Pharmacology Biochemistry & Behavior, Vol. 36, pp. 671–681. © Pergamon Press plc, 1990. Printed in the U.S.A. 0091-3057/90 $3.00 + .00

Neurotoxicologic Evaluation of Rats After 13 Weeks of Inhalation Exposure to Dichloromethane or Carbon Monoxide[1,2]

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Received 11 December 1989

MATTSSON, J. L., R. R. ALBEE AND D. L. EISENBRANDT. *Neurotoxicologic evaluation of rats after 13 weeks of inhalation exposure to dichloromethane or carbon monoxide.* PHARMACOL BIOCHEM BEHAV **36**(3) 671–681, 1990.—Male and female Fischer 344 rats were exposed to dichloromethane (methylene chloride, DCM) or carbon monoxide (CO) for 6 hr/day, 5 days/week, for 13 weeks. Since oxidative metabolism of DCM to CO and CO_2 is a saturable process, DCM exposure concentrations were selected clearly below saturation (50 ppm), just below saturation (200 ppm), and well above saturation (2000 ppm). At saturation of metabolism, metabolic CO causes about 10% carboxyhemoglobinemia (COHb). Therefore, as a control for CO effects, a separate group of rats was exposed to 135 ppm CO to induce approximately 10% COHb. Postexposure functional tests included an observational battery, hindlimb grip strength, and a battery of evoked potentials (flash, auditory brainstem, somatosensory, caudal nerve). After functional tests were completed, rats from all groups were perfused with fixative and a comprehensive set of nervous tissues from the high DCM exposure group and from controls were examined by light microscopy. Although some miscellaneous functional and morphologic variations were recorded, none were related to treatment. Thus, subchronic exposures as high as 2000 ppm DCM or 135 ppm CO had no deleterious effects on any of the measures of this study.

Rats Dichloromethane (methylene chloride) Carbon monoxide Subchronic inhalation exposures
Flash-evoked potentials Click auditory brainstem responses (ABRs) Tone-pip ABRs
Somatosensory-evoked responses Caudal nerve action potentials Perfusion fixation Neuropathology

DICHLOROMETHANE (DCM, methylene chloride) has a diverse number of applications as a chemical solvent. DCM is most commonly used in paint removers and aerosol sprays. Other applications include the production of pharmaceutical products, flexible urethane foams and plastics, as a cleaning agent for metal parts and electronic components, and as a decaffeinating agent and spice extractant (18). The American Conference of Governmental Industrial Hygienists threshold limit value (TLV) is 50 ppm.

Visual-, auditory- and somatosensory-evoked potential and electroencephalographic (EEG) effects of acute, high-concentration exposures of rats to DCM have been well documented (32). Rats were exposed to 5000, 10000, or 15000 ppm DCM for 60 min (recordings were made several times during and after the 60-min exposure). Rather complicated but well defined dose-response and time-response effects were recorded from all measures. The acute effects of DCM were quite different than the acute responses previously noted for acute high-level exposures to toluene (30,31).

Evaluation of the subchronic neurotoxic potential of DCM was complicated by the lack of a clear-cut solvent neurotoxicity syndrome in animals. Demonstration of the relevancy of rats as an experimental model for solvent neurotoxicity (persistent changes vs. acute transient changes in nervous system function) was considered essential. Because of the known neurotoxicity of toluene in solvent abusers, toluene was selected as the solvent for methods validation [see companion paper, (25)]. Rats were exposed to a simulated toluene 'abuse' paradigm of 8000 ppm toluene, 35 min per exposure, 4 exposures per day, 3 days per week for 13 weeks. This subchronic toluene 'abuse' paradigm did cause significant postexposure changes in flash-evoked potentials, auditory brainstem responses, and somatosensory responses. Thus, rats were neurotoxicologically sensitive to toluene, albeit under circumstances of extreme exposure.

In the following study, DCM was evaluated for effects from standard, occupationally relevant exposures, i.e., 6 hr/day, 5 days/week, for 13 weeks. The oxidative metabolism of DCM

[1]This study was sponsored by the Halogenated Solvents Industry Alliance (HSIA), 1225 19th Street, N.W., Suite 300, Washington, DC.
[2]A poster (No. 418) on this DCM/CO study was presented at the 28th Annual Meeting of the Society of Toxicology, Atlanta, GA, February 27 to March 3, 1989.

produces carbon monoxide (CO) and, therefore, carboxyhemoglobin (COHb) (19). Thus, a separate group of rats was exposed to CO to control for COHb. Dose selection was a critical issue, and is addressed in the Method section.

The emphasis on electrophysiologic techniques for the evaluation of DCM was predicated on its general utility in animal neurotoxicology (10,29), its use to characterize the *acute* effects of toluene and DCM (30–33), and our experience with this technology (1, 2, 21–25). Evoked potentials are used in psychiatric diagnostic differentiation (37), are effective for early detection of neurotoxicity (including solvent neurotoxicity) in humans (36), and have readily detected postexposure changes in evoked potentials in toluene 'abuse' rats (25). The purpose of the DCM experiment, therefore, was to expose rats for 13 weeks to metabolically and pharmacologically significant levels of DCM and then perform extensive postexposure neurofunctional and neuropathologic examinations to search for evidence of neurotoxicity.

METHOD

Test Species and Husbandry

Male and female Fischer 344 rats, approximately 16 weeks old, were used in the study. The rats were purchased from the Charles River Breeding Laboratory, Kingston, NY. Rats were housed one per cage in suspended stainless steel cages which have wire-mesh floors. The holding room was maintained at approximately 22°C, 50% humidity, and a 12-hour light-dark cycle.

The animals were stratified by weight and then randomly assigned to an exposure group using a computer program. Purina Certified Rodent Chow (No. 5002) and municipal drinking water were available ad lib throughout the study except during exposure.

Test Material

DCM (CH_2Cl_2), 99.95% pure, lot number TA861111D, was obtained from The Dow Chemical Company, Freeport, TX. The identity of the test material was confirmed by infrared spectroscopy, and gas chromatography/mass spectrometry and its purity evaluated by gas chromatography before and after the study. No significant changes in sample composition were observed. CO, lot number 020185, at a 99.99% minimum specified purity, was obtained from Scott Specialty Gases, Plumsteadville, PA. The identity and purity of CO was confirmed by mass spectrometry before the study.

