TOXICOLOGY AND APPLIED PHARMACOLOGY 81, 1-16 (1985)

The Synergism of *n*-Hexane-Induced Neurotoxicity by Methyl Isobutyl Ketone following Subchronic (90 Days) Inhalation in Hens: Induction of Hepatic Microsomal Cytochrome *P*-450

MOHAMED B. ABOU-DONIA,[1] DANIEL M. LAPADULA, GERALD CAMPBELL,[2] AND PHILLIP R. TIMMONS[3]

Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

Received October 13, 1984; accepted May 3, 1985

The Synergism of *n*-Hexane-Induced Neurotoxicity of Methyl Isobutyl Ketone following Subchronic (90 Days) Inhalation in Hens: Induction of Hepatic Microsomal Cytochrome *P*-450. ABOU-DONIA, M. B., LAPADULA, D. M., CAMPBELL, G., and TIMMONS, P. R. (1985). *Toxicol. Appl. Pharmacol.* **81**, 1-16. The effect of methyl isobutyl ketone (M*i*BK) on *n*-hexane-induced neurotoxicity was investigated via inhalation in seven groups of five hens each for 90 days followed by a 30-day observation period. One group was exposed to vapors containing 1000 ppm *n*-hexane and another group to vapors having 1000 ppm M*i*BK. Four groups were exposed simultaneously to 1000 ppm of *n*-hexane and 100, 250, 500, or 1000 ppm M*i*BK. Another group was exposed similarly to ambient air in an exposure chamber and used as a control. Hens continuously exposed to 1000 ppm M*i*BK developed leg weakness with subsequent recovery, while inhalation of the same concentration of *n*-hexane produced mild ataxia. Hens exposed to mixtures of *n*-hexane and M*i*BK developed clinical signs of neurotoxicity, the severity of which depended on the M*i*BK concentration. Thus, all hens exposed to 1000 ppm *n*-hexane in combination with 250, 500, or 1000 ppm M*i*BK progressed to paralysis. Hens continuously exposed to 1000/100 *n*-hexane/M*i*BK showed severe ataxia which did not change during the observation period. The neurologic dysfunction in hens exposed simultaneously to *n*-hexane and M*i*BK was accompanied by large swollen axons and degeneration of the axon and myelin of the spinal cord and peripheral nerves. The results indicate that the nonneurotoxic chemical M*i*BK synergized the neurotoxic action of the weak neurotoxicant *n*-hexane since the coneurotoxicity coefficient for joint exposure was more than two times the additive effect of each treatment alone. In another experiment, to investigate the mechanism of M*i*BK synergism of *n*-hexane neurotoxicity, continuous inhalation for 50 days of 1000 ppm *n*-hexane had no effect on hen hepatic microsomal enzymes, whereas inhalation of 1000 ppm M*i*BK for 50 days or a mixture of 1000 ppm of each of *n*-hexane and M*i*BK for 30 days significantly induced aniline hydroxylase activity and cytochrome *P*-450 contents in hen liver microsomes. Liver microsomal proteins from these hens and from hens treated with β-naphthoflavone (β-NF) and phenobarbital (PB) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. While β-NF increased the 55-kDa band (1408%), PB, M*i*BK, and M*i*BK/*n*-hexane increased the protein band (49 kDa) (258, 335, and 253%, respectively), indicating that M*i*BK induces chicken hepatic cytochrome *P*-450. The results suggest that the synergistic action of M*i*BK on *n*-hexane neurotoxicity may be related to its ability to induce liver microsomal cytochrome *P*-450, resulting in increased metabolic activation of *n*-hexane to more potent neurotoxic metabolites.

Aliphatic hexacarbon solvents, e.g., *n*-hexane, methyl *n*-butyl ketone (M*n*BK), and methyl isobutyl ketone (M*i*BK), have been used extensively in industry. Hexane is marketed as a 100% *n*-hexane and as technical- or com-

[1] To whom reprint requests should be addressed.

[2] Present address: Department of Pathology, Duke University Medical Center, Durham, N.C. 27710.

[3] Present address: Union Carbide Agricultural Products Company, Inc., Alexander Dr., Durham, N.C. 27709.

0041-008X/85 $3.00

mercial-grade hexane which contains 45 to 86% *n*-hexane, with isohexanes and cyclopentanes comprising the remaining constituents (Kirk-Othmer, 1966). Commercial-grade hexane may also contain small quantities of benzene, toluene, xylene, acetone, or chlorinated hydrocarbons. It is interesting that M*i*BK, which is used as a solvent, has become an environmental contaminant, resulting from improper disposal of industrial waste (Sheldon and Hites, 1978). M*i*BK also occurs naturally in oranges and grapes (Furia and Bellanca, 1975). The U.S. threshold limit value (TLV) for *n*-hexane or M*i*BK in work place air is 150 ppm.

n-Hexane was implicated in producing central-peripheral distal axonopathy in industrial workers and "glue sniffers" (Yamada, 1964; Spencer *et al.*, 1980). Continuous inhalation or prolonged subcutaneous injection of *n*-hexane produced neurotoxicity in rats (Schaumburg and Spencer, 1976). In 1972, a solvent mixture containing 90% methyl ethyl ketone (MEK) and M*n*BK was introduced in the printing of polyvinyl fabrics at a plant in Columbus, Ohio. A year later, workers in this plant developed neuropathy, and M*n*BK was suspected as the causative agent (Billmaier *et al.*, 1974). M*n*BK is also produced as a metabolite *in vivo* of *n*-hexane in various species (Kramer *et al.*, 1974; Abdel-Rahman *et al.*, 1976). All of these metabolic products are more potent in producing neuropathy in laboratory animals than the parent compound, *n*-hexane (Spencer *et al.*, 1978; Abdo *et al.*, 1982; Abou-Donia *et al.*, 1982). A prominent feature of aliphatic hexacarbon-induced neuropathy is the formation of large swellings in the affected tracts of the spinal cord in rats (Schaumburg and Spencer,1976; Cavanagh, 1982) and in the hen (Abdo *et al.*, 1982; Abou-Donia *et al.*, 1982). Distal portions of the peripheral nerves also exhibit axonal swelling and degeneration. By contrast, despite claims that M*i*BK causes neuropathy in humans (Oh and Kim, 1976; AuBuchon *et al.*, 1979), carefully performed laboratory studies have failed to produce neuropathy in rats (Spencer *et al.*, 1975) or in cats (Spencer and Schaumburg, 1976).

The joint effect of combining drugs has been studied as early as 1910 by Burgi who stated, "In combining drugs with the same end-effect, the resulting activity is additive when the sites of action of the components are identical and superadditive if these are different." He also introduced the term "potentiation" to describe the augmentative effect of one of the components by the other. Unfortunately, there are disagreements in the use of the terms "synergism" and "potentiation" in the current literature (Hodgson, 1980). In this study, the nomenclature of Rentz (1932) is used. Synergism or potentiation takes place when the combined effect of two or more chemicals is more than the algebraic summation of the effects of each chemical when used alone. Synergism occurs when one component has little or no intrinsic effect when used alone, while potentiation occurs when both compounds possess the same type of activity.

Because the industrial solvents *n*-hexane and M*i*BK are commonly used, concurrent inhalation of their vapors by workers is possible in the work place. Therefore, the effect of M*i*BK on the neurotoxicity of *n*-hexane was investigated as an example of joint action of aliphathic solvents. Also, the mechanism of their joint neurotoxic action was studied

METHODS

Chemicals

Methyl isobutyl ketone (4-methyl-2-pentanone, 99.95%) was provided by Eastman Kodak Company (Kingsport, Tenn.) and *n*-hexane (99%) was purchased from Phillips Petroleum Company (Bartlesville, Okla.). Sodium phenobarbital (PB) and β-naphthoflavone (β-NF) were obtained from Sigma Chemical Company (St. Louis, Mo.) and Mallinckrodt, Inc. (St. Louis, Mo.), respectively.

Birds

Adult leghorn laying hens (*Gallus gallus* domesticus), 14 months old and weighing 1.62 ± 0.03 kg (mean ± SE) were used (Featherdown Farm, Raleigh, N.C.). The hens

were specified pathogen free and chemical medication free. Hens were kept in single tier stainless-steel inhalation chambers in a room controlled for humidity (40 to 60%) and temperature (21 to 23°C) with a 12-hr light/dark cycle before and during the experiment. The chickens were kept in this environment to acclimatize for 1 week before being randomly assigned to control and treatment groups. They were supplied with feed (Layena Chicken Feed, Ralston Purina Co., St. Louis, Mo.) and water *ad libitum*.

