulation of phosphorylated neurofilaments in the cell body. Immunostained cells were examined by phase microscopy for 2 indicators of chromatolysis: [1] SMI31-positive cell bodies, and [2] eccentric nuclei. The histograms presented in Fig. 4 represent the pooled data from all experiments with 2 mM styrene. DRG or motor neurons in cultures exposed to 2 mM styrene for up to six weeks did not undergo chromatolysis. The number of eccentric nuclei in either motor or DRG neurons was not increased relative to DMSO controls (Fig. 4A,C), nor was there an significant increase in the number of perikarya immunoreactive with SMI31, except in DRG after four weeks of exposure (Fig. 4B,D). An unforseen finding, however, was that DMSO was neurotoxic to both motor and DRG neurons. When the DMSO data were compared with untreated controls, a chromatolytic effect of DMSO

(0.05%) was detected in measures of eccentric nuclei in motor neurons (Fig. 4A), and SMI31-positive somata in DRG neurons (Fig. 4D).

Fig. 5 illustrates the same parameters introduced in Fig. 4 (indicators of chromatolysis), but here, the histograms represent the pooled data from all long-term experiments with 0.2 mM styrene oxide. In cultures treated with styrene oxide for up to five weeks, there was a significantly increased number of DRG neurons with SMI31-immunoreactive somata, compared to vehicle (DMSO) controls (Fig. 5D). This increase in SMI31-immunoreactivity was not observed in motor neurons (Fig. 5B). Neither DRG or motor neurons treated with styrene oxide exhibited an elevated number of eccentric nuclei relative to DMSO controls (Fig.5 A,C). As observed in experiments with 2 mM styrene, DMSO induced a significant degree of chromatolysis in motor and DRG neurons (Fig.5A, C, D), even at a much lower concentration (0.006%). The number of motor neurons exhibiting SMI31-positive somata was not increased after DMSO treatment, except after 1 week of exposure (Fig. 5B).

No toxicity was observed in cultures exposed to 10 mM styrene glycol for up to six weeks (Fig. 6). Although DMSO (0.04%) was used as a solubility vehicle for styrene glycol, experiments were carried out in unsealed culture dishes since styrene glycol was not volatile at 37°C. However, since DMSO is volatile at this temperature, it is not unreasonable to presume that the absence of DMSO toxicity in experiments with styrene glycol may be attributed to the loss of DMSO through evaporation.

The data for 0.006% DMSO, 0.05% DMSO, and untreated controls presented in

Figs. 4, 5, and 6 are replotted together in Fig. 7, to more clearly illustrate the dose-response relationship between DMSO concentration and number of chromatolytic neurons. At most time points shown in Fig. 7A through 7D, as the concentration of DMSO increases, so does the number of neurons exhibiting chromatolysis.

1.2.2. ***Morphology of neuronal processes***

Neuronal processes were examined for signs of axonopathy. Since SMI31 immunolabelling renders neuronal processes highly visible, abnormalities of axonal calibre (swellings and degeneration) could be revealed by their SMI31-immunoreactivity. Focal axonal swellings labelled by SMI31 were observed in cultures treated with 2.0 mM styrene or 0.2 mM styrene oxide after three weeks of exposure. However, these swellings were attributed to the vehicle (DMSO), since they were just as prevalent in cultures treated with 0.006% DMSO or higher (Fig. 8B). As indicated in Fig. 8A, swellings were not evident in untreated cultures. No abnormalities in the morphology of non-neuronal cells were noted.

1.2.3. ***Membrane electrical properties***

Representative APs recorded from DRG and motor neurons are shown in Fig. 9. RMPs, and evoked and spontaneously generated APs were recorded from both motor and DRG neurons, before and after chemical exposure. However, only traces recorded from DRG cells were measured and compared statistically, since the extreme variability of motor neuron responses precluded any meaningful statistical comparisons of their AP amplitudes, AP durations, or RMPs. This variability is illustrated by comparing the

differences between RMPs, AP amplitudes, and frequency of firing in the traces shown in Figs. 9C and 9D. The differences are still well within normal limits for motor neurons. As is evident from the recordings shown in Figs. 9A and 9B, responses of DRG neurons are much more predictable and therefore easier to quantitate.

In motor neurons, styrene did not induce any qualitative changes in AP parameters, or alteration in RMP (Fig. 9C, D). After 30 minutes of exposure to either 8 mM styrene or its DMSO-treated counterpart, only a marginally significant change in AP amplitude (but not AP duration or RMP) of surviving DRG neurons was detected (Fig. 9A,B and Table 1). 8 mM styrene was the upper limit tested because of cytotoxicity. Similar cytotoxicity without significant changes in AP production or RMP were observed in surviving DRG neurons exposed to 4 mM styrene for 4 days (Table 1). As with shorter exposures, long-term treatment of cultures with 2.0 mM styrene, 0.2 mM styrene oxide or 10 mM styrene glycol (or DMSO controls) for 8 weeks, 8 weeks, and 4 weeks, respectively, did not induce any obvious changes in electric properties in either motor or sensory neurons (data not shown).

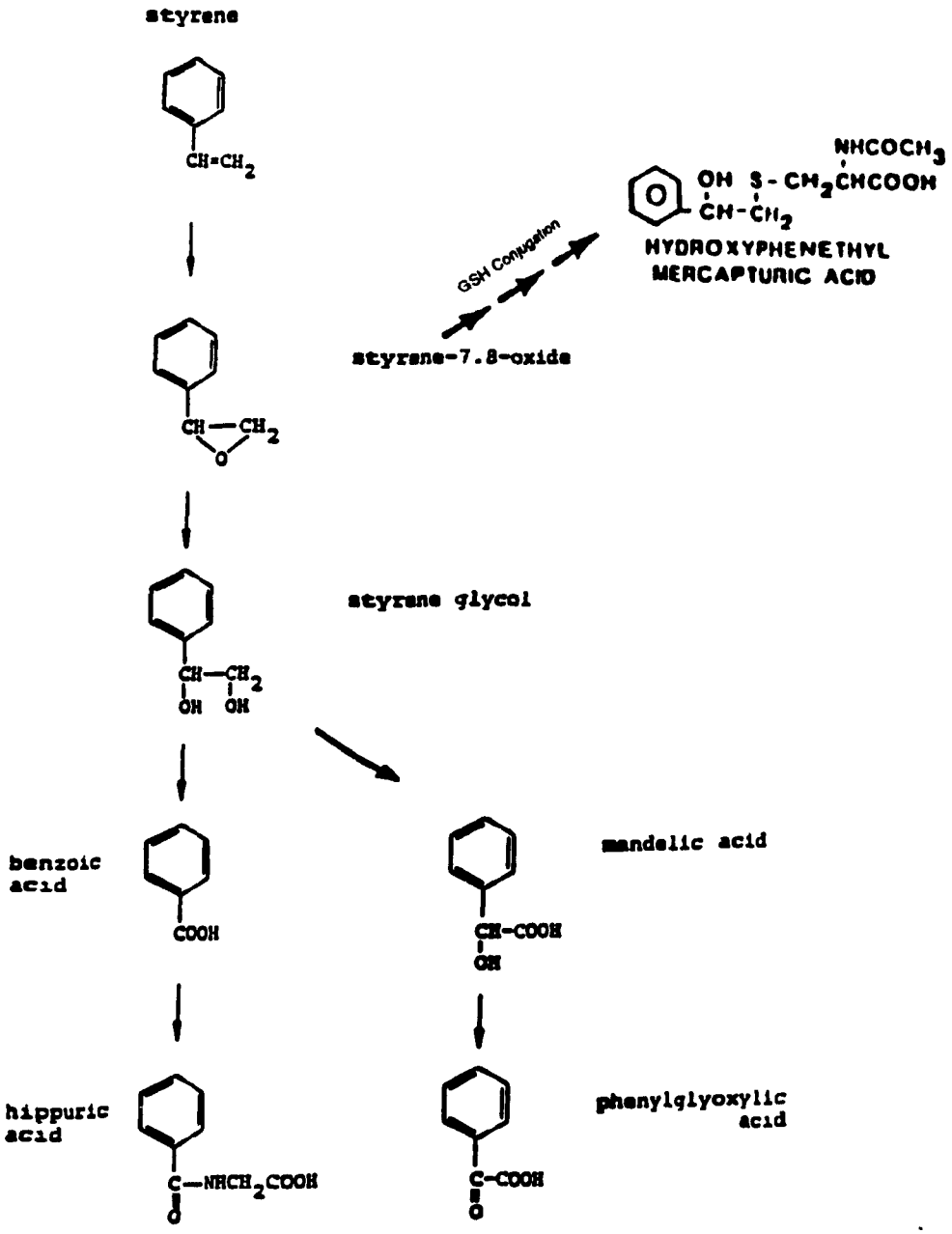

FIG.1. Metabolic Pathway of Styrene [Figure adapted from Arlien-Søborg, 1992].

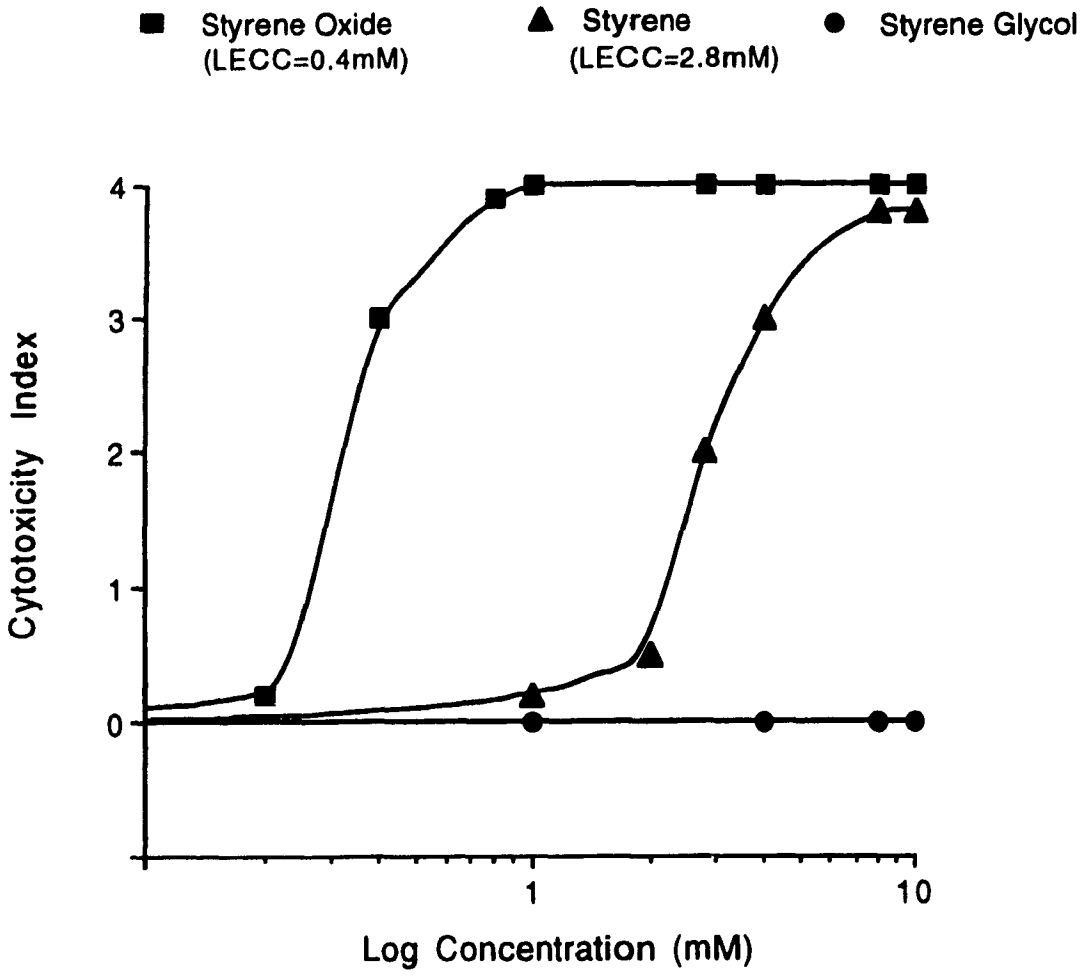

FIG 2. Cytotoxicity index (see text) illustrating the dose-related cytotoxic effects of styrene, styrene oxide, and styrene glycol after 4 days exposure. The lowest effective cytotoxic concentration (LECC) for each chemical is indicated.

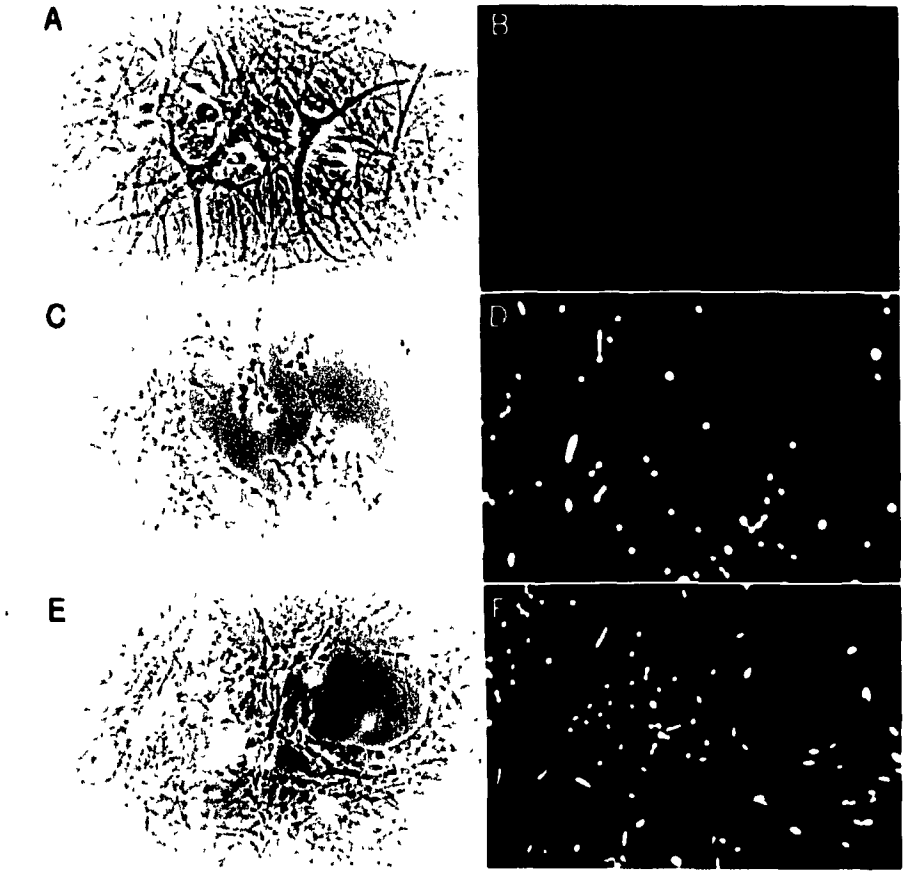

FIG 3. Cytotoxicity observed by phase microscopy (A,C,E) and ethidium uptake (B,D,F) in cultures of spinal cord-DRG-skeletal muscle after 4 days exposure to 0.05% DMSO (A,B), 4.0 mM styrene (C,D), and 0.4 mM styrene oxide (E,F). Scale bar = 30 μm.

