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# The effect of exposure regimen and duration on benzene-induced bone-marrow damage in mice

## I. Sex comparison in DBA/2 mice

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### Summary

In the mouse. the concurrent evaluation of micronuclei frequencies in peripheral blood polychromatic erythrocytes **(PCE)** and normochromatic erythrocytes **(NCE)** permits an assessment of both recently-induced and chronicallyaccumulated bone-marrow damage. This assay system was used to evaluate on a weekly basis the effect of exposure duration **(1–13** weeks. 6 h per day) and exposure regimen (Regimen 1 : 5 exposure days per week: Regimen **2** : **3** exposure days per week) on the ability of 300 ppm benzene to induce genotoxic damage in the bone marrow of male and female DBA/2 mice. In addition. an analysis of the percentage of **PCE** in peripheral blood **was** used to evaluate benzene-induced alterations in the rate of erythropoiesis. Exposure to benzene induced a marked increase in the frequency of micronucleated **PCE** (MN-PCE), an effect which was considerably greater in male mice than in female mice. In both sexes. the induction of MN-PCE was independent of exposure regiment and of exposure duration. Exposure to benzene also resulted in an exposure duration-dependent increase in the frequency of MN-NCE. The frequency **of** MN-NCE increased more slowly in female than in male mice and. within each sex. more slowly in Regimen **2** animals. Apparent steady-state conditions for MN-NCE frequencies were attained by about the fifth week of exposure in female mice exposed by either regimen and in male mice exposed by Regimen **2.** Steady-state conditions for **MN-NCE** frequencies in male mice exposed to benzene by Regimen 1 did not occur during the duration of the study. **An** analysis of **SPCE** data revealed an initial severe depression in the rate of erythropoiesis in both sexes. with the return in the production of PCE to control levels being dependent on both sex and exposure regimen. Suppression of **PCE** production occurred throughout the course of the study in Regimen 2 males. while the percentage of **PCE** returned to control levels sporadically after **5** weeks in Regimen 1 males and wittun 5 weeks in females. regardless of

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regimen. Thus. while the sex-dependent induction of genotoxic damage by multiple exposures to benzene over a 13-week period was independent of exposure regimen and duration. the induction of cytotoxic damage was both sex- and regimen-dependent. The most severe depression of erythropoiesis occurred in male DBA/2 mice exposed to benzene by the more intermittent regimen (i.e., 3 days/week versus 5 days/week). The results of this study underscores the complexity of the peripheral blood micronucleus assay and emphasizes the utility of evaluating micronucleated PCE and micronucleated NCE frequencies and the percentage of PCE in the same animals across time in multiple exposure situations.

Benzene is a common ingredient in gasoline and is used extensively in industry as a solvent and as an intermediate in the synthesis of a variety of chemical products (e.g., detergents. explosives. pharmaceuticals. dyestuffs) (Sittig, 1979: IARC. 1982). Benzene also presents many risks in regard to environmental contamination and human health. Numerous studies have been conducted which associate occupational exposure to benzene with a variety of adverse health effects. including an increased risk for leukemia and aplastic anemia (reviewed in IARC. 1982; Aksoy. 1985; Snyder. 1987). In animal studies. benzene has been shown to be carcinogenic (Maltoni and Scarnato. 1979; Snyder et al., 1980: Cronkite et al., 1984: NTP. 1985: Stoner et al.. 1986), and to induce genotoxic damage as measured by the induction of sister-chromatid exchanges (SCE), chromosomal aberrations and micronuclei (reviewed in IARC. 1982: Dean. 1969. 1985). Although there is no doubt that exposure to benzene is hazardous to human health. there is much debate as to what constitutes safe occupational and environmental exposure limits (White et al., 1980; Federal Registry, 1985: Dean. 1985). Consequently, information relating to whether different exposure situations. especially those involving inhalation (the primary route of exposure to humans), modulate the in vivo genotoxic and cytotoxic effects induced by benzene would be valuable.

Several studies have suggested that the levels of genotoxic damage induced in bone marrow (the principle target of benzene-induced genotoxic and cytotoxic damage) diminish with increasing exposure duration (Anderson and Richardson, 1981; Tice et al., 1984: Dean and Clare. 1985: Choy et al., 1985: Barale et al.. 1985). In addition. several investigators have concluded that. for the same duration of exposure. chronic exposure conditions were more harmful than intermittent ones to bone-marrow integrity and function (Coffin et al.. 1977: Gill et al.. 1980: Toft et al.. 1982). However. Irons (1983) demonstrated that intermittent treatments of mice with metabolites of benzene (hydroquinone and phenol) induced more bone-marrow damage (i.e., loss in cellularity) than chronic treatments at the same doses.

The mouse peripheral blood micronucleus (MN) assay enables an evaluation of both acute and chronic bone marrow damage in the same animal (MacGregor et al., 1980: Schlegel and MacGregor. 1982: Tice and Ivett. 1985). Since the lifetime of a newly formed polychromatic erythrocyte (PCE) in the mouse is about 24 h (Jenssen and Ramel. 1978: Cole et al.. 1981: Hayashi et al.. 1984). while that of a normochromatic erythrocyte (NCE) is around 30 days (Schalm et al., 1975; Schlegel and MacGregor. 1982). the presence of MN-PCE in the peripheral blood are indicative of damage that occurred in the bone marrow 1–2 days prior to sampling, while the frequency of MN-NCE indicates the accumulation of damage in this cell population throughout the course of the exposure period. Due to the lack of information on benzene-induced genotoxic damage under multiple exposure conditions. a study was designed using the peripheral blood MN assay to evaluate the effect of exposure regimen and duration on the ability of inhaled benzene to induce genotoxic damage in the bone marrow of male and female mice. An evaluation in the peripheral blood of the percentage of PCE among total erythrocytes was included to provide an assessment of the rate of erythropoiesis and thus a measure of bone marrow cytotoxicity (Heddle et al., 1983).

In conducting this study. it was decided to expose mice to 300 ppm benzene for 13 weeks, for either 5 exposure days per week or for 3 exposure

lays per week. This concentration of benzene was ısed because of published data demonstrating its ıbility to induce adverse health effects (e.g. eukopenia. anemia. cancer) (Snyder et al.. **1978.** .981; Green et al., **1981;** Cronkite et al., **1984)** and genotoxic damage (Tice et al.. **1982:** Cortina et al.. 984) in exposed mice. This concentration of ben:ene was also not expected to induce overt mortalty over the course of the study (Cronkite et al.. **1984).** Furthermore. **300** ppm benzene was beieved to be within the linear portion of the dose ange for the absorption of inhaled benzene in nice (Wells and Nerland. **1984).** DBA/2 mice were used to enable direct comparison with previous genotoxicity studies **on** benzene (Tice et al.. 1980. **1982).** An exposure duration of **13** weeks was used to provide data over approximately **2-3** NCE lifetimes (Schalm et al., **1975:** Schlegel and MacGregor. **1982).** The selection of the regimens was based on studies by Irons **(1983),** in which more cytotoxic damage occurred when mice were treated with various metabolites of benzene on **3** days per week as compared to daily treatments. Finally, because the genotoxlc and carcinogenic activity of benzene is highly sex-dependent (reviewed by Dean, 1985). both male and female mice were included in the study.

## Materials and methods

***Chemicals.*** Benzene (CAS No. **71-43-2).** "reagent grade". "thiophene free", was obtained from Mallinckrodt Chemical Co. (Paris. KY), while acridine orange (AO) was obtained from Sigma Chemical **Co.** (St. Louis, **MO).**

***Animals.*** Male and female DBA/2 mice were obtained from Jackson Laboratories (Bar Harbor. ME) at **9** weeks of age. After receipt. the mice were acclimated for a period of **2** weeks in standard laboratory stainless steel cages with corn cob bedding (Bed **O Cobs,** J.R. Nielson and Sons. Inc.). The population density was kept at no greater than one animal per 90 $cm^2$, under controlled laboratory conditions [i.e., **22** & *2°C.* 50 k 15% relative humidity, **20** air exchanges per hour and a **12-h** photoperiod (light: 0700–1900)]. Food (Purina Lab Rodent Chow 5001) and water were provided ad libitum. The initial weight range at the onset of exposure was: male. 26–30 g; female. **20–22** g.

***Exposure protocol.*** Groups of male and female DBA/2 mice ($n = 6$) were exposed to either 300 ppm benzene or to ambient air for a period of 13 weeks. using one of two exposure regimens (Regimen 1: live consecutive days exposed. two consecutive days unexposed: Regimen 2: three consecutive days exposed. four consecutive days unexposed). Regimen 1 animals were exposed Mondays through Fridays and Regimen **2** animals were exposed Tuesdays through Thursdays. Exposure was for 6 h a day. generally from 0900 to 1500 hours. The mice were housed in stainless steel mesh cage packs measuring 44 cm x 51 cm that were divided into 8 equal cubicles (2 rows of **4** each). **3** mice of the same sex were housed in each cubicle. Each cage pack housed all animals of a particular exposure group. Animals were housed continuously in these cage packs for the **13** weeks of exposure. with clean cages provided on a weekly basis. Temperature. humidity. air exchange. lighting, food and water were maintained as was described for their acclimation period. except during the actual hours of exposure. Coded toe clipping enabled identification of individual animals throughout the experiment.

Exposure to benzene was carried out in stainless steel and lucite chambers. Filtered compressed air was bubbled through liquid benzene and diluted to produce the desired concentration of benzene vapor. Benzene concentration was measured every **30** min by **a** gas chromatograph (Packard. model **417,** equipped with **a** column of 10% silicone **SE-30** on chromosorb W-HP). with **an** automatic sampling valve. Temperature in the chambers ranged between 21 and **25°C,** with the benzene chamber averaging about 1"*C* higher than the control chamber. Relative humidity **was** maintained at 50 ± 15% with ~ 15 **air** exchanges per hour. Food was not provided during the 6-h exposure period. although water was provided ad libitum. Control animals were treated in a manner identical to the exposed animals. excluding the presence of benzene. The time weighted average (TWA) concentration of benzene for Regimen 1

and Regmen 2 was 299.8 ppm (63 exposure days) and 300.4 (39 exposure days), respectively.

***Sampling, slide preparation, staining and analysis.*** Peripheral blood smears were made from all animals prior to the initiation of the first exposure and weekly thereafter. Both regimen groups were sampled on the morning after the third day of each weekly exposure period (i.e., Regmen 1 on Thursdays. Regimen 2 on Fridays), to allow for equal expression of damage in the peripheral blood. The smears (two per mouse) were prepared using blood obtained by snipping off approximately 1 mm from the end of the tail. Slides were fixed in absolute methanol for about 10 min, air dried. and stored in slide boxes until stained for analysis. Slides were stained for 8 min with AO (0.02 mg/ml in pH 7.3 phosphate buffer) following the protocol of Kato et al. (1974) for SCE analysis. and rinsed in buffer for 10 min. Coverslips were floated onto the wet slides, and blotted to remove excess buffer. The slides were analyzed at 800 x magnification using epi-illuminated fluorescence microscopy (450–490 nm excitation. 520-nm emission) for the number of micronucleated cells per 1000 NCE. the number of micronucleated cells per 1000 PCE. and the number of PCE per 1000 erythrocytes. Although PCE with as many as six MN were observed. all data are presented as the number of micronucleated cells. not as the number of micronuclei. Scoring was restricted to areas of the slide with no overlapping cells. In some instances. PCE formation in the bone marrow of benzene exposed mice was suppressed to such an extent that scoring 1000 PCE was impossible. In some cases, 500 PCE were counted for the presence of micronuclei. and the number adjusted to equal the frequency of micronucleated PCE per 1000.

