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Effect of Hydroxyl Radical Scavengers on Microsomal Oxidation of Alcohols and on Associated Microsomal Reactions†

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ABSTRACT: The possibility that oxidation of alcohols by liver microsomes represents a system potentially dependent upon the interaction of the alcohols with hydroxyl radicals was evaluated. Mannitol, benzoate, and dimethyl sulfoxide, three compounds that react rapidly with hydroxyl radicals, inhibited microsomal oxidation of ethanol. Whereas only slight inhibition was observed in the absence of a catalase inhibitor, azide, all three scavengers strikingly decreased microsomal oxidation of ethanol in the presence of azide. The inhibition was competitive with respect to ethanol. These agents had no effect on xanthine oxidase-mediated (H_2O_2-mediated) oxidation of ethanol, nor did they inhibit microsomal electron transport (NADH- or NADPH-cytochrome *c* reduction, NADH- or NADPH-dependent oxygen uptake) or the metabolism of aminopyrine or aniline. The oxidation of l-butanol, which is not an effective substrate for the peroxidatic activity of catalase, was inhibited by dimethyl sulfoxide even in the absence of azide. Whereas microsomal oxidation of ethanol was inhibited 15–25% by azide, butanol oxidation was doubled in the presence of azide. The addition of H_2O_2 in the presence of azide resulted in a stimulation of microsomal oxidation of ethanol and butanol in short term experiments. These results are consistent with a mechanism of microsomal oxidation of alcohols which involves the interaction of the alcohols with hydroxyl radicals that are generated from the microsomal electron transfer pathway. Differences in the metabolism of ethanol and l-butanol may reflect the fact that, whereas ethanol can be oxidized by both a catalase-dependent pathway and a hydroxyl radical pathway, butanol is oxidized only by the latter.

The oxidation of ethanol to acetaldehyde by isolated rat liver microsomes requires NADPH and is similar in many regards to the oxidation of drugs by the microsomal mixed function oxidase system (Lieber & DeCarli, 1968, 1970, 1972). The presence of catalase in isolated microsomes provides for an alternate route for the production of acetaldehyde, namely, the peroxidatic activity of catalase (Keilin & Hartree, 1945; Tephley et al., 1961; Thurman et al., 1972; Thurman, 1973). The H_2O_2 required for peroxidatic activity arises from the oxidation of NADPH by NADPH oxidase (Gillette et al., 1957; Thurman, 1973). Other experiments, however, have shown that the catalase pathway cannot account for all of the NADPH-dependent, ethanol-oxidizing activity of isolated microsomes (Lieber, 1975; Teschke et al., 1974, 1976). Recent experiments have strongly implicated a role for a cytochrome P-450 dependent pathway in the oxidation of ethanol by microsomes (Joly et al., 1977; Ohnishi & Lieber, 1977; Teschke et al., 1977).

Alcohols are well known scavengers of hydroxyl radicals (Anbar & Neta, 1967; Dorfman & Adams, 1973). Recent reports have suggested that hydroxyl radicals are generated by the NADPH-dependent microsomal electron transfer system (Fong et al., 1973; Lai & Piette, 1977). It therefore seemed possible that the microsomal oxidation of ethanol to acetaldehyde could represent, at least in part, the hydroxyl radical scavenging activity of ethanol. In a preliminary communication (Cederbaum et al., 1977), we reported that four scavengers of hydroxyl radicals, namely, dimethyl sulfoxide (Me_2SO),[1] benzoate, mannitol, and thiourea, inhibited the catalase-independent pathway for the oxidation of ethanol by rat liver microsomes. Urea, a compound that reacted poorly with hydroxyl radicals, did not inhibit the oxidation of ethanol and served as a negative control. In the latter studies, azide was added to the system in order to inhibit the oxidation of ethanol by microsomal catalase. In the present study, the effects of hydroxyl radical scavengers are contrasted in the presence and absence of azide. Additional experiments in which H_2O_2 was added directly and experiments with l-butanol as substrate help to clarify the roles of H_2O_2 and of hydroxyl radicals in the microsomal alcohol-oxidizing system.

