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The Temporal Relationship between Behavioral and Hematological Effects of Inhaled Benzene[1]

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The Temporal Relationship between Behavioral and Hematological Effects of Inhaled Benzene. DEMPSTER, A. M., EVANS, H. L., AND SNYDER, C. A. (1984). *Toxicol. Appl. Pharmacol.* **76**, 195–203. The time–effect relationship of the behavioral and hematological changes caused by inhaled benzene was investigated in C57BL mice. Mice were exposed to air, or 100, 300, 1000, or 3000 ppm benzene in standard inhalation chambers employing dynamic vapor exposure techniques. Mice were exposed for 6 hr/day for the number of days necessary to achieve a minimum concentration × time (Ct) product of 3000 ppm–days. The intermittent exposure regimen of 6 hr/day was employed to simulate occupational exposure. The most sensitive behavioral index (milk-licking) was affected by the lowest concentration tested (100 ppm), while homecage food intake, hindlimb grip strength, and body weight were reduced only at 1000 and 3000 ppm. Some of these previously undocumented behavioral changes occurred as rapidly as hematological changes that have been considered hallmarks of benzene toxicity. A significant decrease in circulating lymphocytes occurred after exposure to all concentrations. Circulating red blood cells were variably affected by benzene, in that anemia resulted after 10 days exposure to 100 ppm and after 3 days exposure to 300 ppm but not after 3 days exposure to 1000 ppm or a single exposure of 3000 ppm. The data indicate a departure from Ct relationships, and suggest that exposure duration as well as daily dose may be an important factor in benzene toxicity.

Benzene, a widely used solvent, has long been recognized as an important health hazard. The critical effect of acute benzene inhalation, depression of central nervous system (CNS) function and narcosis, most likely ensues directly from benzene itself rather than from metabolites (Bergman, 1979; Brief *et al.*, 1980; Haley, 1977). Brief inhalation of approximately 4000 ppm benzene causes symptoms such as giddiness, euphoria, headache, and nausea in humans; continued exposure produces unconsciousness (Haley, 1977; Brief *et al.*, 1980). Extremely high concentrations of benzene (25,000 ppm) are fatal within minutes (Brief *et al.*, 1980). Chronic industrial exposure to benzene also causes neurological abnormalities, including reduced peipheral nerve conduction velocity (Baslo and Aksoy, 1982).

In spite of this clinical evidence of the neurotoxicity of benzene, few experiments have examined behavioral changes in benzene-exposed animals. Rats, given po intubation of liquid benzene as neonates, exhibited increased locomotor activity and a decreased response to *d*-amphetamine when

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tested as adults (Tilson *et al.*, 1980). However, neonatal po exposure to benzene is probably not directly comparable to occupational exposure in adults. Furthermore, inhalation of benzene may cause different effects than exposure by other routes (Snyder *et al.*, 1980). In another study, inhalation of 300 or 900 ppm benzene increased the occurrence of eating and grooming, and reduced the number of mice that were sleeping or resting (Evans *et al.*, 1981). Because these reports suggest that locomotor activity and appetitive behavior are influenced by benzene, these endpoints were examined in the present study. We compared these two indices with hindlimb grip strength, an index of neurotoxicity produced by *n*-hexane (Schaumburg and Spencer, 1979) and by acrylamide (Teal and Evans, 1982) but not previously used to evaluate benzene inhalation.

The hematopoietic system is regarded as an important target of chronic exposure to benzene, with pancytopenia and its variants reported in both animals and humans (Snyder and Kocsis, 1975; Haley, 1977; Laskin and Goldstein, 1977). Pluripotent stem cells, and other precursors of mature hematological cells, are adversely affected by benzene (Green *et al.*, 1981b), and there is an association between chronic benzene exposure and tumors of the hematopoietic organs (Laskin and Goldstein, 1977; Aksoy, 1981).

