TOXICOLOGY AND APPLIED PHARMACOLOGY 75, 571–575 (1984)

SHORT COMMUNICATION

Neurotoxic Properties of Musk Ambrette

Neurotoxic Properties of Musk Ambrette. SPENCER, P. S., BISCHOFF-FENTON, M. C., MORENO, O. M., OPDYKE, D. L., AND FORD, R. A. (1984). *Toxicol. Appl. Pharmacol.*, **75**, 571–575. Musk ambrette (2,6-dinitro-3-methoxy-4-*tert*-butyltoluene), a nitro-musk compound widely used as a fixative in fragrance formulations and found to a lesser degree in flavor compositions, produces hindlimb weakness when administered in the diet or applied to skin of rats for periods up to 12 weeks. Underlying neuropathologic changes consist of primary demyelination and distal axonal degeneration in selected regions of the central and peripheral nervous system. Murine neurological disease induced by musk ambrette occurs at doses well above estimated maximum daily human exposure. Lifetime experimental neurotoxicology studies using lower concentrations of musk ambrette for prolonged periods would be needed for the estimation of human risk.

Musk ambrette (2,6-dinitro-3-methoxy-4-*tert*-butyltoluene) is one of the most frequently used artificial musks. Since the 1920s, the estimated amount of musk ambrette (MA) used in fragrance formulations in the USA has exceeded 100,000 lb/year (Arctander, 1969). MA has a sweet, heavy musk odor with a bitter taste, except at low concentrations (1 ppm). The compound is used as a fixative in fragrance formulations where it may be present in concentrations of 1 to 15+%. MA is also used to a minor degree in flavor compositions such as cherry, nut, spice, vanilla, and mint. Here, the normal concentration in the finished product is 0.01 to 10 ppm. MA is the only nitro-musk regarded as safe for food flavors in the USA. The Council of Europe considers MA suitable for use in concentrations of 1 ppm as an artificial flavoring additive in foodstuffs (Opdyke, 1979), and, in 1965, the Flavoring Extract Manufacturer's Association (FEMA) gave MA GRAS (generally regarded as safe) status.

Little has been reported on the toxicity of MA, and studies of adverse human health effects have focused on photosensitivity and dermal reactions. Screening tests in human subjects have failed to demonstrate photosensitive reactions to MA (Kligman, 1966, 1972), although there have been reports of dermal reactions in individuals using colognes containing MA (Giovinazzo *et al.*, 1980). The oral LD50 of MA in rats is 339 mg/kg (Jenner *et al.*, 1964), and the dermal LD50 is in excess of 2 g/kg (Opdyke, 1979). Subchronic feeding studies in rats receiving 500 to 4000 ppm (25 to 200 mg/kg) caused growth retardation and testicular atrophy (at 2500 ppm), with progressive paralysis of the hindlimbs (at 1500 ppm) beginning at 12 to 15 weeks (Davis *et al.*, 1967). Complete hindlimb paralysis was seen after 16 to 40 weeks of treatment with the higher doses. Female animals, which were more susceptible than males to the toxic effects of MA, also showed depressed erythrocyte counts, hemoglobin values, and icteric plasma when treated with doses greater than 1500 ppm. Decreased clotting time was observed in all groups of exposed females and in males treated with 1500 and 2500 ppm. Postmortem and histologic studies of paralyzed animals revealed brittle bones, and enlarged adrenal glands in females, testicular atrophy in males and muscle atrophy; the nervous system was not examined (Davis *et al.*, 1967).

We have employed contemporary histopathologic methods to examine the effects of topical and oral administration of MA on the nervous system of 46 male or female rats. Routine clinical and histopathologic procedures were also done.

0041-008X/84 $3.00

METHODS

A total of 220 young adult male and female Sprague–Dawley CD rats (Charles River Laboratories, Wilmington, Mass.), was divided into seven groups of 15 males and 15 females each, and treated either with 1500 ppm of MA in their diet (Purina Laboratory Rat Chow 5001) or topically with a solution of MA in phenylethyl alcohol (PEA) applied to the shaven back at concentrations equivalent to 10, 40, 80, and 240 mg/kg. The two lower doses of MA were applied to skin as a 5% solution in PEA, the two higher doses as 20% solutions. Doses were adjusted weekly based on body weight. Control animals treated topically with PEA received a dose (1.2 ml) equivalent to that used for animals treated with the larger doses of musk ambrette. An oral control group received untreated diet. Animals were maintained in controlled housing conditions (22 ± 2°C, 55 to 65% relative humidity, and 12/12 hr light/dark cycle). During the treatment period animals were monitored daily for signs of toxicity and dermal reactions. Body weights and food consumption were recorded weekly. Animals were necropsied and prepared for routine histologic examination at the end of the test period. Hematology, clinical chemistry, and urinalysis were performed on representative animals from each group after 6 and 12 weeks.

