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Triphenyl Phosphite-Induced Ultrastructural Changes in Bovine Adrenomedullary Chromaffin Cells

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Primary cultures of bovine adrenomedullary chromaffin cells were treated with the phosphorus acid ester triphenyl phosphite (TPP), a chemical capable of producing Type II organophosphorus compound-induced delayed neurotoxicity (OPIDN), and the morphological changes were assessed by transmission electron and scanning microscopy. Following a 24-hr incubation with 100 μM TPP nearly all mitochondria were either disrupted or swollen and glycogen buildup within the cytoplasm was evident. The viability of cells treated with TPP and cultured on coverslips for scanning electron microscopy was very low. By scanning electron microscopy, the filopodia of these cells appeared contracted. The surface texture was very irregular and giant globular bodies were evident. Parallel studies were carried out with the cholinergic compound *O,O*-diethyl 4-nitrophenyl phosphate (paraoxon) and the Type I delayed neurotoxicant *O,O*-diisopropylphosphorofluoridate (DFP). Transmission and scanning electron microscopy revealed that treatment with these organophosphorus compounds did not produce the ultrastructural effects that were seen with TPP. The morphological data were confirmed biochemically by assessing the viability of the mitochondria via measurement of [^{3}H]adenosine incorporation into ATP. Treatment with 100 μM TPP for 4 or 24 hr caused a marked inhibition (90% relative to controls) of adenosine incorporation. Neither 100 μM paraoxon nor 100 μM DFP had an inhibitory effect on incorporation. The effect of TPP was time-dependent with significant biochemical effects as early as 60 min. In contrast, ultrastructural changes were not seen until 24 hr. Morphologically, the 60-min incubations showed no perturbation in mitochondrial integrity. Our results support a specific effect of the triphenylphosphite, TPP, a Type II OPIDN compound, not a general toxic effect of organophosphorus compounds since the cholinergic agent paraoxon and the Type I delayed neurotoxic compound DFP did not alter the cells ultrastructurally or compromise the mitochondria biochemically. The apparent target for TPP toxicity is the mitochondria. © 1992 Academic Press, Inc.

INTRODUCTION

The organophosphorus acid ester triphenyl phosphite (TPP) is currently used in both industry (US EPA, 1986) and agriculture and has long been known to produce delayed neurotoxicity in experimental animals. TPP was originally shown by Smith and colleagues (Smith *et al.*, 1930; Lillie and Smith, 1932; Smith *et al.*, 1933) to be neurotoxic to both cats and rats following a single sc injection or chronic dosing, respectively. Clinical signs in the cat are manifested as ataxia and paresis accompanied by CNS degeneration (Smith *et al.*, 1933); whereas, rats display dysfunctions such as hind limb paralysis and circling (Veronesi *et al.*, 1986). Neuropathological damage in the rat consisted of degeneration of the ventrolateral and ventral columns of the spinal cord as well as some moderate peripheral nerve damage (Veronesi and Dvergsten, 1987). More recently, a single sc dose of TPP was neurotoxic in the hen (Carrington and Abou-Donia, 1988; Carrington *et al.*, 1988). Pathognomonic of organophosphorus compound-induced delayed neurotoxicity (OPIDN), TPP produced ataxia and pathological lesions in both the CNS and the PNS following a delay period of approximately 6–14 days. The histopathological lesions produced by TPP were, however, also characterized by neuronal necrosis in the spinal gray matter and swollen axons in the brain stem (Tanaka *et al.*, 1990), thus indicating that TPP affects the cell body as well as the axon. Since delayed neurotoxicity induced by tri-*o*-cresyl phosphate (Smith *et al.*, 1930, Abou-Donia, 1981) is distinct from that produced by TPP, they have been segregated into two classes of OPIDN: Type I and Type II, respectively (Abou-Donia and Lapadula, 1990; Abou-Donia, 1991).

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To investigate TPP's neurotoxic effect on the cell body in isolation we used primary cultures of bovine adrenomedullary cells. Chromaffin cells develop from the neural crest stem cell and have often been viewed as a truncated sympathetic neuron because they lack axonal-like projections (Anderson and Abou-Donia, 1991). Consequently, the chromaffin cell provides an exemplary system for studying the morphology of the cell body and the physiologically related synaptic events in isolation (Livett, 1984).

Until recently, TPP's effect on a mechanistic level had only been investigated with regard to its inhibition of the enzyme neurotoxic esterase (NTE) (Padilla *et al.*, 1987; Carrington and Abou-Donia, 1988). NTE was originally proposed as the putative target for the neurotoxic compounds which produce OPIDN (Johnson, 1982). Although TPP has been shown to significantly inhibit NTE in the hen (Carrington and Abou-Donia, 1988), it only minimally inhibits NTE in the rat (Veronesi *et al.*, 1986).

Konno *et al.* (1989) investigated the distribution and pharmokinetics of TPP in various tissues and assessed its effect on various mitochondrial enzymes in hen skeletal muscle following iv treatment. They found that both creatine kinase and succinate dehydrogenase activities in mitochondria isolated from the adductor magnus and soleus muscle were inhibited significantly 24 to 48 hr postdosing. Creatine kinase has been implicated in energy transfer from mitochondria to the myofibrils in skeletal muscle (Apple and Rogers, 1986). Inhibition of the enzymatic activity associated with these proteins undoubtedly adversely affects intracellular energy transport in skeletal muscle. On the basis of their finding, they suggested that a likely target for TPP toxicity is the mitochondria (Konno *et al.*, 1989).

We recently found that TPP inhibits the exocytotic secretion of catecholamines from chromaffin cells in both a time- and a dose-dependent manner (Abou-Donia and Knoth, 1989). Although maximum inhibition of secretion is seen with nicotine, inhibition is independent of the choice of secretagogue. Also, TPP inhibited calcium uptake into the cells when the cells were stimulated with the secretagogue, nicotine, or high potassium (Abou-Donia *et al.*, 1990). An in-

