

Fig. 4B.



Fig. 4C.

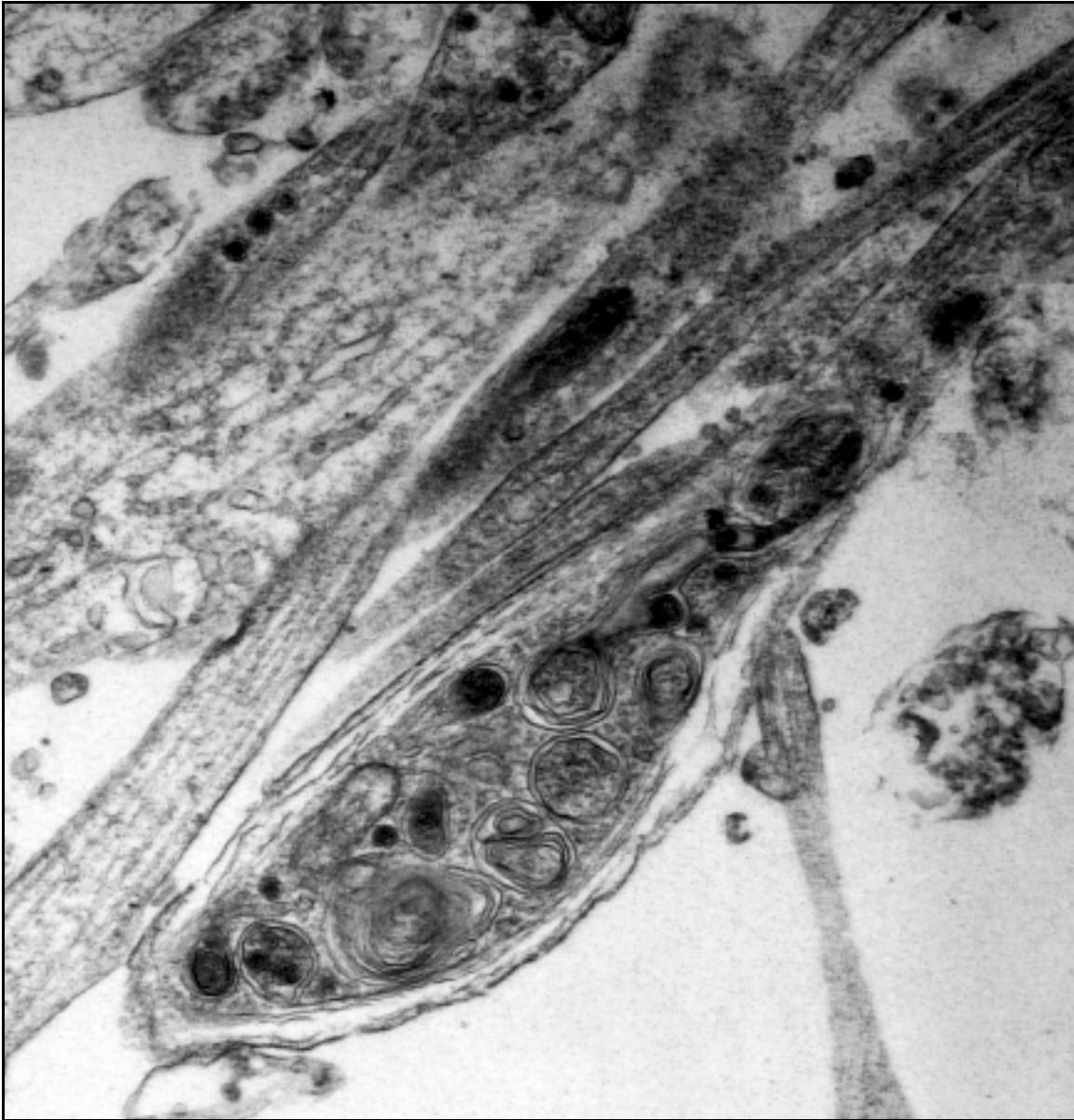


Fig. 4D.