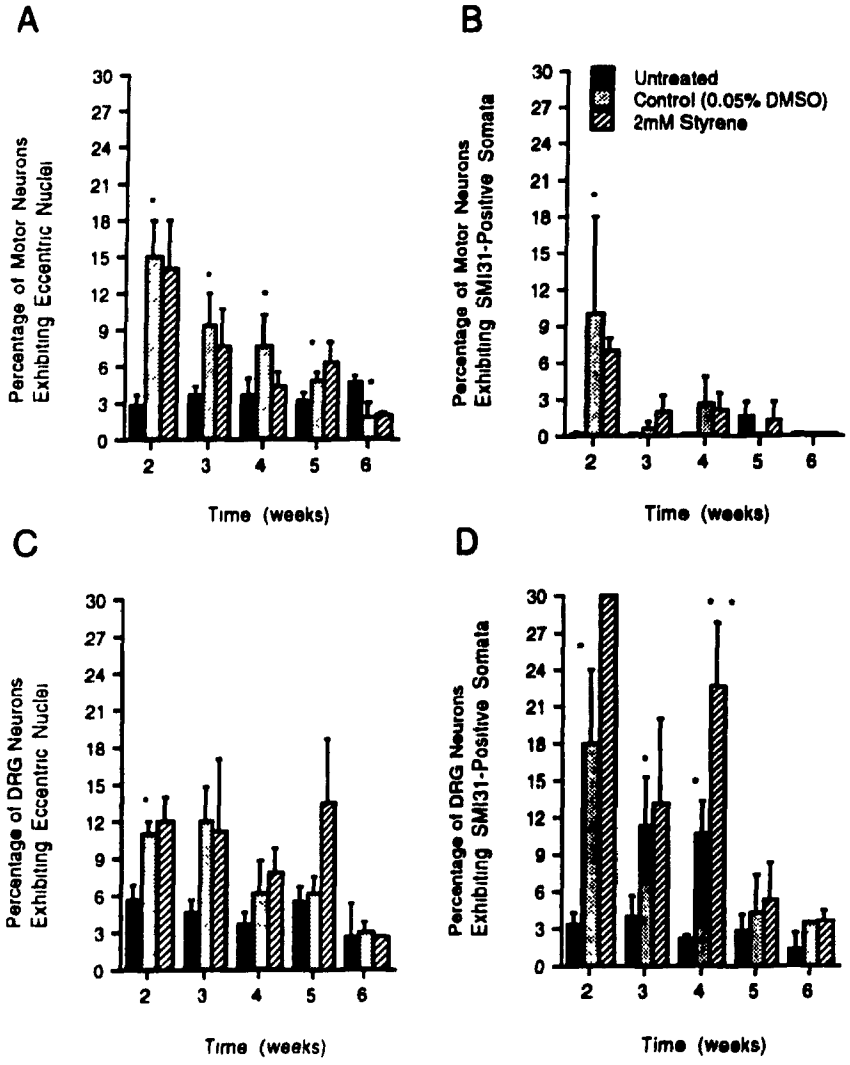

FIG 4. Measures of chromatolysis in perikarya of motor neurons (A,B) and DRG neurons (C,D) of cultures exposed to normal culture medium, 0.05% DMSO or 2.0 mM styrene for up to 6 weeks. The percentage of motor and DRG neurons with eccentric nuclei (A,C) and the percentage of perikarya immunoreactive with SMI31 (B,D) are presented. * = significant difference between means of DMSO and untreated controls; ** = significant difference between means of styrene-treated and DMSO controls, ($p \leq 0.05$).

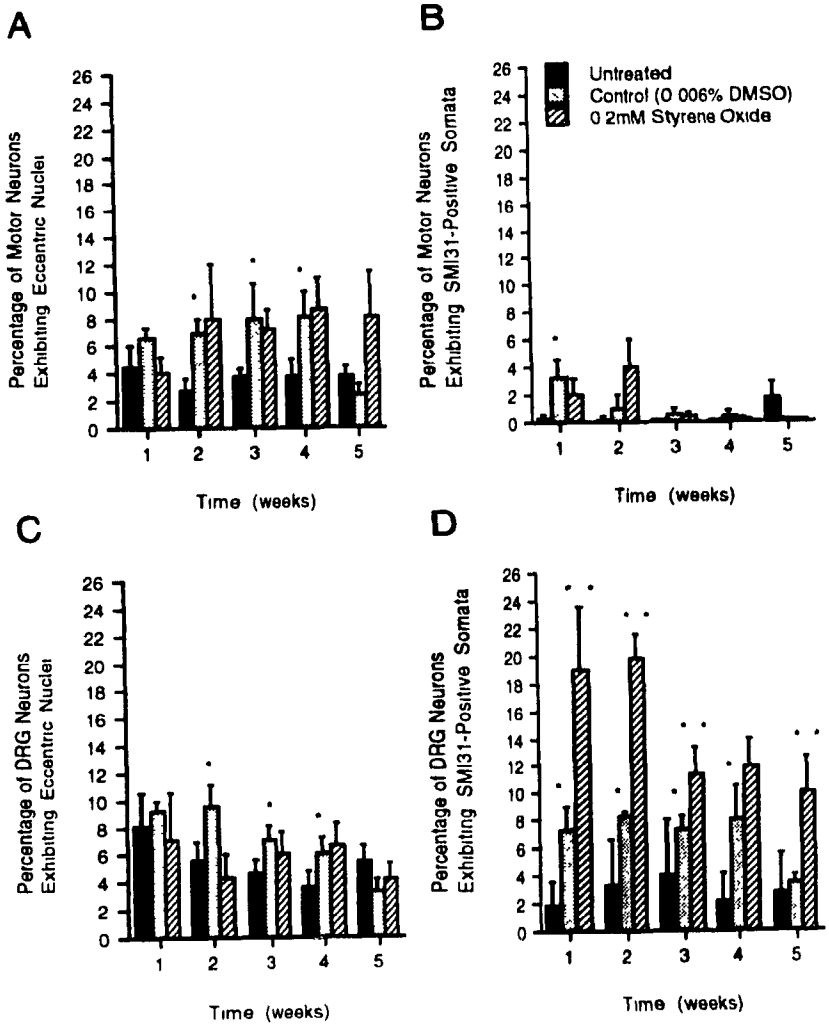

FIG 5. Measures of chromatolysis in perikarya of motor neurons (A,B) and DRG neurons (C,D) of cultures exposed to normal culture medium, 0.006% DMSO, or 0.2 mM styrene oxide for up to 5 weeks. The percentage of motor and DRG neurons with eccentric nuclei (A,C) and the percentage of perikarya immunoreactive with SMI31 (B,D) are presented. * = significant difference between means of DMSO and untreated controls; ** = significant difference between means of styrene oxide-treated and DMSO controls, ($p \leq 0.05$).

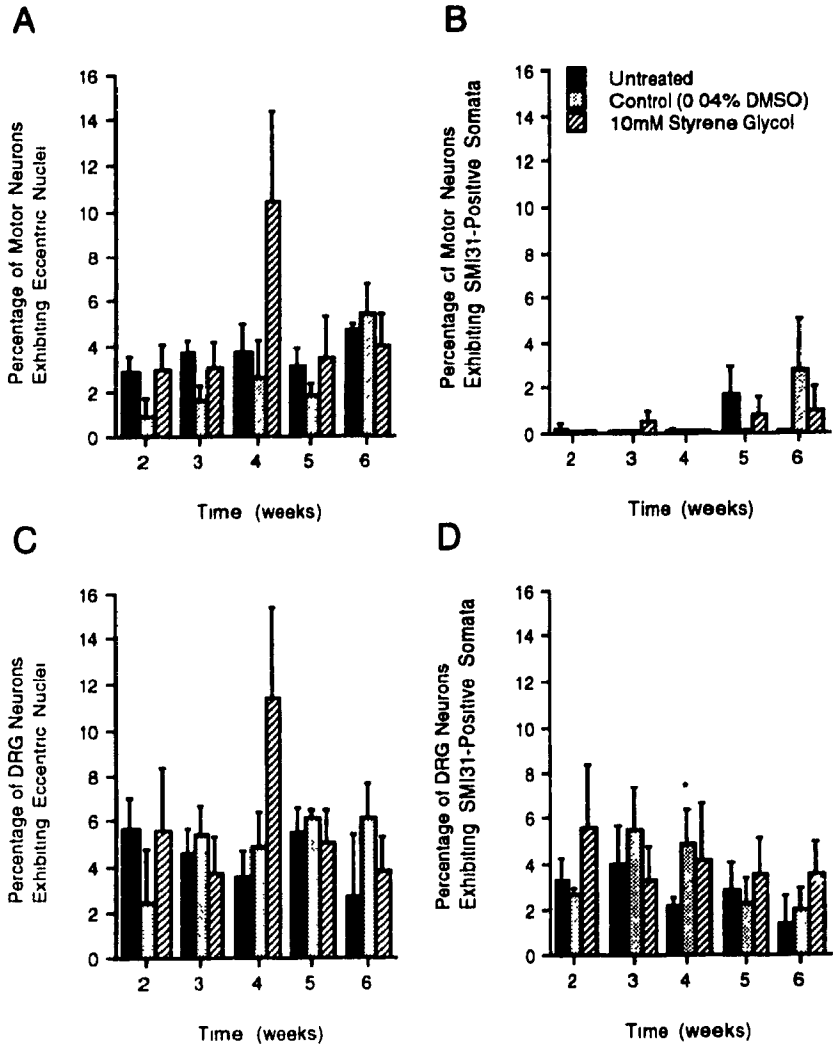

FIG 6. Measures of chromatolysis in perikarya of motor neurons (A,B) and DRG neurons (C,D) of cultures exposed to normal culture medium, 0.04% DMSO, or 10 mM styrene glycol for up to 5 weeks. The percentage of motor and DRG neurons with eccentric nuclei (A,C) and the percentage of perikarya immunoreactive with SMI31 (B,D) are presented. * = significant difference between means of DMSO and untreated controls; ** = significant difference between means of styrene glycol-treated and DMSO controls, ($p \leq 0.05$).

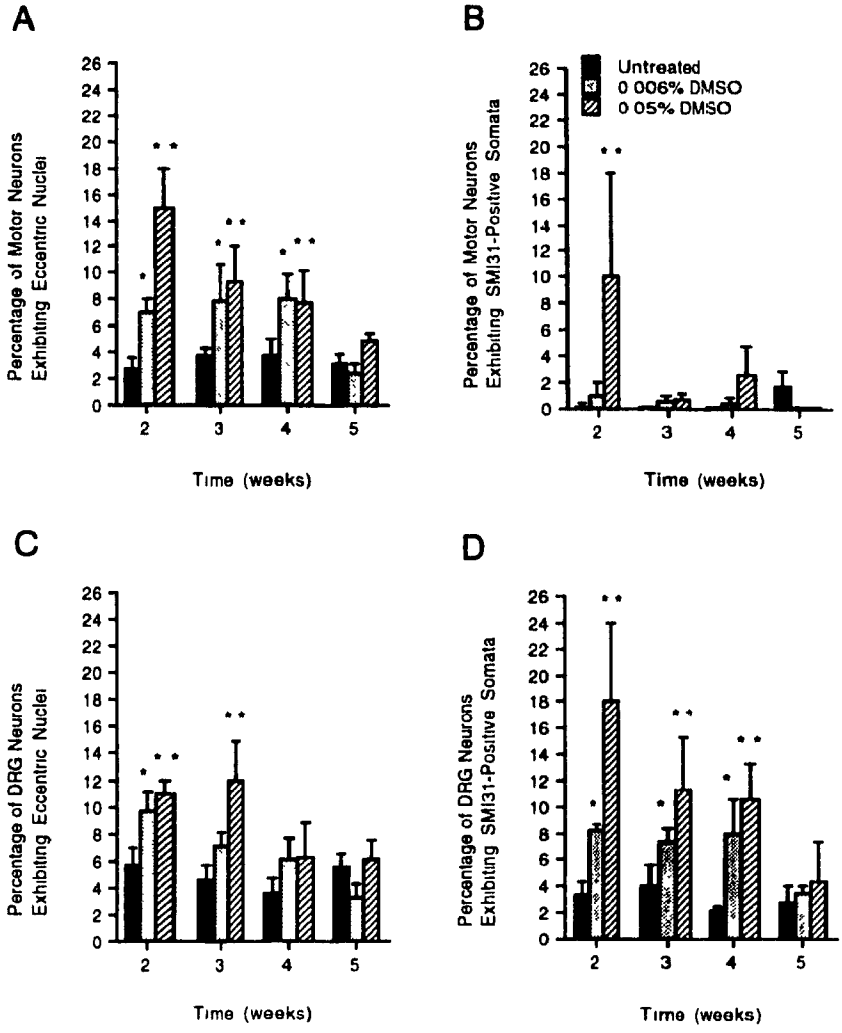

FIG 7. Dose-related chromatolytic effect of DMSO on motor and DRG neurons. DMSO (0.006% DMSO, 0.05% DMSO) and untreated control data from Figs. 4, 5, and 6 are replotted together to illustrate the dose-related effect of DMSO on measures of chromatolysis. The percentage of neurons with eccentric nuclei (A,C) and the percentage of perikarya immunoreactive with SMI31 (B,D) are presented. * = significant difference between means of 0.006% DMSO and untreated controls; ** = significant difference between means of 0.05% DMSO and untreated controls, ($p \leq 0.05$).

FIG 8. Phase contrast micrographs of fixed cultures labelled with SMI31 antibody to neurofilament proteins. (A) An untreated control culture. (B) A culture exposed to 0.006% DMSO for three weeks. A chain of swellings is present in a process emerging from the right of the motor neuron. Both the neuronal perikaryon and swellings are labelled by SMI31. Scale bar = 30 μm.

