

## EFFECTS OF TOLUENE AND ITS METABOLITES ON CEREBRAL REACTIVE OXYGEN SPECIES GENERATION

CARA J. MATTIA, CARL P. LeBEL\* and STEPHEN C. BONDY†

Department of Community and Environmental Medicine, University of California, Irvine,  
CA 92717, U.S.A.

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**Abstract**—The effects of toluene on lipid peroxidation and rates of reactive oxygen species (ROS) formation have been studied in isolated systems and *in vivo*. The induction of reactive oxygen species was assayed using the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). Toluene exposure (1 g/kg, 1 hr, i.p.) did not stimulate cortical lipid peroxidation as evaluated by measurement of conjugated dienes. Exposure to toluene, however, both *in vivo* and *in vitro*, caused a significant elevation of ROS formation within cortical crude synaptosomal fractions (P2) and microsomal fractions (P3). The ROS-inducing properties of toluene were blocked *in vivo* in the presence of a mixed-function oxidase inhibitor, metyrapone. This suggested that a metabolite of toluene may catalyze reactive oxygen formation. Both benzyl alcohol and benzoic acid, *in vitro*, were found to have free radical quenching properties, while benzaldehyde exhibited significant induction of ROS generation. It appears that benzaldehyde is the metabolite responsible for the effect of toluene in accelerating reactive oxygen production within the nervous system. Benzaldehyde may also contribute to the overall neurotoxicity of toluene.

It is widely accepted that the organic solvent toluene is a neurotoxic chemical based on reports of both its neurobehavioral [1–3] and electrophysiological [4] effects in humans and rats. The production of toluene, maintained at approximately six billion pounds per year [5], demonstrates the potential for widespread occupational exposures. The euphoria-producing characteristics of toluene have also led to its abuse [6, 7].

From a mechanistic viewpoint, several recent studies have centered around the ability of toluene to alter neuronal membrane fluidity [8–11], levels of intracellular  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -regulated events [11, 12], and neuronal and astrocytic culture morphology [13]. Although it is unclear which of these events is causal to the neurotoxicity of toluene, these studies provide strong evidence to suggest that membrane-associated phenomena are integral to the mechanism of action of this solvent. Further support for this concept comes from the fact that toluene has a high degree of lipophilicity [14].

Traditionally, lipid peroxidation has been used as a measure of reactive oxygen species (ROS)-induced damage in several tissues. The reactive species, such as superoxide anion, ferryl ion and the hydroxyl ion, are believed to initiate this process [15, 16]. The difficulty of establishing the nature of the reactive species produced in the CNS is due to the short half-life and rapid interconvertability of many of the

putative key species. Recently, ROS have been reported to play a role in the neurotoxic mechanisms of methylmercury and trimethyltin [17, ‡]. These studies were performed using the fluorescent dyes 2',7'-dichlorofluorescein and rhodamine 123, markers that have proven to be more sensitive measures of oxidative stress than the traditional thiobarbituric acid assay [17, 18]. A ferryl-ion or peroxo-metal complex may be the reactive species directly responsible for the oxidation of non-fluorescent dichlorofluorescein to highly fluorescent dichlorofluorescein [19].

There is some evidence that organic solvents may express their toxicity by way of ROS-induced cell damage. Toluene has been shown to induce lipoperoxidation in pulmonary alveolar macrophages [20], while *p*-xylene, a structural analogue, caused similar increases in lung microsomes [21]. Given these findings, this study was undertaken to determine whether reactive oxygen species and related membrane events play a role in the neurotoxicity that results from toluene exposure.

### METHODS

**Animals and treatment.** Male CD rats (Charles River Laboratories, Wilmington, MA) weighing 175–200 g were employed in this study. Rats were housed six per cage with wood-chip bedding and maintained on a 12-hr light/dark cycle in a temperature-controlled ( $20 \pm 1^\circ$ ) room. Food (Purina Laboratory Chow, St. Louis, MO) and tap water were provided *ad lib*.

Toluene and benzene were dissolved in corn oil and administered in single doses of 1.0 g/kg body weight, intraperitoneally, in a volume of 4 mL/kg. Animals were killed 1 hr following exposure. The rationale for the doses and time-course used was based on previous studies that demonstrated maximal

\* Current address: Arthur D. Little, Inc., Toxicology Unit, Chemical and Life Sciences, Acorn Park, Cambridge, MA 02140.

