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FREE RADICAL INDUCTION IN THE BRAIN AND LIVER BY PRODUCTS OF TOLUENE CATABOLISM

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Abstract—Toluene and its metabolites have been studied with respect to their reactive oxygen species-enhancing potential in isolated systems and *in vivo*. The induction of reactive oxygen species (ROS) production was assayed using the probe 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA). Intraperitoneal injection of toluene, benzyl alcohol or benzaldehyde caused a significant elevation in the rate of ROS formation within hepatic mitochondrial fractions (P2). In the brain, only toluene induced ROS formation, while benzyl alcohol and benzaldehyde did not have any effect. Glutathione (GSH) levels were depressed in liver and brain regions from toluene-treated rats. However, no such depression was evident in brains treated with toluene metabolites. P2 fractions from phenobarbital-pretreated rats exhibited a heightened ROS response when challenged with toluene, *in vitro*. Pretreatment of rats *in vivo* with 4-methylpyrazole, an alcohol dehydrogenase inhibitor, or sodium cyanamide, an aldehyde dehydrogenase inhibitor, prior to exposure to toluene, caused a significant decrease and increase, respectively, in toluene-stimulated rates of ROS generation in the CNS and liver. Electron spin resonance spectroscopy, employing the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), was conducted. Incubation of the spin trap with P2 fractions and toluene or benzaldehyde elicited a spectrum corresponding to the hydroxyl radical. Incubation of benzaldehyde with aldehyde dehydrogenase produced a strong signal that was blocked completely by superoxide dismutase and inhibited partially by catalase, suggesting the presence of superoxide radicals and the involvement of the iron-catalyzed Haber–Weiss reaction leading to the production of hydroxyl radicals. Thus, ROS generation during toluene catabolism may occur at two steps: cytochrome P450 oxidation and aldehyde dehydrogenase oxidation. In addition, GSH may play an important role in protection against the induction of ROS generation in the CNS and liver following exposure to toluene.

Toluene is an organic solvent with widespread industrial and commercial uses and is also the most widely abused inhaled solvent [1]. Due to the highly lipophilic nature of toluene, an important health concern for humans from industrial, occupational or intentional exposure is the potentially harmful effects of this agent on the nervous system.

In the past decade, a number of studies have been conducted on the acute neurotoxic effects of toluene in an attempt to elucidate the mechanisms behind its toxicity. Animal studies have suggested that extended toluene exposure may induce long-lasting morphological changes in the frontal cortex [2] and hippocampal and cerebellar regions [3]. Studies on specific biochemical changes effected by toluene have reported neuronal membrane alterations involving enhanced Ca^{2+}/Mg^{2+}-ATPase activity [4], increased intracellular Ca^{2+} [5] and membrane fluidity [6], and alterations in receptor binding and neurotransmitter content [2].

Recently, we found evidence that the toxicity of toluene may be related, in part, to induction of excess reactive oxygen species (ROS§) [7, 8]. Both *in vivo* and *in vitro* exposure to toluene produced a broad systemic elevation in the rate of ROS formation, that persisted in the CNS long after toluene had been eliminated from the blood. The present study is focused upon elucidating the mechanism underlying this property of toluene. The products of toluene catabolism, benzyl alcohol and benzaldehyde, and the enzymes involved in these transformations have been studied with respect to their ROS-enhancing potential. In addition, ESR spectroscopy was used to determine the nature of the oxygen radical being generated during toluene catabolism.

MATERIALS AND METHODS

Chemicals

Toluene, benzyl alcohol, and benzaldehyde were obtained from Fisher Scientific (Tustin, CA). Sodium cyanamide and 5,5-dimethyl-1-pyrroline *N*-oxide (DPMO) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). 4-Methylpyrazole, phenobarbital, glutathione (GSH), glutathione-*S*-transferase, aldehyde dehydrogenase, nicotinamide adenine coenzyme, bovine erythrocyte superoxide dismutase (SOD), catalase and diethylenetriaminepentaacetic acid (DETAPAC) were from the Sigma Chemical Co. (St. Louis, MO). 2′,7′-

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§ Abbreviations: DCFH, 2′,7′-dichlorodihydrofluorescin; DCFH-DA, DCFH diacetate; DCF, 2′,7′-dichlorofluorescein; DETAPAC, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; GSH, glutathione; MBCl, monochlorobimane; ROS, reactive oxygen species; and SOD, superoxide dismutase.

