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ROLE OF CATALASE AND HYDROXYL RADICALS IN THE OXIDATION OF METHANOL BY RAT LIVER MICROSOMES*

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Abstract—In view of the presence of adventitious catalase in isolated microsomes, and the requirement for H_2O_2, it has been suggested that NADPH-dependent oxidation of methanol by rat liver microsomes was mediated exclusively by the peroxidatic activity of catalase. However, H_2O_2 may also serve as a precursor of the hydroxyl radical, and methanol reacts with hydroxyl radicals to produce formaldehyde. Inhibition of H_2O_2 production should therefore decrease methanol oxidation by either a hydroxyl radical-dependent pathway or a catalase-dependent pathway. To attempt to clarify some of the controversies concerning the roles of H_2O_2 and catalase in the microsomal pathway of oxidation of short chain alcohols, studies were carried out to determine the nature of the pathway responsible for methanol oxidation by isolated microsomes. In the absence of the catalase inhibitor azide, methanol may be oxidized primarily by the peroxidatic activity of catalase since there was little effect on methanol oxidation by competing hydroxyl radical scavengers. Azide, which inhibited catalase activity > 95%, inhibited NADPH-dependent oxidation of methanol by 30–50%. The azide-insensitive (catalase-independent) pathway of methanol oxidation was inhibited by scavengers of hydroxyl radicals. The inhibition of the scavengers reflected the rate constant for interaction with hydroxyl radicals and was greater at lower concentrations of methanol than at higher concentrations, suggesting competition between the scavengers and methanol. The addition of H_2O_2 stimulated the oxidation of methanol in the presence of azide; H_2O_2 may serve as a precursor of the hydroxyl radical. Iron–EDTA, which is known to increase hydroxyl radical production, stimulated the oxidation of methanol in the absence and presence of azide. The stimulation by iron–EDTA was blocked by the competing hydroxyl radical scavengers even in the absence of azide, suggesting that the added iron–EDTA competes favorably with microsomal catalase for H_2O_2 to produce hydroxyl radicals (or a species with the oxidizing power of the hydroxyl radical). These results suggest that in microsomes, depending on the absence or presence of azide, methanol may be oxidized by two primary pathways, one involving the peroxidatic activity of catalase, and the other in which hydroxyl radicals, generated from microsomal electron transfer, play a role. In view of the crucial role played by H_2O_2 in both pathways, inhibition of H_2O_2 formation should not be interpreted solely as evidence for a role for catalase in the microsomal oxidation of alcohols.

The ability of microsomes to oxidize short chain alcohols such as methanol and ethanol was first demonstrated by Orme-Johnson and Ziegler [1]. This pathway of alcohol oxidation by microsomes was characterized in detail by Lieber and DeCarli [2–4]. The oxidation of ethanol by liver microsomes was found to require NADPH and oxygen, was inhibited by carbon monoxide, and was similar in many regards to the pathway of oxidation of drugs by the microsomal mixed function oxidase system [3]. The presence of adventitious catalase in isolated microsomes, however, suggested the possibility that the oxidation of ethanol may reflect the peroxidatic activity of catalase [5, 6]. Methanol is also a good substrate for the peroxidatic activity of catalase [7, 8]. The addition of azide, a catalase inhibitor, was found to inhibit microsomal oxidation of methanol by 60–70% [9] or to the background rate of formaldehyde production observed in the absence of methanol [10]. Methanol oxidation by microsomes from acatalasemic mice was 60–70% lower than controls [11]. It was concluded that the NADPH-dependent oxidation of methanol was mediated exclusively by catalase present in the microsomes [9–11].

Recent studies by Teschke *et al.* [12] indicated that 1 mM azide blocked methanol oxidation by microsomes by only 20–30%. The rate of NADPH-dependent methanol oxidation by microsomes from acatalasemic mice was the same as controls [13]. Moreover, purified cytochrome P-450, devoid of the activities of catalase and alcohol dehydrogenase, oxidized methanol in the presence of NADPH [14]. These results clearly indicate a catalase-independent pathway of methanol oxidation by isolated liver microsomes [14].