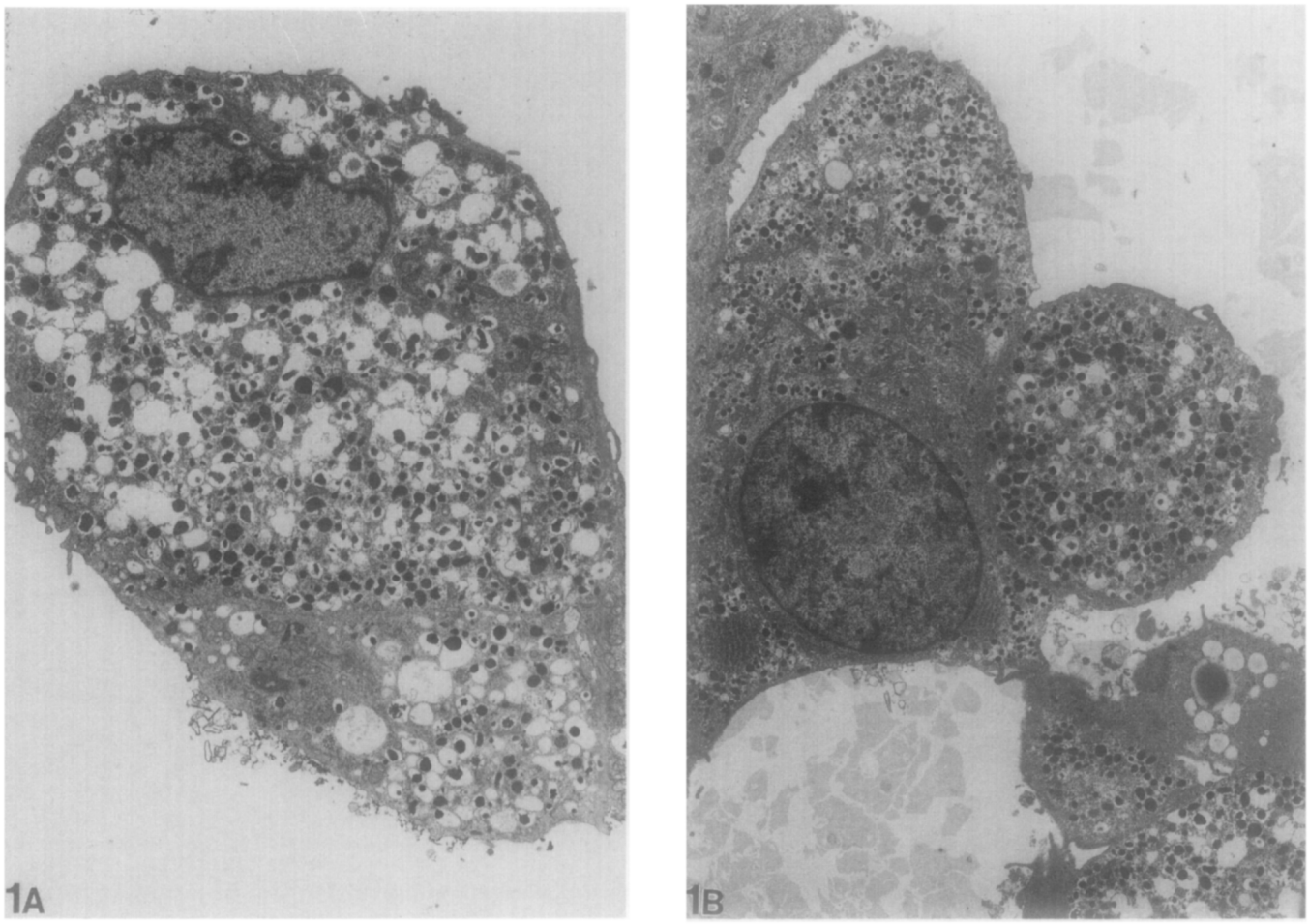

FIG. 1. Electron micrographs of bovine adrenomedullary chromaffin cells 4 days in culture. Noradrenaline-containing cells (A) are differentiated from adrenaline-containing cells (B) by the shape and electron density of their storage vesicles. Electron microscopy; (A) 4320× (B) 3020×.

