JOURNAL OF THE AMERICAN COLLEGE OF TOXICOLOGY
Volume 10, Number 6, 1991
Mary Ann Liebert, Inc., Publishers

Behavioral Toxicology of Volatile Organic Solvents V. Comparisons of the Behavioral and Neuroendocrine Effects Among *n*-Alkanes

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ABSTRACT

Four homologous *n*-alkanes were compared for their ability to impair performance and stimulate hypothalamic–pituitary activity in mice. Performance was assessed using operant responding maintained under a fixed interval 60-sec schedule of milk presentation. Cumulative concentration–effect functions for octane, heptane, hexane, and pentane were obtained by incrementally increasing exposure concentrations until responding was abolished. Recovery from these rate-decreasing effects was determined 30 min after exposure to the highest concentration. Rate-decreasing potency (EC_{50}) was greatest for octane (2474 ppm), and progressively less for heptane (3872 ppm), hexane (7051 ppm), and pentane (36130 ppm). Responding recovered completely 30 min after exposure for pentane and hexane, to 75% of pre-exposure levels for heptane, but to only 15% of pre-exposure levels for octane. The risk of obtaining a small effect with these agents (the concentration expected to decrease performance 10% in 1 out of 1000 mice) exhibited a similar order. The effect was predicted to occur at 227 ppm for octane, 331 ppm for heptane, and 1429 ppm for pentane. However, this prediction occurred at an unusually low dose for hexane (68 ppm). These *n*-alkanes also stimulated up to 2000-fold increases in adrenocorticotropin hormone (ACTH) release. *n*-Hexane was slightly more potent and produced larger effects. These studies demonstrate a direct relationship between aliphatic carbon chain length and the potency of *n*-alkanes in impairing performance.

INTRODUCTION

ACUTE INHALATIONAL EXPOSURE TO ORGANIC solvents can impair normal behavioral functioning.[1–3] The primary means of assessing this impairment is to characterize a dose–effect function and then use this information to assess risks of effects at lower doses.[4] While the intercept (no-effect) of this function has drawn considerable attention in toxicity measurement, the slope also provides valuable information. A recent comparison of the ability of several unrelated solvents to disrupt normal behavioral functioning noted differences in the slope of the dose–effect functions and questioned the role of dose-spacing in risk assessments.[5] Since different solvents may exhibit different effects due to a wide range of other properties (e.g., physiocochemical), the present report compared the ability of several related agents to disrupt behavior. These effects were then used to characterize the risks of a behavioral effect at much lower concentrations, using a dose–effect approach to risk assessment. Lastly,

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since several recent reports have described the ability of solvents to activate the hypothalamic–pituitary axis,[6] the ability of these solvents to simulate ACTH release also was studied.

MATERIALS AND METHODS

Subjects

Experimentally naive, adult male CD-1 mice, weighing between 35.0 and 40.0 g were used. For behavioral experiments, mice were food-deprived to 80% of their free-feeding weight, and then maintained at that weight throughout the studies by supplemental, postsession feeding. For the study of neuroendocrine effects, additional mice, maintained at *ad libitum* weight, were used.

Apparatus

A modified 24-L pressure cooker (Wisconsin Aluminum Foundry, Manitowoc, WI) served as the inhalation chamber.[7] Agents were injected through a septum-sealed port onto an evaporating dish within the chamber. A heater under the dish quickly vaporized the agent and the contents of the chamber were mixed with a fan. All electrical contacts were insulated to prevent the occurrence of sparks. The chamber was placed within a hood and maintained at room temperature. Previous studies have characterized the stability of concentrations of similar solvents within the chamber and have demonstrated that levels remain relatively constant when the chamber was kept closed.[8]

An operant chamber (9 × 9 cm high), suspended in the middle of the inhalational chamber, confined mice individually during experiments. The behavioral response was defined as the interruption of a light beam located behind a 1-cm nose-poke hole centered 2 cm above the floor on the front wall of the operant chamber. Evaporated milk (0.025 mL) was delivered to a receptacle located below the nose-poke hole. Food delivery intermittently followed responses (nose-pokes) which occurred in the presence of discriminative stimuli. Three small green lights located over the poke hole and a lack of white noise served as discriminative stimuli. The same chamber was used for neuroendocrine exposure assessments.