Treatment Protocol

Exposure of hens to n-hexane and MiBK. One group of five hens was exposed to vapors containing 1000 ppm *n*-hexane and one group of five hens was exposed to 1000 ppm M*i*BK, each for 90 days. Four groups (five hens each) were exposed simultaneously to 1000 ppm *n*-hexane in combination with 100, 250, 500, or 1000 ppm M*i*BK. A group of five hens was exposed to ambient air in an exposure chamber for 90 days and served as a control. Moribund hens were killed before the end of the experimental period. Surviving birds were kept out of the chambers for a 30-day observation period. Body weights were monitored weekly, and hens were examined daily for neurological deficits.

Induction of liver microsomal enzymes. In another experiment, groups of five hens were continuously exposed to 1000 ppm *n*-hexane, 1000 ppm M*i*BK, or a mixture of their vapors in inhalation chambers. Another group of five hens was exposed to ambient air. One hen from the *n*-hexane/M*i*BK group died after 30 days and the remaining birds were killed on that day. The other groups were killed after 50 days of exposure. A group of four hens was treated with a consecutive 3-day ip dose of 80 mg/kg sodium phenobarbital in saline; another group of four hens received 80 mg/kg β-naphthoflavone in 5 ml of corn oil for 3 consecutive days. A group of four hens was similarly treated with corn oil and used as a control. Treated and control hens were anesthetized by CO_2 and killed by decapitation 24 hr after the last dose.

Generation of n-Hexane and MiBK Vapors

A portable-type stainless-steel animal inhalation chamber (Young and Bertke, Co., Cincinnati, Ohio), described previously (Abdo *et al.*, 1982), was used to expose the chickens to *n*-hexane and M*i*BK vapors (Fig. 1). Vapors were generated in a 1-liter three-necked round-bottomed flask half filled with either solvent. One neck was connected to a tank of compressed pure breathing air, another to a 500-ml separatory funnel that served as a reservoir for the solvent, and the third to a 400-mm condenser. The condenser was connected to the chamber with Teflon tubing. Both the condenser and the Teflon tubing were wrapped with heating tape that was heated to 50°C to prevent condensation of the solvent vapor. The vapor was swept into the chamber by the airflow from the air tank. The desired concentration of vapor was maintained by adjusting the

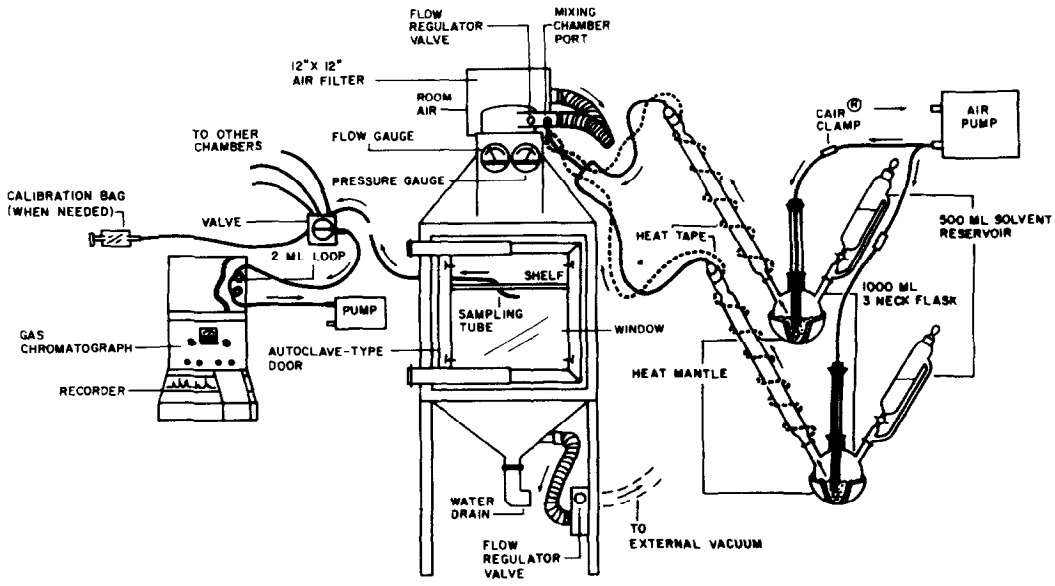

FIG. 1. Exposure system for *n*-hexane/M*i*BK vapors: right, generation of *n*-hexane and M*i*BK vapors; center, inhalation exposure chamber; and left, monitoring of vapor concentration. For a detailed description see Abdo *et al.* (1982).

heating temperature and the airflow through the solvent. No heat was needed to generate *n*-hexane or low concentrations of M*i*BK. However, for 500 and 1000 ppm M*i*BK, the solvent flask was heated by heating the mantel to 65°C. To obtain a mixture of *n*-hexane and M*i*BK vapors, two generating systems were used for each solvent (Fig. 1). The Teflon tubings from each condenser were connected through a T tube into the mixing port of the chamber. A Gow-Mac gas chromatograph (Series 750, Gow-Mac Instruments, Bound Brook, N.J.), equipped with a flame ionization detector and a 2 m × 2 mm o.d. coiled glass column packed with Tenax GC 60/80 mesh, was used to measure *n*-hexane and M*i*BK concentrations (Abdo *et al.*, 1982). The retention times for *n*-hexane and M*i*BK were 0.5 and 1.8 min, respectively. The vapor concentration in the chamber varied a maximum 10%.

Evaluation of Neurologic Deficits

Blind observations of hens were made daily inside the exposure chamber and when moving freely outside the chamber. The neurologic deficits were classified into four grades of ataxia prior to onset of paralysis: mild (T_1), gross (T_2), severe (T_3), and near paralysis (T_4) (Abou-Donia, 1978). For graphical presentation, the clinical condition was assigned numerical values of 0 to 5; 1 to 4 correspond to the T_1 to T_4 stages of ataxia, and 5 corresponds to paralysis. Zero corresponds to the clinical condition of the normal hens.

Histopathological Procedures

Hens were anesthetized with CO_2, followed by opening the thorax and perfusing 10% neutral phosphate-buffered Formalin through a cannula into the ascending aorta via the left ventricle with a pump (Manostat Varistalic Pump, Fisher Scientific, Raleigh, N.C.). Cervical, thoracic, and lumbosacral regions of the spinal cord along with the sciatic, tibial, and peroneal nerves and their branches were fixed for at least a week in the buffered Formalin solution (Abou-Donia and Preissig, 1976). Cross and parasaggital longitudinal sections near the midline were prepared from all regions of the spinal cord and peripheral nerves. Following dehydration in graded ethanol, tissues were embedded in paraffin or glycol methacrylate. Paraffin sections (8 μm) from the spinal cord and peripheral nerves were stained with hematoxylin and eosin (H and E) combined with Luxol fast blue (LFB) or Glees stain. Peripheral nerve sections were stained with Holmes' stain. The glycol methacrylate sections of the spinal cord and peripheral nerves (1 to 2 μm) were stained with toluidine blue. Histopathological examination was done blindly.

Measurement of Liver Microsomal Enzymes

Immediately after decapitation of the hen, the liver was removed. Hepatic microsomes were prepared, washed, frozen, and stored at −70°C (Schenkman and Cinti, 1978). Cytochrome *P*-450 was measured and expressed as micromoles of cytochrome *P*-450 per milligram of protein (Omura and Sato, 1964). Aniline hydroxylase activity was measured by the Mazel (1971) method, with 1.5 μmol of NADPH replacing the NADPH-generating system. Liver microsomal proteins were determined by the Lowry *et al.* (1951) method with bovine serum albumin as standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Aliquots from liver microsomal homogenates of hens treated with *n*-hexane, M*i*BK, or a mixture of their vapors along with those treated with PB and β-NF and control hens were analyzed by SDS–PAGE. A 20-well 0.1% SDS–polyacrylamide vertical slab gel (10 × 16 × 0.15 cm) (Bio–Rad Laboratories) was used to resolve microsomal proteins by a modified procedure of Laemmli (1970), employing 4% acrylamide in the stacking gel and a 6 to 10% gradient of acrylamide in the resolving gel. Final concentrations of 25 μg protein/50 μl samples of microsomal proteins were prepared containing 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, and 0.125 M Tris–HCl, pH 6.8, followed by heating for 5 min in a 90°C water bath. The following protein molecular weight markers were used: carbonic anhydrase (30,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase B (92,500), and β-galactosidase (116,200) (Bio–Rad Laboratories). Electrophoresis of 150-μl samples was carried out under constant power (7 W/slab) until the tracking dye reached the bottom of the gel. The solvent mixture of acetic acid/methanol/water (10/45/45, v/v/v) was used to fix the gels which were stained with 0.1% Coomassie blue in the same solvent system and destained in fixing solution. Gels were soaked for 1 hr in a solvent of glycerol/acetic acid/water (1/10/90, v/v/v) and dried on dialysis membrane. Quantification of each protein band was performed by an LKB laser densitometer with a recording integrator (LKB Instruments, Inc., Brommo, Sweden).

