Extrapolating the Acute Behavioral Effects of Toluene from 1- to 24-h Exposures in Rats: Roles of Dose Metric and Metabolic and Behavioral Tolerance

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Recent research on the acute effects of volatile organic compounds suggests that extrapolation from short (~1 h) to long durations (up to 4 h) may be improved by using estimates of brain toluene concentration (Br[Tol]) instead of cumulative inhaled dose (C × t) as a metric of dose. This study compared predictions of these two dose metrics on the acute behavioral effects of inhaled toluene in rats during exposures up to 24 h in duration. We first evaluated estimates of Br[Tol] with a physiologically based toxicokinetic (PBTK) model for rats intermittently performing an operant task while inhaling toluene for up to 24 h. Exposure longer than 6 h induced P450-mediated metabolism of toluene. Adjusting the corresponding parameters of the PBTK model improved agreement between estimated and observed values of Br[Tol], and also at 1 and 6 h afterexposure. Effects of toluene were better predicted by Br[Tol] than by C × t. However, even using Br[Tol] as the dose metric (after accounting for metabolic induction), acute dose-effect functions during 24-h exposures were shifted to the right relative to 1-h exposures, indicating that a dynamic behavioral tolerance also developed during prolonged exposure to toluene.

Key Words: neurotoxicity; PBTK model; volatile organic compound; toluene; signal detection behavior; attention; extrapolation; metabolism induction; behavioral tolerance.

The toxicity of a chemical is often assumed to be proportional to the product of the concentration (C) and the duration (t) of exposure or C × t (Atherley, 1985; Doull and Rozman, 2000; Miller et al., 2000). This relationship, frequently termed Haber’s Rule (Gaylor, 2000), provides an approach to setting risk assessment guidelines when data are not available for the specific exposure scenario of concern. Whereas Haber’s Rule can accurately predict effects of chronic exposure when the cumulative dose of a chemical determines its toxicity (e.g., for many carcinogens) (Gaylor, 2000), it is less appropriate for acute dosing scenarios, including neurotoxicological effects of volatile organic compounds (VOCs) (Boyes et al., 2005). Specifically, using Haber’s Rule to extrapolate across exposure durations for these effects underestimates toxicity when adjusting from long to short exposure durations (Bushnell, 1997) and overestimates toxicity when adjusting from short to long durations (Crofton and Zhao, 1997).

Studies of this nature indicate that concentration exerts a stronger effect on acute toxicity than does time; therefore, the rule is often modified by raising C to some power, as proposed by ten Berge et al. (1986), with the value of the exponent (n) derived empirically from existing concentration-duration relationships. Guidelines that use this rule, Cn × t, include Acute Exposure Guideline Levels for one-time exposures of short durations (10 min–8 h) (http://www.epa.gov/opptintr/aegl/; NRC, 2001) and Provisional Advisory Levels for one-time chemical exposures of durations of 24 h, 30 days, or 2 years (Adeshina et al., 2009).

However, whereas these temporal extrapolations can be improved by the ten Berge equation, deriving the value of n requires adequate data regarding dose and duration, and its value depends upon the specific measured effect. For example, a dose-time analysis of the acute effects of inhaled trichloroethylene (TCE) on signal detection behavior in rats yielded n values of 2.2 for sensitivity (a measure of accuracy) and 7.1 for response latency (RL) when extrapolating from a 60-min exposure to a 20-min exposure, despite the fact that both effects were measured concurrently in the same animals in the same experiment (Bushnell, 1997).

Alternatively, the acute effects of VOCs are closely related to the internal dose. For example, the concentration of a VOC in the brain provides a more predictive dose metric for acute
neurophysiological effects of TCE (Boy es et al., 2000) and toluene (Boy es et al., 2007) and for acute behavioral effects of toluene (Benignus et al., 1998; Bushnell et al., 2007b; Miyagawa et al., 1986), and 1,1,1-trichloroethane (Warren et al., 2000) than does the C × t product, although results on this question are mixed for perchloroethylene (Boy es et al., 2009; Oshiro et al., 2008). Furthermore, cross-species extrapolation for VOCs can be facilitated by comparing dose-effect relationships as a function of internal dose (Benignus et al., 2009). However, this work is based on VOC exposures of short duration (≤ 4 h). It is unknown whether internal dose provides more accurate estimates of acute effects in the longer duration exposures required by many acute exposure guidelines.

The current study used toluene to compare the accuracy of two dose metrics (exposure C × t and concentration of toluene in the brain, Br[Tol]) for predicting acute behavioral effects in rats inhaling toluene for up to 24 h. The unmodified C × t relationship was used because extrapolations from short to long durations typically set n = 1 by default. Given that the effects of 1-h toluene exposures are accurately determined by Br[Tol] and not by the C × t of exposure (Boy es et al., 2007; Bushnell et al., 2007b), we hypothesized (1) that the magnitude of the acute effects of inhaled toluene would be predictable from Br[Tol] at any exposure duration and (2) that the magnitude of these effects would be better predicted by Br[Tol] than by inhaled C × t. These predictions were tested using the behavior of rats performing a visual signal detection task (SDT) previously shown to detect dose- and duration-related acute effects of toluene (Bushnell et al., 1994, 2007b; Oshiro et al., 2007) and other VOCs (Boy es et al., 2010; Bushnell, 1997; Oshiro et al., 2008). A physiologically based toxicokinetic (PBTK) model (Kenyon et al., 2008) was used to estimate Br[Tol] in rats under the various conditions of exposure and physical activity that were required for these tests.