Dose Selection

Selection of DCM exposures was complicated by the fact that DCM is metabolized by two pathways, an oxidative pathway that yields carbon monoxide (CO) and considerable amounts of carbon dioxide (CO_2), and a glutathione (GSH)-dependent pathway that produces CO_2, but no CO (3). The oxidative pathway is characterized by high-affinity and low-capacity for DCM, while the GSH pathway is characterized by low-affinity and high-capacity. Therefore, the high-affinity oxidative pathway predominates when DCM exposures do not cause saturation of oxidative metabolism. Oxidative metabolic saturation occurs at roughly 200 to 500 ppm. The low-affinity GSH pathway yields S-chloromethylglutathione, formaldehyde, formic acid and ultimately CO_2. The GSH pathway does not appear to saturate as does the oxidative pathway (at least at concentrations <4000 ppm), and thus may become more important at higher exposure concentrations of DCM.

A meaningful range of exposures, based on pharmacokinetics, would include concentrations below saturation, near saturation, and above saturation of metabolism of DCM to CO. Thus, a physiologically based pharmacokinetic profile of COHb production in rats from 6 hr of DCM exposure was utilized (computer model developed by M. E. Andersen and R. H. Reitz). According to the model, exposure to 50 ppm DCM is clearly below saturation, and should cause about 4 to 5% COHb. Published data (19) indicate that Sprague-Dawley rats have a COHb somewhat higher than 3% when exposed to 50 ppm DCM. Therefore, the low exposure level was selected at 50 ppm. The middle exposure level was selected at 200 ppm (about 8.5% COHb), a level just below saturation. A COHb concentration of about 9% was estimated for all exposures above 300 ppm. Actual data were in close agreement with this estimate. Male Fischer 344 rats, exposed to 2000 ppm DCM for 6 hr, had approximately 10% COHb (unpublished report, The Dow Chemical Company). The high-exposure concentration selected for this study was 2000 ppm, which is 10 times greater than the middle-exposure level (200 ppm), and thus was far above metabolic saturation.

Selection of 2000 ppm as the high-exposure concentration also was based on consideration of the known CNS depressant effects of DCM. *Pilot* studies (unpublished report, The Dow Chemical Company) have shown clearly altered neurophysiological function of rats *during* exposure to 2000 ppm DCM. Both EEG and somatosensory-evoked potentials were affected. The EEG had reduced power (MANOVA of power in four frequency bands, covering 0.5 to 25 Hz; $p=0.009$), and somatosensory-evoked potentials (Fig. 1) had altered shape and reduced power (MANOVA for latency, power, and shape, $p=0.005$). The acute (2000 ppm) somatosensory-evoked potential changes were similar to but milder than those observed by Rebert *et al.* (32) in rats exposed acutely to DCM at 5000 ppm. Winneke (40) reported EEG changes in animals at about 1000 ppm DCM, and decreased vigilance performance in humans at about 300 ppm DCM.

A high-exposure level of 2000 ppm should be well tolerated in the context of classical toxicologic effects. Rats have tolerated up to 4000 ppm DCM in chronic studies (6,28). These chronic toxicity/oncogenicity studies have shown mild alterations in liver histology at dose levels ranging from 500 to 4000 ppm DCM. Hepatocellular vacuolization, consistent with fatty changes, and multinucleated hepatocytes were common observations. A low incidence of hepatocellular necrosis also occurred. Exposure levels of DCM for the current experiment were, therefore, sufficient to have pharmacologic effects on the nervous system, and according to previous studies (6,28), sufficient to have a mild effect on the liver.

Because the rats were exposed to both exogenous DCM and endogenous CO, etiologic interpretation of possible toxicologic findings would be difficult. Therefore, our study had a separate group of rats exposed to 135 ppm CO in the absence of DCM. Pilot studies (unpublished report, The Dow Chemical Company) have shown that rats exposed to 135 ppm CO exposure for 2.5 hr will have about 10% COHb, which approximates that of rats exposed to 2000 ppm DCM. Rats tested during exposure to 135 ppm CO had no statistically identified changes in visual- or somatosensory-evoked potentials, but did have a significant increase in EEG power at the low frequencies ($p=0.008$).

Test Animals

Twelve rats of each sex (24 rats per treatment group, 120 total) were selected randomly for exposure to 0, 50, 200 or 2000 ppm DCM (0, 0.17, 0.70, or 7.0 mg/l) or to 135 ppm CO for 6 hr/day, 5 days/week, for 13 weeks. In case of unplanned losses, two extra rats per sex per group also were added. The amount of work (implantation, testing, perfusion) required that the rats be divided

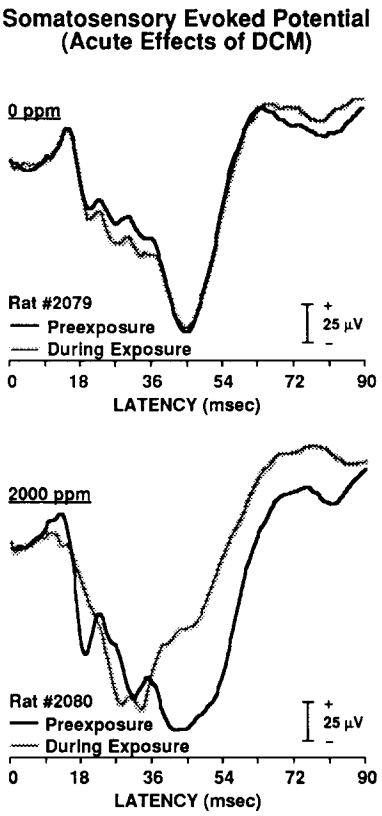

FIG. 1. Representative individual somatosensory-evoked potentials recorded from the sensory cortex (SEP-S) *during* exposure to 2000 ppm DCM in a pilot study of the acute effects of DCM. Note the minimal change in the SEP-S from the rat exposed to air for approximately 3 hr, and the large changes in the waveform of the rat exposed to 2000 ppm DCM for a comparable time. Changes were principally a reduction in power of the early components, and an appreciable alteration in waveform shape. Overall (n = 8/group) DCM vs. control differences in latency, power, and shape had MANOVA $p = 0.005$.

into two subgroups that started on study one week apart. The two subgroups were counterbalanced across exposure levels and sexes. Electrophysiological data were collected after 13 weeks of exposure. Subsequently, six rats of each sex/dose level were randomly selected, perfused and necropsied.

Exposure Conditions

Exposures were conducted in stainless steel and glass 4.1 m^3 Rochester-type chambers with the airflow maintained at 800 l/min. Concentrations of DCM in the chambers were monitored 1–2 times per hour with Miran 1A infrared spectrophotometers at wavelengths of 7.95 (2000 ppm only) or 13.35 μm. The mean (± SD) analytical concentrations of DCM in the chambers during exposures were 50.0 ± 1.2, 200 ± 3, and 2000 ± 33 ppm. Concentrations of CO in the exposure chamber were monitored 1 to 2 times per hour with a Miran 1A infrared spectrometer at a wavelength of 4.65 μm. The mean analytical exposure concentration of CO was 134 ± 3 ppm. Mean temperature values during DCM and CO exposures ranged from 21.3 to 23.2°C, and relative humidity values ranged from 58.8 to 65.4%.