Procedures

Responding was maintained under a fixed-interval (FI) 60-sec schedule of milk presentation.[9] In the presence of flashing green lights, the first response to occur after the elapse of a 60-sec interval produced milk. A 5-sec timeout (TO) followed each milk presentation. After a series of 8 consecutive FI 60-sec TO 5-sec cycles, or 10 min (whichever came first), there was an 1800 sec (30 min) interseries TO. During TO the green lights were not illuminated, white noise was on, and responding had no consequences. Experimental sessions were run 5 days a week, each daily session consisted of 9 FI series. The total number of responses in an FI series was divided by the time required to complete that series, providing a rate of responding. Responding in the first series of a session was excluded. The mean rate of the second and third daily series provided a control value for that session.

For cumulative concentration–effect functions, the agent was introduced into the chamber at the end of the third FI series (and hence 30 min before the fourth series). Rates of responding during the fourth series served as the measure of the behavioral effect of that exposure. Immediately following the fourth series, the concentration was increased to the next value studied; responding during the fifth series, 30 min later, thus reflected the effects of that exposure.

Concentrations were increased incrementally in a similar manner until responding was abolished. Immediately following the first series in which responding was abolished, the cover of the chamber was removed for the next 30 min and then replaced before the next series occurred. Responding during this "air" series reflected the degree of recovery 30 min after exposures had been terminated. Concentration–effect curves were determined twice for each agent in each mouse. Agents were tested in order of decreasing potency. Solvent exposures were separated by at least one week. Eight mice were studied.

For neuroendocrine exposure assessments, the mice were housed singly and exposed to a single concentration of one of the four solvents for 30 min. Mice then were withdrawn from the chamber, immediately sacrificed with

trunk blood collected in chilled EDTA-spiked collection vials. Blood samples were pooled for each concentration for a particular solvent, centrifuged, and the serum was frozen for subsequent analysis. Four to six mice were studied per concentration.

Data analysis

The effects of exposures are expressed as the mean of the proportional effect obtained at each concentration for each mouse. Behavioral decrement also was assessed by a recently developed method of risk assessment.[10] For each experiment, a linear regression function was fit to the portion of the data bound by the highest (ln) concentration to produce less than a 20% decrement in responding, and the lowest concentration to produce more than an 80% decrement. The concentration expected to decrease responding 10% in 1 out of 10, 1 out of 100, and 1 out of 1000 such experiments was determined using the mean and standard deviation of an estimated 10% effect on each function and the expected variability based on the Student's *t* distribution (see refs. 4 and 10).

Analytical procedures

Gas samples were taken from the chamber using a gas-tight syringe and analyzed on line by flame ionization in a Hewlett Packard 5830A Gas Chromatograph and HP 18850A terminal to compare gas samples with external standards. For neuroendocrine experiments, an amount calculated from the standard curves obtained during the behavioral experiments was added to the chamber to produce the desired concentration. Serum levels of adrenocorticotropin hormone (ACTH) were determined by the use of a standard radioimmunoassay kit (ICN Biomedicals, Inc., Costa Mesa, CA). Generally, 4 to 6 mice were used for each concentration. Detection limits were slightly less than 20 pg/mL. Inter- and intra-assay control variabilities were 4.4% and 10.7%, respectively

Agent

n-Alkanes, of purity greater than 99.5% (Fluka AG, spectrophotometric reagent grade) were used.

RESULTS

Control performances

Mean control rates of responding ranged from 0.253 to 2.163 resp/sec for individual mice; the mean variability (SD, as a fraction of control) between control exposures for individual mice across the different experiments was 0.11. No consistent trend in rate over control performances was obtained over the course of exposures. Serum ACTH levels varied from the detection limit (20 pg/mL) to slightly greater levels (24.4 pg/mL) across control groups for the different solvents.