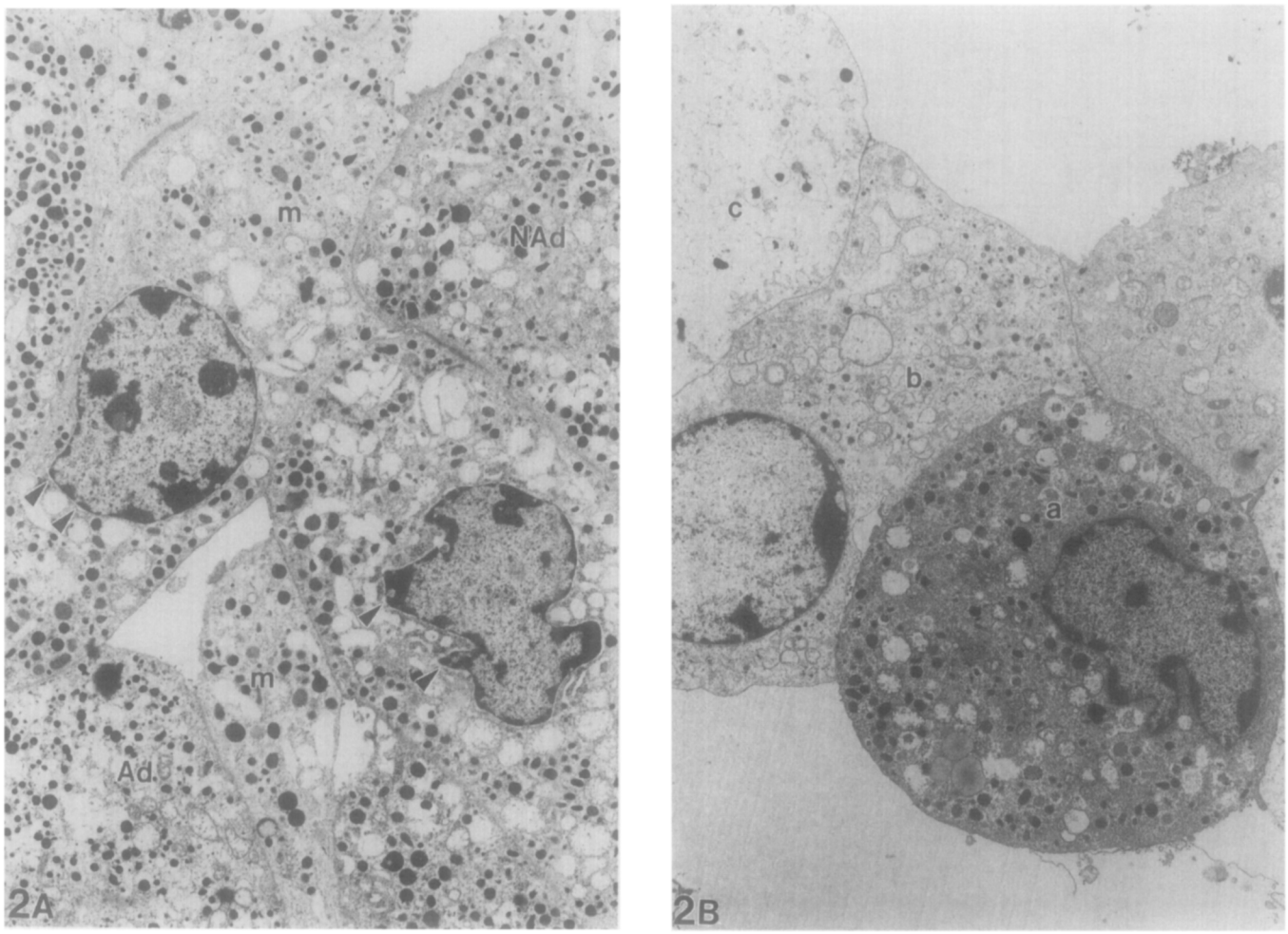

FIG. 2. Chromaffin cells treated for 24 hr with TPP displayed massive disruption of the mitochondria (m). (A) Damaged mitochondria were seen in both adrenergic (Ad) and noradrenergic (NAd) cells. A nuclear clearing (arrowheads) often characterized TPP-treated cells. (B) Chromaffin cells appeared differentially affected by TPP. Although all cells displayed disrupted mitochondria, they varied with respect to the depletion of their secretory vesicles. Some cells retained their vesicles (a) while other cells appeared partially depleted of their vesicles (b) while the remaining appeared necrotic (c). Electron microscopy; (A) 3890× (B) 4320×.

crease in intracellular calcium is critical to the secretory process (Knight, 1986). Thus, one possible mechanism by which TPP operates is via interference with calcium homeostasis. This study was designed to define the ultrastructural histopathologic lesion which corresponds to TPP's adverse biochemical effects on the cell body. A preliminary account of this study has been presented (Anderson *et al.*, 1991).

METHODS

Cell Culturing

Chromaffin cells were isolated from bovine adrenal medullar and maintained in primary cultures according to the methods of Wilson and Kirshner (1983). Cells were plated at a density of 5.2×10^5 cells/cm^2 in 50% Dulbecco's modified Eagle's medium, 50% F12 nutrient mixture, 5 mM Hepes, 10% fetal bovine serum, and antibiotics (100 units/ml penicillin, 40 μg/ml gentamycin, 0.1% amikacin). Serum levels were reduced to 1% on Day 2 after plating to minimize fibroblast growth and to maximize drug treatment efficacy. Cell cultures were maintained at 37°C in a 5% CO_2/95% air mixture. Experiments were performed 4–10 days following plating.

Electron Microscopy

Transmission electron microscopy. Cells, cultured on six well plates, were treated at 37°C with 100 μM TPP, *O,O*-diisopropylphosphorofluoridate (DFP), *O,O*-diethyl 4-nitrophenyl phosphate (paraoxon), or 1% vehicle (ethanol, H_2O, DMSO, respectively) for either 24 or 1 hr. The medium was removed by aspiration and the cells were washed three times with 0.1 M cacodylate (dimethylarsinate) buffer, pH 7.4. Cells were fixed with 2.5% glutaraldehyde (v/v) in 0.1 M cacodylate buffer for 1 hr at 4°C. Cells were postfixed with Dalton's 2% osmium tetroxide for 1 hr at 4°C and then dehydrated for 15 min in a graded series of ethanol (i.e., 50, 70, 80, 95, and 100%) and infiltrated with ratios of ethanol and Poly/Bed 812 followed by several changes of pure resin. Inverted BEEM capsules were placed over the cells and polymerized at 45°C for 12 hr before final polymerization for 48 hr at 60°C. The resulting blocks were sectioned on a Sorvall MT 6000 ultramicrotome, and silver sections (70 μm) were collected on 200-mesh copper grids. The sections were stained with uranyl acetate and lead citrate and examined with a Philips 410 electron microscope.

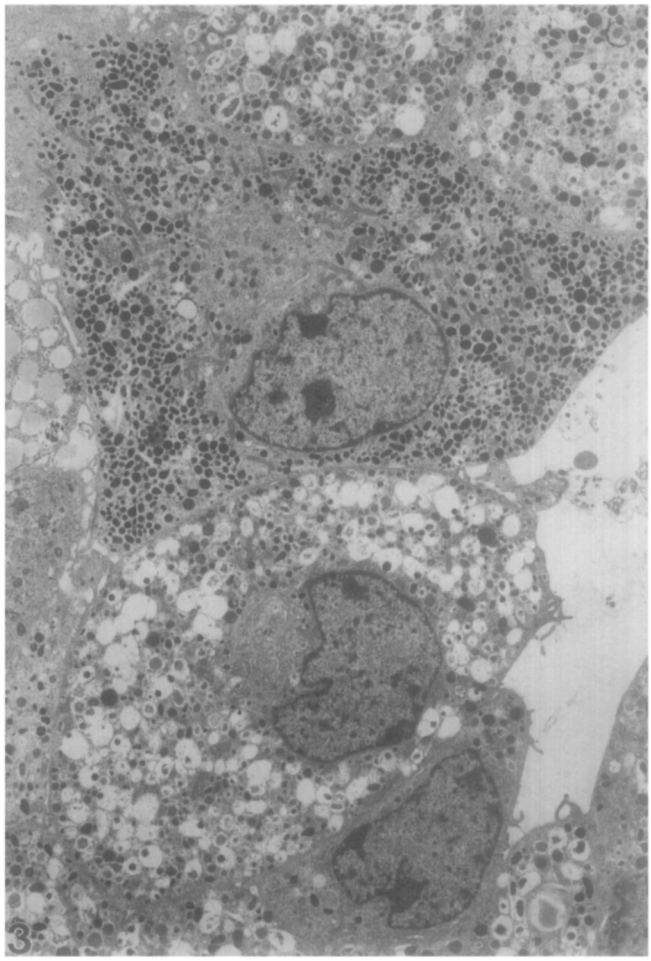

FIG. 3. Chromaffin cells from cultures exposed to DFP for 24 hr retained their chromaffin vesicles and displayed normal ultrastructural appearance of all organelles. Electron microscopy; 3170×.

