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OXIDATIVE DEMETHYLATION OF t-BUTYL ALCOHOL BY RAT LIVER MICROSOMES

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SUMMARY

Tertiary butyl alcohol has often been used experimentally as a "non-metabolizable" alcohol. In this report, evidence is presented that t-butanol serves as a substrate for rat liver microsomes and that it is oxidatively demethylated to yield formaldehyde. The apparent K_m for t-butanol is 30 mM while V_{max} is about 5.5 nmol per min per mg microsomal protein. Formaldehyde production is stimulated by azide, which prevents destruction of H_2O_2 by catalase. Hydroxyl radical scavenging agents, such as benzoate, mannitol, and 2-keto-4-thiomethylbutyrate, suppress formaldehyde production. Therefore, the microsomal reaction pathway appears to involve the interaction of t-butanol with hydroxyl radicals generated from H_2O_2 by the microsomes. Formaldehyde is also produced when t-butanol is incubated with model hydroxyl radical-generating systems such as the iron-EDTA-stimulated oxidation of xanthine by xanthine oxidase or the iron-EDTA-catalyzed autoxidation of ascorbate. These results indicate that t-butanol cannot be used to distinguish metabolically-linked from non-metabolically-linked actions of ethanol.

INTRODUCTION

Tertiary-butyl alcohol ($(CH_3)_3C$-OH) has been popularly used as a "non-metabolizable" alcohol (e.g., 1-5). t-Butanol cannot form an aldehyde or ketone by dehydrogenation and it does not serve as a substrate for alcohol dehydrogenase (6,7). Hence, t-butanol has been utilized in efforts to distinguish biologic events that may represent direct actions of alcohols (e.g., effects on membranes) from indirect actions that require metabolism of the alcohol. t-Butanol has been employed most frequently in conjunction with studies of the effects of ethanol, in attempts to distinguish those actions that might be ascribed to acetaldehyde (1) or to redox changes (i.e., a shift in the $NADH/NAD^+$ ratio) induced by the metabolism of ethanol (5).

We now report that t-butanol serves as a substrate for rat liver microsomes and that it is oxidatively demethylated to yield formaldehyde, a congener of acetaldehyde.

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The reaction pathway appears to involve hydroxyl radicals (•OH) generated during microsomal electron transfer reactions. Recently, McComb and Goldstein (8) noted an increased rate of elimination of t-butanol from the bloodstream in animals previously exposed to t-butanol. The increased elimination of t-butanol may reflect an induction of the smooth endoplasmic reticulum, which is known to occur after exposure to ethanol (9). Baker et al. (10) observed that rats injected with t-butanol excrete acetone in their urine. Since the formation of formaldehyde represents a fragmentation of the t-butanol molecule, the production of other products, such as acetone, would not be unexpected (eq. 1):

$$(CH_3)_3\text{-C-OH} + \cdot OH \longrightarrow CH_2{=}O + (CH_3)_2\text{-C=O} + \text{other products} \quad (1)$$

A portion of t-butanol is also excreted as a glucuronic acid conjugate (11). The production of formaldehyde and its subsequent metabolism and/or reaction with cellular constituents means that t-butanol cannot be used to distinguish metabolically-linked from non-metabolically-linked actions of alcohols.

METHODS

Rat liver microsomes were prepared from Sprague-Dawley rats (12) and were incubated in sealed Erlenmeyer flasks at 37°C. The reaction mixture consisted of 100 mM potassium phosphate, pH 7.4, 10 mM sodium pyrophosphate, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM $NADP^+$ and about 5 mg microsomal protein in a final volume of 3.0 ml. The final concentration of t-butanol was usually 35 mM. The reaction was initiated by the addition of 10 mM glucose-6-phosphate plus 7 units of glucose-6-phosphate dehydrogenase. The reaction was terminated by the addition of 1.0 ml of 17.5% w/v trichloroacetic acid. After centrifugation, formaldehyde was determined in aliquots of the supernatant by the method of Nash (13). Two model systems were used to generate •OH in the absence of microsomes. One system consisted of 100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 35 mM t-butanol and 0.167 mM iron-EDTA (1:2 mixture of 5 mM ferrous ammonium sulfate and 10 mM EDTA) in a final volume of 3.0 ml. The reaction was initiated by the addition of ascorbate to a final concentration of 2.0 mM. The second system consisted of 100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 35 mM t-butanol, 0.083 mM iron-EDTA (1:2 mixture, as above) and 1 mM xanthine in a final volume of 3.0 ml. The reaction was initiated by the addition of 0.10 units of xanthine oxidase. Reactions were terminated with acid and analyzed for formaldehyde. All values were corrected for zero time blanks, which generally constituted 10% or less of the optical density at 30 min. Stock solutions of t-butanol were prepared in water. The buffer and appropriate solutions were passed through a chelex-100 resin (BioRad Labs.) to remove metal contaminants.