GENERAL DISCUSSION AND CONCLUSIONS

PART V: CHAPTER 7

DISCUSSION

The present study is the first time that the effects of neuropathy-inducing OP compounds were determined using chick DRG neuronal cell cultures as an *in vitro* model system for study of pathogenic mechanisms associated with OPIDN. The adult hen is considered the most reliable animal model for the study of OPIDN (Abou-Donia, 1981, 1986; US EPA, 1991); therefore, this *in vitro* neuronal cell model is unique because it allows investigation of OP compounds in a target species. The DRG, although removed from chicks, which are less susceptible to OPIDN than adults (Funk *et al.*, 1994), could be used to measure axonal growth cones in relatively mature (14 days) neuronal cultures. These cultures developed very long neurites (axons) which were much longer than those seen in immortalized cell lines (Taylor *et al.*, 1995; Argiro *et al.*, 1985; Castellanos *et al.*, 2001; Doane *et al.*, 1992; Kawa *et al.*, 1988; Oorshot *et al.*, 1991; Saxod and Bizet, 1988; Nishiyama *et al.*, 1994; Antuned-Madeira *et al.*, 1994; Pope *et al.*, 1995). The DRG cell model is, therefore, more appropriate for study of the effects of neuropathy-inducing compounds because immortalized cell lines do not allow sufficient neurite outgrowth to truly represent axonal degeneration observed *in vivo*. In fact, there have been some questions whether all neurites of cell lines contain the same cytoskeletal elements as do true axons (Taylor *et al.*, 1995). This primary cell culture preparation is the closest *in vitro* representation for neuronal effects occurring *in vivo*; therefore, DRG cell cultures should be used in the future to extrapolate morphological alterations and mechanisms of axonal damage of OPIDN at the cellular level to events occurring *in vivo*. However, in future studies, the dosing paradigm may need alterations from the 12-hr exposure to 1 μ M used in the studies reported here. Higher dosages or more prolonged exposure may be more appropriate since chicks are not as susceptible as adult to the effects of neuropathy-inducing OP compounds (Funk *et al.*, 1994).

The present study verifies the hypothesis that neuropathy-inducing OP compounds modify the energy production. This was first noted *in vivo* (Part IV, chapter 3), when ATP production was demonstrated to be altered in sciatic nerves of hens given PSP. This was also noted *in vitro*, using DRG cell cultures (Part IV, chapter 5). ATP production and the structure of mitochondria responsible for producing ATP were altered. These mitochondrial changes could lead to axonal degeneration in these high energy-demanding cells. Mitochondrial effects of neuropathy-inducing OP compounds have not previously been extensively explored in neuronal cell cultures from the US EPA-approved target species (US EPA, 1991). This study, therefore, provides new insight on the early mechanisms involved in the development of OPIDN.

The alterations in mitochondrial functions following exposure to neuropathy-inducing OP compounds included disruption of electron transport chain reactions (Part IV, chapter 5) and opening of mitochondrial permeability transition pores (Part IV, chapter 4), as proposed in the statement of hypothesis for this dissertation. These mitochondrial modifications are likely to be the responsible for rapid fluctuations in mitochondrial membrane potentials ($\Delta\Psi_m$), and variation of ionic gradient across its membrane, which resulted in a wide range of mitochondrial matrix swelling that was seen (Part IV, chapter 6). This study strongly suggests that alterations in mitochondrial functions were crucial for the ultimate well-described neuronal degeneration observed in OPIDN.

The findings reported in this study confirm that OP compounds causing delayed neuropathy reduced ATP production and MPT 12 hours post-exposure. This time-point was chosen for both methods of evaluation since it was the same time as when mitochondrial Ψ_m alterations were detected in previous studies using SH-SY5Y cells (Carlson and Ehrich, 1999). The lower energy state seen in this study can be explained by depolarization of the mitochondrial membrane potentials observed by Carlson and Ehrich

(1999). In our study, alterations in ATP concentrations occurred as early as 12 hours post-exposure to OP compounds, which is considerably earlier than morphological alterations measured in sciatic nerves of hens treated with neuropathy-inducing OP compounds (Massicotte *et al.*, 2001, part IV, chapter 3). It is also earlier than the time points at which OP-exposed DRG cultures were examined for morphological changes (Part IV, chapter 6). In studies done both *in vivo* and *in vitro*, modifications in ATP concentrations suggested dysregulation of mitochondrial-associated cellular energy production.

Results reported in this study support other *in vitro* and *in vivo* models that demonstrated the toxic effects of OP compounds on mitochondria (Carlson and Ehrich, 1999; Mochida *et al.*, 1988; Veronesi *et al.*, 1993). This study is unique in that it specifically evaluates the mitochondrial respiratory chain as an intracellular target for physiological concentrations of neuropathy-inducing compounds in living cells. These alterations in energy metabolism demonstrated in our investigation support other studies showing variations in mitochondrial energy metabolism as an effect of exposure to toxicants. For example, mitochondrial membrane depolarization (Holmuhamedov *et al.*, 1996; Carlson and Ehrich, 1999; Poppe *et al.*, 2001), inhibition of mitochondrial oxygen consumption and respiration, and reduction of oxidative phosphorylation have been documented following exposure to OP compounds and other toxicants (Spetale *et al.*, 1976; Sitkiewick *et al.*, 1980; Skonieczna *et al.*, 1980). Mitochondrial membrane depolarization could modify the H⁺ proton pump across the mitochondrial membrane, causing failure of ATP coupling, and disruption of the mitochondrial respiratory chain reactions (Rustin *et al.*, 1994). Other metabolic pathways affected by toxicants include oxidation of succinate, α -glycerophosphate, and pyruvate/malate substrates, all of which are ATP dependent reactions (Holmuhamedov *et al.*, 1996). Therefore, it is likely that the alterations in ATP concentrations observed in our study are relevant events that could contribute to the pathogenesis of OPIDN observed *in vivo*.