Methods

Liver microsomes were prepared from male Sprague-Dawley rats as previously described (Cederbaum et al., 1976), washed once, and suspended in 125 mM KCl. Protein was determined by the method of Lowry et al. (1951).

Microsomal oxidation of ethanol or l-butanol was assayed as previously described (Cederbaum et al., 1977), with the use of flasks containing 0.6 mL of 15 mM semicarbazide in the center well. In most experiments, the final concentration of ethanol or butanol was 51 mM. The reaction was initiated with the NADPH-generating system and was terminated after 15 min at 37 °C by the addition of trichloroacetic acid (final

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[1] Abbreviation used: Me_2SO, dimethyl sulfoxide; SEM, standard error of the mean.

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TABLE I: Effect of Dimethyl Sulfoxide, Mannitol, and Benzoate on Microsomal Oxidation of Ethanol.[a]

		− azide			+ azide		
additions	concn (mM)	ethanol oxidation (nmol/(min mg of protein))	effect (%)	P	ethanol oxidation (nmol/(min mg of protein))	effect (%)	P
control		10.65 ± 0.66			9.05 ± 1.03		
dimethyl	4.7	10.09 ± 1.14	−5	NS	8.13 ± 0.34	−10	NS
sulfoxide	14.0	9.28 ± 0.71	−13	NS	6.27 ± 0.57	−31	<0.05
	23.5	8.93 ± 1.13	−16	NS	4.92 ± 0.56	−46	<0.01
	47.0	8.39 ± 0.79	−21	<0.05	3.81 ± 0.64	−58	<0.002
	94.0	7.89 ± 0.75	−26	<0.05	2.71 ± 0.57	−70	<0.001
control		11.35 ± 0.90			8.54 ± 0.90		
mannitol	3.3				8.63 ± 0.75	+1	NS
	10.0	10.95 ± 1.21	−4	NS	7.00 ± 0.39	−18	<0.05
	33.0	10.23 ± 1.18	−10	NS	5.91 ± 1.05	−31	<0.05
	67.0	9.56 ± 1.31	−16	0.10 > P >0.05	4.71 ± 0.76	−45	<0.01
	100.0	7.71 ± 0.22	−32	<0.05	3.34 ± 0.41	−61	<0.01
benzoate	3.3				7.69 ± 0.42	−10	NS
	10.0	11.31 ± 1.22	0	NS	6.83 ± 0.68	−20	<0.05
	33.0	10.85 ± 0.90	−4	NS	5.20 ± 0.75	−39	<0.01
	67.0	10.13 ± 0.90	−11	NS	4.28 ± 0.58	−50	<0.001
	100.0	9.04 ± 0.70	−20	<0.05	2.78 ± 0.69	−67	<0.001

[a] NADPH-dependent microsomal oxidation of ethanol (final concentration of 51 mM) was assayed as described in Methods. Results are from either five (mannitol and benzoate) or four (Me_2SO) experiments in the absence of azide, and from four experiments in the presence of azide (0.05 mM).

concentration 5%). After an overnight diffusion period, the optical density of the semicarbazone complex was determined at 224 nm and compared with standards. Blanks included zero time controls and flasks without microsomes.

