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PRODUCTION OF FORMALDEHYDE AND ACETONE BY HYDROXYL-RADICAL GENERATING SYSTEMS DURING THE METABOLISM OF TERTIARY BUTYL ALCOHOL*

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Abstract—*t*-Butyl alcohol is not a substrate for alcohol dehydrogenase or for the peroxidatic activity of catalase and, therefore, it is used frequently as an example of a non-metabolizable alcohol. *t*-Butyl alcohol is, however, a scavenger of the hydroxyl radical. The current report demonstrates that *t*-butyl alcohol can be oxidized to formaldehyde plus acetone by hydroxyl radicals generated from four different systems. The systems studied were: (a) two chemical systems, namely, the iron catalyzed oxidation of ascorbic acid and the Fenton reaction between H_2O_2 and iron; (b) an enzymatic system, the coupled oxidation of xanthine by xanthine oxidase; and (c) a membrane-bound system, NADPH-dependent microsomal electron transfer. The oxidation of *t*-butyl alcohol appeared to be mediated by hydroxyl radicals, or by a species with the oxidizing power of the hydroxyl radical, because the production of formaldehyde plus acetone was (a) inhibited by competing scavengers of the hydroxyl radical; (b) stimulated by the addition of iron–EDTA; and (c) inhibited by catalase. The last observation suggests that H_2O_2 served as the precursor of the hydroxyl radical in all three systems. A possible mechanism is hydrogen abstraction to form the alkoxyl radical ($(CH_3)_3$—C—O·), spontaneous fission of the alkoxyl radical to produce acetone and the methyl radical (CH_3·), interaction of the methyl radical with O_2 to form the methyl peroxy radical (CH_3OO·), and decomposition of the later to formaldehyde. These results extend the alcohol oxidizing capacity of the microsomal alcohol oxidizing system to a tertiary alcohol. Since *t*-butyl alcohol is not a substrate for alcohol dehydrogenase or catalase, the ability of microsomes to oxidize *t*-butyl alcohol lends further support for a role for hydroxyl radicals in the microsomal alcohol oxidation system. In view of the production of formaldehyde, and the reactivity as well as further metabolism of this aldehyde, caution should be used in interpreting experiments in which *t*-butyl alcohol is used as a presumed "non-metabolizable" alcohol. *t*-Butyl alcohol may be a valuable probe for the detection of hydroxyl radicals in intact cells and *in vivo*.

t-Butyl alcohol cannot form an aldehyde or ketone by dehydrogenation, and it does not serve as a substrate for alcohol dehydrogenase [1, 2]. Studies with rats indicate that *t*-butyl alcohol is not metabolized, as reflected by an almost constant blood level [3, 4], while in hepatocytes *t*-butyl alcohol does not change significantly the lactate/pyruvate ratio [5]. As a consequence, *t*-butyl alcohol has been employed frequently as a prime example of a non-metabolizable alcohol [3, 4, 6–9]. The effects of *t*-butyl alcohol are often compared to those of ethanol in order to evaluate whether the actions of ethanol reflect a direct effect of ethanol or require its metabolism, with associated changes in the cellular redox state and/or the production of acetaldehyde. An important assumption in formulating these conclusions is that *t*-butyl alcohol is not metabolized to an aldehyde.

While it is clear that *t*-butyl alcohol may not be metabolized rapidly, other results indicate that it is not as inert metabolically as usually assumed. Kamil *et al.* [10] had reported the presence of a glucuronide in the urine of rabbits after a single injection of *t*-butyl alcohol. More recently, McComb and Goldstein [11] found that a single dose of *t*-butyl alcohol (8.1 mmoles/kg) is eliminated in 8–9 hr; however, after 3 days of exposure to *t*-butyl alcohol, the elimination rate is increased, i.e. a metabolic adaptation to *t*-butyl alcohol develops. Baker *et al.* [12] found that acetone appeared in the urine and expired air of rats receiving 0.75 to 2.0 g/kg of *t*-butyl alcohol. The acetone excretion is not due to a disturbance of fatty acid metabolism by the administered *t*-butyl alcohol. Studies with [^{14}C]- and [^{13}C]-*t*-butyl alcohol yielded labeled acetone and demonstrated directly the fragmentation of the *t*-butyl alcohol molecule [12].

t-Butyl alcohol is a scavenger of the hydroxyl radical*, with a rate constant equal to $8 \times 10^8 M^{-1}$ sec^{-1} [13]. In chemical systems that generate ·OH (H_2O_2 plus metal salts), *t*-butyl alcohol is oxidized

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* Abbreviations: ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; Me_2SO, dimethyl sulfoxide; KTBA, 2-keto-4-thiomethylbutyric acid; DETAPAC, diethylenetriaminepentaacetic acid; and $O_2^{\cdot-}$, superoxide anion radical.