***Statistical analysis.*** Temporal averages for MN-PCE, MN-NCE and the percentage of PCE were calculated for each animal by summing the values across time (except for the MN-NCE data. week 0 was omitted from the exposed mouse calculations) and dividing by the number of samples evaluated. Animals that died prior to the 12th week of exposure were omitted from this analysis. In cases were it was impossible to score MN-NCE or the percentage of PCE and the statistical analysis required a complete data set, the missing data was estimated by averaging the two nearest data points for that animal. Using temporal averages. a two-way Brown–Forsythe analysis of variance (ANOVA), based on separate group variances (BMDP, 1985). with dose and regimen as factors was conducted for each sex. Where a significant sex by dose interaction term was found (at an alpha level of 0.05). group mean data were compared using Student's *t* test based on separate variances after the alpha level had been Bonferroni corrected for the appropriate number of multiple comparisons. To evaluate sex differences. a two-way Brown–Forsythe ANOVA with sex and dose as factors, was conducted for each endpoint defined by regimen. unless differences between regimens were found to be nonsignificant, in which case the appropriate regmen data were pooled. In the event of a significant sex by dose interaction term, group mean data were compared using Student's t test based on separate variances after the alpha level had been Bonferroni corrected for the appropriate number of multiple comparisons. Student's *t* tests were also used to determine at which sample times the percentage of peripheral blood PCE in the benzene-exposed mice were significantly different from control values. To evaluate for an exposure duration-dependent alteration in the induction of genotoxic damage in the bone marrow of benzene-exposed mice. weekly frequencies of MN-PCE in each animal were analyzed by multiple regression analysis. In this analysis. MN-PCE data were evaluated for a nonsignificant trend. a significant positive trend, or a sigmficant negative trend across time. As with the calculation of temporal averages, pre-exposure MN-PCE data were omitted from each analysis of benzene-exposed mouse data.

## Results

### *Frequency of MN-PCE*

***Males.*** There was a dramatic increase (between 50- and 100-fold) in the number of MN-PCE observed in the peripheral blood of benzene-exposed male mice sampled at the end of the first

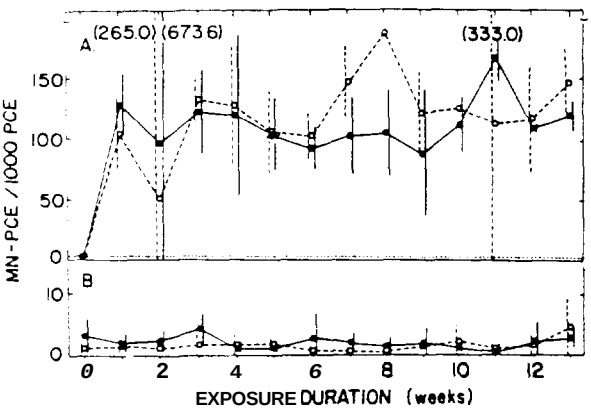


Fig. 1 Regimen companson of the group mean peripheral blood MN-PCE frequency across time for male DBA/2 mice. (A) Data on benzene-exposed mice. (B) Data on control mice. ●, for Regimen 1: ○, for Regimen 2. Error bars indicate 95% confidence limits. The dashed line in Panel A indicates the upper 95% confidence limit for MN-PCE frequency in control mice. The values in parenthesis indicate the upper bound of the 95% confidence limits for that weekly mean frequency. Note the difference in scale for the Y axis between Panels A and B. See Appendix 1A for individual and group mean data.

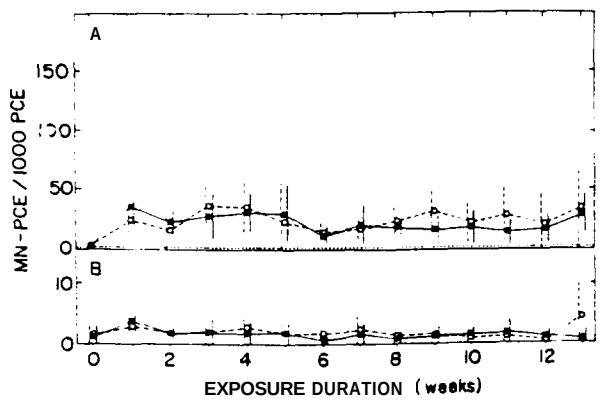


Fig. 2. Regimen comparison of the group mean penpheral blood MN-PCE frequency across time for female DBA/2 mice. (A) Data on benzene-exposed mice. (B) Data on control mice. ●, for Regimen I: ○, for Regmen 2. Error bars indicate 95% confidence limits. The dashed line in Panel A indicates the upper 95% confidence limit for MN-PCE frequency in control mice. Note the difference in scale for the Y axis between Panels A and B. See Appendix 1B for individual and group mean data.

week of exposure (Fig. 1; see Appendix 1A for individual animal data and weekly group means). A two-way ANOVA of temporal averages with regmen and dose as factors revealed a significant effect due to exposure to benzene without a significant regimen difference (Table 1). Regression analyses to evaluate for an exposure duration-dependent difference in MN-PCE levels among the control or exposed animals revealed one control male in Regmen 2 with a significant negative slope and one exposed male in Regimen 1 with a significant positive slope (Appendix 14). The lack of a significant slope (negative or positive) among the majority of the exposed mice indicates that the frequency of benzene-induced MN-PCE remained relatively constant throughout the 13-week exposure period.

*Females.* Both regimens produced a significant increase in MN-PCE frequency, with the MN-PCE levels increasing approximately 15-fold above control levels (Fig. 2; see Appendix 1B for individual animal data and weekly group means). A two-way ANOVA comparison of temporal aver-

TABLE 1

TWO-WAY ANOVA OF MN-PCE TEMPORAL AVERAGES FOR MALE MICE. WITH REGIMEN AND DOSE AS FACTORS

| Regmen | Opprn | N | 300 ppm | N |
|---|---|---|---|---|
| 1 | 2.05f0.131 | 6 | 107.85 ± 5.529 | 6 |
| 2 | 1.53 ± 0.150 | 6 | 121.23 ± 1.700 | 6 |
| Brown–Forsythe ANOVA | | | P values | |
| Regmen | | | 0.0768 | |
| Dose | | | 0.0001 * | |
| Interaction | | | 0.0615 | |

Mean number of MN-PCE per 1000 PCE ± the standard error of the mean among N animals.

* Significant at $\alpha = 0.05$.

TABLE 2

TWO-WAY ANOVA OF MN-PCE TEMPORAL AVERAGES FOR FEMALE MICE. WITH REGIMEN AND DOSE AS FACTORS

| Regimen | Oppm | N | 300 ppm | N |
|---|---|---|---|---|
| 1 | 1.5520.211 | 6 | 20.93 ± 3.173 | 6 |
| 2 | 1.90 ± 0.188 | 6 | 24.40 ± 4.454 | 5 |
| Brown–Forsythe ANOVA | | | P values | |
| Regmen | | | 05450 | |
| Dose | | | 0.0001 * | |
| Interaction | | | 0.6286 | |

Mean number of MN-PCE per 1000 PCE ± the standard error of the mean among N animals.

* Significant at $\alpha = 0.05$.

TABLE 3

TWO-WAY ANOVA OF MN-PCE TEMPORAL AVERAGES FOR DBA/2 MICE. WITH SEX AND DOSE AS FACTORS

| Sex | 0 ppm | N | 300 ppm | N |
|---|---|---|---|---|
| Male | 1.79 ± 0.123 | 12 | 114.54 ± 3.417 | 12 |
| Female | 1.72 ± 0.145 | 12 | 22.51 ± 2.579 | 11 |
| **Brown–Forsythe ANOVA** | | | ***P* values** | |
| Sex | | | 0.0001 * | |
| Dose | | | 0.0001 * | |
| Interaction | | | 0.0001 * | |

Mean number of MN-PCE per 1000 PCE ± the standard error of the mean among N animals
* Significant at $\alpha = 0.05$

ages (Table 2) indicated a significant benzene effect which was independent of the regimen used. A regression analysis of individual female MN-PCE frequencies against exposure duration revealed the absence of a significant slope among all control and exposed animals (Appendix 1B).

*Males vs. females.* A visual comparison of Figs. 1 and 2 illustrates the sex-dependent difference in the frequency of MN-PCE in the peripheral blood of male and female mice exposure to benzene. Since regimen differences in the temporal averages of MN-PCE were not significant within each sex. MN-PCE data were pooled between regimens before analyzing by two-way ANOVA for sex differences in the frequency of MN-PCE in unexposed and exposed mice (Table 3). Because of the significant sex by dose-interaction term. pairwise comparisons were conducted using Student's *t* test. with the alpha level Bonferroni corrected for 4 pairwise comparisons, to evaluate for sex differences among control and exposed groups. A significant difference in MN-PCE levels between male and female control mice was not present ($P = 0.7287$). However, in the benzene-exposed groups, male mice exhibited about a 5-fold greater level of damage than female mice ($P = 0.0001$).

### *Frequency of MN-NCE*

*Males.* There was an exposure duration-dependent increase in the frequency of MN-NCE in the male mice exposed to benzene (Fig. 3; see

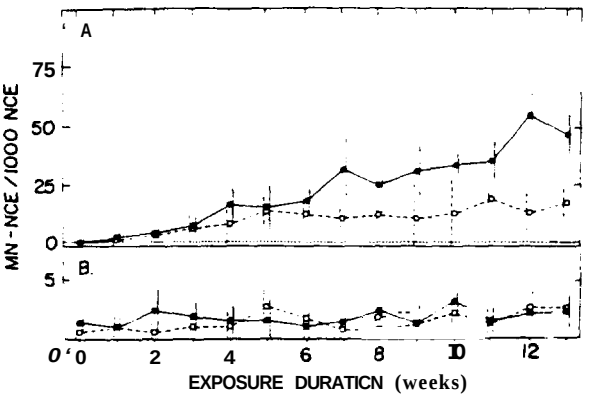


**Fig. 3. Regiment comparison of the group mean penpheral blood MN-NCE frequency across time for male DBA/2 mice. (A) Data on benzene-exposed mice. (B) Data on control mice. ●, for Regimen 1: ○, for Regmen 2. Error bars indicate 95% confidence limits. The dashed line in Panel A indicates the upper 95% confidence limit for MN-NCE frequencv in control rmce. Note the difference in scale for the Y axis between Panels A and B. See Appendix 2A for individual and group mean data.**

Appendix 2A for individual animal data and weekly group means). At about week 7, a significant difference between regimens in the accumulation of MN-NCE in exposed mice is apparent. with the Regimen 1 animals exhibiting the greater frequency of MN-NCE (week 13 group average MN-NCE levels being 47 and 17 MN-NCE/1000 NCE for Regimen 1 and Regimen 2 mice. respectively). Examination of the results obtained from a two-way ANOVA of temporal averages (Table 4) with benzene exposure and regimen as factors revealed a highly significant interaction

**TABLE 4**

**TWO-WAY ANOVA OF MN-NCE TEMPORAL AVERAGES FOR MALE MICE. WITH REGIMEN AND DOSE AS FACTORS**

| Regimen | Oppm | N | 300 ppm | N |
|---|---|---|---|---|
| 1 | 1.70 ± 0.157 | 6 | 23.47 ± 1.040 | 6 |
| 2 | 1.35 ± 0.034 | 6 | 10.55 ± 0.633 | 6 |
| **Brown–Forsythe ANOVA** | | | ***P* values** | |
| Regimen | | | 0.0001 * | |
| Dose | | | 0.0001 * | |
| Interaction | | | 0.0001 * | |

**Mean number of MN-NCE per 1000 NCE ± the standard error of the mean among N animals.**
*** Significant at $\alpha = 0.05$.**

term ( $P = 0.0001$). Conducting pairwise comparisons using Student's $t$ test, with the alpha level Bonferroni corrected for **4** pairwise comparisons. indicated that while there was not a significant difference between regimens in control data ( $P = 0.0771$). a significant difference due to benzene within a regmen ( $P < 0.0001$) and for exposed groups between regimens ( $P < 0.0001$) existed. Also. while there was no evidence in male mice exposed by Regimen 1 of a plateauing over time in the frequency of MN-NCE, steady-state conditions in the level of peripheral blood MN-NCE appears to have occurred in the Regmen 2 mice by about the iourth week of exposure (see Fig. 3).

*Females.* The kinetics of the exposure duration-dependent increase in the frequency of MN-NCE in the penpheral blood of female mice was similar to that observed in males. but not nearly as dramatic (Fig. 4). Although there was not a great difference in MN-NCE frequency between regimens in the exposed animals at any one sampling time. the animals exposed by Regmen 1 (i.e., for 5 days a week) consistently exhibited higher frequencies of MN-NCE than mice exposed by Regimen 2 (i.e., for 3 days a week). **An** analysis of MN-NCE temporal averages supported this inference (Table 5), revealing both a significant regimen and a significant benzene effect. An evaluation of pairwise differences among the MN-NCE data using Student's $t$ test. with the alpha level Bonferroni corrected for **4** pairwise comparisons. indicated the lack of a significant regimen difference between MN-NCE frequencies in control mice ( $P = 0.3558$). Among exposed female mice. a saturation of MN-NCE levels appears to have occurred in both exposure regimens by about the fifth week of exposure.