Aniline hydroxylase was assayed in a reaction system containing 83 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 10 mM glucose 6-phosphate, 0.3 mM NADP, 7 units of glucose-6-phosphate dehydrogenase, 10 mM aniline, and about 5 mg of microsomal protein in a final volume of 3.0 mL. The reaction was initiated by the addition of aniline and was terminated after 20 min in air at 37 °C by the addition of 20 mL of ice-cold ether. The assay for *p*-aminophenol was performed as described by Kato & Gillette (1965). Aminopyrine demethylase activity (Cochin & Axelrod, 1959) was assayed using the same reaction mixture described for aniline hydroxylase. The reaction was initiated by the addition of aminopyrine (final concentration of 5 mM) and after 15 min at 37 °C was terminated with trichloroacetic acid. Formaldehyde was detected with the Nash reagent (Nash, 1953). NADPH and NADH-cytochrome *c* reductase activities were assayed as described by Phillips & Langdon (1962). NADPH or NADH-dependent oxygen uptake was measured by following the increase in oxygen consumption when 3 mM NADPH or NADH was added to a microsomal suspension in 83 mM phosphate (pH 7.4) with 10 mM $MgCl_2$. Oxygen uptake was assayed with a Clark oxygen electrode and a Yellow Springs oxygen monitor.

Statistics. All values refer to mean ± SEM. Statistical analysis was performed by Student's *t* test. The number of experiments is stated in the table or figure legends.

Results

Effect of Me_2SO, Mannitol, and Benzoate on Microsomal Oxidation of Ethanol. In the presence of an NADPH-generating system, the addition of ethanol to liver microsomes resulted in the production of acetaldehyde. Ethanol oxidation was linear over the 15-min-reaction period and with respect to microsomal protein concentration. A number of hydroxyl radical scavengers were tested for their ability to inhibit the oxidation of ethanol (51 mM) by microsomes. The bimolecular rate constants for the reaction of various scavengers with hydroxyl radicals are (M^{-1} s^{-1}): ethanol, 0.7–1.1 × 10^9; l-butanol, 2.2 × 10^9; Me_2SO, 7 × 10^9; mannitol, >10^9; benzoate, 3.3–3.8 × 10^9 (Dorfman & Adams, 1973; Anbar & Neta, 1967).[2] Mannitol, benzoate and Me_2SO were not very effective as inhibitors of the microsomal oxidation of ethanol (absence of azide, Table I); significant inhibition was observed only at higher concentrations of these agents.

Azide is a potent inhibitor of catalase and has been used in attempts to differentiate microsomal oxidation of ethanol from the peroxidatic activity of catalase. The oxidation of ethanol by catalase can also be supported by replacing the NADPH-generating system with xanthine plus xanthine oxidase. Sodium azide, at concentrations varying from 0.3 to 1 mM, almost completely blocked the xanthine oxidase-dependent oxidation of ethanol, e.g., in the presence of xanthine (3 mM) plus xanthine oxidase (0.03 unit) control rates of ethanol oxidation were 12.02 ± 1.82 nmol of acetaldehyde produced per min per mg of microsomal protein, whereas, in the presence of 0.5 mM sodium azide, the rate was 0.74 ± 0.46 (−94%, $p < 0.001$, $n = 6$). These results emphasize that catalase plays the major role when ethanol oxidation by crude liver microsomes is supported by xanthine plus xanthine oxidase. With the NADPH-generating system, on the other hand, 0.5 mM azide blocked the microsomal oxidation of ethanol by only 15–25% (Table I). In the presence of azide, the addition of Me_2SO, mannitol, or benzoate to liver microsomes led to progressive inhibition of ethanol oxidation (Table I). The extent of inhibition was much greater than that seen in the absence of azide. Significant inhibition of ethanol oxidation was observed when the scavengers were added in concentrations of 10 mM or higher.

In concentrations up to 100 mM, the scavengers had no ef-

[2] The rate constant for mannitol was estimated by comparison with othe polyhydroxylic compounds such as erythritol and pentaerythritol.