The current study was carried out to investigate the nature of this catalase-independent pathway of methanol oxidation. Recent studies have implicated a role for a species with the oxidizing power of the

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hydroxyl radical (·OH)* in the liver microsomal pathway for oxidation of ethanol [15–17]. In the presence of NADPH, liver microsomes oxidized several ·OH-scavenging agents by a mechanism consistent with the production of ·OH or a species with the oxidizing power of ·OH [18–20]. Since methanol is a scavenger of ·OH [21, 22], the possibility that the catalase-independent pathway of methanol oxidation may involve, at least in part, the interaction of methanol with ·OH generated by the microsomes was evaluated.

MATERIALS AND METHODS

Liver microsomes were prepared from male Sprague–Dawley rats (200–300 g) as previously described [15]. The microsomes were washed once and suspended in 125 mM KCl. The oxidation of methanol was assayed at 37° in 25-ml Erlenmeyer flasks containing 100 mM potassium phosphate (pH 7.4), 10 mM potassium pyrophosphate, 10 mM $MgCl_2$, 10 mM glucose-6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, 0.4 mM $NADP^+$, 0.1 mM EDTA, and about 4–5 mg microsomal protein in a final volume of 3.0 ml. The final concentration of methanol in most experiments was 80 mM. Azide, when added, was present at a final concentration of 1.0 mM. The reaction was initiated with glucose-6-phosphate plus glucose-6-phosphate dehydrogenase and was terminated by the addition of trichloroacetic acid (final concentration of 4.5%). The samples were centrifuged in a clinical centrifuge and formaldehyde was determined on a 1.5-ml aliquot by the method of Nash [23]. Standard curves containing known amounts of formaldehyde were carried out in an identical method. In most experiments, activity was calculated using an extinction coefficient of 8.0 mM^{-1} cm^{-1}. All values were corrected for zero time controls which contained the acid added before the microsomes. In some experiments, the NADPH-generating system was replaced by an H_2O_2-generating system, which consisted of 0.4 mM xanthine plus 0.040 units of xanthine oxidase.

All chemicals were of the highest grade from commercial sources. All buffers and solutions were prepared from water passed through a Millipore system and subsequently glass distilled. The buffers and solutions (except $MgCl_2$) were passed through a Chelex-100 column (35 × 3 cm, Kontes). An iron–EDTA solution was prepared by dissolving 5 mM ferrous ammonium sulfate in 10 mM EDTA.

All values refer to mean ±S.E.M. Statistical analysis was performed by Student's *t*-test.

RESULTS

Oxidation of methanol by rat liver microsomes. The oxidation of methanol by rat liver microsomes was studied in the absence or presence of azide, an inhibitor of catalase. Methanol oxidation was supported by either an NADPH-generating system or an H_2O_2-generating system (xanthine plus xanthine oxidase). In the absence of azide, the rates of NADPH-supported and H_2O_2-supported methanol oxidation were similar (Fig. 1). The addition of 1.0 mM azide led to an almost complete inhibition of the H_2O_2-supported oxidation of methanol (Fig. 1). This result indicates that the H_2O_2-supported oxidation of methanol is a catalase-dependent pathway. In contrast, the addition of azide led to only a 30–45% decrease in the rate of the NADPH-supported oxidation of methanol (Fig. 1). Similar results were obtained when the concentration of azide was varied from 0.2 to 2.0 mM. The inhibition of catalase by azide has been shown to be dependent on the ratio of catalase heme to rate of H_2O_2 generation [24]. In these experiments, the units of xanthine oxidase activity were adjusted so that comparable rates of NADPH-supported and H_2O_2-supported oxidation of methanol would be obtained. This suggests the possibility that, in the presence of azide and the NADPH-generating system, methanol is oxidized, in part, by a catalase-independent mechanism.