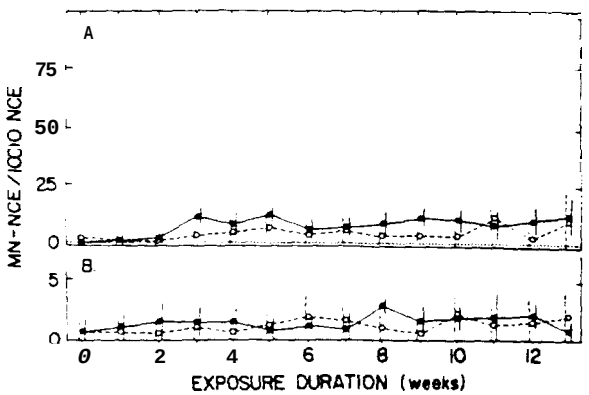


Fig. 4. Regimen comparison of the group mean peripheral blood MN-NCE frequency across time for female DBA/2 mice. (A) Data on benzene-exposed mice. (B) Data on control mice. **a.** for Regimen 1: ○, lor Regimen 2. Error bars indicate 95% confidence **limits.** The dashed line in Panel **A** indicates the upper 95% confidence limit for MN-NCE frequency in control mice. Note the difference in scale for the Y **axis** between Panels A and B. See Appendix 2B for individual and group mean data.

TABLE 5

TWO-WAY ANOVA **OF** MN-NCE TEMPORAL AVERAGES FOR FEMALE **MICE. WITH** REGIMEN **AND** DOSE **AS** FACTORS

| Regmen | Oppm | $N$ | 300 ppm | $N$ |
|---|---|---|---|---|
| 1 | 1.35 ± 0.102 | 6 | 7.72 ± 0.995 | 6 |
| 2 | 1.15 ± 0.177 | 6 | 4.98 ± 0.497 | 5 |
| Brown–Forsythe ANOVA | | | $P$ values | |
| Regimen | | | **0.0325** • | |
| Dose | | | 0.0001 • | |
| Interaction | | | **0.0548** | |

Mean number of MN-NCE per 1000 NCE ± the standard error of the mean among $N$ animals.
• Significant at $\alpha = 0.05$.

***Males vs. females.*** **A** visual comparison of Figs. 3 and **4** illustrates the sex-dependent difference in the peripheral blood levels of MN-NCE in response to the exposure to benzene. The frequency of MN-NCE in females exposed to benzene for 5 days a week (Regmen 1) was much less than that which occurred in similarly-exposed males. The difference in MN-NCE frequency between male and female mice was not **as** striking for the animals exposed for **3** days a week (Regimen 2). However, the peripheral blood MN-NCE frequencies in females were consistently lower than **MN-NCE** levels in males. **A** two-way **ANOVA** of temporal averages for each regimen **using** pooled control animal data. **with** sex and exposure as factors, revealed a significant sex by exposure interaction term (Table **6).** Temporal averages of MN-NCE frequencies in control mice pooled between regimens were not significantly different between the sexes ( $P = 0.8289$) while, for both regmens. the MN-NCE temporal averages among exposed mice

TABLE 6

TWO-WAY ANOVA OF MN-NCE TEMPORAL AVERAGES FOR DBA/2 MICE. WITH **SEX AND** DOSE AS FACTORS

***(A) Regimen*** I

| Sex | 0 ppm | *N* | 300 ppm | *N* |
|---|---|---|---|---|
| Male | 1.52 ± 0.093 | 12 | 23.47 ± 1.040 | 6 |
| Female | 1.25 ± 0.102 | 12 | 7.72 ± 0.995 | 6 |
| Brown–Forsythe ANOVA | | | *P* values | |
| Sex | | | 0.0001 * | |
| Dose | | | 0.0001 * | |
| Interaction | | | 0.0001 * | |

Mean number of MN-NCE per 1000 NCE ± the standard error of the mean among *N* animals. Control data are pooled due to lack of regmen difference.
* Significant at $\alpha = 0.05$.

***(E)*** **Regimen** *2*

| Sex | 0 ppm | *N* | 300 ppm | *N* |
|---|---|---|---|---|
| Male | 1.52 ± 0.093 | 12 | 10.55 ± 0.633 | 6 |
| Female | 1.25 ± 0.102 | 12 | 4.98 ± 0.497 | 5 |
| Brown–Forsythe ANOVA | | | *P* values | |
| Sex | | | 0.0001 * | |
| Dose | | | 0.0001 * | |
| Interaction | | | 0.0001 * | |

Mean number of **MN-NCE** per 1000 NCE ± the standard error of the mean among *N* animals.
* Sigruficant at $\alpha = 0.05$.

were significantly higher in male mice ($P < 0.0001$).

### *PCE frequency*

***Males.*** Exposure to 300 ppm benzene resulted in a marked depression of PCE levels in the peripheral blood of male mice (Fig. **5B:** see Appendix 3A for individual animal data and weekly group means), with the magnitude and the duration of the depression being strikingly dependent upon the exposure regmen. In both regmens, the frequency of PCE in the exposed male mice declined from ~ 3.0% to almost 0% by the completion of the first week of exposure. The percentage of PCE in male mice exposed to benzene by Regimen 2 remained significantly depressed below control levels throughout the duration of the study, while the percentage of PCE in the peripheral blood of Regimen 1 exposed males returned to control levels at week 6 and exceeded control levels at week 9 after the start of the study. A two-way ANOVA of temporal averages using dose and regmen as factors resulted in a significant dose by treatment interaction term (Table 7). From a pairwise comparison analysis, based on Student's *t* test with the alpha level Bonferroni corrected for 4 pairwise comparisons. a significant benzene effect occurred in both regimens ($P < 0.0002$). with the depression in Regimen 2 being significantly greater than that in Regimen 1 ($P = 0.0016$). Although the group SPCE temporal averages in the peripheral blood of the control mice were not significantly different between regimens ($P = 0.8223$) and remained relatively constant throughout the course of the study. there was a surprising amount of intersample variability among individual mice in the percentage of PCE in their peripheral blood. This was particularly evident near the final weeks of the study. This within animal variability was even more pronounced among the ben-

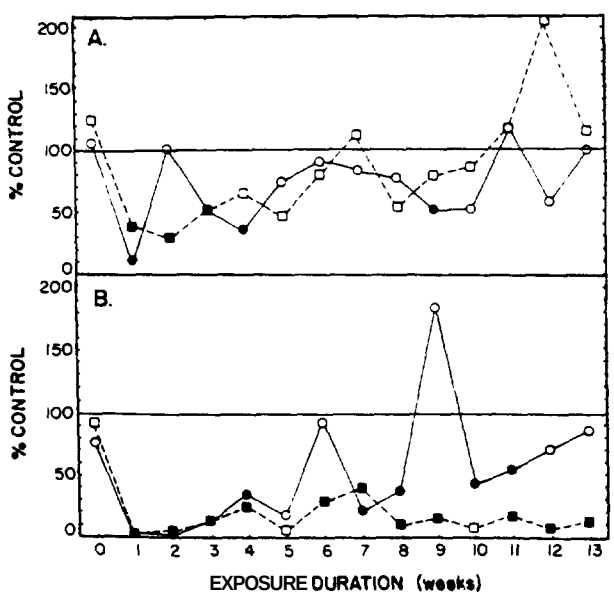


Fig. 5. Group mean frequency of peripheral blood PCE in DBA/2 mice across time. presented as the percentage of the corresponding control group data. **(A)** Female mice. (B) Male mice. ○, for Regimen 1; □, for Regmen 2. Solid symbols indicate statistically significant differences between control and benzene-exposed mice as determined by Student's *t* test at $\alpha = 0.05$. See Appendix 3A for individual and group mean data.

TABLE 7

TWO-WAY ANOVA OF %PCE TEMPORAL AVERAGES FOR MALE MICE. WITH REGIMEN AND DOSE AS FACTORS

| Regimen | Oppm | N | 300 pm | N |
|---|---|---|---|---|
| 1 | 3.49 ± 0.152 | 6 | 1.48 ± 0.264 | 6 |
| | 3.56f0.236 | 6 | 0.48 ± 0.072 | 6 |
| Brown-Forsythe ANOVA | | | *P* values | |
| Regimen | | | 0.0324 * | |
| Dose | | | 0.0001 * | |
| Interaction | | | 0.0173 * | |

Mean percentage of PCE per 1000 erythrocytes ± the standard error of the mean among N animals.
* Significant at $\alpha = 0.05$.

lene-exposed rmce. with the percentage of PCE varying as much as 20-fold (from 0.6% to 11.6%) within a single animal from one week to the next (Appendix **3A).**

*Females.* Although the percentage of PCE in the penpheral blood of exposed female mice were significantly depressed by the completion of the first week of exposure to benzene, they returned to control levels within the next few weeks (Fig. **5A:** see Appendix 3B for individual animal data and weekly group means). **A** two-way **ANOVA** of the temporal averages with exposure **and** regimen as factors indicated that while there was a significant benzene effect. there was not a significant difference between regimens (Table 8). The same within animal variability in the percentage of PCE in the peripheral blood that was observed in the male mice during the study was also observed in the female mice (Appendix 3[redacted]

TABLE 8

TWO-WAY ANOVA OF %PCE TEMPORAL AVERAGES FOR FEMALE MICE. WITH REGIMEN AND DOSE AS FACTORS

| Regmen | Oppm | *N* | 300 ppm | *N* |
|---|---|---|---|---|
| 1 | 3.51 ± 0.129 | 6 | 2.40 ± 0.316 | 6 |
| 2 | 3.83 ± 0.178 | 6 | 3.09 ± 0.520 | 5 |
| Brown–Forsythe ANOVA | | | *P* values | |
| Regimen | | | 0.1523 | |
| Dose | | | 0.0201 * | |
| Interaction | | | 0.5733 | |

Mean percentage of PCE per 1000 erythrocytes ± the standard error of the mean among N animals.
• Significant at $a = 0.05$.

***Males*** cs. *females.* ,Although animals of both sexes experienced a marked suppression of PCE production by the end **of** the first week of exposure, the effect was more pronounced and was much more persistent in male **mice** (Fig. 5). A two-way **ANOVA** of temporal averages for each regimen with sex and exposure as factors (with control data pooled between regimens) resulted in both a significant sex and and a significant dose effect for Regimen 1 (Table **9A).** and a significant interaction term for Regimen 2 (Table 9B). Pairwise comparisons of the various groups revealed no difference between the percentage of PCE in

**TABLE** *9*

**TWO-WAY ANOVA** OF %PCE **TEMPORAL AVERAGES FOR DBA/2 .MICE.** WITH **SEX AND DOSE AS FACTORS**

***(A) Regimen*** *1*

| Sex | 0 ppm | *N* | 300 ppm | *N* |
|---|---|---|---|---|
| Male | 3.53 ± 0.134 | 12 | 1.48 ± 0.264 | 6 |
| Female | 3.67 ± 0.115 | 12 | 2.40 ± 0.316 | 6 |
| Brown–Forsvthe **ANOVA** | | | ***P*** values | |
| Sex | | | 0.033' • | |
| Dose | | | 0.0001 • | |
| Interaction | | | 0.1167 | |

Mean percentage of **PCE** per 1000 erythrocytes ± the standard error of the mean among *N* animals. Control data are pooled due to lack **of** regmen difference.
• Significant at $\alpha = 0.05$.

***(B) Regimen 2***

| Sex | 0 ppm | *N* | 300 ppm | *N* |
|---|---|---|---|---|
| Male | 3.53 ± 0.134 | 12 | 0.48 ± 0.072 | 6 |
| Female | 3.67 ± 0.115 | 12 | 3.09 ± 0.520 | 5 |
| Brown–Forsythe **ANOVA** | | | ***P*** values | |
| Sex | | | 0.0040 * | |
| Dose | | | 0.0011 • | |
| Interaction | | | 0.0067 • | |

Mean percentage of **PCE** per 1000 erythrocytes ± the standard error of the mean among *N* animals. Control data are pooled due to lack of regimen difference.
• Significant at $a = 0.05$.

the peripheral blood of male and female control mice ($P = 0.4292$). and greater suppression of erythropoiesis in exposed male mice than in exposed female mice in Regimen 2 ($P = 0.0072$).