The hematological effects of benzene have been associated with longer exposures to lower benzene concentrations than the behavioral effects reviewed above. Only one study has directly compared hematological and behavioral effects of inhaled benzene. Mice, exposed to either 10 or 100 ppm benzene for 6 hr/day for 20 days, exhibited decreased wheel-running activity without a significant change in white and red blood cells (Horiuchi *et al.*, 1967). Since many of the hematotoxic effects of chronic low-concentration benzene exposure are delayed (Snyder *et al.*, 1980), the experiment reported here examined the possibility that behavioral change may be an earlier indicator of benzene toxicity. Therefore, we directly compared the dose–effect and time–effect relationships between the known hematological end-points and the previously undetermined behavioral effects of inhaled benzene.

A second goal of the present experiment was to determine whether brief exposure to high concentrations could produce a profile of effects similar to those produced by repeated exposure to lower concentrations which characterize human occupational exposures. Thus, the present experiment examined whether the interaction of four different benzene concentrations and exposure durations would produce a constant concentration × time relationship, as predicted by Haber's Law (Andersen, 1981).

METHODS

Animals. Adult male C57BL/6J mice[3] were housed in groups of five in clear-plastic cages containing wood-chip bedding[4] with wire tops. Powdered feed[5] was continuously available in a glass feeder[6] to prevent scattering of feed, and tap water was available *ad libitum.* Mice were quarantined for the first 2 weeks after arrival to the laboratory, adapted to a 12-hr light/dark cycle, and then randomly assigned to treatment groups. Care and use of the mice conformed to contemporary standards (NIH, 1980).

Exposure conditions. All inhalation exposures were conducted in either a 1.3- or a 0.13-m^3 stainless-steel Laskin chamber (Drew and Laskin, 1973). Control mice were exposed to filtered air in other chambers concurrently with test mice. Mice were exposed in stainless-steel wire cages during the light part of their daily cycle. The mean (±SD) temperature and relative humidity of the exposure chambers were 22 ± 1°C and 57.7 ± 7%, respectively. All mice received at least 1 week of sham exposures prior to the start of the experiment.

Benzene[7] atmospheres were generated by vaporizing

[3] Jackson Labs., Bar Harbor, Maine.

[4] Beta Chip, Northwestern Products Corporation, Warrensburg, N.Y.

[5] Ralston Purina Company, St. Louis, Mo.

[6] Model LC-207/A, Wahmann Corporation, Timonium, Md.

[7] Chromatoquality-grade benzene, verified for purity by gas chromatography, was used (Alltech Associates, Ann Arbor, Mich.).

benzene through a Laskin nebulizer and by heating the saturated vapor to 27°C to prevent precipitation. The resultant vapor was diluted with filtered air to achieve the desired concentration in the exposure chamber. The chamber atmosphere was monitored every 30 min by a infrared gas analyzer[8] calibrated at 9.8 μm.

Exposures were conducted for 6 hr/day up to 5 days/week for the number of exposures required to yield a minimum concentration by time product (Ct) of 3000 ppm–days. Since each daily exposure lasted 6 hr, 3000 ppm–days is equivalent to 18,000 ppm–hr. The nominal exposure concentrations (with the actual concentration ± SD of the daily averages) for each of the four exposures were 1 exposure to 3000 (3122) ppm; 3 exposures to 1000 (1006 ± 3.9) ppm; 10 exposures to 300 (301 ± 2.8) ppm; and 30 exposures to 100 (100 ± 1.2) ppm.

Hematological parameters. Blood samples were taken from the tail vein of 10 test and 10 control mice within 2 hr after inhalation exposure. Each mouse was bled no more than once weekly to avoid artifacts due to repetitive bleeding. Total red and white blood cells were counted by a blood cell counter.[9] Peripheral blood smears were stained with Wright–Giemsa, and 100 white blood cells were counted on each slide to determine differential white cell counts. Individual absolute granulocyte and lymphocyte numbers were determined by multiplying the percentage of each type of white cell in the differential count for a particular mouse by the total white cell count for that mouse.