Evaluation of the Joint Neurotoxic Action

Neurotoxicity index (NTI). Neurotoxicity of each treatment was quantified by calculating the neurotoxicity index described previously (Abou-Donia *et al.*, 1982). The NTI was calculated by ranking the hens, starting with minimal changes in the following three parameters: (1) the time of onset of neurologic dysfunction, (2) the severity of neurologic deficit, and (3) the severity and frequency of histopathologic lesions (Jonckheere, 1954). The NTI was calculated as the mean of the three ranks of hens in each of these three areas.

Coneurotoxicity coefficient (CNC). The following equation was used to determine the coneurotoxicity coefficient for two or more neurotoxic agents:

$$\text{CNC} = \frac{\text{experimentally determined NTI for chemicals 1, 2, . . . , } n.}{\text{NTI}_1 + \text{NTI}_2 + \cdots + \text{NTI}_n} \quad (1)$$

NTI_1, NTI_2, and NTI_n are the neurotoxicity indices for chemical 1, 2, and n, respectively.

When the CNC value is larger than 1, synergism or potentiation is indicated, when the CNC is less than one, antagonism occurs. The additive effect takes place when CNC equals one.

The joint neurotoxic action produced by exposing hens simultaneously to *n*-hexane and M*i*BK is measured by calculating $\text{CNC}_{n\text{-hexane,MiBK}}$ as

$$\text{CNC}_{n\text{-hexane,MiBK}} = \frac{\text{experimentally determined NTI}_{n\text{-hexane,MiBK}}}{\text{NTI}_{n\text{-hexane}} + \text{NTI}_{\text{MiBK}}} \quad (2)$$

where $\text{NTI}_{n\text{-hexane}}$ is the neurotoxicity index when *n*-hexane was used alone, NTI_{MiBK} is the neurotoxicity index when M*i*BK was used alone, and $\text{NTI}_{n\text{-hexane,MiBK}}$ is the neurotoxicity index when *n*-hexane and M*i*BK were used simultaneously.

Statistics

Significance of the difference between the weight and liver microsomal cytochrome *P*-450 of control and treated hens was assessed by the two-way ANOVA test. A p value of 0.05 or less was considered significant.

RESULTS

Body Weight Changes

Hens exposed to 1000 ppm M*i*BK weighed 90.5 ± 7.3% of their initial weights when they developed leg weakness. These hens regained most of the lost weight by the end of the 90-day exposure period and continued to gain weight during the 30-day observation period (Table 1). Exposure to 1000 ppm *n*-hexane caused significant loss of weight at onset of ataxia in treated hens. These hens regained all the lost weight and weighed 111.9 ± 1.7% of their initial weight at termination. Hens exposed to mixtures of *n*-hexane and M*i*BK vapors lost weight at onset of ataxia, and except for the group given 1000/100 ppm *n*-hexane/M*i*BK, the loss of weight continued throughout the experiment. The weight of control hens that were exposed to ambient air did not significantly change throughout the experiment.

Clinical Assessment of Neurotoxicity

Continuous inhalation of 1000 M*i*BK caused leg weakness in hens after 44 ± 2 days

TABLE 1

CHANGE IN WEIGHT OF CONTROL AND TREATED HENS FOLLOWING CONTINUOUS INHALATION OF *n*-HEXANE, M*i*BK, OR A MIXTURE OF *n*-HEXANE AND M*i*BK[a]

Concentration (ppm)			% of initial weight at		
n-Hexane	M*i*BK	Initial wt (kg)	Onset of ataxia	End of exposure	Termination
0	1000	1.60 ± 0.07	—	97.9 ± 3.9	110.0 ± 3.3*
1000	0	1.47 ± 0.07	89.1 ± 7.5*	87.7 ± 3.2*	111.9 ± 1.7*
1000	1000[b]	1.43 ± 0.09	87.8 ± 5.3*	70.9 ± 5.7**	68.5 ± 5.9**
1000	500	1.69 ± 0.05	80.7 ± 3.4**	67.5 ± 4.8**	67.5 ± 4.8**
1000	250	1.75 ± 0.06	76.6 ± 7.0**	65.2 ± 7.4**	65.2 ± 7.4**
1000	100	1.69 ± 0.06	83.2 ± 1.4*	80.0 ± 2.3**	94.8 ± 2.1
0	0[c]	1.62 ± 0.03	—	98.3 ± 1.5	97.1 ± 0.8

[a] Hens were continuously exposed to *n*-hexane, M*i*BK, or a mixture of *n*-hexane and M*i*BK vapors in inhalation chambers for 90 days. Surviving birds were kept out of the chambers for a 30-day observation period.

[b] One hen was killed on Day 17 and another on Day 31 when its condition deteriorated.

[c] Control hens were exposed to ambient air in an exposure chamber for 90 days and then kept outside the chamber for a 30-day observation period.

* Significant difference from control $p < 0.05$.

** Significant difference from control $p < 0.02$.

(mean ± SE). These hens recovered after the daily exposure was discontinued. Hens exposed to 1000 ppm *n*-hexane showed mild ataxia (Fig. 2). The condition of these birds did not change during the 30-day observation period.

All hens continually exposed to mixed vapors of 1000 ppm *n*-hexane and to 100, 250, 500, or 1000 ppm M*i*BK developed ataxia after a latent period of 10 to 29 days, depending on M*i*BK concentration (Fig. 2). The severity and progression of clinical signs also depended on M*i*BK concentration. All hens exposed to 1000 ppm *n*-hexane and 250, 500, or 1000 ppm M*i*BK developed all stages of ataxia and progressed to paralysis. One hen exposed to 1000/1000 ppm *n*-hexane/M*i*BK was killed on Day 17 and the remaining four were killed on Day 31 when their conditions deteriorated. Hens continually exposed to 1000/100 ppm *n*-hexane/M*i*BK showed severe ataxia and their clinical condition did not change during the observation period. Ambient air/control hens remained normal.

Necropsy Observations

All tissues were grossly examined at termination. When tissues of treated and control birds were compared for size, shape, and color, no differences were observed.

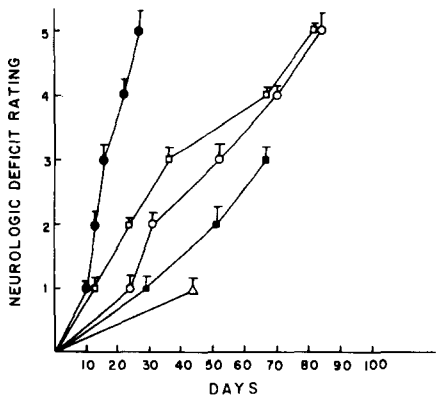

FIG. 2. Neurologic deficits in hens exposed to 1000 ppm *n*-hexane (△), and simultaneous exposure to vapors of 1000 ppm *n*-hexane and the following concentrations (ppm) of M*i*BK: 100 (■), 250 (○), 500 (□), and 1000 (●). Neurologic deficit rating is described under Methods. The results show days before onset of each stage of neurologic deficit and represent $\bar{x}$ ± SE from three hens.

Histopathological Changes

Tissues from spinal cord and peripheral nerves of control and treated hens were histologically examined (Table 2). Sections from control hens were normal. Lesions in nervous tissues depended on the treatment, concentration, and duration of exposure. Continuous inhalation of 1000 ppm M*i*BK for 90 days did not induce any histopathological changes in the spinal cord or peripheral nerves of treated hens.

