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Behavioral teratology investigation of tertiary-butanol administered by inhalation to rats

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Abstract. Two concentrations of tertiary-butanol (t-butanol; 6000 and 12,000 mg/m^3) were administered by inhalation to groups of 15 pregnant Sprague-Dawley rats for 7 hr/day on gestation days 1 - 19; groups of 18 male rats were similarly exposed for 7 hr/day for 6 weeks, and mated to unexposed females. Litters were culled to 4 female and 4 male pups at birth, and were fostered to untreated controls. From days 10-90, offspring were tested for neuromotor coordination (ascent on a wire mesh screen, rotorod), activity (open field, photoelectrically-monitored activity, and running wheel), and learning (avoidance conditioning and operant conditioning). In addition, brains from 10 offspring/group at 21 days of age were dissected into cerebrum, cerebellum, brainstem, and midbrain. Each sample was assayed for protein and the neurotransmitters acetylcholine, dopamine, norepinephrine, serotonin, met-enkephalin, B-endorphin, and substance P. The results indicated that the high concentration of t-butanol was maternally toxic, reducing feed intake and maternal weight gain. Few differences, however, were found from controls in either the behavioral or neurochemical measures.

Key Words. Alcohols. t-butanol. t-butyl alcohol. Tertiary alcohols. Developmental neurotoxicology. Behavioral teratology. Fetal alcohol syndrome.

Alcohols are widely used as industrial solvents. The vast literature for ethanol (e.g., Able, 1981, 1982) discusses both structural malformations and behavioral teratogenicity. Because of the demonstrated teratogenicity of ethanol and the lack of evidence, either positive or negative, of the developmental toxicity of other structurally-related alcohols used in the industrial setting, a large study was undertaken to assess both types of endpoints for several of these industrial alcohols (Nelson et al., 1990). With inhalation being the primary route for occupational exposures, we selected this as the exposure route. After completing developmental toxicology assessments of these alcohols, behavioral teratology evaluations have been completed for ethanol (Nelson et al., 1985, 1988b), 1-propanol (Nelson et al., 1989b), and 1-butanol (Nelson et al., 1989c). This report presents our results for tertiary-butanol, the fourth (and final) alcohol examined by us for behavioral teratogenicity.

Tertiary-butyl alcohol (t-butanol or 2-methyl-2-propanol) is used: (a) for the removal of water from substances; (b) in the manufacture of perfumes, flotation agents, flavors, cellulose esters, plastics, lacquers, and paint removers; (c) in the extraction of drugs; and, (d) as a solvent (Rowe & McCollister, 1982). Since the oral LD50 of t-butanol in rats is 3.5 g/kg, it is considered relatively nontoxic. Exposure standards for t- butanol are 300 mg/m^3 [100 ppm] for both the Permissible Exposure Limit established by the Occupational Safety and Health Administration (OSHA; 29 CFR 1910.1000) and the Threshold Limit Value recommended by the American Conference of Governmental Industrial Hygienists (ACGIH, 1987).

Since t-butanol is not metabolized by alcohol dehydrogenase, and is oxidized slowly in vivo, it is used occasionally to investigate ethanol's mechanism of action (viz., to determine if ethanol or a biotransformation product of ethanol is the proximate toxicant). For example, Daniel and Evans (1982) compared the effects of ethanol and t-butanol on postnatal development of mice. Administering 3.6% ethanol-derived calories, or 0.5, 0.75, or 1.0% t-butanol in liquid diet to groups of 15 Swiss Webster (Cox) mice on gestation days 6-20, they tested offspring from 8 litters/group on five tests of weanling behavior. They observed that "... t-butanol was approximately 5 times more potent than ethanol in producing a developmental delay in postparturition physiological and psychomotor performance scores."