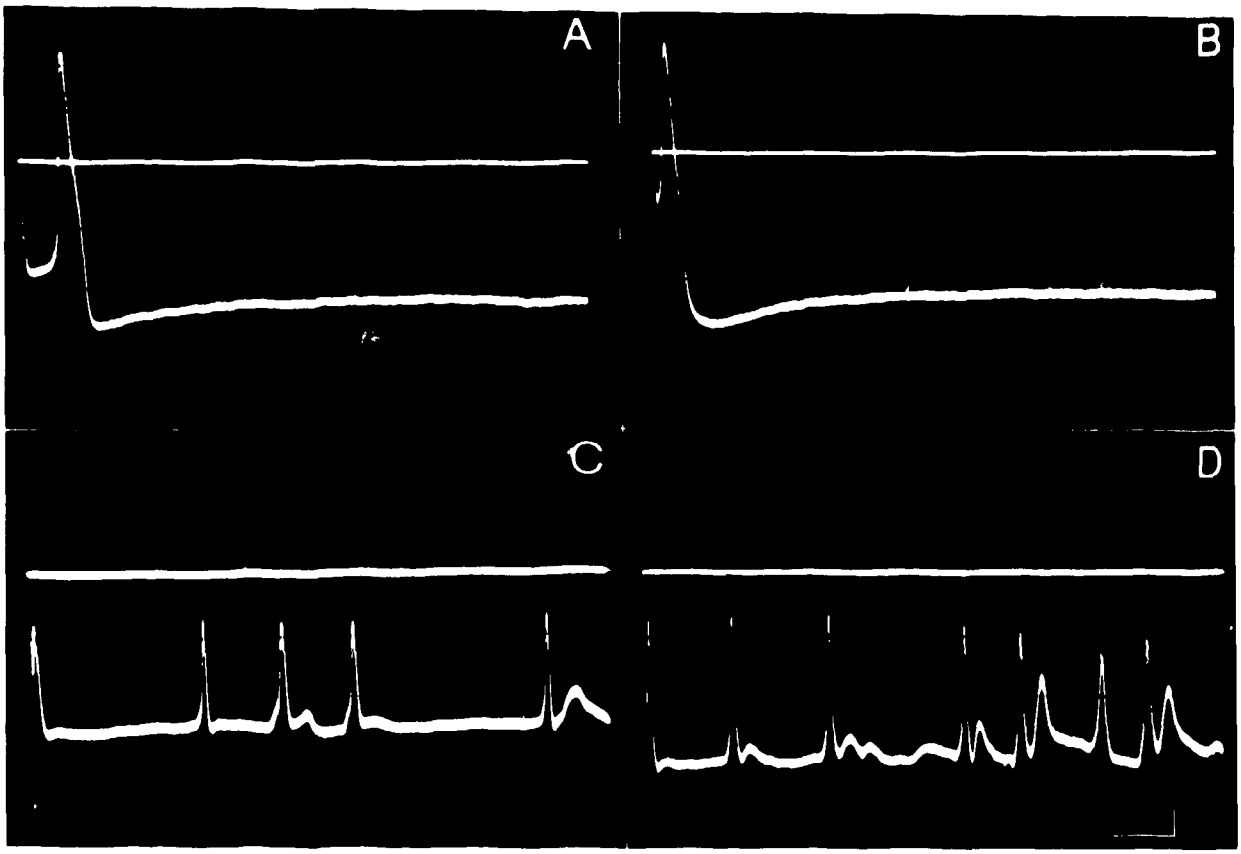

FIG 9. Intracellular recordings of action potentials obtained from DRG neurons and motor neurons. Action potentials from a DRG neuron before (A) and 30 minutes after (B) exposure to 8 mM styrene. Action potentials recorded from a motor neuron in a culture treated with 0.05% DMSO (C) and a culture treated with 4 mM styrene (D) for 4 days. In C and D, the first action potential was evoked by depolarizing stimulation, whereas the others occurred spontaneously. The horizontal line transecting each field represents zero membrane potential. Scale bars (time base) = 5 msec/20 mV (A,B), and 10 msec/20 mV (C,D).

TABLE 1. EFFECT OF STYRENE TREATMENT ON MEMBRANE ELECTRICAL PROPERTIES OF DRG NEURONS.

CONDITION	PARAMETER		
		ACTION POTENTIAL	
ACUTE (30 min) EXPOSURE	RMP(mV)	AMPLITUDE(mV)	DURATION(msec)
Before DMSO Control (17)	- 46 ± 8.1	69.7 ± 9.5	10.2 ± 2.3
After 30 min in DMSO (17)	- 47 ± 9.4	63.1 ± 10.8 *	10.8 ± 2.9
Before 8 mM Styrene (39)	- 46 ± 8.9	74.2 ± 16.1	11.6 ± 3.5
After 8 mM Styrene (21)	- 48 ± 4.9	69.1 ± 16.5 *	10.8 ± 2.0
4 DAYS OF TREATMENT			
DMSO Control (19)	- 48 ± 5.2	73.6 ± 6.1	11.9 ± 2.5
4.0 mM Styrene (22)	- 47 ± 7.4	72.5 ± 11.5	10.4 ± 3.0

Values are Means ± S.D. In parentheses are the number of cells, pooled from 2 separate experiments. Statistical comparisons were made using the Student's t-Test, two-tailed, paired or unpaired as was appropriate; * = significantly decreased relative to control ($p \leq 0.05$).

CHAPTER 4

DISCUSSION

CONCLUSIONS

FUTURE CONSIDERATIONS

1. DISCUSSION

Although the neurotoxicity of styrene has been widely investigated, there has been no definitive explanation of the mechanisms responsible. In this study, assessment of the toxicity of styrene and its major metabolites *in vitro* provided the opportunity to differentiate between toxic effects on neurons and other types of cells, to establish the relative potencies of these chemicals, and obtain further clues concerning mechanisms of neurotoxic damage. This study offers some evidence that mechanisms underlying the general cytotoxic effects of styrene and the more potent styrene oxide, may be responsible for damage to cells of the nervous system, as well as damage to cells of other target organs.

1.1. *Xenobiotic metabolism in nervous tissue and expression of toxicity*

The metabolism of xenobiotics converts lipophilic molecules to more polar compounds either through modifications to their chemical structure, or via conjugation reactions. As indicated in Chapter 1, many of the adverse effects of styrene have been attributed to the accumulation of the major metabolite of styrene, styrene oxide, in tissues (Barale, 1991; Bond, 1989). Styrene and styrene oxide have both been found in brain, since they are highly lipophilic and cross the blood-brain-barrier (Savolainen and Vainio, 1977; Löf *et al.*, 1983; Trenga *et al.*, 1991). Styrene metabolites may also be produced locally in nervous tissue, since the P-450 enzymes responsible for the oxidation of styrene to styrene oxide are expressed in the nervous system, although in much lower amounts

than in liver (Köhler *et al.*, 1988; Minn *et al.*, 1991; Ghersi-Egea *et al.*, 1993). Several other drug-metabolizing enzymes have been demonstrated in brain and also in cerebral microvessels of laboratory animals (Ghersi-Egea *et al.*, 1988; Minn *et al.*, 1991). This may be related to the protective function of these enzymes both in brain tissue itself, and in the endothelial cells that form the blood-brain-barrier (Ghersi-Egea, 1988). Although the activity of epoxide hydrolase is low in brain, fairly high levels of styrene glycol have been measured in mouse brain after systemic administration of styrene (Löf *et al.*, 1983). The glutathione (GSH) group of enzymes are present in neural tissue, and many compounds that are toxic via reactive intermediates will affect cellular levels of GSH. Since GSH conjugation plays an important role in lessening cell damage resulting from oxidative stress, its depletion may contribute to toxicity exerted by reactive molecules. Both styrene and styrene oxide have been shown to deplete GSH stores in cultured Pc12 cells (Dypbukt *et al.*, 1992) and in rat brain (Dixit *et al.*, 1982; Katoh *et al.*, 1989; Trenga *et al.*, 1991), where it is localized predominantly in glia (Philbert *et al.*, 1991; Trenga *et al.*, 1991). Thus, the relative levels of styrene and its various metabolites that are present in nervous tissues after systemic exposure will depend on the same factors that determine their distribution in other extrahepatic tissues (Dixit *et al.*, 1982), and the expression of neurotoxicity will thus depend on the relative potencies of these compounds at target sites. Since P-450 enzymes are not expressed to any significant degree in dissociated cultures, the present study does not rule out the possibility that short-lived metabolic intermediates of styrene may be responsible for the more obvious neurotoxic effects. It is possible that

such metabolites would be produced within neurons *in situ* since the presence of P-450 enzymes in their perikarya have been demonstrated in tissue sections (Hansson *et al.*, 1990; Reuhl and Lowndes, 1992).

1.2. *Styrene toxicity: possible mechanisms of reversible and irreversible effects*

Styrene may exert either reversible or irreversible effects on the nervous system. Some organic solvents exert a depressant or anaesthetic effect by acting directly on the neuronal membrane, inducing nonspecific alterations in membrane permeability and thus decreases in membrane excitability (Bridges *et al.*, 1983). This is manifested as inhibition of AP generation and conduction. Parkki *et al.* (1976) suggested that the depressant effect of styrene could be mediated, at least partially, by styrene glycol, since very high doses of styrene glycol (750 mg/kg) had an anaesthetic effect on rats. Since AP production was not altered in our experiments, even in cultures exposed to 10 mM styrene glycol, it is unlikely that anaesthetic effects on membranes are responsible for CNS depression under more realistic conditions of exposure to styrene.

It is possible that more subtle effects on neurotransmission may contribute to the reversible neurotoxicity of styrene. To fully understand its mechanism of neurotoxicity, it may be necessary to evaluate styrene's actions on neuron-specific enzymes or neurotransmitters, as prior studies indicate that styrene may interfere with brain chemistry in exposed workers or laboratory animals. Decreased levels of serotonin (Kishi *et al.*, 1992) and decreased dopamine receptor binding have been reported in brain tissue of rats

exposed to styrene or styrene oxide (Zaidi *et al.*, 1985). Low dopamine levels have been measured in striatum (Mutti *et al.*, 1984*b*, 1985, 1988) and hypothalamus-median eminence [tuberoinfundibular area] (Mutti et al., 1984*b*, 1988) of rabbits exposed to styrene by inhalation. In exposed workers, interference with dopaminergic neurotransmission has received the most attention. Occupational styrene exposure has been associated with neuroendocrine effects linked to dopamine depletion. Prolactin secretion is normally under inhibitory control by dopamine. Since workers exposed to styrene have exhibited increases in prolactin levels (Mutti *et al.*, 1984*c*; Arfini *et al.*, 1987), and prolactin secretion is under the control of tuberoinfundibular dopaminergic activity, Arfini *et al.* (1987) have speculated that a styrene-mediated decrease in hypothalamic dopamine may be associated with the hyperprolactinemia observed in styrene-exposed humans. They concluded that this might explain the subjective symptoms and behavioural disturbances that are often recorded among styrene exposed workers.

Besides the reversible effects on neuronal physiology, exposure to styrene can also induce permanent damage to cells of the nervous system. Neuronal loss, indicated by an increase in astrocytic glial fibrillary acidic protein, has been demonstrated in sensory and motor cortex and hippocampus of rats exposed chronically to styrene by inhalation (Rosengren and Haglid, 1989). The authors hypothesized that autoxidative injury by styrene or styrene oxide was responsible.

1.3. *Cytotoxicity of styrene and styrene oxide: mechanisms of oxidative stress*

In this study, neurons were not noticeably more sensitive to cytotoxic effects of styrene or styrene oxide than were non-neuronal cells in the cultures, nor did sublethal concentrations of these chemicals have specific effects on neuronal morphology or membrane electrical properties. In neural cells, as in cells of other organs, styrene oxide is a more potent cytotoxicant than styrene by almost an order of magnitude. No differential cytotoxic effects on specific subclasses of neurons was evident. The concentrations of these agents that were cytotoxic to primary CNS neurons in this study are similar to those that are cytotoxic to other types of cultured cell lines and primary cells. These include the epithelial cell lines CG5 (breast carcinoma) and HEp-2 (laryngeal carcinoma) (Malorni *et al.*, 1988), Pc12 cells (Dypbukt *et al.*, 1992), lymphocytes (Sharma *et al.*, 1981; Zhang *et al.*, 1993) and human skin fibroblasts (Cherry and Durham, unpublished observations).

Styrene and styrene oxide have been shown to bind covalently to proteins, DNA, and lipids (Byfält-Nordqvist *et al.*, 1985). The cytotoxicity observed in response to styrene and styrene oxide is typical of that produced by chemicals that induce oxidative stress or form adducts with protein thiols, and was expressed as plasma membrane blebbing, cytoplasmic vacuolation, cell rounding, detachment from the substratum, and cell death. Oxidative-stress-related changes such as these represent the multiple consequences of ATP depletion, increases in intracellular Ca^{2+}, and alterations in cytoskeletal structure and organization (Bellomo and Mirabelli, 1992; Nicotera *et al.*, 1992; Orrenius *et al.*, 1992;

Verity, 1992).

One of the earliest signs of irreversible cell injury and impending cell death that is attributed to oxidative stress is the appearance of multiple protrusions (blebs) on the cell surface (Orrenius *et al.*, 1992). Membrane blebbing, changes in shape, and detachment from the substratum are effected through changes in the cytoskeleton. The polymerization of cytoskeletal proteins into cytoskeletal structures is very sensitive to the oxidation of thiol groups and to increased levels of intracellular calcium. Oxidative stress leads to depletion of sulfhydryl groups of various cytoskeletal proteins and disulphide bridging between molecules, including actin (Bellomo and Mirabelli, 1992). Changes in the microfilament network and detachment of the membrane from the subaxolemmal cytoskeleton has been demonstrated to occur during bleb formation induced by oxidative stress (Bellomo and Mirabelli, 1992). Direct binding of electrophilic chemicals such as styrene oxide to thiol groups, would disrupt the normal function of multiple cellular proteins, including cytoskeletal proteins and rate limiting enzymes involved in ATP generation. If ATP were to become depleted, intracellular calcium homeostasis would be disrupted. Elevation of intracellular calcium is a pathological process common to mechanisms of cell death induced by many toxic agents. In Pc12 cells, styrene oxide has induced a progressive decrease in intracellular ATP and a sustained increase in cytosolic free calcium, along with depletion of GSH (Dypbukt *et al.*,1992). Since this mechanism is common to a number of toxic chemicals, it possible that disturbed calcium homeostasis is responsible for the cytotoxic effects observed in this study.

The collapse of multiple cytoskeletal element around the nucleus (intermediate filaments, microfilaments, microtubules and extracellullar matrix) is a common manifestation of cytotoxicity in cultured cells (Durham 1986, 1990*a*; Bellomo and Mirabelli, 1992). In human skin fibroblasts treated with styrene, rounding of cell shape and detachment from the substratum was accompanied by simultaneous aggregation of intermediate filaments, microtubules and microfilaments to a juxtanuclear position (Cherry and Durham, unpublished observations). Rounding and detachment of cells would also be expected to disrupt intercellular communications and important interactions with the extracellular matrix (Reuhl and Lowndes, 1992). In neurons, as in other types of cells, intracellular transport of cytoskeletal elements can be affected by interference with essential cellular components. However, neither styrene, styrene oxide, or styrene glycol induced specific changes in the cytoskeleton of cultured neuronal cells that would indicate specific impairment of axonal transport.

1.4. *Neurotoxicity and abnormal perikaryal neurofilament phosphorylation*

Toxicant-induced neuronal injury shares many common features with axotomy-induced neuronal injury, which is known to initiate a cell body response, and alter neuronal metabolism (Sterman, 1984; Sterman and Delannoy, 1985). One such common feature is pathological neurofilament phosphorylation, which has been demonstrated in perikarya after neurotoxicant-induced injury (Bizzi and Gambetti, 1986; Troncoso *et al.*, 1986; Gold *et al.*, 1988; Hugon and Vallat, 1990; Gold and Austin, 1991). This finding

was exploited in this study, to measure neuronal stress. In neuronal cells, the C-terminal extensions of the high and middle molecular weight neurofilament proteins (NF-H and NF-M) contain several repeats of the amino acid sequence KSP. Variants of such neurofilament proteins exist according to the degree of phosphorylation of the serine residues. Phosphorylation begins in the cell body, but in normal neurons, the most highly phosphorylated neurofilaments are demonstrable only in axons (Black and Lee, 1988; Nixon *et al.*, 1989). The antibody SMI31 reacts with NF-M and NF-H when they are in the conformation conferred by an intermediate state of phosphorylation (Lee *et al.*, 1988). These forms are present in both axons and perikarya of motor and sensory neurons under normal condition. In unfixed cultures or tissue sections, SMI31 labels both axonal and perikaryal neurofilaments, but in fixed specimens, significant perikaryal labelling is found only in subpopulations of small neurons and DRG neurons (Durham, 1990*b*). After injury induced by axotomy, neurotoxicants or neurodegenerative disease, neuronal perikarya in both fixed and unfixed preparations become highly immunoreactive to antibodies against phosphorylated epitopes (Goldstein *et al.*, 1987; Nixon and Sihag, 1991). This may be due to the aberrant phosphorylation of perikaryal neurofilaments associated with the chromatolytic response to injury.