Surgery

Surgical implantation of epidural electrodes was conducted 10 weeks into the study. Anesthesia was by intramuscular injection (posterior-lateral thigh) of a combination of xylazine (13 mg/kg) and ketamine (87 mg/kg). The somatosensory electrode was placed 2.0 mm posterior and 2.0 mm lateral left of bregma, the visual cortex electrode (used also for auditory brainstem responses) was placed 6.8 mm posterior and 3.0 mm lateral right of bregma, and the cerebellar electrode was located on the midline and 12.0 mm posterior of bregma. A reference electrode was placed 7.0 mm anterior and 1.0 mm lateral left of bregma.

Body Weights

Body weights were measured prior to initiation of the study and weekly thereafter. Body weights also were measured at the time of hindlimb grip strength testing.

Weekly Clinical Observations

Each rat was removed weekly from its cage and physically examined for changes in general health. Specific observations were made for: muscle tone, tremor (or other abnormal muscular movements), skin and haircoat condition, salivation, lacrimation, urine staining and fecal staining. Surgery was conducted during week 10, and clinical observations were not recorded for that week.

Functional Tests

The following data were collected beginning 65 or more hours after 13 weeks of exposure: functional observation battery (FOB), grip strength, flash-evoked potential (FEP), cortical flicker fusion (CFF), auditory brainstem response to clicks (ABR_c), auditory brainstem response to tone-pips at 10 kHz (ABR_{10}) and 30 kHz (ABR_{30}), somatosensory-evoked potentials recorded from the sensory cortex (SEP-S) and from the cerebellum (SEP-C), and caudal nerve action potentials to single ($CNAP_1$) and paired stimuli ($CNAP_2$). Rats were physically restrained during electrophysiological testing, which took about 35 min for the test battery.

Functional observation battery. Each rat was removed from its cage and the handler evaluated the evasive behavior. The handler then presented the rat to an observer in such a way that the observer did not know the identity of the rat or the exposure concentration. The observer held the rat in-hand and classified the following events as normal, increased, decreased, or as present or absent, as appropriate: muscle tone, tremor, haircoat condition, salivation, lacrimation, pupil size, urine staining, fecal staining. The rat was then placed into a 50 × 50 cm clear plastic box and locomotor behavior was described if abnormal, and then responsiveness to touch, sharp noise, and tail pinch were evaluated as normal, increased or decreased.

Hindlimb grip strength. Hindlimb grip strength for each rat was measured according to the procedure described by Mattsson *et al.* (26). Briefly, the rats were selected in a random manner and given to the observer in such a way that the observer did not know the treatment status of the animal. The observer then placed the rat's forelegs on a bench and the hindfeet were set on a horizontal screen attached to a strain gauge. The observer smoothly but firmly pulled backward on the tail until the rat's grip on the screen was broken. The strain gauge recorded the rat's resistance to the pull in grams. The strongest response of three trails was used for statistical analysis.

Electrophysiological tests. The electrophysiological system was a Nicolet Pathfinder II (Nicolet Biomedical Instruments, Madison, WI). Data sweeps (msec segments of EEG) were digitally sampled 512 times and averaged by an online computer. Rectal or tail temperature was recorded prior to each electrophysiological test.

TABLE 1

PHYSICAL PARAMETERS OF ELECTROPHYSIOLOGICAL TESTS

Test	Stimulus	Stimulus Intenstiy	Stimulus Rate/Sec	Amplifier Filters (Hz)	Sweep Duration (msec)	Number of Sweeps
FEP*	Flash	0.5 cd-sec/m^2	0.9	0.5–1500	150 and 750	200
CFF	Flash	1.3 cd-sec/m^2	about 48	0.5–1500	about 25	200
ABRc†	Click	80 dB linear	29.1	100–8000	10	2,000
ABR10†	10 kHz tone	55 dB linear	29.1	100–8000	10	2,000
ABR30†	30 kHz tone	73 dB linear	29.1	100–8000	10	2,000
SEP	Electrical	3 mA, 50 μsec	1.1	1–1500	35 and 150	200
CNAP	Electrical	3 mA, 20 μsec	10.1	1–3000	20	200

*Calibrated with a United Detector Technology 350 photodetector (plus 111 filter and lumilens 1153) placed in the same position as the rat, facing a white reflective surface (cubicle wall) opposite the strobe.

†The distance from speaker to ears was about 17 cm. Sound pressure level calibrated on linear scale with a Bruel & Kjaer Model 2230 with ¼ inch condenser microphone model 4135 placed in the restrainer at the location of the ears (rat removed).

The physical parameters for the different evoked potential tests are listed in Table 1. Rats are physically restrained during electrophysiological testing. Tests conducted were:

1. Flash-evoked potential (FEP, low intensity).
2. Cortical flicker fusion (CFF): The maximum rate of flash that elicits a synchronized cortical response was determined by increasing or decreasing the flash rate, in 2 Hz or larger steps, from 48 Hz.
3. Auditory brainstem response to clicks (ABR_c).
4. Auditory brainstem response to tone-pips (ABR_{10} and ABR_{30}): Auditory brainstem response to tone-pips were tested at middle and high frequencies (10 kHz and 30 kHz). Each tone-pip had a 2.25-msec rise/fall ramp and no plateau (4.5 msec total duration).
5. Somatosensory-evoked potentials (SEP-S and SEP-C): Ventrolateral caudal nerves were stimulated at the base of the tail and a response was recorded at the somatosensory cortex (SEP-S) and from the cerebellum (SEP-C). The stimulating electrodes were small needles set into the bottom of a plastic tray that fit the tail (29).
6. Caudal nerve action potentials (CNAP): Ventrolateral caudal nerves were stimulated near the tip of the tail and mixed nerve action potentials from single stimuli ($CNAP_1$) were recorded at the base of the tail (29). The stimulating and recording electrodes (separated by 9 cm) were mounted in a plastic tray. Subsequently, the nerves were stimulated with a pair of pulses ($CNAP_2$ with interstimulus interval of 3 msec).

Digital Filtering

A computer routine (Nicolet Biomedical Instruments, Madison, WI) digitally filtered each of the waveforms that were collected with a broad-band analog filter. Digital filter settings were as follows: FEP 1–250 Hz; ABR 150–3000 Hz; SEP-S and SEP-C with a 35-msec data sweep filtered at 1–750 Hz; SEP-S and SEP-C recorded with a 150-msec data sweep filtered at 1–500 Hz.