Exposure effects

Behavioral Effects: Figure 1 shows the mean effects of each of the four *n*-alkanes on FI responding and the degree of recovery 30 min after the cumulative exposures were terminated.

Octane: Concentrations greater than 1000 ppm decreased responding in a dose-related manner. Responding was decreased slightly more than 50% at about 3000 ppm, and almost completely abolished at 5600 ppm. The number of food presentations generally did not decrease until exposures of 5600 ppm. Following exposures of 10,000 ppm, mice were observed to be engaged in circular locomotive activity. Responding recovered to 15% of control levels 30 min after 10,000 ppm octane exposures were terminated.

Heptane: Concentrations of 1000–3000 ppm of *n*-heptane occasionally increased rates of responding, while the next largest concentration studied, 5600 ppm, virtually abolished responding. Thus, responding was decreased 50% at about 4500 ppm. Following 10,000 ppm mice were prostrate. Responding recovered to 75% of control levels 30 min following the removal of 10,000 ppm heptane.

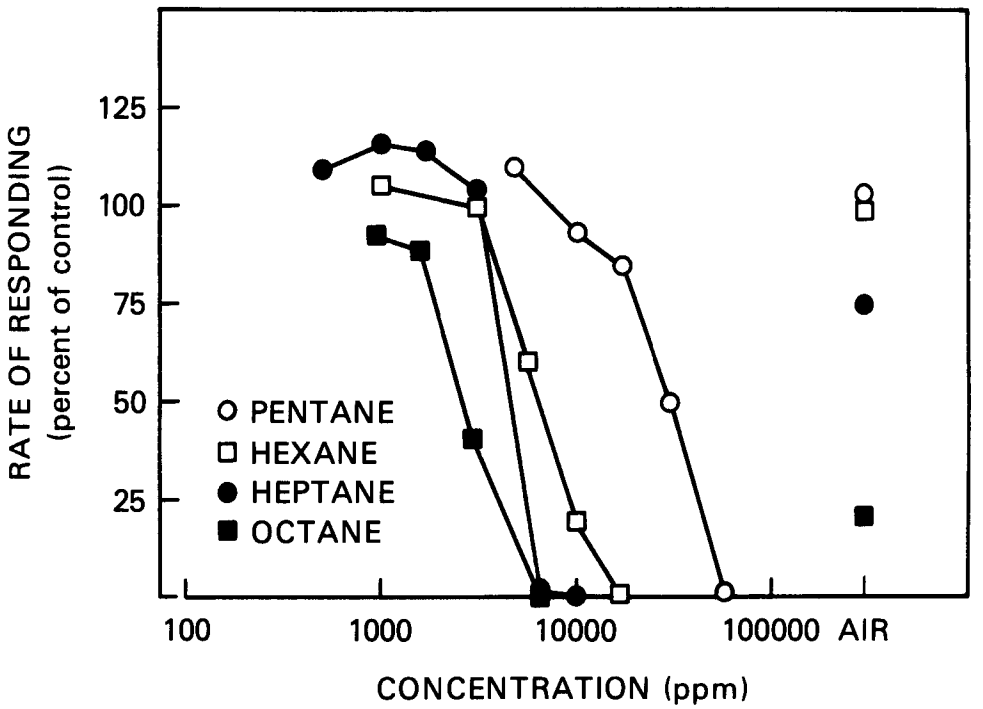

FIG. 1. The mean effects of different concentrations of *n*-octane, *n*-heptane, *n*-hexane, and *n*-pentane on schedule-controlled responding of mice. Responding was maintained under FI 60-sec schedules of milk presentation. Individual concentration effect functions were obtained by comparing control (pre-exposure) levels of responding to responding after 30-min exposures of incrementally increased solvent concentrations. Concentration was increased until responding abolished, then the chamber was "aired" for 30 min and recovery of responding was assessed. Each curve is the mean effect of eight experiments. Recovery functions for each agent are shown on the right side of the figure. Abscissa, concentration in ppm. Ordinate, mean effect expressed as a percentage of the individual control rate of responding.

