PAPER NO. 3

INTERACTIONS OF KETONES AND HEXACARBONS

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INTRODUCTION

Methyl ethyl ketone (MEK), a widely used commercial solvent, is frequently combined with other aliphatic and aromatic hydrocarbons in paint thinners, lacquers, and solvents for technical coatings (NIOSH, 1978). Although MEK itself is considered an innocuous compound, the above products can contain n-hexane and methyl n-butyl ketone (MnBK), which are known neurotoxic solvents. While MEK itself appears devoid of neurotoxic properties (Saida et al., 1976; Spencer and Schaumburg, 1976; Altenkirch et al., 1977; Egan et al., 1980), recent clinical and experimental findings indicate that this ketone can enhance the neurotoxic properties of both n-hexane and MnBK. This property was first suspected in the 1973 outbreak of occupational neuropathy among Ohio textile workers exposed to mixtures of MEK and MnBK (Allen et al., 1974; Billmaier et al., 1974) and later demonstrated experimentally in rats continuously exposed to these mixtures (Saida et al., 1976; Couri et al., 1977). MEK-related neurotoxic potentiation was again suspected in an outbreak of n-hexane-induced neuropathy among solvent abusers (glue-sniffers,

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"huffers") in West Berlin (Altenkirch et al., 1977) and subsequently confirmed in rats exposed to high levels of both n-hexane and MEK (Altenkirch et al., 1978). The picture emerging from these studies is that MEK appears to accelerate the onset and severity of the "dying-back" neuropathy produced by hexacarbons. The mechanism underlying this potentiation is unknown.

It was our intention to replicate and investigate this interaction in tissue culture using organotypic explants of mouse spinal cord with attached dorsal root ganglia and striated muscle. At maturity, this complex displays morphological and bioelectrical features typical of mammalian neuromuscular tissues in vivo. This type of tissue culture has been used for many years in experimental neurobiology (Peterson and Crain, 1972) and neuropathology (Raine, 1973) and is amenable to neurotoxicological inquiries. It has been especially valuable in addressing the pathogenic and metabolic events of subchronic exposure to aliphatic hexacarbons (Veronesi et al., 1980, 1983).

MATERIALS AND METHODS

ESTABLISHMENT OF ORGANOTYPIC CULTURES

Cross sections of fetal mouse spinal cord (14 day in utero), with meningeal covering and attached dorsal root ganglia (DRG), were individually explanted onto collagen-coated coverslips. A 6-10 mm strip of skeletal muscle taken from the thigh of an adult mouse was positioned on the coverslip approximately 1 mm from the ventral cord region. Explants were provided with a drop of nutrient fluid containing Eagle's minimum essential medium with glutamine (56%), human placental serum (33%), chick embryo extract (10%), and glucose (6 mg/100 ml). The nerve-muscle complex was positioned in a sterile Maximow chamber (Maximow, 1925), sealed in its concave well, and incubated at 34-35°C. The chamber was opened twice weekly, the explant removed and washed for 10 min with Hank's basic salt solution (Gibco, Long Island, NY), fed with a fresh drop of nutrient medium, and returned to a sterile chamber. Cultures maintained in this fashion matured in approximately 8-10 weeks and were then selected for treatment by the following criteria: an agranular spinal cord with abundant myelinated central nervous system (CNS) fibers; DRG neurons housing centrally located nuclei; numerous, well- myelinated sensory and motor peripheral nervous system (PNS) fibers; and muscle tissue with well-defined cross-striations and synchronized contractions. Details of this technique are given elsewhere (Bornstein, 1973).

CHEMICALS

Test chemicals were obtained from a commercial supplier (Eastman Kodak Co., Rochester, NY) and their purity assessed by gas chromatography-mass spectrometry (courtesy of Dr. G. DiVincenzo, Eastman Kodak Co., Rochester, NY). n-Hexane was determined to be 97% pure, containing 2% methyl isobutyl ketone

and 1% unidentified ingredients as contaminants. MEK was assessed to be 98% pure, containing 1% 2-butanol and 1% unidentified ingredients as contaminants.