Moreover, results reported in this study demonstrate that effects on MPT were unique to neurons (Part IV, chapter 4), which also supports other *in vivo* studies reporting primary neuronal lesions in OPIDN (Ehrich and Jortner, 2001). In Schwann cells, OP compounds used in this study failed to alter mitochondrial function because MPT and TMRM-induced mitochondrial fluorescence were similar in exposed and control cultures. Therefore, these findings demonstrate that mitochondrial structural changes are significant in chick embryo neuronal populations but minimal in Schwann cell cultures, as can be expected for neuropathy inducing OP compounds. This further supports the value of this culture system for studies of pathogenic mechanisms of OPIDN at the cellular level. In addition, MPT was unique to neuropathy-inducing OP-compounds since exposure of neuronal cells to paraoxon, an OP that does not cause OPIDN, failed to alter MPT. According to these results, mitochondria were a primary target in neuronal populations affected by OPIDN.

The current study used several endpoints to demonstrate the toxic effects of OP-compounds on mitochondria. For example, this study demonstrated that mitochondrial permeability transition (MPT) occurred in neuronal DRG cell cultures exposed to OP toxicants (Part IV, chapter 4). When mitochondria undergo MPT in a cell, ATP becomes progressively depleted secondary to uncoupling of oxidative phosphorylation and accelerated ATP hydrolysis by mitochondrial ATP synthase (Park *et al.*, 2001). ATP depletion has also been noted in other cell cultures exposed to other toxicants (Berman *et al.*, 2000; Crompton, 1999; Farber, 1982; Lemaster *et al.*, 1979, 1999; Lofrumento *et al.*, 1991; Parks *et al.*, 2001). Direct comparison of the effects of neuropathy-inducing OP compounds with effects of these toxicants is not possible at present, however, because complex-specific effects were not determined and studies were done in other cell systems under different conditions of exposure. Energy depletion can lead to mitochondrial swelling, with secondary opening of mitochondrial permeability transition pores. Alterations of MPT state has also been associated with the progression of apoptotic cell death, and the release of

apoptotic factors (Bernadi *et al.*, 1996; Cai *et al.*, 1998; Lemasters *et al.*, 1998; Qian *et al.*, 1999). Therefore, the low energy state observed in previous *in vivo* studies (Massicotte *et al.*, 2001, part IV, chapter 3) suggest that alteration of free ATP production could lead to cell death by necrosis and apoptosis, secondary to MPT alterations. Furthermore, opening of the MPT pore following exposure to neuropathy-inducing OP compounds causes uncoupling of mitochondrial respiration and profound ATP depletion (Part IV, chapter 5), suggesting the release of intramitochondrial ions and apoptotic proteins within the cytoplasm (Berman *et al.*, 2000; Crompton, 1999; Farber, 1982; Lemaster *et al.*, 1979, 1999; Lofrumento *et al.*, 1991; Massicotte *et al.*, 2001 a, b; Parks *et al.*, 2001). Therefore, we suggest that alteration in MPT structure is an early event involved in mechanisms contributing to mitochondrial swelling observed in neurons exposed to neuropathy-inducing OP compounds.

Electron and confocal microscopy performed on the neuronal DRG cell cultures used in this study provided high resolution of cellular structures in the perikarion and axons (Part IV, chapters 4 and 6). The high degree of resolution obtained with confocal microscopy permitted visualization of *in situ* cellular structures and mitochondrial function, including MPT. This study is also unique because it used morphometric measurements in a living neuronal DRG cell culture model (Part IV, chapter 6). Lesions of neuronal growth cones damaged by other treatments described in the literature were used as references for the present study (Bray and Bunge, 1981; Bunge and Bunge, 1984; Cheville, 1983; Dyck *et al.*, 1993; Fadic *et al.*, 1985; Fried *et al.*, 1970; Ghadially, 1988; Gray, 1975; Lee and Cleveland, 1994; Liem *et al.*, 1978; Peters *et al.*, 1991; Westrum *et al.*, 1976). Once more, our findings demonstrate that the mitochondria are a primary target in the development of OPIDN, using several morphological approaches.

CONCLUSIONS

In conclusion, the lesions that we observed in DRG cell cultures were similar to morphological changes described *in vivo* with OPIDN. DRG cultures appear to provide the closest *in vitro* model to evaluate the effects of neuropathy-inducing compounds since axonal structures were well developed.