Behavioral parameters. Behavioral tests were always conducted immediately after removal from the inhalation chambers, and blood samples were always obtained after the behavioral tests. Behavioral measurements, which are nondestructive, were often determined from the same animal on consecutive days. For each concentration of benzene, a group of benzene-exposed mice was paired with a group of age-matched air-exposed mice; thus, each benzene group had its own control group given the same sequence of behavioral and hematological tests.

Mice were placed individually in black-lucite test cages (Teal and Evans, 1982), in which milk-licking and locomotor activity were measured for 15 min. By licking a stainless-steel spout protruding from one wall, mice could drink a solution of 10% sweetened milk.[10] Licking was recorded by a capacitance-operated touch detector,[11] connected to a PDP8 computer through a SKED interface.[12] Locomotor activity was recorded by similar touch detectors attached to two steel bars in the cage floor, located 6 and 12 cm from the drinking spout. Locomotor activity was thus measured by the frequency of contacts with these sensitive bars.

Immediately after the milk-licking test, hindlimb grip strength was measured by the method of Meyer *et al.* (1979) as adapted for the mouse (Teal and Evans, 1982). The mouse was held by the tail, and steadily pulled backward through a trough until both hind paws grasped a wire triangle. The experimenter continued to pull until the mouse's reflexive grip on the triangle was broken. Trials were recorded only if the mouse gripped with both feet; triplicate trials were averaged for each mouse each day.

Body weight, and food and water consumption were measured daily. All mice were weighed after benzene exposure and before testing. Water bottles and feeders were weighed after mice were removed from their home cages.

Experimental protocol. (100 ppm) Forty-five test mice inhaled 100 ppm benzene, and 45 control mice concurrently inhaled air for 30 exposures. Behavioral and hematologic parameters were determined after the first and last exposure of each week until significant hematological changes were observed. Thereafter, these parameters were determined once per week. *(300 ppm)* Mice inhaled either air (n = 30) or 300 ppm benzene (n = 30) for 10 exposures. During Week 1, behavioral and hematological tests were conducted after the second, third, and fifth exposures. During Week 2, behavioral tests were conducted after the seventh, eighth, ninth, and tenth exposures, but hematological parameters were determined only after the tenth exposure. *(1000 ppm)* Mice inhaled either air (n = 40) or 1000 ppm benzene (n = 40) for four exposures. Mice underwent behavioral tests after each of the exposures, and hematological assays after the first and third exposures. *(3000 ppm)* Mice inhaled air (n = 10) or 3000 ppm benzene (n = 30) for one 6-hr exposure. Behavioral and hematologic tests were performed after the exposure.

Statistics. Statistical significance was evaluated by analyses of covariance for single measures and for repeated measures using BMDP computer programs (Dixon *et al.*, 1981). Both test and control mice received sham exposures prior to the start of benzene, and these baseline data were used as the covariate. All significant F values are presented in Table 1. When treatment effects were significant ($p < 0.05$), pairs of control and benzene data points were further analyzed with a two-tailed Student's t test. Effects of each benzene concentration were analyzed separately using data from the specific group of control mice that had been exposed to air simultaneously with the benzene group.

RESULTS

The most consistent effect of benzene exposure was lymphocytopenia, i.e., a decrease

[8] MIRAN 1A, Foxboro Analytical, South Norwalk, Conn.

[9] Model ZB-1, Coulter Electronics, Hialeah, Fla.

[10] Eagle Brand, Borden Company, St. Louis, Mo.

[11] Sobco Corporation, Cambridge, Mass.

[12] State Systems, Kalamazoo, Mich.