RESULTS

The production of formaldehyde by rat liver microsomes acting upon t-butanol is illustrated in Figure 1. The addition of 0.5 mM azide to the system increased the yield of

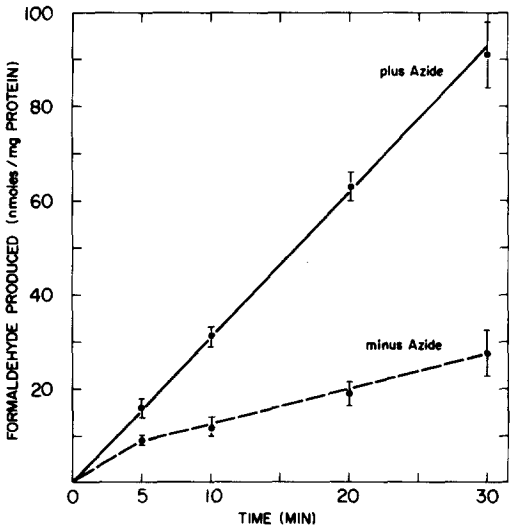


FIGURE 1

Time course for the production of formaldehyde from 35 mM t-butanol by rat liver microsomes. Reactions were carried out as described in Materials and Methods in the absence and presence of 0.50 mM azide. Results are the mean ± SEM from four experiments.

formaldehyde (Figure 1). A similar action of azide had been noted previously on the yield of products from other •OH scavengers, namely dimethylsulfoxide, methional and 2-keto-4-thiomethylbutyrate (14, 15); the products in the latter experiments were hydrocarbon gases (methane or ethylene). The action of azide is attributed to its ability to inhibit microsomal catalase, which normally decomposes the H_2O_2 generated during NADPH-dependent electron transport (15). H_2O_2 itself serves as a precursor of •OH. t-Butanol is a known scavenger of •OH ($k = 8 \times 10^8\ M^{-1}\ sec^{-1}$) (16, 17). Increased accumulation of H_2O_2 in the presence of azide should increase the production of •OH and, consequently, augment the rate of oxidation of t-butanol.

In other experiments, H_2O_2 was added directly to the microsomal system in concentrations up to 0.33 mM (presence of azide); glucose-6-P was omitted in the latter experiments in order to eliminate microsomal electron flow. Under these conditions, the formation of formaldehyde at 30 minutes was less than 15% of control values. Therefore, it can be concluded that the mere production of H_2O_2 by microsomes does not account for the yield of formaldehyde and that electron flow initiated by NADPH is

required. Similarly, boiled microsomes (azide and glucose-6-P added) produced only small amounts of formaldehyde (10% of control). When t-butanol was omitted, some formaldehyde was produced (10% of control values), perhaps due to the presence of endogenous substrates for the microsomal system. In the absence of microsomes, no formaldehyde was produced.

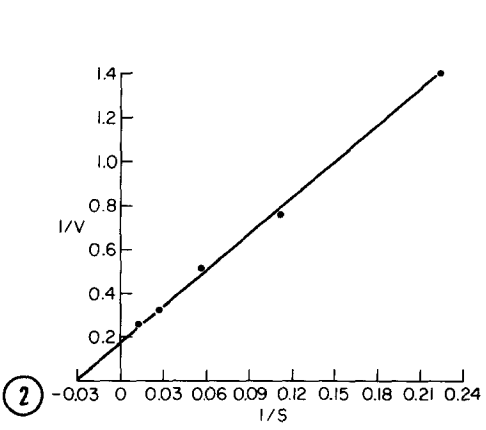
The rate of formation of formaldehyde was dependent upon the concentration of t-butanol used (Fig. 2). A Lineweaver-Burk plot was linear. The apparent K_m for t-butanol was about 30 mM while V_{max} was about 5.5 nmol/min/mg microsomal protein (Figure 2).

Three competing •OH scavengers were tested to see if they would block the production of formaldehyde from 35 mM t-butanol. Mannitol, benzoate and 2-keto-4-thiomethylbutyrate, at concentrations of 33 mM inhibited the production of formaldehyde 31%, 59% and 69%, respectively ($p<0.05$, Student "t"-test).

Other •OH-generating systems were also studied. The oxidation of xanthine by xanthine oxidase produces •OH as a by-product (18), especially in the presence of iron-EDTA (19, 20). Similarly, the iron-EDTA-ascorbate system produces hydroxyl radicals (15, 21). These two systems generated formaldehyde from t-butanol in a time-dependent manner (Fig. 3). In both systems, the yield of product was suppressed by benzoate and mannitol, two competing •OH scavengers (data not shown).