Using an artificial rearing technique, Grant and Samson (1982) found that neonatal rats given ethanol or t-butanol became microcephalic. These alcohols produced visible intoxication at blood concentrations of approximately 250 mg ethanol/100 ml blood or 50 mg t-butanol/100 ml blood. In addition, these same levels of ethanol and t-butanol produced comparable microcephaly, without reductions in total body, liver, or heart weights. In a developmental toxicology evaluation of t-butanol (Nelson et al., 1989a), 10,000 ppm [30,000 mg/m^3] t-butanol was severely toxic, killing 5 of 6 exposed rats. Consequently, concentrations of 2000, 3500, or 5000 ppm [6000, 10,500 or 15,000 mg/m^3, respectively] t-butanol were administered to groups of approximately 15 pregnant Sprague-Dawley rats for 7 hr/day on gestation days 1-20. The high concentration of 5000 ppm [15,000 mg/m^3] t-butanol produced narcosis in the exposed animals. In the developmental toxicology study, all three concentrations produced intoxication as evidenced by unsteady gait in the maternal animals. In addition, maternal feed consumption and weight gain were inhibited at 5000 ppm (15,000 mg/m^3] t-butanol, but objective maternal effects were not seen at the two lower concentrations. Although teratogenicity was not observed at any concentration, fetotoxicity was apparent at all three concentrations as manifested by a dose-dependent reduction in fetal weights and an increase in skeletal variations. Nelson et al. (1989a) speculated that this fetotoxicity may be manifested postnatally by an increase in functional deficits and/or postnatal deaths, similar to that reported by Daniel and Evans (1982).

Accordingly, the present study was undertaken to investigate the behavioral teratogenicity of t-butanol administered by inhalation to rats. The concentrations were selected based on the study just cited, with 4000 ppm [12,000 mg/m^3] selected as the high concentration and 2000 ppm [6000 mg/m^3] as the low concentration.

Materials and Methods

The procedures used for generation of t-butanol vapors have been described previously (Nelson et al., 1989a). Briefly, a micrometering pump controlled injection of a specified amount of reagent grade tertiary-butanol (MCB Chemicals; Curtin Matheson Scientific Co.; Florence, KY) into one inlet of a three-way valve. Heated, compressed air was introduced into a second inlet of the valve, and the liquid-air mixture was forced through the valve outlet into a Greensmith impinger for evaporation and mixing. The concentration was controlled by modifying the flow of chemical and the temperature of the compressed air. This vapor mixture was introduced into a 0.5 m^3 Hinners exposure chamber (Charles Spengler and Associates; Cincinnati, OH) upstream from an orifice plate, and the resulting turbulence provided uniform mixing of the vapor and air before the mixture entered the chamber (as determined in unpublished previous research). Air flow was approximately 0.5 m^3/minute. Because t-butanol freezes near room temperature, it was necessary to insulate and heat the lines into the chamber, resulting in chamber temperatures which consistently ran at a predetermined maximum of 80 degree F; relative humidity was maintained at 50 ± 10%.

Chamber concentrations near the animals' breathing zone were continuously monitored by a Miran 1A infrared analyzer (Wilkes/Foxboro Analytical; South Norwalk, CN) that had been calibrated previously at the concentrations used in this study. Calibration checks were performed daily prior to, and after, the exposure. In addition to being connected to a stripchart recorder for continuous monitoring, the infrared analyzer was interfaced with an Apple II+ computer which calculated hourly means from chamber atmosphere samples collected every five minutes throughout the 7-hour exposures. Daily means were calculated using the hourly readings taken directly from the infrared analyzer and the hourly means calculated by the computer. These two daily means were used to calculate independent overall study mean (± SD) concentrations.

Concentrations of 0 (sham-exposed controls), 6000, and 12,000 mg/m^3 t-butanol were administered for 7 hr/day by inhalation to groups of 15 pregnant Sprague-Dawley rats (VAF+, Charles River Laboratories, Wilmington, MA) on gestation days 1-20 (sperm = day 0). In addition, groups of 18 male Sprague-Dawley rats (initial mean weights approximately 450g) were exposed 7 hr/day for 6 weeks to the same concentrations of t-butanol. These male rats were subsequently mated to non-exposed females (one per male).

On the day of birth (defined as day 0 for purposes of scheduling tests), litters were culled to 4 females and 4 males (± 1), and fostered to untreated controls. Offspring were weighed individually each week through 5 weeks of age. On postnatal day 10, one male and one female per litter were randomly assigned to one of four groups and tested using procedures described previously (Nelson et al., 1988b, 1989b). Briefly, this testing included: Ascent on a wire mesh screen (days 10, 12, 14); activity in an open field and a photoelectrically-monitored activity device (days 16, 17, 18; 30, 31, 32; 44, 45, 46; 58, 59, and 60); running wheel activity (days 32,33); avoidance conditioning (separate groups tested beginning on days 34 and 60); and, operant conditioning (beginning day 40). In addition, brains from 10 offspring/group at 21 days of age (one female and one male/litter) were collected after microwave fixation, and dissected into four general brain regions (cerebrum, cerebellum, brainstem, and midbrain; as previously described by Nelson et al., 1984). These samples were analyzed, in separate batches for the two concentrations, as described previously (Nelson et al., 1988b, 1989b) for steady-state levels of protein and the neurotransmitters acetylcholine, dopamine, norepinephrine, serotonin or 5-hydroxytryptamine, metenkephalin, B-endorphin, and substance P.