This study demonstrated clearly that alterations in ATP production and morphological changes noted in nerves from exposed hens would also occur in primary neuronal cultures of chick embryos. Depletion in ATP concentration occurred early and only after exposure to compounds capable of causing OPIDN. Neuronal mitochondria were the primary site of injury, and these mitochondria were being targeted at several levels, including MPT pore and mitochondrial respiratory chain. We suggest that the induction MPT first caused rapid collapse of $\Delta\Psi_m$, followed by redistribution of soluble calcium within the mitochondrial matrix, mitochondrial swelling and release of apoptotic factors and proteases within the cytoplasm, in this order. Since MPT induces depolarization of $\Delta\Psi_m$, uncoupling of the respiratory chain can occur at any complex levels, especially complex-I as demonstrated in this study, resulting in alteration in mitochondrial ATP production. These mitochondrial changes could eventually lead to the axonal degeneration observed in OPIDN.

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PART VI

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PART VII: APPENDIX

APPENDIX 1: Percentage of NTE inhibition in chick DRG cell cultures

Percentage of NTE inhibition 24 hours post-exposure to PSP

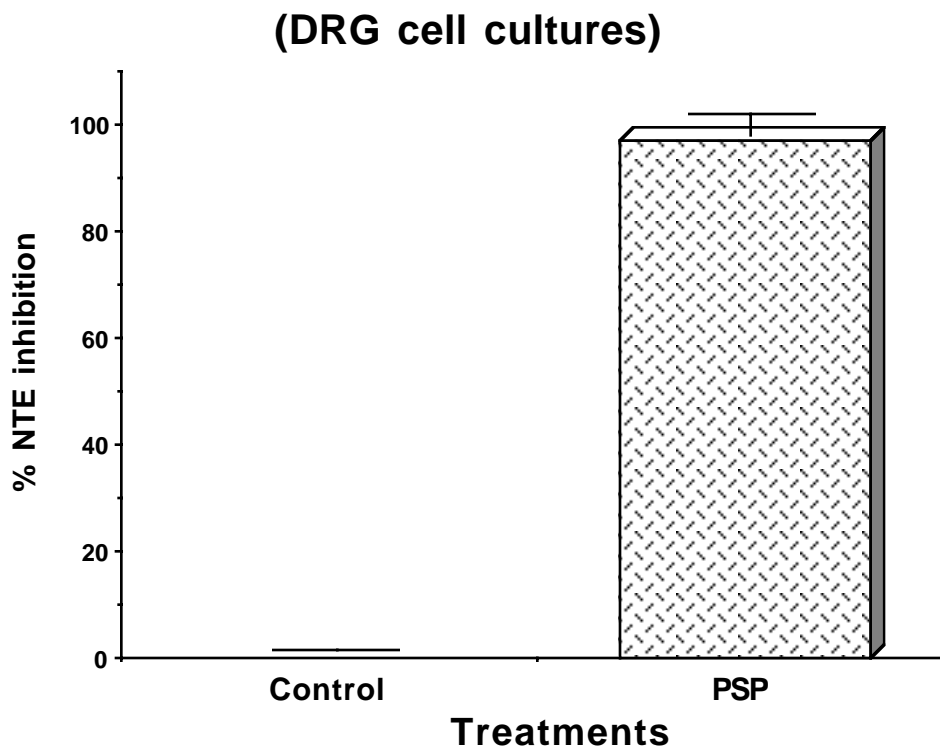


Fig. 1. Percentage of neuropathy target esterase (NTE) inhibition in chick dorsal root ganglia (DRG) 24 hours post-exposure to PSP. This graph shows that NTE activities were dramatically decreased in PSP treated DRG neuronal population compared to controls (mean \pm SEM). Acetylcholinesterase (AChE) was not inhibited by this compound.

PART VIII: VITA

Vita

Christiane Massicotte was born on June 3, 1970 in Montreal, Canada. After studying piano for 15 years, she obtained a Post-Laureate in piano interpretation in 1989. She then taught piano interpretation for 2 years at Vincent d'Indy (University of Montreal). She graduated from Jean de Brebeuf in May 1990 with an International Baccalaureate Degree in Science, Major in Biochemistry. Christiane then attended University of Montreal to pursue the Doctor in Veterinary Medicine program, and graduated in May, 1994. In 1994-1995 she was an intern at the Veterinary Teaching Hospital of the University of Minnesota. Following this internship (in 1995), she entered a neurology residency in veterinary medicine and obtained a MS degree in Veterinary Medical Sciences at VA-MD Regional College of Veterinary Medicine in 1998. Recently, she obtained her board certification from ACVIM (neurology), and will be pursuing a career as clinical neurologist and neurotoxicology researcher at the University of Pennsylvania, School of Veterinary Medicine.