Three experiments were conducted to test these predictions. Experiment 1 evaluated the accuracy of predictions of the PBTK model for Br[Tol] in rats periodically performing behavioral tasks while inhaling toluene for up to 24 h. Experiment 2 provided data to refine metabolic and physiological parameters for the PBTK model in rats performing those tasks. These results were used to design experiment 3, which tested the primary hypotheses of this study. In experiment 3, rats were trained to perform the SDT and then were exposed to toluene at concentrations of 0, 1125, and 1450 ppm for 24 h and to 1660 ppm for 21 h during four 30-h exposure sessions. During each toluene exposure, the animals were tested at times predetermined to yield equivalent C × t products (2900, 8700, 27,000, and 34,800 ppm-h) but different values of Br[Tol], which were estimated by the PBTK model to reach 80–140 mg/l at the end of exposure. These Br[Tol] values were selected because previous 1-h toluene inhalation studies showed that they caused significant and graded impairment of behavior in the SDT (Bushnell et al., 2007a; Oshiro et al., 2007). Signal detection behavior was also assessed 1 and 6 h after toluene exposure ended to determine whether behavioral effects were related to Br[Tol] during clearance of the compound from the brain.

Finally, to evaluate whether the effects observed during the 24-h exposure could be predicted quantitatively from data obtained from a 1-h exposure, the results of experiment 3 were compared with published data using the same behavioral assay in a 1-h exposure scenario (Bushnell et al., 2007b). This comparison showed that effects of 24-h exposure were substantially less severe than that of 1-h exposure, for given concentrations of inhaled toluene and Br[Tol]. Thus, extrapolating from 1- to 24-h exposures, using either C × t or internal dose methods, can yield erroneously high estimates of hazard because of apparent compensatory kinetic and dynamic processes that are induced by prolonged exposure to toluene.

MATERIALS AND METHODS

Subjects

Male Long-Evans rats were obtained from Charles River Laboratories (Raleigh, NC) at ~85 days of age and were weight maintained at 350 ± 10 g by scheduled home-cage feeding (Ali et al., 1992). The rats were individually housed in polycarbonate cages with kiln-dried pine shaving bedding (Northeastern Products, Warrensburg, NY) and had ad libitum access to tap water. The animal colony was controlled to an ambient temperature of 22°C ± 2°C and relative humidity 50 ± 10%, with a 12:12 h light:dark cycle (lights on at 6:00 AM). The animal housing facility was fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International according to National Institutes of Health guidelines. Animal research protocols were reviewed and approved by the U.S. Environmental Protection Agency (EPA), Office of Research and Development, National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee which ensured conformance with the 1996 National Research Council “Guide for the Care and Use of Laboratory Animals,” the Animal Welfare Act, and Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

During all toluene exposure sessions, animals lived in operant-inhalation chambers from 1 to 30 h, depending on the experiment and exposure condition. Water was available ad libitum inside the chambers at all times, and the animals earned food during performance of the operant tasks at a rate that maintained their weights within the target range of 350 ± 10 g. Temperature (23°C ± 2°C) and humidity (51 ± 7%) within each chamber was monitored using a LogTag Recorder (Model #HAXO-8; MicroDAQ.com, Contoocook, NH) for the duration of the exposures. A covered incandescent bulb (houselight), mounted inside the operant chamber, maintained the 12:12 h light:dark cycle (lights on at 6 A.M.) during all exposure sessions.

In experiment 2, 14 rats were implanted with radio telemeters in the abdominal cavity to monitor heart rate, core temperature, and motor activity (transmitter model TA11ICTA-F40; Data Sciences Int., St Paul, MN). The transmitters were surgically implanted by Charles River Laboratories at an age of 77 days; 8 days prior to the date when animals were shipped to the EPA facility. The target weight for maintenance of these animals was increased to 360 (± 10 g) to account for the weight of the telemeter.

Operant-Inhalation Chambers

Four 32.9-1 operant-inhalation chambers were constructed as previously described (Bushnell et al., 2007b). Briefly, each chamber was made of stainless steel and glass for the assessment of operant performance of rats inhaling controlled concentrations of solvent vapors. Each chamber contained two retractable response levers, a food cup for delivery of reinforcers for correct responses, a loudspeaker for generating background noise (65 dB[A]), a houselight, and a signal light. Signal intensities and background illumination...
in the chambers were measured using a photometer with cosine probe attachment (models DR2000 and D2000, respectively; EG&G, Inc., Salem, MA). Four signal intensities, ranging from 0.3 to 5.2 lux, were presented above background illumination from the signal light of 0.15 lux and a houselight illumination of 0.2 lux. MED PC software (Med Associates, St Albans, VT) controlled all behavioral testing, signal stimuli, and vapor concentration monitoring (see below).

Some modifications were made to the four operand-inhalation chambers to accommodate the radio-telemetered rats used in experiment 2. The radio signals from the telemetric units are normally detected by a receiver board placed beneath a rat’s cage (Model RPC-1; Data Sciences Int.). Because the boards were too large to fit in the inhalation chambers, the receiver coils in the receiver boards were removed and secured to a smaller platform that fit within the ceiling of the inhalation chamber and provided excellent detection of the radio-transmitter signal. The telemetry data were collected at 2-min intervals and stored and analyzed on the telemetry acquisition system (ART Gold 4.0; Data Sciences Int.).

**Inhalation Exposures**

Toluene (99.5% pure, ACS grade; Sigma–Aldrich Chemical Co., St Louis, MO) vapor was generated within each operand-inhalation chamber and monitored as previously described (Bushnell, 1997; Oshiro et al., 2007). Liquid toluene was dispensed by a fluid metering pump (Model# RHO0CCLF; Fluid Metering Inc., Syosset, NY) into a heated stream of zero grade nitrogen gas, yielding concentrated toluene vapor that was mixed with filtered air. Inlet air was cleaned with carbon and high efficiency particulate air filters. Airflow rates through these exposure chambers were ~20 1/min. The rise time (ty) of toluene vapor concentrations was 6 min. Behavioral tests began at variable times during the exposure sessions (see below). Each rat was always tested and exposed in the same chamber. An infrared spectrophotometer (MIRAN 1A; The Foxboro Co., Bridgewater, MA) was used to monitor toluene vapor concentrations sequentially in each chamber throughout the sessions. Target vapor concentrations for experiment 1 (775 and 1125 ppm), experiment 2 (0 and 1125 ppm), and experiment 3 (0, 1125, 1450, and 1600 ppm) included the nominal toluene concentration ± 10%.