The only morphological indicator of neurotoxicity in cultures treated with styrene or metabolites was a slight chromatolytic reaction in DRG neurons, and only after exposure to styrene oxide. This was manifested as an increase in the number of perikarya immunoreactive with SMI31, relative to vehicle controls. No other changes in perikaryal,

dendritic or axonal morphology were observed in either motor neurons or DRG cells in response to styrene, styrene oxide, and styrene glycol, even after 6 weeks of exposure.

The effect on DRG neurons (increased perikaryal neurofilament phosphorylation) may be due to greater sensitivity of sensory neurons, or a subpopulation of these neurons, to toxic insult, compared to motor neurons. This is consistent with clinical observations of sensory neuropathies in persons occupationally exposed to styrene (Behari *et al.*, 1986). Alternatively, SMI31 immunoreactivity may be a more sensitive endpoint in DRG neurons compared to motor neurons. Goldstein *et al.* (1987) found that chromatolysis and alterations of phosphorylation-dependent immunoreactivity were much less apparent in motor than in sensory neurons after axotomy in rats. A study by Mansour *et al.* (1989) yielded similar results: they showed that motor neurons axotomized close to the cell body did not always exhibit perikaryal neurofilament phosphorylation whereas DRG neurons did. From their findings, they concluded that perikaryal neurofilament phosphorylation does not invariably occur after neuronal injury. If chromatolysis is not detectable in both motor and DRG neurons after neuronal injury (or if it does not always occur post-trauma), this also raises the possibility that it may not be the most sensitive endpoint for the characterization of neuronal injury. The reason why motor neurons remained unaffected by styrene oxide is unclear, but it is possible that they are not as sensitive to this kind of toxic injury than are sensory neurons.

1.5. *Effect of styrene and metabolites on neurophysiological properties*

Since there was little evidence of morphological damage after long-term exposure to styrene or its metabolites, styrene's effects on neuronal physiology were evaluated. Our screening methods were designed to detect gross changes in overall excitability that could indicate a solvent effect on neuronal membranes. Since our focus was on screening, we did not look for more subtle disturbances in specific ion channel function. Styrene had no apparent effect on RMP or AP duration. A minor decrease in AP amplitude of DRG neurons was observed after 30 minutes in either 8 mM styrene or its DMSO control. Since this slight decrease was apparent in both styrene and DMSO treated cells, this effect was probably due to DMSO (0.2%). Higher concentrations of DMSO (0.5-1%) have been shown to reversibly block membrane currents in neuroblastoma-glioma hybrid cells (Jourdon *et al.*, 1986). Since styrene did not alter AP production, even in cells exposed to cytotoxic concentrations, it is unlikely that anaesthetic effects on membranes are responsible for CNS depression in humans. The use of more sophisticated neurophysiological tests using more specific electrophysiological endpoints might reveal effects on neuronal physiology that our general screen was not sufficiently sensitive to detect.

1.6. *Neurotoxicity of DMSO*

Although the concentrations of DMSO used in these experiments were not cytotoxic in short-term tests, an unanticipated finding was that DMSO was selectively

neurotoxic with long-term exposure. It was discovered that chromatolytic neurons occurred more frequently in vehicle (DMSO) controls than in untreated cells when the pooled data from experiments with 2.0 mM styrene and 0.2 mM styrene oxide were plotted (Figs.4,5,6).

At concentrations of 0.006% or higher, DMSO induced chromatolysis and axonal swellings in motor and DRG neurons, as well as a small decrease in AP amplitude of DRG neurons. The degree of toxicity (as evidenced by the number of chromatolytic neurons) was proportional to concentration. Similar chromatolytic changes have been observed in DRG neurons of whole animals intoxicated with chemicals that induce axonal swelling and degeneration (Sterman, 1984; Gold *et al.*, 1988). Neurons appear to be particularly sensitive to DMSO, since the other cell types in the cultures were not affected at these concentrations. DMSO's neurotoxicity was surprising, because the concentrations employed in these experiments are routinely used to solubilize hydrophobic compounds in tissue culture studies; even higher concentrations (up to 0.1%) are ordinarily used. DMSO toxicity was also unexpected because cytotoxicity was not apparent in DMSO-treated cultures when examined by phase optics.

Axonal swellings labelled by SMI31 were detected after 3 weeks of exposure to either 2.0 mM styrene or 0.2 mM styrene oxide. However, swellings were also evident in the corresponding vehicle controls [0.05% DMSO (6 mM) and 0.006% DMSO (0.7 mM), respectively], suggesting that DMSO interferes with axonal transport of neurofilament proteins. Whether the axonal swellings observed in this study are due to

a primary effect of DMSO on neurofilaments, or are secondary to changes in the integrity of microtubules remains to be determined. Alterations in neurofilament transport have been demonstrated secondary to depolymerization of microtubules (Sahenk *et al.*, 1987), but DMSO has been shown to stabilize microtubules and promote their assembly in some cell types in culture (Katsuda *et al.*, 1988). However, another agent that stabilizes microtubules, taxol, has no effect on neurofilament transport (Komiya and Tashiro, 1988). Whether DMSO's primary effect is on microtubules or on neurofilaments could be resolved in experiments that examine the dynamics of these proteins.

It is also interesting to note that at higher concentrations, DMSO can act as an oxygen and hydroxyl free radical scavenger, and it is therefore not inconceivable to speculate that this cytoprotective property of DMSO could somehow confer on it a neuroprotective role, rather than a neurotoxic one, depending on its level of exposure (short-term exposure to a high concentration).

2. CONCLUSIONS

Using morphological and neurophysiological endpoints, we have found that:

1) Styrene and styrene oxide were cytotoxic to all cell types in the cultures. These chemicals produced qualitatively similar cytotoxic effects, but styrene oxide was almost one order of magnitude more potent at inducing toxicity. Styrene glycol was not acutely toxic to either neurons, glial cells, or fibroblasts.

2) Sublethal concentrations of styrene and metabolites (2.0 mM styrene, 0.2 mM styrene oxide or 10 mM styrene glycol) had no observable effects on the morphology of neuronal cells, with the exception of a small increase in perikaryal SMI31 immunoreactivity induced by 0.2 mM styrene oxide in DRG neurons. This is suggestive of a sensory type of neuropathology, and is supported by clinical observations of peripheral neuropathy with sensory involvement.

3) DMSO, at a concentration of 0.006% or higher was neurotoxic, provoking chromatolysis and focal axonal swellings after long-term exposure. Swellings were suggestive of an interference with slow axonal transport of neurofilament proteins. Thus, the upper limit of DMSO recommended for use in long-term studies with primary neuronal cultures is less than 0.006%.

4) No effects of styrene or its metabolites were apparent on membrane electrical properties in either short or long-term tests, but a minor effect of DMSO on action potential amplitude in DRG neurons was observed. Thus *in vitro*, styrene and its metabolites do not exert obvious depressant effects on neuronal membranes.

In conclusion, we have found evidence only of a non-specific, cytotoxic effect of styrene and styrene oxide on motor and sensory neurons. The results support the hypothesis that mechanisms of oxidative stress that are induced by styrene in other target

organs may also be responsible for damage to cells of the nervous system. Oxidation of cellular proteins and lipids could disrupt neuronal homeostasis, and adduct formation with DNA could alter synthesis of essential proteins or change responsiveness to transcription factors. Consistent with this postulated mechanism of cellular injury is the finding of Dypbukt *et al.* (1992) that subpopulations of Pc12 cells exposed to styrene oxide lost their ability to differentiate in response to the neurotrophic factor, nerve growth factor.

Although this study did not demonstrate that styrene's neurotoxicity was mediated by an effect on some neuron-specific aspect, it did show that styrene and styrene oxide were cytotoxic, and we therefore surmised that the neuronal damage resulting from styrene exposure might be the result of oxidative-stress-induced cytotoxicity. However, the major metabolite, styrene oxide, was neurotoxic (to DRG neurons) as well as cytotoxic. If it is taken into account that at high concentrations, some neurotoxicants may act as cytotoxicants, the *concentration* of toxicant (in this case styrene oxide) may determine whether *cytotoxicity* (expressed as cell death) or *neurotoxicity* (expressed as neuronal damage) is the ultimate endpoint, even though the *underlying mechanism* (in this case, oxidative-stress) *may be the same.* In other words, whether neurons die or only become damaged after styrene oxide exposure may depend on the dose, but both these manifestations of toxicity may be mediated by a common mechanism. The finding that styrene oxide induced both cytotoxicity and a chromatolytic response in DRG neurons may fit this premise and might be interpreted as follows: if styrene interferes with cytoskeletal organization via oxidative-stress-related mechanisms, neurofilament

organization and metabolism could be affected at low doses; if these parameters are affected, this may be why chromatolysis was observed in DRG neurons at low concentrations of styrene oxide. The absence of a chromatolytic effect in motor neurons may be due to a differential sensitivity, as alluded to previously. If this line of speculation is now taken one step further, the chromatolysis observed in DRG neurons after styrene oxide exposure might correspond to the clinical observations of sensory rather than motor neuropathy. Since it is chemically reactive molecules that provoke oxidative stress, styrene oxide, rather than styrene itself, is most likely to be responsible for the consequences of oxidative stress *in vivo*, which again corresponds to the clinical situation.

3. FUTURE CONSIDERATIONS

Upon analysis of methods used in this series of experiments, there are some methodological modifications that are worth exploring for future *in vitro* studies of this kind. The first (and most obvious) change should be in the solubility vehicle, DMSO, as it was shown in this study to be neurotoxic *in vitro*, at low concentrations.

Mammalian species (and strains) exhibit differential sensitivities to toxicants both *in vitro* and *in vivo*; this is usually a confounding variable when interpreting results of toxicity studies. Thus, this series of experiments should be repeated with similar cultures prepared from different species of rodents and rabbit, to take these intrinsic properties of cells into account.

Other mechanisms and endpoints should be investigated *in vitro* to acquire a

meaningful profile of styrene's neurotoxicity. Future studies should focus on specific aspects of chemical neurotransmission, and the particular mechanisms associated with chemically-induced oxidative stress. Finally, although this study investigated the major metabolites of styrene for their neurotoxic potential, no metabolic activation system was integrated into the test protocols. Our original experimental design had incorporated a biotransformation system, but methodological problems ultimately precluded its use with cultured neurons for generating metabolites of styrene. It is therefore conceivable that other metabolites of styrene are responsible for neurotoxicity, particularly those that require metabolic activation to become neurotoxic. A short discussion of metabolic activation systems in neurotoxicology follows in Chapter 5, and improvements to the methodology for biotransformation *in vitro* are described in the Appendix.

CHAPTER 5

METABOLIC ACTIVATION SYSTEMS IN NEUROTOXICOLOGY

METABOLIC ACTIVATION SYSTEMS IN NEUROTOXICOLOGY

In vitro methods for assessing chemical neurotoxicity must take into account that the neurotoxic properties of many xenobiotics are not always intrinsic to the chemicals themselves, but may reside in one or more of their metabolites. In the intact animal, the most important system for xenobiotic biotransformation is the cytochrome P-450 mixed function oxidase system. Since neurons do not express P-450 enzymes to any significant degree, a metabolic activation system must be incorporated into neurotoxicity test protocols, if any degree of biotransformation is to be achieved *in vitro*.

A good source of P-450 enzymes for *in vitro* neurotoxicity studies is the S9 (9000 x g supernatant) microsomal fraction, a subcellular preparation of homogenized liver. Other methods used to generate metabolites *in vitro* include co-culturing neurons with hepatic cell lines or isolated hepatocytes, or co-culture with mammalian cell lines that express human P-450 genes. A major problem with using isolated hepatocytes or hepatic cell lines is the rapid loss of their metabolizing capacity. Most hepatic cell lines have lost much of their ability to express P-450, and even freshly prepared, isolated hepatocytes have only between 33 and 90% of the P-450 content of intact liver cells (Flint, 1988). This value rapidly drops to about 18% after 24 hours of being cultured (Paine and Legg, 1978; Acosta *et al.*, 1979). A cell line which has been genetically engineered to provide human hepatic bioactivation and deactivation is the MCL-5 human β-lymphoblastoid cell line, which has been transfected with 5 cDNAs of the human P-450 genome (Crespi *et al.*, 1990, 1991; Gonzalez and Gelboin, 1991). Although this line has definite potential

for neurotoxicity testing, their use is not economically feasible for our laboratory, and considerable work remains to validate this model in comparison to liver and enzyme preparations.

For this investigation, we had originally planned to use rat liver S9 microsomes in order to generate metabolites of styrene in long-term tests. However, when we prepared the subcellular fraction and applied it to our cultures, we found that it was cytotoxic when used in quantities sufficient for metabolic activation. Experiments were then continued with the commercially available metabolites of styrene, styrene oxide and styrene glycol. However, we reasoned that there must be some means of overcoming this methodological problem, and therefore further investigated the cytotoxicity of S9. Ultimately, we demonstrated that the cytotoxicity of S9 was attributable to its particulate nature (Kohn and Durham, 1993; see Appendix), and that toxicity could be attenuated when it was separated from the cultures by a microporous membrane. The published account of these experiments is presented in the appendix, as it appeared in Neurotoxicology 14(4): 381-386, 1993.

CHAPTER 6

REFERENCES

REFERENCES

Acosta D, Anuforo DC, McMillin R, Soine WH, Smith RV (1979). Comparison of cytochrome P-450 levels in adult rat liver, postnatal rat liver, and primary cultures of postnatal rat hepatocytes. ***Life Sci*** 25: 1413-1418.

Arfini G, Mutti A, Vescovi P, Ferroni C, Ferrari M, Giaroli C, Passeri M, Franchini I (1987). Impaired dopaminergic modulation of pituitary secretion in workers occupationally exposed to styrene: Further evidence from PRL response to TRH stimulation. ***J Occup Med*** 29: 826-830.

Arlien-Søborg, P (1992). ***Solvent Neurotoxicity***. CRC Press Inc, Boca Raton, Chapter 4 (Styrene), pp. 129-153.

Augusti-Tocco G, Sato G (1969). Establishment of functional clonal lines of neurons from mouse neuroblastoma. ***Proc Nat Acad Sci USA*** 64: 311-315.

Barale R (1991). The genetic toxicity of styrene and styrene oxide. ***Mutat Res*** 257: 107-126.

Behari M, Choudhary C, Roy S, Maheshwari MC (1986). Styrene induced peripheral neuropathy. ***Eur Neurol*** 25: 424-427.