Dichlorodihydrofluorescin diacetate (DCFH-DA) and monochlorobimane (mBCl) were obtained from Molecular Probes, Inc. (Eugene, OR), and 2',7'-dichlorofluorescein (DCF) was purchased from Polysciences, Inc. (Warrington, PA).

Animals and treatment

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 175–200 g, were utilized in the following studies. Rats were housed six per cage with wood chip bedding and maintained on a 12-hr light/dark cycle in a temperature-controlled (20 ± 1°) room. Food (Purina Laboratory Chow, St. Louis, MO) and water were provided *ad lib.*

Relative intensity of enhancement of reactive oxygen species formation by toluene and its metabolites. The animals were injected intraperitoneally with toluene (1.5 g/kg body wt), benzyl alcohol (0.5 g/kg body wt) or benzaldehyde (0.5 g/kg body wt). All exposure times were for 2 hr. The selection of the dose and the duration of exposure to toluene were based on an earlier study [8]. Control animals were treated with a corresponding volume of 0.9% saline.

Inhibition of enzymes participating in the degradation of toluene. Enzyme inhibition studies were performed both in the intact animal and in isolated subcellular systems. Animals were dosed intraperitoneally with the aldehyde dehydrogenase inhibitor sodium cyanamide (100 mg/kg body wt) or the alcohol dehydrogenase inhibitor 4-methylpyrazole (75 mg/kg body wt), 30 min prior to toluene treatment (1.5 g/kg body wt). Both inhibitors were made up in 0.9% saline, and control animals for each group received saline injections. All animals were decapitated 2 hr following exposure to toluene. Dose and time course values for sodium cyanamide were selected from the report of Deitrich *et al.* [9], and those for 4-methylpyrazole were from studies by Feierman and Cederbaum [10] and Rydberg and Neri [11]. Cerebrocortical crude synaptosomal fractions were incubated *in vitro* with each inhibitor for 15 min prior to exposure to toluene. The concentrations of 4-methylpyrazole and sodium cyanamide used in the *in vitro* studies were selected from earlier reports [12, 13], respectively.

Enzyme induction studies. Animals were pretreated with intraperitoneal injections of sodium phenobarbital (75 mg/kg body wt) daily for 4 days. On day 5, the animals were killed. Dose and time course were based on prior experiments by Ikeda and Ohtsuji [14]. Control animals received injections of 0.9% saline.

Preparation of morphological fractions

Rats were decapitated, the brains were excised quickly on ice and, where needed, the cortex, cerebellum, hippocampus and striatum were dissected out. In addition, the liver was removed and frozen at −70°. All of the brain regions were placed into sterile, screw-capped microcentrifuge tubes, stored at −20° for 24 hr and subsequently stored at −70° until preparation. A relatively slow freezing rate was intended to maintain the integrity of synaptosomal structure [15]. Each tissue was weighed and homogenized in 10 vol. of 0.32 M sucrose and centrifuged at 1,800 *g* for 10 min. The resulting supernatant fraction was then centrifuged at 31,500 *g* for 10 min to yield the crude hepatic mitochondrial or cerebral synaptosomal pellet (P2). The P2 pellet was resuspended in HEPES buffer to a concentration of 0.037 g-eq/mL. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10; pH 7.4.

Assay for reactive oxygen species

P2 fractions were diluted 1:10 with 40 mM Tris (pH 7.4) and loaded with 5 μM DCFH-DA (0.5 mM in ethanol; stored at −70°) for 15 min at 37°, during which time esteratic activity resulted in the formation of the nonfluorescent compound 2',7'-dichlorodihyrofluorescin (DCFH) [16]. Following loading, the formation of DCF, the fluorescent oxidized derivative of DCFH, was recorded and incubation continued for an additional 60 min, when the fluorescence was again determined [17]. For the *in vitro* studies, the agents were added, at various concentrations, to P2 fractions after the initial fluorescence reading. Samples were then incubated for 60 min, and the final fluorescence reading was recorded. Fluorescence was monitored at excitation wavelength 488 nm (bandwidth 5 mm) and emission wavelength 525 nm (bandwidth 20 nm). The cuvette holder was maintained thermostatically at 37°. 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 of 0.05 to 1.0 μM.