Waveform Analyses

All waveforms were evaluated by visual examination, and waveforms from DCM high-concentration and control rats were evaluated by an automated computer technique. Because of the absence of DCM treatment-related effects at the high concentration, waveforms from animals exposed to lower concentrations and those exposed to CO were evaluated by visual examination but not by computer. The computer analyzed the high concentration and control FEP, SEP, ABR, and CNAP data by quantification of differences from a template in waveform shape, latency and power [for more details, see Mattsson and Albee, (21)]. A template for each type of response was created by making a 'grand average' of all the control records for each sex. A window (starting and ending point in msec) was established for each type of response. The window widths for automated computer scoring were: FEP early components = 28.5 to 130.5 msec; FEP late components = 132.0 to 300.0 msec; ABR_c = 1 to 5 msec; ABR_{10} = 1.8 to 7.0 msec; ABR_{30} = 1.5 to 5.5 msec; SEP-C early components = 3.5 to 24.0 msec; SEP-C late components = 24.3 to 96.0 msec; SEP-S early components = 4.0 to 25.0 msec; SEP-S late components = 25.2 to 85.5 msec; CNAP single pulse = 2.8 to 7.4 msec; and CNAP paired pulse = 6.0 to 10.0 msec.

The analyses included optimal correlation of an individual waveform to the template waveform, the latency difference (phase shift in msec required to reach optimal correlation), and total power in the window (RMS volts).

Statistical Analyses

Each variable was tested for homogeneity of variance by the F-max test, alpha = 0.01 (5). When heterogeneity occurred, and was attributed to one or two outlying data points, these data were removed from parametric analysis and the data were reported separately.

Subsequent parametric analyses were conducted after homogeneity of variance was assured. Electrophysiology data (optimal correlation, latency difference and power) were analyzed by a multivariate analysis of variance (MANOVA), using the general linear model procedure of SAS (SAS Institute Incorporated, Cary, NC). The factors were treatment, sex and group (rats were put on study in two groups, one week apart). The interaction of treatment-by-sex also was assessed. Probabilities were calculated by an F-test based on Hotelling-Lawley trace statistics.

Because of the importance of body temperature (38) and body weight (2) on measurements of nervous system function, the data also were analyzed with temperature and body weights as covari-

ates (MANCOVA). The statistical procedure also provided analogous univariate analyses (ANOVA/ANCOVA) for the 3 dependent variables of the MANOVA: optimal correlation, latency difference and power.

Temperatures, taken before each electrophysiological test, were analyzed by factorial MANOVA (treatment and sex), with analogous step-down ANOVAs for temperature for each test. Single variable measures (body weight, grip strength, CFF rate) were tested with the factorial univariate analysis of variance (ANOVA).

This study was exploratory and numerous measurements were statistically compared in the same group of animals. Because of the large number of statistical calculations, the overall false positive rate (Type I error) was greater than the cited alpha would suggest and a firm statement of statistical probability cannot be made. For this reason, a modest alpha correction was made to adjust for repetitive testing. The correction approximates that recommended by Tukey *et al.* (39) wherein the class alpha (0.05) was divided by the square root of the number of variables in that class. The class in this instance was the group of evoked potential tests. Analysis of high-concentration vs. control data required 11 MANOVAs within this class. Thus, MANOVA alpha = 0.05 was divided by the square root of 11 to provide an alpha criterion of 0.015, rounded to MANOVA alpha = 0.02. Likewise, there were 33 ANOVAs of individual variables within this class, yielding a corrected alpha = 0.009, rounded to ANOVA alpha = 0.01. Body weight and grip strength were each a class of one with an alpha = 0.05. The temperature MANOVA had an alpha criterion of 0.05, with step-down ANOVA alphas of 0.02.

Necropsy

Necropsies were performed after completion of 13 weeks of exposure and after neurologic testing, on 6 randomly selected rats of each sex per exposure level. Each rat was heparinized and anesthetized with methoxyflurane. Tissues were preserved by whole-body perfusion with phosphate-buffered 1.5% glutaraldehyde/4% formaldehyde solution (pH 7.4, 540 mOsM). Gross pathological examination by a veterinary pathologist was as thorough as possible for perfused tissues.

Microscopic Pathology

Neural tissues from the control and DCM high-concentration groups that were necropsied after the 13-week study were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, luxol fast blue/periodic acid-Schiff-hematoxylin, and Sevier-Munger silver. Additional microscopic sections were stained with cresyl echt violet and gallocyanin. Tissues examined by light microscopy were forebrain, midbrain, cerebellum and pons, medulla oblongata, optic nerves, eyes, spinal cord (cervical and lumbar), dorsal root ganglia, dorsal and ventral spinal roots, Gasserian ganglia, proximal sciatic nerve and tibial nerve. Although tissues from lower DCM concentration groups and the CO group were collected and preserved in fixative, the absence of treatment-related lesions in the DCM high-concentration group negated the need to histopathologically examine these tissues. Thus, tissues from lower concentration groups and from the CO group were not processed for light microscopy. Also, tissues from animals that were moribund or died prior to scheduled necropsy were not processed for light microscopy.

RESULTS

Overall Perspective

There were no treatment-related alterations in this study. All animals were examined clinically at weekly intervals, and were tested at the end of the exposure period by functional observational battery, grip strength, body weight, temperature, and by sensory-evoked potentials. Although all waveforms and all data were examined visually, statistical analyses were performed only on data from 2000 ppm DCM and controls. An exception was the N_1 wave of the FEP. Amplitudes of the N_1 were analyzed statistically for all treatment groups, but no statistically significant changes were discovered.

Although statistical power for MANOVA is unknown, reasonable statistical power was expected based on the coefficients of variation and sample sizes. When coefficients of variation (CV) for all evoked potential tests were averaged, the average CV was about 4% for latency differences, about 8% for correlations (shape of waveforms), and about 27% for waveform power. Sex-by-treatment interactions were not present, so sex was retained as a factor in the analyses and sample sizes were n = 24 except for FEPs, where n = 20 (see below re: light-induced retinopathy in 4 males/group).

Gross pathologic examinations were conducted on all rats, and nervous tissues from rats exposed to 2000 ppm DCM and from controls were examined histopathologically. In the absence of histopathologically identified treatment effects in the 2000 ppm DCM rats, tissues from rats exposed to CO and DCM lower dose groups were not examined by light microscopy.

Although no DCM or CO effects were identified in this study, persistent muscular weakness in one hind leg from an intramuscular injection of ketamine and xylazine (anesthesia for cranial implant surgery) was readily recognized during weekly clinical examinations and on the FOB.