Hexane: Concentrations less than 3000 ppm *n*-hexane had no effect, and larger concentrations decreased responding in a concentration-related manner. Responding was decreased slightly less than 50% at 5600 ppm, and about 80% at 10,000 ppm. Concentrations of 17,000 ppm abolished responding. The number of food presentations generally did not decrease until exposures of 10,000 ppm were obtained. Responding recovered fully 30 min following the removal of 17,000 ppm hexane.

Pentane: Concentrations less than 10,000 ppm *n*-pentane slightly increased responding. Larger concentrations decreased responding in a concentration-related manner, with 30,000 and 56,000 ppm decreasing responding approximately 50% and 100%, respectively. Responding recovered fully 30 min following the removal of 56,000 ppm pentane.

Estimation of risks: When a line was fit to the descending limb of individual functions, the mean (ln) concentration to result in a 50% (EC_{50}) and 10% (EC_{10}) decrement in responding was determined (Table 1). Based on individual EC_{10}s, the concentrations expected to produce the same effect in 1 out of 10 ($p = 0.1$), 100 ($p = 0.01$), and 1000 ($p = 0{,}001$) mice also were determined for each agent and are listed in Table 1. With decreasing proportions of the population set as the acceptable risk level, concentrations of each agent expected to produce an EC_{10} decreased. These concentrations maintained a SAR similar to that seen with the EC_{50}, with the exception of *n*-hexane.

Neuroendocrine effects: Serum ACTH levels increased in a dose-dependent manner for each agent studied, although large increases were not obtained until 3000–10,000 ppm were assessed. Although pentane generally

TABLE 1. MEAN (± SD) FOR EC_{50}, EC_{10} AND THE CONCENTRATIONS EXPECTED TO PRODUCE AN EC_{10} IN LESS THAN 0.1, 0.01, AND 0.001 OF THE PROPORTION OF THE POPULATION STUDIED FOR EACH SOLVENT

	$EC_{50} \pm SD$	$EC_{10} \pm SD$	$p < 0.1$	$p < 0.01$	$p < 0.001$
n-octane	2,474 ± 496	1,508 ± 543	898	517	277
n-heptane	3,872 ± 917	2,945 ± 1,199	1,459	727	331
n-hexane	7,051 ± 3,138	4,537 ± 3,490	1,092	295	68
n-pentane	36,130 ± 9,531	26,553 ± 12,980	10,226	4,057	1,429

produced the smallest increases in ACTH at concentrations up to 3000 ppm, and octane produced the largest increase at 10,000 ppm, little other data supported a direct relationship between alkane chain length and either potency or efficacy in increasing ACTH levels. Rather, hexane generally appeared to produce the largest increases in ACTH (Fig. 2).

DISCUSSION

The potency to disrupt normal behavioral functioning is directly related to the carbon chain length of for *n*5-*n*8 aliphatic alkanes. The rate-decreasing effect and the recovery from that effect exhibited a structure–activity relationship (SAR) such that the longer the chain length, the more potent and long-lasting the agent. These results