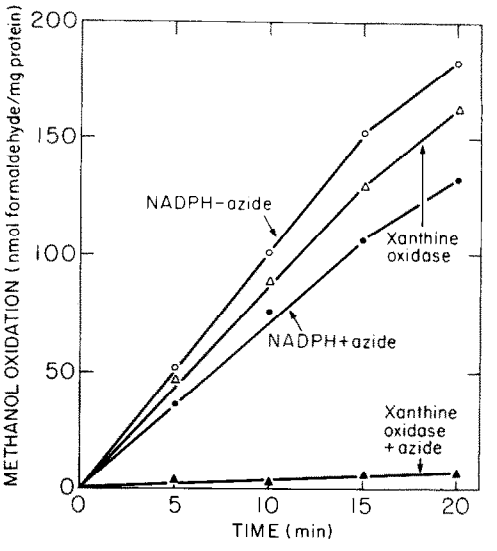

Fig. 1. Time course for the oxidation of methanol by rat liver microsomes. The oxidation of 80 mM methanol was assayed under four reaction conditions: (○), NADPH-generating system in the absence of azide; (●), NADPH-generating system in the presence of azide; (△), H_2O_2-generating system (0.4 mM xanthine plus 0.04 units xanthine oxidase) in the absence of azide; and (▲), H_2O_2-generating system in the presence of azide. When present, the final concentration of azide was 1.0 mM.

Effect of hydroxyl radical scavenging agents on microsomal oxidation of methanol. Since methanol is an effective scavenger of ·OH, the azide-insensitive pathway of methanol oxidation may involve interaction of methanol with ·OH generated by the microsomes. The ability of several competing ·OH scavenging agents to affect microsomal oxidation of methanol was studied in the absence and presence of azide. The scavengers employed were KTBA,

* Abbreviations: ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; KTBA, 2-keto-4-thiomethylbutyric acid; and Fe–EDTA, ferrous ammonium sulfate dissolved in EDTA in a 1:2 molar complex.

Table 1. Effect of hydroxyl radical scavenging agents on NADPH-dependent microsomal oxidation of methanol*

Scavenger	Concn (mM)	− Azide: Methanol oxidation [nmoles · min^{-1} · (mg protein)$^{-1}$]	− Azide: Effect of scavenger (%)	+ Azide: Methanol oxidation [nmoles · min^{-1} · (mg protein)$^{-1}$]	+ Azide: Effect of scavenger (%)
Control		13.57 ± 0.78		7.20 ± 0.46	
KTBA	10	11.99 ± 0.39	−12	4.72 ± 0.18	−34†
	33	11.30 ± 0.51	−17	3.50 ± 0.20	−51†
	67	10.44 ± 0.36	−23‡	2.92 ± 0.23	−59†
Mannitol	10	14.11 ± 0.88	+4	6.96 ± 0.52	−3
	33	13.69 ± 1.07	+1	5.79 ± 0.40	−20‡
	67	13.26 ± 1.17	−2	5.10 ± 0.50	−29§
1-Butanol	16.5	12.04 ± 0.39	−11	5.47 ± 0.41	−24‡
	33	12.45 ± 0.66	−8	4.67 ± 0.46	−35§
	67	12.58 ± 0.43	−7	3.86 ± 0.25	−46†
Ethanol	50	8.34 ± 0.84	−39†	4.84 ± 0.34	−33§
	100	7.59 ± 0.67	−44†	4.13 ± 0.24	−43†

* The NADPH-dependent oxidation of 80 mM methanol was assayed as described in Materials and Methods in the absence or presence of 1.0 mM azide. Results are from four experiments in the absence of azide and six experiments in the presence of azide.
† $P < 0.001$.
‡ $P < 0.05$.
§ $P < 0.01$.

mannitol, 1-butanol and ethanol. These compounds were shown previously to inhibit the metabolism of one another by liver microsomes [15, 19]. In the absence of azide, mannitol and 1-butanol had no effect on methanol oxidation, while KTBA was slightly inhibitory (Table 1). Ethanol, however, significantly inhibited methanol oxidation (Table 1). In this context, ethanol, but not 1-butanol, has been shown to serve as an effective substrate for the peroxidatic activity of catalase in liver microsomes [14]. This was confirmed in the current studies by assaying the oxidation of methanol, ethanol and butanol in the presence of xanthine plus xanthine oxidase as the H_2O_2-generating system. Rates of product formation [nmoles · min^{-1} · (mg microsomal protein)$^{-1}$] were: methanol, 10.13; ethanol, 9.41; and butanol, 0.56; (N = 2). These results suggest that the inhibition of methanol oxidation by ethanol reflects competition by these alcohols for oxidation by the catalase-dependent pathway.