to a dimer

$$\left((CH_3)_2—\underset{\displaystyle OH}{\underset{|}{C}}—CH_2\right)_2$$

in the presence of iron, or to the carbinol

$$(CH_3)_2—\underset{\displaystyle OH}{\underset{|}{C}}—CH_2—OH$$

in the presence of copper [14]. A preliminary report showing the production of formaldehyde from *t*-butyl alcohol by rat liver microsomes has been presented [15]. In view of the production of both acetone [12] and formaldehyde [15] from *t*-butyl alcohol, it is likely that interaction of *t*-butyl alcohol with a potent oxidizing species such as $^{\cdot}OH$ results in a demethylation to produce acetone and a methyl radical ($^{\cdot}CH_3$) $(CH_3)_3—C—OH + {}^{\cdot}OH \rightarrow (CH_3)_2—C=O + {}^{\cdot}CH_3$ The methyl radical is the source of formaldehyde. Dimethyl sulfoxide (Me_2SO) is known to react with $^{\cdot}OH$ to produce $^{\cdot}CH_3$ [16–18]. The interaction of Me_2SO with $^{\cdot}OH$ has been shown recently to produce formaldehyde as a major product [19].

The objective of this report is to demonstrate that *t*-butyl alcohol can be oxidized to formaldehyde plus acetone by various systems known to generate $^{\cdot}OH$.

EXPERIMENTAL PROCEDURES

Liver microsomes were prepared from male Sprague–Dawley rats (200–300 g) as previously described [20], washed once, and resuspended in 125 mM KCl. The oxidation of *t*-butyl alcohol was assayed at 37° in center-well flasks containing 0.6 ml of 15 mM semicarbazide–HCl in 180 mM potassium phosphate, pH 7.4, in the center-well. The reaction mixture contained 100 mM potassium phosphate, pH 7.4, 10 mM sodium pyrophosphate, 10 mM $MgCl_2$, 10 mM glucose-6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, 0.3 mM $NADP^+$, 0.1 mM EDTA and about 5 mg of microsomal protein in a final volume of 3.0 ml. The final concentration of *t*-butyl alcohol in most experiments was 35 mM. Azide, when added, was present at a final concentration of 1.0 mM. The reaction was initiated by the addition of the NADPH-generating system and was terminated by the addition of trichloroacetic acid (final concentration of 4.5%). In most cases (except for Fig. 3), the reaction period was for 30 min. After an overnight diffusion period at room temperature, an aliquot of the center-well was diluted with H_2O to 3.0 ml, and the optical density of the acetone-semicarbazone complex was determined at 224 nm as previously described for the oxidation of isopropyl alcohol to acetone [21]. Formaldehyde is not measured in this procedure. The contents of the main compartment were centrifuged, and an aliquot of the clear supernatant fraction was then assayed for formaldehyde by the method of Nash [22]. In some experiments, separate flasks were utilized to analyze for acetone (center-well flasks) and for formaldehyde (25-ml Erlenmeyer flasks) in order to ensure that each product did not interfere with the measurement of the other. Blanks included zero-time controls (trichloroacetic acid added before the NADPH-generating system), boiled microsomes, and flasks lacking either microsomes, *t*-butyl alcohol or the NADPH-generating system. Extinction coefficients of 9.4 and 8.0 $mM^{-1}cm^{-1}$ were used to calculate the amounts of acetone and formaldehyde, respectively, which were produced from the oxidation of *t*-butyl alcohol.

Two model systems were used to generate $^{\cdot}OH$ in the absence of microsomes. One was the iron-catalyzed oxidation of ascorbic acid [23, 24]. The reaction mixture contained 100 mM potassium phosphate, pH 7.4, to which was added 0.10 mM EDTA, a 0.167 mM ferrous ammonium sulfate in 0.33 mM EDTA mixture, and 35 mM *t*-butyl alcohol in a final volume of 3.0 ml. After incubating at 37° for 2–3 min, the reaction was initiated by the addition of ascorbate (final concentration of 2 mM) and was quenched at various time intervals by the addition of trichloroacetic acid. Center-well flasks as described for the microsomal experiments were utilized. Zero-time controls contained the acid added before the ascorbate. Formaldehyde and acetone were assayed as described above. The second system was the coupled oxidation of xanthine oxidase [23, 25]. The reaction mixture contained 100 mM potassium phosphate, pH 7.4, 0.10 mM EDTA, a 0.083 mM ferrous ammonium sulfate in 0.167 mM EDTA mixture, 35 mM *t*-butyl alcohol and 0.18 units of xanthine oxidase in a final volume of 3.0 ml. The reaction was initiated at 37° by the addition of xanthine (final concentration of 1.0 mM) and was terminated by the addition of trichloroacetic acid. Zero-time controls contained the acid added before the xanthine. Reactions were determined as described above.

In some experiments, the formation of acetone from *t*-butyl alcohol was confirmed by gas chromatography. Microsomal experiments were carried out in regular 25-ml Erlenmeyer flasks sealed with tight serum caps. The reactions were terminated by injection of acid through the caps. The flasks were incubated at 60°, and 1-ml samples from the head space were injected directly into a Hewlett–Packard model 5750 gas chromatograph for the measurement of acetone. A Porapak N (50–80) column (6 ft) was used at 60°. Operating conditions were similar to those previously described [26] except that the injection temperature was 130°. The retention time for acetone under these conditions was 11.5 min.