## Discussion

Interpretation of peripheral blood MN-NCE data requires the integration of the results of analysis on MN-PCE frequencies with an understanding of the kinetics of the hematopoietic system. The frequency of MN-NCE is a Function of: (i) the daily production of micronucleated cells in the differentiating cell population which leads to the formation of MN-PCE. (ii) the daily production of PCE. (iii) the transit time between enucleation and identification as an NCE. and (iv) the lifespan of both the normal and micronucleated NCE under different exposure conditions (see Hayashi et al.. 1984). Theoretically. under multiple exposure conditions. MN-NCE frequencies should attain steady-state conditions after the duration of the exposure has exceeded the average NCE lifetime. In the mouse. damaged erythroid precursors in the bone marrow take about 6–12 h to give rise to MN-PCE. and approximately one day to be detected in the peripheral blood as MN-NCE (Jensen and Ramel, 1978; Cole et al., 1981; Salamone and Heddle, 1983; Hart and Hartley-Asp, 1983: Hayashi et al.. 1984). The lifetime of NCE in the mouse is ~30 days under normal conditions (Schlegel and MacGregor, 1982). Therefore. in following an extended exposure protocol. steady-state conditions for MN-NCE frequencies should be attained by about 5 weeks after the first exposure to benzene. Barale et al. (1985) observed in Swiss CD-1 mice steady-state conditions for peripheral blood MN-NCE frequencies after about 4 weeks of exposure to benzene by gavage in male mice administered low doses and in female mice administered low or high doses. but not in male mice administered benzene at a high dose. Barale and his colleagues suggested that the lack of steady-state conditions and the apparently diminished MN-NCE response at extended exposure times in male mice administered high doses of benzene by gavage could be due to the selection of resistant cells, an increased detoxification of benzene. and/or a decreased ability of the animals to metabolize benzene. Similarly, Tice et al. (1984) and Choy et al. (1985) failed to observe steady-state conditions for MN-NCE frequencies in the peripheral blood of C57B1/6 and B6C3F1 mice exposed to benzene for 16 weeks by inhalation or for 2 years by gavage, respectively. In all cases. a diminished level of MN-NCE as a function of exposure duration appeared to support an interpretation that the level of genotoxic damage induced by benzene diminished with increasing exposure duration. In agreement with this interpretation. metabolic studies on benzene have demonstrated an increased rate of benzene clearance/ metabolism in rodents under repeated exposure conditions (Snyder et al.. 1967: Gonasun et al.. 1973; Snvder et al.. 1981: Post and Snvder. 1983: Driscoll and Snyder. 1984: Pathiratne et al.. 1986).

In the present study, apparent steady-state conditions for MN-NCE levels were attained after about 5 weeks in female mice exposed to benzene by both regimens and in male mice exposed to benzene for 3 days per week (Figs. 3 and 4). However, in male mice exposed to benzene 5 days per week. the frequency of MN-NCE continued to increase over the duration of the study. As opposed to the other peripheral blood MN studies discussed earlier. this temporal pattern of increasing frequencies of MN-NCE in male DBA/2 mice exposed to 300 ppm benzene for 5 days per week suggests that the induction of genotoxic damage by benzene increases with increasing exposure duration. By examining the effect of exposure duration on benzene-induced MN-PCE frequencies. this question of an exposure duration-dependent alteration in the induction of genotoxic damage in bone marrow can be examined more critically.

Inhalation of benzene produced a marked increase in the frequency of MN-PCE in the peripheral blood of exposed mice. particularly in males. The level of damage was independent of the exposure regimen and of the exposure duration. The constant frequency of MN-PCE in the peripheral blood of these mice over a 13-week period, regardless of the kinetics of MN-NCE accumulation. strongly suggests that modulation of sensitivity and/or metabolism did not occur or. at least, did not affect the induction of micronucleated

erythrocytes by benzene and/or its metabolites. This lack of an exposure duration-dependent alteration in benzene-induced levels of genotoxic damage in the bone marrow of DBA/2 mice appears to conflict with the chromosomal aberration data of Anderson and Richardson **(1981)** and of Dean and Clare **(1985).** and the peripheral blood micronucleated erythrocyte data of Tice et al. (1984), Barale et al. **(1985).** and Choy et al. **(1985).** However. the chromosomal aberration studies were conducted over an exposure period of less than a week in rats. suggesting the possibility for species and/or endpoint differences in response. The previous peripheral blood micronucleated erythrocyte studies conducted in mice were limited to an analysis of NCE populations only. involved different strains of mice and. in two studies. a different route of exposure. Either the effect is mouse strain/ route of exposure-specific or benzene-induced. exposure duration-dependent differences in the rate of erythropoiesis may be responsible for the apparent time-dependent differences in **MN-NCE** levels observed in these peripheral blood studies. In this context. it should also be noted that the age of the animal can have a profound effect on the induction of genotoxic damage induced by single acute exposures to benzene (Tice et al., **1982).** suggesting that age-related differences in sensitivity and/or metabolism may confound the interpretation of exposure duration-related data.

The suppression of PCE production by exposure to benzene comes as no surprise in view of the large number of studies demonstrating the toxic effects of benzene on the hematopoietic system (reviewed in **IARC. 1982;** Dean. **1985:** Snyder. 1987). Barale et al. **(1985)** also reported **a** decrease in the percentage of peripheral blood PCE due to ,benzene. when administered chronically by gavage, but did not present data on temporal or sex-dependent variability. Particularly striking, and a finding unique to this study, was the regmen-dependent persistence of PCE suppression in male mice. Male mice exposed to benzene for only 3 days per week exhibited suppressed erythropoiesis throughout most of the 13-week exposure period. while male mice exposed 5 days per week exhibited an apparent recovery in hematopoietic activity to control levels by about the sixth week of exposure. These data are in agreement with the benzene metabolite studies of Irons (1983) and provide the first expenmental evidence that the extent of benzene-induced toxicity can be inversely related to the number of exposure days per week. This finding is in contrast to the studies of Coffin et al. **(1977).** Gill et al. **(1980).** and Toft et al. **(1982).** for which it was concluded that chronic exposures were more harmful to bone marrow function and integrity than intermittent ones? However, these investigators used different endpoints (bone-marrow cellularity. the onset of leucopenia). other species (rats) or other strains of mice (C57B1/6, NMRI). and different exposure regimens. It is not obvious why greater suppression of erythropoiesis occurred in male mice exposed to 300 ppm benzene for *3* days per week. However, since proliferating cells appear to be much more susceptible to the cytotoxic actions of benzene than are the normally-quiescent stem cells and since benzene appears to inhibit stem cell proliferation. the 3-day exposure regmen may provide a greater opportunity for stem cell mobilization in response to a benzene-induced depletion of differentiated cells and. thus. a greater opportunity for toxicity (R.D. Irons. **1983;** Snyder. 1987).

The increased variability of PCE frequency encountered among individual control and exposed animals during the later weeks of exposure is of concern, particularly in terms of extended exposure studies. Infections among the animals. due to the repeated blood samplings, and fighting which occurred among some of the mice was probably responsible for such variability. These are factors that need to be addressed in future experiments of this kind. perhaps necessitating increased care in obtaining the blood samples and **by** caging the animals individually.

The observation that the ability of benzene to suppress erythropoiesis and to induce micronucleated erythrocytes was less pronounced in female than in male mice was not unexpected. There are numerous reports indicating that male mice are more susceptible than are female mice to the genotoxic and carcinogenic effects of benzene (reviewed in Dean. **1985).** Siou and Conan (1980) implicated the influence of testosterone in male mice on the metabolism of benzene as the basis for the sex difference in sensitivity.

The induction of genotoxic and cytotoxic damage in the hematopoietic system of the mouse following multiple exposures to benzene has proven to be a complex interaction of many variables. An analysis of MN-PCE frequencies throughout the 13 weeks of exposure revealed that the level of genotoxlc damage induced by benzene remained fairly constant during the course of the extended exposure period. These data indicate that interpretations of bone marrow sensitivity based simply on an evaluation of peripheral blood MN-NCE frequencies may be inadequate. Since this observation may in part be mouse strain and route of exposure specific. studies to compare the time-dependent induction of MN-PCE/MN-NCE in other strains of mice (see Luke et al., 1987) and by other routes of exposure appear warranted. It was of interest to find that while the induction of genotoxic damage was independent of exposure regimen. the induction of cytotoxic damage was not. The fact that exposure to benzene for 3 days per week as opposed to 5 davs per week resulted in greater bone-marrow cytotoxicity has implications in the extrapolation of animal data to human exposure situations. However, the concentration of benzene (300 ppm) used in this study greatly exceeds the current and recent occupational exposure limits (a time-weighted average of 1 and 10 ppm. respectively). Furthermore, although 300 ppm benzene was believed to be within the linear portion of the dose range for the absorption of inhaled benzene in mice (Wells and Nerland. 1984). more recent data indicate that this dose lies above the linear portion of the dose–response curve for inhaled benzene metabolism (Sabourin et al., 1987). These factors suggest the need to evaluate this observation at lower concentrations of benzene. Regardless. the effects of benzene exposure on PCE production has a number of implications in regard to the interpretation of MN-PCE and MN-NCE data resulting from such exposure situations. The results of this study underscore the need for an analysis of PCE frequency in peripheral blood studies of MN induction and indicate some future directions for research on the chronic effects of benzene and other agents.

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## References

Aksoy. M. (1985) Benzene as a leukemogenic and carcinogenic agent. Am. J. Indust. Med., **8. 9-20.**

Anderson. D.. and C.R. Richardson **(1981)** Issues relevant to the assessment of chemically induced chromosome damage in vivo and their relationship to chemical mutagenesis. Mutation Res.. **90. 261**–272.

Barale. R. F. Giorgelli. L. Migliore. R. Ciranni. **D.** Casini. D. Zucconi and N. Loprieno **(1985)** Benzene induces micronuclei in circulating erythrocytes of chronically treated mice. Mutation Res.. **144. 193-196.**

BMDP Statistical Software **(1985)** W.J. Dixon (Ed.). University Press. Berkeley. California.

Choy. W.N.. J.T. MacGregor. M.D. Shelby and R.R. Maronpot (1985) Induction of micronuclei by benzene in B6C3F1 mice: Retrospective analysis of peripheral blood smears from the NTP carcinogenesis bioassay. Mutation Res.. **143. 55-59.**

Coffin. D.L.. D.E. Gardner. G.I. Sidorenko and M.A. Pinigin **(1977)** Role of time as a factor in the toxicity of chermcal compounds in intermittent and continuous exposures. II. Effects of intermittent exposure. J. Toxicol. Environ. Health. **3. 821-828.**

Cole. R.J.. **N.** Taylor. J. Cole and C.P. Arlett (1981) Short-term tests for transplacentally active carcinogens. 1. Micronucleus formation in fetal and maternal mouse erythroblasts. Mutation Res.. **89, 141-157.**

Cortina. T.A.. **E.W.** Sica. N.E. McCarroll. W. Coate. A. Thakur and M.G. Farrow **(1984)** Inhalation cytogenetics in mice and rats exposed to benzene. in: H.N. MacFarland. C.E. Holdsworth. J.A. MacGregor, **R.W.** Call and M.L. Lane (Eds.). Applied Toxicology of Petroleum Products. Adv. Modern Environ. Toxicol.. Vol.. 6, Princeton Scientific Publishers. Princeton. NJ. pp. **81-88.**

Cronkite. E.P.. **J.E.** Bullis. T. Inoue and R.T. Drew **(1984)** Benzene inhalation produces leukerma in mice. Toxicol. Appl. Pharmacol.. **75. 358-361.**

Dean. B.J. **(1969)** Chemical-induced chromosome damage. Lab. Anim.. 3. 157–174.

Dean. **B.H.** (1985) Recent findings on the genetic toxicology of benzene. toluene. xylenes and phenols. Mutation Res.. 154. 153–181.

Dean. B.J.. and M.J. Clare (1985) p. 168. cited in Dean. B.J. (1985) Recent findings on the genetic toxicology of benzene. toluene. xylenes and phenols. Mutation Res.. 154. 153–181.

Driscoll. K.E.. and C.A. Snyder (1984) The effects of ethanol injection and repeated benzene exposures on benzene pharmacokinetics. Toxicol. Appl. Pharmacol.. 73. 525–535.

Federal Register (1985. Occupational exposure to benzene: Proposed rule and notice of heanng. 50. 50512–50586.

Gill. D.P.. V.K. Jenkins. R.R. Kempen and S. Ellis (1980) The importance of plunpotential stem cells in benzene toxicity. Toxicology. 16. 163–171.