Further evidence for this suggestion was provided by studying the effect of the ·OH scavengers on H_2O_2-dependent (catalase-dependent) microsomal oxidation of methanol (Table 2). This methanol oxidation system was unaffected by mannitol or 1-butanol; KTBA was slightly inhibitory at the highest concentration, whereas ethanol again significantly inhibited methanol oxidation (Table 2). These results are identical to those found for the NADPH-supported oxidation of methanol in the absence of azide (Table 1) and support the suggestion that these microsomal methanol oxidation systems reflect the peroxidatic activity of catalase.

In the presence of azide, the NADPH-dependent

Table 2. Effect of hydroxyl radical scavenging agents on H_2O_2-dependent microsomal oxidation of methanol*

Scavenger	Concn (mM)	Methanol oxidation [nmoles · min^{-1} · (mg protein)$^{-1}$]	Effect of scavenger (%)
Control		10.22 ± 1.18	
KTBA	10	11.09 ± 1.86	+9
	33	9.43 ± 0.96	−8
	67	8.03 ± 1.13†	−21†
Mannitol	33	10.77 ± 0.77	+5
	67	11.41 ± 1.52	+12
1-Butanol	33	10.56 ± 1.35	+3
	67	10.83 ± 1.91	+6
Ethanol	50	7.39 ± 1.79	−28†
	100	5.85 ± 1.63	−43‡

* H_2O_2-dependent oxidation of 80 mM methanol was assayed by replacing the NADPH-generating system with 0.4 mM xanthine plus 0.040 units of xanthine oxidase. Results are from three experiments carried out in the absence of azide. When 1.0 mM azide was added, the control rate decreased to 0.52 ± 0.30 nmoles · min^{-1} · (mg protein)$^{-1}$.
† $P < 0.05$.
‡ $P < 0.01$.

Table 3. Effect of methanol concentration on the inhibition of methanol oxidation by hydroxyl radical scavenging agents*

Addition	Concentration of methanol (mM) 20		80		160	
	Methanol oxidation	Effect of scavenger (%)	Methanol oxidation	Effect of scavenger (%)	Methanol oxidation	Effect of scavenger (%)
Control	3.17 ± 0.38		5.48 ± 0.92		5.98 ± 1.03	
Mannitol, 10 mM	2.50 ± 0.48	−21	5.22 ± 0.83	−5	6.29 ± 0.99	+5
33 mM	2.16 ± 0.40	−32	4.15 ± 0.70	−24	5.28 ± 0.83	−12
67 mM	1.84 ± 0.31	−42	3.46 ± 0.82	−36	4.71 ± 1.14	−21
Benzoate, 10 mM	2.31 ± 0.67	−27	4.38 ± 1.30	−20	5.21 ± 0.73	−13
33 mM	2.05 ± 0.47	−35	3.77 ± 1.34	−31	4.69 ± 0.74	−22

* NADPH-dependent microsomal oxidation of methanol (20, 80 or 160 mM) was assayed in the presence of 1.0 mM azide and the indicated concentrations of hydroxyl radical scavenging agent. Results are from three experiments. Methanol oxidation refers to nmoles formaldehyde produced · min^{-1} · (mg microsomal protein)$^{-1}$.