As the two concentrations of t-butanol were run at different times (separated by approximately three months), data were compared only against the respective control group and comparisons between the two concentrations are not appropriate. For behavioral and other data sets with multiple dependent measures, statistical analyses employed multivariate analysis of variance (MANOVA), followed by analysis of variance (ANOVA) if the MANOVA was significant (Dunn & Clark, 1974; Dixon, 1985; Freeman, 1987). MANOVA was used to reduce the probability of making Type I errors when multiple dependent measures are analyzed. Where single dependent measures were obtained, only ANOVA was used. For all ANOVAs, the degrees of freedom of all within-litter main effects and interactions were corrected with the Greenhouse-Geisser estimate of Box's epsilon, since ANOVA is not robust when there are repeated measurements of an experimental unit and the measure-to-measure correlations are unequal. The major independent variable of interest was treatment group. Following a significant ANOVA for the main effect of group or an interaction involving group, two preplanned contrasts were made, one for maternally-exposed animals vs controls and the other for paternally-exposed animals vs controls, each with alpha = 0.05.

Neurochemical data were analyzed initially using MANOVAs on the log-transformed data (with the four dependent variables in each MANOVA being the log of the levels for a given chemical in each of the four brain regions). This transformation conformed to the assumptions required for MANOVA (equal covariance matrices) and ANOVA (normality and homogeneity of variance). These MANOVAs were followed by univariate tests for each brain region and chemical. An ANOVA was used where the residuals were judged to be normal (Shapiro-Wilk test p <0.05) and the variance appeared to be homogeneous (Levene's test p <0.05); Sheffe's test was used for multiple comparisons. The Kruskal-Wallis test was used where the residuals were judged to be nonnormal, but the variance was homogeneous; Dunn's test was used for multiple comparisons. The median test was used where the criteria for normality and homogeneity of variance were not met (Hollander and Wolfe, 1973).

Since a large number of differences between protein levels of treated and control groups were noted in these analyses (unrelated to exposure concentration), and all other chemicals were measured as a ratio to protein (viz., amount of chemical/ug protein), the log of the protein level was treated as a covariate in subsequent analyses. An 'F' test was first used to establish that the regression lines (of the log of the chemical concentration as a function of the log of the protein level) were parallel for the different treatments. Also, the data were analyzed to determine if the log of the chemical concentration and the log of the protein level were substantially correlated (viz., <0.3; see Dunn & Clark, 1974). Both conditions (parallel regression lines and the substantial correlation of chemical concentration with protein level) were met in most cases. For these cases, the adjusted means were tested for equality and then compared using pairwise 't' tests (BMDP program IV; Dixon, 1985). To ensure an overall 5% confidence level in making the three possible pairwise comparisons (i.e., chemical X region X specific exposure level), the significance level for a single comparison was established at p <0.017.

Results

Vapor concentrations of t-butanol are not easily generated because of its low volatility and the tendency for t-butanol to freeze at 25.5 degree C (near room temperature). Nonetheless, using heated and insulated generation lines, concentrations remained quite stable. Mean exposure concentration (± SD), as determined from hourly infrared analyzer readings, over the 126- and 107-day exposure periods were 12,000 (± 140) mg/m^3 and 6000 (± 60) mg/m^3 for target concentrations of 12,000 mg/m^3 and 6000 mg/m^3, respectively. Daily mean exposure concentrations derived from the computer-logged, five-minute samples were 12,098 (± 267) mg/m^3 and 6027 (± 284) mg/m^3), for target concentratins of 12,000 and 6000 mg/m^3, respectively. Mean charcoal tube results for 195 samples at 12000 mg/m^3 were 12000 (± 160) mg/m^3, and for 145 samples at 6000 mg/m^3, the mean results were 5970 (± 270) mg/m^3. Since all mean values are within 1% of the target concentrations, the target concentrations are cited throughout this paper. Chamber temperatures over the exposure periods averaged 80.6 (± 1.0) degree F and 80.2 (± 1.3) degree F, and the relative humidity averaged 44 (± 3)% and 38 (± 5)%, for the high and low concentrations, respectively. Control chamber temperatures corresponding to these same concentrations were 75.0 (± 1.1) degree F and 76.3 (± 1.8) degree F, and the relative humidity averaged 51 (±4)% and 42 (± 7)%, respectively.