The spinal cord of one hen exposed to 1000 ppm *n*-hexane showed equivocal histologic changes in the lumbar region (Fig. 3). These lesions were occasional swollen axons without fragmentation, phagocytosis, or myelin loss. Although these changes may be early histopathologic changes in *n*-hexane neurotoxicity, they have rarely been seen in the spinal cords of normal birds, and thus may not be related to treatment. Another hen of this group exhibited unequivocal degeneration of the axons and myelin in the ventral columns of the thoracic spinal cord. No changes were seen in the peripheral nerves.

Lesions in the nervous tissues of hens exposed to mixtures of 1000 ppm *n*-hexane and different concentrations of M*i*BK were dependent on M*i*BK concentration, period of exposure, and duration of intoxication. Hens exposed to 1000/1000 ppm *n*-hexane/M*i*BK were killed after 18 ± 3 days (mean ± SE) of onset of intoxication; only one of these hens exhibited equivocal lesions in the ventral columns of the thoracic spinal cord. By contrast, hens exposed to 1000/500 ppm *n*-hexane/M*i*BK survived longer; all exhibited signs of neurotoxicity for 108 ± 1 days, and all five hens showed unequivocal changes in all columns at all levels of the spinal cord. Swollen and degenerated axons can be identified in the ventral columns of the thoracic spinal cord (Fig. 4). Also, extensive degeneration of the

TABLE 2
DURATION OF INTOXICATION AND HISTOPATHOLOGICAL CHANGES IN NERVOUS TISSUES FROM HENS FOLLOWING CONTINUOUS INHALATION OF *n*-HEXANE, M*i*BK, OR MIXTURES OF *n*-HEXANE AND M*i*BK[a]

Concentration (ppm)			Number of hens showing histopathological changes[c]								
			Cervical			Spinal cord, thoracic			Lumbar			
n-hexane	M*i*BK	Days of intoxication[b]	DC	VC	LC	DC	VC	LC	DC	VC	LC	Peripheral nerves
0	1000	0	0	0	0	0	0	0	0	0	0	0
1000	0	76 ± 1	0	0	0	0	1±	0	0	1±	0	0
1000	1000	18 ± 3	0	0	0	0	1±	0	0	0	0	1±
1000	500	108 ± 1	1±,1±	5+	5+	1±	3+,2++	5+	0	3+,2++	1+	4+
1000	250	96 ± 2	0	3+	4+	0	4+,1±	3+,1±	0	3+,1±	2+	2+
1000	100	91 ± 1	0	1+	5+	0	5+	5+	0	5+	2+	2±

[a] The exposure schedule is listed in Table 1.
[b] Days between onset of mild ataxia and termination.
[c] The following symbols are used: ±, equivocal changes (rare swollen axons without fragmentation, phagocytosis, or loss of myelin staining); +, mild to moderate degeneration of axons and myelin; ++, lesions are termed severe where there is almost complete destruction of axons and myelin in a given tract, such as anterior columns or without extensive areas of peripheral nerve; VC, ventral column; LC, lateral column; DC, dorsal column.

axons and myelin is seen in the longitudinal (Fig. 5) and cross (Fig. 6) sections of the lumbar spinal cord. Four hens from this group showed unequivocal histopathological lesions in peripheral nerves, and one hen showed markedly swollen dystrophic and degenerated axons (Fig. 7) in the longitudinal section of the peripheral nerve. Hens exposed to 1000/250 *n*-hexane/M*i*BK showed clinical signs of neurotoxicity for 96 ± 2 days; one of these hens exhibited equivocal changes in the spinal cord, and the other four exhibited unequivocal lesions in the ventral and lateral columns but not in the dorsal columns of the spinal cord. Two hens from this group showed unequivocal changes in the peripheral nerves. Hens exposed to 1000/100 ppm *n*-hexane/M*i*BK developed signs of neurotoxicity for a mean of 91 ± 1 days and showed degeneration of the axons and myelin in the ventral and lateral columns of the spinal cord. Only two hens from this group exhibited equivocal histopathologic changes in the peripheral nerves.

Quantification of Neurotoxic Effects

The neurotoxicity index was calculated for each treatment in order to compare the neurotoxic effect produced by exposure to *n*-hexane vapor alone and that to simultaneous exposure to *n*-hexane in combination with various concentrations of M*i*BK vapors (Table 3). The NTI values for exposure to 1000 ppm of *n*-hexane or 1000 ppm M*i*BK vapor alone were 6.2 and 4.2, respectively. When hens were simultaneously exposed to 1000 ppm *n*-hexane, the NTI value depended on the concentration of M*i*BK. Therefore, concurrent exposure to 1000 ppm *n*-hexane and 100, 250, 500, or 1000 ppm M*i*BK produced NTI values of 19.2, 24.3, 28.1, and 24.6, respectively.

The coneurotoxicity coefficient was calculated to determine the joint neurotoxic action of 1000 ppm *n*-hexane and 1000 ppm M*i*BK as described under Methods:

$$\text{CNC} = \frac{24.6}{4.2 + 6.2} = \frac{24.6}{10.4} = 2.3.$$

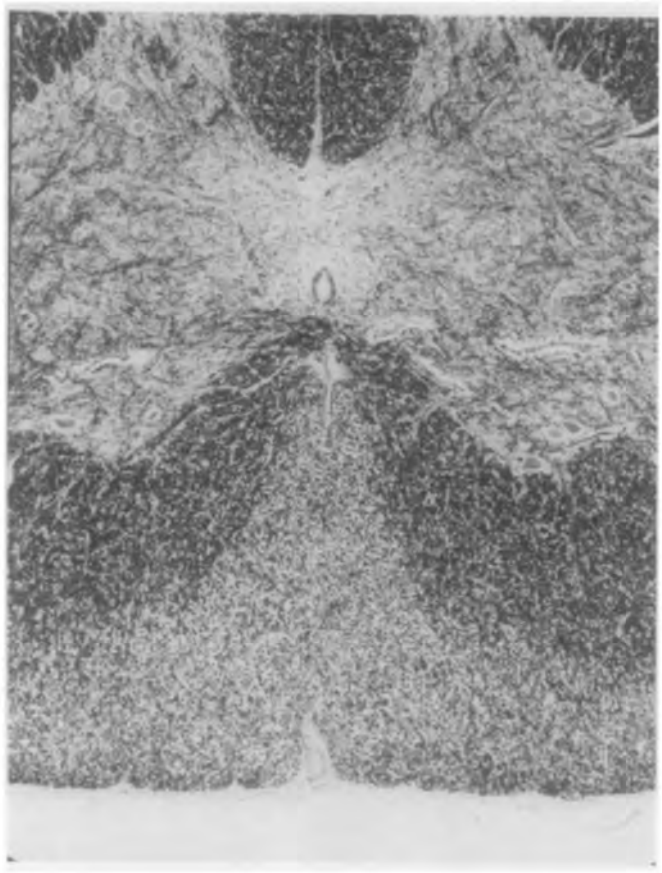

FIG. 3. Lumbar spinal cord from a hen continuously exposed to 1000 ppm *n*-hexane vapor for 90 days. This cross section displays a single axonal swelling in the ventral columns. A paraffin-embedded section, H and E–LFB, × 52.

Liver Microsomal Enzymes

Cytochrome P-450. A small amount, 0.19 ± 0.03 nmol, of cytochrome *P*-450 per milligram of protein, was found in liver from control hens (Table 4). Inhalation of 1000 ppm *n*-hexane for 50 days resulted in a small (23%) increase of cytochrome *P*-450 that was not statistically significant from control hens. By contrast, exposure of hens to the same level of M*i*BK significantly increased cytochrome *P*-450, i.e., 558% of control. Inhalation of an *n*-hexane/M*i*BK mixture for only 30 days induced liver microsomal cytochrome *P*-450 to 378% of control levels.