TABLE 1

SIGNIFICANT F VALUES FROM ANALYSIS OF COVARIANCE[a]

	Benzene (ppm)						
	100		300		1000		3000
	B	DB	B	DB	B	DB	B
Lymphocytes	[a]8.09 (1, 15)	3.62 (5, 80)	47.34 (1, 17)	NS	[b]15.50 (1, 17)		34.74 (1, 17)
RBCs	17.17 (1, 15)	3.92 (5, 80)	15.72 (1, 17)	14.70 (1, 18)	[b]10.31 (1, 17)		NS
Milk-licking	NS	2.26 (11, 374)	14.86 (1, 16)	8.29 (1, 34)	NS	NS	NS
Grip strength	NM		NS	NS	16.85 (1, 20)	NS	14.03 (1, 27)
Food intake	NS	NS	NS	NS	6.97 (1, 15)	NS	NS

Note. NS, not significant; NM, not measured; B, benzene effect; DB, days × benzene interaction.

[a] Significant ($P < 0.05$) F values with df in parentheses below.

[b] Baseline vs Day 3—no repeated measure.

in the number of circulating lymphocytes (Fig. 1). The number of lymphocytes decreased to 50% or less of control within two exposures to benzene concentrations of 300 ppm or greater. Lymphocyte numbers decreased to 68% of control after the fifth exposure to 100 ppm benzene, and decreased further with additional exposures. None of the benzene concentrations could reduce the peripheral lymphocytes to less than 45% of control, suggesting that a maximal response of lymphocytes was attained. Lymphocyte counts remained depressed throughout each exposure series.

Circulating red blood cell (RBC) counts were not depressed as early as lymphocyte counts in response to benzene exposure (Fig. 1). The peripheral RBC count fell to 90% of control after the tenth exposure to 100 ppm benzene, and then to 85% near the end of the exposure. RBCs declined steadily with repeated exposure to 300 ppm benzene, reaching a minimum of 70% by the end of exposure period. RBCs were unaltered after one exposure to 3000 ppm, and were not consistently affected by 1000 ppm benzene.

Granulocyte counts were not consistently affected by benzene (data not shown). One exposure to 1000 and 3000 ppm benzene reduced the number of circulating granulocytes; however, the granulocyte count recovered after the third exposure to 1000 ppm. Granulocyte numbers were not consistently affected by exposure to 300 or 100 ppm. No atypical blood cell morphology was observed at any exposure.

Licking of sweetened milk was increased after exposure to 100 and 300 ppm benzene (Fig. 2 and Table 1). Licking was significantly increased by the end of the first week of exposure to 300 ppm, and continued throughout the second week. Licking increased during the first week of exposure to 100 ppm, and then gradually declined to control values in the succeeding weeks. No changes in licking were observed after the one exposure to 3000 ppm; however, a trend toward an increase in licking ($p = 0.056$)