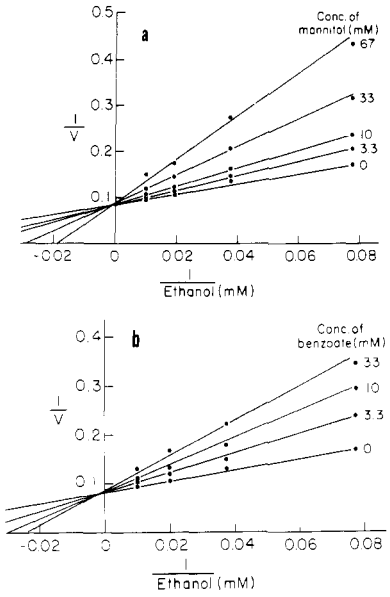

FIGURE 1: Double-reciprocal plot showing the kinetics of the inhibition of microsomal oxidation of ethanol by mannitol and benzoate. NADPH-dependent microsomal oxidation of ethanol (concentrations of 12.8, 25.5, 51, and 102 mM) was assayed in the presence of 0.50 mM azide. V refers to nmoles acetaldehyde produced per min per mg of microsomal protein. Results are from four experiments.

TABLE II: Effect of Ethanol Concentration on the Inhibition of Microsomal Ethanol Oxidation by Dimethyl Sulfoxide, Mannitol, and Benzoate in the Presence of Azide.[a]

additions	concn (mM)	percent inhibition concn of ethanol (mM) 12.8	25.5	51	102
dimethyl sulfoxide	5	28	19	9	6
	14	53	44	32	23
	23.5	63	56	49	34
	47	78	75	58	53
	94	84	83	71	64
mannitol	3.3	17	4	+1	3
	10	27	14	8	9
	33	43	33	24	21
	67	61	50	39	38
benzoate	3.3	29	12	10	7
	10	42	24	20	19
	33	51	39	36	27
	67	60	53	49	40

[a] Microsomal oxidation of ethanol was assayed in the presence of the indicated concentrations of ethanol and hydroxyl radical scavenging agent. Azide was present at a final concentration of 0.50 mM. Results are from four experiments. Control rates of ethanol oxidation (nmoles acetaldehyde produced/(min mg of microsomal protein)) were: 12.8 mM ethanol, 5.84 ± 0.64; 25.5 mM ethanol, 7.32 ± 0.48; 51 mM ethanol, 9.40 ± 0.70; 102 mM ethanol, 10.70 ± 0.49.

fect on the NADPH-generating system (glucose-6-phosphate dehydrogenase activity), nor did they interfere with recoveries of known amounts of acetaldehyde (500–1500 nmol) added to the system.

The effect of the hydroxyl radical scavengers on xanthine oxidase-dependent (catalase-dependent) oxidation of ethanol was studied (control rate = 12.99 ± 2.18 nmol of acetaldehyde produced per min per mg of microsomal protein). At concentrations of scavengers identical with those listed in Table I, no significant inhibition was observed (−1 to −14%, $p > 0.05$) except with 100 mM benzoate (−35%, $p < 0.01$).

Kinetics of the Inhibition of Microsomal Ethanol Oxidation by Me_2SO, Mannitol, and Benzoate. The concentration of ethanol was varied from 12.8 to 102 mM in order to study the kinetics of the inhibition by Me_2SO, mannitol, and benzoate in the presence of azide. The amount of acetaldehyde produced increased as the concentration of ethanol was increased (Figure 1). The apparent K_m for ethanol based on the data plotted in Figure 1 was 12–14 mM. The extent of inhibition of microsomal ethanol oxidation by Me_2SO, mannitol, and benzoate varied with the concentration of ethanol; greater inhibition was found at the lower concentrations of ethanol (Table II). Scavenger concentrations as low as 3.3 to 5 mM produced significant inhibition of ethanol oxidation at ethanol concentrations of 12.8 mM, but not at 51 mM (Table II). Double-reciprocal plots (Figure 1) showed that mannitol and benzoate increased the K_m for ethanol without changing the maximum velocity. Similar data were previously reported for Me_2SO (Cederbaum et al., 1977). In the presence of azide, Me_2SO, mannitol, and benzoate were competitive inhibitors of microsomal ethanol oxidation.