Aniline hydroxylase. The effect of *n*-hexane and M*i*BK vapors on liver microsomal aniline hydroxylase activity paralleled the effect of *n*-hexane and M*i*BK vapors on cytochrome *P*-450 content (Table 4). The largest induction of this enzyme was produced by inhalation of M*i*BK (362% of control), whereas simultaneous exposure to *n*-hexane and M*i*BK for a shorter duration resulted in enzymatic activity of 309% of control levels. On the other hand, inhalation of 1000 ppm *n*-hexane for 50 days did not affect hepatic microsomal aniline hydroxylase activity.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Microsomal protein profiles from control hens and from hens exposed to 1000 ppm of *n*-hexane, M*i*BK, or both exhib-

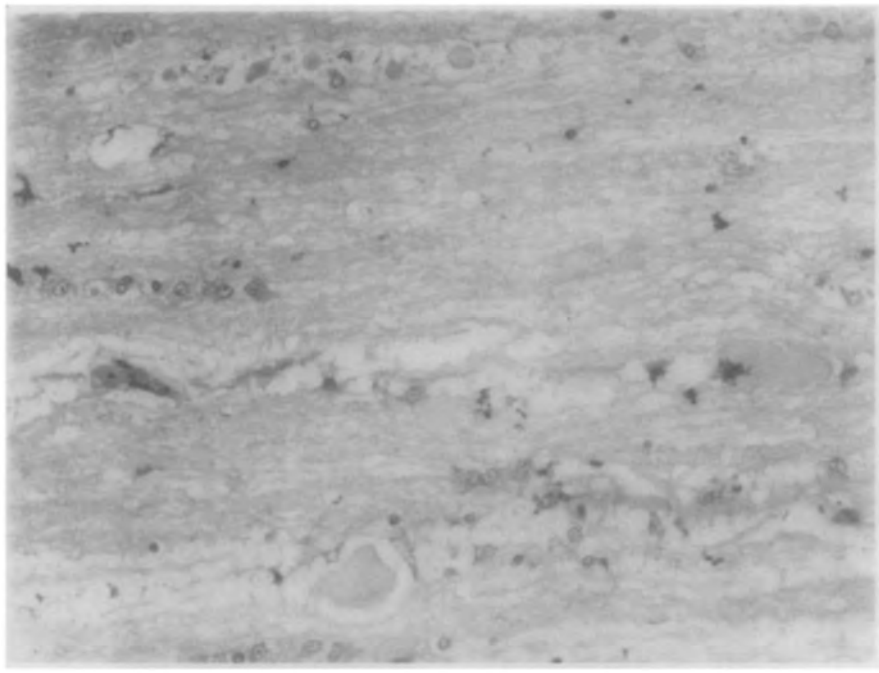

FIG. 4. Thoracic spinal cord from a hen simultaneously exposed to 1000 ppm *n*-hexane and 500 ppm M*i*BK vapors for 90 days. This longitudinal section of the ventral columns shows swollen dystrophic axons (lower half) as well as axon tracts occupied by phagocytes indicative of Wallerian-type degeneration (upper half). A methacrylate-embedded section, toluidine blue, ×325.

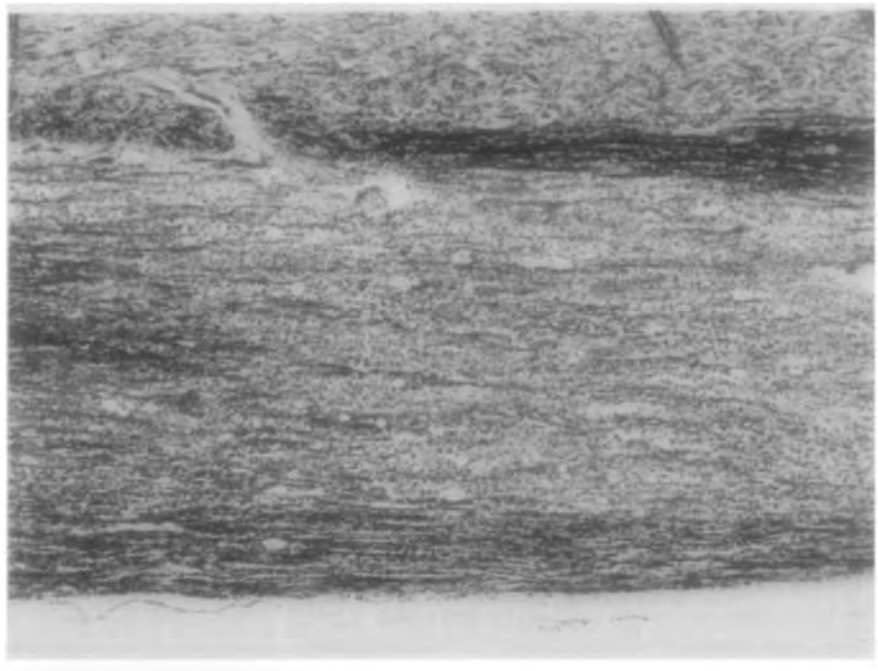

FIG. 5. Lumbar spinal cord from a hen simultaneously exposed to 1000 ppm *n*-hexane and 500 ppm M*i*BK vapors for 90 days. This longitudinal section of ventral columns documents massive degeneration of axons and myelin. A paraffin-embedded section, H and E–LFB, ×100.

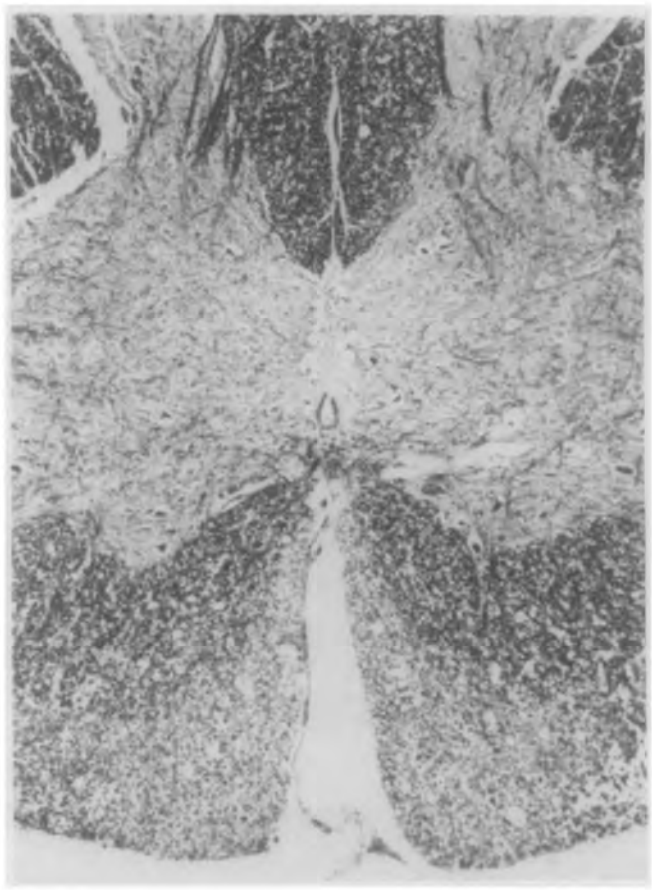

FIG. 6. Lumbar spinal cord from a hen simultaneously exposed to 1000 ppm *n*-hexane and 500 ppm M*i*BK vapors for 90 days. This cross section reveals extensive degeneration of the ventral columns. A paraffin-embedded section, H and E–LFB, ×52.

ited distinct differences (Fig. 8). Microdensitometry demonstrated that *n*-hexane exposure produced a quantitatively and qualitatively similar hepatic microsomal protein profile to that of control hens. Phenobarbital produced a significant increase in protein bands of 49,000 and 52,000 daltons (Table 5). β-Naphthoflavone significantly increased the 52,000 and 55,000 bands. Exposure to M*i*BK or a mixture of *n*-hexane and M*i*BK vapors resulted in a significant increase of the protein bands that were induced by PB, suggesting an induction of cytochrome *P*-450 (Fig. 8).

Liver microsomes from control and all treated hens were scanned for cytochrome *P*-450 content. The λ_{max} of the Soret peak of the reduced cytochrome *P*-450-CO complex in PB-, M*i*BK-, and *n*-hexane/M*i*BK-treated animals was determined to be 450 nm, whereas it was 448 nm in β-NF-treated animals and 451 nm in control hens.