Another nontreatment-related but significant finding was that male rats caged on the top tier of the cage rack had very poor flash-evoked potentials (FEPs), and had severe histopathologic retinal degeneration. The translucent plastic cover of one of the overhead fluorescent lights in the holding room for male rats had become dislodged, and top-tier male rats were exposed directly to the lights. Since rats were in unlighted exposure chambers for 6 hr/day, 5 days/week, and in a holding room the remainder of the time, this unusual exposure to light would have been 6 hr/day, 5 days/week, and 12 hr/day, 2 days/week, for an uncertain duration. Rats were housed one per cage, but the cages were built in sets of four. These four-cage units were portable so the cages could be moved from the holding rack to the exposure chamber and back without having to handle the rats. The four-cage units were placed on the cage rack in the holding room in a counterbalanced order, so that four male rats from the control group and from each DCM treatment level were affected by the light. FEP data from these rats were excluded from analysis. No male CO rats were housed on the top tier. Female rats were housed in another room and were not affected.

Weekly Clinical Observations

Rats remained in very good health throughout the study with one minor exception. Beginning at week 11, routine hand-held examinations revealed muscular weakness in the leg that was intramuscularly injected with anesthetic the previous week. Slightly more than half the rats (52%) were affected at the 11th week (one week postsurgery), and 16% at the 13th week.

Postexposure Body Weights

Rats exposed to 2000 ppm DCM weighed slightly more than controls, about 3% more for males and 4% for females. These weight differences were not statistically significant (mean ± SD;

TABLE 2

CONTROL VS. 2000 PPM DCM STATISTICAL SUMMARY FOR EVOKED POTENTIAL MAIN EFFECTS OF TREATMENT*

Test	MANOVA/ MANCOVA	ANOVA/ANCOVA Lat. Diff.	Correl.†	Power
FEP early	0.220/0.467	0.221/0.892	0.878/0.463	**0.048/0.146**
FEP late	0.597/0.798	0.827/0.657	0.927/0.783	0.246/0.357
ABR_{10}	0.808/0.891	0.607/0.853	0.798/0.999	0.320/0.455
ABR_{30}	0.209/0.152	0.409/0.161	0.063/0.084	0.962/0.995
ABR_c	0.771/0.537	0.658/0.604	0.795/0.514	0.290/0.156
SEP-C early	0.797/0.720	0.366/0.281	0.644/0.715	0.593/0.483
SEP-C late	0.960/0.917	0.793/0.490	0.667/0.786	0.800/0.909
SEP-S early	0.888/0.914	0.673/0.752	0.934/0.947	0.695/0.657
SEP-S late	0.957/0.929	0.909/0.988	0.976/0.721	0.573/0.539
$CNAP_1$ single	0.298/0.455	0.420/0.681	0.143/0.232	0.103/0.159
$CNAP_2$ paired	0.126/0.216	0.447/0.608	**0.043/0.136**	0.052/0.058

*Covariates were body weight and body (or tail) temperature. Note covariate effects on p-values. Probability values were considered statistically significant only if MANOVA $p<0.02$ and ANOVA $p<0.01$. Although not regarded as statistically significant, the FEP early components and $CNAP_2$ had a treatment level ANOVA $p<0.05$ (regarded as statistically suggestive). These p-values increased to $p>0.05$ with use of covariates.

†Correlation data were transformed by Arc Sine for statistical analysis.

TABLE 3

DCM FLASH-EVOKED POTENTIAL P_1–N_1 AMPLITUDES AND STATISTICAL ANALYSES (BODY WEIGHT AND RECTAL TEMPERATURE USED AS COVARIATES*)

Level of Analysis	ANOVA/ ANCOVA ($p=$)
Treatment (overall)	0.102/0.172
Group	0.147/0.218
Sex	0.351/0.035
Treatment × Sex	0.931/0.776
/Body Weight	NA/**0.016**
/Temperature	NA/0.395

	Sex	n=	P_1–N_1 Ampl. (μV, mean ± SD)	Mean Weight	Mean Temp.	ANOVA/ANCOVA ($p=$)
0 ppm DCM	F	12	31.3 ± 28.6	196.9	36.6	NA
	M	8	44.3 ± 28.5	318.2	36.7	
50 ppm DCM	F	11	40.7 ± 27.7	199.8	36.7	0.805/0.948
	M	8	42.3 ± 16.7	324.6	36.6	
200 ppm DCM	F	12	43.7 ± 27.2	206.2	36.4	0.364/0.583
	M	8	49.8 ± 24.0	325.4	37.0	
2000 ppm DCM	F	12	52.2 ± 30.3	205.4	36.8	**0.032/0.079**
	M	9	60.4 ± 40.7	331.0	37.2	
135 ppm CO	F	12	35.2 ± 23.3	197.1	36.7	0.803/0.620
	M	12	34.9 ± 18.0	329.9	36.9	

*Note the effect of the covariates on the statistical significance of P_1–N_1 amplitude. Body weight appears to be the most important covariate.

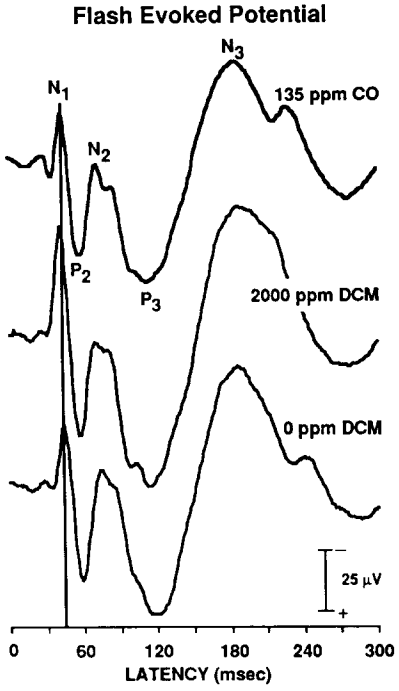

FIG. 2. Group composite flash-evoked potentials (FEPs, low intensity flash) after three months of treatment. Males and females were combined for purposes of illustration. As a point of reference, a vertical line was drawn through the N_1. DCM and CO FEPs were virtually unchanged by treatment, with the possible exception of a larger N_1 of DCM rats (amplitude ANOVA $p = 0.032$). The slightly greater body weights of DCM rats were associated with their increased N_1 amplitudes; when body weight was used as a covariate, then ANCOVA $p = 0.079$ for N_1 amplitudes (Table 3).

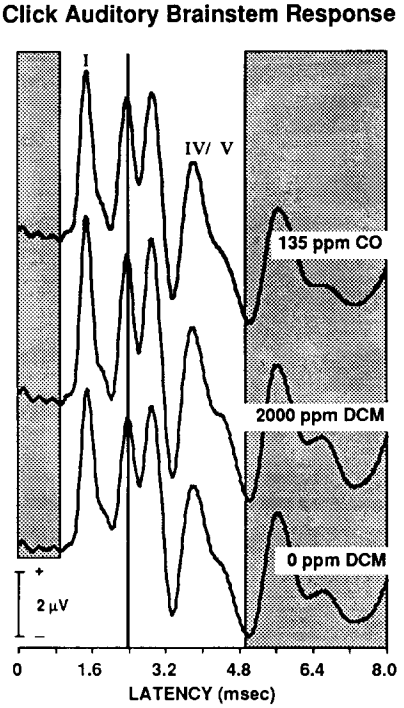

FIG. 3. Group composite click auditory brainstem responses (ABR_c): The analysis window is the section of waveform between the shaded areas. As a point of reference, a vertical line was drawn through peak II. Neither DCM nor CO had a discernable effect on ABR_c. Waveforms from tone-pips were similarly unaffected, but are not shown.