Determination of reduced glutathione

The method for determination of GSH levels was a modification of the methods of Shrieve *et al.* [18] and Rice *et al.* [19]. The principle behind the assay is that mBCl, itself a nonfluorescent compound, reacts with GSH to form a fluorescent adduct. Rice *et al.* [19] showed that there is very little reaction between mBCl and protein sulfhydryl groups. mBCl was dissolved in ethanol to a concentration of 5 mM and stored at 0–5° in the dark. mBCl was added to 2 mL of a given P2 suspension to a final concentration of 20 μM, after which the suspension was incubated for 15 min at 37° and then centrifuged for 10 min at 31,500 *g*. The fluorescence of the final supernatant was read on an Aminco-Bowman spectrophotofluorometer at excitation wavelength 395 nm and emission wavelength 470 nm. The tissue GSH concentration was determined by reference to a GSH standard curve.

Electron spin resonance spectroscopy

Reaction mixtures contained 200 μL of crude cerebral cortical mitochondrial fractions, various concentrations of toluene, benzyl alcohol or benzaldehyde, and 100 mM DMPO in 250 μL of phosphate buffer (pH 7.4). All solutions were incubated for 15 min at 37°. ESR spectra were performed on mixtures of aldehyde dehydrogenase and benzaldehyde in the absence of mitochondrial

Table 1. Rate of ROS formation in various tissues following *in vivo* exposure to toluene and its metabolites

Tissue	Treatment	DCF (nmol formed/g-eq/min)
Liver	Control	2.51 ± 0.03
	Toluene	4.43 ± 0.13*
	Benzaldehyde	5.32 ± 0.17*†
	Benzyl alcohol	6.30 ± 0.20*†
Brain		
Hippocampus	Control	4.20 ± 0.16
	Toluene	5.60 ± 0.20*
	Benzaldehyde	4.01 ± 0.20
	Benzyl alcohol	4.30 ± 0.16
Cerebellum	Control	3.32 ± 0.07
	Toluene	4.31 ± 0.10*
	Benzaldehyde	3.20 ± 0.13
	Benzyl alcohol	3.50 ± 0.12
Striatum	Control	2.30 ± 0.07
	Toluene	2.80 ± 0.10*
	Benzaldehyde	2.36 ± 0.06
	Benzyl alcohol	2.32 ± 0.10

Data are means ± SEM of 5–6 rats. Doses: toluene, 1.5 g/kg body wt; benzaldehyde, 0.5 g/kg body wt; benzyl alcohol, 0.5 kg body wt. All exposure times were for 2 hr.

* Significant difference from control value ($P < 0.05$).

† Significant difference from toluene-treated rats ($P < 0.05$).

fractions. These incubations contained 0.8 U/mL of aldehyde dehydrogenase, 0.14 M KCl, 0.38 mM NAD^+ and 100 mM DMPO in 0.1 M glycine, 0.1 mM DETAPAC buffer (pH 8.3). Where indicated, incubations contained 0.1 mg/mL of SOD and catalase. Spectra were recorded with a Bruker model ECS 106 ESR spectrometer under the following conditions: power, 20 mW; frequency, 100 kHz; time constant, 1.3 sec; modulation amplitude, 1 G; sweep time, 335 sec. All solutions were placed in 1 mL quartz flat cells prior to introduction in the ESR.

Statistical analysis

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 ability of toluene or its metabolic products to induce ROS generation was studied using the DCF-generating assay. In the three brain regions studied and the liver, toluene (1.5 g/kg body wt) induced oxidative activity (Table 1). Liver mitochondrial fractions from benzaldehyde- and benzyl alcohol-treated rats (both administered at 0.5 g/kg body wt) also exhibited a significant elevation in the rate of ROS generation (Table 1). In contrast, benzyl alcohol and benzaldehyde did not have any effect on basal ROS levels in cerebral P2 fractions.

Tissue levels of GSH from the above study were determined. Liver fractions from rats exposed to toluene, benzyl alcohol or benzaldehyde contained less GSH than controls (Table 2). Toluene-treated hippocampus, cerebellum and striatum also exhibited significant decreases in GSH levels. However, the

Table 2. Levels of GSH in P2 fractions from various tissues following exposure to toluene or its metabolites

Tissue	GSH (μmol/g-eq tissue)			
	Control	Toluene	Benzyl alcohol	Benzaldehyde
Liver	6.54 ± 0.24	4.55 ± 0.16*	4.06 ± 0.09*	4.37 ± 0.08*
Brain				
Hippocampus	1.58 ± 0.04	1.27 ± 0.03*	1.56 ± 0.05	1.64 ± 0.06
Cerebellum	1.72 ± 0.06	1.37 ± 0.06*	1.75 ± 0.06	1.71 ± 0.04
Striatum	1.85 ± 0.04	1.62 ± 0.02*	1.88 ± 0.06	1.79 ± 0.05

Data are means ± SEM derived from 5–6 rats.