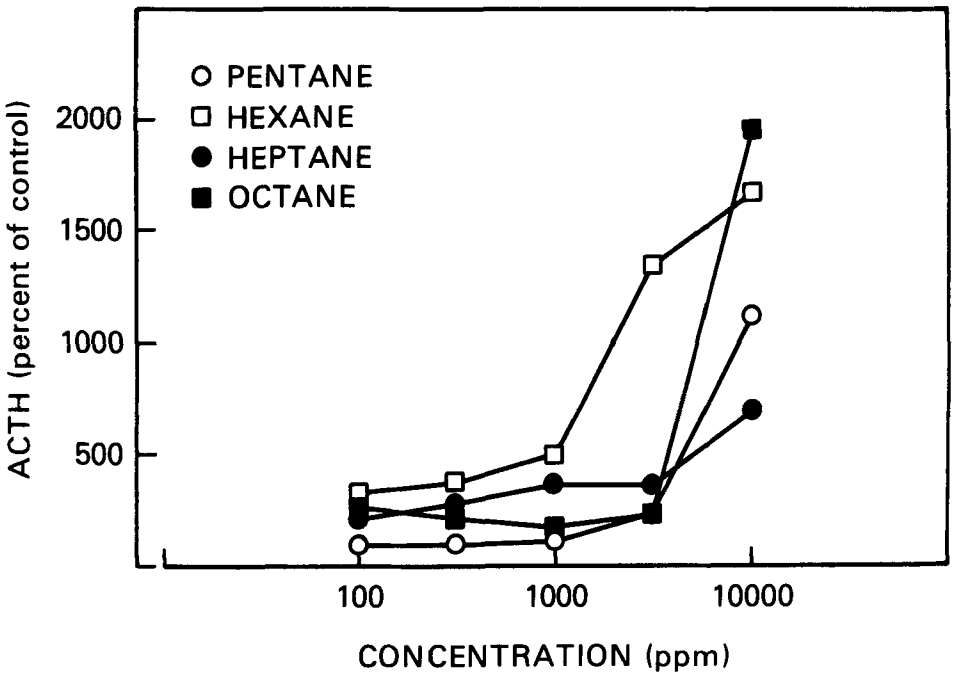

FIG. 2. The mean effects of different concentrations of *n*-octane, *n*-heptane, *n*-hexane, and *n*-pentane on plasma ACTH. Each curve is the mean effect in four-six mice exposed to a single concentration. Abscissa, concentration in ppm. Ordinate, effect expressed as a percentage of the control levels of ACTH (i.e., ~ 20–24 pg/mL).

suggest that SAR may be useful in determining risks of exposure to lower levels of volatile agents. However, two additional analyses questioned this conclusion. When risk assessments were compiled using the variability in the data, *n*-hexane appeared more likely to produce effects at low doses than would be expected based on structure. In addition, *n*-hexane resulted in greater elevations in ACTH than expected strictly on the basis of SAR. Thus, these results maintain a cautious acceptance of SAR in predicting risks of effects across classes of toxicants.

The present results are consistent with previous studies of the behavioral effects of exposure to increasing concentrations of solvents. Both rate-decreasing effects and recovery from those effects have been observed for several solvents.[5,7] For example, toluene produced concentration-dependent decreases in responding following cumulative exposures to 1000–4000 ppm, and these effects were well replicated with single exposures to comparable levels.[7] More toxic agents, such as carbon disulfide, clearly decrease responding at lower concentrations, and recovery is prolonged.[11] The present results are also consistent with a previous report of *n*-octane effects on responding of mice.[12] In that study the ED_{50} was determined to be 2844 ppm, as compared with the 2474 ppm determined in the present study. This study also assessed the effects of 4-h exposures to single concentrations of octane. As in the earlier studies with toluene, single (4 h) exposures to *n*-octane had effects comparable to those seen with cumulative 30-min exposures. While 500 ppm had little effect over the entire exposure period, concentrations of about 2000 ppm resulted in a moderate, yet consistent, rate-increasing effect. Concentrations of about 4000 ppm decreased responding almost completely. Such results suggest that steady-state effects were obtained.

Previous comparisons of the rate-decreasing effects of representative agents from different classes of solvents[5] suggested a relatively consistent relationship between their established threshold limit values (TLVs) and their ability to impair performance. While the present results are consistent with this conclusion, the TLV for pentane is 1000 ppm but TLVs for the other three alkanes are all 500 ppm.[1] Thus, these data suggest that a re-examination of the basis for the TLV for octane, and possibly hexane, may be warranted.