microsomal oxidation of methanol was inhibited by all the ·OH scavenging agents tested (Table 1). Although the rate constant for the interaction of KTBA with ·OH is not known, it appears to be similar to that of dimethylsulfoxide (about $7 \times 10^9 M^{-1}$ sec^{-1}). The inhibition of microsomal oxidation of methanol by the scavengers follows the ability of the scavengers to interact with ·OH, i.e. KTBA > 1-butanol > mannitol (Table 1). Previous experiments indicated that the scavengers did not interfere with microsomal drug metabolism or microsomal electron transfer [15, 19]. A substrate concentration curve with methanol (in the presence of azide) is shown in Table 3. V_{max} was about 7.5 nmoles · min^{-1} · (mg microsomal protein)$^{-1}$, while the apparent K_m for methanol was about 23 mM (Lineweaver–Burk plots). Published K_m values for NADPH-dependent methanol oxidation by microsomes are 22 mM [12] and 33 mM [9]. The inhibition of microsomal oxidation of methanol by either mannitol or benzoate was greater at 20 mM methanol than that found at 160 mM methanol (Table 3). These results suggest the possibility that the residual oxidation of methanol by liver microsomes which persists in the presence of azide involves interaction of methanol with ·OH generated by microsomes, and that the inhibition of this system of methanol oxidation by ·OH scavengers reflects competition for the metabolically generated ·OH.

Effect of H_2O_2 on methanol oxidation. In previous experiments with ethanol, 1-butanol and isopropanol, H_2O_2 (added in the presence of azide) was found to stimulate the oxidation of these alcohols, especially at shorter incubation times [15, 25]. H_2O_2 serves as a precursor of ·OH, and it was suggested that increased generation of ·OH from the added H_2O_2 was responsible for the increase in oxidation of the alcohols, i.e. accumulation of H_2O_2, especially at shorter incubation periods, was limiting production of ·OH [15, 25]. Similar experiments were

Table 4. Effect of hydrogen peroxide on the oxidation of methanol by rat liver microsomes*

Reaction time (min)	Concn of H_2O_2 (mM)	− Azide: Methanol oxidation (nmoles/mg protein)	− Azide: Effect of H_2O_2 (%)	+ Azide: Methanol oxidation (nmoles/mg protein)	+ Azide: Effect of H_2O_2 (%)
2.5	0	38.8 ± 7.3		19.8 ± 4.5	
	0.1	43.3 ± 7.3	+12	27.4 ± 5.0	+38†
	0.3	48.2 ± 8.1	+24	35.2 ± 8.2	+78†
	1.0	53.2 ± 9.0	+37	59.0 ± 7.5	+198‡
5.0	0	66.3 ± 5.2		43.1 ± 4.0	
	0.1	66.0 ± 6.8	0	49.0 ± 4.1	+14
	0.3	72.3 ± 6.6	+9	61.4 ± 3.1	+42†
	1.0	71.9 ± 7.0	+8	82.8 ± 7.0	+92‡
10	0	132.5 ± 7.4		86.5 ± 6.8	
	0.1	132.8 ± 10.7	0	89.0 ± 8.9	+3
	0.3	135.1 ± 9.2	+2	106.5 ± 9.3	+23
	1.0	136.1 ± 12.7	+3	128.8 ± 12.5	+49†

* NADPH-dependent oxidation of 80 mM methanol was assayed in the presence of the indicated concentrations of H_2O_2, and for the listed reaction periods (2.5, 5 or 10 min). Experiments were carried out in the absence or presence of 1.0 mM azide. Results are from four experiments.

† $P < 0.05$.

‡ $P < 0.01$.

carried out with methanol. In the absence of azide, H_2O_2 had a slight stimulatory effect (2.5 min reaction) or had no effect on methanol oxidation (Table 4). Presumably the added H_2O_2 is rapidly decomposed by the catalytic activity of catalase. In the presence of azide, H_2O_2 stimulated methanol oxidation in a concentration-dependent manner (Table 4). Stimulation by H_2O_2 was greatest at the shorter incubation periods, consistent with a precursor role for H_2O_2. In the absence of NADPH-generating system, but in the presence of azide, H_2O_2 was ineffective in supporting methanol oxidation [rates of methanol oxidation less than 1 nmole · min^{-1} · (mg protein)$^{-1}$]. Thus, the stimulation by H_2O_2 required simultaneous electron transport as provided by the oxidation of NADPH.