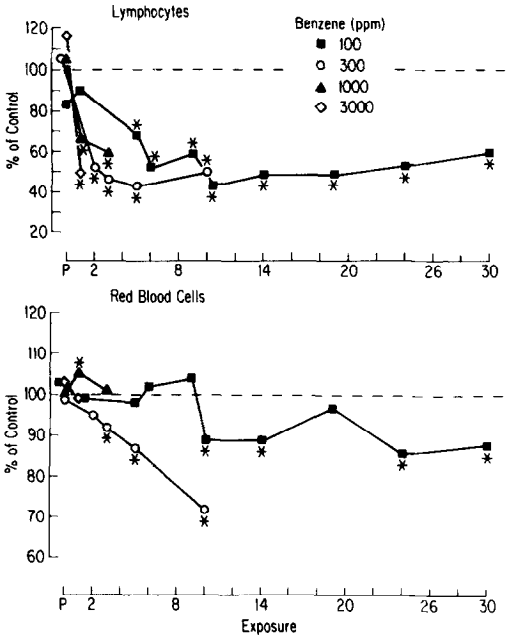

FIG. 1. Effect of benzene on peripheral blood lymphocytes (top panel) and on red blood cells (bottom panel). Note that the reduction in lymphocyte number reaches a plateau as the dose is increased to 300 ppm and as the duration of exposure is increased to 10 days. Anemia is seen as a decline in RBCs in response to 100 and 300 ppm benzene. Asterisks indicate a significant departure from values of air-exposed control mice. P = data collected prior to the first benzene exposure. x Axis indicates the number of 6-hr exposures which usually were conducted on a 5-day/week basis. Thus, the number of calendar days from the beginning of exposure is greater than the number of exposure days shown in the figure. A different group of air-exposed mice (N = 20) was tested with mice from each of the four benzene concentrations (N = 20). The average number of circulating lymphocytes for air-exposed mice was 7060/mm^3 blood. Average number of peripheral RBCs for the air-exposed mice was 10×10^6 cells/mm^3 blood.

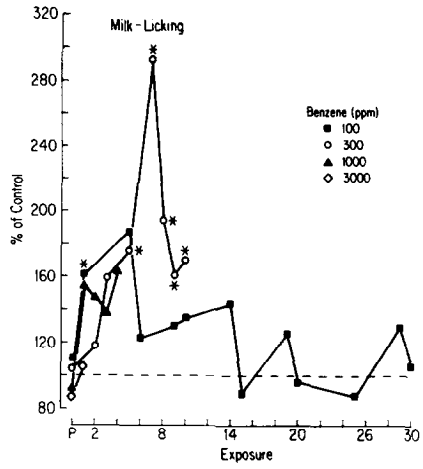

FIG. 2. Effect of benzene on licking of sweetened milk. Note the increase of licking at 100 and 300 ppm. Symbols are as in Fig. 1. The air-exposed control mice averaged 492 licks per 15-min session.

was observed during the three exposures to 1000 ppm.

Hindlimb grip strength decreased to 90% of control in mice exposed to either 1000 or 3000 ppm benzene (Fig. 3 and Table 1). No significant changes in grip strength were observed in animals exposed to 300 ppm benzene. Grip strength was not measured in mice exposed to 100 ppm. Other signs of CNS toxicity, such as body tremors, were observed in mice exposed to 3000 ppm. Mice exhibited tremors while in the exposure chamber, but these tremors subsided within 30 min after exposure had ended. Locomotor activity, measured simultaneously with milk-licking, was unaltered by benzene exposure.

Homecage food and water consumption were not severely affected by benzene. Food intake was only decreased after exposure to 1000 ppm benzene (Fig. 4). Homecage water intake was reduced after three exposures to

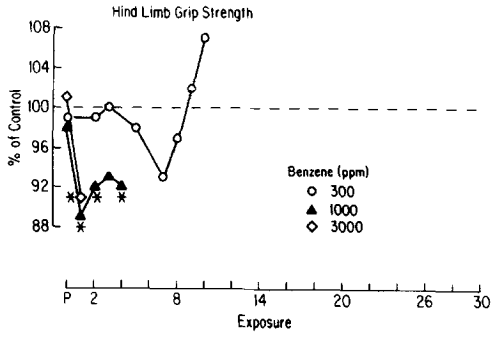

FIG. 3. Effect of benzene on hindlimb grip strength. Note the decrease in grip strength at 1000 and 3000 ppm. Data were not collected for mice exposed to 100 ppm. Symbols are as in Fig. 1. The average grip strength of air-exposed mice was 87 g.

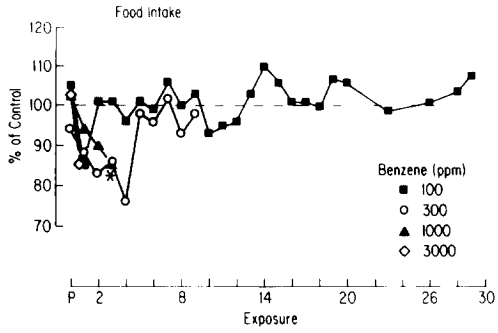

FIG. 4. Effect of benzene in homecage food intake. Note that the intake was significantly decreased only at 1000 ppm. Symbols are as in Fig. 1. Air-exposed control mice ate an average of 3.4 g of powdered food daily.