Bellomo G and Mirabelli F (1992). Oxidative stress and cytoskeletal alterations. ***Ann NY Acad Sci***: 663: 97-109.

Bizzi A, Gambetti P (1986). Phosphorylation of neurofilaments is altered in aluminum intoxication. ***Acta Neuropathol (Berl)*** 71: 154-158.

Black MM, Lee VM-Y (1988). Phosphorylation of neurofilament proteins in intact neurons: Demonstration of phosphorylation in cell bodies and axons. ***J Neurosci*** 8: 3269-3305.

Bond JA (1989). Review of the toxicology of styrene. ***CRC Crit Rev Toxicol*** 19: 227-249.

Borenfreund E, Babich H (1987). *In vitro* cytotoxicity of heavy metals, acrylamide, and organotin salts to neural cells and fibroblasts. ***Cell Biol Toxicol*** 3: 63-73.

Bridges JW, Benford DJ, Hubbard, SA (1983). Mechanisms of toxic injury. ***Ann NY Acad Sci*** 407: 42-63.

Byfält Nordqvist M, Löf A, Osterman-Golkar S, Walles SAS (1985). Covalent binding of styrene and styrene-7,8-oxide to plasma proteins, hemoglobin and DNA in the mouse. ***Chem-Biol Interact*** 55: 63-73.

Camurri L, Codeluppi S, Pedroni C, Scarduelli L (1983). Chromosomal aberrations and sister-chromatid exchanges in workers exposed to styrene. ***Mutat Res*** 119: 361-369.

Cantoni L, Salmona M, Facchinetti T, Pantarotto C, Belvedere G (1978). Hepatic and extrahepatic formation and hydration of styrene oxide *in vitro* in animals of different species and sex. ***Toxicol Lett*** 2: 179-186.

Cherry N, Waldron HA, Wells GG, Wilkinson RT, Wilson HK, Jones S (1980). An investigation of the acute behavioural effects of styrene on factory workers. ***Br J ind Med*** 37: 234-240.

Cherry N, Rogers B, Venables H, Waldron HA, Wells GG (1981). Acute behavioural effects of styrene exposure: a further analysis. ***Br J ind Med*** 38: 346-350.

Cherry N and Gautrin D (1990). Neurotoxic effects of styrene: further evidence. ***Br J Ind Med*** 47: 29-37.

Conti B, Maltoni C, Perino G, Ciliberti A (1988). Long-term carcinogenicity bioassays on styrene administered by inhalation, ingestion, and injection and styrene oxide administered by ingestion in Sprague-Dawley rats, and para-methylstyrene administered by ingestion in Sprague-Dawley rats and Swiss mice. ***Ann NY Acad Sci*** 534: 203-235.

Crespi CL, Langenbach R, Penman BW (1990). The development of a panel of human cell lines expressing human cytochrome P450 cDNAs. ***Prog Clin Biol Res*** 340B: 97-106.

Crespi CL, Gonzalez FJ, Steimel DT, Turner TR, Gelboin HV, Penman BW, Langenbach R (1991). A metabolically competent human cell line expressing Five cDNAs encoding procarcinogen-activating enzymes: application to mutagenicity testing. ***Chem Res Toxicol*** 4: 566-572.

Davenport CJ, Williams DA, Morgan KT (1989). Neurotoxicology using cell culture. ***CIIT Activities - Chemical Industry Institute of Toxicology*** 9, No. 1.

DeMeester C, Poncelet F, Roberfroid M, Rondelet J, Mercier M (1977). Mutagenicity of styrene and styrene oxide. ***Mutat Res*** 56: 147-152.

Dixit R, Das M, Mushtaq M, Srivastava SP, Seth PK (1982). Depletion of glutathione content and inhibition of glutathione-S-transferase and aryl hydrocarbon hydroxylase activity of rat brain following exposure to styrene. ***NeuroToxicology*** 3: 142-145.

Durham HD (1986). The effect of beta, beta'-iminodipropionitrile (IDPN) on cytoskeletal organization in cultured human skin fibroblasts. ***Cell Biol Int Rep*** 10: 599-610.

Durham HD (1988). Aggregation of intermediate filaments by 2,5-hexanedione: Comparison of effects on neurofilaments, GFAP-filaments and vimentin-filaments in dissociated cultures of mouse spinal cord-dorsal root ganglia. ***J Neuropathol Exp Neurol*** 47: 432-442.

Durham HD, Salera I, Dahrouge S (1989). Evidence that formation of an intermediate filament-protein complex plays a primary role in aggregation of neurofilaments and vimentin-filaments by 2,5-hexanedione. ***J Neuropathol Exp Neurol*** 48: 197-211.

Durham HD (1990*a*). Endpoints for assessment of neurotoxicity in cultured cells. ***NeuroToxicology*** 11: 145.

Durham HD (1990*b*). Demonstration of hyperphosphorylated neurofilaments in neuronal perikarya *in vivo* by microinjection of antibodies into cultured spinal neurons. ***J Neuropathol Exp Neurol*** 49: 582-590.

Dypbukt JM, Costa LG, Manzo L, Orrenius S, Nicotera P (1992). Cytotoxic and genotoxic effects of styrene-7,8-oxide in noradrenergic Pc12 cells. ***Carcinogenesis*** 13: 417-442.

Ekwall B (1983*a*). Screening of toxic compounds in mammalian cell cultures. ***Ann NY Acad Sci*** 407: 64-77.

Ekwall B (1983*b*). Correlation between cytotoxicity *in vitro* and LD_{50}-values. ***Acta Pharmacol Toxicol*** 52 (Suppl 2): 80-99.

Fabry L, Léonard A, Roberfroid M (1978). Mutagenicity tests with styrene oxide in mammals. ***Mutat Res*** 51: 377-381.

Flint OP (1988). ***In vitro*** toxicology: a commercial proposition? ***Xenobiotica*** 18: 715-724.

Flodin U, Ekberg K, Andersson L (1989). Neuropsychiatric effects of low exposure to styrene. ***Br J Ind Med*** 46: 805-808.

Frazier JM (1993). In vitro models for toxicological research and testing. ***Toxicol Lett*** 68: 73-90.

Gamberale F, Hultengren M (1974). Exposure to styrene. II. Psychological functions. ***Work Environ Health*** 11: 86-93.

Ghersi-Egea J-F, Minn A, Siest G (1988). A new aspect of the protective function of the blood-brain-barrier: activities of four drug-metabolizing enzymes in isolated rat brain microvessels. ***Life Sci*** 42: 2515-2523.

Ghersi-Egea J-F, Perrin R, Leininger-Muller B, Grassiot M-C, Jeandel C, Floquet J, Cuny G, Siest G, Minn A (1993). Subcellular localization of cytochrome P450, and activities of several enzymes responsible for drug metabolism in the human brain. ***Biochem Pharmacol*** 45: 647-658.

Gilioli R (1993). EURONEST: A concerted action of the European community for the study of organic solvents neurotoxicity. ***Environ Res*** 62: 89-98.

Gobba F, Galassi C, Imbriani M, Ghittori S, Candela S, Cavalleri A. Acquired dyschromatopsia among styrene exposed workers (1991). ***J Occup Med*** 33: 761-765.

Gold BG, Price DL, Griffin JW, Rosenfeld J, Hoffman PN, Sternberger NH, Sternberger LA (1988). Neurofilament antigens in acrylamide neuropathy. ***J Neuropathol Exp Neurol*** 47: 145-157.

Gold BG, Austin DR (1991). Regulation of aberrant neurofilament phosphorylation in neuronal perikarya. I. Production following colchicine application to the sciatic nerve. ***J Neuropathol Exp Neurol*** 50: 615-626.

Gold BG, Griffin JW, Price DL (1992). Somatofugal axonal atrophy precedes development of axonal degeneration in acrylamide neuropathy. ***Arch Toxicol*** 66: 57-66.

Goldberg AM (1980). Mechanisms of neurotoxicity as studied in tissue culture systems. ***Toxicology*** 17: 201-208.

Goldberg AM, Brookes N, Burt DR (1980). The use of spinal cord cell cultures in the study of neurotoxicological agents. ***Toxicology*** 17: 233-235.

Goldstein ME, Cooper HS, Bruce J, Carden MJ, Lee VM-Y, Schlaepfer WW (1987). Phosphorylation of neurofilament proteins and chromatolysis following transection of rat sciatic nerve. ***J Neurosci*** 7: 1586-1594.

Gonzalez FJ, Gelboin HV (1991). DNA-expressed human cytochrome P-450s: a new age of molecular toxicology and human risk assessment. ***Mutat Res*** 247: 113-127.

Götell P, Axelson O and Lindelhof B (1972). Field studies of human styrene exposure. ***Work Environ Health*** 9: 76-83.

Grisham JW and Smith GJ (1984). Predictive and mechanistic evaluation of toxic responses in mammalian cell culture systems. ***Pharmacol Rev*** 36: 151S-171S.

Hamprecht B (1977). Structural, electrophysiological, biochemical and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. ***Int Rev Cytol*** 49: 99-170.

Hansson T, Tindberg N, Ingelman-Sundberg M, Köhler C (1990). Regional distribution of ethanol-inducible cytochrome P450 IIE1 in the rat central nervous system. ***Neuroscience*** 34: 451-463.

Härkönen H (1977). Relationship of symptoms to occupational styrene exposure and to findings of electroencephalographic and psychological examinations. ***Int Arch Occup Environ Health*** 40: 231-239.

Härkönen H, Lehtniemi A and Aitio A (1984). Styrene exposure and the liver. ***Scand J Work Environ Health*** 10: 59-61.

Högstedt B, Akesson B, Axell K, Gullberg B, Mitelaran F, Pero RW, Skerfving S, Welinder H (1983). Increased frequency of lymphocyte micronuclei in workers producing reinforced polyester resin with low exposure to styrene. ***Scand J Work Environ Health*** 9: 241-246.

Holtzman D, Olson JE, De Vries C, Bensch K (1987). Lead toxicity in primary cultured astrocytes and cerebellar granular neurons. ***Toxicol Appl Pharmacol*** 89: 211-225.

Hooisma J, DeGroot DM, Magchielse T, Muijser H (1980). Sensitivity of several cell systems to acrylamide. ***Toxicology*** 17: 161-167.

Hotz P, Guillemin MP and Lob M (1980). Study of some hepatic effects (induction and toxicity) caused by occupational exposure to styrene in the polyester industry. ***Scand J Work Environ Health*** 6: 206-215.

Hugon J, Vallat JM (1990). Abnormal distribution of phosphorylated neurofilaments in neuronal degeneration induced by kainic acid. ***Neurosci Lett*** 119: 45-48.

Husain R, Srivastava SP, Mushtaq M, Seth PK (1980). Effect of styrene on levels of serotonin, noradrenaline, dopamine, and activity of acetylcholinesterase and monoamine oxidase in rat brain. ***Toxicol Lett*** 7: 47-50.

Husain R, Srivastava SP, Seth PK (1985). Some behavioral effects of early styrene intoxication in experimental animals. ***Arch Toxicol*** 57: 53-55.

International Agency for Research on Cancer (1979). ***IARC Monogr Eval Carcinog Risk Chem Hum: Some monomers, plastics and synthetic elastomers, and acrolein.*** Lyon: IARC, volume 19, pp. 231-283.

International Agency for Research on Cancer (1984). Berlin A, Draper M, Hemminki K and Vainio H (Eds). ***Methods of monitoring human exposure to carcinogenic and mutagenic agents***. IARC Sci Publ No. 59.

International Agency for Research on Cancer (1987). ***IARC Monogr Eval Carcinog Risk Chem Hum: Styrene (Group 2B)***. Lyon: IARC, suppl. 7, pp. 345-347.

Jennings CGB, Burden SJ (1993). Development of the neuromuscular synapse. ***Curr Opin Neurobiol*** 3: 75-81.

Jourdon P, Berwald-Netter Y, Dubois JM (1986). Effects of dimethylsulfoxide on membrane currents of neuroblastoma x glioma hybrid cell. ***Biochim Biophys Acta*** 856: 399-402.

Katoh T, Higashi K, Inoue N (1989). Sub-chronic effects of styrene and styrene oxide on lipid peroxidation and the metabolism of glutathione in rat liver and brain. ***J Toxicol Sci*** 14: 1-9.

Katsuda S, Okada Y, Nakanishi I, Tanaka J (1988). Inhibitory effect of dimethyl sulfoxide on the proliferation of cultured arterial smooth muscle cells: relationship to the cytoplasmic microtubules. ***Exp Molec Pathol*** 48: 48-58.

Kishi R, Katakura Y, Ikeda T, Chen BQ, Miyake H (1992). Neurochemical effects in rats following gestational exposure to styrene. ***Toxicol Lett*** 63: 141-146.

Köhler C, Hansson T, Eriksson LG, Gustafsson J, Warner M (1988). Immunohistochemical localization of cytochrome P-450 in the rat brain. ***Neurosci Lett*** 84: 109-114.

Kohn J, Durham HD (1993). S9 liver fraction is cytotoxic to neurons in dissociated culture. ***NeuroToxicology*** 14: 381-386.

Komiya Y, Tashiro T (1988). Effects of taxol on slow and fast axonal transport. ***Cell Motility and the Cytoskeleton*** 11: 151-156.

Korpela M, Tähti H (1986). The effect of selected organic solvents on intact human red cell membrane acetylcholinesterase *in vitro*. ***Toxicol Appl Pharmacol*** 85: 257-264.

Kulig BM (1989). The neurobehavioural effects of chronic styrene exposure in the rat. ***Neurotoxicol Teratol*** 10: 511-517.

Laerum OD, Steinsvåg S, Bjerkvig R (1985). Cell and tissue culture of the central nervous system: Recent developments and current applications. ***Acta Neurol Scand*** 72: 529-549.

Larsby B, Tham R, Ödkvist LM, Hydén D, Bunnfors I, Aschan G (1978). Exposure of rabbits to styrene. Electronystagmographic findings correlated to the styrene level in blood and cerebrospinal fluid. ***Scand J Work environ Health*** 4: 60-65.

Lee VM-Y, Otvos LJ, Carden MJ, Hollosi M, Dietzschold B, Lazzarini RA (1988). Identification of the major multiphosphorylation site in mammalian neurofilaments. ***Proc Natl Acad Sci USA*** 85: 1998-2002.

Leibman KC (1975). Metabolism and toxicity of styrene. ***Environ Health Perspect*** 11: 115-119.

Leibman KC, Ortiz E (1968). Styrene epoxide - An intermediate in microsomal oxidation of styrene to its glycol. ***Pharmacologist*** 10: 203 (abstract #277).

Leibman KC, Ortiz E (1969). Oxidation of styrene in liver microsomes. ***Biochem Pharmacol*** 18: 552-554.

Lieberman AR (1971). The axon reaction: a review of the principal features of the perikaryal responses to axon injury. ***Int Rev Neurobiol*** 14: 49-124.

Lijinsky W (1986). Rat and mouse forestomach tumours induced by chronic oral administration of styrene oxide. ***J Natl Cancer inst*** 77: 471-476.

Lilis R, Lorimer WV, Diamond S and Selikoff IJ (1978). Neurotoxicity of styrene in production and polymerization workers. ***Environ Res*** 15: 133-138.