Effect of Fe–EDTA *on microsomal oxidation of methanol.* In model ·OH generating systems, Fe–EDTA was shown to increase the rate of ·OH production [26, 27]. This stimulation was suggested to result as a consequence of an "iron-catalyzed" Haber–Weiss reaction:

$$Fe^{3+} - EDTA + O_2^{\cdot-} \rightarrow Fe^{2+} - EDTA + O_2 \quad (1)$$

$$O_2^{\cdot-} + HO_2\cdot + H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} - EDTA + H_2O_2 \rightarrow Fe^{3+} - EDTA + OH^- + \cdot OH \quad (3)$$

Fe–EDTA was shown to increase the rate of ·OH production in the microsomal system [28]. However, microsomal drug metabolism (aminopyrine demethylase, aniline hydroxylase) was not affected by Fe–EDTA. The effect of Fe–EDTA on microsomal oxidation of methanol was studied. In the absence of azide, the rate of methanol oxidation was 12.71 nmoles · min^{-1} · (mg protein)$^{-1}$ in the absence of Fe–EDTA, and 14.17, 20.33 and 25.78 in the presence of 8.5, 17 and 50 μM Fe–EDTA, respectively. In the presence of azide, the rate of methanol oxidation was 7.0 nmoles · min^{-1} · (mg protein)$^{-1}$ in the absence of Fe–EDTA, and 12.31, 16.25 and 20.59 in the presence of 8.5, 17 and 50 μM Fe–EDTA respectively. Fe–EDTA stimulated the rate of methanol oxidation in the absence as well as in the presence of azide.

To confirm that the stimulation of methanol oxidation by Fe–EDTA involved ·OH, the effect of ·OH scavenging agents on this stimulation was evaluated. The stimulation by Fe–EDTA, in the presence as well as the absence of azide, was inhibited by KTBA, mannitol, 1-butanol and ethanol (Table 5). That the stimulation by Fe–EDTA was blocked by the ·OH scavengers can be determined by comparing the results of Tables 1 and 5, e.g. in the presence of azide, 67 mM KTBA decreased the control rate of methanol oxidation by 4.28 nmoles · min^{-1} · mg^{-1} (7.20–2.92), while in the presence of Fe–EDTA plus azide, 67 mM KTBA decreased the control rate of methanol oxidation by 15.39 nmoles · min^{-1} · mg^{-1} (21.66–6.27). Similar calculations can be made for the other scavengers as well. Thus, the scavengers blocked the control rate as well as the Fe–EDTA-stimulated rate of methanol oxidation.

DISCUSSION

The NADPH-dependent oxidation of methanol by rat liver microsomes appears to be mediated by two pathways, one dependent upon the peroxidatic activity of catalase and the other dependent on the interaction of methanol with ·OH generated by the

Table 5. Effect of hydroxyl radical scavenging agents on microsomal oxidation of methanol in the presence of Fe–EDTA*

		− Azide		+ Azide	
Scavenger	Concn (mM)	Methanol oxidation [nmoles · min^{-1} · (mg protein)$^{-1}$]	Effect of scavenger (%)	Methanol oxidation [nmoles · min^{-1} · (mg protein)$^{-1}$]	Effect of scavenger (%)
Control		27.36 ± 1.55		21.66 ± 1.19	
KTBA	10	21.52 ± 1.93	−21†	14.41 ± 1.18	−33‡
	33	14.85 ± 1.63	−46‡	8.44 ± 0.93	−61§
	67	11.94 ± 1.47	−56§	6.27 ± 0.84	−71§
Mannitol	10	26.09 ± 1.67	−5	19.56 ± 1.20	−10
	33	22.65 ± 1.24	−17†	16.18 ± 1.68	−26†
	67	20.59 ± 1.71	−25†	13.52 ± 1.70	−38‡
1-Butanol	16.5	22.44 ± 2.18	−18	15.85 ± 0.98	−27‡
	33	20.30 ± 2.22	−26†	13.66 ± 1.27	−37‡
	67	18.74 ± 2.07	−32†	11.24 ± 1.19	−48§
Ethanol	50	17.16 ± 2.80	−37†	14.18 ± 1.95	−35‡
	100	14.01 ± 2.21	−49‡	12.45 ± 1.52	−43‡