DISCUSSION

These data indicate that rat liver microsomes metabolize t-butanol to formaldehyde via a mechanism involving oxidation of NADPH, microsomal electron flow, and the generation of •OH from H_2O_2. Formaldehyde is also produced from dimethylsulfoxide via a similar mechanism (22). Although an important role for electron transfer reactions is the production of H_2O_2 (23, 24), exogenous H_2O_2 in the absence of electron transfer (i.e., with glucose-6-P omitted to eliminate the production of NADPH) is ineffective. Therefore, some reduced component of the electron transfer chain is required. It seems probable that the generation of •OH involves a Fenton-type reaction



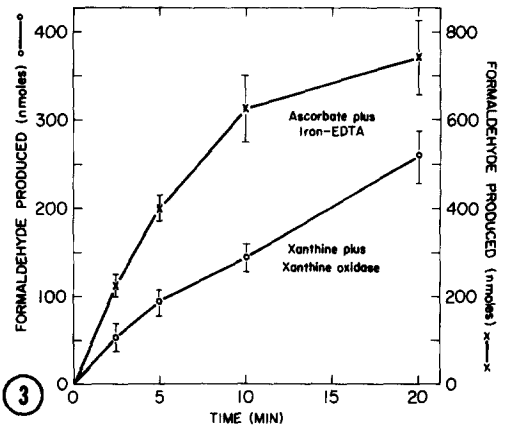


FIGURE 2

Lineweaver-Burk plot of the reciprocal of the velocity versus the reciprocal of the substrate concentration during the oxidation of t-butanol. The concentration of t-butanol was varied from 4.5 to 70 mM. Reactions were carried out for 30 min in the presence of 0.50 mM azide. Results are from four experiments.

FIGURE 3

Time course for the production of formaldehyde from 35 mM t-butanol by two model hydroxyl radical-generating systems viz., iron-EDTA-ascorbate and iron-EDTA-xanthine oxidase. Reactions were carried out as described in Methods. Results are the mean ± SEM from either six (ascorbate) or four (xanthine oxidase) experiments.

(17) or an "iron-catalyzed Haber-Weiss reaction" (19, 20), perhaps with cytochrome P-450 serving as the iron-chelate. It should be emphasized that the buffers, reagents and H_2O were passed through a chelex-100 resin to remove possible iron contaminants.

The reactions of •OH with alcohols proceed by hydrogen abstraction to produce either hydroxyalkyl radicals (generally at the alpha carbon R-$\dot{C}$H-OH) or alkoxy radicals (R-CH_2O•) (16). t-Butanol does not have an alpha hydrogen and, therefore, hydrogen abstraction would yield either •CH_2-$C(CH_3)_2$-OH by beta-hydrogen abstraction or $(CH_3)_3$C-O•, the corresponding alkoxy radical. The production of final stable products from these reaction intermediates depends upon the oxidizing or reducing properties of the free radicals and their interaction with constituents in the immediate environment. The reducing hydroxyalkyl radical formed from ethanol (CH_3-$\dot{C}$H-OH), for example, can be oxidized to acetaldehyde by oxygen or NAD^+ (25) or by ferric chelates (26). The properties of the free radical intermediates formed by t-butanol and the fragmentation pathway(s) that yield formaldehyde are not clear at the present time.

The amounts of formaldehyde produced from 35 mM t-butanol are approximately 30% that formed from 10 mM aminopyrine (27) with comparably prepared microsomes. In experiments with animals, blood levels of t-butanol in the range 25-32 mM have been reported (1). Therefore the production of formaldehyde cannot be inconsequential from a metabolic point of view. Among the classes of reactions to be considered for formaldehyde are condensation reactions with biogenic amines to form alkaloids of the tetrahydroisoquinoline and beta-carboline classes (28), condensation reactions with sulfhydryl groups (29), including ring closure reactions to form thiazolidine rings (29, 30), reversible Schiff's base formation with amino groups, as well as further metabolism of formaldehyde via incorporation into the one-carbon pool or via oxidation to formate and carbon dioxide. Current evidence indicates that the oxidative demethylation of dimethylsulfoxide (22) by liver microsomes proceeds via the same •OH-dependent pathway as t-butanol, whereas demethylation of more classical substrates for microsomes, such as aminopyrine, does not (27).

In addition to liver microsomes, other sites of cytochrome P-450 linked electron transport chains, such as in kidney, lung and adrenals, may similarly be capable of carrying out the oxidative demethylation of t-butanol. Additional sources of hydroxyl radicals are the oxidation of xanthine by xanthine oxidase (18), the autoxidation of ascorbate (31), and the specialized metabolism linked to the bactericidal activity of phagocytic cells (32-34). The metabolism of t-butanol by liver and by other tissues in the animal organism is deserving of further investigation.

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