Twenty-three male animals and twenty-three female animals were selected randomly for extensive morphological examination of the nervous system. Two representative male and female animals treated with 1500 ppm in the diet, and one animal of each sex receiving a normal diet were terminated for morphological examination after 4, 8, and 12 weeks of dosing; rats receiving 240 mg/kg/day MA or PEA topically were examined after 10 ($n = 6 + 2$, respectively) and 12 weeks ($n = 4 + 2$); two male and two female animals treated with 10, 40, or 80 mg/kg or PEA were examined after 12 weeks. Sampled animals were anesthetized and systemically perfused via intracardiac cannulation with 4% paraformaldehyde followed by 5% glutaraldehyde, each in a 0.1 M phosphate buffer (pH 7.4). Brain, spinal cord, and peripheral nerves were removed; tissue segments were obtained from the sciatic nerve and its branches, lumbar dorsal root ganglia, corresponding dorsal and ventral roots, lumbar, thoracic, and cervical spinal cord, medulla oblongata, cerebellar vermis, lateral geniculate nuclei, cerebral cortex, and optic nerve. Tissue samples were postfixed in 2% Dalton's chrome osmium tetroxide, dehydrated in increasing concentrations of ethanol, cleared in acetone, infiltrated and embedded in epoxy resin, and processed for light microscopy. One-micrometer-thick epoxy cross sections were stained with borate-buffered 1% toluidine blue and examined by bright-field microscopy. Thin (50 nm) sections of selected areas were stained with uranyl acetate followed by lead citrate and examined by transmission electron microscopy.

RESULTS

Animals receiving MA applications displayed no adverse skin reactions throughout the test period. Hematology, clinical chemistry, and urinalysis parameters in treated animals were comparable to control animals at each time point tested. Body-weight gain was depressed in male animals receiving 80 and 240 mg/kg applied to the shaven back, and in female animals receiving 1500 ppm in the diet. Body-weight gain in the remaining animals treated with MA was similar to controls. By the end of the treatment period (12 weeks), hindlimb weakness was observed in 1/30 animals treated with 40 mg/kg/day, 15/30 animals treated with 80 mg/kg/day, and all animals treated with 240 mg/kg/day percutaneously, and 20/40 animals receiving 1500 ppm in the diet.

Upon necropsy, depressed testicular weight was noted in animals treated with 240 mg/kg/day. Examination of paraffin-embedded tissues revealed testicular tubular degeneration in these animals. Tubular degeneration was also observed in some animals receiving MA in the diet or treated with 80 mg/kg/day.

Neuropathologic changes observed in epoxy-embedded tissues of the central and peripheral nervous system were of two types, primary demyelination and distal axonal degeneration of long myelinated nerve fibers. Both types of abnormality appeared more or less simultaneously in affected areas of the nervous system, and were qualitatively similar in dietary and topically treated animals. Primary demyelination first appeared, and was most pronounced, in sampled regions of the spinal roots and corresponding spinal ganglia. The myelin sheaths of many large-diameter dorsal- and ventral-root fibers underwent conspicuous vacuolation (Fig. 1), leaving axons in affected regions denuded and attenuated. Intramyelinic phagocytes participated in the removal of damaged myelin, leaving demyelinated axonal segments. Affected axon