For the microsome experiments, all solutions except for the $MgCl_2$ and the microsomes themselves were passed through a Chelex-100 column to remove extraneous metals. Diethylenetriaminepentaacetic acid (DETAPAC), ascorbic acid, and 2-keto-4-thiomethylbutyric acid (KTBA) were obtained from the Sigma Chemical Co. (St. Louis, MO), xanthine was from the Eastman Kodak Co. (Rochester, NY), and xanthine oxidase (0.45 units/mg protein) and catalase (65,000 units/mg protein) were from Boehringer Mannheim (Indianapolis, IN). Superoxide dismutase was obtained as a lyophilized powder from the Biotics Research Corp. (Houston, TX). Chelex-100 resin (200–400 mesh) was from BioRad Laboratories (Richmond, CA), and *t*-butyl alcohol, sodium azide, H_2O_2 and most other agents were from the Fisher Chemical Co. (Springfield, NJ). An iron–EDTA solution was prepared by dissolving

5 mM ferrous ammonium sulfate in 10 mM EDTA (1:2 iron–EDTA mixture).

All values refer to mean ± S.E.M. Statistical analysis was performed by Student's *t*-test. The number of experiments is indicated in the legends to figures or tables.

RESULTS

Oxidation of t-*butyl alcohol by ascorbate–Fe–EDTA.* The iron-catalyzed oxidation of ascorbic acid was used as a model 'OH-generating system [23, 24]. *t*-Butyl alcohol was oxidized to formaldehyde plus acetone by this system (Fig. 1). No product was formed when *t*-butyl alcohol or ascorbate was omitted. Production of formaldehyde or acetone was lowered about 75% when Fe–EDTA was omitted from the system.

Superoxide dismutase had no significant effect on the production of formaldehyde and acetone by the ascorbate–Fe–EDTA system (Fig. 1). This suggests the absence of a major role for the superoxide anion in generating 'OH by this system. In contrast, catalase produced strong inhibition of the production of formaldehyde plus acetone (Fig. 1). This indicates that H_2O_2 played a major role as the precursor of 'OH. The inhibition by catalase also indicates that *t*-butyl alcohol did not serve as an effective substrate for the peroxidatic activity of catalase.

The oxidation of *t*-butyl alcohol was inhibited by competing 'OH scavenging agents. The amount of formaldehyde produced in 5 min by the control ascorbate–Fe–EDTA system was 414 ± 51 nmoles, whereas the amounts produced in the presence of 50 mM benzoate, 67 mM mannitol or 35 mM 1-butanol were 27 ± 12 (−93%), 29 ± 28 (−93%) or 98 ± 79 (−76%) nmoles/5 min respectively (N = 3). The amount of acetone produced in 5 min by the control system was 276 ± 70 nmoles, whereas the amounts produced in the presence of 67 mM mannitol or 33 mM dimethyl sulfoxide were lowered to 96 ± 67 (−65%) and 30 ± 14 (−89%) nmoles/5 min respectively (N = 3).

Oxidation of t-*butyl alcohol by a Fenton system.* *t*-Butyl alcohol was also metabolized to acetone when a Fenton system was studied. The system consisted of 3 mM H_2O_2, 35 mM *t*-butyl alcohol and 1 mM ferrous ammonium sulfate alone or a 1 mM ferrous ammonium sulfate–2 mM DETAPAC chelate. The reactivity of ferrous–DETAPAC in Fenton-type oxidations has been reported [27]. The amount of acetone produced was 220 and 460 nmoles in the iron alone or iron–DETAPAC system respectively (N = 2).

Oxidation of t-*butyl alcohol during the coupled oxidation of xanthine by xanthine oxidase.* The coupled oxidation of xanthine by xanthine oxidase was used as a second model 'OH-generating system [23, 25]. The production of 'OH by this system is probably mediated by the interaction of $O_2^{\cdot-}$ and H_2O_2 in an iron-catalyzed Haber–Weiss type of reaction [28, 29]. This system oxidizes other 'OH scavengers, e.g. ethylene gas is produced from methional [25], acetaldehyde from ethanol [23], and formaldehyde from dimethyl sulfoxide [19]. When *t*-butyl alcohol was the substrate, both formaldehyde and acetone were produced (Fig. 2). Neither product was produced when either xanthine or xanthine oxidase was omitted. Product formation was also markedly lowered when Fe–EDTA was omitted. Production of formaldehyde and acetone was inhibited by superoxide dismutase and by catalase (Fig. 2). These results confirm that both $O_2^{\cdot-}$ and H_2O_2 are required to generate 'OH [25]. Product formation was also inhibited by the competing 'OH scavenging agents, benzoate and mannitol (data not shown).