Scanning electron microscopy. Cells cultured on coverslips were treated for 24 hr with 100 μM TPP, DFP, paraoxon, or 1% vehicle. The medium was removed by aspiration and the cells were washed three times with 0.1 M cacodylate buffer, pH 7.4. Cells were fixed and dehydrated as described for transmission electron microscopy. The cells were critical point dried using liquid carbon dioxide in a Parr bomb critical point dryer with Freon 113 as the exchange solvent. The resulting coverslips were mounted on aluminum studs and coated with a 20-nm layer of gold:palladium in a 60:40 ratio using a Denton vacuum evaporator. Cells were examined with a Cambridge 5200 scanning electron microscope. Micrographs were taken with Polaroid type 55 positive/negative film.

[^{3}H]Adenosine Incorporation into ATP

Cells cultured on 24 well plates were treated for 0.25, 0.5, 1, 4, or 24 hr with 100 μM TPP, DFP, paraoxon, or 1% vehicle. Cells were washed three times with about 2 ml of a balanced salt solution (150 mM NaCl, 4.2 mM KCl, 1.0 mM NaH_2PO_4, 11.2 mM glucose, 10.0 mM Hepes, 0.7 mM $MgCl_2$, 2.0 mM $CaCl_2$, pH 7.4). Cells were labeled (2 μCi/well) for 1 hr at 37°C in a modified Locke's solution (154 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM 2-deoxyglucose, 5 mM Hepes, pH 7.4) containing [2,8-^{3}H]adenosine (28 Ci/mmol; 2 μCi/ml). The solution was then aspirated and the cells were washed three times (at 37°C) with 1 ml modified Locke's for 5 min/wash.

Cell extracts were prepared by adding 0.5 ml of 0.4 M perchloric acid to each well and neutralizing the extract with a mixture of 1 M KH_2PO_4 and 1 M KOH. The sample was subjected to a freeze–thaw cycle and the precipitate was removed by centrifugation.

[^{3}H]Adenosine incorporation into adenosine triphosphate was determined by thin layer chromatography coupled with liquid scintillation counting. Adenosine nucleotides were separated and identified by comparison to standards using TLC with polyethyleneimine cellulose as the solid phase and 0.75 M KH_2PO_4 as the mobile phase (Corcoran *et al.*, 1986). The chromatographed standards were visualized by exposure to uv light and parallel samples were cut and counted for radioactivity in a Beckman liquid scintillation counter.

Materials

Triphenyl phosphite (97%) and *O,O*-diethyl 4-nitrophenyl phosphate (99%) were purchased from Aldrich Chemical Co., and *O,O*-diisopropylphosphorofluoridate (99%) was purchased from Sigma Chemical Co. [2,8-^{3}H]Adenosine (25–50 Ci/mmol) was obtained from ICN and the polyethyleneimine-coated plastic sheet (CEL 300 PEI) from Brinkman Instruments. All other chemicals were reagent grade. Electron microscopy materials were obtained from Polyscience, Inc.

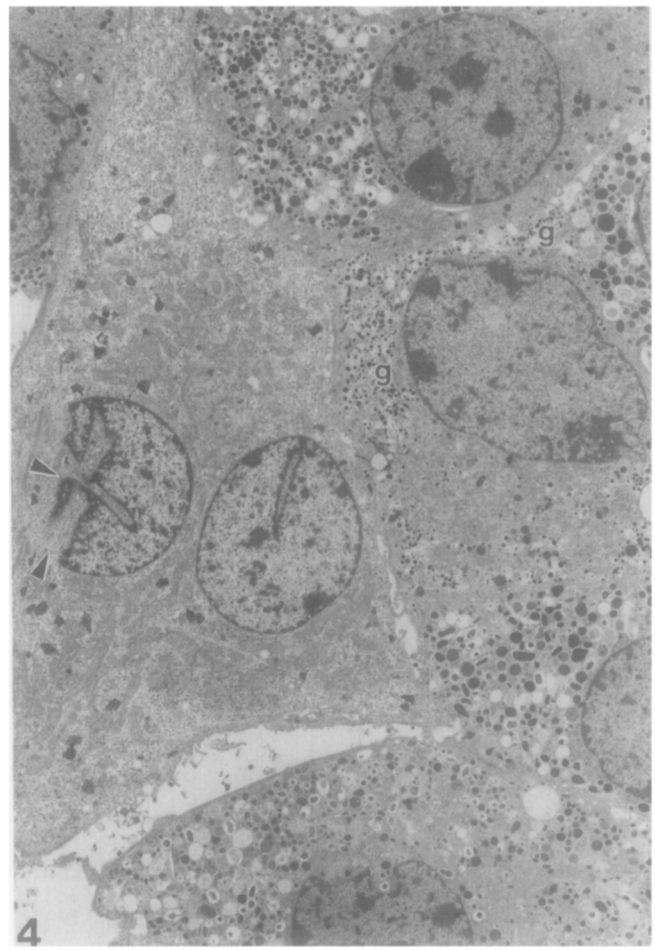

FIG. 4. Cultures exposed to paraoxon for 24 hr characteristically displayed numerous microvesicles (g) and crenated nuclei (arrowheads). Electron microscopy; 2880×.