* Significant difference from control value ($P < 0.05$).

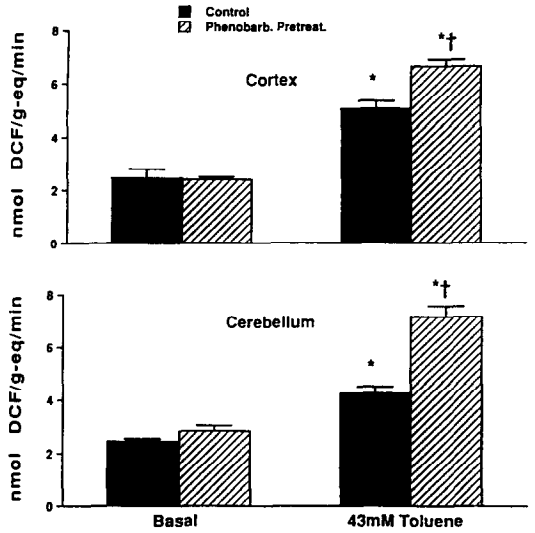

Fig. 1. Rate of formation of ROS in synaptosomal fraction (P2) from cortex and cerebellum following pretreatment, *in vivo* with phenobarbital (75 mg/kg body wt, i.p.) for 4 consecutive days prior to exposure to toluene, *in vitro*. Data are the means ± SEM of 6 independent experiments. Key: (*) significant difference from basal control values ($P < 0.05$), and (†) significant difference from toluene without phenobarbital samples ($P < 0.05$).

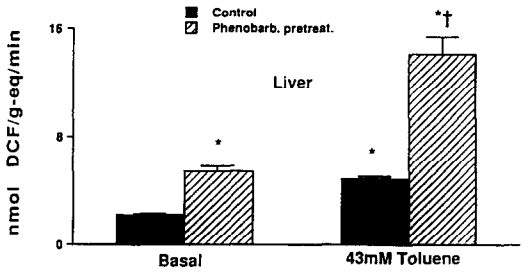

Fig. 2. Rate of formation of ROS in liver mitochondrial fractions following pretreatment, *in vivo* with phenobarbital (75 mg/kg body i.p.) for 4 consecutive days prior to incubation with toluene, *in vitro*. Data are the means ± SEM of 6 independent experiments. Key: (*) significant difference from basal control values ($P < 0.05$), and (†) significant difference from toluene without phenobarbital samples ($P < 0.05$).

brain regions from animals treated with the toluene metabolites did not shown any change in GSH concentrations.

Studies involving the induction or inhibition of various enzymes involved in toluene catabolism helped to clarify further the role that toluene metabolites play in the enhancement of ROS production. Rats were dosed with a mixed-function oxidase inducer, phenobarbital (75 mg/kg body wt, i.p.), for 4 consecutive days. Basal levels of ROS in control and treated brain P2 fractions were similar. However, when challenged with toluene *in vitro*, induction of ROS in the cerebellum and cerebrocortex was significantly higher in fractions from phenobarbital-treated rats (Fig. 1). In contrast, basal levels of ROS in liver mitochondrial fractions pretreated with phenobarbital were higher than those from control animals (Fig. 2). When liver fractions from phenobarbital-pretreated rats were exposed to toluene, *in vitro*, the rates of ROS generation were

Table 3. ROS induction following toluene exposure

Tissue	Treatment	DCF (nmol formed/g-eq/min)
Liver	Control	3.67 ± 0.08
	Toluene	7.44 ± 0.60*
	Cyanamide + toluene	9.32 ± 0.50*†
	4-Methylpyrazole + toluene	6.24 ± 0.23*
Brain		
Hippocampus	Control	3.78 ± 0.04
	Toluene	5.62 ± 0.12*
	Cyanamide + toluene	6.20 ± 0.04*†
	4-Methylpyrazole + toluene	4.33 ± 0.20*†
Cerebellum	Control	3.41 ± 0.14
	Toluene	4.22 ± 0.13*
	Cyanamide + toluene	4.70 ± 0.17*†
	4-Methylpyrazole + toluene	4.00 ± 0.09*
Striatum	Control	2.02 ± 0.07
	Toluene	3.20 ± 0.13*
	Cyanamide + toluene	4.10 ± 0.22*†
	4-Methylpyrazole + toluene	2.32 ± 0.17†