Effects of Hydroxyl Radical Scavengers on Microsomal Drug Oxidation and Associated Reactions. The NADPH-mediated microsomal oxidation of ethanol is believed to involve, at least in part, some of the components which participate in the pathways of microsomal drug oxidation (Lieber & DeCarli, 1970). The effect of Me_2SO, mannitol, and benzoate on reactions associated with the microsomal electron transfer pathway was evaluated. Neither NADPH- or NADH-cytochrome *c* reductase activities were affected by concentrations of these agents which inhibited microsomal ethanol oxidation. Similarly, the scavengers had no effect on microsomal oxygen uptake with either NADPH or NADH as substrates, e.g., in a typical experiment with NADPH as substrate, the rate of oxygen consumption was 14.5 natoms per min per mg of microsomal protein in the absence of Me_2SO, and 13.5, 15, 14, 14, and 14.5 natoms per min per mg of microsomal protein in the presence of 4.7, 14, 23.5, 47, and 94 mM Me_2SO, respectively. Since benzoate, mannitol, and Me_2SO had no effect on some of the partial reactions involved in microsomal drug oxidation, the effect of these agents on the overall oxidation of drugs was studied. Aniline was used as a typical type 2 binder, while aminopyrine was representative of a type 1 binder (Schenkman et al., 1967; Schenkman, 1970). Neither the activities of aminopyrine demethylase nor of aniline hydroxylase were inhibited by concentrations of the scavengers which inhibited microsomal ethanol oxidation (Table III).[3]

Effect of Azide and Me_2SO on Microsomal Oxidation of l-Butanol. l-Butanol is a substrate for the microsomal alcohol oxidizing system (Lieber, 1975; Teschke et al., 1974), whereas the peroxidatic activity of catalase with l-butanol is negligible (Keilin & Hartree, 1945; Tephly et al., 1961; Teschke et al., 1974). In our studies, in the presence of xanthine–xanthine oxidase, l-butanol was oxidized at only 2–3% of the ethanol oxidation rate. In the presence of NADPH, l-butanol was oxidized by hepatic microsomes (Table IV). Since l-butanol is not an effective substrate for the peroxidatic activity of catalase, azide would be expected to have little or no effect on the NADPH-dependent oxidation of l-butanol. However, concentrations of azide which inhibited the rate of ethanol oxidation (Table I), actually doubled the rate of l-butanol oxi-

[3] Benzoate incubated with microsomes in the absence of aniline resulted in the production of a compound which was extracted by ether and showed absorbance at 412 nm. Absorbance was proportional to the concentration of benzoate. When corrected for this absorbance, benzoate had no effect on aniline hydroxylase activity (Table III).

TABLE III: Effect of Mannitol, Benzoate, and Dimethyl Sulfoxide on the Activities of Aminopyrine Demethylase and Aniline Hydroxylase.[a]

addition	concn (mM)	aminopyrine demethylase (nmol/(min mg of protein))	effect (%)	aniline hydroxylase (nmol/(min mg of protein))	effect (%)
control		7.61 ± 0.34		0.49 ± 0.03	
mannitol	3.3	7.83 ± 0.62	+3	0.60 ± 0.09	+22
	10.0	8.20 ± 0.77	+8	0.55 ± 0.08	+12
	33.0	8.35 ± 0.95	+9	0.60 ± 0.08	+22
	67.0	8.18 ± 0.77	+7	0.59 ± 0.09	+20
benzoate	3.3	7.65 ± 1.00	+1	0.53 ± 0.07	+8
	10.0	7.20 ± 0.57	−5	0.55 ± 0.07	+12
	33.0	7.62 ± 0.70	0	0.54 ± 0.04	+11
	67.0	8.03 ± 0.58	+6		
control		7.13 ± 0.38		0.52 ± 0.02	
dimethyl sulfoxide	14.0	7.04 ± 0.61	−1	0.55 ± 0.06	+6
	23.5	7.35 ± 0.48	+3	0.58 ± 0.03	+12
	47.0	6.95 ± 0.60	−3	0.52 ± 0.02	0
	94.0	7.03 ± 0.52	−1	0.48 ± 0.04	−8