DISCUSSION

This report establishes that simultaneous subchronic exposure to *n*-hexane and M*i*BK vapors markedly increased the neurotoxic action of the weak neurotoxicant *n*-hexane. Continuous inhalation of 1000 ppm M*i*BK for 90 days failed to produce neurotoxicity in hens; this finding is in agreement with previous findings in rats (Spencer *et al.*, 1975) and cats

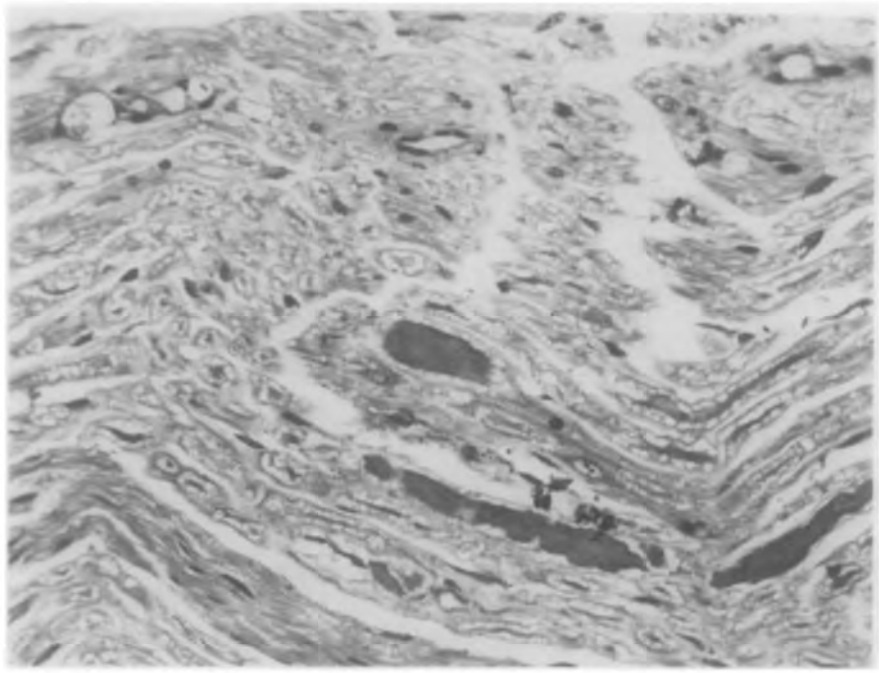

FIG. 7. Peripheral nerve from a hen simultaneously exposed to 1000 *n*-hexane and 500 ppm M*i*BK vapors for 90 days. A branch of sural nerve is seen in longitudinal section. Several swollen, dystrophic axons are present in the lower right field, while an axon undergoing Wallerian degeneration is present in the upper left corner. A methacrylate-embedded section, toluidine blue, ×400.

(Spencer and Schaumburg, 1976). On the other hand, while inhalation of 1000 ppm *n*-hexane produced mild neurotoxicity, simultaneous exposure to 1000 ppm of *n*-hexane and 250, 500, or 1000 ppm M*i*BK resulted in increased neurotoxic action.

TABLE 3

NEUROTOXICITY INDEX OF VARIOUS TREATMENTS OF *n*-HEXANE AND/OR M*i*BK IN HENS[a]

Concentration (ppm)		Group ranking within (mean ± SE)[b]			
n-Hexane	M*i*BK	Clinical condition	Onset of clinical signs	Severity of histopathological changes	NTI[c]
0	1000	3.0 ± 0.7	3.0 ± 0.7	6.5 ± 0.7	4.2 ± 1.2
1000	0	8.0 ± 0.7	8.0 ± 0.7	9.6 ± 1.9	6.2 ± 2.6
1000	1000	33.0 ± 0.7	33.0 ± 0.7	7.9 ± 1.4	24.6 ± 8.4
1000	500	28.0 ± 0.7	26.0 ± 1.4	30.4 ± 1.4	28.1 ± 1.3
1000	250	23.0 ± 0.7	24.2 ± 1.2	25.8 ± 2.5	24.3 ± 0.8
1000	100	18.0 ± 0.7	18.0 ± 0.7	21.6 ± 1.6	19.2 ± 1.2

[a] The exposure schedule is listed in Table 1.

[b] Hens were assigned ranks within the following categories: clinical condition, onset of clinical signs, and severity of histopathological changes.

[c] Neurotoxicity index was calculated as $\bar{x} \pm$ SE of the three ranks of hens of each of the three factors. Standard error-type calculations were made to provide an indication of the variability among the values contributing to each mean.

TABLE 4

EFFECT OF CONTINUOUS EXPOSURE TO *n*-HEXANE, M*i*BK, OR A MIXTURE OF *n*-HEXANE AND M*i*BK VAPORS ON CHICKEN HEPATIC MICROSOMAL CYTOCHROME *P*-450 CONTENT AND ANILINE HYDROXYLASE ACTIVITY[a]

Concentration (ppm)			Cytochrome *P*-450 content		Aniline hydroxylase	
n-Hexane	M*i*BK	Days of exposure	μmol/mg of protein ± SE	%	μmol PAP/mg of protein	%
0	0	50	0.19 ± 0.03	100 ± 16	0.09 ± 0.01	100 ± 15
1000	0	50	0.24 ± 0.02	123 ± 13	0.09 ± 0.01	99 ± 13
0	1000	50	1.80 ± 0.05	558 ± 29*	0.33 ± 0.04	362 ± 40*
1000	1000	30[b]	0.70 ± 0.06	378 ± 31*	0.29 ± 0.04	309 ± 44*

[a] Each treatment consisted of five hens.

[b] One hen from this group died after 30 days of simultaneous exposure to *n*-hexane and M*i*BK. The other four were morbid and were killed on that day.

* Significant difference from control $p < 0.01$.

It is interesting to speculate explanations for the synergistic effect of M*i*BK on *n*-hexane neurotoxicity. *n*-Hexane, a straight six-carbon and 14-hydrogen chemical, is chemically unreactive and remains unchanged after treatment with concentrated sulfuric acid, boiling nitric acid, or molten sodium hydroxide (Kirk-Othmer, 1966). *n*-Hexane undergoes oxidation by microsomal enzymes to alcohols, primarily 2-hexanol (Frommer *et al.*, 1974). 2-Hexanol is then further oxidized to 2,5-hexanediol, 2-hydroxy-5-hexanone, 2-hexanone (M*n*BK), and 2,5-hexanedione (2,5-HD) (Kramer *et al.*, 1974; Abdel-Rahman *et al.*, 1976; DiVincenzo *et al.*, 1977). All of these biotransformation products of *n*-hexane are more neurotoxic than the parent compound. The metabolic product 2,5-HD is particularly interesting since it is the most persistent metabolite found in serum *in vivo;* it is also the most potent neurotoxic hexacarbon of all *n*-hexane-type chemicals (Spencer *et al.*, 1978; Abou-Donia *et al.*, 1982). In fact, it is believed that 2,5-HD is the neurotoxic agent responsible for *n*-hexane's and its related chemicals' neurotoxicity. This may explain the weak neurotoxic effect of *n*-hexane in the chicken compared to the rat; in the hen liver microsomes, cytochrome *P*-450 content is approximately 25% of that in the rat (Lasker *et al.*, 1982; Lapadula *et al.*, 1984). Furthermore, the fact that M*i*BK (4-methyl-2-pentanone) cannot be metabolized to 2,5-HD may also explain its nonneurotoxic properties.

The synergistic action of *n*-hexane by M*i*BK depended upon (1) M*i*BK concentration, (2) duration of exposure, (3) length of time between onset of neurotoxicity signs and termination, and (4) severity of the clinical condition. All of these factors were incorporated into the neurotoxicity index that was used to quantify the neurotoxic effect of each treatment. The NTI value was least in hens given M*i*BK followed by *n*-hexane alone. On the other hand, the NTI was greatly increased by concurrent exposure to the two solvents. Although NTI was generally proportional to the concentration of M*i*BK, duration of exposure and the length of the intoxication period were important factors.