At the high concentration of t-butanol (12,000 mg/m^3), mean male weights increased from 484 (± 30)g to 532 (± 31)g over the six-week exposure period. Weight gain in the maternally-exposed pregnant rats was significantly lower than that of controls, particularly during the first week of exposure (exposed = 266 ± 18g, 254 ± 16g, 278 ± 19g, and 351 ± 32g vs control = 260 ± 18g, 278 ± 25g, 308± 29g, and 380 ± 39g, [means ± SD for days 0, 7, 14, and 21, respectively]). Feed consumption among maternally-exposed pregnant rats was similarly lower than for controls in the first week of exposure (79[± 21] g vs 130 [± 26] g; Kruskal Wallis X^2 = 22.83, p = 0.001). Water intake was higher for the maternally-exposed group during the third week of pregnancy than for controls (466[± 87]g vs 304 [± 58]g; ANOVA F[2, 38] = 18.81, p = 0.0001). At the lower concentration of t-butanol (6000 mg/m^3), mean male weight increased from 450(± 40)g to 548(± 45)g over the six weeks of exposure. Weight gain, feed intake, and water consumption for the maternally-exposed pregnant rats were apparently unaffected by t- butanol exposure.

For the behavioral data, few differences were seen between the control group and either the maternally-exposed or the paternally-exposed groups (data not shown). At the highest concentration of t-butanol (12,000 mg/m^3), the main effect for group was significant for rotorod performace (F[2,41] = 20.09, p = 0.0001). Both the maternal group mean of 26 revolutions per minute (rpm) (F[1,41] = 4.82, p = 0.039) and the paternal group mean of 20 rpm (F[1,41] = 39.79, p = 0.0001) were significantly higher than the control mean of 16 rpm. For open field testing, the main effect of treatment group (on latency to reach the outer circles of the field) was significant (F[2,41] = 4.45, p = 0.018). The maternally-exposed group mean latency did not differ significantly from the control group mean latency (p = 0.10), but the paternally-exposed group mean latency of 115 seconds was significantly lower than the mean latency for the control group of 210 seconds (p = 0.005). No other group comparisons were significant at the higher concentration of t-butanol, although significant sex and day differences were seen with some tests (unrelated to treatment group).

At the lower concentration of t-butanol (6000 mg/m^3), the only significant group effect was in performance on the ascent test (MANOVA lambda = 0.699; F(4,80) = 3.92; p = 0.006). The ANOVA for the time the animals held the wire was not significant (p = 0.63), but the ANOVA for the distance the animals climbed on the screen was significant (F[2,41] = 5.67; p = 0.007). The difference between the paternally-exposed and the control groups was not significant (p = 0.08), but the mean time the animals held the wire (10 sec) for the maternally-exposed group was significatly less than the mean time (16 sec) for the control group (F[1,41] = 11.33, p = 0.002). Some significant sex and day differences were apparent (unrelated to treatment group), but no other group comparisons were significant at the lower concentration of t-butanol.

For the neurochemistry evaluations, only a few statistically significant differences were seen between exposed and control groups using analysis of covariance (with protein level as the covariate). At the highest concentration of t-butanol (12,000 mg/m^3), group differences were significant for norepinephrine in the cerebellum (control 3.03 ± 1.24 vs maternal 1.41 ± 0.26 and paternal 1.84 ± 0.34 pg/ug protein; $F[2, 26] = 6.33$, $p = 0.006$), met-enkephalin in the cerebrum (control 0.630 ± 0.314 vs maternal 0.268 ± 0.075 and paternal 0.381 ± 0.127 pg/ug protein; $F[2, 26] = 4.26$, $p = 0.025$), and B-endorphin in the cerebellum (control 0.159± 0.070 vs maternal 0.062 ± 0.05 and paternal 0.046 0.046 pg/ug protein; $F[2, 26] = 4.95$, $p = 0.015$). At the lower concentration of t-butanol (6000 mg/m^3), significant differences were found for serotonin in the midbrain (control 49.3 ± 12.8 vs maternal 16.5± 3.46 and paternal 26.1 ± 31.4 pg/ug protein; $F[2,28] = 1.31$, $p = 0.009$) and met-enkephalin in the cerebrum (control 1.524 ± 0.758 vs maternal 0.260± 0.073 and paternal 0.381 ± 0.157 pg/ug protein, $F[2, 28] = 8.90$, $p = 0.001$). Although the differences seen in cerebral met-enkephalin levels were in the same direction at both concentrations of t-butanol, the reductions were not dose-related.