Gonasun. L.M.. C.M. Witmer. J.J. Kocsis and R. Snyder (1973) In vivo metabolism in mouse liver microsomes. Toxicol. Appl. Pharmacol.. 26. 398–406.

Green. J.D.. C.A. Snyder. J. LoBue. B.D. Goldstein and R.E. Albert (1981) Acute and chronic dose/response effect of benzene inhalation an the penpheral blood. bone marrow. and spleen cells of CD-I male mice. Toxicol. Appl. Pharmacol.. 59. 204–214.

Hart. J.W.. and B. Hadel;-Asp (1983) Induction of micronuclei in the mouse. Revised timing of the final stage of enthropoiesis. Slutation Res. 120. 127–132.

Hayashi. M.. T. Sofuni and M. Ishidate Jr. (1984) Kinetics of micronucleus formation in relation to chromosomal aberrations in mouse bone marrow. Mutation Res.. 127. 129–137.

Heddle. J.A.. M. Hite. B. Kirkhart. K. Mavournin. J.T. MacGregor. G.W. Newell and M.F. Salamone (1983) The induction of rmcronuclei as a measure of gcnotoxicitl;. A report of the C.S. Environmental Protection Agency Gene-Tox Program. Mutation Res., 123. 61–118.

IARC (1982) Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 29. World Health Organization International Agency for Research in Cancer. Lyon. France. pp. 93–148.

Irons. R.D. (1983) Metabolism of benzene and mechanisms of its toxicity. Collegium Ramazzini International Conference on Benzene. November 3–4. Grand Hyatt Hotel. New York.

Jenssen. D.. and C. Ramel (1978) The micronucleus test as part of a short-term mutagenicity test program for the prediction of carcinogenicitv evaluated by 143 agents tested. Mutation Res.. 75. 191–202.

Kato. H. (1974) Spontaneous sister chromatid exchanges detected by a BudR-labeling method. Nature (London). 251. 70–72.

Luke. C.A.. R.R. Tice and R.T. Drew (1988) The effect of exposure regimen and duration on benzene-induced bone marrow damage in mice. II. Strain compansons involving B6C3F1. C57B1/6 and DBA/2 male mice. Mutation Res.. 203. 273–295.

MacGregor. J.T.. C.M. Wehr and D.H. Could (1980) Clastogen-induced micronuclei in peripheral blood erythrocytes: The basis of an improved micronucleus test. Environ. Mutagen.. 2. 509–514.

Maltoni. C.. and C. Scarnato (1979) First expenmental demonstration of the carcinogenic effects of benzene: Long-term bioassays on Sprague–Dawley rats by oral administration. Med. Lav.. 70. 353–357.

NTP Technical Report 289 (1985) NTP Technical Report on the toxicology and carcinogenesis studies of benzene in F244/N rats and B6C3F1 mice (gavage studies). NIH Publication No. 84-2545. U.S. Department of Human Services.

Pathiratnr. A.. R.L. Puyear and J.D. Brammer (1986) A comparative study of the effects of benzene. toluene. and xvlenes on their in vitro metabolism and drug-metabolizing enzvrnes in rat liver, Toxicol. Appl. Pharmacol.. 82. 272–280.

Post. G.B.. and R. Snyder (1983) Effects of enzyme induction on mucrosomal benzene induction. J. Toxicol. Environ. Health. 11. 811–825.

Sabounn. P.J.. T.-H. Chen. G. Lucier. L.S. Birnbaum. E. Fisher and R.F. Henderson (1987) Effect of dose on the absorption and excretion of $^{14}C$ benzene administered orally or hv inhalation in rats and mice. Toxicol. Appl. Pharmacol.. 87. 325–336.

Salamone. M.F.. and J.A. Heddle (1983) The bone marrou micronucleus assay: Rationale for a revised protocol. in: F.J. de Serres and A. Hollaender (Eds.). Chemical Mutagens. Principles and Methods for Their Detection. Vol. 8. Plenum. New York. pp. 111–149.

Schalm. O.W.. N.C. Jain and E.J. Carrol (1975) Vetennary Hematology. 3rd edn.. Lea and Febiger. Philadelphia. p. 390.

Schlegel. R.. and J.T. MacGregor (1982) The persistence of rmcronuclei in peripheral blood erythrocytes: detection of chronic chromosome breakage in mice, Mutation Res.. 104. 367–369.

Siou. G.. and L. Conan (1980) Contribution à la connaissance de l'effet mutagene du benzene. I. Influence de la Matunte sexuelle sur la sensibilite de la souris a l'action mutagene du benzene. Cahiers Notes Docum. INRS. 101. 531–534.

Sittig. M. (Ed.) (1979) Benzene. in: Hazardous and Toxic Effects of Industnal Chemicals. Noyes Data Corp.. Park Ridge. NY. pp. 52–59.

Snyder. C.A. (1987) Benzene. in: R. Snyder (Ed.). Ethyl Browning's Toxicity and Metabolism of Industnal Solvents. Elsevier, Amsterdam. pp. 3–37.

Snyder. C.A.. B.D. Goldstein. A. Sellakumar. S.R. Wollman. I. Bromberg. M.N. Erlichman and S. Laskin (1978) Hematotoxicity of inhaled benzene to Sprague-Dawley rats and AKR mice at 300 ppm, J. Toxicol. Environ. Hlth.. 4. 605–618.

Snyder. C.A., B.D. Goldstein. A.R. Sellakumar. I. Bromberg. S. Laskin and R.E. Albert (1980) The inhalation toxicology of benzene: incidence of hematopoietic neoplasms and hematoxicity in AKR/J and C57B1/6J mice. Toxicol. Appl. Pharmacol.. 54, 323–331.

Snyder. C.A.. M.N. Erlichman. S. Laskin. B.D. Goldstein and R.E. Albert (1981) The pharmacokinetics of repetitive benzene exposures at 300 and 100 ppm in AKR mice and Sprague–Dawley rats. Toxicol. Appl. Pharrnacol.. 57, 164–171.

Snyder. R.. F. Uzuki. L. Gonasun. E. Bromfield and A. Wells (1967) The metabolism of benzene in vitro. Toxicol. Appl. Pharrnacol.. 11. 346–360.

Stoner. G.D.. P.B. Conran. E.A. Greisiger. J. Stoher. M. Morgan

and M A Pereira (1986) Comparison of two routes of chemical administration on the lung adenoma response in strain A/J rmce. Toxicol. Appl. Pharrnacol.. **82.** 19–31.

ce. R R.. and J.L. Ivett (1985) Cytogenetic analysis of bone marrow damage. in: R.D. Irons **(Ed.).** Toxicology of the Blood and Bone Marrow. Raven. New York. pp. 119–140.

Tice. R R D.L. Costa and R.T. Drew (1980) Cytogenetic effects of inhaled benzene in murine bone marrow: Induction of sister chromatid exchanges. chromosomal aberrations. and cellular proliferation inhibition in DBA/2 mice. Proc. Natl. Acad. Sci. (U.S.A.). 77. 2148–2152.

Tice. K.R.. T.F. Vogt and D.L. Costa (1982) Cytogenetic effects of inhaled benzene in murine bone marrow. in: R.R. Tice. D.L. Costa and **K.M.** Schaich (Eds.). Genotoxic Effects of Airborne Agents. Plenum. Yew York. pp. 257–275.

Tice. R.R.. M.J. Sawey, R.T. Drew and E.P. Cronkite (1984) Benzene-induced micronuclei in the peripheral blood of rmce: A retrospective analysis. Environ. Mutagen.. **6.** 421.

Toft. K.. T. Olofsson. **A.** Tunek and **M.** Berlin (1982) Toxic effects on mouse bone marrow caused by inhalation of benzene. Arch. Toxicol.. 51. 295–302.

Wells. **M..** and D.E. Nerland (1984) Nonlinear relationships between benzene exposure and blood levels of benzene in mice. Fed. Proc.. 43. 759.

White. M.C.. P.F. Infante and **B.** Walker Jr. (1980) Occupational exposure to benzene: A review of carcinogenic and related health effects following the **U.S.** Supreme Court Decision. Am. J. Indust. Med.. 1. 233–243.

APPENDIX 1

INDIVIDUAL ANIMAL DATA FOR MN-PCE FREQUENCY

*(A) DBA/2 Male Mice*

| Animal number | Week 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Temporal average (Mean ± SEM) | Regression *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Regimen 1*, B | | | ati | | | | | | | | | | | | | |
| 1 | 3 | 1 | 3 | 4 | 3 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 8 | 2 | 2.1 ± 0.60 | 0.9643 |
| 2 | 0 | 0 | 3 | 5 | 0 | 2 | 2 | 4 | 4 | 7 | 2 | 0 | 0 | 4 | 2.4 ± 0.60 | 0.5087 |
| 3 | 1 | 3 | 2 | 5 | 1 | 1 | 10 | 3 | 1 | 2 | 0 | 0 | 1 | 4 | 2.4 ± 0.70 | 0.6493 |
| 4 | 2 | 0 | 1 | 4 | 0 | 1 | 0 | 2 | 2 | 1 | 1 | 3 | 3 | 2 | 1.6 ± 0.33 | 0.2960 |
| 5 | 7 | 2 | 5 | 0 | 1 | 2 | 3 | 2 | 0 | 0 | 2 | 0 | 1 | 3 | 2.0 ± 0.54 | 0.0837 |
| 6 | 6 | 3 | 1 | 7 | 1 | 1 | 1 | 0 | 2 | 0 | 1 | 0 | 1 | 1 | 1.8 ± 0.58 | 0.0226 ** (−) |
| Mean | 3.2 | 1.5 | 2.5 | 4.2 | 1.0 | 1.3 | 2.8 | 2.0 | 1.5 | 1.8 | 1.2 | 0.5 | 2.3 | 2.7 | | |
| SEM | 1.1 | 0.56 | 0.62 | 0.95 | 0.45 | 0.21 | 1.49 | 0.58 | 0.62 | 1.08 | 0.31 | 0.05 | 1.20 | 0.49 | | |
| *Regimen 1*, Benzene concentration = 300 ppm | | | | | | | | | | | | | | | | |
| 13 | 4 | 126* | – | 124 | 140 | 154 | 112* | 104 | 116 | 70 | 110 | 171 | 100 | 93 | 118.3 ± 7.90 | 0.3190 |
| 14 | 5 | – | – | – | 62 | 104 | 74 | 136 | 81 | 107 | 119 | – | 87 | 122 | 99.1 ± 8.21 | 0.2470 |
| 15 | 1 | 130 | 501 | 146 | – | 99 | 106 | 110 | 99 | 94 | 151 | 202 | 102** | 128 | 118.1 ± 10.86 | 0.2822 |
| 16 | 1 | – | – | 93 | 117 | 82 | 72 | 104 | 101 | 80 | 89 | 149 | 90 | a | 97.7 ± 7.02 | 0.5619 |
| 17 | 5 | – | – | – | 157 | 103 | 95 | 90 | 92 | 100 | – | 143 | 188** | 140 | 123.1 ± 11.69 | 0.2301 |
| 18 | 1 | | | | | | | 55 | 51 | 77 | 84 | – | 87 | 117 | 90.8 ± 8.72 | 0.3501 |
| Mean | 2.8 | 128.0 | 95.5 | 121.2 | 119.0 | 103.8 | 90.3 | 101.5 | 8. | 88.0 | 110.6 | 16.2 | 99.0 | 120.0 | | |
| SEM | 0.83 | 2.00 | 45.50 | 10.87 | 20.69 | 10.86 | 6.82 | 11.99 | 9. | 16.02 | 7.76 | 5. | 2.17 | 3.77 | | |

*Regimen 2*, Benzene concentration = 0 ppm

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7 | 0 | 1 | 1 | 2 | 2 | 1 | 1 | 0 | ) | 1 | 2 | 1 | 0 | 3 | 1.1 ± 0.25 | 0.5141 |
| 8 | 3 | 0 | 2 | 0 | 0 | 3 | 0 | 1 | ) | 1 | 2 | 1 | 3 | 0 | 1.1 ± 0.33 | 0.9193 |
| 9 | 3 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 2 | u | 1 | 2 | 13 | 1.8 ± 0.97 | 0.0760 |
| 10 | 1 | 3 | 2 | 4 | 1 | 0 | 2 | 0 | 2 | 2 | u | 1 | 1 | 7 | 2.0 ± 0.52 | 0.4674 |
| 11 | 0 | 3 | 1 | 1 | 1 | 2 | 0 | 0 | 1 | 1 | 5 | 2 | 3 | 3 | 1.6 ± 0.39 | 0.0948 |
| 12 | 1 | 2 | 1 | 3 | 5 | 2 | 1 | 2 | ) | 2 | 0 | 0 | 1 | 2 | 1.6 ± 0.36 | 0.2511 |
| Mean | 1.3 | 1.5 | 1.2 | 1.7 | 1.5 | 1.7 | 0.7 | 0.5 | 1.7 | 1.5 | 2.2 | 1.0 | 1.7 | 4.7 | | |
| SEM | 0.56 | 0.56 | 0.31 | 0.67 | 0.76 | 0.42 | 0.33 | 0.34 | 0.33 | 0.22 | 1.03 | 0.26 | 0.49 | 1.91 | | |