N = 12 for all groups): male controls vs. 2000 ppm DCM were 318.0 ± 23.0 g vs. 326.6 ± 17.4 g; female controls vs. 2000 ppm DCM were 196.8 ± 14.7 g vs. 205.4 ± 9.3 g. No effects of CO on body weight were detected.

Functional Observational Battery

No treatment-related effects were seen on the FOB. There was a possibility that lacrimation was slightly increased in female rats exposed to DCM; the relationship to DCM was unconvincing, however, since the observation also was present in female CO and control rats, did not show orderly dose response, and was not seen in male rats exposed to 2000 ppm DCM. As noted during weekly clinical observations, a low incidence of persistent muscular weakness was seen in the leg used for anesthetic injection. Some rats showed minor locomotor changes that were confined to one rear leg (the anesthetic injected leg).

Hindlimb Grip Strength

The grip strength of male rats exposed to 2000 ppm DCM was about 9% greater than controls, and grip strength of female rats exposed to 2000 ppm DCM was about 3% less than controls. These differences were not statistically significant (mean ± SD; N = 12 for all groups): male controls vs. 2000 ppm DCM were 590.0 ± 136.9 g vs. 642.5 ± 113.2 g; female controls vs. 2000 ppm DCM were 446.7 ± 105.3 g vs. 431.7 ± 57.3 g. There is a possibility that residual (anesthetic injection related) hindlimb weakness may have slightly increased the coefficient of variation of this test. The coefficients of variation for control rats were about

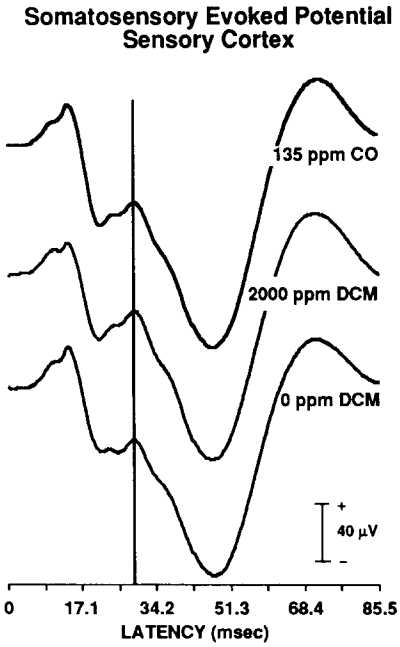

FIG. 4. Group composite somatosensory-evoked potentials-sensory cortex (SEP-S): As a point of reference, a vertical line was drawn at about 28 msec. Neither DCM nor CO had a discernable effect on the SEP-S. Recordings from over the cerebellum (SEP-C) were similarly unaffected, but are not shown.

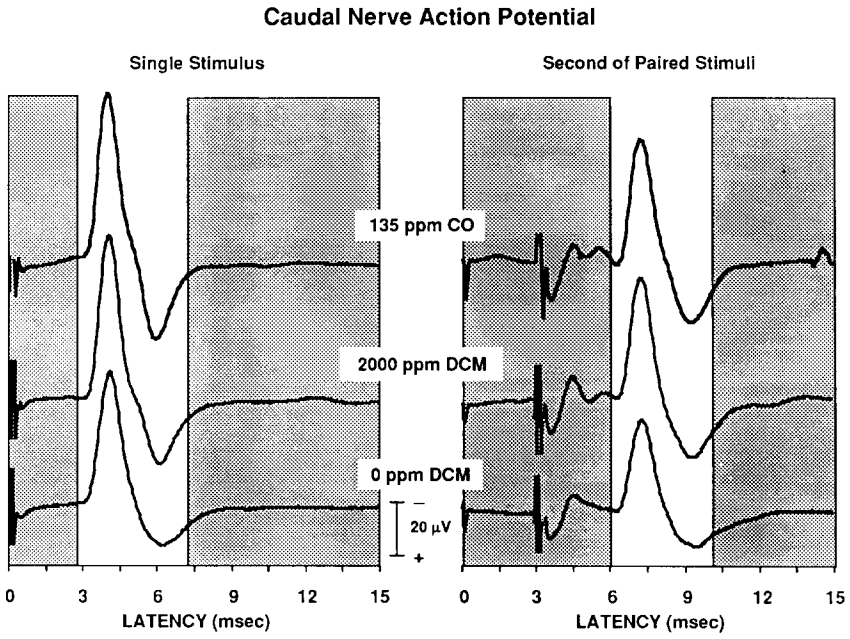

FIG. 5. Group composite caudal nerve action potentials (CNAP): CNAP responses to single ($CNAP_1$) and paired ($CNAP_2$) stimuli were slightly but not significantly more robust in DCM-treated rats. This waveform difference was somewhat more apparent for the $CNAP_2$ (correlation ANOVA $p=0.043$, Table 2). DCM rats were slightly larger than controls, and this may have resulted in slightly more robust waveforms. When body weight was used as a covariate, the $CNAP_2$ correlation ANCOVA $p=0.136$. The analysis window is the section of waveform between the shaded areas. On the right side (paired stimuli), the second stimulus occurred at 3 msec (stim. artifact), and $CNAP_1$ was subtracted to facilitate analysis of $CNAP_2$.

23%, and values near or below 20% were expected based on previous experience.

Body and Tail Temperatures

Body temperatures were taken just before each electrophysiological test. Body temperatures of rats from the 2000 ppm DCM group were slightly higher than that of controls (up to 0.4°C higher, depending on sex and test). Tail temperatures of male rats from the 2000 ppm DCM group were the same as controls, and those from treated female rats were 0.2°C less than controls. These differences were not statistically significant.

Flash-Evoked Potentials (FEPs)

No treatment-related differences were discovered (Table 2, Fig. 2). Although no statistically significant findings occurred (MANOVA p-values were greater than 0.02 and ANOVA p-values were greater than 0.01), the control vs. 2000 ppm DCM differences in power of the FEP early components had ANOVA $p=0.048$. When the statistical analyses accounted for the influences of body weight and body temperature (covariate analysis), the statistical significance of this power difference decreased to ANCOVA $p=0.146$.