[a] The activities of aminopyrine demethylase and aniline hydroxylase were assayed as described in Methods. Results are from three experiments with each scavenger. In all cases, there were no significant effects ($p > 0.05$).

dation (Table IV). In the absence or presence of azide, Me_2SO inhibited l-butanol oxidation (Table IV). In contrast to results obtained with ethanol (Table I), the extent of inhibition of l-butanol oxidation by Me_2SO was only slightly enhanced in the presence of azide (Table IV). The inhibition of l-butanol oxidation by Me_2SO in the absence or presence of azide was, however, considerably less than the inhibition of ethanol oxidation in the presence of azide (compare Table I and IV).

Effect of Hydrogen Peroxide on Microsomal Oxidation of Ethanol and l-Butanol. Hydrogen peroxide appears to play a central role in the oxidation of ethanol by microsomes. It serves as a substrate in catalase-mediated oxidation of ethanol and it can also serve as a precursor of hydroxyl radicals (see Discussion). To evaluate the possible requirement for H_2O_2 in microsomal oxidation of ethanol and l-butanol, the effect of externally added H_2O_2 on ethanol and l-butanol oxidation in the presence of 1.0 mM azide was studied. That catalase was inoperative under these conditions was verified by the observation that 1.0 mM azide almost completely inhibited (>98%) xanthine oxidase mediated oxidation of ethanol, and that this inhibition persisted in the presence of externally added (0.1–1.0 mM) H_2O_2. Microsomal oxidation of ethanol and 1-butanol was stimulated by the addition of H_2O_2 (Table V). This stimulation was limited to reaction periods of short time intervals, i.e., the extent of stimulation was less as the reaction time increased from 2.5 to 5 min (Table V). In view of the toxic properties of H_2O_2 and hydroxyl radicals, longer periods of exposure of the microsomes particularly at the higher H_2O_2 concentrations may result in damage to the microsomes and thereby mask the stimulation found at the early time periods.

TABLE IV: Effect of Dimethyl Sulfoxide on Microsomal Oxidation of l-Butanol in the Absence and Presence of Azide.[a]

azide	concn of dimethyl sulfoxide (mM)	l-butanol oxidation (nmol/(min mg of protein))	effect (%)	P
−	0	3.56 ± 0.28		
	14.0	3.12 ± 0.26	−12	NS
	23.5	2.92 ± 0.28	−18	$0.10 > P > 0.05$
	47.0	2.49 ± 0.14	−30	<0.01
	94.0	2.14 ± 0.21	−40	<0.005
+	0	7.24 ± 0.29		
	14.0	6.06 ± 0.46	−16	<0.05
	23.5	5.37 ± 0.42	−26	<0.005
	47.0	4.48 ± 0.47	−38	<0.001
	94.0	3.88 ± 0.32	−46	<0.001

[a] Microsomal oxidation of butanol (final concentration of 51 mM) was assayed as described in Methods. When present, azide was added at a final concentration of 1.0 mM. Results are from 5 (+ azide) or 6 (− azide) different preparations.

Discussion

In the presence of concentrations of azide that block almost totally the catalase-dependent oxidation of ethanol, high rates of the NADPH-dependent pathway are still maintained. This latter pathway is inhibited by three powerful scavengers of hydroxyl radicals, namely, mannitol, benzoate, and Me_2SO. The inhibition is competitive with respect to ethanol (Table II and Figure 1). The effects of the scavengers are rather specific as various other reactions, e.g., xanthine oxidase mediated oxidation of ethanol, microsomal electron transfer reactions, as well as the metabolism of two typical substrates for the mixed function oxidase system are not affected by concentrations of the scavengers which block microsomal oxidation of ethanol and l-butanol. Studies with other substrates, e.g., benzphetamine, benzopyrene, would help to further evaluate the specificity of action of the scavengers. In addition, it remains to be determined whether or not the scavengers themselves produce detectable binding spectra or modify the binding spectra of other drugs.