*Regimen 2*, Benzene concentration = 300 ppm

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | 2 | 113 | – | 146 | – | 100 | 106 | 128 | 186 * | 111 | u | 101 | – | 124 | 123.9 ± 9.18 | 0.9706 |
| 20 | 2 | – | 32 | 132 | 168 | – | 109 | 129 | – | 156 | 123 | – | 100 * | 138 | 120.8 ± 13.13 | 0.4679 |
| 21 | 1 | 133 | – | 134 | – | 134 * | 102 | 158 | – | 68 | – | 103 | 134 * | 124 | 121.1 ± 8.76 | 0.4974 |
| 22 | 0 | 102 | 66 | 124 | 123 | – | – | 177 | – | 113 | – | – | – | 186 | 127.3 ± 15.85 | 0.0551 |
| 23 | 1 | 66 * | – | 148 | 96 * | 100 | 78 | 135 | – | 144 | – | 134 | – | 173 | 119.3 ± 11.93 | 0.0329 ** (+) |
| 24 | 0 | 100 * | – | 100 * | 118 * | 84 * | 115 | 142 | – | 130 | – | – | – | 131 | 115.0 ± 6.85 | 0.0822 |
| Mean | 1.0 | 102.8 | 49.0 | 130.7 | 126.2 | 104.5 | 102.0 | 144.8 | 186.0 | 120.3 | 123.0 | 112.7 | 117.0 | 146.0 | | |
| SEM | 0.36 | 10.91 | 17.00 | 7.15 | 15.10 | 10.53 | 6.36 | 7.85 | 0.00 | 12.66 | 0.00 | 10.68 | 17.00 | 10.93 | | |

*(B) DBA/2 Female Mice*

*Regimen 1*, Benzene concentration = 0 ppm

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 0 | 5 | 1 | 3 | 4 | 0 | 0 | 2 | 0 | 1 | 1 | 5 | 1 | 3 | 1.9 ± 0.49 | 0.9457 |
| 26 | 3 | 3 | 3 | 4 | 0 | 1 | 1 | 4 | 1 | 1 | 1 | 0 | 3 | 0 | 1.8 ± 0.40 | 0.0741 |
| 27 | 3 | 6 | 3 | 2 | 2 | 5 | 0 | 0 | 1 | 4 | 1 | 2 | 1 | 1 | 2.2 ± 0.48 | 0.0660 |
| 28 | 1 | 2 | 0 | 1 | 0 | 2 | 1 | 1 | 1 | 0 | 1 | 2 | 0 | 0 | 0.9 ± 0.21 | 0.4112 |
| 29 | 1 | 2 | 2 | 0 | 1 | 1 | 0 | 2 | 0 | 1 | 3 | 0 | 1 | 0 | 1.0 ± 0.26 | 0.4700 |
| 30 | 1 | 4 | 1 | 1 | 2 | 1 | 1 | 0 | 2 | 1 | 2 | 1 | 2 | 2 | 1.5 ± 0.25 | 0.8682 |
| Mean | 1.5 | 3.7 | 1.7 | 1.8 | 1.5 | 1.7 | 0.5 | 1.5 | 0.8 | 1.3 | 1.5 | 1.7 | 1.3 | 1.0 | | |
| SEM | 0.50 | 0.67 | 0.49 | 0.60 | 0.62 | 0.72 | 0.22 | 0.22 | 0.31 | 0.56 | 0.34 | 0.76 | 0.42 | 0.52 | | |

APPENDIX 1 (continued)

| Animal number | Week 0 | 1 | | | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Temporal average (Mean ± SEM) | Regression *P* value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Regimen 1*, Benzene concentration = 300 ppm | | | | | | | | | | | | | | | |
| 37 | 0 | 28 | 17 | 18 | 16 | 18 | 12 | 9 | 25 | 9 | 7 | 9 | 5 | 21 | 14.9 ± 1.98 | 0.0745 |
| 38 | 1 | 53 | 28 | 51 | 38 | 19 | 19 | 13 | 10 | 32 | 33 | 22 | 32 | 53 | 31.0 ± 4.06 | 0.0541 |
| 39 | 1 | 32 | 30 | 19 | 30 | 28 | 12 | 11 | 13 | 9 | 11 | 4 | 17 | 29 | 18.8 ± 2.70 | 0.0674 |
| 40 | 0 | 48 | 27 | 41 | – | 70 | 9 | 46 | 15 | [illegible] | 27 | 19 | 13 | 25 | 29.3 ± 5.32 | 0.0869 |
| 41 | 1 | 29 | 11 | 17 | 41 | 24 | 4 | 19 | 20 | [illegible] | 10 | 44 | 11 | 18 | 20.1 ± 3.30 | 0.1196 |
| 42 | 0 | 16 | 9 | 10 | 17 | 11 | 3 | 8 | 17 | 7 | 14 | 12 | 9 | 16 | 11.5 ± 1.21 | 0.9365 |
| Mean | 0.5 | 34.3 | 20.3 | 26.0 | 28.4 | 28.3 | 9.8 | 17.7 | 16.7 | 13.7 | 17.0 | 18.3 | 14.5 | 27.0 | | |
| SEM | 0.22 | 5.61 | 3.76 | 6.58 | 5.18 | 8.66 | 2.41 | 5.89 | 2.17 | 3.77 | 4.23 | 5.79 | 3.86 | 5.54 | | |
| *Regimen 2*, Benzene concentration = 0 ppm | | | | | | | | | | | | | | | |
| 31 | 3 | 2 | 1 | 5 | 3 | 2 | 2 | 5 | 1 | 3 | 1 | 2 | 0 | 2 | 2.3 ± 0.38 | 0.2264 |
| 32 | 0 | 3 | 1 | 0 | 4 | 4 | 0 | 2 | 3 | 0 | 3 | 1 | 0 | 12 | 2.4 ± 0.85 | 0.2116 |
| 33 | 2 | 1 | 4 | 1 | [illegible] | 1 | 3 | 3 | 0 | 2 | 0 | 0 | 1 | 0 | 1.4 ± 0.34 | 0.0808 |
| 34 | 2 | 7 | 1 | 2 | [illegible] | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 8 | 1.9 ± 0.65 | 0.9081 |
| 35 | 3 | 1 | 1 | 1 | 4 | 3 | 3 | 3 | 3 | 2 | 1 | 1 | 2 | 2 | 2.1 ± 0.28 | 0.7147 |
| 36 | 0 | 2 | 3 | 3 | 2 | 0 | 0 | 0 | 1 | 1 | u | 3 | 0 | 2 | 1.3 ± 0.35 | 0.8010 |
| Mean | 1.7 | 2.7 | 1.8 | 2.0 | 2.5 | [illegible] | 1.5 | 2.3 | 1.3 | 1.5 | 1.2 | 1.3 | 0.5 | 4.3 | | |
| SEM | 0.56 | 0.92 | 0.54 | 0.73 | 0.56 | [illegible] | 0.56 | 0.72 | 0.56 | 0.43 | 0.49 | 0.42 | 0.34 | 1.89 | | |
| *Regimen 2*, Benzene concentration = 300 ppm | | | | | | | | | | | | | | | |
| 43 | 2 | 24 | 5 | 24 | 25 | 29 | 9 | 16 | 3 [illegible] | 31 | 12 | 17 | 9 | 20 | 19.4 ± 2.44 | 0.7222 |
| 44 | 1 | 38 | 26 | 48 | 56 | – | 10 | 16 | – | 57 | 34 | 60 | – | 64 | 40.9 ± 6.04 | 0.2627 |
| 45 | 1 | 19 | – | 41 | 41 | 29 | 16 | 17 | 25 | 22 | 22 | 24 | a | | 25.6 ± 2.84 | 0.3223 |
| 46 | a | | | | | | | | | | | | | | | |
| 47 | 0 | 14 | – | 43 | 15 | 5 | 4 | 6 | 18 | 13 | u | 9 | 19 | 20 | 15.1 ± 3.27 | 0.7244 |
| 48 | 4 | 14 | 8 | 20 | 33 | u | 21 | 21 | 16 | 26 | 13 | 26 | 30 * | 24 | 21.0 ± 2.12 | 0.1645 |
| Mean | 1.6 | 21.8 | 13.0 | 35.2 | 34.0 | 21.0 | 12.0 | 15.2 | 22.5 | 29.8 | 20.2 | 27.2 | 19.3 | 32.0 | | |
| SEM | 0.68 | 4.45 | 6.56 | 5.54 | 6.99 | 8.00 | 2.95 | 2.48 | 3.43 | 7.41 | 5.10 | 8.73 | 6.06 | 10.71 | | |

Number of MN-PCE per 1000 PCE with means and standard error of the means (SEM) calculated for each exposure group at each sample time. The mean response ± the SEM across time for each mouse is presented as a temporal average. The *P* value for having a significant time-dependent slope, as determined from multiple linear regression analysis is presented for each mouse.

*Abbreviations*: a, animal died; u, slides unscorable due to technical reasons; * 500 cells scored due to a limited number of PCE; –, less than 500 PCEs available for analysis; ** Significant slope at $\alpha = 0.05$, (+) or (–) indicates direction of slope.