† Correspondence: Dr. Stephen C. Bondy, Department of Community and Environmental Medicine, University of California at Irvine, Irvine, CA 92717.

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cerebral levels of toluene within the brain, concurrent with pronounced sedative effects under these conditions [8–10]. Metyrapone (200 mg/kg, i.p., 4 mL/kg) was also dissolved in corn oil and given 1 hr prior to toluene.

**Chemicals.** Toluene, benzene, benzaldehyde, benzoic acid and benzyl alcohol were obtained from Fisher Scientific (Tustin, CA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes, Inc. (Eugene, OR), and 2',7'-dichlorofluorescein (DCF) was purchased from Polysciences, Inc. (Warrington, PA).

**Preparation of morphological fractions.** Rats were decapitated, the brains were excised quickly on ice, and the cortex was dissected out. The brain sections were placed into a microcentrifuge tube, stored at  $-20^{\circ}$  for 24 hr and subsequently stored at  $-70^{\circ}$  until preparation. This slow freezing rate was intended to maintain synaptosomal integrity [22]. Each cortex was weighed and homogenized in 10 vol. of 0.32 M sucrose and centrifuged at 1800 g for 10 min. The resulting supernatant fraction was then centrifuged at 31,500 g for 10 min to yield the crude mitochondrial pellet (P2). The P2 pellet was resuspended in HEPES buffer to a concentration of 0.037 g-equivalents/mL. The supernatant was further centrifuged at 250,000 g for 30 min, and the resulting microsomal pellet (P3) was resuspended to a concentration of 0.2 g-equivalents/mL in HEPES buffer. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5;  $\text{NaH}_2\text{PO}_4$ , 1.2;  $\text{MgCl}_2$ , 0.1;  $\text{NaHCO}_3$ , 5.0; glucose, 6.0;  $\text{CaCl}_2$ , 1.0; and HEPES, 10, pH 7.4.

**Assay for reactive oxygen species.** P2 and P3 fractions were diluted 1:10 with 40 mM Tris (pH 7.4) and loaded with 5  $\mu\text{M}$  DCFH-DA (0.5 mM in methanol) for 15 min at  $37^{\circ}$ , during which time esteratic activity results in the formation of the nonfluorescent compound DCFH [23]. Following loading, the fluorescence was recorded and incubation continued for an additional 60 min, when the fluorescence was again determined [24]. For the *in vitro* studies, the agents were added, at various concentrations, to P2 or P3 fractions after the initial fluorescence reading. Samples were then incubated for 60 min and the final fluorescence reading was recorded. The formation of DCF, the fluorescent-oxidized derivative of DCFH, was monitored at excitation wavelength 488 nm (bandwidth 5 nm) and emission wavelength 525 nm (bandwidth 20 nm). The cuvette holder was maintained thermostatically at  $37^{\circ}$ . Prior to calculating the rate of formation of DCF, corrections were made for any autofluorescence of fractions. This correction was always less than 6% of values in the presence of DCFH. DCF formation was quantified from a standard curve over the range 0.05 to 1.0  $\mu\text{M}$ .

**Assay for lipid peroxidation.** Samples were prepared for conjugated diene analysis as described by Recknagel *et al.* [25]. Briefly, P2 suspensions (200  $\mu\text{L}$ ) were added to microcentrifuge tubes, followed by the addition of 1 mL of chloroform:methanol (2:1, v/v). The samples were vortexed and incubated at  $37^{\circ}$  for 15 min. After this incubation, the samples were centrifuged for 3 min at 16,000 g, and the upper aqueous layer was discarded. Next, the remaining organic phase was