1000 ppm, but was unaffected by exposure to 100, 300, or 3000 ppm benzene. At the end of the first week of exposure to 300 ppm, body weight was decreased, but recovered in the second week of exposure. Body weight was decreased following all three exposures to 1000 ppm, but was not altered by exposure to 100 or 3000 ppm benzene.

To summarize concentration by time interaction, the data obtained after a Ct of 3000 ppm–days were plotted as a percentage of concurrent air-exposed controls for each of the four exposure regimens (Fig. 5). The four different exposure regimens produced a constant effect only in the decrease of lymphocytes. Neither the milk-licking, grip strength, nor RBC data exhibited a constant Ct relationship. Depression of RBC counts required long exposure duration, whereas reduction of grip strength was seen only at the highest benzene concentration. Licking of milk was maximally increased by the 10 exposures to 300 ppm, thus reflecting an inverted-U function.

To determine the reversibility of benzene effects, mice exposed to 100 and 300 ppm were retested 60 to 70 days following cessation of exposure to benzene. None of the behavioral or hematological parameters differed significantly from the pre-exposure baseline, indicating complete recovery from the effects described above.

DISCUSSION

Both behavioral and hematological changes provided early evidence of benzene toxicity following 1 or 2 days of exposure. Circulating lymphocytes were the most sensitive and consistent indicator (Fig. 1). The onset of lymphocytopenia was concentration dependent; it occurred after 1 to 5 days of exposure, depending on the benzene concentration. This onset is the earliest report of benzene-induced lymphocytopenia (compare with Green *et al.*, 1981a). Neither of the other two hematological parameters, circulating RBCs (Fig. 1) or granulocytes, appeared to be as sensitive as lymphocyte levels to benzene exposure.

Of the behavioral measures, increased milk-licking at 100 and 300 ppm benzene was the most sensitive (Fig. 2). Short-term exposures to higher concentrations did not increase licking. The milk-licking data reflect an inverted-U concentration–effect relationship, in which the maximal response occurred with 300 ppm. This finding is compatible with the increased ingestion of both food and bedding by mice immediately following benzene inhalation (Evans *et al.*, 1981). In the present experiment, enhanced consumption of milk

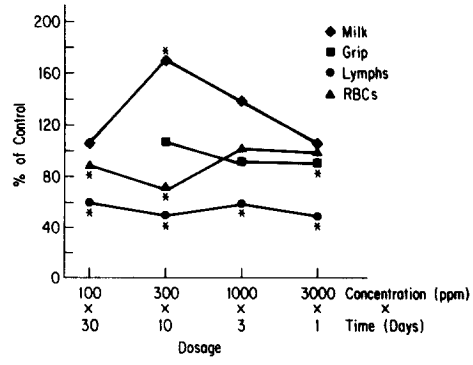

FIG. 5. Effect of a Ct of 3000 ppm–days benzene in each of the four exposure regimens on milk consumption, grip strength, peripheral lymphocytes, and red blood cells. Grip strength was not tested in mice exposed to 100 ppm. Note that only the lymphocyte data portray a constant Ct relationship. Asterisks indicate a significant departure from values of air-exposed control mice.

was not accompanied by a significant change in 24-hr food consumption in the home cage (Fig. 4), indicating that the enhanced milk-licking was not simply a matter of benzene-induced changes in hunger.

Inhalation of either *n*-hexane or toluene (Evans and Dempster, 1984) and injection of acrylamide (Teal and Evans, 1982) also caused enhanced milk-licking similar to that produced by benzene inhalation. Benzene and other toxicants may act as a stressful stimulus (*e.g.* Teal and Evans, 1982), functionally similar to the stimulus of a painful tail pinch which increases the rat's chewing of both food and nonfood substances (Rowland and Antelman, 1976). This increased consummatory behavior also may reflect altered sensation of taste or smell.