Previous studies have noted that lethality (LD_{50}) data and righting reflex functions obtained for aliphatic alkanes also exhibit a similar structure-activity relationship as the one demonstrated here.[13–15] Generally, the toxicity of alkanes increases with increasing numbers of carbon atoms up to octane. With further increases in carbon chain length, toxicity decreases, presumably due to decreased absorption, volatility, and/or solubility. However, while these properties suggest toxicokinetic parameters exhibit order, few studies have systematically examined other effects that may be relevant, for example the irritant properties of alkanes.

The recovery data were interesting because they suggested that the potency of these solvents in decreasing rates is related to the rate of elimination of the solvent. The more potent the agent, the longer its rate-decreasing effects lasted. Thus, while steady-state behavioral effects were assumed to obtain with 30-min exposures, the elimination of the solvent (or its residual effects) appeared to require longer periods for the more potent agents. If steady state had not been obtained with 30-min exposures, then these agents may be even more potent.

There were also subtle differences between these agents. For example, *n*-heptane produced rate-increasing effects over a moderate range of concentrations, while the other agents generally did not. Octane had previously been reported to increase responding[12] but the magnitude of effect was smaller than currently seen with heptane. *n*-Heptane also appeared to have a much steeper concentration–effect function than the other agents. Differences in the consistency of the concentration–effect functions between agents were also noted. For example, the variability in the EC_{50} generally was about 20–26% of the mean, except for hexane (which was about 45%). The variability for hexane was due mostly to the results of two individual experiments, one resulting in a high estimate and one a rather low estimate. The large variability in effect of hexane resulted in a conservative figure for the current method of risk assessment, as the method is based on individual differences. As such, these results amplify previous observations that the utility of the current risk assessment model depends on the reproducibility of effects.

The use of single subject design allows toxicity assessment using a relatively small number of animals. The present results support previous conclusions regarding the reproducibility of effects using the single subject design to assess behavioral toxicity of volatile organic solvents.[5] In that report, the effect of a potent solvent, ethyl acetate, was assessed after exposure to four other agents. When compared to an ethyl acetate-only-exposed group, the effects were almost identical. Such methods are clearly efficient in the use of animals and time, and allow the repeated observation of baseline performances to assess residual or cumulative toxicity long after exposure. As in the earlier report, the availability of functions determined independently (such as that described for *n*-octane)

allows further assessment of effect. However, it should not be assumed that prior exposure to agents with unknown effects will not have any residual effect, without direct replication.

The neuroendocrine data illustrate that these solvents share the ability to activate the hypothalamic–pituitary–adrenal (HPA) axis. This type of effect has been recognized for diethyl ether,[6] and may be of general concern for agents with solvent-like properties. For example, ethanol and drugs with behavioral effects similar to those of ethanol (such as the benzodiazepines and barbiturates) also increase circulating levels of plasma corticosterone.[16] Since these agents share similar behavioral properties with solvents, such as anticonvulsant, antianxiety, and discriminative effects, common neurobiological effects are of interest. On the other hand, less order was currently seen in the SAR for neuroendocrine effect in mice. Some of that lack of order may have been due to the difficulty in blood extraction procedures in mice. Further studies using comparable exposure techniques (i.e., within-, or between-subject designs) should be used to compare the sensitivity and risk figures produced using behavioral or neuroendocrine end points. One study to directly compare these effects in mice for diethyl ether concluded that behavioral impairment and HPA activation were closely associated in the low concentration range, but could not determine relative causality.[6]

In conclusion, the present methods appear quite suitable for the assessment of behavioral toxicity resulting from exposure to volatile organic solvents. The methods outlined for assessing risk should be considered for further development in neurotoxicological risk assessment.

ACKNOWLEDGMENT

The author gratefully acknowledges the assistance of J.J. Holland and M.E. Natale in the conduct of these experiments. The author also expresses his thanks to P.B. Dews for his contributions to the development of this work and my interests in solvent effects. This research was supported in part by U.S. Public Health Service Grants OH00706, MH07658, MH14275, DA02658, and RR00168.

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