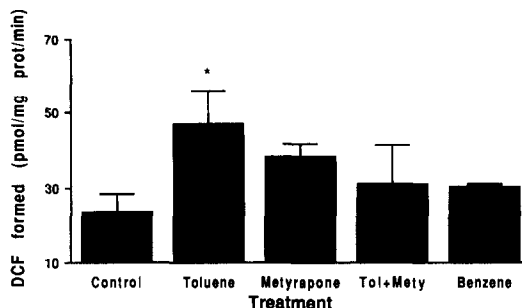


Fig. 1. Effects of *in vivo* exposure to organic solvents and metyrapone on the formation rate of reactive oxygen species in cerebrocortical synaptosomes (P2). Data are means  $\pm$  SEM derived from five to six rats. Doses and exposure times: toluene, 1 g/kg, 1 hr; metyrapone, 200 mg/kg, 1 hr prior to toluene; benzene, 0.5 g/kg, 1 hr. Key: (\*) statistically different from control value,  $P < 0.05$ .

washed twice with 0.5 mL of acidified water (pH 2.6) and the samples were again vortexed. After removal of the upper aqueous layer, 0.3 mL of chloroform was evaporated to dryness under  $\text{N}_2$ , and reconstituted in 2 mL of cyclohexane. The absorbance of each sample, a measure of conjugated dienes, was read at 233 nm against a cyclohexane blank. Conjugated dienes were quantitated by use of the molar extinction coefficient  $2.5 \times 10^4$  [25].

**Statistical analyses.** Differences between groups were assessed by one-way analysis of variance followed by Fisher's Least Significant Difference Test. The acceptance level of significance was  $P < 0.05$  using a two-tailed distribution. Five to six rats were used in the determination of each data point.

## RESULTS

The effects of toluene administration on rat cerebrocortical lipid peroxidation were studied by quantitation of conjugated dienes. There was no difference in lipid peroxidation between crude synaptosomes isolated from the cortices of control animals, or those from animals treated with toluene (1 g/kg body weight) for 1 hr (data not shown). Basal and toluene-treated values were  $0.26 \pm 0.03 \mu\text{mol/mg protein}$ .

The effect of toluene treatment on the rate of formation of reactive oxygen species was also studied, using the DCF-generating assay. Crude cerebrocortical synaptosomes (P2) and microsomes (P3), isolated from toluene-treated animals, showed significantly higher rates of ROS formation when compared to controls (Figs. 1 and 2). Treatment of animals for 1 hr with benzene (0.5 g/kg), a structurally related organic solvent with no known neurotoxic potential, did not alter the generation of ROS in the rat P2 or P3 fractions (Figs. 1 and 2).

To determine the relationship of toluene metabolism to ROS generation, a cytochrome P450 inhibitor, metyrapone, was given to rats by intraperitoneal injection (200 mg/kg), 1 hr prior to

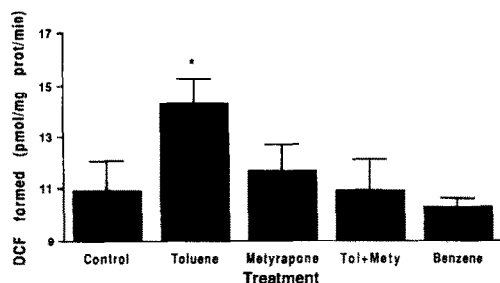


Fig. 2. Effects of *in vivo* exposure to organic solvents and metyrapone on the formation rate of oxygen reactive species in cerebrocortical microsomes (P3). Data are means  $\pm$  SEM derived from five to six rats. Doses and exposure times: toluene, 1 g/kg, 1 hr; metyrapone, 200 mg/kg, 1 hr prior to toluene; benzene, 0.5 g/kg, 1 hr. Key: (\*) statistically different from control value,  $P < 0.05$ .

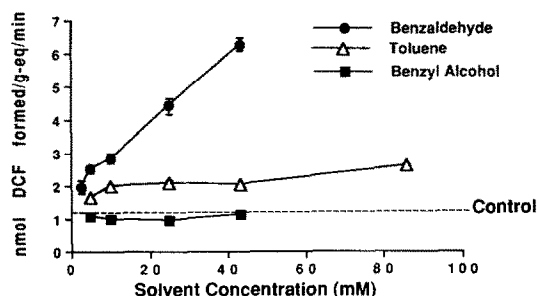


Fig. 3. Formation rates of reactive oxygen species in cerebral P2 fractions following *in vitro* incubation with various concentrations of toluene, benzaldehyde and benzyl alcohol. Data are means  $\pm$  SEM of five to eight independent experiments. Standard error values are generally too small to be visible in the graph.

toluene administration. This pretreatment completely blocked the toluene-induced reactive oxygen increase in both morphological fractions (Figs. 1 and 2). Metyrapone itself was without significant effect *in vivo*.