Data are means ± SEM for P2 fractions from 5–6 rats. Dose and exposure times: toluene, 1.5 g/kg body wt, 2 hr; sodium cyanamide, 100 mg/kg body wt, 30 min prior to toluene; 4 methylpyrazole, 75 mg/kg body wt, 30 min prior to toluene.

* Significant difference from control values ($P < 0.05$).

† Significant difference from toluene-treated rats ($P < 0.05$).

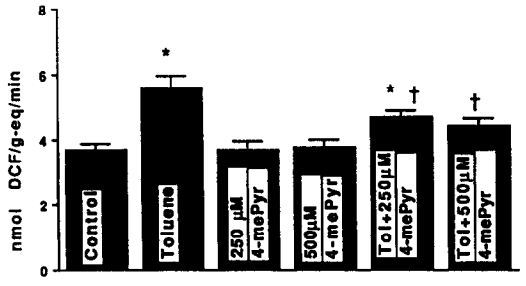

Fig. 3. Rate of formation of reactive oxygen species in cerebrocortical crude synaptosomal fractions (P2) pretreated with 4-methylpyrazole. Fractions were pre-incubated with 4-methylpyrazole 15 min prior to a 1-hr incubation with toluene (43 mM). Data are the means ± SEM of 5–8 independent experiments. Key: (*) significant difference from control values ($P < 0.05$), and (†) significant difference from toluene-treated samples ($P < 0.05$).

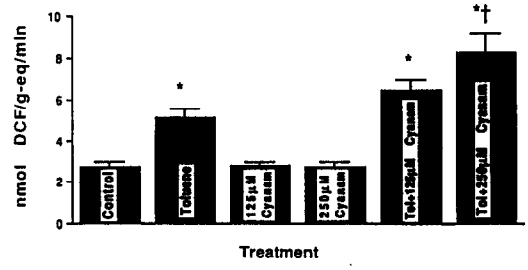

Fig. 4. Rate of formation of ROS in cerebrocortical crude synaptosomal fractions (P2) pretreated with sodium cyanamide. Fractions were preincubated with sodium cyanamide 15 min prior to a 1-hr incubation with toluene (43 mM). Data are the means ± SEM of 5–8 independent experiments. Key: (*) significant difference from control values ($P < 0.05$), and (†) significant difference from toluene-treated samples ($P < 0.05$).

also greater than those from rats not receiving this pretreatment.

Pretreatment, *in vivo* with 4-methylpyrazole, 30 min prior to exposure to toluene, caused a decrease in toluene-stimulated rates of ROS generation in striatum and hippocampus. A similar non-significant trend was apparent in cerebellum and liver (Table 3). Pretreatment with sodium cyanamide resulted in a significant increase in the magnitude of toluene-stimulated ROS, in all brain regions studied and in rat liver (Table 3). Administration of the inhibitory agents alone showed no change from control values (data not shown). Toluene-effected increases in ROS formation in isolated P2 fractions from the cerebrocortex were inhibited by pretreatment with 4-methylpyrazole and enhanced by prior administration of higher doses of sodium cyanamide (Figs. 3 and 4). These pretreatments also had no effect on basal rates of ROS generation (Figs. 3 and 4).

To investigate further the specific oxygen radical responsible for the oxidative damage induced by toluene or related metabolites, ESR spectroscopy employing the spin-trap DMPO was conducted. P2 fractions were incubated at 37° with toluene (22 mM), benzyl alcohol (48 mM) or benzaldehyde (12 mM) for 15 min (see Materials and Methods). When P2 fractions were incubated without organic solvent or with benzyl alcohol, no signal indicating the presence of an oxygen radical was observed. However, a weak signal corresponding to the hydroxyl radical was obtained following incubation with toluene, and a stronger spectrum was observed when benzaldehyde was incubated with the P2 fractions (Fig. 5). To identify the enzymatic step involved in the production of free radicals, the interaction of benzaldehyde and aldehyde dehydrogenase in the absence of crude mitochondrial fractions was studied. Metabolism of benzaldehyde by aldehyde dehydrogenase produced a very strong ESR spectrum that was inhibited almost completely by the addition of SOD and inhibited partially by the addition of catalase (Fig. 5). This implied the presence of superoxide radicals, primarily, and hydroxyl radicals, secondarily. Aldehyde dehydrogenase in the absence of a substrate did not generate any oxygen radicals.