APPENDIX 2

INDIVIDUAL ANIMAL DATA FOR MN-NCE FREQUENCY

*(A) DBA/2 Male Mice*

| Animal number | Week 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Temporal average (Mean ± SEM) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Regimen 1.* **Benzene concentration = 300 ppm** | | | | | | | | | | | | | | | |
| 1 | 1 | 2 | 2 | 4 | 2 | 2 | 2 | 1 | 4 | 1 | 3 | 1 | 4 | 3 | 2.3 ± 0.30 |
| 2 | 2 | 1 | 0 | 2 | | 2 | 0 | 1 | 2 | 2 | 2 | 0 | 0 | 4 | 1.5f0.33 |
| 3 | 2 | 0 | 1 | 2 | 0 | 1 | 0 | 1 | 2 | 0 | 3 | 1 | 1 | 3 | **1.2±0.28** |
| 4 | 1 | 2 | 3 | 1 | 1 | 0 | 1 | 1 | 3 | 3 | 3 | 3 | 2 | 1 | 1.8 ± 0.28 |
| 5 | 1 | 0 | 5 | 1 | 2 | 0 | 3 | 2 | 0 | 2 | 2 | 1 | 1 | 1 | 1.5 ± 0.36 |
| 6 | 1 | 1 | 2 | 1 | 1 | 4 | 0 | 2 | 3 | 0 | 5 | 2 | 4 | 1 | 1.9 ± 0.41 |
| Mean | 1.3 | 1.0 | 2.2 | 1.8 | 1.5 | 1.5 | 1.0 | 1.3 | 2.3 | 1.3 | 3.0 | 1.3 | 2.0 | 2.2 | |
| SEM | 0.21 | 0.36 | 0.70 | 0.48 | 0.43 | 0.62 | 0.52 | 0.21 | 0.56 | 0.49 | 0.45 | **0.42** | 0.68 | 0.54 | |
| *Regimen 1.* **Benzene concentration = 300 ppm** | | | | | | | | | | | | | | | |
| 13 | **1** | **2** | **9** | 6 | 1 7 | 2 0 | IS | **43** | **17** | 50 | 35 | 35 | 52 | **43** | 24.8 ± 4 76 |
| 14 | 3 | 3 | 2 | 4 | 9 | 13 | 21 | 14 | 28 | 23 | 39 | **44** | **19** | 48 | 22.2 5.04 |
| 15 | 0 | | 2 | 15 | 16 | 30 | 0 | 34 | **44** | **22** | 26 | 32 | 81 | 59 | 27.6 ± 6.00 |
| 16 | 1 | 2 | 4 | 4 | 12 | 12 | 15 | 16 | 19 | 33 | 31 | **41** | 50 | (46)a | 20.4 ± **4** 49 |
| **17** | 1 | 4 | 5 | 8 | 1 9 | 5 | **17** | **45** | **41** | **32** | 37 | 37 | 37 | 47 | 23.9 ± **4.58** |
| 18 | 0 | 4 | 9 | 8 | 2 7 | 1 1 | 74 | 35 | **17** | 24 | 33 | 23 | 53 | **40** | *22.0* ± 4.01 |
| **Mean** | 1.0 | 3.3 | **5.2** | 7.5 | 16.7 | 15.2 | 19.2 | 31.2 | 27.7 | 30.7 | 33.5 | 35.3 | 55.3 | 47.4 | |
| SEM | 0.45 | 0.49 | 1.30 | 1.67 | 2.54 | 3.55 | 1.30 | 5.41 | 4.99 | **4.32** | 1.89 | 3.02 | 5.92 | 3.23 | |
| *Regimen 2.* **Benzene concenrrarion** = *0* **ppm** | | | | | | | | | | | | | | | |
| 7 | 0 | 2 | 1 | 1 | 0 | **4** | 7 | I | 1 | 2 | 1 | 1 | 2 | 2 | 1.4 ± 0.27 |
| 8 | *2* | *2* | *0* | I | 0 | 4 | 0 | 1 | 1 | I | 2 | 1 | 3 | 2 | 1.4 ± 0.31 |
| 9 | 0 | 0 | 0 | 1 | 3 | *2* | *2* | 0 | 1 | 1 | (2)u | *2* | *3* | *3* | 1.4t0.31 |
| 10 | 1 | 1 | 0 | 2 | 1 | 2 | **2** | 1 | **1** | 0 | (0)u | 0 | 3 | 3 | 1.2 ± 0.28 |
| 11 | 0 | 0 | 2 | 1 | 2 | 1 | 0 | 2 | 0 | 1 | 4 | 1 | 2 | 3 | 1.420.32 |
| **12** | 0 | 1 | 0 | 0 | 0 | 3 | 4 | 0 | 0 | 2 | 1 | 3 | 2 | 1 | 1.2 ± 0.37 |
| **Mean** | **0.5** | 1.0 | 0.5 | 1.0 | 1.0 | 2.7 | **1.7** | 0.8 | 0.7 | 1.2 | 2.0 | 1.3 | 2.5 | 2.3 | |
| **SEM** | 0.34 | 0.36 | 0.34 | 0.26 | 0.52 | 0.49 | 0.62 | 0.31 | 0.21 | 0.31 | 0.71 | 0.42 | 0.22 | 0.33 | |
| *Regimen 1.* **Benzene concentration** = 300 **ppm** | | | | | | | | | | | | | | | |
| 19 | 0 | 2 | 1 | 3 | 7 | **14** | 19 | 11 | 11 | 15 | (17)u | 19 | 13 | 14 | 10.4 ± 1.78 |
| 20 | **0** | 4 | 5 | 8 | 9 | 8 | 9 | 6 | *22* | 11 | 19 | 20 | 11 | 13 | 10.4 ± 1.69 |
| 21 | 0 | 3 | 3 | 5 | 10 | 9 | 10 | 14 | 3 | 7 | 13 | 21 | 13 | 26 | 9.8 ± 1.95 |
| 77 | 0 | 3 | 9 | 16 | 11 | **19** | **21** | 6 | 13 | 10 | 9 | **21** | 25 | 23 | 13.3 ± 2.07 |
| 23 | *2* | 2 | I | 3 | 5 | 19 | 10 | 10 | 18 | 18 | IO | **20** | 16 | 17 | **10.8 ± 1.92** |
| 74 | 1 | 1 | 4 | 4 | **9** 1 | **6** | 7 1 | 6 1 | 0 | 4 1 | 4 1 | 6 | **4** | 14 | 8.6 ± 1.58 |
| **Mean** | 0.5 | 2.5 | **3.8** | 6.5 | 8.5 | 14.2 | **12.7** | 10.5 | 12.8 | 10.8 | 13.0 | 19.5 | 13.7 | 17.8 | |
| SEM | 0.34 | 1.02 | **1.22** | 2.04 | 3.47 | 1.96 | 2.38 | 1.67 | **2.70** | **2.09** | 1.76 | 0.76 | 2.80 | 2.21 | |

**APPENDIX** 2 (continued)

*(B) DBA/2 Female Mire*

| Animal number | Week 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Temporal average (Mean ± SEM) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Regimen 1***, Benzene concentration = 0 pprn | | | | | | | | | | | | | | | |
| 25 | 0 | 0 | 3 | 1 | 2 | 2 | 1 | 0 | 3 | 2 | 1 | 5 | 3 | 1 | 1.7 ± 0.38 |
| 26 | 2 | 1 | 0 | 2 | 2 | 1 | 1 | 0 | 2 | 1 | 3 | 0 | 1 | 1 | 1.2 ± 0.24 |
| 27 | 0 | 2 | 1 | 1 | 1 | 1 | 0 | 1 | 2 | 1 | 1 | 1 | 2 | 2 | 1.1 ± 0.18 |
| 28 | 1 | 2 | 2 | 1 | 0 | 0 | 1 | 0 | 3 | 0 | 3 | 0 | 3 | 0 | 1.1 ± 0.33 |
| 29 | 0 | 0 | 3 | 0 | 1 | 0 | 1 | 3 | 3 | 3 | 3 | 2 | 2 | 1 | 1.5 ± 0.33 |
| 30 | 1 | 1 | 0 | 3 | 2 | 1 | 2 | 1 | 3 | 2 | 1 | 3 | 1 | 0 | 1.5 ± 0.27 |
| Mean | 0.7 | 1.0 | 1.5 | 1.3 | 1.3 | 0.8 | 1.0 | 0 8 | 2.7 | 1.5 | 1.8 | I 8 | 2.0 | 0.8 | |
| SEM | 0.33 | 0.36 | 0.56 | 0.42 | 0.33 | 0.31 | 0.26 | 0.48 | 0.21 | 0.43 | 0.40 | 0.79 | 0.36 | 0.31 | |
| ***Regimen 1***, Benzene concentration = 300 pprn | | | | | | | | | | | | | | | |
| 37 | 0 | 0 | 1 | 14 | 5 | 14 | 6 | 3 | 9 | 4 | | 7 | 9 | 13 | 6.6 ± 1.29 |
| 38 | 0 | 3 | 2 | 9 | 11 | 14 | 11 | 12 | 17 | 13 | 15 | IS | 17 | 22 | 11.7 ± 1.71 |
| 39 | 2 | 1 | 4 | 16 | 6 | 10 | 4 | 9 | 7 | 1 3 | 1 0 | 7 | 5 | 5 | 7.1 ± 1.12 |
| 40 | 0 | 2 | 5 | 10 | 16 | 16 | 11 | 3 | 7 | 14 | 4 | 10 | 17 | 20 | 9.6 ± 1.69 |
| 41 | 0 | I | 2 | 11 | 7 | 10 | 2 | 6 | 4 | 12 | 9 | 5 | 7 | 7 | 5.9 ± 1.01 |
| 42 | 0 | 1 | 2 | 6 | 6 | 9 | 2 | 1 | 1 5 | 7 | 7 | 5 | 7 | 8 | 5.4 ± 0.85 |
| Mean | 0.3 | 1.3 | 2.7 | 11.0 | 8.5 | 12.2 | 6.0 | 7.3 | 8.2 | 10.5 | 8.7 | 8.7 | 10.3 | 12.5 | |
| **SEM** | 0.33 | 0.42 | 0.62 | 1.46 | 1.73 | 1.17 | 1.69 | 1.61 | 1.90 | 1.65 | 1.52 | 2.01 | 2.17 | 2.91 | |
| ***Regimen 2***, Benzene concentration = 0 pprn | | | | | | | | | | | | | | | |
| 31 | 0 | 2 | 0 | 0 | 2 | 2 | 2 | 2 | 0 | 2 | 4 | 4 | I | 1 | 1.6 ± 0.36 |
| 32 | 1 | 1 | 0 | 2 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 2 | 0.9 ± 0.18 |
| 33 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 2 | 0 | 1 | 1 | 4 | 0 | 1.0 ± 0.28 |
| 34 | 0 | 0 | 0 | 0 | 0 | 2 | 5 | 3 | 2 | 0 | 2 | 1 | 0 | 3 | 1.3 ± 0.42 |
| 35 | 0 | 0 | 2 | 2 | 1 | 1 | 1 | 2 | 1 | 0 | 2 | 2 | 3 | 5 | 1.6 ± 0.36 |
| 36 | 2 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | u | 0 | 0 | 1 | 0.5 ± 0.18 |
| Mean | 0.7 | 0.7 | 0.5 | 1.0 | 0.5 | 1.2 | 1.8 | 1.5 | 1.0 | 0.5 | 2.0 | 1.3 | 1.5 | 2.0 | |
| **SEM** | 0.33 | 0.33 | 0.34 | 0.36 | 0.34 | 0.31 | 0.65 | 0.43 | 0.36 | 0.34 | 0.55 | 0.62 | 0.67 | 0.73 | |
| ***Regimen 2***, Benzene concentration = 300 ppm | | | | | | | | | | | | | | | |
| 43 | 2 | 0 | 1 | 2 | 3 | 3 | 6 | 5 | 1 | 4 | 3 | 10 | 3 | 4 | 3.4 ± 0.67 |
| 44 | 3 | 0 | 1 | 6 | 4 | I2 | 1 | 4 | 7 | 3 | 2 | 17 | 4 | 22 | 6.1 ± 1.73 |
| 45 | 1 | 3 | 2 | 2 | 9 | 9 | 8 | 1 | 3 | 3 | 5 | 8 | 6 | (7)a | (6)a 5.9 ± 0.91 |
| 46 | a | | | | | | | | | | | | | | |
| 47 | 3 | 3 | 2 | 3 | 2 | 5 | 5 | 4 | 6 | 6 | (8)u | 9 | 6 | 9 | 5.1 ± 0.64 |
| 48 | 1 | 1 | 0 | 3 | 9 | 6 | 3 | 7 | 3 | 5 | 3 | 12 | 3 | 6 | 4.4 ± 0.89 |
| Mean | 2.0 | 1.4 | 1.2 | 3.2 | 5.4 | 7.0 | 4.6 | 6.6 | 4.0 | 4.0 | 4.6 | 10.8 | 4.0 | 10.2 | |
| **SEM** | 0.45 | 0.68 | 0.37 | 0.74 | 1.50 | 1.58 | 1.21 | 1.69 | 1.10 | 0.71 | 0.51 | 1.83 | 0.71 | 4.05 | |

Number of MN-NCE per 1000 NCE with means and standard error of the means (SEM) calculated for each exposure group at each sample time. The mean response ± the SEM across time for each mouse is presented as a temporal average.

*Abbreviations*: a animal died; u, slides unscorable due to technical reasons: ( ), estimated value by averaging two nearest data points, these values used in the calculation of the temporal averages but not in the calculation of the weeklymeans.