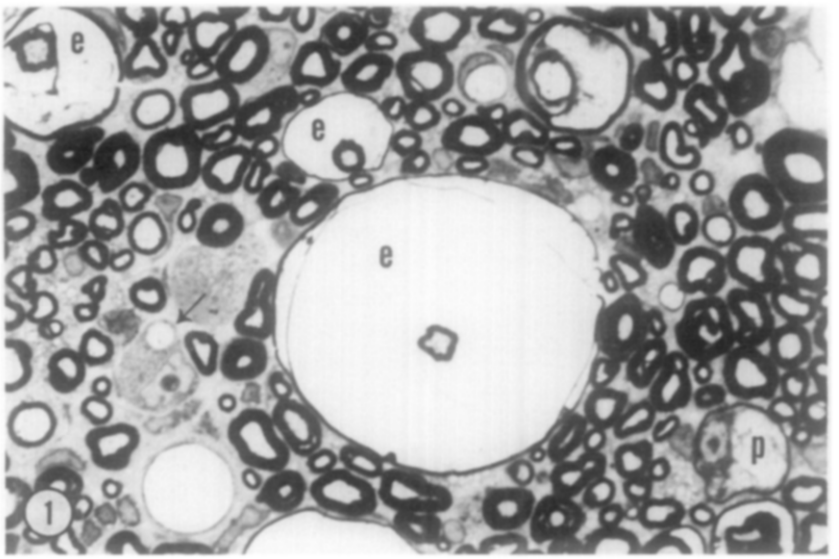

FIG. 1. Cross section of a lumbar ventral root taken from an animal treated with 240 mg/kg/day MA for 12 weeks. Several myelinated fibers display pathological changes, including splitting and intramyelinic edema (e), phagocytic penetration of the myelin sheath (p), and axonal denudation (arrow). One-micrometer epoxy cross section stained with toluidine blue (×560).

segments were sometimes associated with proliferated Schwann cells, some of which participated in remyelination of denuded segments. Scattered myelin vacuolation was observed in sampled nerves, throughout the white matter of the spinal cord, and occasionally in the cerebellar vermis. Primary axonal degeneration of myelinated fibers first appeared symmetrically in the gracile tract at the level of the medulla oblongata, and in tibial nerve branches supplying the calf muscles. In animals with greater degrees of hindlimb weakness, more proximal regions of these fiber tracts displayed axonal degeneration. The most severely affected animals showed axonal degeneration in the gracile tracts to the level of the lumbar spinal cord, and sciatic nerve up to the sciatic notch.

Neuropathologic changes were most severe in the central and peripheral nervous system of all animals receiving 240 mg/kg/day topically. A similar, but less severe, pattern of changes was encountered in tissues taken from animals receiving 1500 ppm (75 mg/kg) in the diet for 12 weeks. No clear pattern of differential sensitivity to MA with respect to sex was noted. Scattered early changes were found in animals treated with 80 mg/kg/day; some animals receiving 40 mg/kg/day showed isolated abnormalities, and tissue removed from rats dosed with 10 mg/kg/day revealed no pathologic alterations.

DISCUSSION

Subchronic dermal and dietary treatment of male and female rats with MA induces a complex neuropathologic picture of distal axonopathy and myelinopathy. Distal axonopathy is seen in a number of inherited and acquired diseases whose clinical expression is peripheral neuropathy characterized by a stocking-and-glove pattern of sensory loss and motor weakness (Spencer and Schaumburg, 1976). Myelinopathy, especially of dorsal roots, is a common accompaniment of advanced age in rats, humans, and other species (Griffin and Price, 1981; Spencer and Ochoa, 1981).

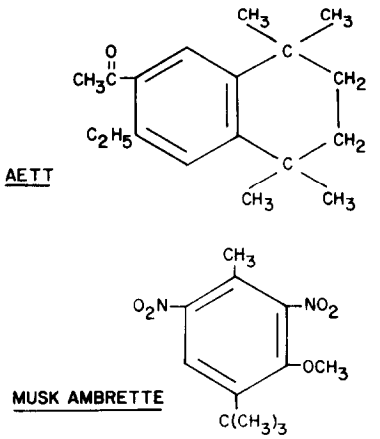

FIG. 2. Chemical structures of AETT and Musk Ambrette.

Demonstration of neurotoxic damage in rats receiving repeated dermal applications of MA in phenylethyl alcohol follows an earlier report of murine neurotoxicity from topically applied acetylethyltetramethyltetralin (AETT) (Spencer *et al.*, 1979), another musk agent which, until withdrawn from use in 1978, was widely used in fragrance formulations. Despite their disparate chemical structures (Fig. 2), MA and AETT both induce primary demyelination in experimental animals. These agents disrupt preformed myelin and yet fail morphologically to disturb remyelination, an event observed to progress unimpeded in the presence of either agent. There are, however, important differences in the neuropathologic responses of rats to the two synthetic musks; AETT additionally induces pigmentation and degeneration of CNS neurons of experimental rats, an irreversible event likely to precipitate permanent neurological deficit. MA appears to cause a central-peripheral distal axonopathy in addition to demyelination, both of which are, to some extent, reversible.