TREATMENT

To establish dose:response data, n-hexane was dissolved in nutrient fluid in concentrations of 25, 50, 80, 100, or 250 ug/ml. In spite of n-hexane's low water solubility, these concentrations readily dissolved in the nutrient fluids. At least 8 mature cultures were exposed to each dose of n-hexane for periods up to 56d. Cultures were examined twice weekly for morphologic changes by brightfield microscopy. Those doses of n-hexane that induced giant axonal swellings, the morphologic hallmark of hexacarbon neuropathology, within the exposure period were defined as "neurotoxic." The duration of exposure needed for 75% of the cultures to develop distinct axonal swellings was defined as the time-to-onset (TTO) of specific pathologic response and was recorded in days (d). Those concentrations of n-hexane which were unable to induce axonal swellings within the defined treatment period were labeled "no-response" (NR) doses.

To establish dose-response data for MEK, the solvent was dissolved in nutrient fluids in concentrations of 10, 25, 50, 200, 300, 400, or 600 ug/ml. At least 8 mature cultures were exposed to each dose of MEK for periods up to 49d. Again, individual cultures were monitored twice weekly by brightfield microscopy for morphologic changes. Concentrations of MEK that produced nonspecific cellular breakdown of the explant were defined as pancytotoxic. Noncytotoxic doses were those allowing the culture to retain normal morphology throughout the treatment period.

Neurotoxic and "no-response" doses of n-hexane were individually combined with noncytotoxic doses of MEK and dissolved in nutrient fluid in effective n-hexane:MEK mixtures of 25:50, 25:100, 50:10, 50:50, 50:100, 100:10, 100:50, 100:100, 250:25, or 250:50 ug/ml. Additional groups of mature cultures (minimum of 6 cultures per group) were individually exposed to these mixtures for periods up to 56 d. Age-matched, untreated cultures served as negative controls for all test groups. Explants were monitored microscopically and the TTO for 75% of the cultures to develop giant axonal swellings was recorded. These data were also evaluated by probit analysis (SAS, 1982) at the 28-day time point and by Cox's linear model for proportional hazards (Cox, 1972; SAS, 1982). Both models evaluated MEK, n-hexane and their multiplication product at various doses.

MICROSCOPIC EXAMINATION

Living cultures were examined within the Maximow chamber twice weekly for the appearance of distinct axonal changes. To examine the explant microscopically, a special long-working-distance, fluorite 40x oil-immersion lens with a high numerical aperture was used to penetrate optically the various glass coverslips used in the Maximow assembly. Areas of interest were photographed with Panasonic X (ASA 32) film. For electron microscopy, selected cultures were removed from their Maximow chambers, fixed in Millonig's-buffered 2.5% glutaraldehyde, followed by Millonig's-buffered 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol, and cleared in propylene oxide. The explant and its collagen bed were gently removed from the glass coverslip, infiltrated with epoxy-resin (Epon 812), sandwiched between two Teflon-coated coverslips (Polyscience 4505), and polymerized in a 60°C oven. This treatment produced a thin epoxy-wafer that could readily be examined with a dissecting microscope. Areas of interest were identified, trimmed from the wafer, re-embedded, and sectioned for light and electron microscopy. One-micrometer-thick epoxy sections were stained with borate-buffered 1% toluidine blue. Thin (50 nm) sections (based on interference colors) were cut with a diamond knife, collected on 200-mesh copper grids, stained with 2% uranyl acetate followed by 1% aqueous lead citrate and examined with a Zeiss 10 A transmission electron microscope.