APPENDIX 3

INDIVIDUAL ANIMAL DATA FOR PCE FREQUENCY

*(a) DBA/2 Male Mice*

| Animal number | Week 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Temporal average (Mean ± SEM) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Regimen 1*. Benzene concentration = 0 ppm | | | | | | | | | | | | | | | |
| 1 | 31 | 64 | 30 | 29 | 26 | 34 | 5 | 46 | 60 | 15 | 42 | 21 | 16 | 35 | 33.1 ± 4.46 |
| 2 | 36 | 52 | 67 | 41 | 17 | 114 | 8 | 42 | 50 | 7 | 41 | 46 | 49 | 23 | 42.4 ± 7.20 |
| 3 | 73 | 14 | 38 | 62 | 32 | 18 | 10 | 62 | 23 | 37 | 59 | 35 | 6 | 52 | 33.6 ± 5.14 |
| 3 | 33 | 38 | 40 | 33 | 26 | 48 | 14 | 25 | 51 | 33 | 33 | 44 | 12 | 34 | 33.1 ± 3.00 |
| 5 | 26 | 43 | 48 | 32 | 20 | 10 | 13 | 46 | 64 | 34 | 77 | 36 | 24 | 15 | 34.8 ± 5.19 |
| 6 | 46 | 49 | 37 | 34 | 22 | 25 | 14 | 30 | 24 | 14 | 34 | 57 | 5 | 65 | 32.6 ± 4.58 |
| Mean | 34.2 | 43.3 | 43.3 | 38.5 | 23.8 | 41.5 | 10.7 | 41.8 | 45.3 | 23.3 | 47.7 | 39.X | 18.7 | 37.3 | |
| SEM | 3.57 | 6.89 | 5.29 | 4.97 | 2.17 | 15.46 | 1.50 | 5.37 | 7.24 | 5.22 | 6.99 | 4.98 | 6.70 | 7.53 | |
| *Regimen 1*. Benzene concenrrarion = 300 ppm | | | | | | | | | | | | | | | |
| 13 | 37 | 3 | 0 | 5 | 14 | 7 | 0 | 15 | 17 | 77 | 27 | 14 | 10 | 12 | 15.5 ± 5.54 |
| 14 | 36 | 1 | 0 | 1 | 8 | 19 | 17 | 12 | 37 | 29 | 23 | 11 | 9 | 9 | 13.5 ± 3.10 |
| 15 | 28 | 0 | 3 | 8 | 7 | 10 | 10 | 12 | 24 | 74 | 28 | 39 | 9 | 110 | 25.7 ± 8.92 |
| 16 | 2 | 6 | 0 | 0 | 4 | 8 | 2 | 8 | 3 | 5 | 48 | 39 | 35 | 36 | (36)a 17.4 ± 3.61 |
| 17 | 15 | 0 | 0 | 2 | 10 | 4 | 18 | 4 | 16 | 11 | 0 | 27 | 9 | 23 | 9.5 ± 2.62 |
| 18 | 1 | 1 | 2 | 2 | 8 | 5 | 5 | 7 | 8 | 16 | 18 | 9 | 5 | 6 | 6 | 7.5 ± 1.31 |
| Mean | 75.5 | 1.0 | 0.8 | 4.7 | 8.7 | 7.8 | 10.0 | 9.3 | 19.2 | 42.8 | 21.0 | 21.8 | 13.2 | 32.0 | |
| SEM | 4.36 | 0.52 | 0.54 | 1.20 | 1.26 | 2.50 | 2.14 | 1.78 | 4.35 | 11.53 | 5.79 | 5.65 | 4.60 | 19.71 | |
| *Regrmen 2*. Benzene concentration = 0 ppm | | | | | | | | | | | | | | | |
| 7 | 31 | 40 | 83 | 40 | 21 | 21 | 8 | 13 | 34 | 40 | 14 | 27 | 16 | 22 | 30.9 ± 5.22 |
| 8 | 32 | 44 | 55 | 28 | 19 | 11 | 8 | 45 | 26 | 40 | 20 | 39 | 6 | 116 | 36.1 ± 7.89 |
| 9 | 25 | 35 | 31 | 57 | 31 | 25 | 20 | 19 | 12 | 20 | (30)u | 41 | 26 | 104 | 34.05 6.13 |
| 10 | 23 | 71 | 77 | 55 | 7 | 64 | 10 | 43 | 22 | 50 | (36)u | 23 | 14 | 155 | 46.4 ± 10.34 |
| 11 | 24 | 32 | 31 | 47 | 37 | 7 | 25 | 42 | 18 | 24 | 29 | 52 | 18 | 41 | 30.6 ± 3.58 |
| 12 | 37 | 45 | 63 | 54 | 18 | 4 | 13 | 27 | 30 | 47 | 61 | 27 | 24 | 73 | 35.55 5.57 |
| Mean | 28.7 | 44.5 | 56.7 | 46.8 | 23.2 | 22.0 | 14.0 | 31.5 | 23.7 | 36.8 | 31.0 | 34.8 | 17.3 | 85.2 | |
| SEM | 2.26 | 5.68 | 9.07 | 4.56 | 4.37 | 9.03 | 2.86 | 5.61 | 3.28 | 4.98 | 10.46 | 4.52 | 2.95 | 20.22 | |
| Regimen 2. Benzene concentration = 300 ppm | | | | | | | | | | | | | | | |
| 19 | 3 | 7 | 1 | 1 | 7 | 1 | 3 | 4 | 3 | 3 | 6 | (8)u | 11 | I | 17 | 5.1 ± 1.32 |
| 20 | 21 | 0 | 5 | 3 | 7 | 1 | 7 | 2 | 2 | 3 | 8 | 1 | 4 | 7 | 3.8 ± 0.75 |
| 21 | 17 | 0 | 0 | 24 | 2 | 1 | 0 | 38 | 1 | 11 | 3 | 5 | 0 | 8 | 7.2 ± 3.18 |
| 22 | 26 | 3 | 13 | 1 | 16 | 0 | 0 | 15 | 9 | 5 | 0 | 5 | 1 | 11 | 6.1 ± 1.67 |
| 23 | 2 | 8 | 0 | 0 | 1 | 1 | 2 | 5 | 9 | 0 | 3 | 1 | 14 | 1 | 22 | 4.5 ± 1.85 |
| 24 | 3 | 4 | 0 | 0 | 3 | 3 | 0 | 2 | 9 | 0 | 6 | 1 | 0 | 1 | 3 | 2.2 ± 0.76 |
| Mean | 27.2 | 0.7 | 3.2 | 6.5 | 5.0 | 1.2 | 3.0 | 12.7 | 2.5 | 5.7 | 2.6 | 6.0 | 1.3 | 11.3 | |
| SEM | 3.09 | 0.49 | 2.12 | 3.61 | 2.38 | 0.48 | 1.15 | 5.42 | 1.38 | 1.20 | 1.44 | 2.25 | 0.56 | 2.86 | |

APPENDIX 3 (continued)

*(A) DBA/2 Female Mice*

| Animal number | Week 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Temporal average (Mean ± SEM) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Regimen 1.*** Benzene concentration = 0 ppm | | | | | | | | | | | | | | | |
| 25 | 18 | *50* | 38 | 51 | 24 | 16 | 15 | 27 | 18 | 42 | 48 | 21 | 7 | 68 | 31.6 ± 4.77 |
| 26 | 31 | 77 | 30 | 41 | 36 | *46* | 10 | 34 | 43 | 30 | 33 | 27 | 17 | 68 | 37.4 ± 4.74 |
| 27 | 23 | 43 | 40 | 45 | 24 | 24 | 26 | 30 | 46 | 40 | 27 | 39 | 5 | 77 | 34.9 ± 4.43 |
| 28 | 18 | 45 | 37 | 86 | 27 | 15 | 24 | 45 | 14 | 56 | 19 | 13 | 12 | 41 | 33.0 ± 5.48 |
| 29 | 24 | 64 | 40 | 49 | 42 | 15 | 18 | 47 | 39 | 57 | 32 | 36 | 18 | *82* | 40.2 ± 5.08 |
| 30 | 33 | *55* | 34 | 48 | 30 | 19 | 29 | 31 | 45 | 28 | *25* | 43 | 37 | 22 | 33.5 ± 2.80 |
| Mean | 24.5 | 55.7 | 36.5 | 53.3 | 30.5 | 22.5 | 20.3 | 35.7 | 34.2 | 2.2 | 30.7 | 31.5 | 14.3 | 59.7 | |
| SEM | 2.59 | 5.26 | 1.59 | 6.69 | 2.94 | 4.90 | 2.95 | 3.40 | 5.85 | 5.05 | 4.04 | 3.70 | 3.30 | 950 | |
| ***Regimen 1.*** Benzene concentration = 300 ppm | | | | | | | | | | | | | | | |
| 37 | 32 | 14 | *46* | 23 | 8 | 10 | *5* | *5* | 14 | 25 | 10 | 69 | 5 | 140 | 28.8 ± 10.62 |
| 38 | 40 | 0 | 44 | 1 | 7 | 9 | 1 5 | 16 | 22 | 14 | 16 | 28 | io | 52 | 18.0 ± 4.28 |
| 39 | 22 | 3 | 41 | 33 | 9 | *5* | 25 | 19 | 28 | 18 | 10 | 23 | 3 | 37 | 19.55 3.60 |
| 40 | 16 | 2 | 26 | 6 | 3 | 11 | 9 | 34 | 31 | 6 | 30 | 15 | 12 | 36 | 17.0 ± 3.48 |
| 41 | 30 | 21 | 36 | 35 | 15 | 58 | 38 | 68 | 16 | 58 | 5 | 53 | 11 | *68* | 37.1 ± 6.17 |
| 42 | 16 | *5* | 28 | 63 | 24 | 9 | 7 | 32 | 49 | 7 | 24 | 31 | 8 | 20 | 23.62 4.88 |
| Mean | 26.0 | 7.5 | 36.8 | 26.8 | 11.0 | 17.0 | 16.5 | 29.0 | 26.7 | 21.3 | 15.8 | 36.5 | 8.2 | 58.8 | |
| SEM | 3.93 | 3.35 | 3.41 | 9.18 | 3.04 | 8.24 | 5.21 | 8.94 | 5.21 | 7.88 | 3.88 | 8.32 | 1.45 | 17.54 | |
| ***Regrmen 2.*** Benzene concentration = 0 ppm | | | | | | | | | | | | | | | |
| 31 | 31 | *50* | 29 | 36 | 39 | 11 | 45 | 26 | 38 | 49 | 13 | 27 | 7 | 100 | 35.8 ± 6.11 |
| 32 | 19 | 35 | 23 | 48 | 30 | 30 | 26 | 22 | 21 | 40 | 36 | 7 | 17 | 182 | 38.3 ± 11.40 |
| 33 | 17 | 33 | 34 | 50 | 14 | 19 | 26 | 40 | 14 | 39 | 16 | 33 | 19 | 91 | 32.2 ± 5.82 |
| 34 | 37 | 42 | 40 | 57 | 21 | 28 | 25 | 68 | 12 | 61 | 49 | 37 | 14 | 134 | 44.6 ± 8.24 |
| 35 | 23 | *48* | 61 | 50 | 27 | 16 | 29 | 22 | 13 | 33 | 32 | 29 | 14 | 124 | 37.2 ± 7.66 |
| 36 | 21 | 41 | 53 | 53 | 13 | 23 | 38 | 30 | 26 | 44 | (47)u | *50* | *5* | 137 | 41.5 ± 8.38 |
| Mean | 24.7 | 41.5 | 40.0 | 49.0 | 24.0 | 21.2 | 31.5 | 34.7 | 20.7 | 44.3 | 29.2 | 30.5 | 12.7 | 129.0 | |
| SEM | 3.16 | 2.77 | 5.93 | 2.90 | 4.08 | 2.96 | 3.33 | 7.20 | 4.11 | 3.98 | 6.64 | 5.76 | 2.26 | 12.63 | |
| ***Regmen 2.*** Benzene concentration = 300 ppm | | | | | | | | | | | | | | | |
| 43 | 40 | 3 | 20 | 48 | 14 | 2 | 16 | I5 | 16 | 58 | 13 | 61 | 3 | 141 | 31.5 ± 10.70 |
| 44 | 42 | 14 | 2 | 13 | 19 | 1 | 20 | 54 | 0 | 13 | 32 | 21 | 0 | 118 | 23.6 ± 8.90 |
| 45 | 24 | 9 | 1 | 10 | 10 | 2 | 7 | 43 | 7 | 31 | 22 | 18 | (20)a | (19)a | 15.3 ± 3.32 |
| 46 | a | | | | | | | | | | | | | | |
| 47 | 23 | 23 | 1 | 9 | 11 | 42 | *46* | 52 | 11 | 59 | (54) | 49 | 99 | 82 | 41.41 8.20 |
| 48 | 25 | 33 | 36 | *50* | 24 | 1 | 36 | 29 | 21 | 14 | 33 | 31 | 2 | 242 | 42.5 ± 17.06 |
| Mean | 30.8 | 16.4 | 12.0 | 26.0 | 15.6 | 9.6 | 25.0 | 38.6 | 11.0 | 35.0 | 25.0 | 36.0 | 26.0 | 145.8 | |
| SEM | 4.19 | 5.29 | 7.01 | 9.42 | 2.62 | 8.10 | 7.04 | 7.37 | 3.62 | 10.11 | 4.71 | 8.27 | 24.34 | 34.30 | |

Number of PCE per 1000 erythrocytes with means and standard error of the means (SEM) calculated for each exposure group at each sample time. The mean response ± the SEM across time for each mouse is presented as a temporal average.
*Abbreviations*: a. animal died: u, slides unscorable due to technical reasons: ( ), estimated value by averaging two nearest data points, these values used in the calculation of the temporal averages but not in the calculation of the weeklv means.