The inhibition of toluene-stimulated increases in ROS generation by metyrapone suggested the involvement of some metabolites of toluene. Thus, P2 fractions prepared from untreated rats were incubated with various concentrations of toluene, benzaldehyde, benzyl alcohol and benzoic acid (Fig. 3). Both toluene and benzaldehyde, *in vitro*, caused marked concentration-dependent increases in ROS formation; however, the stimulation by benzaldehyde was much greater than that effected by toluene. Benzyl alcohol quenched ROS formation slightly, while benzoic acid was without significant effect (data not shown). Metyrapone pretreatment *in vitro* blocked the stimulation of ROS generation; however, these results were complicated by the fact that metyrapone itself depressed basal levels of generation (data not shown).

#### DISCUSSION

The main purpose of the present study was to

characterize the effect of toluene on lipid peroxidation and on the stimulation of reactive oxygen species formation, both *in vivo* and *in vitro*, and also to identify what metabolites of toluene may contribute to the neurotoxicity of the solvent. Increased ROS generation and lipid peroxidation have been suggested to be responsible for the toxic actions of a wide range of compounds, including solvents such as carbon tetrachloride [26]. While free radical reactions may be associated with many toxic processes, there is little data concerning the reactive oxygen-generating potential of aromatic solvents. The occurrence of lipid peroxidation in biological membranes can cause impaired membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased non-specific permeability to ions such as  $\text{Ca}^{2+}$  [for review, see Ref. 27].

The first part of this study showed that *in vivo* exposure to toluene did not appear to stimulate lipid peroxidation, as evaluated by measurement of conjugated dienes. The conjugated diene experiment in this study investigated toluene exposure at one time point. It is unclear whether lipid peroxidation occurs in the brains of rats exposed to toluene at alternate time points; therefore, studies are underway to address this question. The basal levels of conjugated dienes obtained here are in agreement with those values published in the literature [28]. The conjugated diene assay is a widely used means of analyzing oxidative products of lipid degradation. The fluorometric techniques employed in these studies appear to be more sensitive than the former method. This may be due to the fact that reactive oxygen is assayed directly by DCFH, while other assays, such as conjugated dienes or formation of colored thiobarbituric acid reactive materials, are directed at secondary cellular products of oxygen radical attack. Using the DCFH probe, both *in vivo* and *in vitro* exposures to toluene were found to cause a significant elevation of reactive oxygen species formation in isolated cell fractions.

Toluene undergoes biotransformation through the mixed-function oxidase system to benzyl alcohol which, in turn, undergoes oxidation to the aldehyde. Benzaldehyde is oxidized rapidly to benzoic acid, which is conjugated with glycine to form hippuric acid, a byproduct largely excreted in the urine. The ROS-inducing properties of toluene both *in vivo* and *in vitro* can be blocked by the addition of a mixed-function oxidase inhibitor, metyrapone. These findings suggested that a metabolite of toluene may catalyze ROS formation. This led us to investigate the effects of various metabolites of toluene in an *in vitro* system. Both benzyl alcohol and benzoic acid were found to have free radical quenching properties, *in vitro*, whereas benzaldehyde was a potent inducer of reactive oxygen species. Therefore, benzaldehyde appears to be the metabolite responsible for the effect of toluene in accelerating the production of ROS within the nervous system. Benzene was not found to induce ROS formation similar to toluene, either *in vivo* or *in vitro*. This may be due to the fact that benzene catabolism does not occur predominantly by way of aldehyde formation which would involve scission of the aromatic ring. However,

a benzene metabolite, *trans,trans*-muconaldehyde has been suggested to play a role in benzene-induced free radical events [29].

Future experiments are planned which will employ mixed-function oxidase inducers and alcohol and aldehyde dehydrogenase inhibitors in order to selectively accelerate and block various steps of the catabolism of toluene. The use of these agents may lead to the accumulation of specific metabolites of toluene. Such studies may further clarify the role of toluene breakdown products in effecting enhancement of reactive oxygen species production.

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