**Behavioral Tests**

**Lever-pressing task.** Rats used in experiments 1 and 2 were trained to perform a simple lever-pressing task (LPT) that produced levels of physical activity and heart rate equivalent to those required for the SDT (Bushnell et al., 2007c; Kenyon et al., 2008) used to assess attention in experiment 3. The task timing and presentation of stimuli were the same as the SDT (described below), but no discrimination training was involved (to shorten training time). For the LPT, only one of the two levers was inserted into the chamber on each trial. A press on this lever was always a correct response. Rats thus performed this task with 100% accuracy and received a timeout (as described below) only for failure to press the lever. The reinforcement rate for the LPT was reduced to 70% of responses to keep food intake similar to that received by rats performing the SDT.

**Signal detection task.** Rats used in experiment 3 were trained to perform a visual SDT as previously described (Bushnell, 1997; Bushnell and Rice, 1999). Briefly, after a variable intertrial interval, selected randomly from a list of delays ranging from 0.30 to 24.39 s, rats were required to report the occurrence or nonoccurrence of a signal (a 300-ms light flash) by pressing one of the two response levers. Both levers were inserted at variable time (2–4 s) after the signal period during each trial and retracted when either was pressed. Signal and blank trials were intermixed unpredictably in equal number during each test and differed only in that no signal occurred during the signal period on a blank trial. One lever was designated as the signal lever and the other lever as the blank lever. Each correct response, i.e., a press on the signal lever on a signal trial or a press on the blank lever on a blank trial caused illumination of the food cup light (2 s) and delivery of a food pellet (Poy Noyes Co., Lancaster, NH) on 80% of these trials. This rate of food delivery prevented satiating the rat. After each incorrect response (i.e., a press on the signal lever on a blank trial or a press on the blank lever on a signal trial) or after a response failure, the houselight was turned off for 3 s (timeout period) and no food was delivered. Both levers were retracted when either lever was pressed or the rat failed to respond within 5 s (response failure). Trials lacking a response were not repeated. During training and to acquire baseline performance, the task was conducted in daily tests at a rate of approximately five trials per minute for a 48-min test.

During the exposure assessments, each SDT or LPT test lasted 48 min. The number of tests given depended upon the experiment and time point for the exposure. Two minutes prior to the start of each test, the rat was alerted that a test was about to start by illuminating the food cup and delivering pellets into it. Rats earned ~7 g of food during each test.

**Direct Behavioral Measures**

**Lever-pressing task.** Rats were recorded as in the SDT (see below), however, these measures were not taken as behavioral endpoints, and the task was used solely to increase the rats’ activity for the PBTK model and metabolic and physiological evaluation studies.

**Signal detection task.** The number of “hits” (signal-lever presses on signal trials), “correct rejections” (blank-lever presses on blank trials), “false alarms” (signal-lever presses on blank trials), and “misses” (blank-lever presses on signal trials) were recorded in five 9.6-min blocks during the 48-min test. RLs for each response type were also recorded in each block and were defined as the time between insertion of the levers into the chamber and the time at which a lever press was recorded. The number of hits and misses during signal trials were also recorded for each signal intensity. Finally, the number of trial completions, defined as trials ending with a choice response, was recorded for each 9.6-min block.

**Calculated Behavioral Measures (for SDT only)**

The primary outcome measures for experiment 3 (behavior assessment) were overall accuracy, calculated as the proportion of correct responses (P(Cr) = (hits + correct rejections)/(hits + misses + correct rejections + false alarms)) and overall mean RL, defined as the cumulative RL for all response types divided by the total number of trials completed. These measures were calculated for each of five 48-min tests that the rat performed during the 30-h exposure session (three tests during toluene exposures and two tests after exposures terminated). P(Cr) and RL data from tests containing fewer than 25 trials were eliminated from the analysis.

**Experiment 1: Evaluation of the PBTK Model**

**Study design.** Forty rats were trained to perform the LPT and then between the ages of 7.5 and 8.5 months of age were exposed to toluene vapor at concentrations of 775 or 1125 ppm for 1, 6, or 24 h. Lever-pressing tests were administered during the exposure sessions to simulate the activity of rats performing the SDT (Table 1). Blood and brains were collected after 1, 6, and 24 h of exposure and after 6 h in air following a 24-h exposure (n = 5 per time point). LPT testing coincided with these tissue collection times, with each 48-min test ending at the tissue collection time. One additional LPT test was given that ended up to five LPT tests (active period) during the 30-h session.

**TABLE 1**

<table>
<thead>
<tr>
<th>Exposure length (h)</th>
<th>Active period(s) (h of exposure session)</th>
<th>Brain/blood collection time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2–1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0.2–1 and 5.2–6</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>0.2–1, 5.2–6, and 23.2–24</td>
<td>24</td>
</tr>
<tr>
<td>24</td>
<td>0.2–1, 5.2–6, 23.2–24, 24.2–25, and 29.2–30</td>
<td>*30</td>
</tr>
</tbody>
</table>

Note. Rats inhaled toluene (775 or 1124 ppm) for up to 24 h. Rats performed up to five LPT tests (active period) during the 30-h session.

*30 Indicates a sample taken 6 h after a 24-h exposure.
Three rats were eliminated from the PBTK evaluation study. One rat was found in a moribund state on the day of exposure and was thus eliminated from the 1125 ppm 6-h exposure group. Two more rats were eliminated from the 1125 ppm 6-h exposure group. Two more rats were found in a moribund state on the day of exposure and were thus removed from the study.

Results. Three rats were eliminated from the PBTK evaluation study. One rat was found in a moribund state on the day of exposure and was thus eliminated from the 1125 ppm 6-h exposure group. Two more rats were eliminated due to toluene pump failure during the exposure: one rat each from the 1125 ppm 30-h and the 775 ppm 1-h time points. This left an n = 4 for each of these conditions. The mean exposure concentration for each of the timer conditions fell within 10% of its nominal concentration and the results are listed in Supplement 3 of this report.