Lindström K, Härkönen H and Hernberg S (1976). Disturbances in Psychological functions of workers occupationally exposed to styrene. ***Scand J Work Environ Health*** 2: 129-139.

Linnainmaa K, Meretoja T, Sorsa M, Vainio H (1978*a*). Cytogenetic effects of styrene and styrene oxide. ***Mutat Res*** 58: 277-286.

Linnainmaa K, Meretoja T, Sorsa M, Vainio H (1978*b*). Cytogenetic effects of styrene and styrene oxide on human lymphocytes and *Allium cepa*. ***Scand J Work Environ Health*** 4 (Suppl 2): 156-162.

Löf A, Gullstrand E, Byfält-Nordqvist M (1983). Tissue distribution of styrene, styrene glycol and more polar styrene metabolites in the mouse. ***Scand J Work Environ Health*** 9: 419-430.

Loprieno N, Abbondandolo A, Barale R, Baroncelli S, Bonatti S, Bronzetti G, Cammellini A, Corsi C, Corti G, Frezza D, Leporini C, Mazzaccaro A, Nieri R, Rossellini D, Rossi AM (1976). Mutagenicity of industrial compounds: Styrene and its possible metabolite styrene oxide. ***Mutat Res*** 40: 317-324.

Maki-Paakkanen J (1987). Chromosome aberrations, micronuclei and sister chromatid exchanges in blood lymphocytes after occupational exposure to low levels of styrene. ***Mutat Res*** 189: 399-406.

Malorni W, Formisano G, Iosi F (1988). *In vitro* effects of styrene on cytoskeletal apparatus: An immunocytochemical study. ***Drug Chem Toxicol*** 11: 419-431.

Manetto V, Sternberger NH, Perry G, Sternberger LA, Gambetti P (1988). Phosphorylation of neurofilaments is altered in amyotrophic lateral sclerosis. ***J Neuropathol Exp Neurol*** 47: 642-653.

Mansour H, Bignami A, Labkovsky B, Dahl D (1989). Neurofilament phosphorylation in neuronal perikarya following axotomy: A study of rat spinal cord with ventral and dorsal root transection. ***J Comp Neurol*** 283: 481-485.

Marniemi J, Suolinna EM, Kaartinen N, Vainio H (1977). Covalent binding of styrene oxide to rat liver macromolecules in vivo and in vitro. In: ***Microsomes and Drug Oxidation***. Ulrich V, Roots I, Hildebrandt A, Estabrook RW, Conney AH (eds), Pergammon Press Inc, Oxford, pp. 698-702.

Matikainen E, Forsman-Grönholm L, Pfäffli P, Juntunen J (1993). Nervous system effects of occupational exposure to styrene: A clinical and neurophysiological study. ***Environ Res*** 61: 84-92.

Mattsson JL, Eisenbrandt, DL, Albee RR (1990). Screening for neurotoxicity: Complementarity of functional and morphologic techniques. ***Toxicol Pathol*** 18: 115-127.

McFarlane Abdulla E, Campbell IC (1993). *In vitro* tests of neurotoxicity. ***J Pharmacol Toxicol Methods*** 29: 69-75.

Milvy P, Garro A (1976). Mutagenic activity of styrene oxide (1,2-epoxyethylbenzene), a presumed styrene metabolite. ***Mutat Res*** 40: 15-18.

Minn A, Ghersi-Egea J-F, Perrin R, Leininger B, Siest G (1991). Drug metabolizing enzymes in the rat brain and cerebral microvessels. ***Brain Res Rev*** 16: 65-82.

Morgan DL, Mahler JF, O'Connor RW, Price HC, Adkins B (1993*a*). Styrene inhalation toxicity studies in mice. I. Hepatotoxicity in B6C3F1 mice. ***Fund Appl Toxicol*** 20: 325-335.

Morgan DL, Mahler JF, Dill JA, Price Jr HC, O'Connor RW, Adkins B (1993*b*). Styrene inhalation toxicity studies in mice. II. Sex differences in susceptibility of B6C3F1 mice ***Fund Appl Toxicol*** 21: 317-325.

Mutti A, Mazzuchi A, Rustichelli P, Figeri G, Arfini G and Franchini I (1984*a*). Exposure-effect and exposure-response relationships between occupational exposure to styrene and neuropsychological functions. ***Am J Ind Med*** 5: 275-286.

Mutti A, Falzoy M, Romanelli A, Franchini I (1984*b*). Regional alteration of brain catecholamines by styrene exposure in rabbits. ***Arch Toxicol*** 55: 173-177.

Mutti A, Vescovi PP, Falzoy M, Arfini G, Valenti G, Franchini I (1984*c*). Neuroendocrine effects of styrene on occupationally exposed workers. ***Scand J Work Environ Health*** 10: 225-228.

Mutti A, Romanelli A, Falzoi M, Lucertini S, Franchini I (1985). Styrene metabolism and striatal dopamine depletion in rabbits. ***Arch Toxicol*** Suppl 8: 447-450.

Mutti A, Falzoi M, Romanelli A, Bocchi MC, Ferroni C, Franchini I (1988). Brain dopamine as a target for solvent toxicity: effects of some monocyclic aromatic hydrocarbons. ***Toxicology*** 49: 77-82.

Nakano I, Donnenfeld H, Hirano A (1983). A neuropathological study of amyotrophic lateral sclerosis: with special reference to central chromatolysis and spheroid in the spinal ventral horn and some pathological changes of the motor cortex. ***Neurol Med*** 18: 136-144

National Cancer Institute [NCI] (1979). ***Bioassay of Styrene for Possible Carcinogenicity (Tech Rep Ser No 185; DHEW Publ No (NIH) 79-1741)***, Washington DC, US Government Printing Office.

National Institute of Occupational Safety and Health [NIOSH] (1983). ***Criteria for a recommended standard: Occupational exposure to styrene***. DHHS (NIOSH) Publication No. 83-119.

Nicotera P, Bellomo G, Orrenius S. Calcium-mediated mechanisms in chemically induced cell death (1992). ***Annu Rev Pharmacol Toxicol*** 32: 449-470.

Nixon RA, Lewis SE, Dahl D, Marotta CA, Drager UC (1989). Early posttranslational modifications of the three neurofilament subunits in mouse retinal ganglion cells: Neuronal sites and time course in relation to subunit polymerization and axonal transport. ***Mol Brain Res*** 5: 93-108.

Nixon RA, Sihag RK (1991). Neurofilament phosphorylation: A new look at regulation and function. ***TINS*** 14: 501-506.

Nordensen I, Beckman L (1984). Chromosomal aberrations in lymphocytes of workers exposed to low levels of styrene. ***Hum Hered*** 34: 178-182.

Norppa H, Sorsa M, Pfäffli R, Vainio H (1980). Styrene and styrene oxide induce SCEs and are metabolized in human lymphocyte cultures. ***Carcinogenesis*** 1: 357-361.

Norppa H, Hemminki K, Sorsa M, Vainio H (1981). Effects of monosubstituted epoxides on chromosome aberrations and SCE in cultured human lymphocytes. ***Mutat Res*** 91: 243-250.

Norppa H, Vainio H (1983*a*). Induction of sister-chromatid exchanges by styrene analogues in cultured human lymphocytes. ***Mutat Res*** 116: 379-387.

Norppa H, Vainio H (1983*b*). Genetic toxicity of styrene and some of its derivatives. ***Scand J Work Environ Health*** 9: 108.

Ödkvist LM, Larsby B, Tham R, Åhlfeldt H, Andersson B, Eriksson B, Liedgren SRC (1982). Vestibulo-oculomotor disturbances in humans exposed to styrene. ***Acta Otolaryngol*** 94: 487-493.

Ohashi Y, Nakai Y, Ikeoka H, Koshimo H, Esaki Y, Horiguchi S, Teramoto A (1985). Electron microscopic study of the respiratory toxicity of styrene. ***Osaka City Med J*** 31: 11.

Ohashi Y, Nakai Y, Ikeoka H, Koshimo H, Nakata J, Esaki Y (1986). Degeneration and regeneration of respiratory mucosa of rats after exposure to styrene. ***J Appl Toxicol*** 10: 405.

Ohtsuji H and Ikeda M (1971). The metabolism of styrene in the rat and the stimulatory effect of phenobarbital. ***Toxicol Appl Pharmacol*** 18: 321-328.

Orrenius S, Burkitt MJ, Kass GE, Dypbukt JM, Nicotera P (1992). Calcium ion and oxidative cell injury. ***Ann Neurol*** 32: S33-S42.

Paine AJ, Legg F (1978). Apparent lack of correlation between the loss of cytochrome P-450 in hepatic parenchymal cell culture and the stimulation of haem oxygenase activity. ***Biochem Biophys Res Commun*** 81: 672-679.

Parkki MG, Marniemi J, Vainio H (1976). Action of styrene and its metabolites styrene oxide and styrene glycol on activities of xenobiotic biotransformation enzymes in rat liver *in vivo*. ***Toxicol Appl Pharmacol*** 38: 59-70.

Philbert MA, Beiswanger CM, Waters DK, Reuhl K, Lowndes HE (1991). Cellular and regional distribution of reduced glutathione in the nervous system of the rat: Histochemical localization by mercury orange and *o*-Phthaldialdehyde-induced histofluorescence. ***Toxicol Appl Pharmacol*** 107: 215-227.

Ponomarkov V, Cabral JRP, Wahrendorf J, Galengo D (1984). A carcinogenicity study of styrene-7,8 oxide in rats. ***Cancer Lett*** 24: 95-101.

Pryor GT, Rebert CS and Howd RA (1987). Hearing loss in rats caused by inhalation of mixed xylenes and styrene. ***J Appl Toxicol*** 7: 55-61.

Rebert CS, Boyes WK, Pryor GT, Svensgaard DJ, Kassay KM, Gordon GR, Shinsky N (1993). Combined effects of solvents on the rat's auditory system: styrene and trichloroethylene. ***Int J Psychophysiol*** 14: 49-59.

Reuhl KR, Lowndes HE (1992). Factors influencing morphological expression of neurotoxicity. In: ***Target Organ Toxicology Series - Neurotoxicology***; Tilson HA, Mitchell CL, (eds), Raven Press Ltd, New York, Chapter 4 , pp 67-81.

Riopelle RJ, Dow KE, Eisenhauer E (1984). Some observations on vinca alkaloid and ethanol neurotoxicity using dissociated neuronal cultures. ***Can J Physiol Pharmacol*** 62: 1032-1036.

Rosen I, Haeger-Aronsen B, Rehstrom S and Welinder H (1978). Neurophysiological observations after chronic styrene exposure. ***Scand J Work Environ Health*** 4(Suppl 2): 184-194.

Rosengren LE, Haglid KG (1989). Long-term neurotoxicity of styrene. A quantitative study of glial fibrillary acidic protein (GFA) and S-100. ***Br J Ind Med*** 46: 316-320.

Rowan AN, Goldberg AM (1985). Perspectives on alternatives to current animal testing techniques in preclinical toxicology. ***Ann Rev Pharmacol Toxicol*** 25: 225-247.

Ryan AJ, James MO, Ben-Zvi Z, Law FCP, Bend JR (1976). Hepatic and extrahepatic metabolism of ^{14}C-styrene oxide. ***Environ Health Perspect*** 17: 135-144.

Ryan AJ, Bend JR (1977). Metabolism of styrene oxide in the isolated perfused rat liver. Identification and quantitation of major metabolites. ***Drug Metab Dispos*** 5: 363-367.

Sahenk Z, Mendell JR (1979). Ultrastructural study of zinc pyridinethione-induced peripheral neuropathy. ***J Neuropathol Exp Neurol*** 38: 532-550.

Sahenk Z, Brady ST, Mendell JR (1987). Studies on the pathogenesis of vincristine-induced neuropathy. ***Muscle and Nerve*** 10: 80-84.

Sass JB, Ang LC, Juurlink BHJ (1993). Aluminum pretreatment impairs the ability of astrocytes to protect neurons from glutamate mediated toxicity. ***Brain Res*** 621: 207-214.

Savolainen H, Pfäffli P (1977). Effects of chronic styrene inhalation on rat brain protein metabolism. ***Acta Neuropath (Berl)*** 40: 237-241.

Savolainen H, Vainio H (1977). Organ distribution and nervous system binding of styrene and styrene oxide. ***Toxicology*** 8: 135-141.

Savolainen H, Pfäffli P (1978). Accumulation of styrene monomer and neurochemical effects of long-term inhalation exposure in rats. ***Scand J Work Environ Health*** 4 (Suppl 2): 78-83.

Savolainen H, Helojoki M, Tengen-Junnila M (1980). Behavioral and glial effects of inhalation exposure to styrene vapour and special reference to interactions of simultaneous peroral ethanol intake. ***Acta Pharmacol Toxicol*** 46: 51-56.

Schrier BK (1982). Nervous system cultures as toxicologic test systems. In: ***Nervous System Toxicology***, Tilson H., Mitchell C. L. (eds); Raven Press Ltd., New York; pp. 337-348.

Schubert D, Humphreys S, Baroni C, Cohn M (1969). *In vitro* differentiation of a mouse neuroblastoma. ***Proc Natl Acad Sci USA*** 64: 316-323.

Segal LM, Fedoroff S (1989*a*). The acute and subchronic effects of organophosphorus and carbamate pesticides on cholinesterase activity in aggregate cultures of neural cells from the foetal rat brain. ***Toxicol In Vitro*** 3: 111-122.

Segal LM, Fedoroff S (1989*b*). Cholinesterase inhibition by organophosphorus and carbamate pesticides in aggregate cultures of neural cells from the foetal rat brain: The effects of metabolic activation and pesticide mixtures. ***Toxic In Vitro*** 3: 123-128.

Seil FJ, and Lampert PW (1969). Neurofibrillary spheroids induced by aluminum phosphate in dorsal root ganglia neurons *in vitro*. ***J Neuropathol Exp Neurol*** 28: 74-85.

Seppäläinen AM (1978). Neurotoxicity of styrene in occupational and experimental exposure. ***Scand J Work Environ Health*** 4 (Suppl 2): 181-183.

Seppäläinen AM, Härkönen H (1976). Neurophysiological findings among workers occupationally exposed to styrene. ***Scand J Work Environ Health*** 3: 140-146.

Shahar A, Bidder M, David Y, Amir A, Shainberg A (1987). Autologous neuronal and muscle cocultures: A model for neurotoxicology. ***Prog Clin Biol Res*** 253: 45-58.

Sharma R, Smith FA, Gehring PJ (1981). Styrene epoxide intermediate as a possible stimulant of lymphocytic function. ***J Immunopharmacol*** 3: 67-78.

Skoog KO, Nilsson SEG (1981). Changes in the c-wave of the electroretinogram and in the standing potential of the eye after small doses of toluene and styrene. ***Acta Ophthalmol*** 59: 71-79.

Spencer HC, Irish DD, Adams EM and Rowe VK (1942). The response of laboratory animals to monomeric styrene. ***J Ind Hyg Toxicol*** 24: 295-301.

Spencer PS, Schaumburg HH (1977). Ultrastructural studies of the dying-back process. III. The evolution of experimental peripheral giant axonal degeneration. ***J Neuropath Exp Neurol*** 36: 276-299.