RESULTS

CONTROL CULTURES

The nerve-muscle explants matured into structurally and functionally coupled co-cultures within 8-10 weeks. At maturation, the entire living complex measured approximately 5mm^2 (Figure 1). Typically, a mature control culture was composed of an agranular spinal cord containing neurons and numerous thinly myelinated CNS fibers. Clearly defined dorsal and ventral spinal roots combined to form sensory-motor nerve tracts. Large DRG (sensory) neurons contained prominent, centrally located nuclei and a cytoplasm rich in Nissl substance and other organelles. Heavily myelinated PNS fibers exhibited well-defined nodes of Ranvier and Schmidt-Lanterman clefts. Numerous unmyelinated fibers were grouped with myelinated fibers in small fascicles delimited by cells of fibroblast-type (Figure 2). Skeletal muscle tissue, which initially had been incorporated into the system as adult tissue, degenerated, regenerated, and eventually differentiated into finely striated, synchronously twitching muscle fibers. The morphologic and physiologic viability of the muscle tissues in vitro depended on motor fiber innervation. By electron microscopy, PNS and CNS myelinated axons typically contained 10-nm neurofilaments, 24-nm microtubules, longitudinally oriented mitochondria, smooth endoplasmic reticulum, and scattered vesicles. Small groups of unmyelinated axons were

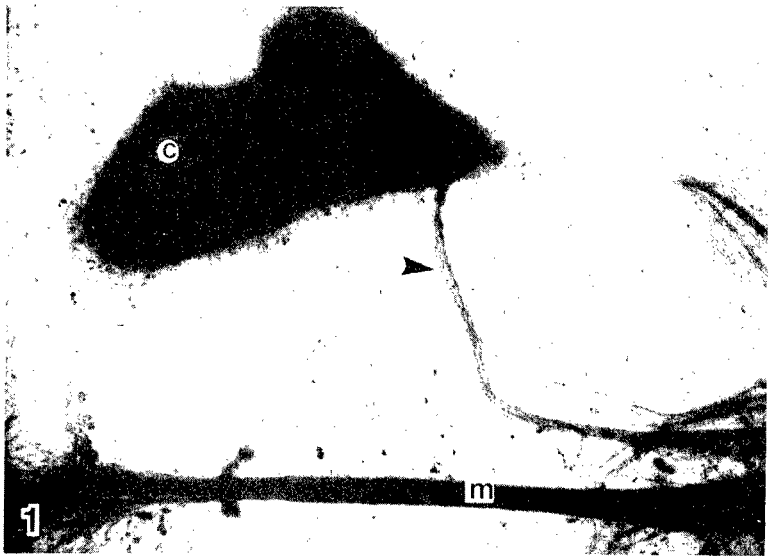

Figure 1. Mature organotypic co-culture of spinal cord (c) and muscle (m) explants. Note fascicles of peripheral nerve fibers (arrow). Brightfield, living culture X20.

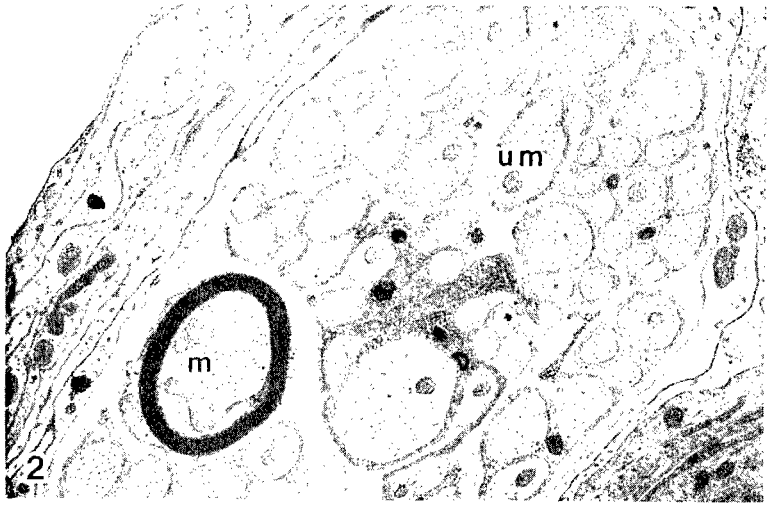

Figure 2. Peripheral nerve fascicle from control culture showing myelinated (m) and unmyelinated (um) axons. Electron micrograph X9600.