Simulations using the previously published PBTK model (Kenyon et al., 2008) fell within the range of the measured Br[Tol] values after 1 and 6 h of exposure but underestimated Br[Tol] levels at the 24-h time point and at 6-h postexposure (Fig. 1A). Further detail concerning the model is provided in Supplement 3 of this report.

Given that hepatic cytochrome P450 enzyme induction in rats has been reported in conjunction with toluene exposures of shorter duration and higher concentration (Wang et al., 1993), we hypothesized that increased metabolism lowered Br[Tol] to values below those estimated by the model at the later time points. Note that Wang et al. (1993) reported induction of both CYP 2E1 and 2B in rats exposed to toluene for 6 h at concentrations of 500, 1000, 2000, and 4000 ppm. Furthermore, it is also possible that toluene caused lethargy in the rats immediately after exposure, which would reduce their activity levels and thus the likelihood of toluene exposure in the environment.

Table 2: Study Design for Metabolic and Physiological Evaluations

<table>
<thead>
<tr>
<th>Exposure length</th>
<th>Active periods</th>
<th>Liver collection</th>
<th>Physiological monitoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>0.2–1, 5.2–6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>0.2–1, 5.2–6, 23.2–24</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>0.2–1, 5.2–6, 23.2–24, 24.2–25, and 29.2–30</td>
<td>30</td>
<td>h30</td>
</tr>
</tbody>
</table>

Note. Rats inhaled air or toluene (1125 ppm) for 6 or 24 h. Rats performed up to five LPT tests during the 30-h session (active periods). 30 indicates a sample taken 6 h after a 24-h exposure. h30 indicates monitoring occurred throughout a 24-h exposure plus 6 h after exposure.
animals and thus reduced breathing and heart rates, thus reducing uptake of toluene in the animals. These possibilities were investigated in experiment 2.

**Experiment 2: Metabolic and Physiological Assessments**

**Study design.** Thirty-two rats were trained to perform the LPT. Fourteen of these animals had been previously implanted with radio telemeters (see Subjects, above) that report physical activity, heart rate, and core body temperature. All animals, aged 4–5 months, were then exposed in operant-inhalation chambers to 0 (air) or 1125 ppm toluene for 6 or 24 h, while also performing the LPT at designated times (Table 2). Rats were killed as in experiment 1 after 6 or 24 h of exposure to clean air or toluene or after 6 h of inhaling clean air after a 24-h exposure (30-h time point) \((n = 6\) per group). Livers were collected, weighed, and the right lateral lobe was removed and immediately flash frozen in liquid nitrogen. Microsomal fractions were prepared from this tissue as described previously (De Vito et al., 1993). Microsomal protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin (Sigma) as the standard. Hepatic microsomal pentoxyresorufin-O-dealkylase (PROD) activity (a marker of CYP 2B activity) was assayed according to De Vito et al. (1993). We chose to evaluate PROD because this marker demonstrated a stronger dose-dependent induction compared with other CYPs reported to be induced by toluene exposure (Wang et al., 1993) and also because CYP 2B is the enzyme predominantly involved in toluene metabolism at higher exposure concentrations. Measurements of the extent of enzyme induction were undertaken in experiment 2 at specific time points of interest to obtain biological support for our hypothesis that longer term continuous exposure to toluene (24 h) results in induction that is persistent for at least 6 h after exposure ends.

Six of the telemetered rats were placed in each of the two groups assigned to 30-h exposure sessions (0 or 1125 ppm) to allow physiological monitoring throughout the entire exposure and postexposure periods. Two additional telemetered animals were exposed to toluene for 24 h, one at 0 ppm and the other at 1125-ppm toluene. These rats served as extras in case of telemetry failure prior to exposures.

**Results.** The mean toluene concentration for each timed condition in experiment 2 fell within 10% of the target concentration and all rats were used in the evaluations (see Supplement 3 of this manuscript for these details).

**FIG. 2.** PROD activity in rat livers collected from air-exposed (0 ppm) and toluene-exposed (1125 ppm) rats after 6 and 24 h of exposure and 6 h after a 24 h exposure. At 24 h, a 4.5-fold increase in activity was observed in toluene-exposed animals compared with air-exposed animals. Activity remained elevated (4.4-fold increase) 6 h after the 24-h exposure ended (*indicates significant difference from control \(p < 0.05\)).

**FIG. 3.** Physiological responses to air and toluene during a 30-test session. Counts of physical activity (A) during LPT testing and dormant periods were similar for air- and toluene-exposed rats during the 30-h exposure period. Heart rate (B) was elevated in the toluene-exposed animals by ~10% above controls throughout the 24-h toluene exposure. Heart rate returned toward control levels when the toluene exposure ended (25 h–30 h). Core temperature (C) in toluene-exposed animals was elevated above controls during the first hour of exposure, then varied below or above controls during the rest of the toluene exposure; as with heart rate, core temperature returned to control levels after the exposures ended at 24 h (25–30 h). The black bar on the x-axes represents the dark phase of the animal’s 24-h circadian cycle.