* NADPH-dependent oxidation of 80 mM methanol was assayed in the presence of 50 μM Fe–EDTA, and in the absence or presence of 1.0 mM azide. Results are from four experiments. The control rates of methanol oxidation [nmoles · min^{-1} · (mg protein)$^{-1}$] in the absence of 50 μM Fe–EDTA were: no azide, 13.16 ± 0.65; plus azide, 6.93 ± 0.54 (N = 4). The stimulation by Fe–EDTA was significant ($P < 0.001$) in the presence as well as in the absence of azide.

† $P < 0.05$.

‡ $P < 0.01$.

§ $P < 0.001$.

microsomes. The two pathways share a common intermediate, H_2O_2. In the catalase-dependent pathway, H_2O_2 serves as a substrate; in the ·OH-dependent pathway, H_2O_2 may serve as a precursor of ·OH. Therefore, inhibition of H_2O_2 production should result in a decrease in methanol oxidation by either the ·OH-dependent or the catalase-dependent pathways. The ability of metyrapone to inhibit microsomal oxidation of methanol was suggested to reflect an inhibition of the rate of H_2O_2 production rather than a direct effect on cytochrome P-450 [10]. Similar considerations may apply to the ability of carbon monoxide to depress microsomal oxidation of methanol [10, 14, 29]. In light of the above discussion, inhibition of H_2O_2 formation should not be interpreted solely as proof for a role for catalase in the microsomal oxidation of alcohols, since ·OH production would also be suppressed.

Of considerable interest is the observation that the ·OH scavengers which are ineffective inhibitors of methanol oxidation in the absence of azide (Table 1) are inhibitory in the presence of Fe–EDTA even in the absence of azide (Table 5). When the results of Table 1 are subtracted from those of Table 5, the net rate of methanol oxidation represents the net increment in methanol oxidation produced by 50 μM Fe–EDTA. These results are shown in Table 6 for the controls as well as for the activities found in the presence of the ·OH scavengers. Results in the presence and absence of azide are shown. Two observations are apparent. First, the net increments in methanol oxidation produced by Fe–EDTA are the same in the presence and absence of azide. This suggests that the added Fe–EDTA competes favorably with catalase for H_2O_2. Second, the inhibition of this Fe–EDTA-stimulated rate of methanol oxidation by competing ·OH scavengers is the same in the presence and absence of azide (Table 6). These results strongly suggest a role for ·OH in the Fe–EDTA-mediated stimulation of methanol oxidation which occurs in the absence, as well as in the presence, of azide. A possible explanation for these results may involve the following:

NADPH-dependent microsomal electron transfer

$$\rightarrow O_2^{\cdot -} \rightarrow H_2O_2 \quad (4)$$

$$H_2O_2 \xrightarrow[\text{cytochrome P-450?}]{O_2^{\cdot -}\text{? Fe?}} \cdot OH \quad (5)$$

$$\text{Methanol} + \cdot OH \rightarrow \text{formaldehyde} \quad (6)$$

$$\text{Methanol} + H_2O_2 \xrightarrow{\text{catalase}} \text{formaldehyde} \quad (7)$$

The utilization of H_2O_2 via one pathway could influence its availability via an alternative pathway [15, 28], i.e. the utilization of H_2O_2 by microsomal catalase may limit ·OH production. In the absence of azide, most of the H_2O_2 may react with catalase to form the catalase Compound 1 species, which then oxidizes methanol. Hence there is little effect on methanol oxidation by competing ·OH scavengers. However, in the presence of an iron-chelate, H_2O_2 may be capable of generating ·OH even in the presence of catalase, i.e. Fe–EDTA may compete effectively with catalase for the generated H_2O_2.

$$\cdot OH \xleftarrow{Fe^{2+}\text{–EDTA}} H_2O_2 \xrightarrow[\text{catalase}]{} \text{Compound 1}$$

Consequently, the ·OH scavengers block methanol oxidation in the presence of Fe–EDTA even though azide is not added to inhibit catalase. When azide is present to inhibit catalase, the accumulated H_2O_2 may serve to generate ·OH. Under these conditions, methanol may be oxidized primarily by an ·OH-dependent pathway, and therefore be susceptible to inhibition by competing ·OH scavengers.