partially or completely enclosed in Schwann cell cytoplasm and typically contained a higher proportion of microtubules to neurofilaments relative to their myelinated counterparts.

n-HEXANE-TREATED CULTURES

Cultures treated with neurotoxic doses of n-hexane developed giant axonal swellings that first appeared in distal paranodal regions of large-diameter nerve fibers (Figure 3). A progression of axonal pathology was recorded in these fibers that included nodal elongation, swelling of the paranodal axon, retraction of paranodal myelin, and eventual degeneration of the fiber length distal to the swelling. By electron microscopy, the giant axonal swelling was characterized by an accumulation of 10-nm neurofilaments, peripheral displacement of neurotubules and mitochondria, and a disproportionately thin or absent myelin sheath. The pathogenesis of hexacarbon neuropathy in vitro has been detailed elsewhere (Veronesi et al., 1980, 1983).

Giant axonal swellings of this type were seen in cultures exposed to n-hexane in concentrations of, or greater than, 100 ug/ml. The duration of time (TTO) required for axonal swellings to develop in 75% of explants treated with 100 and 250 ug/ml (neurotoxic levels) was 43 and 28 d, respectively. Axonal swellings were not seen in cultures treated with 25, 50, or 80 ug/ml (NR levels) for periods up to 56 d. These data are summarized in Table 1.

TABLE 1. DOSE RESPONSE FOR n-HEXANE[a]

n-Hexane (ug/ml)	Response (TTO)	Incidence of Effects[b]
25	NC	0/8
50	NC	0/8
80	NC	0/8
100	43	6/8
250	28	8/8

[a] Explants (8 per dose) were treated for 56 days with 25-250 ug/ml of n-hexane dissolved in nutrient fluid.

[b] Tissue treated with 100 ug/ml displayed no pathological change (NC). Explants treated with doses of 100 ug/ml or greater developed giant axonal swellings in nerve fibers at the times stated in days. TTO: Time-to-onset of axonal swellings in 75% of cultures.

MEK-TREATED CULTURES

Organotypic explants treated with 600 ug/ml of MEK displayed a generalized cellular breakdown (pancytotoxic response) within 28 days of treatment. This nonspecific response was characterized by granularity of the spinal cord, bubbling of CNS and PNS myelin sheaths, and rapid breakdown of motor and sensory PNS fibers. In contrast, those explants treated with 10-400 ug/ml (non-cytotoxic levels) remained viable throughout the exposure period. However, after 38-49 days of treatment, intra-axonal rectilinear inclusions (Figure 4) developed in several cultures treated with 200-400 ug/ml MEK. These structures were often associated with axoplasmic granularity, although overt fiber degeneration was rarely seen. Electron microscopy revealed the granularity to correspond to foci of degraded axoplasm (Figure 5). Such foci were not noted in age-matched controls nor in n-hexane-treated cultures. Cultures treated with lower doses of MEK (10, 25, 50, 100 ug/ml) for similar periods and age-matched controls did not develop these foci (Table 2).

TABLE 2. DOSE RESPONSE FOR MEK[a]

MEK (ug/ml)	Response[b]	Incidence of Effects
10	NC	0/8
25	NC	0/8
50	NC	0/8
100	NC	0/8
200	RI (49)	3/8
300	RI (42)	5/8
400	RI (38)	5/8
600	Pancytotoxic	8/8

[a] Explants (8 per dose) were treated for 49 days with 10-600 ug/ml of MEK dissolved in nutrient fluid.