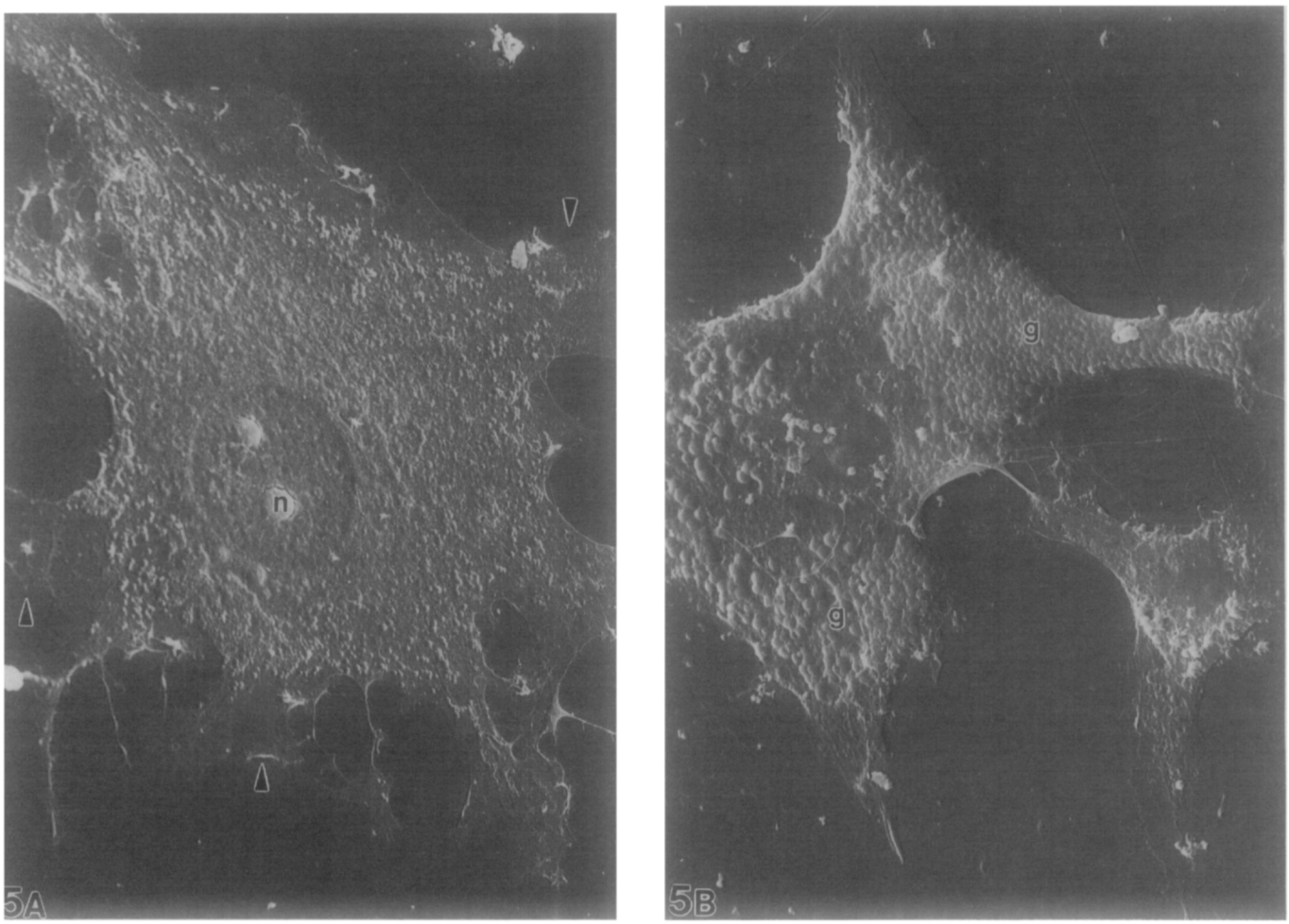

FIG. 5. Scanning electron micrographs (SEM) of control bovine adrenomedullary chromaffin cells 4 days in culture. (A) SEM of untreated chromaffin cells showed numerous cells with prominent nuclei and nucleoli (n) and numerous sheet-like filopodia (arrowheads). (B) Cells were also characterized by many chromaffin vesicles (g) arranged perinuclearly and along the filopodia. Scanning electron microscopy; (A) 1205× (B) 725×.

RESULTS

Electron Microscopy

Figures 1A and 1B depict the two types of chromaffin cells which can be isolated from bovine adrenal medulla and maintained in primary culture (Fenwick *et al.*, 1978). The distinguishing feature lies in the contents of the membrane-bound electron-dense vesicles. Noradrenaline-containing cells (Fig. 1A) housed uniformly dense but irregularly shaped vesicles whereas adrenaline-containing cells (Fig. 1B) contained more uniformly shaped round vesicles of irregular electron densities. Adrenaline cells appeared to be the predominant cell type in the 4-day-old cultures.

The plasma membrane of the untreated cells (Figs. 1A and 1B) exhibited filopodial-like prominences along the cell perimeter. The cells often displayed a thin layer of basement membrane. The nuclei of control cells were often irregular in shape and typically housed evenly dispersed chromatin. Within their cytoplasm the major organelles consisted of elongated mitochondria with lamellar cristae, Golgi apparati, bodies of rough and smooth endoplasmic reticulum, and membrane-bound vesicles. The Golgi complex consisted of loosely arranged or aggregated smooth-surfaced membranes with numerous coated and translucent vesicles. Numerous pockets of free ribosomes could be seen throughout the cytoplasm. Lysosomes, however, were rarely observed. The vesicles were characteristically located in the apical perinuclear region.

Figures 2A and 2B are representative of cells treated for 24 hr with 100 μM TPP. Cells treated for 1 hr (data not shown) exhibited a morphology characteristic of vehicle (ethanol) or untreated cells. Ethanol and DMSO vehicle treatments for TPP and paraoxon, respectively, had no effect on the cells either morphologically or biochemically. Dramatic morphological changes could be observed ultrastructurally following a 24-hr treatment with TPP. The primary target for TPP toxicity appeared to be the mitochondria. Virtually every mitochondria appeared disrupted or swollen.