Spencer PS, Crain SM, Bornstein MB, Peterson ER (1986). Chemical neurotoxicity: Detection and analysis in organotypic cultures of sensory and motor systems. ***Fd Chem Toxicol*** 24: 539-544.

Spencer PS, Ross SM, Nunn PB, Roy DN, Seelig M (1987). Detection and characterization of plant-derived amino acid motorsystem toxins in mouse CNS cultures. ***Prog Clin Biol Res*** 253: 349-361.

Srivastava SP, Das M, Mushtaq M, Chandra SV, Seth PK (1982). Hepatic effects of orally administered styrene in rats. ***J Appl Toxicol*** 2: 219.

Sterman AB (1982). Cell body remodelling during dying-back axonopathy: DRG changes during advanced disease. ***J Neuropathol Exp Neurol*** 41: 400-410.

Sterman AB (1984). Hexacarbon neuropathy: Cell body changes are early, dynamic, and specific. ***Ann Neurol*** 16: 343-348.

Sterman AB, Delannoy MR (1985). Cell body responses to axonal injury: Traumatic axotomy versus toxic neuropathy. ***Exp Neurol*** 89: 408-419.

Stewart RD, Dodd HC, Baretta ED and Schaeffer AW (1968). Human exposure to styrene vapour. ***Arch Environ Health*** 16: 656-662.

Storey ND, Smith RA, Orr DJ (1987). Effects of ethanol and acetaldehyde on primary cultured adult mouse DRG neurons. ***J Anat*** 152: 43-55.

Trenga CA, Kunkel DD, Eaton DL, Costa LG (1991). Effect of styrene oxide on rat brain glutathione. ***NeuroToxicology*** 12: 165-178.

Triebig G, Schaller KH and Valentin H (1985). Investigations on neurotoxicity of chemical substances at the workplace. VII. Longitudinal study with determination of nerve conduction velocities in persons occupationally exposed to styrene. ***Int. Arch Occup Environ Health*** 56: 239-247.

Triebig G, Lehrl S, Weltle D, Schaller KH, Valentin H (1989). Clinical and neurobehavioral study of the acute and chronic neurotoxicity of styrene. ***Br J Ind Med*** 46: 799-804.

Troncoso JC, Sternberger NH, Sternberger LA, Hoffman PN, Price DL (1986). Immunocytochemical studies of neurofilament antigens in the neurofibrillary pathology induced by aluminum. ***Brain Res*** 364: 295-300.

Turchi G, Bonatti S, Citti L, Gervasi PG, Abbondandolo (1981). Alkylating properties and genetic activity of 4-vinylcyclohexene metabolites and structurally related epoxides. ***Mutat Res*** 83: 419-430.

United States International Trade Commission [USITC] (1988). **Synthetic Organic Chemicals, U.S. Production and Sales, 1988,** US Government Printing Office, Washington [USITC Publication 2219].

Vainio H, Pääkkönen R, Rönnholm K, Raunio V, Pelkonen O (1976). A study on the mutagenic activity of styrene and styrene oxide. ***Scand J Work Environ Health*** 3: 147-151.

Vainio H, Norppa H, Hemmincki K, Sorsa M (1981). Metabolism and Genotoxicity of styrene. ***Adv Exp Med Biol*** 136A: 257.

Vainio H, Norppa H, Hemminki K, Sorsa M (1982). Metabolism and genotoxicity of styrene. In: ***Biological Active Intermediates II- Chemical mechanisms and biological effects***. Snyder R, Parke DV, Kocsis J, Jollow J, Gibson G, Witmer C (Eds), Part A, Plenum Press, New York, pp. 257-274.

Valic F (1983). International Programme on Chemical Safety, ***Styrene: Environmental Health Criteria Document 26***, World Health Organization.

Verity MA (1992). Ca^{2+}-dependent processes as mediators of neurotoxicity. ***NeuroToxicology*** 13: 139-148.

Veronesi B, Peterson ER, Bornstein MB, Spencer PS (1983). Ultrastructural studies of the dying back process. VI. Examination of nerve fibers undergoing giant axonal degeneration in organotypic culture. ***J Neuropath Exp Neurol*** 42: 153-165.

Veronesi B, Lington A, Spencer PS (1984). A tissue culture model of methyl ethyl ketone's potentiation of n-hexane neurotoxicity. ***NeuroToxicology*** 5: 43-52.

Veronesi B (1992). The use of cell culture for evaluating neurotoxicity. In: ***Target Organ Toxicology Series - Neurotoxicology***; Tilson HA, Mitchell CL (eds), Raven Press Ltd, New York, Chapter 2, pp. 21-49.

Wakayama I (1992). Morphometry of spinal motor neurons in amyotrophic lateral sclerosis with special reference to chromatolysis and intracytoplasmic inclusion bodies. ***Brain Res*** 586: 12-18.

Walles SAS, Norppa H, Osterman-Golkar S, Maki-Paakkanen J (1988). Single-strand breaks in DNA of peripheral lymphocytes of styrene exposed workers. ***IARC Sci Publ*** 89: 223-226.

Walum E, Hansson E, Harvey AL (1990). *In vitro* testing of neurotoxicity. ***ATLA*** 18: 153-179.

Watabe T, Hiratsuka A, Ozawa N, Isobe M (1981). Glutathione-S-conjugates of phenyloxirane. ***Biochem Pharmacol*** 30: 390-392.

Wigaeus E, Löf A, Bjurstrom R, Nordqvist MB (1983). Exposure to styrene, uptake, distribution, metabolism, and elimination in man. ***Scand J Work Environ Health*** 9: 479-488.

Withey JR (1976). Quantitative analysis of styrene monomer in polystyrene and foods including some preliminary studies of the uptake and pharmacodynamics of the monomer in rats. ***Environ Health Perspect*** 17: 125-133.

Wolf MA, Rowe VK, McCollister DD, Hollingsworth RL, Oyen F (1956). Toxicological studies of certain alkylated benzenes and benzene. ***AMA Arch Ind Health*** 14: 387-398.

Yager JW, Paradisin WM and Rappaport SM (1993). Sister-chromatid exchanges in lymphocytes are increased in relation to longitudinally measured occupational exposure to low concentrations of styrene. ***Mutat Res*** 319: 155-165.

Zagoren JC, Seelig M, Bornstein MB, Spencer PS (1984). The evolution of cellular degeneration in dorsal root ganglia exposed to doxorubicin in tissue culture. ***J Neuropath Exp Neurol*** 43: 384-394.

Zaidi NF, Agrawal AK, Srivastava SP (1985). Effect of styrene oxide on dopamine receptors in rats. ***Bull Environ Contamin Toxicol*** 35: 602-607.

Zhang XX, Chakrabarti S, Malick AM, Richer CL (1993). Effects of different styrene metabolites on cytotoxicity, sister-chromatid exchanges and cell-cycle kinetics in human whole blood lymphocytes *in vitro*. ***Mutat Res*** 302: 213-218.

APPENDIX

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S9 Liver Fraction is Cytotoxic to Neurons in Dissociated Culture

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Abstract JUDITH KOHN AND HEATHER D DURHAM S9 Liver Fraction is Cytotoxic to Neurons in Dissociated Culture *Neurotoxicology* 14(4) 381-386, 1993 *Methods to evaluate the neurotoxicity of chemicals in vitro must take into account that many xenobiotics exert their toxic effects through metabolites S9 fraction of liver homogenate has been used in cultures of bacterial and mammalian cells as a system for metabolic activation The suitability of the S9 activation system for long-term neurotoxicity studies in vitro was investigated in dissociated cultures of murine spinal cord-dorsal root ganglia. Exposure to amounts of S9 greater than 0 07 mg S9 protein/ml of culture medium for 4 days or longer was cytotoxic to all types of neurons in the cultures Non neuronal cells tolerated higher exposures, but contained numerous cytoplasmic inclusions when 0 35 mg S9 protein was included in the medium. It was demonstrated that cytotoxicity was caused by the particulate, microsomal fraction of S9 It is concluded that direct incorporation of S9 fraction in the growth medium (0 07 mg S9 protein/ml or greater) is not a suitable method of generating metabolites in dissociated cultures of central nervous system when several days are required to elicit a biological response Cytotoxicity can be prevented by using tissue culture inserts to separate cultured cells from S9 particulate fraction by a microporous membrane* © *1993 Intox Press, Inc*

Key Words: In Vitro Models, Metabolism, P450, Neurotoxicity, Cytotoxicity, Cell Culture, Activation Systems

INTRODUCTION

An important consideration in development of *in vitro* techniques for evaluation of the neurotoxic potential of xenobiotics is that many toxic effects of chemicals on the nervous system are mediated through metabolites rather than the parent compound Various *in vitro* activation systems have been developed based on the premise that the most important system in the generation of metabolites in the intact animal is the P450 mixed function oxidase system The most widely used preparation is the 9,000 x g supernatant of homogenized liver (S9) (Maron and Ames, 1983) Although developed for use with the Salmonella mutagenicity test, S9 fraction has been used successfully to generate metabolites in cultures of mammalian cells It has been demonstrated that inclusion of S9 in the culture medium for periods ranging from 30 min to two days is compatible with survival of dissociated cultures of various non-neuronal cells (Borenfreund and Puerner, 1987, Fry, 1982, Krahn and Heidelberger, 1977, Machanoff *et al*, 1981, Phillips, 1974, Pratt and Willis, 1985, Weinstein *et al*, 1977), aggregate cultures of fetal rat brain (Segal and Fedoroff, 1989) and whole embryo cultures (Hales and Slott, 1987, Kitchin *et al*, 1981) With some chemicals, longer exposures of up to several days may be required to distinguish generalized cytotoxic from specific neurotoxic effects, particularly when morphological endpoints are used (Durham, 1990, Veronesi, 1992).

We have investigated the suitability of S9 fraction as an activation system for long term use in cultures of neuronal cells Since cultures of dissociated, embryonic nervous tissue are commonly used for neurotoxicity studies in our laboratory and others, we have utilized dissociated cultures of murine E13 spinal cord-dorsal root ganglia (DRG) as the culture model

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MATERIALS AND METHODS

Induction of Rat Liver Enzymes

Three days before preparation of the S9 fraction 7-8 week old male Sprague Dawley rats (Charles River, Canada, St Constant, QUE) were pretreated with 80 mg/kg sc ß-Naphthoflavone (Aldrich Chemical Co., Milwaukee, WI) dissolved in corn oil, and 50 mg/kg ip phenobarbitone (Allen and Hanburys, Toronto, ONT, Canada) as inducers of P450 enzymes. Administration of phenobarbitone was repeated 24, and 48 hr after the initial treatment.

Preparation of S9 Fraction

S9 fraction was prepared according to the method of Maron and Ames (1983). Twelve hr before sacrifice, food but not water was removed. Twenty-four hr after the last injection, rats were killed by an overdose of sodium pentobarbital (Sigma Chemical Company, St. Louis, Missouri) and intracardiac perfusion was carried out using cold, sterile isotonic saline in order to remove residual blood from the livers. Livers were excised aseptically, weighed, and washed three times in cold, sterile 0.15 M KCl. All solutions, glassware, and instruments were sterile, and maintained at 0 to 4°C. Washed livers were placed in a beaker containing 3 volumes of KCl, finely minced with scissors, and homogenized with a Potter-Elvehjem homogenizer, using a teflon pestle. The homogenate was centrifuged for 10 min at 9000 x g in a Sorval RC-5B centrifuge and the supernatant (S9 fraction) was removed. The preparation was kept on ice, and used within 2 hours. Unused portions were aliquoted promptly, and stored at -70°C.

Measurement of Cytochrome P450

Cytochrome P450 content of the S9 preparation was determined according to the method of Omura and Sato (1964), by spectrophotometric measurement of the difference spectrum (450-480 ηm) after conversion to its carbon monoxide derivative form. Test samples of S9 were bubbled with carbon monoxide, reduced with sodium dithionite (BDH, QUE, Canada), and rebubbled with carbon monoxide. Reference samples were treated the same way, except they were not exposed to carbon monoxide. Protein concentration was determined using the Pierce BCA Protein Assay (Professional Diagnostic Inc., Montreal, QUE, Canada).

Preparation and Treatment of Cultures

Primary cultures of dissociated spinal cord and DRG were prepared from E13 mouse embryos. Spinal cords with DRG attached were excised, finely minced, and enzymatically dissociated using 0.25% trypsin. The tissue was then further dissociated mechanically by trituration. Cells were plated at a density of 175,000 per well in Nunclon 4-well culture dishes (Gibco Canada Inc., Burlington, ONT) containing 13 mm diameter round glass coverslips (J.B. EM Services, St. Laurent, QUE, Canada) coated with poly-D-lysine (Collaborative Research Inc., Bedford, Massachusetts). On day 4, cultures were treated for 24 hr with cytosine-ß-D-arabinofuranoside (Calbiochem-Behring Corp., La Jolla, California), to reduce proliferation of non-neuronal cells. Cultures were maintained in N3 medium plus 3% horse serum as previously described (Durham, 1988) and were used for experiments 5-8 weeks post-plating. Cells were treated for up to 19 days with 1, 2, 5, 10, 15, and 30 µl S9 fraction/ml media.

In other experiments, cultures received the same amounts of S9 fraction, contained within Nunc 10 mm tissue culture inserts (Gibco). Cultures on coverslips were transferred into 35mm glass culture dishes (Bellco Glass Incorporated, Vineland, New Jersey). An insert was positioned in each dish beside two coverslips, the S9 fraction was placed inside the inserts. The microporous ANOPORE® membrane that forms the bottom of these inserts, has been designed to allow free exchange of fluid and soluble molecules between the culture dish and the inserts. Any soluble S9 components would be free to diffuse out of the insert into the growth medium surrounding the cells, whereas the particulate fraction would be retained within the insert.

Neuronal and non-neuronal cells were examined daily by phase contrast microscopy for generalized cytotoxic manifestations. Morphological endpoints included cell membrane blebbing, vacuolation, granularity, detachment from the substratum, and cell death. On day 4, loss of cell viability was assayed by nuclear uptake of ethidium homodimer (Molecular Probes Incorporated, Eugene, Oregon). Cultures were treated with 4.0 µM ethidium homodimer in minimum essential medium (Gibco) for 15 minutes and examined by epifluorescence microscopy for nuclear uptake of the ethidium reagent. Observations were made in duplicate cultures, in each of 3 separate experiments.