[b] MEK in doses of 600 ug/ml produced pancytotoxic effects whereas cultures treated with 10-100 ug/ml showed no morphological changes (NC) within the test period. Explants treated with 200-400 ug/ml developed rectilinear inclusions (RI) along the length of myelinated fibers. The time of their appearance was recorded in days.

Figure 3. Typical giant axonal swelling (arrow) seen in distal end of myelinated fiber in culture treated with n-hexane (250 ug/ml) for 42 days. Brightfield, living culture X1800.

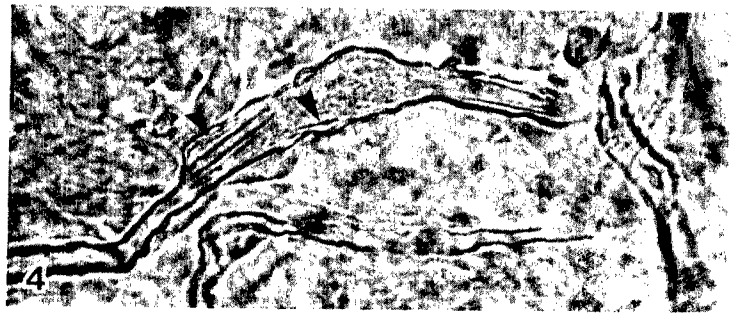

Figure 4. Intra-axonal rectilinear structures (arrows) are shown. Culture treated with MEK (300 ug/ml) for 42 days. Note axoplasmic granularity. Brightfield, living culture X1200.

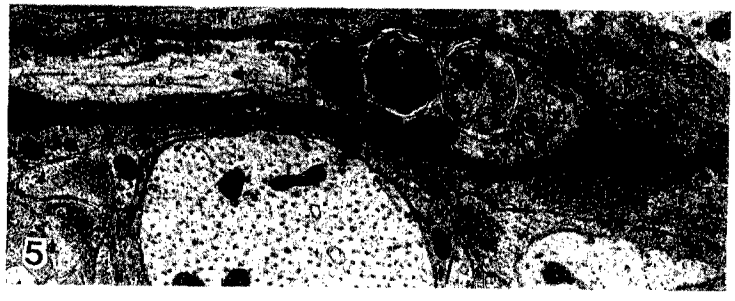

Figure 5. Electron microscopy of rectilinear structures revealed them to be multiple foci of axoplasmic debris. Culture treated with MEK (300 ug/ml) for 42 days. Electron micrograph X12,000.

n-HEXANE: MEK-TREATED CULTURES

Table 3 reports the TTO of giant axonal swellings in the 10 groups of cultures treated with n-hexane:MEK mixtures. Irrespective of concentration, MEK (10-100 ug/ml) induced "NR" doses of n-hexane (25, 50 ug/ml) to produce typical giant axonal swellings within 21 days of exposure. These levels of MEK, in combination with neurotoxic doses of n-hexane (100, 250 ug/ml), accelerated the TTO of axonal pathology when compared with n-hexane treatment alone.

TABLE 3. DOSE RESPONSE FOR n-HEXANE:MEK[a]

n-Hexane \ MEK	0	10	25	50	100
0	NC	/	/	NC	NC
25	NC	/	/	11	22
50	NC	14	/	12	18
100	43	31	/	19	25
250	28	/	21	27	/

[a] Explants (6 per dose) were treated for 4-8 weeks with n-hexane alone (0-250 ug/ml), MEK alone (0-100 ug/ml), or selected combinations thereof. The TTO for the appearance of axonal swellings to occur in 75% of the cultures is given in days. In all mixture-treated cultures, MEK was observed to enhance the TTO. NC: no change/: not tested.