It is proposed that ethanol oxidation by microsomes can proceed by two pathways, one involving catalase (reaction 1) and the other involving hydroxyl radicals (reaction 2b).

$$H_2O_2 + \text{ethanol} + \text{catalase} \rightarrow \text{acetaldehyde} \quad (1)$$

TABLE V: Effect of Hydrogen Peroxide on Microsomal Oxidation of Ethanol and Butanol.[a]

reaction time (min)	concn of hydrogen peroxide (mM)	ethanol oxidation (nmol/(mg of protein))	effect (%)	P	butanol oxidation (nmol/(mg of protein))	effect (%)	P
2.5	0	36.88 ± 4.88			22.24 ± 3.29		
	0.1	48.79 ± 3.92	+32	=0.05	34.01 ± 5.37	+53	<0.05
	0.3	54.24 ± 7.68	+47	<0.02	48.12 ± 7.05	+116	<0.05
	1.0	48.52 ± 2.77	+32	<0.05	46.65 ± 5.95	+110	<0.05
5.0	0	72.16 ± 3.95			54.00 ± 5.98		
	0.1	89.71 ± 3.48	+24	<0.01	64.34 ± 5.92	+19	NS
	0.3	91.10 ± 6.40	+26	<0.02	86.57 ± 8.44	+60	<0.05
	1.0	80.76 ± 4.86	+12	NS	79.12 ± 2.98	+47	<0.05
15.0	0	189.87 ± 11.20			144.37 ± 9.61		
	0.1	189.64 ± 9.82	0	NS	145.94 ± 11.67	+1	NS
	0.3	170.88 ± 13.61	−10	NS	138.95 ± 12.84	−4	NS
	1.0	131.10 ± 17.61	−31	<0.05	127.40 ± 11.54	−12	NS

[a] NADPH-dependent oxidation of ethanol and butanol was assayed for the indicated reaction periods in the presence of the listed concentrations of hydrogen peroxide. Azide was present at a final concentration of 1.0 mM. Results are from five (ethanol) or three (butanol) experiments.

$$H_2O_2 \rightarrow \cdot OH \quad (2a)$$

$$\cdot OH + \text{ethanol or l-butanol} \rightarrow \text{acetaldehyde or l-butyraldehyde} \quad (2b)$$

In the absence of azide, there is only slight inhibition by the scavengers (Table I) because ethanol oxidation can still proceed via the catalase-mediated peroxidative pathway. In the presence of azide, the catalase pathway is blocked and ethanol oxidation proceeds via the hydroxyl radical pathway. Hence, hydroxyl radical scavengers appear more effective as inhibitors of ethanol oxidation when azide is present.

Hydrogen peroxide is essential for both the catalase-mediated oxidation of ethanol and for the generation of hydroxyl radicals (reactions 1 and 2a). The central role played by H_2O_2 in the noncatalase pathway (presence of azide) was verified by the observation that oxidation rates for ethanol and l-butanol were increased when exogenous H_2O_2 was added (Table V). In these latter experiments, the absence of the catalase-mediated pathway was confirmed by an absence of acetaldehyde production when an H_2O_2-generating system (xanthine plus xanthine oxidase) replaced the NADPH-dependent system.

The utilization of H_2O_2 via one pathway could influence its availability via an alternate pathway, e.g., competition between the "decomposition" of hydrogen peroxide by catalase and the oxidation of ethanol by catalase (Laser, 1955), or the competition between catalase and glutathione peroxidase in intact erythrocytes (Cohen & Hochstein, 1963). Similarly, the utilization of H_2O_2 by microsomal catalase should limit the hydroxyl radical pathway for the oxidation of ethanol. Inhibition of catalase by azide, on the other hand, should augment the accumulation of H_2O_2 and, hence, the formation of hydroxyl radicals (see reactions described below).