The earliest behavioral effects of benzene were the reduction in hindlimb grip strength, which occurred after the first 6-hr exposure to 1000 or 3000 ppm (Fig. 3), and the enhanced milk-licking following the first exposure to 100 ppm (Fig. 2). This report is the first quantification of a significant reduction in grip strength following benzene inhalation, and may indicate an immediate effect of benzene upon neuromuscular function. Hindlimb weakness has been described following exposure to *n*-hexane and other solvents (Spencer *et al.*, 1980) and to acrylamide, (Teal and Evans, 1982). Thus, reduced grip strength may provide early functional evidence of changes which eventually result in peripheral neuropathy of the type described by Spencer *et al.* (1980).

Motor activity was not affected by benzene, whether in a special test cage used in this experiment or in the homecage (Evans *et al.*, 1981). The reported decrease in wheel-running of mice following exposure to benzene (Horiuchi *et al.*, 1967) suggests that this more strenuous exercise may be more sensitive to benzene exposure than the other indices of activity.

Metabolites of benzene are thought to be responsible for its hematotoxic effect (Snyder *et al.*, 1967; Green *et al.*, 1981a), whereas benzene itself is thought to produce acute neurotoxicity (Bergman, 1979). The increased milk-licking may be caused by cytotoxic metabolites of benzene, since the maximal increase in licking at 300 ppm followed the same time course as the hematological changes. The immediate maximal decrease in grip strength after the first 6-hr exposure to 1000 and 3000 ppm (Fig. 3) may indicate either a direct effect or a rapid conversion to a sufficient concentration of metabolites.

Another facet of the present study was to examine whether there is an interaction of dose and duration of exposure in benzene-induced toxicity. However, an important constraint was the use of intermittent (6 hr/day) rather than continuous (24 hr/day) exposures to create the total duration of exposure. The intermittent exposure was selected as the better model of industrial exposure. All test mice were exposed to a Ct of 3000 ppm–days or more. Haber's Law predicts that, over a narrow range of concentrations, the Ct necessary to produce a specific response will be constant (Andersen, 1981). Our lymphocyte data exhibited a constant Ct relationship, but the other behavioral and hematological measures did not (Fig. 5). These latter end-points may require a "threshold" duration as well as a "threshold" concentration of benzene, as has been reported for methylmercury (Evans *et al.*, 1977).

In a capacity-limited metabolic system, such that may exist for benzene, a more appropriate expression for the Ct product would be duration of exposure $\times$ dose at target tissue or rate of metabolism rather than inspired concentration (Andersen, 1981). The present results conform with this interpretation as well as with other experiments in which continuous inhalation of benzene has been found to cause toxic signs not observed with intermittent exposures even when the same Ct is employed (Coffin *et al.*, 1977; Gardner *et al.*, 1977). Possibly greater toxicity and a more consistent Ct relationship might have been observed if continuous ex-

posure (24 hr/day, 7 days/wk) had been employed. Similarly, the hematoxicity of 100 ppm benzene for 5 days was not duplicated by intermittant exposure to 10 ppm for 50 days (Green *et al.*, 1981a). At lower doses, benzene may be detoxified before injuring target tissue. Data are not sufficient to evaluate Ct interactions in humans (Haley, 1977).

In summary, both hematological and behavioral changes occurred in temporal proximity with benzene concentrations as low as 100 ppm. The coincidence of behavioral and hematological changes suggest that common mechanisms may be involved, but additional work will be needed to document the specific mechanisms. The behavioral changes demonstrated here are more suitable for laboratory experiments than are the subjective indices of neurotoxicity in humans, e.g., euphoria, giddiness, headache, and nervous irritability. In the present study, benzene-induced milk-licking, tremors, and hindlimb weakness indicate that mice exhibit early neurobehavioral as well as hematological effects of benzene.

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