Differences in control and exposed groups were compared for liver weights, microsomal protein and PROD activity using t-tests in PROC MEANS (SAS Version 9.1; SAS Institute, Cary, NC). Liver weights and total microsomal protein levels did not differ between control- and toluene-exposed rats (data not shown). PROD activity was not changed after 6 h of exposure but was significantly \((p < 0.05)\) increased after 24-h continuous toluene exposure (4.5-fold) and remained significantly \((p < 0.05)\) elevated (4.4-fold over control) 6 h after the end of exposure (Fig. 2).
Raw telemetry data (motor activity, heart rate, and core temperature) were edited to remove any extraneous signals. This was done using the clipping routine imbedded in the telemetry software that eliminates nonphysiological heart rates and core temperatures (i.e., HR < 200 beats/min or >500 beats/min; T < 34°C or > 41°C). The data were then averaged across each 60-min interval throughout the 30-h monitoring period for each rat (Fig. 3). No differences were observed in the activity level between the toluene-exposed and air-exposed (0 ppm) rats during the LPT tests or during the resting periods throughout the exposure session (Fig. 3A). Heart rate of rats exposed to toluene was consistently ~10% higher than that of controls during both rest and work. For the first 9 h in the chamber, prior to the dark phase, body temperature of rats exposed to toluene was below controls during resting periods, but the difference was negligible during the behavioral tests, when the temperature of both groups increased. On the other hand, there was no effect of toluene on body temperature during the dark phase and subsequent light phase (Fig. 3C). It is notable that heart rate, body temperature, and motor activity were generally higher during the light phase. While this would normally be unexpected in the rat, a nocturnal species, rats maintained on a weight maintenance regimen and fed during the day develop a circadian pattern of diurnal activity (Gordon and Padnos, 2002).

These physiological data were not consistent with the surprisingly low Br[Tol] values. The lack of effect of toluene on physical activity suggests that toluene concentration used in the present study did not induce lethargy that could have led to a reduction in uptake of the vapor. The relatively mild effects (MEs) of toluene on body temperature would lead one to expect little effect on Br[Tol]. A suppressed heart rate during toluene exposure would be consistent with lower Br[Tol], whereas the observed elevation in rate in toluene-exposed rats would be more likely to elevate Br[Tol]. Thus, toluene-induced changes in these parameters are unlikely to have caused overestimation of Br[Tol] by the PBTK model.

In contrast, the elevation of PROD activity shown in Figure 2 suggests that toluene exposure in excess of 6-h duration induced its metabolism via the low-affinity high-capacity P450 isozyme CYP 2B. Metabolic induction was therefore incorporated into the PBTK model as a series of graded fold-increases in VmaxC (maximum rate of toluene metabolism in liver) used in the original model (Kenyon et al., 2008). Increases were applied sequentially at 3- to 5-h intervals, beginning 2 h after the start of exposure, with a total increase of sixfold at the end of 24 h (see Supplement 1, Table S3). These changes improved the accuracy of the model’s predictions of Br[Tol] values from experiment 1 (Fig. 1B). The revised model was then used to design experiment 3 (Fig. 4) to achieve the main goals of this study.

**Experiment 3: Behavioral Assessments to Evaluate Dose Metrics**

**Study design.** Sixteen rats were trained to perform the SDT and were exposed between ages 11 and 13 months to toluene at concentrations of 0, 1125, and 1450 ppm for 24 h and to 1660 ppm for 21 h during exposure sessions lasting 30 h. Three 48-min SDT tests (T1, T2, and T3) were administered during each exposure session at times that differed depending on the concentration. That is, tests were scheduled to end at times that yielded pairs or triplicates of comparable C × t products (Table 3). For 0 and 1450 ppm, SDT tests ended at 2, 6, and 24 h of exposure; for 1125 ppm, tests ended at 2.6, 7.7, and 24 h of exposure; and for 1660 ppm, SDT tests ended at 1.8, 16.3, and 21 h of exposure. Together, these concentrations and assessment times produced five comparable C × t products (0, 2900, 8700, 27,000, and 34,800 ppm-h) with two or three observations for each product. Note that these times also yielded different Br[Tol] values as estimated by the PBTK model (Fig. 4). Additionally, for each of these concentrations, two more 48-min SDT tests (T4 and T5) were conducted at 25 h (1-h postexposure) and 30 h (6-h
postexposure) for the 0, 1125, and 1450 ppm concentrations and equivalently at 22 and 27 h for the 1660 ppm concentration. These tests provided data on the effects of falling Br[Tol] values and the expected recovery of SDT behavior.

All rats were tested under all test conditions and were allowed to recover between each toluene exposure for at least 2 weeks. This long washout period reduced the likelihood of carryover effects during the course of the assessments. The sequence of exposure concentrations was randomized and counter-balanced across rats and test chambers. During each of these exposure assessments, the rats were exposed to toluene for 15 min prior to starting the exposures, which began at 9 A.M. for 0, 1125, and 1450 ppm and at 12 P.M. for 1660 ppm toluene (see Table 3 and Fig. 4). During the 1660 ppm toluene exposures, animals sat in their operant-inhalation chambers from 9 A.M. to 15 min prior to the start of exposure. All toluene exposures (T1, T2, and T3) were analyzed in separate ANOVAs using Proc Mixed analyses (SAS, V 6.0; SAS Institute). The two dose metrics—Br[Tol] product (ppm-h) and estimated Br[Tol] (0–145 mg/l)—were used as independent variables in separate repeated-measures designs. Because four analyses were run, \( p < 0.0125 \) was considered significant for each analysis. Akaike’s Information Criterion of best fit with correction for small n size (AIC_C) was used to identify the statistical model (dose metric) that better accounted for the toluene-induced changes in each behavioral measure. This criterion provides an unbiased tool for evaluating goodness-of-fit across statistical models of specific datasets where the best model is not obvious. Smaller values of AIC_C indicate better fits (Burnham and Anderson, 2004).

Results. Four rats were eliminated from experiment 3 due to the following reasons. Three rats did not achieve acceptable baseline SDT performance levels prior to the start of exposure (proportion of false alarms > 0.30 and/or proportion of hits < 0.80 at the brightest signal intensity) and one rat experienced mechanical failures during the exposures; this resulted in an n of 12 for the behavioral assessment. The mean (± SD) of the actual toluene concentrations inhaled by these rats is listed in Supplement 3 of this manuscript.