Visual inspection of the FEPs (Fig. 2) indicated that the N_1 peak may account for most of the FEP power differences between 2000 ppm DCM rats and controls. Since an early-component analysis window that included several peaks (P_1, N_1, P_2, N_2 and P_3) may have diluted the ability to statistically detect a treatment-related change confined only to N_1, the FEP was reanalyzed for only the valley to peak amplitude of P_1–N_1 (Table 3). The statistical results were similar to the previous results of FEP early-components analysis; control vs. 2000 ppm differences in amplitudes of N_1 had ANOVA $p=0.032$, which increased to $p=0.079$ when ANCOVA statistically accounted for the influences of body weight and temperature.

All FEPs from male rats that were overexposed to light on the top tier of the cage rack were abnormally small and poorly formed. Rats were caged in the rack in a counterbalanced order, so that four rats from the control group and from each DCM treatment level were affected. FEPs from these male top tier rats were not included in the composite FEP waveform and were not analyzed.

Cortical Flicker Fusion

When compared to controls, CFF of rats exposed to 2000 ppm DCM was about 3% lower for males and 0.5% higher for females. These differences were not statistically significant.

Auditory Brainstem Responses

Neither visual examination nor statistical analyses of ABR_c, ABR_{10} or ABR_{30} waveforms revealed any evidence of treatment (Fig. 3).

Somatosensory-Evoked Potentials

Neither visual examination nor statistical analyses of SEP-Ss or SEP-Cs revealed any evidence of treatment (Fig. 4).

Caudal Nerve Action Potentials

Neither visual examination nor statistical analyses of CNAPs (single or paired pulses) revealed any evidence of treatment-

related effects (Fig. 5). Although no statistically significant findings occurred (MANOVA p-values were greater than 0.02, and ANOVA p-values were greater than 0.01), the control vs. 2000 ppm DCM correlation values of the second (paired) CNAP had ANOVA $p=0.043$. When the statistical analyses accounted for the influences of body weight and tail temperature (covariate analysis), the statistical significance of this correlation difference decreased to ANCOVA $p=0.136$.

Outliers

No treatment-related pattern was discerned in data from outliers. One control male rat had poorly shaped, slow and small ABRs for clicks and tones, one control female rat had a poorly shaped and slow SEP-C late component, one 2000 ppm DCM male rat had a very poorly shaped SEP-C early component, and one 2000 ppm DCM female rat had a poorly shaped and small SEP-S early component. Data from these specific waveforms from these specific animals were not included in the statistical analyses.

Gross Pathology

No DCM- or CO-induced gross pathologic alterations were observed.

Microscopic Pathology

Observations in control and treated animals were similar in incidence, and none of the histopathologic observations in rats exposed to 2000 ppm DCM for 13 weeks were attributed to the test compound. Degeneration of the retina in light-overexposed control and high-concentration males was characterized by a reduction in photoreceptor cells and was typical of that expected of light-induced injury.

Swollen axons in control and treated rats were common in the medial aspect of the nucleus gracilis adjacent to the area postrema. Generally the swollen axons were bilaterally symmetrical and the incidence was influenced to some degree by plane of section. The swollen axons appeared as spheroids (axonal bodies, giant axonal swellings, dystrophic axons) and were most apparent in sections stained with hematoxylin and eosin. The swollen axons were eosinophilic, round to ovoid, variable in size, and some contained small clefts. A few swollen axons also were detected in the spinal cord.

Spontaneous degeneration of individual nerve fibers was present in the central and peripheral nervous systems of both control and treated animals. Generally, the degeneration was very mild and occurred in single fibers scattered randomly in the affected tracts or nerves. The degeneration was characterized by digestion chambers that consisted of disrupted myelin, myelin degeneration, vacuolation and axonal fragmentation. These observations were considered normal for the age of these rats.

DISCUSSION

There were no apparent clinical, observational battery, evoked potential, or pathologic changes in DCM-exposed rats. While the literature indicates that exposures to DCM have little to no potential to cause brain injury, DCM does have sedative and anesthetic properties which account for its use as an anesthetic gas many years ago (4, 16, 17). Anesthesia with DCM was reported to be different from other gases in that analgesia and unconsciousness could be achieved without loss of muscle tone. As would be expected, sedative concentrations of DCM can temporarily alter both human and animal task performance. These effects were reviewed by Winneke (40). The fact that DCM can be given to humans and animals at anesthetic concentrations without evidence of brain injury indicated that this particular solvent does not share the acute neurotoxic properties of some other solvents, such as carbon disulfide and methyl chloride.

The potential for brain injury in humans from occupational exposure to organic solvents (including DCM) has been the subject of several studies. Some publications have suggested that long-term exposure of workers to some organic solvents can cause neurological and behavioral dysfunction [e.g., (11,35)]. Other reports, however, have either equivocal or negative findings (7,20). One study dealt specifically with DCM (8); no evidence was found of long-term damage that could be attributed to exposure to DCM. A recent critique of the Danish solvent-neurotoxicity literature suggests that extreme care be taken in the interpretation of the solvent-neurotoxicity literature, since many of the positive findings may have been caused by methodological flaws such as incorrect selection of controls (12,13). Indeed, one research group has recently reclassified as normal many patients previously diagnosed as having solvent-induced toxic encephalopathy (14).

Small but statistically significant changes were reported for selection brain chemistries of gerbils four months after exposure to dichloromethane for 24 hr/day for 2 months (34). Several anatomic sites in the brain were evaluated for DNA, and glia cell marker proteins S-100 and GFAP. This study is difficult to interpret because: 1) There was excessive mortality given the level of exposure. Exposures to 700 ppm were terminated after 7 weeks because most of the exposed gerbils died. Exposures to 350 ppm killed about half the test gerbils. In contrast, rats readily survive a life-time exposure up to 4000 ppm, although these exposures were 6 hr/day, 5 days/week (6,28). One wonders, therefore, whether the survival differences are due to exposure paradigm, species, or other unidentified variables. 2) The relationship of near-lethal exposures and non-CNS toxicity to the changes in brain chemistry were not defined. 3) The treated vs. control differences were small, and many appeared to be within the variation demonstrated between different control groups. 4) Alpha corrections were not performed for the inflated number of false positive findings expected from repetitive use of statistics.

Experimental data from other studies indicate a lack of neuropathologic effects of DCM. Both the US government and industry have conducted medium- and long-term rodent DCM studies, and the brain, spinal cord and peripheral nerves were histopathologically examined in most of these studies (6, 27, 28). These DCM studies involved daily exposure up to 4000 ppm of DCM for up to the life-time of the animals. No treatment-related changes in the nervous system were reported. The results of our neurologic and neuropathologic assessment of rats exposed to DCM for 13 weeks are in agreement with the above literature. No statistically significant changes occurred in the 43 dependent variables measured in this study (MANOVA $\alpha=0.02$; ANOVA $\alpha=0.01$), and no treatment-related histopathologic changes were noted in the brain, spinal cord, peripheral nerves or other nervous tissues.