Oxidation of t-*butyl alcohol by rat liver microsomes.* NADPH-dependent electron transfer by rat liver microsomes was used as a membrane-bound 'OH-generating system [26]. Previous experiments indicated that the molecular mechanism for the oxidation of primary aliphatic alcohols by rat liver microsomes involved, at least in part, the interactions of the alcohols with 'OH generated during micro-

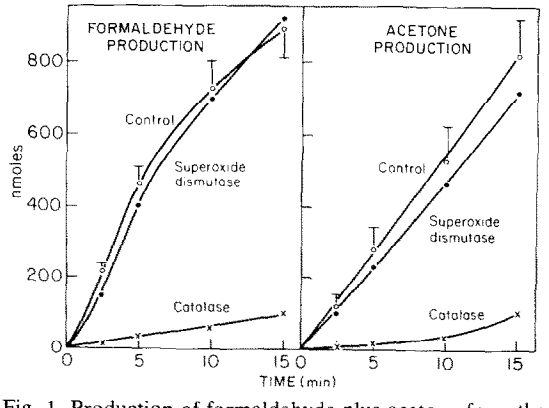

Fig. 1. Production of formaldehyde plus acetone from the oxidation of *t*-butyl alcohol by the iron–ascorbate 'OH-generating system. The oxidation of 35 mM *t*-butyl alcohol was assayed as described in Experimental Procedures. Results are from six experiments for the controls and from two experiments for the catalase and superoxide dismutase experiments. Final concentrations of catalase and superoxide dismutase were 67 and 60 μg/ml respectively.

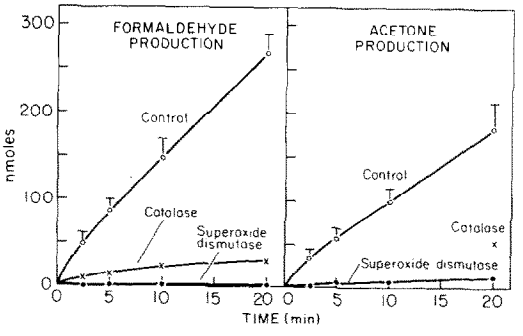

Fig. 2. Production of formaldehyde plus acetone from the oxidation of *t*-butyl alcohol by the xanthine–xanthine oxidase 'OH-generating system. The oxidation of 35 mM *t*-butyl alcohol was assayed in the presence of 0.083 mM iron–0.167 mM EDTA as described in Experimental Procedures. Results are from five experiments for the controls and from two experiments for the catalase (67 μg/ml) and superoxide dismutase (60 μg/ml) experiments.

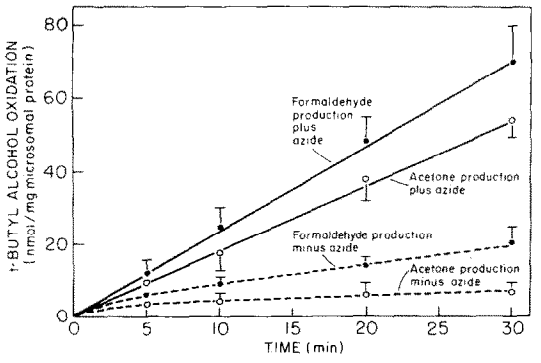

Fig. 3. Time course of the oxidation of *t*-butyl alcohol by rat liver microsomes. The production of formaldehyde plus acetone from the oxidation of 35 mM *t*-butyl alcohol during NADPH-dependent microsomal electron transfer was assayed in the absence or presence of 1.0 mM azide as described in Experimental Procedures. Results are from eight experiments for the formaldehyde plus azide curve and from four experiments for all the other curves.

somal electron transfer [20, 30, 31]. Evidence has been presented recently that a branch chain alcohol, isopropyl alcohol, also serves as a substrate [21]. Similar experiments have now been carried out with *t*-butyl alcohol (Fig. 3). Rat liver microsomes become contaminated with catalase during their preparation. Therefore, the production of formaldehyde plus acetone from *t*-butyl alcohol was assayed in both the absence and presence of azide, an inhibitor of catalase (Fig. 3). Azide increased the rate of oxidation of *t*-butyl alcohol (Fig. 3). Inhibition of catalase by azide prevented the enzymatic decomposition of H_2O_2. The increased yield of product in the presence of azide is consistent with H_2O_2 serving as a precursor of $^{\cdot}OH$ in the microsomal system; this is analogous to the precursor role for H_2O_2 in the ascorbate–Fe–EDTA and xanthine—xanthine oxidase systems.

Control experiments indicated that the production of formaldehyde plus acetone was markedly lowered in the absence of either microsomes, *t*-butyl alcohol or the NADPH-generating system (data not shown). Boiled microsomes did not support the oxidation of *t*-butyl alcohol. Hydrogen peroxide, in the absence of the NADPH-generating system, did not effectively support the oxidation of *t*-butyl alcohol. The latter experiment indicated that the production of formaldehyde plus acetone by the microsomes, as well as the increase in product formation produced by azide, was not due to a direct interaction between *t*-butyl alcohol and H_2O_2. The inability of H_2O_2 to replace the NADPH-generating system shows that microsomal electron transfer is required to generate $^{\cdot}OH$.