This study provides conclusive evidence that repeated dietary or topical treatment of rats with MA causes central and peripheral nervous system damage characterized by degeneration of myelin and selected distal axons. These toxic effects were seen in animals treated with MA at concentrations of, or greater than, 1500 ppm (diet) or 80 mg/kg/day (dermal). Estimated maximum human exposure to MA in fragrances from all possible sources is 0.15 to 0.32 mg/kg/day (Opdyke, 1981). While the doses producing consistent neurotoxic effects in rats are therefore well above (50 to 250×) the estimated maximum human exposure, borderline changes were seen in certain animals treated with 40 mg/kg/day. Most significantly, it has not been determined if lower concentrations of MA administered for longer periods induce nervous system damage (commonly observed with other neurotoxic agents that induce similar patterns of degeneration of myelin and/or distal axons). Given that lifetime human dietary and dermal exposure to consumer products containing musk ambrette is widespread, additional testing of the safety of this compound is warranted.

ACKNOWLEDGMENTS

The authors thank Louise Tedesco and Richard Robertson for technical assistance. Supported in part by the Research Institute for Fragrance Materials, Englewood Cliffs, New Jersey, NIOSH Grant OH00851, and NINCDS Grant NS 19611.

REFERENCES

ARCTANDER, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals).* Arctander, Montclair, New Jersey.

DAVIS, D. A., TAYLOR, J. M., JONES, W. I., AND BROUWER, J. B. (1967). Toxicity of musk ambrette. *Toxicol. Appl. Pharmacol.* **10,** 405.

GIOVINAZZO, V. J., HARBER, L. C., ARMSTRONG, R. B., AND KOCHEVAR, I. E. (1980). Photoallergic contact dermatitis to musk ambrette. Clinical report of two patients with persistent light reactor patterns. *J. Amer. Acad. Dermatol.* **3,** 384.

GRIFFIN, J. W., AND PRICE, D. L. (1981). Demyelination in experimental beta, beta'-iminodipropionitrile and hexacarbon neuropathies: Evidence for axonal influence. *Lab. Invest.* **45,** 130–141.

JENNER, P. M., HAGAN, E. C., TAYLOR, J. M., COOK, E. L., AND FITZHUGH, O. G. (1964). Food flavourings and compounds of related structure. *Food Cosmet. Toxicol.* **2,** 327.

KLIGMAN, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. Invest. Dermatol.* **47,** 393.

KLIGMAN, A. M. (1972). *Report to Research Institute for Fragrance Materials.* May 2.

OPDYKE, D. L. J. (1979). *Monographs on Fragrance Raw Materials.* Pergamon, New York.

OPDYKE, D. L. (1981). *Research Institute for Fragrance Materials,* NBB Information Bulletin 1, October 29.

SPENCER, P. S., AND OCHOA, J. (1981). The mammalian peripheral nervous system in old age. In *Aging and Cell Structure.* (J. E. Johnson, ed.), p. 35. Plenum, New York.

SPENCER, P. S., AND SCHAUMBURG, H. H. (1976). Central and peripheral distal axonopathy—The pathology of dying-back polyneuropathies. In *Progress in Neuropathology.* (H. M. Zimmerman, ed.), Vol. 3, p. 253. Grune & Stratton, New York.

SPENCER, P. S., STERMAN, A. B., HOROUPIAN, D. S., AND FOULDS, M. M. (1979). Neurotoxic fragrance produces ceroid and myelin disease. *Science (Washington, D.C.)* **204,** 633.

PETER S. SPENCER
MONICA C. BISCHOFF-FENTON

Institute of Neurotoxicology,
Departments of Neuroscience and Pathology,
Rose F. Kennedy Center for Research in Mental Retardation and Human Development,
Albert Einstein College of Medicine,
Bronx, New York 10461

OSCAR M. MORENO

M B Research Laboratories, Inc.,
Spinnerstown, Pennsylvania

DONALD L. OPDYKE
RICHARD A. FORD

Research Institute for Fragrance Materials, Inc.,
Englewood Cliffs, New Jersey
Received January 19, 1984