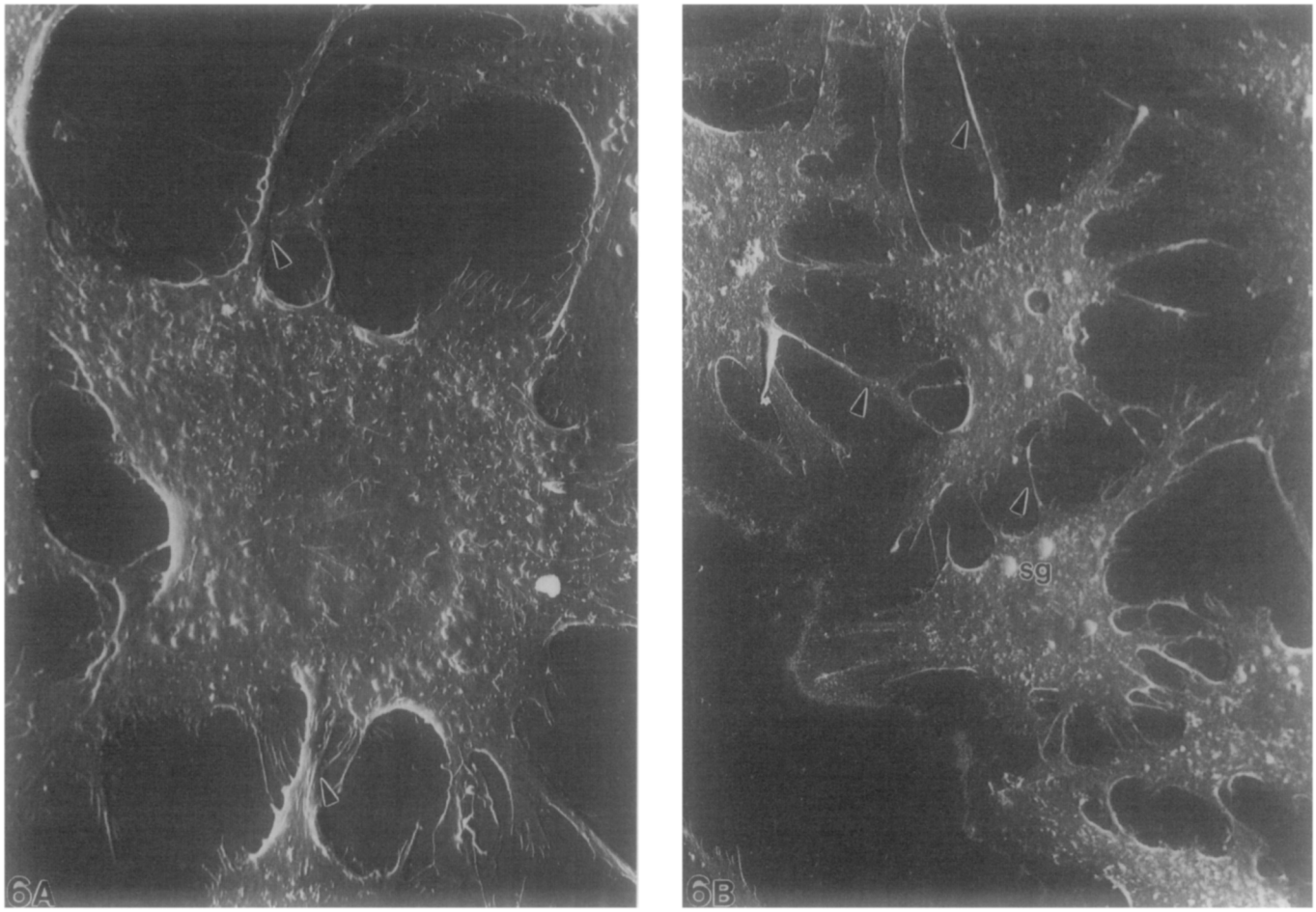

FIG. 6. (A) Under SEM, cells exposed to TPP for 24 hr appear contracted and condensed, with both spiked (arrowheads) and fan-like filopodia. (B) Examination at lower magnification suggested that the spiked and contracted filopodia are a predominate feature of these cells. Large cytoplasmic spherical globules (sg) were also evident. Scanning electron microscopy; (A) 1603× (B) 840×.

The nuclear envelope of affected cells was indented and surrounded by a band of translucent materials indicative of heightened nuclear RNA synthesis (Winkler, 1977).

Cells treated with DFP (100 μM final concentration) for 24 hr (Fig. 3) or 1 hr (data not shown) did not differ significantly from control or untreated cells. Most importantly, the mitochondria appeared normal and not swollen. Paraoxon-treated cells were similar to control or vehicle-treated cells (Fig. 4). As seen with DFP-treated cells, paraoxon-treated cells also displayed abnormal or crenated nuclei. Paraoxon-treated cells, however, also displayed numerous microvesicles which probably reflects newly synthesized chromaffin vesicles.

Scanning Electron Microscopy

Control or untreated chromaffin cells were characterized by prominent nuclei and nucleoli, extensive filopodia, and numerous cytoplasmic inclusions corresponding to chromaffin vesicles (Figs. 5A and 5B). The filopodia often appeared sheet-like and contained visible clumps of chromaffin vesicles.

Chromaffin cells treated with TPP displayed varying degrees of cellular damage. The nuclear membrane and nucleoli of TPP-treated cells did not appear as pronounced or as indented as in controls or DFP or paraoxon treatments (see below). The vesicles did not seem as numerous nor as uniformly arranged as evident in other treatment groups and instead, giant globular bodies appeared in the cytoplasm of TPP-treated cells. Unlike controls, which showed a flattened, almost sheet like appearance, the filopodia of TPP-treated cells appeared spindle-shaped (Figs. 6A and 6B).

DFP (Fig. 7) and paraoxon (Fig. 8) treated cells were similar in appearance to untreated and vehicle-treated cells. In both cases prominent nuclei and nucleoli were evident. Numerous cytoplasmic pits and an extensive network of filopodia interconnecting cells could be seen.

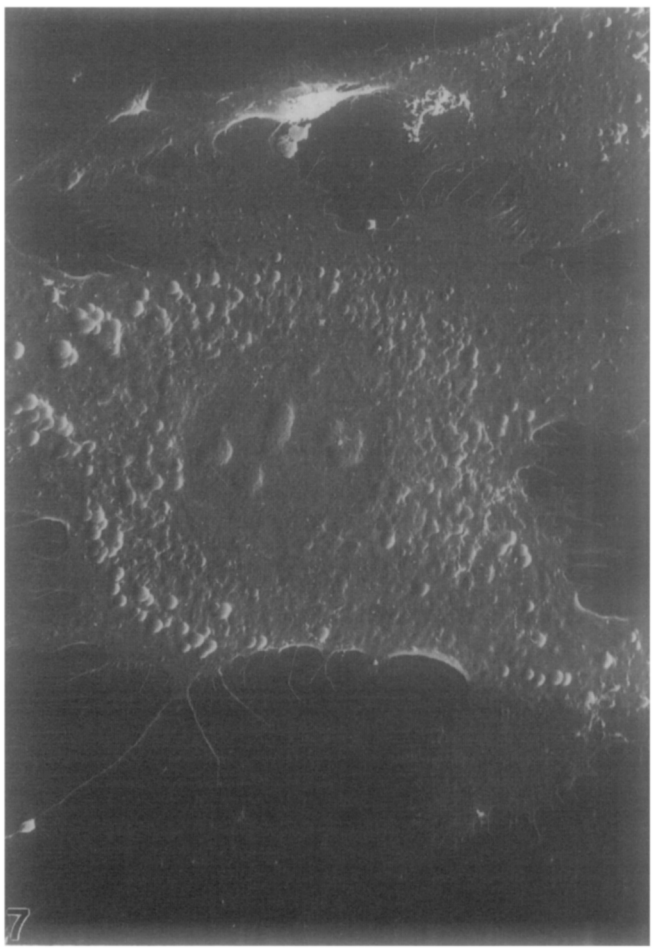

FIG. 7. DFP-treated cells (24 hr) retained their vesicles, displayed numerous sheet-like filopodia, and, in general, appeared similar to vehicle controls. Scanning electron microscopy; 1334×.