Behavioral data [P(Cr) and RL] from the SDT tests performed during the toluene exposures (T1, T2, and T3) were analyzed in separate ANOVAs using Proc Mixed analyses (SAS, V 6.0; SAS Institute). The two dose metrics—\( C \times t \) product (0, 2900, 8700, 27,000, and 34,800 ppm-h) and estimated Br[Tol] (0–145 mg/l)—were used as independent variables in separate repeated-measures designs. Because four analyses were run, \( p < 0.0125 \) was considered significant for each analysis. Akaike’s Information Criterion of best fit with correction for small n size (AIC_C) was used to identify the statistical model (dose metric) that better accounted for the toluene-induced changes in each behavioral measure. This criterion provides an unbiased tool for evaluating goodness-of-fit across statistical models of specific datasets where the best model is not obvious. Smaller values of AIC_C indicate better fits (Burnham and Anderson, 2004).

To evaluate performance during falling Br[Tol] concentrations, the results of the two SDT tests that were administered after exposures ended (T4 and T5) are plotted alongside the results of T1–T3, as a function of Br[Tol] for P(Cr) and RL (Fig. 5). In addition, data from a previously reported experiment (Bushnell et al., 2007b) are plotted in Figure 6, showing the effects of toluene exposures at concentrations of 1200–2400 ppm for about 1 h.

TABLE 3
Study Design for the Behavioral Assessments (dose-metric comparisons)

<table>
<thead>
<tr>
<th>Toluene (ppm)</th>
<th>Exposure length (h)</th>
<th>SDT test</th>
<th>Start of SDT</th>
<th>End of SDT</th>
<th>( C \times t ) product (ppm-h)</th>
<th>Estimated Br[Tol] mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24</td>
<td>T1</td>
<td>1.2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1125</td>
<td>24</td>
<td>T1</td>
<td>1.8</td>
<td>2</td>
<td>2.6</td>
<td>2925</td>
</tr>
<tr>
<td>1450</td>
<td>24</td>
<td>T2</td>
<td>6.9</td>
<td>6</td>
<td>7.7</td>
<td>8700</td>
</tr>
<tr>
<td>1660</td>
<td>21</td>
<td>T3</td>
<td>23.2</td>
<td>24</td>
<td>27,000</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T4</td>
<td>24.2</td>
<td>25</td>
<td>34,800</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T5</td>
<td>29.2</td>
<td>30</td>
<td>34,800</td>
<td>14</td>
</tr>
</tbody>
</table>

Note. Each animal was exposed to each toluene concentration in individual 30-h exposure sessions for 24 or 21 h. Five SDT sessions (T1-T5) were delivered during the course of each 30-h session (behavior test periods). Values used in the statistical comparisons of the two dose-metrics are in bold.

Figure 6 also shows that, when plotted as a function of Br[Tol], the magnitude of the effects of inhaling toluene for 24 h on both P(Cr) and RL is greatly reduced as a function of Br[Tol], the magnitude of the effects of inhaling toluene for 1 h (Fig. 5). However, the differences in fit were not large and, while substantial for RL, are probably inconsequential for P(Cr).

Centerline (mean) for each toluene exposure for up to 24 h (Fig. 5). However, the differences in fit were not large and, while substantial for RL, are probably inconsequential for P(Cr).
metabolic clearance of toluene because it is clearly apparent as a function of Br[Tol], which accounts for the increased metabolism. Furthermore, Figure 6 also shows that P(Cr) was reduced during tests administered after exposures had ended, when Br[Tol] was falling. In fact, accuracy was more disrupted in these tests during "recovery" than it had been at the higher Br[Tol] concentrations that were achieved during the tests while toluene was being inhaled. Further, during these recovery tests, toluene reduced P(Cr) at concentrations that were relatively ineffective in 1-h exposures. In contrast to this pattern for P(Cr), RL values observed during this recovery period fell closely in line with the values observed during the 24- and 1-h exposure scenarios.

DISCUSSION

These results support previous observations that the concentration of toluene in the brain more accurately predicts its acute neurological effects than does the cumulative inhaled dose ($C \times t$), and they extend the duration for which this dose metric applies to exposures lasting up to 24 h. Specifically, the data show that when $C \times t$ and Br[Tol] were compared directly, Br[Tol] yielded a better statistical fit to the acute effects of inhaled toluene, though the difference was modest and more evident for RL than for accuracy.

More importantly, the data also demonstrate that the acute effects of 24-h exposure to toluene cannot be accurately predicted from the effects of a 1-h exposure by use of either dose metric. Two factors complicate extrapolation across these time intervals. First, toluene induced its own metabolism, as shown by the increase in PROD activity in livers of rats exposed for longer than 6 h (Fig. 2). This elevated rate of metabolism explains why the PBTK model with uninduced metabolism overestimated the 24-h Br[Tol] values in experiment 1 (Fig. 1A). Second, in addition to this elevated metabolism, dose-effect relationships based on Br[Tol] for acute behavioral effects of toluene during the 24-h exposure were shifted to the right in comparison to 1-h exposures (Fig. 6), suggesting the influence of compensatory processes in the central nervous system (CNS) that reduced the potency of circulating toluene. These compensations—metabolic induction and behavioral tolerance—have implications for approaches to extrapolating effects across durations of exposure, as will be discussed below.

Comparing the two dose metrics required that the PBTK model estimate Br[Tol] accurately under the conditions of the behavioral assessments. Experiment 1 demonstrated that the PBTK model (Kenyon et al., 2008) originally developed and validated for shorter term exposures, overestimated Br[Tol] after 24-h continuous exposure and at 6 h after the end of exposure (Fig. 1A). Results from experiment 2 provided a biological basis for incorporating enzyme induction into the PBTK model as a series of graded fold increases in the rate of metabolism ($V_{maxC}$) as described in detail in Supplement 1. Furthermore, a ~10% increase in heart rate produced by physical activity in the
LPT, independent of toluene exposure and reported previously during SDT tests (Kenyon et al., 2008), justified increasing ventilation and cardiac output parameters during the active periods. These changes resulted in model predictions that were consistent with values of Br[Tol] obtained in experiment 1 (Fig. 1B), thus adding confidence to the estimates of Br[Tol] used to design experiment 3 (Fig. 4). The additional 10% increase in heart rate observed in the toluene-exposed rats (Fig. 3B) was not applied to the kinetic model because of (1) uncertainty regarding its possible effect on ventilation rate and thus the uptake of toluene and (2) the fact that the model had been evaluated against other toluene-concentration datasets without consideration for the possible effect of toluene on physiological parameters of the model.