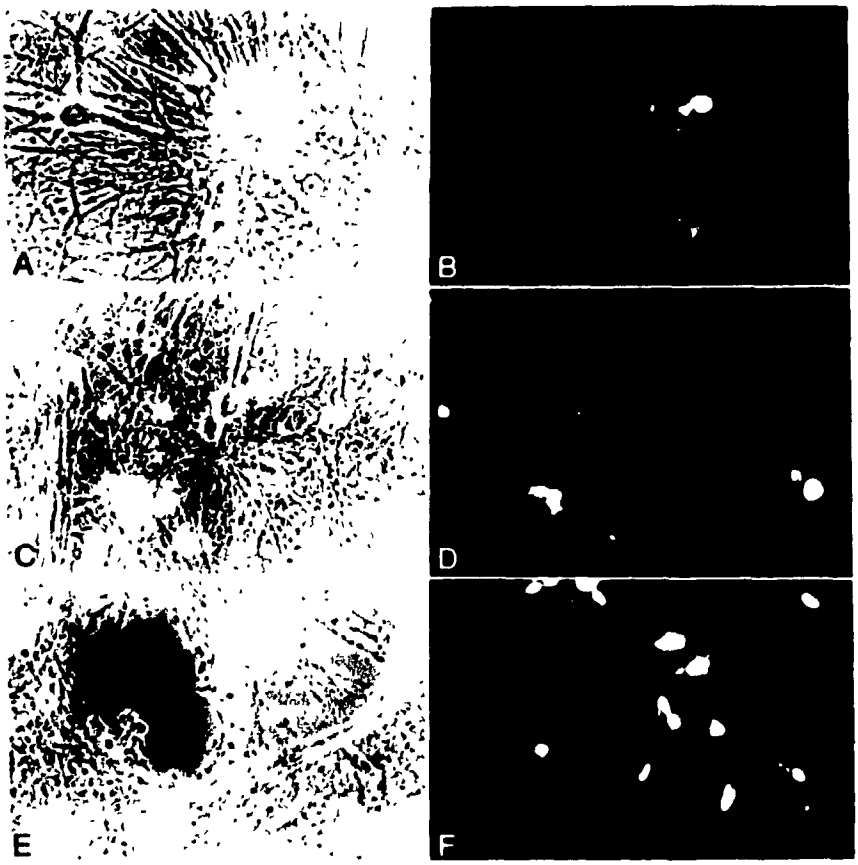

FIG 1. (A, C, E) Phase contrast micrographs of dissociated spinal cord-DRG cultures 4 days after addition of S9 fraction to the medium, and (B, D, F) fluorescence micrographs of ethidium homodimer uptake into nuclei of the same cultures (A, B) Untreated control culture (C, D) Culture receiving 5 µl S9/ml medium (E, F) Culture exposed to 10 µl S9/ml medium Scale bar = 30 µm

RESULTS

The protein concentration of the S9 fraction was 33 mg/ml. Therefore, 1, 2, 5, 10, 15 and 30 µl S9/ml of culture medium contained 0 035, 0.07, 0 17, 0 35, 0.7, and 1.05 mg protein/ml of medium, respectively P450 content was measured as 0.62 ηM P450/mg protein.

Addition of S9 fraction directly to the culture medium induced a dose- and time-dependent cytotoxic response, particularly in neuronal cells Fig 1 illustrates morphology of cells by phase microscopy, and ethidium uptake in cultures exposed to 0, 5 and 10 µl S9/ml of medium for 4 days Cultures exposed to 1 or 2 µl of S9/ml of medium (not shown) were similar to control cultures (Fig. 1A, B) In cultures exposed to 5 µl S9/ml medium, the neuronal processes were granular and irregular in shape (Fig 1C). Uptake of ethidium into nuclei was observed in small clumps of degenerating cells (Fig. 1C, D) Background, non-neuronal cells contained phase-dense inclusions, but remained viable as demonstrated by absence of ethidium uptake (Fig 1D) In cultures exposed to 10 µl S9/ml medium (Fig 1E, F), cells of all types were full of granular inclusions and vacuoles, and were beginning to detach from the substratum Comparable results were observed with two other batches of S9, prepared from different animals

Cultures directly exposed to 5 µl S9/ml for 11 days resembled those exposed to 10 µl S9/ml for 4 days Signs of neuronal cytotoxicity (granular processes, intracellular inclusions) also were observed in cultures exposed to 2 µl S9/ml for 19 days

In other experiments, similar cytotoxicity of S9 was observed when nicotinamide adenine dinucleotide phosphate (NADP) and R-D-glucose-6 phosphate (G-6-P), the cofactors for microsomal enzymes, were also included in the culture medium (final concentrations were 4 mM NADP and 5 mM G-6-P, both supplied by Aldrich)

Fig 2 illustrates the protective effect of tissue culture inserts against S9-induced cytotoxicity Addition of 15 µl S9/ml directly to the culture medium resulted

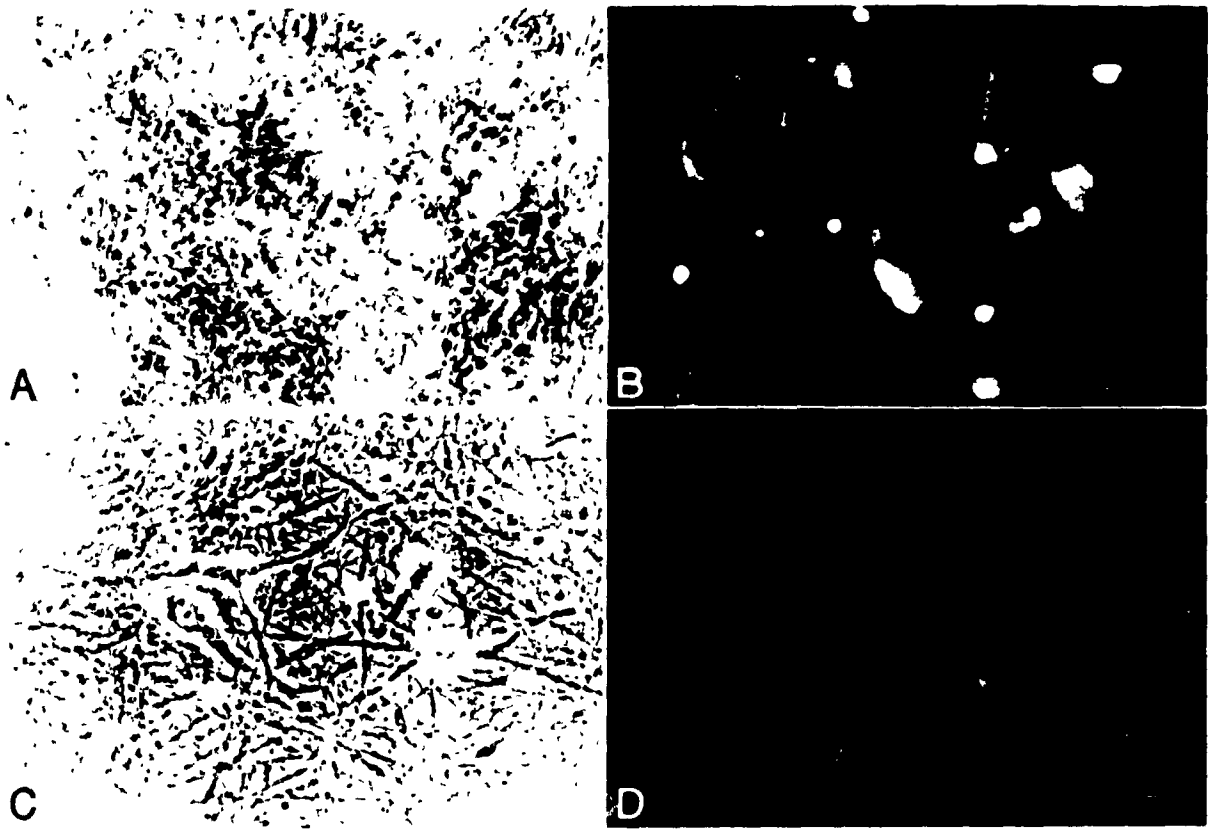

FIG 2. Protective effect of tissue culture inserts against S9 induced cytotoxicity in dissociated spinal cord-DRG cultures (A) phase contrast micrograph of a culture exposed to 15 µl S9/ml in the medium for 4 days, and (C) a culture receiving the same volume of S9 added to a tissue culture insert (B, D) ethidium uptake into nuclei of the same cells illustrated in A and C, respectively Scale bar = 30 µM

in generalized cytotoxicity and cell death within 4 days (Fig. 2A, B) When the same volume of S9 was contained within a tissue culture insert, no morphological signs of cytotoxicity or significant uptake of ethidium were observed (Fig 2C, D)

DISCUSSION

In dissociated cultures of fetal mouse spinal cord-DRG, more than 2 µl of S9 fraction/ml of medium [equivalent to 0.07 mg S9 protein/ml] was cytotoxic to neuronal cells when incubation times were longer than 4 days and the S9 fraction was placed directly into the growth medium This volume is below the concentrations previously used for shorter durations in other culture systems [0 1 to 4 mg S9/ml] (Borenfreund and Puerner, 1987, Fry, 1982, Hales and Slott, 1987, Kitchin *et al*, 1981, Krahn and Heidelberger, 1977, Machanoff *et al*, 1981, Phillips, 1974; Pratt and Willis, 1985, Segal and Fedorott, 1989; Weinstein *et al*, 1977) The appropriate amount of S9 to use depends on the enzyme activity per mg of protein and on the concentration of xenobiotic to be metabolized Generally, 0.15 to 0.4 mg S9/ml have been shown to induce sufficient metabolism to achieve measurable biological effects of metabolites in bacterial and mammalian cell culture systems: In the study by Maron and Ames (1983), 0.15 mg S9/ml was the minimal amount required to achieve measurable metabolic conversion of chemicals to mutagens in the plate incorporation test for mutagenicity Machanoff *et al* (1981) found that 0 1 to 0 2 mg S9/ml was required to increase the mutation frequency in Chinese hamster ovary cells in the presence of benzo[a]pyrene These amounts of S9 were cytotoxic to neurons in our dissociated cultures within 4 days unless the S9 fraction was contained within tissue culture inserts

The toxicity of S9 appeared to be related to its particulate nature, rather than a soluble component The number of intracellular inclusions increased with the duration of exposure to S9 and the degree of cytotoxicity Cytotoxicity, including increase in intracel-

lular inclusions, was prevented by containing S9 fraction within tissue culture inserts. Under these conditions, the cultures were exposed only to soluble factors that could permeate the microporous membrane, and not to particulate constituents of S9. That similar results were obtained using the 105,000 x g microsomal pellet, also indicates that the microsomes, not a soluble component of S9, was responsible for the cytotoxicity (data not shown).

Cells in dissociated culture may be more vulnerable to cytotoxicity induced by particulate components than are those in organotypic or aggregate cultures. Chick embryo extract, which is particulate in nature, induces intracellular inclusions and cytotoxicity when incorporated into the medium of dissociated spinal cord cultures (Durham, unpublished observations); however, 10% chick embryo extract has been used without toxicity in long-term organotypic cultures of nervous tissue (Veronesi *et al.*, 1983).

Neuronal cells in the mixed cultures were more sensitive to S9 than were non-neuronal cells such as astrocytes and fibroblasts, but higher concentrations of S9 [0.35 mg/ml] were toxic to these cell types as well. This is consistent with the findings of others. Borenfreund and Puerner (1987) reported that 0.25 mg S9/ml for 18 hr induced some toxicity in cultured 3T3 fibroblasts. Cooper and Goldstein (1976) found that cultures of human skin fibroblasts exposed to 105,000 x g liver microsomal fraction showed some morphological changes at the end of 18 hr and grew markedly more slowly than untreated cultures upon refeeding with growth medium.

It is concluded that addition of S9 liver fraction directly to the culture medium is not a suitable method of generating metabolites in dissociated neuronal cultures because of toxicity induced by the particulate fraction. This problem can be overcome by separating the microsomes from the cells by a microporous membrane. Alternatives such as co-culture with immortalized hepatocyte lines (Bayard *et al.*, 1991) or cell lines transfected with cDNAs encoding human P450 enzymes (Crespi *et al.*, 1990; Gonzalez and Gelboin, 1991) may also be appropriate for long term studies of xenobiotic metabolism in cultures of the nervous system.

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REFERENCES

Bayard J, Bagrel D, Sabolovic N, Magdolou J, Siest G. Expression and regulation of drug metabolizing enzymes in an immortalized rat hepatocyte cell line *Biochem Pharmacol* 1991, 42.1345-1351

Borenfreund E, Puerner JA. Short-term quantitative in vitro cytotoxicity assay involving an S-9 activating system *Cancer Lett* 1987, 34 243-248

Cooper JT, Goldstein S. Toxicity testing *in vitro* II Use of a microsome-cultured human fibroblast system to study the cytotoxicity of cyclophosphamide *Can J Physiol Pharmacol* 1976, 54 546-550

Crespi CL, Langenbach R, Penman BW. The development of a panel of human cell lines expressing specific human cytochrome P450 cDNAs *Prog Clin Biol Res* 1990, 340B:97-106

Durham HD. Aggregation of intermediate filaments by 2,5-hexanedione. comparison of effects on neurofilaments, GFAP-filaments and vimentin-filaments in dissociated cultures of mouse spinal cord-dorsal root ganglia. *J Neuropathol Exp Neurol* 1988, 47:432-442

Durham HD. Endpoints for assessment of neurotoxicity in cultured cells. *Neurotoxicology* 1990, 11:145

Fry JR. Activation systems in tissue culture toxicity studies. *Toxicology* 1982, 25 1-12

Gonzalez FJ, Gelboin HV. DNA-expressed human cytochrome P450s. a new age of molecular toxicology and human risk assessment *Mutat Res* 1991, 247:113-127

Hales BF, Slott VL. The role of reactive metabolites in drug-induced teratogenesis. *Prog Clin Biol Res* 1987; 253:181-191

Kitchin KT, Schmid BP, Sanyal MK. Teratogenicity of cyclophosphamide in a coupled microsomal activating/embryo culture system *Biochem Pharmacol* 1981; 30.59-64

Krahn DF, Heidelberger C. Liver homogenate-mediated mutagenesis in Chinese hamster V79 cells by polycyclic aromatic hydrocarbons and aflatoxins. *Mutat Res* 1977, 46 27-44

Machanoff R, O'Neill JP, Hsie AW. Quantitative analysis of cytotoxicity and mutagenicity of benzo[a]pyrene in mammalian cells (CHO/HGPRT system). *Chem-Biol Interactt* 1981, 34 1-10

Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test *Mutat Res* 1983, 113:173-215

Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes *J Biol Chem* 1964;239 2370-2378

Phillips BJ. A simple, small scale cytotoxicity test, and its uses in drug metabolism studies. *Biochem Pharmacol* 1974; 23:131-138

Pratt RM, Willis WD. *In vitro* screening assay for teratogens using growth inhibition of human embryonic cells. *Proc Natl Acad Sci USA* 1985; 82:5791-5794

Segal , LM, Fedoroff S. Cholinesterase inhibition by organophosphorus and carbamate pesticides in aggregate cultures of neural cells from the foetal rat brain: The effects of metabolic activation and pesticide mixtures. *Toxicol In Vitro* 1989; 3:123-128

Veronesi B, Peterson ER, Bornstein MB, Spencer PS. Ultrastructural studies of the dying-back process. VI. Examination of nerve fibers undergoing giant axonal degeneration in organotypic culture. *J Neuropathol Exp Neurol* 1983; 42:153-65

Veronesi B. *In vitro* screening batteries for neurotoxicants. *Neurotoxicology* 1992; 13:185-196

Weinstein D, Katz ML, Kazmer S. Chromosomal effects of carcinogens and non-carcinogens on WI-38 after short term exposures with and without metabolic activation. *Mutat Res* 1977; 46:297-304