Further indication of an indirect role for H_2O_2 in the oxidation of *t*-butyl alcohol was provided by the ability of externally-added H_2O_2 to stimulate the production of formaldehyde plus acetone over the control rates found in the presence of the NADPH-generating system alone (Table 1). Azide was present in these latter experiments to inhibit catalase and prevent the catalytic decomposition of H_2O_2. Therefore, although H_2O_2 alone was not effective in supporting the oxidation of *t*-butyl alcohol, H_2O_2 did increase product formation in the presence of microsomal electron transfer.

The rate of oxidation of *t*-butyl alcohol was dependent on the concentration of *t*-butyl alcohol used (Fig. 4). The apparent V_{max} value (nmoles per min per mg microsomal protein) was either 4.1 (calculated from the formaldehyde data) or 3.4 (calculated from the acetone data). The concentration of *t*-butyl alcohol that gave half-maximal yield of product under these conditions was about 24 mM as calculated from the formaldehyde production data and about 22 mM based on the acetone production data.

Further support linking the oxidation of *t*-butyl alcohol to the production of $^{\cdot}OH$ by microsomes was provided by studying the effects of several $^{\cdot}OH$ scavenging agents on the oxidation of *t*-butyl alcohol. Mannitol, KTBA and benzoate inhibited the production of formaldehyde plus acetone in a concentration-dependent manner (Table 2). These compounds do not affect other microsomal functions such as the activities of aniline hydroxylase and aminopyrine demethylase [20].

Effect of iron on the oxidation of t*-butyl alcohol by rat liver microsomes.* In biological systems, iron may stimulate $^{\cdot}OH$ generation by a modified Haber–Weiss reaction or the Fenton reaction. Iron–EDTA stimulates $^{\cdot}OH$ production by the xanthine oxidase system [28, 29]. The oxidation of

Table 1. Effect of H_2O_2, in the presence of NADPH, on microsomal oxidation of *t*-butyl alcohol to formaldehyde and acetone*

Conc of H_2O_2 (mM)	Oxidation of *t*-butyl alcohol		Stimulation by H_2O_2	
	Formaldehyde production (nmoles/min/mg protein)	Acetone production (nmoles/min/mg protein)	Formaldehyde production (%)	Acetone production (%)
0	3.38 ± 0.38	2.84 ± 0.44		
0.1	4.28 ± 0.24	3.80 ± 0.39	27	34
0.3	4.73 ± 0.47	4.44 ± 0.31	40†	56†
1.0	5.09 ± 0.66	4.57 ± 0.70	51†	61†

* Oxidation of *t*-butyl alcohol by rat liver microsomes to formaldehyde plus acetone was assayed in the presence of the indicated concentrations of H_2O_2. Azide was present at a final concentration of 1.0 mM. Results are from either five (acetone production) or four (formaldehyde production) experiments.

† $P < 0.05$.

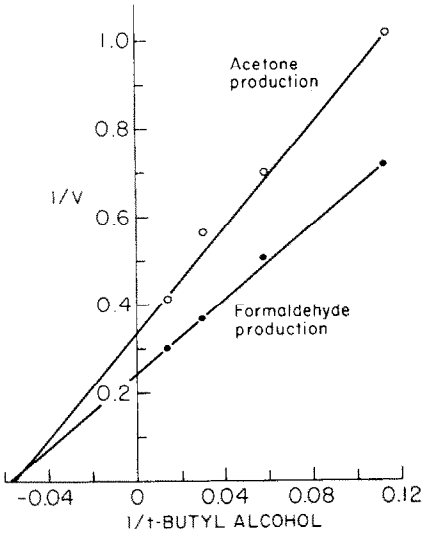

Fig. 4. Lineweaver–Burk plot of the substrate dependence of *t*-butyl alcohol oxidation by rat liver microsomes. The oxidation of 9.0, 17.5, 35 and 70 mM *t*-butyl alcohol to formaldehyde plus acetone was assayed in the presence of 1 mM azide. Results are from six to nine experiments.

ethanol and 1-butanol by rat liver microsomes is also stimulated by Fe–EDTA and blocked by competitive ·OH scavengers [32]. It follows that if ·OH were responsible for the oxidation of *t*-butyl alcohol, an increase in the rate of ·OH generation would result in increased production of formaldehyde and acetone. The addition of Fe–EDTA increased the production of formaldehyde and acetone to comparable extents, in a concentration-dependent manner (Table 3). As a control, addition of EDTA alone (beyond the 100 μM EDTA already present in the basic reaction mixture) had no effect on the oxidation of *t*-butyl alcohol (data not shown).

Of considerable interest is the fact that Fe–EDTA increased the formation of product in the presence as well as in the absence of azide (Table 3). This suggests that the added Fe–EDTA competes favorably with microsomal catalase for the metabolically-generated H_2O_2.