Previous experiments have shown that the metabolism of typical ·OH scavengers such as 1-butanol, dimethylsulfoxide, isopropanol, *t*-butanol and KTBA was increased in the presence of azide [15, 19, 20, 25], consistent with the above suggestion that, once catalase is inhibited, accumulated H_2O_2 may serve to generate ·OH. The probable reason why the oxidation of methanol is not increased in

Table 6. Effect of hydroxyl radical scavenging agents on the net increment of microsomal oxidation of methanol produced by Fe–EDTA*

		− Azide		+ Azide	
Scavenger	Concn (mM)	Methanol oxidation [nmoles · min^{-1} · (mg protein)$^{-1}$]	Effect of scavenger (%)	Methanol oxidation [nmoles · min^{-1} · (mg protein)$^{-1}$]	Effect of scavenger (%)
Control		13.79		14.46	
KTBA	10	9.53	−31	9.69	−33
	33	3.55	−74	4.94	−66
	67	1.50	−89	3.35	−77
Mannitol	10	11.98	−13	12.60	−13
	33	8.96	−35	10.29	−29
	67	7.33	−47	8.42	−42
1-Butanol	16.5	10.40	−25	10.38	−28
	33	7.85	−43	8.99	−39
	67	6.16	−55	7.38	−49
Ethanol	50	8.82	−36	9.34	−35
	100	6.42	−53	8.32	−42

* Results were obtained by subtracting the appropriate values of Table 1 from those in Table 5, e.g. in the absence of azide, 27.36 (Table 5) −13.57 (Table 1) = 13.79; in the presence of azide, 21.66 (Table 5) − 7.20 (Table 1) = 14.46. The values obtained represent the net increase in methanol oxidation produced by 50 μM Fe–EDTA.

the presence of azide alone is that inhibition of catalase by azide would be expected to decrease the peroxidatic pathway of methanol oxidation but simultaneously increase the ·OH pathway of methanol oxidation. The net effect probably depends on the relative contributions made by each pathway, and this may depend on the level of contaminating catalase, rate of H_2O_2 production, microsomal electron transfer, endogenous iron content and the substrate being studied. Similar results were previously found for microsomal ethanol oxidation [15]; ethanol and methanol, unlike 1-butanol, isopropanol, *t*-butyl alcohol, dimethylsulfoxide or KTBA, react with catalase as well as with ·OH.

In summary, the azide-insensitive rate of methanol oxidation by rat liver microsomes appears to reflect, at least in part, the ability of methanol to interact with ·OH or another species with the oxidizing power of ·OH generated from microsomal electron tranfer. H_2O_2 appears to serve as a precursor of ·OH in this system. In the absence of azide, H_2O_2 also participates in the oxidation of methanol via the peroxidatic activity of catalase. In the presence of azide, added H_2O_2 alone is not sufficient to result in the oxidation of methanol. NADPH-dependent electron transfer is also required, presumably to reduce endogenous iron or cytochrome P-450 to the Fe^{2+} state. A Fenton reaction between the H_2O_2 and Fe^{2+} (chelated) or H_2O_2 and reduced cytochrome P-450 may generate ·OH. The nature of the endogenous iron catalyst is unknown but, in view of the chelex treatment of all solutions, the catalyst is probably microsomal iron (either adventitious, non-heme iron or heme iron). Further studies on this point are required, especially in view of the ability of purified cytochrome P-450 to oxidize methanol [14] as well as other alcohols in an NADPH-dependent manner [14, 30–34]. It should be emphasized that the major pathway of methanol oxidation *in vivo* is either via alcohol dehydrogenase (humans, monkeys) or peroxisomal catalase (rats) [35]. Production of ·OH *in vivo* may be too small to play a major significant role in methanol oxidation *in vivo*.

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