Dose-effect functions for the behavioral effects of toluene based on $C_t$ and Br[Tol] from experiment 3 (Fig. 5) showed that response accuracy decreased with increasing dose, with a slightly better model fit for Br[Tol] than for $C_t$. In addition, RL increased as a function of dose using both dose metrics and was considerably better fit by Br[Tol] than by $C_t$. Nevertheless, although latency increased monotonically with dose at the highest exposure concentration, it did not do so at the lower exposure concentrations. Indeed, the trend to fall back toward control values in the third test at these concentrations supports the conclusion that the animals were modifying their behavior to mitigate the slowing effect of toluene during the 24-h exposure period.

These dose-effect functions show that extrapolation from 1- to 24-h exposure is complicated by changes in the potency of toluene in the brain. Thus, Figure 6 shows that, relative to Br[Tol], the magnitude of effects on accuracy and latency observed during 24-h exposures were lower than those observed during 1-h exposures. Two factors likely contributed to this outcome. First, rats develop tolerance to the effects of toluene and TCE on performance of the SDT within 5 days of repeated 1-h exposures when they perform the task during exposure (Bushnell et al., 2000; Oshiro et al., 2007). This “behavioral tolerance” develops independently of induction of metabolism of the chemical. This pharmacodynamic process involves a reduction in the magnitude of a behavioral change induced by a given dose of a chemical and is believed to be driven by food rewards that are lost because of inaccurate choices (Demellweek and Goudie, 1983; Kalant, 1998). This kind of tolerance involves putative compensatory changes in the CNS that mitigate the effects of the chemical and has been shown to develop to the acute effects of many VOCs, including ethanol, toluene, trichloroethylene, m-xylene, and perchloroethylene in rats (Bushnell and Oshiro, 2000; Himnan, 1984; Holloway et al., 1988, 1989; Holloway and King, 1989; Kjellstrand et al., 1990; Oshiro et al., 2007) and in humans (Kalant, 1998; Savolainen et al., 1980).

A second likely cause of the difference in magnitude of effects between the 1- and 24-h studies is the rate at which Br[Tol] was rising during the SDT testing. In the 1-h exposures (Bushnell et al., 2007b), Br[Tol] increased rapidly during toluene inhalation (0–140 mg/l/h at 2400 ppm), during which time, behavior was measured in 12-min intervals. In the present 24-h study, tests were scheduled later during exposure when Br[Tol] was rising more slowly (e.g., from ~50 to 90 mg/l/h at 1660 ppm, beginning after 1.8 h of exposure). It is possible that rapidly rising Br[Tol] values during the 1-h exposure accentuated the effects of inhaled toluene relative to the more gradual change during the 24-h exposures. Rapid changes in the inhaled concentration of two VOCs were shown to affect motor activity in mice more than the concentration of the VOCs themselves (Kjellstrand et al., 1990).
The fact that rats develop tolerance to the behavioral effects of VOCs indicates that the CNS can compensate for their actions, probably by adjusting the activity of affected ion channels to restore a functional balance between the excitatory and inhibitory pathways that mediate the behavior (Bushnell et al., 2005). The greater effect of toluene when internal concentrations are rising (or falling—see below) suggests that the rate at which the CNS can alter that balance in response to changing VOC concentrations is limited. That is, the machinery propelling these compensatory processes operates at a finite rate, and when that rate is exceeded by the rate of change of the VOC concentration in the CNS, behavioral performance is compromised.

The large deficit in accuracy during rapidly falling Br[Tol] levels (Fig. 6A) also supports this contention, but the gradual return to control latency values as a function of Br[Tol] during recovery (Fig. 6B) does not. These differential effects on the two measures suggest that different mechanisms mediate effects of toluene on accuracy and speed. Indeed, a previous study found that rats developed limited tolerance to the slowing effect of toluene on accuracy and speed. Indeed, a previous study found that rats developed limited tolerance to the slowing effect of toluene on accuracy and speed. Indeed, a previous study found that rats developed limited tolerance to the slowing effect of toluene on accuracy and speed.

The telemetered physiological measures showed that toluene-induced changes in physical activity, heart rate, and body temperature did not explain the over-predicted Br[Tol] values by the original PBTK model at 24 h (Fig. 1A). First, the motor activity data (Fig. 3A) suggested that toluene did not alter physical activity during the exposures, despite other findings to the contrary (Bushnell et al., 1985; Hinman, 1987; Kjellstrand et al., 1990). Second, the ~10% toluene-induced elevation of heart rate (Fig. 3B) might explain an elevated Br[Tol]; however, it does not explain the reduced Br[Tol] observed here. Third, the toluene-induced elevation in body temperature during the periods of activity and reduction in temperature when inactive are not likely to be of sufficient magnitude or consistency to affect Br[Tol] (Fig. 3C).

The rightward shifts in the dose-effect functions in Figure 6 suggest that use of the default C observed on accuracy (Oshiro et al., 2005). The greater effect of toluene when internal concentrations are rising (or falling—see below) suggests that the rate at which the CNS can alter that balance in response to changing VOC concentrations is limited. That is, the machinery propelling these compensatory processes operates at a finite rate, and when that rate is exceeded by the rate of change of the VOC concentration in the CNS, behavioral performance is compromised.

Figure 7 illustrates the potential magnitude of the extrapolation error for the effect of toluene on RL. A similar case can be made for accuracy because the shift in its dose-effect function (Fig. 6A) is of the same magnitude as for latency (Fig. 6B).