Weak statistical findings are common in multiparameter studies. Gill (15) estimates that with 41–43 dependent variables, one would need five or more findings at $p<0.05$ to be 95% confident that one or more of the findings represented a true difference. Our DCM study is no exception, and although no statistically significant changes occurred, two ANOVA-level statistical findings were $p<0.05$ and, thus, were considered statistically suggestive.

One of the statistically suggestive findings was ANOVA $p=0.043$ for control vs. 2000 ppm DCM differences in the shape (cross-correlation values) of the paired caudal nerve action potential (Table 2). Examination of the CNAP waveforms (Fig. 5) shows that the 2000 ppm DCM-treated rats had slightly faster, slightly better shaped, and slightly larger CNAPs. These differences were small, and when body weights and tail temperatures

were accounted for by analysis of covariance (ANCOVA), the level of significance decreased from ANOVA $p=0.043$ to ANCOVA $p=0.136$. Since the mean tail tempratures for the control and 2000 ppm DCM male rats were identical (23.6°C), and were only 0.2°C different for females, the effects were more likely due to the slightly greater body weights of the 2000 ppm DCM rats. Although not statistically significant, 2000 ppm DCM rats weighed 3–4% more than controls.

A 4% difference in body weights seems small, but rats that weighed 9% less than controls because of mild dietary restriction have been shown to have mild FEP, ABR and CNAP changes (2). Whereas the 2000 ppm DCM rats in our study weighed 4% more than controls, and had slightly better CNAP cross-correlation coefficients to the template waveform, the rats in the study by Albee *et al.* (2) weighed 9% less and had poorer cross-correlation coefficients to the template waveform. Thus, the CNAP findings are consistent between the DCM and dietary restriction studies, and indicate that body weight has an influence on CNAP waveforms.

The second statistically suggestive finding was ANOVA $p=0.048$ for control vs. 2000 ppm DCM differences in the power of the early components of the flash-evoked potential (Table 2). The early FEP components (P_1 through P_3) of 2000 ppm rats were about 20% larger than controls, and very slightly faster than controls (Fig. 2). When body weight and temperature differences were accounted for by ANCOVA, the level of statistical significance for the power difference decreased to ANCOVA $p=0.146$.

Examination of the FEP waveforms in Fig. 2 indicates that much of the power difference may have occurred in the N_1 peak of the DCM 2000 ppm rats. An N_1 power increase is interesting since Dyer (9) has shown *acute* CO exposure to increase selectively the amplitude of N_1, while Rebert *et al.* (32) have shown *acute* DCM exposures above 5000 ppm to decrease selectively the amplitude of N_1. To examine the possibility that DCM caused a persistent increase in N_1, the amplitude of just the P_1-N_1 for all DCM treatment levels and for CO was statistically analyzed (Table 3). The N_1 peaks for CO rats were comparable to that of controls, and did not show persistent N_1 changes similar to the acute changes seen by Dyer (9).

The FEP amplitudes of N_1 for DCM rats were, in general, larger than controls. Overall, however, the differences were statistically unimpressive (Table 3). The ANOVA for an overall treatment effect was $p=0.102$, while the overall ANCOVA (body weight and temperature as covariates) was $p=0.172$. The significance of body weight as a covariate was $p=0.016$, suggesting body weight may have an important influence on N_1.

When step-down ANOVAs on N_1 were conducted at different DCM dose levels, the 2000 ppm DCM vs. control differences in N_1 amplitude yielded ANOVA $p=0.032$. None of the other treatment levels approached statistical significance (Table 3). When the influence of body weight and temperature were statistically accounted for in the high dose N_1 analysis, the significance decreased from ANOVA $p=0.032$ to ANCOVA $p=0.079$.

Both the CNAP and FEP covariate analyses indicate that body weight is an important factor in the shape of these waveforms. Although not analyzed specifically, an examination of Fig. 2 in the manuscript of Albee *et al.* (2) shows that dietary restriction has a large effect on the FEP N_1 peak. The N_1 of rats with mild dietary restriction (9% body weight decrement) appears to be about half the amplitude of controls, and the N_1 of rats with severe dietary restriction appears to be about a quarter the amplitude of controls.

Our conclusion is that DCM is unlikely to have directly influenced the CNAP and FEP waveforms, but that body weight did have an influence on these waveforms. This conclusion leads to a second question: did DCM have an influence on body weight? Evidence to support a relationship between DCM exposure and increased body weights is weak, but body weights of rats exposed to 2100 ppm in a National Toxicology Program (NTP) 90-day study were about 6% greater than controls (28). As in our study, these differences were not statistically significant. It might be argued that irritative or sedative properties of DCM altered appetitive behavior or the overall level of physical activity, and a small increase in body weight ensued. At this time, these arguments are too tenuous to arrive at a conclusion that DCM affected body weight, and a conservative conclusion would be that the differences in body weights were adventitious.

Concerns of experimental and statistical power exist when a study does not reveal toxicologically significant events. Although statistical power for MANOVA is unknown (due to the complexity of the calculations), our coefficients of variation were reasonably small, and sample sizes reasonably large (see the Results section). The study did reveal effects from intramuscular injection of anesthetic, and FEP and retinopathic effects of overexposure to light in a small group of male rats ($n=4$ per group). The toluene companion paper (25) demonstrates easily recognized and statistically impressive effects on evoked potentials with sample sizes of only 6 per group, in the absence of neuropathologic changes (perfusion, extensive light microscopy). Other rat studies from this laboratory have shown evoked potential effects from 9% weight reduction ($n=12$/group) (2), subchronic exposure to 100 ppm sulfuryl fluoride ($n\leq 14$/group) (23), and very mild congenital hypothyroidism ($n=24$) (1). As with toluene, evoked potential effects were recognized easily in the absence of CNS lesions (light microscopy).

When considering all of the neurofunctional and morphologic data from the current DCM study, the overall conclusion was that 13 weeks of exposure of Fischer 344 rats to DCM at levels up to 2000 ppm, 6 hr/day, 5 days/week, had no persistent discernable effects on either function or structure of the nervous system. A similar conclusion was reached for rats exposed to 135 ppm CO; indirect evidence from the DCM study (morphology and function), and direct information from clinical and electrophysiologic examination of CO-exposed rats, showed no evidence persistent CNS alterations after subchronic exposure.

ACKNOWLEDGEMENTS

This DCM/CO study was complicated and required a large amount of support. In particular, we wish to express our great appreciation to the animal care personnel, to C. M. Streeter and T. S. Gushow for managing the inhalation chambers, and to P. J. Hopkins for neurohistology.

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