DISCUSSION

The purpose of the present study was to establish the events underlying the enhancement of rates of ROS production by toluene, in brain and liver. Exposure to toluene, *in vivo* and *in vitro*, was found previously to result in the induction of both ROS formation and lipid peroxidation in the liver, lung, kidney and various brain regions [7, 8]. Toluene is metabolized through the oxidation of its methyl group by mixed-function oxidases to benzyl alcohol, followed by oxidation to benzaldehyde with alcohol dehydrogenase. Benzaldehyde is rapidly oxidized by aldehyde dehydrogenase to benzoic acid, the majority of which is conjugated with glycine and excreted as hippuric acid [20].

Our results demonstrate that *in vivo* exposure to toluene, benzyl alcohol or benzaldehyde results in significant induction in the rate of ROS generation in the liver. In this organ, benzyl alcohol and benzaldehyde exhibited a greater ROS-enhancing potential than toluene. There are two major aldehyde oxidizing systems in the mammalian cell: the NAD-dependent aldehyde dehydrogenases and the flavin requiring aldehyde oxidases, and both systems are widely distributed in mammalian tissues [21]. Shaw and Jayatilleke [22, 23] have suggested that ethanol-induced changes in lipid peroxidation and glutathione levels imply a role for oxygen free radicals in the pathogenesis of alcohol-induced liver injury. These workers demonstrated that the metabolism of acetaldehyde by aldehyde oxidase produced superoxide-mediated iron mobilization and, consequently, hepatic lipid peroxidation. Hepatic aldehyde oxidase, characterized by Rajagopalan and Handler [24], has a low K_m (1 mM) toward acetaldehyde and produces superoxide anion during the metabolism of various aldehyde-containing substrates [25]. Since acetaldehyde and benzaldehyde have similar affinities