In order to determine the joint neurotoxic action of simultaneous exposure to 1000 ppm of *n*-hexane and 1000 ppm of M*i*BK, the co-neurotoxicity was calculated to be 2.3. Since CNC is more than one and M*i*BK is not neurotoxic per se, the joint neurotoxic action of M*i*BK on *n*-hexane is classified as synergism (Rentz, 1932; Hodgson, 1980). Although the mechanism by which M*i*BK synergizes the neurotoxic effect of *n*-hexane is not known,

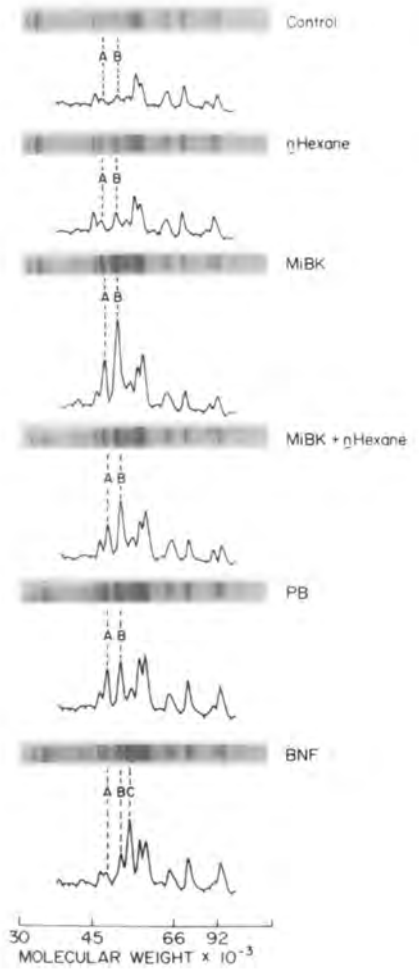

FIG. 8. Gels and microdensitometric scans of liver microsomes from control hens and hens treated with 1000 ppm of *n*-hexane, M*i*BK, or a *n*-hexane/M*i*BK mixture, PB, and β-NF. Band A, M_r = 49,000 is the putative cytochrome *P*-450. In M*i*BK and *n*-hexane/M*i*BK-treated hens, there was an increase in the A band, indicating an induction of cytochrome *P*-450. Band B corresponding to M_r of 52,000 was increased in all treatments. The cytochrome *P*-450 content in these gels was (nmol/mg of protein): control, 0.19; *n*-hexane, 0.24; M*i*BK, 1.09; *n*-hexane/M*i*BK, 0.7; PB, 0.32; and β-NF, 0.61.

the finding that M*i*BK induces chicken hepatic microsomal cytochrome *P*-450 suggests that M*i*BK may enhance the metabolic activation of *n*-hexane to the active metabolites mentioned above, leading to more formation of the ultimate neurotoxic agent 2,5-HD.

The marked induction of cytochrome *P*-450 by exposure to M*i*BK alone or M*i*BK/*n*-hexane but not by *n*-hexane alone was confirmed with SDS–PAGE. Continuous inhalation of *n*-hexane for 50 days did not induce liver microsome cytochrome *P*-450; this finding is in contrast to previous reports that continuous exposure of mice for 24 days to 2.5 to 3% ppm *n*-hexane induced the liver contents of this enzyme (Kramer *et al.*, 1974) and may be attributed to species differences. The induction of cytochrome *P*-450 was dependent on the exposure to M*i*BK. Aniline hydroxylation, which is a cytochrome *P*-450-mediated reaction, also increased with the inhalation of M*i*BK alone or in combination with *n*-hexane. These results are consistent with a previous study which demonstrated that daily ip injections of M*n*BK or technical-grade MBK, for 3 days, equally induced hen liver microsomal cytochrome *P*-450 (Abou-Donia *et al.*, 1985).

M*i*BK induced the same band as phenobarbital, and it is concluded that M*i*BK induced PB-type cytochrome *P*-450. The present study confirms our previous results (Lasker *et al.*, 1982; Lapadula *et al.*, 1984), as well as others (Ehrich and Larson, 1983), that in the chicken the hepatic microsomal cytochrome *P*-450 content is approximately one-fourth that of the rat. The results of this investigation, however, do not show which form of cytochrome *P*-450 was induced by M*i*BK. In rats at least six forms of cytochrome *P*-450 were induced by phenobarbital, in addition to three more proteins present in 3-methylcholanthrene-treated or untreated animals (Guengerich, 1978, 1979). Some of these forms of cytochrome *P*-450 exhibited the same apparent molecular weight on SDS–PAGE. Since very little is known about hepatic microsomal cytochrome *P*-450 in chickens, further work is in progress to characterize the various forms of this enzyme in the hen when untreated and when induced with phenobarbital or M*i*BK. Preliminary results, with rat liver microsomal antibodies, suggest that hen hepatic cytochrome *P*-450b is induced by exposure to

TABLE 5

CHANGES OF PROTEIN BANDS FROM SDS-PAGE OF LIVER MICROSOMAL FRACTIONS FROM TREATED HENS[a]

Protein band	Treatment[b]					
	Control	β-NF	PB	*n*-Hexane	M*i*BK	*n*-hexane/M*i*BK
49,000	100 ± 8	83 ± 17	258 ± 36*	98 ± 17	335 ± 25*	253 ± 27*
52,000	100 ± 10	165 ± 12*	279 ± 23*	130 ± 23	451 ± 45*	492 ± 54*
55,000[c]	100 ± 25	1408 ± 292*	91 ± 42	163 ± 76	139 ± 74	136 ± 85

[a] Results are expressed as percentage of control of microdensitometry scans. Values are $\bar{x} \pm$ SE from five hens except *n*-hexane/M*i*BK where four hens were used.

[b] Treatments: β-NF = 80 mg/kg, ip, for 3 days; PB = 80 mg/kg, ip, for 3 days; *n*-hexane, 1000 ppm, inhalation for 50 days; M*i*BK = 1000 ppm, inhalation for 50 days; *n*-hexane/M*i*BK, 1000 ppm each, inhalation for 30 days.

[c] Except for β-NF treatment, this band was faint in other treatments which resulted in large standard errors when quantified by the densitometer (see Fig. 8).

* Significantly different from control values ± SE, $p < 0.02$, $n = 5$.

M*i*BK (unpublished results). Furthermore, the induction of cytochrome *P*-450 could conceivably have occurred from the action of M*i*BK metabolites, e.g., 4-methyl-2-pentanol and/or 4-hydroxy-4-methyl-2-pentanone, which were reported as metabolites of M*i*BK in guinea pigs (DiVincenzo *et al.*, 1976).

Although the action of M*i*BK on inducing chicken hepatic microsomal cytochrome *P*-450 might provide some insight into the mechanism of M*i*BK-synergized *n*-hexane neurotoxicity, alternate mechanisms may also be operating. For instance, M*i*BK might induce a local trauma to the nervous tissue which, by increasing vascular permeability, accelerates the entry from the circulation of *n*-hexane or its active metabolites, leading to locally enhanced neurotoxic effects similar to those proposed for 2,5-HD (Simonati *et al.*, 1983).

Simultaneous exposure of hens to *n*-hexane and M*i*BK vapors greatly enhanced the neurotoxicity of *n*-hexane alone. These results are consistent with the previous finding that exposure to a mixture of M*n*BK and M*i*BK (7:3) vapors increased the susceptibility of hen nervous tissues to delayed neurotoxicity induced by *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN) (Abou-Donia, 1983; Abou-Donia *et al.*, 1985). Therefore, because of the ability of the nonneurotoxicant M*i*BK to synergize the neurotoxic action of *n*-hexane, it is prudent to minimize human exposure to mixed solvents containing M*i*BK and *n*-hexane or other neurotoxic chemicals prone toward metabolic activation, e.g., EPN (Abou-Donia, 1981). This is particularly important in lieu of the estimate that 436,000 workers in 43,000 plants are potentially exposed to M*i*BK (SRI International, 1978).

ACKNOWLEDGMENTS

The supply of technical methyl isobutyl ketone by Eastman Kodak Company, Kingsport, Tenn., is acknowledged. The authors thank Mrs. Erna S. Daniel for her secretarial assistance in the preparation of this manuscript. This study was supported in part by NIEHS Grant OH00823.

REFERENCES

ABDEL-RAHMAN, M. S., HETLAND, L. B., AND COURI, D. (1976). Toxicity and metabolism of methyl *n*-butyl ketone. *Amer. Ind. Hyg. Assoc.* **37**, 95–102.

ABDO, K. M., GRAHAM, D. G., TIMMONS, P. R., AND ABOU-DONIA, M. B. (1982). Neurotoxicity of continuous (90 days) inhalation of technical grade methyl butyl ketone in hens. *J. Toxicol. Environ.* **9**, 199–215.

ABOU-DONIA, M. B. (1978). Role of acid phosphatase in delayed neurotoxicity by leptophos in hens. *Biochem. Pharmacol.* **27,** 2055–2058.