In the microsomal experiments, the solutions (except for the $MgCl_2$ and the microsomes themselves) were treated with Chelex-100 resin to remove extraneous iron. Since microsomes catalyze the oxidation of *t*-butyl alcohol in the absence of externally-added iron—EDTA, ·OH production may be mediated by adventitious iron in the microsomes or, perhaps, by the iron chelate cytochrome P-450. EDTA itself stimulated the oxidation of *t*-butyl alcohol; rates of formaldehyde production in the absence and presence of 0.1 mM EDTA were 0.72 ± 0.09 and 2.76 ± 0.43 nmoles per min per mg protein respectively ($N = 3$). The stimulation by EDTA probably reflected chelation of microsomal iron. Desferrioxamine, a potent iron chelator that blocks the iron-catalyzed production of ·OH in a variety of systems, inhibited the oxidation of *t*-butyl alcohol by 92% when added at a concentration of 0.33 mM (the system contained 0.1 mM EDTA). These differences in the actions of desferrioxamine and EDTA support a role for iron in the oxidation of *t*-butyl alcohol by the microsomes.

Effect of inhibitors and inducers of cytochrome P-450 on the oxidation of t*-butyl alcohol by rat liver microsomes.* The results with competitive ·OH scavengers, azide, H_2O_2, iron and iron–chelators support a role for ·OH in the mechanism by which microsomes oxidize *t*-butyl alcohol. Can *t*-butyl alcohol be oxidized by cytochrome P-450, acting as a peroxidase? Previous results [20, 32] indicate that competitive ·OH scavengers or iron–EDTA have no effect on the cytochrome P-450-dependent oxidation of aminopyrine or aniline, thus disassociating the effects of these agents on microsomal metabolism of ·OH scavengers from effects on the metabolism

Table 2. Effects of hydroxyl radical scavenging agents on microsomal oxidation of *t*-butyl alcohol to formaldehyde and acetone*

Scavenger	Conc (mM)	Oxidation of *t*-butyl alcohol: Formaldehyde production (nmoles/min/mg protein)	Oxidation of *t*-butyl alcohol: Acetone production (nmoles/min/mg protein)	Inhibition by scavenger: Formaldehyde production (%)	Inhibition by scavenger: Acetone production (%)
Control		2.82 ± 0.31	2.32 ± 0.17		
mannitol	10	2.32 ± 0.29	1.67 ± 0.12	18	27
	33	1.78 ± 0.21	1.10 ± 0.20	37†	52‡
	67	1.42 ± 0.26	0.51 ± 0.04	50†	78§
	100	1.30 ± 0.17	0.28 ± 0.06	54†	88§
KTBA	10	1.12 ± 0.14	0.86 ± 0.15	61‡	63‡
	33	0.88 ± 0.18	0.52 ± 0.24	69‡	77§
Benzoate	10	1.88 ± 0.43	1.72 ± 0.28	34	25
	33	0.90 ± 0.07	1.27 ± 0.16	68‡	45†
	67	0.64 ± 0.05	0.90 ± 0.27	77‡	61‡

* Oxidation of *t*-butyl alcohol by rat liver microsomes to formaldehyde plus acetone was assayed as described in Experimental Procedures. Results are from four experiments.

† $P < 0.05$.

‡ $P < 0.01$.

§ $P < 0.001$.

Table 3. Effects of Fe–EDTA on microsomal oxidation of *t*-butyl alcohol to formaldehyde and acetone in the presence and absence of azide*

Conc of Fe-EDTA (μM)	Conc of azide (mM)	Oxidation of *t*-butyl alcohol		Stimulation by Fe–EDTA	
		Formaldehyde production (nmoles/min/mg protein)	Acetone production	Formaldehyde production (%)	Acetone production
0	1.0	3.30 ± 0.51	2.70 ± 0.51		
3.4		5.48 ± 0.78	3.93 ± 0.38	66†	46
8.5		6.62 ± 0.75	4.92 ± 0.51	101‡	82†
17.0		7.68 ± 0.83	5.61 ± 0.48	133§	108‡
50.0		10.10 ± 1.24	7.10 ± 0.68	206§	161§
0	0	0.79 ± 0.23	0.42 ± 0.06		
3.4		2.20 ± 0.20	1.44 ± 0.40	178†	243†
8.5		3.40 ± 0.16	2.40 ± 0.44	330‡	471‡
17.0		6.31 ± 0.19	2.85 ± 0.49	699§	579§
50.0		10.40 ± 1.02	5.43 ± 0.81	1216§	1193§

* Oxidation of *t*-butyl alcohol by rat liver microsomes to formaldehyde plus acetone was assayed in the presence of the indicated concentrations of Fe–EDTA and azide. Results are from four experiments in the presence of 1.0 mM azide and from three experiments in the absence of azide.
† $P < 0.05$.
‡ $P < 0.01$.
§ $P < 0.001$.

of typical mixed function oxidase substrates. Table 4 shows that the production of formaldehyde from aminopyrine was inhibited by metyrapone and SKF-525A, two typical inhibitors of cytochrome P-450 mediated reactions. However, these two inhibitors had no effect on the production of formaldehyde from *t*-butyl alcohol (Table 4). In other experiments, rats were treated with phenobarbital (80 mg per kg body wt per day for 4 days) or 3-methylcholanthrene (20 mg per kg body wt per day for 3 days) to induce the microsomes. Controls received either saline or corn oil, and microsomes were prepared after an overnight fast. The rates of *t*-butyl alcohol oxidation were (nmoles formaldehyde per min per mg microsomal protein) as follows: saline control, 2.51 ± 0.25; phenobarbital-treated, 1.69 ± 0.23; corn oil control, 3.22 ± 0.34; and 3-methylcholanthrene in corn oil, 3.74 ± 0.47 (N = 4 in all cases). Thus, there was no increase in the metabolism of *t*-butyl alcohol after treatment with inducers of mixed function oxidase activity. These results suggest that *t*-butyl alcohol was not metabolized by a direct peroxidase-like action of cytochrome P-450.