[^{3}H]Adenosine Incorporation into ATP

Figure 9 demonstrates biochemically that, as early as 60 min, the metabolic activity of the mitochondria is inhibited significantly. By 4 hr almost no incorporation of [^{3}H]adenosine into ATP could be measured as compared to untreated or vehicle-treated cells. Values for 4 hr were comparable to those obtained at 24 hr. There was essentially no effect of either paraoxon or DFP on [^{3}H]adenosine incorporation into ATP at 4 and 24 hr. Also, phenol, a metabolite of TPP, did not effect [^{3}H]adenosine incorporation into ATP (data not shown). We chose a concentration of 100 μM based on previous *in vitro* studies with regard to a maximal effect produced by TPP. It should be noted that a large dose of TPP (1000 mg/kg) is required to produce OPIDN *in vivo* in both the rat (Veronesi *et al.*, 1986) and the chicken (Carrington and Abou-Donia, 1988).

DISCUSSION

Triphenyl phosphite, a Type II OPIDN chemical, has long been recognized for its neurotoxic effects, but only recently has it been investigated with regard to its mode of action. This study was designed to investigate the differential neurotoxic effects of DFP (Type I) and TPP (Type II) (Abou-Donia and Lapadula, 1990). In addition to the Wallerian-type degeneration of the axon and myelin which is the only pathologic alteration produced by DFP, neuropathologic lesions in the neuronal cell body are also a major feature of TPP-induced delayed neurotoxicity (Abou-Donia, 1991). We chose chromaffin cells because only they have cell bodies with no other processes, e.g., axons. TPP, but not DFP or paraoxon, produced disruption or swelling in the cell's mitochondria that corresponded with the inhibition of [^{3}H]adenosine incorporation into ATP. We chose a cell system to investigate TPP's neurotoxicity since *in vitro* model systems are uncomplicated by *in vivo* metabolism. This particular cell type affords advantages over other neural crest derivatives in that it can be easily isolated, obtained in large quantities from adrenomedullary tissue, and maintained as primary cultures for up to 2 weeks (Anderson and Abou-Donia, 1991). Since adult adrenomedullary tissue is fully

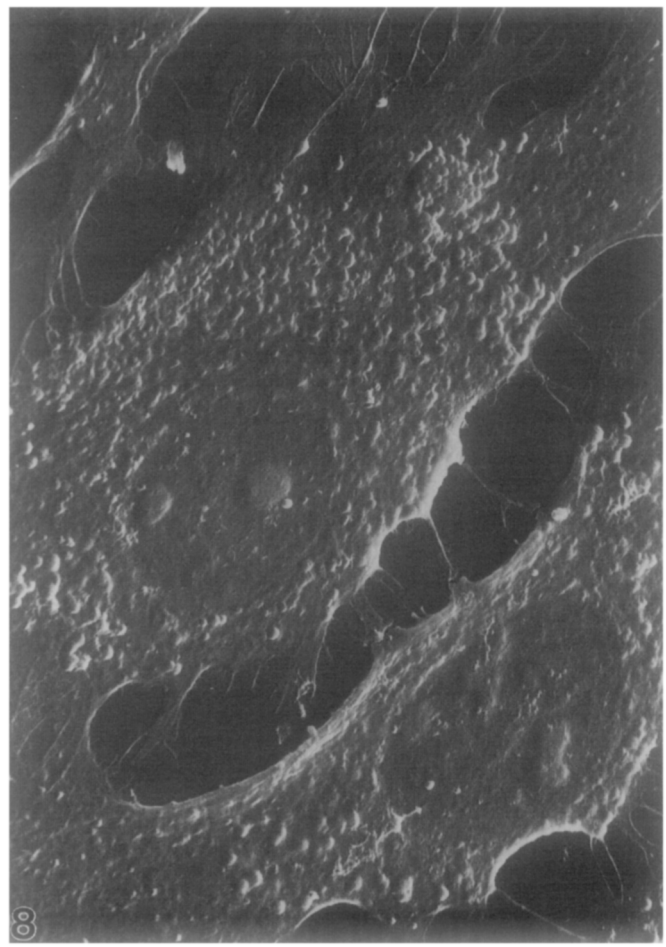

FIG. 8. Cells treated with paraoxon for 24 hr display an appearance similar to vehicle-treated cells. Scanning electron microscopy; 1661×.