Discussion

In this behavioral teratology evaluation of tertiary-butanol administered by inhalation to rats, few statistically significant effects were seen. Since the small number of statistically significant behavioral and neurochemical effects did not provide evidence of a dose-effect relationship or a discernible pattern of effects, this finding suggests that the few effects were likely of little or no biological significance. However, it would seem prudent to investigate t-butanol further before concluding that no effects would be seen in offspring following prenatal exposure. The small number of significant effects may be the result of low blood concentrations of t-butanol, although these blood concentrations were not measured in the present study. We have observed relatively low blood concentrations in rats following inhalation exposure to ethanol (Nelson et al., 1985, 1988b), n-propanol, and isopropanol (Nelson et al., 1988a). For example, exposure of rats to 20,000 ppm ethanol for 7 hr/day produced blood concentrations below 200 mg/dl, whereas administration of 5-6% ethanol in liquid diet generally produces blood concentrations above 200 mg/dl (discussed by Nelson et al., 1985). Consequently, we expect that relatively low blood levels were achieved with these t-butanol exposures. Nonetheless, the high exposure concentration of t-butanol produced maternal toxicity, as evidenced by lower feed intake and weight gain. Since a primary concern in developmental toxicology is toxicity in the developing conceptus relative to toxicity in the adult animal (see, e.g., Johnson, 1984), our results suggest a lack of selective developmental toxicity for t-butanol in rats.

The present results appear to conflict with the t-butanol data reported by Daniel and Evans (1982). Whereas we saw few consistent effects produced by t-butanol, Daniel and Evans reported high mortality rates at the highest concentration (1.0%) t-butanol (administered in liquid diet), and found substantial functional deficits in survivors at that concentration as well as at lower concentrations. Daniel and Evans (1982) observed a relatively high rate of neonatal death (20% at their highest dose), compared to no deaths at the highest dose in the present study; furthermore, Daniel and Evans reported significant weight reductions (approximately 40%), compared to no weight reduction at the highest dose in the present study. Since the Daniel and Evans study used mice, it is possible that some of the effects they reported may not be due to differences in blood concentrations, but may instead indicate that mice are more sensitive than rats to the developmental toxicity of t-butanol. Other dissimilarities between the two studies could account for differences in mortality rates and weight reduction. For example, the Daniel and Evans study: (a) Administered t-butanol in liquid diet (thereby creating the potential for 24 hr/day exposures); (b) fostered only half of the litters (for a total of 7 or 8 litters); and, (c) conducted behavioral observations using only 4 fostered litters and 4 nonfostered litters. In the Daniel and Evans study, lower offspring survival and weight reductions were most severe in nonfostered litters. Our study, which used inhalation exposure of rats for 7 hr/day and fostered all litters, did not assess developmental effects in nonfostered litters. Based on a comparison of blood levels observed with ethanol following inhalation versus oral administration, and on the much more severe effects reported by Daniel and Evans (1982), we speculate that the blood levels seen in their study were much higher than those we attained (although neither study measured blood levels).

Few significant neurochemical effects were found in this study. The small number of significant effects in the present study may be due to the conservative statistical analyses used (i.e., much more conservative than those commonly used for neurochemical analyses). As discussed earlier (see Materials and Methods' above), however, the MANOVA which we used is more appropriate than the ANOVA technique when multiple dependent measures are involved. The low number of significant effects also may be related to the inclusion of all neurochemical assay data in the present analyses. It is a routine practice in neurochemistry to discard data which fall beyond two standard deviations from the mean. (These extreme data are attributed to random error; Massari, personal communication.) In our judgement, however, all data should be used, recognizing that biological variability is normal, and accepting the possibility that experimental treatments may increase the variability seen in the data. No data were discarded in the present study, and the inclusion of extreme values in the present statistical analyses, therefore, may have reduced the chances of observing significant effects.

In summary, 12,000 and 6,000 mg/m^3 of tertiary-butanol were administered by inhalation (a) throughout gestation to pregnant female rats, and (b) for six weeks to adult male rats subsequently mated to control female rats. These procedures did not produce a consistent pattern of effects in the offspring, similar to the lack of behavioral effects we have seen with other alcohols administered by inhalation to rats. While maternal toxicity was apparent at the high concentration of t-butanol used in this study, only limited behavioral or neurochemical effects were noted in the offspring on tests conducted through 90 days of age. T-butanol administered by inhalation to rats, therefore, does not appear to produce remarkable behavioral or neurochemical deviations in offspring at these exposure concentrations.

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Disclaimer: Mention of company or product names does not constitute endorsement by NIOSH.

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