ABOU-DONIA, M. B. (1981). Organophosphorus ester induced delayed neurotoxicity. *Annu. Rev. Pharmacol. Toxicol.* **21,** 511–548.

ABOU-DONIA, M. B. (1983). Interaction between neurotoxicities induced by organophosphorus and long-chain hexacarbon compounds. *Neurotoxicology* **4,** 117–136.

ABOU-DONIA, M. B., LAPADULA, D. M., CAMPBELL, G. M., AND ABDO, K. M. (1985). The joint neurotoxic action of inhaled methyl butyl ketone vapor and dermally applied *O*-ethyl-*O*-4-nitrophenyl phenylphosphonothioate in hens: Potentiating effect. *Toxicol. Appl. Pharmacol.* **79,** 69–82.

ABOU-DONIA, M. B., MAKKAWY, H. M., AND GRAHAM, D. G. (1982). The relative neurotoxicities of *n*-hexane, methyl *n*-butyl ketone, 2,5-hexanediol and 2,5-hexanedione following oral or intraperitoneal administration in hens. *Toxicol. Appl. Pharmacol.* **62,** 369–389.

ABOU-DONIA, M. B., AND PREISSIG, S. H. (1976). Delayed neurotoxicity of leptophos: Toxic effects on the nervous system. *Toxicol. Appl. Pharmacol.* **35,** 269–282.

AUBUCHON, J., ROBINS, IAN, H., AND VISESKUL, C. (1979). Peripheral neuropathy after exposure to methyl *iso*-butyl ketone in spray paint. *Lancet,* Aug. 18, **1,** 363–364.

BILLMAIER, D., YEE, H. T., ALLEN, N., CRAFT, R., WILLIAMS, H., EPSTEIN, F., AND FONTAINE, R. (1974). Peripheral neuropathy in a coated fabric plant. *J. Occup. Med.* **16,** 655–671.

BURGI, E. (1910). Die Wirkung von narcotica-kombinationen. *Dtsch. Med. Wochenschr.* **36,** 20–23.

CAVANAGH, J. B. (1982). The pattern of recovery of axons in the nervous system of rats following 2,5-hexanediol intoxication: A question of rheology. *Neuropathol. Appl. Neurobiol.* **8,** 19–34.

DIVINCENZO, G. D., KAPLAN, C. J., AND DEDINAS, J. (1976). Characterization of the metabolites of methyl *iso*-butyl ketone in guine pig serum and their clearance. *Toxicol. Appl. Pharmacol.* **36,** 511–522.

DIVINCENZO, G. D., HAMILTON, M. L., KAPLAN, C. J., AND DEDINAS, J. (1977). Metabolic fate and disposition of ^{14}C-labeled methyl *n*-butyl ketone in the rat. *Toxicol. Appl. Pharmacol.* **41,** 547–560.

EHRICH, M., AND LARSEN, C. (1983). Drug metabolism in adult white leghorn hens—Response to enzyme induces. *Comp. Biochem. Physiol. C* **64,** 383–386.

FROMMER, V., ULLRICH, V., AND BRRENIUS, S. (1974). Influence of inducers and inhibitors on the hydroxylation pattern of *n*-hexane in rat liver microsomes. *Fed. Eur. Biochem. Soc. Lett.* **41,** 12.

FURIA, T. E., AND BELLANCA V. (eds.). (1975). *Frenaroli's Handbook of Flavor Ingredients,* Vol. 2, 2nd ed., p. 391. CRC Press, Cleveland, Ohio.

GUENGERICH, F. P. (1978). Separation and purification of multiple forms of microsomal cytochrome *P*-450. *J. Biol. Chem.* **253,** 7931–7939.

GUENGERICH, F. P. (1979). Isolation and purification of cytochrome *P*-450, and the existence of multiple forms. *Pharmacol. Ther.* **6,** 99–121.

HODGSON, E. (1980). Chemical and environmental factors affecting metabolism of xenobiotics. In *Introduction to Biochemical Toxicology* (E. Hodgson and F. E. Guthrie, eds.), pp. 143–161. Elsevier, New York.

JONCKHEERE, A. R. (1964). A distribution free *k*-sample test against order alternatives. *Biometrika* **41,** 133–145.

KIRK-OTHMER (1966). Hexanes. In *Encyclopedia of Chemical Technology,* Vol. 11, 2nd ed., pp. 1–5. Wiley, New York.

KRAMER, A., STAUDINGER, H., AND ULLRICH, V. (1974). Effect of *n*-hexane inhalation on the monooxygenase system in mice liver microsomes. *Chem. Biol. Interact.* **8,** 11–18.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the hen of bacteriophage T4. *Nature (London)* **277,** 680–685.

LAPADULA, D. M., CARRINGTON, C. D., AND ABOU-DONIA, M. B. (1984). Induction of hepatic microsomal cytochrome *P*-450 and inhibition of brain, liver, and plasma estrases by an acute dose of *S,S,S*-tri-*n*-butyl phosphorotrithioate (DEF) in the adult hen. *Toxicol. Appl. Pharmacol.* **73,** 300–310.

LASKER, J. M., GRAHAM, D. G., AND ABOU-DONIA, M. B. (1982). Differential metabolism of *O*-ethyl *O*-4-nirophenyl phenylphosphonothiaote by rat and chicken liver microsomes: A factor in species selectivity to delayed neurotoxicity. *Biochem. Pharmacol.* **31,** 1961–1967.

LOWRY, O. H., ROSEBROUGH, N. J., GARR, A. L., AND RANDALL, R. J. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

MAZEL, P. (1971). Experiments illustrating drug metabolism *in vitro*. In *Fundamentals of Drug Metabolism and Drug Disposition* (D. N. LaDu, H. G. Mandel, and E. L. Way, eds.), pp. 546–582. Krieger, Huntington, N.Y.

OH, S. J., AND KIM, J. M. (1976). Giant axonal swelling in "huffers" neuropathy. *Arch. Neurol. (Chicago)* **33,** 583.

OMURA, T., AND SATO, R. (1964). The carbon-monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239,** 2370–2378.

RENTZ, E. (1932). Zur systematik und nomenklatur der kombinationswirkungen. *Arch. Int. Pharmacodyn.* **43,** 337–361.

SCHAUMBURG, H. H., AND SPENCER, P. A. (1976). Central and peripheral nervous system degeneration produced by pure *n*-hexane: An experimental study. *Brain* **99,** 183–192.

SCHENKMAN, J. B., AND CINTI, D. L. (1978). Preparation of microsomes with calcium. In *Methods of Enzymology*

(S. Fleischer and L. Packer, eds.), Vol. 52, pp. 83–89. Academic Press, New York.

SHELDON, L. S., AND HITES, R. A. (1978). Organic compounds in the Delaware River. *Environ. Sci. Technol.* **12,** 1188–1194.

SIMONATI, A., RIZZUTO, N., AND CAVANAGH, J. B. (1983). The effects of 2,5-hexanedione on axonal regeneration after nerve crush in the rat. *Acta Neuropathol.* **59,** 216–224.

SPENCER, P. S., BISCHOFF, M. K., AND SCHAUMBURG, H. W. (1978). On the specific molecular configuration of neurotoxic aliphatic hexacarbon compounds causing central-peripheral distal axonopathy. *Toxicol. Appl. Pharmacol.* **44,** 17–28.

SPENCER, P. S., AND SCHAUMBURG, H. H. (1976). Feline nervous system response to chronic intoxication with commercial grades of methyl *n*-butyl ketone, methyl *iso*-butyl ketone, and methyl ethyl ketone. *Toxicol. Appl. Pharmacol.* **37,** 301–311.

SPENCER, P. S., SCHAUMBURG, H. H., SABRI, M. I., AND VERONESI, B. (1980). The enlarged view of hexacarbon neurotoxicity. *CRC Crit. Rev. Toxicol.* **7,** 279–356.

SPENCER, P. S., SCHAUMBURG, H. H., RALEIGH, R. L., AND TERHAAR, C. J. (1975). Nervous system degeneration produced by the industrial solvent methyl *n*-butyl ketone. *Arch. Neurol.* **32,** 219–222.

SRI International (1978). *Chemical Economics Handbook,* p. 675.6030D. SRI, Menlo Park, Calif.

YAMADA, A. (1964). An occurrence of polyneuritis by *n*-hexane in polyethylene-laminating plants. *Japan J. Ind. Health* **6,** 192–197.