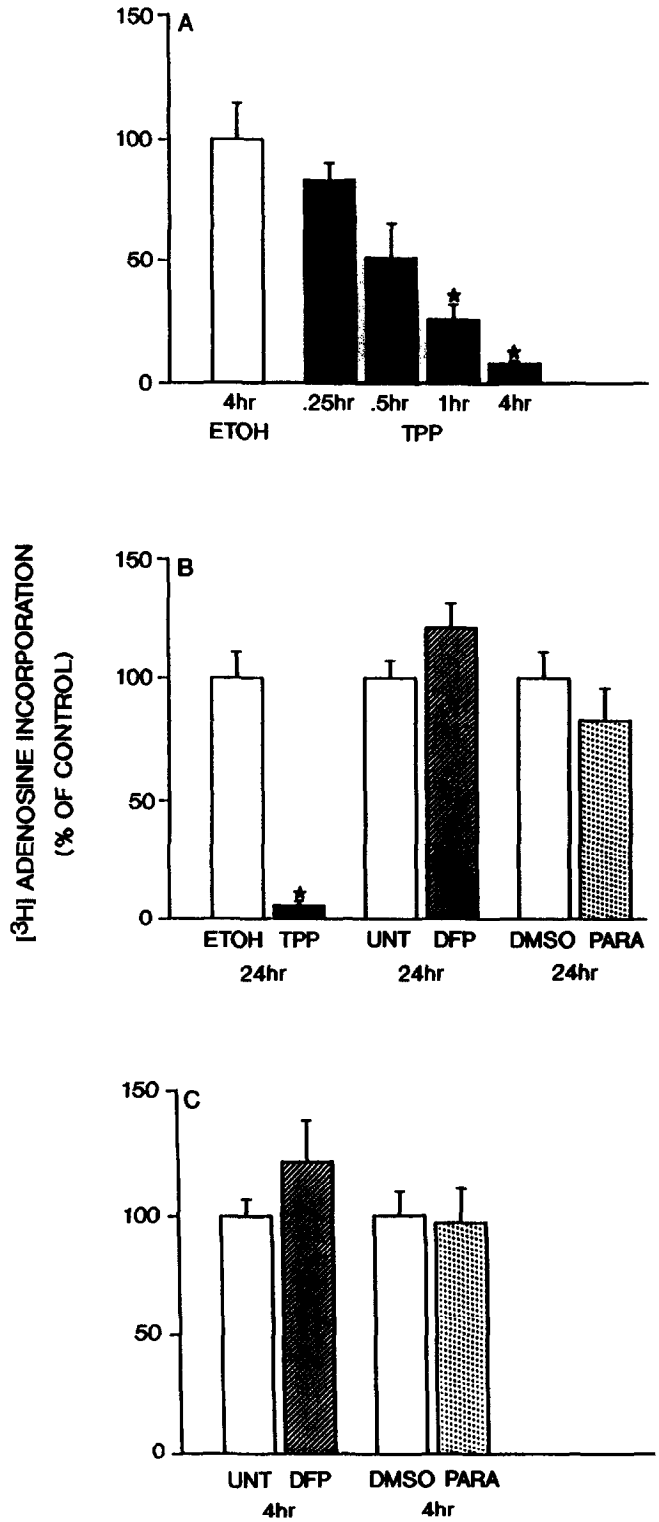

FIG. 9. Effect of TPP, paraoxon, and DFP on [^{3}H]adenosine incorporation into ATP in primary cultures of bovine adrenomedullary cells. Cells were treated for 0.25 to 4 hr (A), 24 hr (B), or 4 hr (C) and labeled as described under Methods. Actual values in dpm's for controls are as follows: (A) 4 hr ETOH, 1823 ± 267; (B) ETOH, 1066 ± 113; UNT, 852 ± 57; DMSO, 1080 ± 131; (C) UNT, 1666 ± 116; DMSO, 2011 ± 203. Each histogram is an average of four samples (n = 4 ± SD; asterisks indicate significant difference from control, $p < 0.05$).

differentiated, a relatively homogenous population of cells is obtained. Chromaffin cells are derived from the neural crest and are sympathetic-like in nature except for their lack of neurite processes (for a comprehensive review, see Livett, 1984). It is this distinguishing feature that allowed us to investigate TPP's neurotoxic effect on the cell body in isolation.

Recently, we reported differential effects of Type I and Type II compounds (DFP and TPP, respectively) on the exocytotic secretion of catecholamine from chromaffin cells (Abou-Donia and Knoth, 1989). TPP exposure to the cell cultures caused a marked inhibition in the secretory response regardless of the secretagogue used. Concominant with inhibition of secretion, was inhibition of $^{45}Ca^{2+}$ uptake into the cells (Abou-Donia *et al.*, 1990). In contrast, DFP, a Type I compound, did not inhibit secretion or $^{45}Ca^{2+}$ uptake into the cells. Since TPP adversely affected exocytotic secretion of catecholamine regardless of the secretagogue used, it most probably interfered with the secretory mechanism at a step beyond the receptor level. This was further supported by TPP's ability to inhibit calcium uptake into the cells. Inhibition of catecholamine secretion and calcium uptake could result from a primary or secondary effect of the neurotoxicant.

Data presented in this communication suggest that the mitochondrion is a primary target for TPP neurotoxicity. Electron micrographs of TPP-treated chromaffin cells consistently revealed that the cell's mitochondria were swollen or disrupted. In support of the ultrastructural changes observed, we found that biochemically the chromaffin cells' ability to synthesize ATP was significantly compromised upon exposure to TPP. Biochemically, however, significant changes were observed as early as 60 min. This is not too surprising since one might expect the onset of biochemical dysfunction to precede gross morphological changes. We feel this is a specific mitochondrial effect of TPP since the organophosphorus compounds DFP (Type I compound) and paraoxon (nondelayed neurotoxicant) did not significantly affect the mitochondria morphologically or biochemically.

Smith *et al.* (1933) showed that the acute convulsive action of TPP was produced by the phenol, a hydrolytic product of TPP. Phenol, however, was ruled out as the cause of the delayed neurotoxicity resulting in axonal degeneration. Likewise, in the present study, phenol did not inhibit ATP synthesis and, consequently, may not be involved in the morphologic changes seen in the mitochondria following TPP treatment.

TPP's effect on the mitochondria seen here is consistent with the recent *in vivo* findings by Konno *et al.* (1989) in that TPP has a direct effect on mitochondrial function. In anoxia-induced mitochondrial swelling, both Beatrice *et al.* (1984) and Tagawa *et al.* (1985) suggested that structural alterations in the mitochondria result from an increase in free calcium which has been released from bound intramitochondrial calcium stores and not from changes in calcium

uptake. In contrast, the mitochondrial swelling reported by Maduh *et al.* (1990) upon cyanide treatment of PC12 cells supports a direct role for calcium uptake. They suggested that mitochondrial swelling resulted from an increase in calcium uptake since the calcium channel blocker diltiazem inhibited swelling. Although their endpoint (i.e., mitochondrial swelling) is similar to ours, the precise role for calcium uptake in producing this change is unclear.

There are many possible mechanisms by which TPP may alter the metabolic activity of the mitochondria. On the basis of its chemical structure, we could speculate that TPP is interfering with oxidative phosphorylation by acting as an uncoupler of the electron transport chain. We are currently investigating this and other possibilities (i.e., inhibition of mitochondrial enzymes) in isolated mitochondria. Nonetheless, inhibition of ATP synthesis leads to changes in ion gradients via reduction in the activity of ATP-dependent ion pumps. A disruption in elemental homeostasis will subsequently lead to changes in the osmolality of the cell and its organelles. This ion deregulation in turn can affect numerous biochemical events (for a review, see LoPachin and Saubermann, 1990).

In light of the metabolic changes presented in this study, TPP's inhibition of the exocytotic process could be directly related to the loss of metabolic ATP since ATP is apparently required for exocytosis (Dunn and Holz, 1983; Wilson and Kirshner, 1983). It could also be related to changes in osmolality since it has been shown that osmotic forces affect secretion of catecholamines as well as nonvesicular components of the cell (Hampton and Holz, 1983; Knoth *et al.*, 1987). Upon exposure to TPP, an arrest in metabolic activity coupled to changes in the ionic equilibrium of the cell may be responsible for the lack of neural communication and the resultant neurotoxicity observed *in vivo*. It is conceivable, however, that TPP, an oxygen scavenger, may produce hypoxia by directly binding to cellular oxygen. Also, the involvement of increased kinase-mediated phosphorylation of cytoskeletal proteins implicated in the development of Type I OPIDN (Abou-Donia and Lapadula, 1990) is being investigated for the mechanism of TPP-induced Type II OPIDN (Abou-Donia and Lapadula, 1990).

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