DISCUSSION

t-Butyl alcohol was oxidized to formaldehyde and acetone by four different systems that produce hydroxyl radicals. The systems were: two chemical systems, the iron-catalyzed oxidation of ascorbic acid and the Fenton reaction of iron with H_2O_2; an enzymatic system, the oxidation of xanthine by xanthine oxidase; and a membrane-bound system, NADPH-dependent microsomal electron transfer. The following observations are consistent with an oxidation of *t*-butyl alcohol mediated by ·OH or by

Table 4. Effects of metyrapone and SKF-525A on microsomal oxidation of aminopyrine and *t*-butyl alcohol*

Addition	Conc (mM)	Rate of formaldehyde production (nmoles/min/mg)			
		Aminopyrine		*t*-butyl alcohol	
		Rate	Effect (%)	Rate	Effect (%)
Control		17.73 ± 2.27		3.85 ± 0.15	
Metapyrone	0.33	9.93 ± 1.11	−44†	3.93 ± 0.29	+2
	1.0	7.17 ± 1.05	−60‡	3.49 ± 0.26	−9
Control		13.26 ± 0.99		2.76 ± 0.32	
SKF-525A	0.1	9.66 ± 0.83	−27‡	2.65 ± 0.32	−4
	1.0	6.78 ± 0.85	−49§	2.43 ± 0.29	−12

* Oxidation of either aminopyrine (10 mM) or *t*-butyl alcohol to formaldehyde was assayed in the absence or presence of metyrapone or SKF-525A. Results are from three experiments.
† $P < 0.05$.
‡ $P < 0.02$.
§ $P < 0.01$.

a species with the oxidizing power of ·OH. The production of both formaldehyde and acetone was inhibited by competing ·OH scavengers; the effect was relatively specific in that the scavengers did not affect microsomal electron transfer or microsomal drug metabolism.The ascorbate and xanthine oxidase systems were inhibited by catalase, while the microsomal system, which contains endogenous catalase, was stimulated by azide, a catalase inhibitor. Since H_2O_2 alone did not oxidize *t*-butyl alcohol, the data indicate an indirect role for H_2O_2; H_2O_2 served as a precursor of ·OH. The oxidation of *t*-butyl alcohol was increased by the addition of iron–EDTA; iron–EDTA is known to increase the rate of generation of ·OH in several systems. Production of ·OH by the xanthine oxidase system involves an $O_2^{\cdot-}$-dependent, iron-catalyzed Haber–Weiss reaction [28, 29]; oxidation of *t*-butyl alcohol by the xanthine oxidase system was inhibited by superoxide dismutase.

A Fenton-type reaction may be responsible for the generation of ·OH in all systems. $H_2O_2 + Fe^{2+}$–chelate $\rightarrow$ ·OH + OH^- + Fe^{3+}–chelate. Since it is iron in the ferrous state which reacts with H_2O_2 to produce ·OH, reduction of ferric iron to ferrous iron is required for continuous activity. In the xanthine oxidase system, the Fe^{3+}–chelate is reduced to the Fe^{2+}–chelate by $O_2^{\cdot-}$; hence, superoxide dismutase was inhibitory (Fig. 2). In the ascorbate system, the Fe^{3+}–chelate can be reduced to the Fe^{2+}–chelate by ascorbate [33]; hence, superoxide dismutase was not inhibitory in this latter system (Fig. 1). In the microsomal system, reduction of iron may occur either via NADPH-dependent electron transfer or via $O_2^{\cdot-}$. The generation of $O_2^{\cdot-}$ during microsomal electron transfer has been reported [34–37]. We have found that the addition of superoxide dismutase (67 μg/ml) did not inhibit oxidation of *t*-butyl alcohol; however, the added superoxide dismutase may not have penetrated to the site of $O_2^{\cdot-}$ generation in the microsomes. Therefore, the nature of the iron present in the microsomes, as well as the pathway of reduction of the iron, remains to be determined.