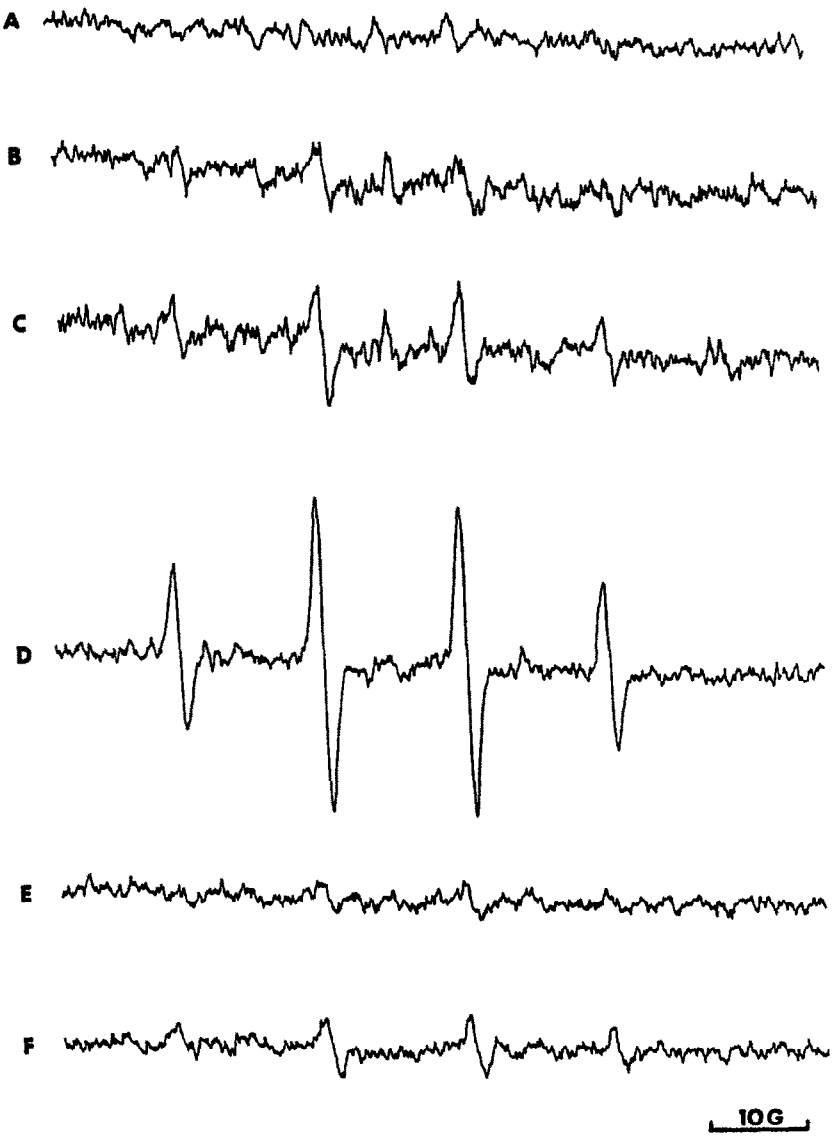

Fig. 5. ESR spectra of cerebrocortical crude synaptosomal fractions (P2). (A) 15-min incubation with 100 mM DMPO in phosphate buffer (pH 7.4); (B) same as (A) with 22 mM toluene added; (C) same as (A) with 12 mM benzaldehyde added; (D) incubation medium contained 12 mM benzaldehyde and 0.8 U of aldehyde dehydrogenase, 0.14 M KCl, 0.38 mM NAD^+ and 100 mM DMPO in 0.1 M glycine, 0.1 mM DETAPAC buffer (pH 8.3); (E) same as (D) with 0.1 mg/mL of SOD added; and (F) same as in (D) with 0.1 mg/mL of catalase added.

for aldehyde dehydrogenase [21], they may also exhibit similar affinities for aldehyde oxidase. The metabolism of benzaldehyde by aldehyde oxidase could thus release superoxide radicals and effect an enhanced rate of oxygen radical formation. In our study, benzyl alcohol exhibited a greater hepatic ROS-enhancing potential than benzaldehyde, suggesting that benzyl alcohol may be rapidly metabolized to benzaldehyde, leading to an accumulation of the aldehyde.

In contrast, oxidative activity in the hippocampus, cerebellum and striatum remained unaltered following exposure to products of toluene breakdown. Only toluene itself was able to increase significantly oxidative activity in these brain regions, with the hippocampus demonstrating the highest level of induction. This finding parallels our earlier results [7] and may imply a special vulnerability of the hippocampus to intoxication by this solvent. Such selectivity would be expected to relate to the deficits in memory and cognition that have been reported following toluene exposure [26]. Since benzyl alcohol and benzaldehyde did not enhance ROS formation in the CNS, it may be that neither substance readily crosses the blood–brain barrier. However, since all treated animals exhibited behavioral changes

(dragging of hind limbs and narcosis), small amounts of each compound must have diffused into the CNS.

Levels of GSH were measured to determine whether toluene exposure influenced cerebral and hepatic protective mechanisms against oxidative damage. Of the three brain regions examined, the striatum had the highest levels of GSH and the hippocampus the lowest. Following exposure to toluene, significantly decreased GSH levels were found in all three brain regions and the liver. Exposure to benzyl alcohol and benzaldehyde resulted in a significant reduction only in liver GSH levels. These latter findings are in agreement with the results of van Doorn *et al.* [27] following intraperitoneal injection of rats with toluene or xylene.

Glutathione is a major cellular defense mechanism against free radical-induced damage [28, 29]. The liver contains high concentrations of GSH and protective enzymes, such as catalase and superoxide dismutase [29], and yet we have found ROS production to be more than doubled by the administration of toluene or its catabolic products.