Probit analysis (Table 4) confirmed this potentiation although there were insufficient or partial responses in some mixtures. Cox's model (Table 5) gives the corresponding coefficients for the various exposure combinations where trials were observed. These coefficients indicate relative effects of each material alone and in combinations as estimated from Cox's linear model for proportional hazards. These data indicate that the coefficient of the product of MEK and n-hexane levels was significantly different from zero when MEK and n-hexane were present in the model. Since this coefficient was highly significant, ($p<.001$) potentiation was indicated.

TABLE 4. PROBIT ANALYSIS OF MIXTURE-TREATED CULTURES[a]

MEK (ug/ml)	n-Hexane (ug/ml)	R/C[b]	EC 50[c]
0	50	0/6	
0	80	0/6	154 (108,335)
0	100	2/6	
0	250	4/5	
50	0	0/6	
50	25	6/6	<100 may be <25[d]
50	50	4/4	
50	100	9/12	
100	0	0/6	
100	25	6/6	<25[d]
100	50	4/5	
100	100	5/5	

[a] SAS, 1982.
[b] R/C: the number of cultures responding after 28 days exposure/the total number of treated cultures.
[c] 95% confidence intervals.
[d] Insufficient or partial responses for probit analysis.

In addition to developing giant axonal swellings, some explants treated with n-hexane:MEK in mixtures of 25:50, 25:100, 50:10, 50:25, 50:50, 100:10, and 100:50 ug/ml, developed the rectilinear inclusions described previously. The timing of development and location of these inclusions were independent of the spatial-temporal characteristics of the n-hexane-induced axonal swellings.

DISCUSSION

This study describes a tissue culture model of MEK's potentiation of n-hexane neurotoxicity and again demonstrates the flexibility and potential usefulness of in vitro systems to evaluate neurotoxic chemicals. Two salient features of MEK's interaction with n-hexane are demonstrated in this study. First, MEK accelerates the onset of specific hexacarbon pathology, an observation that is supported by animal studies. Rats inhaling 9:1 mixtures of n-hexane (9000 ppm) and MEK (1000 ppm) for 8 h/d, 7 d/wk, displayed functional signs of neuropathy after 5 wks, whereas litter mates exposed to 10,000 ppm of n-hexane alone developed neuropathy after 8-10 wks (Altenkirch et al., 1978).

TABLE 5. COX'S STATISTICAL ANALYSIS OF MIXTURE TREATED CULTURES[a]

	MEK (ug/ml)				
	0	10	25	50	100
n-Hexane (ug/ml)					
0	1.0	0.9	0.9	0.7	0.6
25	1.1			1.1	1.1
50	1.2	1.3		1.6	2.1
100	1.5	1.7		3.4	8.0
250	2.6		9.4	34.0	

[a] Cox's general linear model for proportional hazards (Cox, 1972; SAS, 1982) was used to examine the interaction of mixture-treated cultures. The table above gives predictive values of the coefficients of hazards where n-hexane, MEK and their multiplication products are included.

Similarly, in the organotypic culture system, specific neuropathologic changes appeared after 31 days of treatment to mixtures of n-hexane and MEK (100:10) whereas in the absence of MEK, explants treated with 100 ug/ml of n-hexane developed axonal swellings after 43 days of exposure. The second observation is that MEK lowers the neurotoxic threshold of n-hexane in vitro. Although "NR" levels of n-hexane (25, 50 ug/ml) failed to produce axonal swellings after 56 days of exposure, these levels when combined with MEK (10-100 ug/ml), produced the characteristic axonal pathology in 11-12 days. MEK's ability to induce "no-response" levels of n-hexane to express axonal degeneration is an entirely new experimental observation supported in part by clinical reports of solvent addicts in West Berlin. Between 1968 and 1975, these individuals chronically abused a commercial product containing 30% n-hexane without developing neuropathy. However, in October 1975, the n-hexane content of the product was lowered to 16%, and 11% MEK was added to the mixture. Within 6 months, 19 cases of severe hexacarbon neuropathy appeared, followed by 6 additional cases within the next 24 months (Altenkirch et al., 1977). It should be noted that in the reformulated product, the toluene fraction remained unchanged, the ethyl acetate fraction was decreased, and the benzene fraction was increased, raising the possibility that these ingredients might also have influenced

n-hexane's neurotoxicity. Other reports of occupationally-related solvent neuropathy involving commercial mixtures of MEK and n-hexane exist (Barone, 1973; Carapella, 1975; Gaultier and Rancruel, 1973; Goto et al., 1974). Unfortunately, quantitative and qualitative analysis of the product's ingredients were missing in these reports, preventing the identification of the culpable neurotoxic agent(s).