The microsomal pathway for oxidizing primary aliphatic alcohols [38] had been ascribed previously by some investigators to either the peroxidatic activity of catalase [39] or to microsomal alcohol dehydrogenase [40] or to both [41, 42]. Since *t*-butyl alcohol does not serve as a substrate for alcohol dehydrogenase [1, 2] nor was it an effective substrate for the peroxidatic activity of catalase (witness the inhibition by catalase of *t*-butyl alcohol oxidation in the ascorbate and xanthine oxidase systems, and the stimulation by azide in the microsomal system), an alternative mechanism is required. Previous studies demonstrated that rat liver microsomes oxidize several ·OH scavengers to their respective products: ethylene was produced from methional or KTBA [26], methane or formaldehyde from Me_2SO [19, 26], and acetone from isopropyl alcohol [21]. It has been suggested that the oxidation of aliphatic alcohols involves, at least in part, an interaction with ·OH generated by the microsomes [20, 30]. The ability of the microsomes to oxidize *t*-butyl alcohol lends further support to a role for ·OH in the molecular mechanism whereby alcohols are metabolized by microsomes.

The V_{max} for *t*-butyl alcohol (3–4 nmoles per min per mg microsomal protein) was only one-half or one-quarter that found for the microsomal oxidation of methanol and ethanol respectively (Fig. 4; [20, 43]). The rate constants for the interactions of these alcohols with ·OH [13] are ($M^{-1}sec^{-1}$): *t*-butyl alcohol, 0.8×10^9; methanol, 1.1×10^9; and ethanol, 1.8×10^9, i.e. ethanol > methanol > *t*-butyl alcohol, the same order as the V_{max} values. Another contributing factor may be that, whereas primary alcohols are oxidized by a chain reaction (one ·OH can result in the oxidation of more than one alcohol), tertiary alcohols react with ·OH by a non-chain reaction mechanism [44].

The reactions of ·OH with alcohols proceed by hydrogen abstraction to produce either hydroxyalkyl radicals or alkoxyl radicals [13]. *t*-Butyl alcohol would produce either $\cdot CH_2—C(CH_3)_2—OH$, the hydroxyalkyl radical, or $(CH_3)_3—C—O\cdot$, the alkoxyl radical. Walling [14] reported that, in the presence of Fe and H_2O_2,

$$\left(\begin{array}{c}(CH_3)_2—C—CH_2\\ \quad\quad|\\ \quad\quad OH\end{array}\right)_2$$

is produced; this would be the result of dimerization of two hydroxyalkyl radicals. The alkoxyl radical, on the other hand, can undergo spontaneous fission to produce acetone and the methyl radical.

$$(CH_3)_3—C—O\cdot \rightarrow (CH_3)_2—C=O + \cdot CH_3$$

This has been described for di-*tert*-butylperoxide vapor which decomposes spontaneously at 130° into the free *t*-butyloxy radical, which, in turn, splits into acetone and $\cdot CH_3$ in the gas phase [45, 46]. The methyl radical can dimerize to produce ethane, or it can abstract a hydrogen from a suitable donor to produce methane (e.g. Ref. 26).

$$\cdot CH_3 + \cdot CH_3 \rightarrow CH_3—CH_3$$
$$\cdot CH_3 + RH \rightarrow CH_4 + R\cdot$$

Alternatively, the methyl radical can react with molecular oxygen to produce the methyl peroxy radical

$$\cdot CH_3 + O_2 \rightarrow CH_3OO\cdot$$

The further reaction of the methyl peroxy radical, perhaps via a Russell type mechanism [47], may result in the production of formaldehyde: $2CH_3OO\cdot \rightarrow CH_3OH + CH_2O + O_2$. Similarly, dimethyl sulfoxide reacts with ·OH to yield formaldehyde [19]; free methyl radicals have been identified as an intermediate of this interaction [16–18].

The amounts of formaldehyde produced from the oxidation of *t*-butyl alcohol by microsomes are within the range found for the N-demethylation of several typical substrates of the mixed function oxidase system. These data suggest that some caution should be used in interpreting experiments in which *t*-butyl alcohol is used as a 'non-metabolizable' alcohol. *t*-Butyl alcohol is, however, a valuable probe to evaluate metabolic events related to the shift in the cellular redox state which occurs during the metabolism of ethanol via alcohol dehydrogenase. It is intriguing

to speculate that the metabolic adaptation to *t*-butyl alcohol, which develops after 3 days of exposure to *t*-butyl alcohol [11], may involve induction of the microsomal alcohol oxidizing system, in analogy to that found after chronic administration of ethanol [48]. The *in vivo* metabolism of *t*-butyl alcohol (both unlabeled, as well as labeled with ^{14}C or ^{13}C) to acetone has been reported [12]. Baker *et al.* [12] did not observe increased production of acetone from unlabeled *t*-butyl alcohol *in vivo* after administration of the catalase inhibitor 3-amino-1,2,4-triazole. This observation is understandable because H_2O_2 generated in the smooth endoplasmic reticulum is metabolized primarily by glutathione peroxidase rather than by catalase [49] and because a large portion of unlabeled acetone is not derived directly from the administered *t*-butyl alcohol. Although the pathway for the *in vivo* oxidation of *t*-butyl alcohol remains to be determined, it is possible that this alcohol, appropriately labeled with ^{14}C or ^{13}C, can serve as a probe to detect the generation of ·OH-like species by intact cells *in vitro* or *in vivo*.

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