We have attempted to gauge the relative role of mixed-function oxidase, alcohol dehydrogenase and aldehyde dehydrogenase in the induction of ROS. Pretreatment of animals with phenobarbital, a cytochrome P450 inducer, enhanced the rate of ROS generation following *in vitro* toluene exposure in both the brain and liver. Basal levels of ROS formation in the CNS following pretreatment with phenobarbital were unchanged; however, the basal rate of oxidative activity in the pretreated liver was elevated significantly from control values. This suggests that hepatic cytochrome P450 induction can generate radicals in the absence of a xenobiotic substrate. The cytochrome P450 series of enzymes is involved in the oxidation of a wide range of compounds and utilizes molecular oxygen [29]. There is some evidence that the oxygenated intermediates of cytochrome P450 itself can decompose in a minor side reaction and release superoxide anion, which rapidly produces hydrogen peroxide via dismutation. Thus, when amounts of P450 and its reductase are increased by pretreatment of animals with phenobarbital, the rates of H_2O_2 production by liver microsomes become elevated [29]. These results are in accord with our findings of increased rates of ROS formation in liver fractions from phenobarbital-treated rats, both basally and following exposure to toluene. Phenobarbital pretreatment has been shown to increase cytochrome P450 mixed-function oxidase levels in the brain 2-fold [30]. This report also showed that brain P450 levels were one-tenth the corresponding hepatic levels, and this may explain our inability to detect an increase in the basal rate of ROS formation in the CNS. Cortex and cerebellum from phenobarbital-treated rats were more sensitive than control regions to the induction of ROS by toluene *in vitro*, suggesting that P450 levels may influence cerebral rates of ROS formation. Prior studies with phenobarbital have also demonstrated that *in vivo* pretreatment accelerates hepatic metabolism of toluene and benzene, and thereby results in increased tolerance of the rats to the narcotic action of toluene [14]. Pretreatment of rats with metyrapone, a mixed-function oxidase inhibitor, significantly reduces the toluene-stimulated rate of ROS generation in cerebrocortical synaptosomal and hepatic microsomal fractions [7]. This supports our finding that the first step in the catabolism of toluene (by cytochrome P450) can contribute to the enhancement of ROS formation.

These data led us to inquire as to the ROS-enhancing potential of the other enzymes involved in toluene catabolism. Animals were pretreated with an alcohol dehydrogenase inhibitor, 4-methylpyrazole, or an aldehyde dehydrogenase inhibitor, cyanamide. This resulted in either an elevation or a decline, respectively, in the rate of ROS formation in all brain regions studied and liver. The results with cyanamide suggest that the ensuing accumulation of benzaldehyde may lead to its being metabolized by an alternate pathway that enhances ROS formation, presumably by way of aldehyde oxidase. Even though aldehyde oxidase levels have not been directly measured in the brain, Weiner [21] has suggested that the aldehyde oxidizing system is widely distributed in all mammalian tissues. Pretreatment of rats with 4-methylpyrazole, by inhibiting the metabolism of benzyl alcohol to benzaldehyde, may lead to an accumulation of the alcohol, thereby quenching any ROS formation resulting from the cytochrome P450-catalyzed metabolism of toluene.

ESR spectroscopy was performed to determine the specific oxygen radical produced and detected in our system. ESR spectra corresponding to the hydroxyl radical were obtained from mixtures of crude synaptosomal fractions with toluene or benzaldehyde. ESR spectra revealing reactive oxygen species were not obtained from control P2 fractions or from incubations with benzyl alcohol. When benzaldehyde was incubated with aldehyde dehydrogenase, a very strong hydroxyl radical signal was observed. This spectrum was eliminated completely by the addition of superoxide dismutase and decreased slightly by the addition of catalase to the incubation mixture. These results suggest that superoxide anion is the primary, but not the only, radical produced in the metabolism of toluene, with subsequent enzymatic formation of hydroxyl radical. As stated previously, aldehyde oxidase has also been shown to release superoxide anion in the metabolism of ethanol [23], a finding that may also relate to the metabolism of toluene by brain and liver. ROS generation during toluene oxidation may occur in at least two steps: by way of cytochrome P450 enzymes acting directly on toluene and in the oxidation of benzaldehyde.

In conclusion, exposure to toluene results in broad systemic elevation in the normal cellular rate of oxygen radical generation. This appears to be due primarily to the release of superoxide anion generated during the catabolism of toluene by mixed-function oxidase and aldehyde dehydrogenase. Glutathione may play an important role in the protection against the induction of ROS generation in the CNS and liver, subsequent to toluene exposure. These conclusions are strengthened by the close correlation between *in vivo* and *in vitro* findings. Future studies are needed to establish the role that

aldehyde oxidase plays in toluene metabolism. In addition, further characterization of the potential of various exogenous antioxidants to protect against the effects of toluene may aid in the development of therapeutic and preventative approaches to toluene poisoning.

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