To estimate the magnitude of the extrapolation error, assume that the dose-effect data from the 1-h toluene experiment (Bushnell et al., 2007b) (open symbols of Fig. 7) provide the starting point for extrapolating to 24 h. Assume further that mild effects (MEs) occur at about a doubling of the control RL (from 0.43 to 1 s) and that disabling effects (DEs) occur at about triple the control value (1.5 s). Figure 7 shows that in a 1-h exposure scenario, these effects occur at 80 and 120 mg/l Br[Tol], respectively (dotted lines for MEs and dashed lines for DEs). However, in the 24-h scenario, the ME level occurred at a Br[Tol] of 145 mg/l and the DE level was not achieved at all. The horizontal arrows in Figure 7 show the increase in Br[Tol] necessary to achieve RLs corresponding to the ME and DE levels at 24 h of exposure, compared with those levels at 1 h. For MEs, the necessary Br[Tol] increased from 80 mg/l at 1 h to 144 mg/l at 24 h, an increase of 64 mg/l. Because the DE level was not observed in this study after 24-h exposure, its shift cannot be quantified, but assuming an increase in Br[Tol] equivalent to that at the ME level yields a 24-h DE level of about 185 mg/l.

Table 4 compares the estimates of 24-h ME and DE dose values calculated by the default C × t approach and by using the present data for internal dose (Br[Tol], top) and for inhaled dose (ppm, bottom). Using the C × t approach, the ME and DE levels at 1 h are divided by 24 to yield 24-h values of 3.3 and 5 mg/l for ME and DE levels, respectively. In contrast, using the data from the present study, the rightward shift in the dose-effect function indicates that the ME level at 24 h increases to 145 mg/l and the DE level at 24 h, though not actually achieved in the experiment, should fall somewhere in the range of 185 mg/l (Table 4, top).

Similarly, these comparisons can be made for the inhaled concentrations of toluene necessary to yield Br[Tol] values of 80 and 120 mg/l. These concentrations were estimated using the PBTK model (Kenyon et al., 2008); specifically, the 24-h values were taken from the 24-h time points in Figure 1A (model without metabolic induction). Simulations by this model yielded 1-h ME and DE values of 1460 and 2156 ppm, respectively. As above, the C × t approach calculates the 24-h
are 775 and 1125 ppm, respectively (Table 4, bottom).

In contrast, the data-driven estimates of 24-h ME and DE values are 60 and 88 ppm, respectively. In

value by dividing the 1-h value by 24, yielding estimates of 24-h ME and DE values of 80 and 120 mg/l after 1 h and 24 h, as estimated by the PBTK model for toluene (Kenyon et al., 2008).

<table>
<thead>
<tr>
<th></th>
<th>1 h</th>
<th>24-h C × t</th>
<th>24-h data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Using internal dose—brain [Tol], mg/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME level</td>
<td>80</td>
<td>3.3</td>
<td>145</td>
</tr>
<tr>
<td>DE level</td>
<td>120</td>
<td>5</td>
<td>185?</td>
</tr>
<tr>
<td>Using inhaled dose-air [Tol], ppm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME level</td>
<td>1430</td>
<td>60</td>
<td>775</td>
</tr>
<tr>
<td>DE level</td>
<td>2120</td>
<td>88</td>
<td>1125</td>
</tr>
</tbody>
</table>

Note. Using the default method, 24-h values are estimated from 1-h values by dividing the 1-h value by 24. The values for the data-driven method for internal dose are taken from Figure 7; the ? for the DE level indicates extrapolation beyond the range of data. The comparisons based on inhaled dose show airborne toluene concentrations that yield internal doses of 80 and 120 mg/l after 1 h and 24 h, as estimated by the PBTK model for toluene (Kenyon et al., 2008).

It is clear that, based on 1-h data, the default and data-driven approaches yield divergent estimates of effect level values at 24 h for both inhaled dose and internal dose. The $C \times t$ approach decreases the effective dose in proportion to the duration of exposure, under the assumption that the cumulative dose is the appropriate dose metric. In contrast, experimental data (1) show that Br[Tol] more accurately predicts acute effects and (2) by measuring 24-h ME and DE levels empirically, result in higher effective doses due to strong indications that long-duration exposure to toluene induces tolerance to its effects. This empirical approach yields estimates of 24-h effect level values almost two orders of magnitude higher than those estimated by the default approach using Br[Tol] as the dose metric and about one order of magnitude higher than the default approach using inhaled concentration as the dose metric (Table 4). Including the effects of metabolic induction would increase 24-h effect level values above values calculated without induction because induction increases the concentration of inhaled toluene necessary to achieve a given internal dose.

Taken together, these data support the following conclusions:

1. For exposures of toluene lasting up to 24 h, internal dose provides a statistically more consistent prediction of acute behavioral effects of toluene in the rat than does the cumulative inhaled dose ($C \times t$).
2. Induction of toluene metabolism occurs in rats beginning after about 6 h of exposure. This induction reduces its internal dose for a given inhaled concentration.
3. The rightward shift in dose-effect functions for the two behaviors measured here strongly suggests a further effect of behavioral tolerance, which reduces the effect of toluene for a given internal dose.
4. The detrimental effect of toluene on response accuracy after termination of exposure suggest that acute effects on accuracy may be sensitive to changes in internal dose as well as to the specific value of the dose. This mechanism also may contribute to the increased apparent potency of toluene during 1-h exposures, in which higher inhaled concentrations were used, and Br[Tol] increased at a more rapid rate.
5. Extrapolating effective doses from 1 to 24 h using the default $C \times t$ approach yields unnecessarily conservative values for compounds to which animals develop tolerance.
6. The underlying assumption of the $C \times t$ approach, that cumulative exposure is the appropriate metric of dose, should be evaluated prior to applying this approach to extrapolation issues in risk assessments.
7. Similar considerations should be applied to setting exposure guidelines for other compounds like toluene that induce tolerance (e.g., TCE, perchloroethylene, and m-xylene).
8. Behavioral effect levels for 24-h exposures cannot be accurately extrapolated from 1-h data without a consideration of both metabolic induction and behavioral tolerance.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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