The mechanism(s) underlying MEK's influence on n-hexane neurotoxicity is still unknown. Experimental studies have demonstrated that both oral administration (Traiger and Bruckner, 1976) and subchronic inhalation exposure to MEK (Couri et al., 1977) induce rat hepatic microsomal activity. The combination of MEK with hexacarbons might alter the rate at which the parent compound(s) is converted to its neurotoxic metabolite(s) or influence the retention or elimination of the neurotoxic moiety. Another possibility is that MEK is able to displace or alter the binding affinity of neurotoxic hexacarbons to target tissues or enzymes, thus increasing the bioavailability of the toxicant. Another attractive explanation is that MEK's metabolic induction promotes a more rapid conversion of the parent hexacarbons n-hexane and MnBK to their putative primary neurotoxic metabolite 2,5- hexanedione (2,5-HD) which would result in a more rapid onset of neuropathy. Data from two studies, however, argue against this notion. Experiments have shown that animals continuously exposed to mixtures of MEK and MnBK display an elevated blood level of MnBK when compared to animals exposed to MnBK alone (Abdel-Rahman et al., 1976). Other experiments have demonstrated that 2,5-HD levels in the sciatic nerves of rats acutely exposed to n-hexane:MEK mixtures are actually lower than in nerves taken from animals exposed to equivalent concentrations of n-hexane alone (White and Bus, 1980). Note that this study involved single-dose administration of MEK rather than subchronic exposure, a dosing regimen which may not have produced the induction of microsomal activity needed to accelerate n-hexane's conversion to 2,5-HD. To address this possibility, levels of 2,5-HD should be measured in the nerves of animals subchronically exposed to n-hexane:MEK mixtures.

Finally, the appearance of intra-axonal, rectilinear inclusions in cultures treated with MEK alone and in combination with n-hexane merits discussion. Under electron microscopy, these structures were identified as foci of organelle degradation and appear to be a nonspecific response of the axon to toxic injury. Although these changes have not been reported in animals exposed subchronically to MEK (Saida et al., 1976; Spencer and Schaumburg, 1976; Altenkirch et al., 1977; Egan et al., 1980), their development may be ascribed to differences in bioavailability, pharmacokinetics, and biocompartmentation between the in vitro and in vivo systems. An ultrastructural examination of these structures is presented in a companion paper.

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PROCEEDINGS OF THE FOURTEENTH CONFERENCE ON ENVIRONMENTAL TOXCICOLOGY 15, 16, and 17 NOVEMBER 1983

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

BRUCE O. STUART, PhD
Director Toxic Hazards Division
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Paper No. 17 - Page 245

Insert the following four references:

MacFarland, H. N. (1982), Chronic gasoline toxicity, The Toxicology of Petroleum Hydrocarbons, American Petroleum Institute, Publication No. 847-90000, pp. 78-86.

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Papers were presented covering molecular mechanisms of n-hexane neurotoxicity, metabolism of n-hexane and anatomical effects of neurotoxins. The pathological, metabolic, electrophysiological and biochemical characteristics of organophosphorus induced neurotoxicity were discussed in specific presentations. The male reproductive system as a target organ and specific problems associated with mixture toxicology were subjects of individual sessions. Occupational health data bases, early detection of environmental exposure and effects of exposure to Agent Orange were discussed in papers.

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