Because l-butanol is a poor substrate for the peroxidatic activity of catalase (Lieber, 1975; Teschke et al. 1976), experiments with l-butanol can help to clarify some of the complex interrelationships in the microsomal system. l-Butanol is a good scavenger of hydroxyl radicals; it is roughly twice as effective as ethanol (Dorfman & Adams, 1973). In the presence of an H_2O_2 generating system, the oxidation rate of l-butanol by rat liver microsomes (that is, the peroxidatic reaction of catalase) was less than 2% that for ethanol. In contrast, in the presence of the NADPH-dependent pathway, the rate of oxidation of l-butanol was 30–40% the rate for ethanol. Therefore, microsomal oxidation of butanol may proceed by a pathway involving interaction with hydroxyl radicals. Inhibition of catalase by azide should result in increased production of hydroxyl radicals (derived from the increased accumulation of H_2O_2) and should, in turn, promote the oxidation of butanol. Indeed, butanol oxidation was doubled in the presence of azide, a result consistent with increased generation of hydroxyl radicals. The fact that Me_2SO inhibited butanol oxidation to the same extent in the presence or absence of azide suggests that the oxidation of butanol proceeds via interaction with hydroxyl radicals in both of these cases.

Different results are expected with ethanol. Inhibition of catalase by azide would be expected to diminish the peroxidatic pathway but, simultaneously, augment the hydroxyl radical pathway. The net observable effect will depend upon the relative contributions of the two pathways, which may reflect levels of contaminating catalase, the rate of production of H_2O_2 and other factors associated with the microsomal pathways. The complexity to the role of azide (or other catalase inhibitors) may explain aspects of the divergence in results obtained by other investigators who attempted to assess the role of catalase in their experiments. When azide was added in our experiments, a net decrease in ethanol oxidation but a net increase in l-butanol oxidation was observed (Tables I and IV).

The differing quantitative aspects to the effects of hydroxyl radical scavengers on the oxidation of ethanol and l-butanol can be understood in the light of the above discussion. The apparent increase in effectiveness of scavengers on the oxidation of ethanol when azide was present may be attributed to the fact that only the hydroxyl radical pathway was operative in the presence of azide, and that hydroxyl radical production was actually increased under these conditions. With l-butanol, on the other hand, the peroxidative pathway was negligible in either the presence or absence of azide, and so the inhibitory action of Me_2SO was similar under both conditions. Although the rate of l-butanol oxidation was increased in the presence of azide, the percentage inhibition by the hydroxyl radical scavengers was unchanged. The twofold greater sensitivity of ethanol oxidation compared with l-butanol oxidation to inhibition by Me_2SO in the presence of azide (compare Tables I

and IV) probably reflects the faster rate constant exhibited by l-butanol for reaction with hydroxyl radicals; that is, l-butanol was a more effective competitor for hydroxyl radicals. In a similar manner, the greater inhibitory effectiveness of Me_2SO compared with mannitol and benzoate may reflect the faster rate constant exhibited by Me_2SO for reaction with hydroxyl radicals.

A reaction pathway for the production of hydroxyl radicals from NADPH in microsomes has not yet been clarified. However, recent studies with a spin-trapping method have shown formation of hydroxyl radicals from NADPH (Lai & Piette, 1977). In addition, hydroxyl radical scavengers such as ethanol, benzoate, and mannitol protected lysosomes from lysis induced by a combination of microsomes, NADPH, and a Fe^{3+}-ADP complex (Fong et al., 1973). Okita et al. (1974) suggested that the purified microsomal NADPH-cytochrome b_5 reductase oxidizes ethanol in the presence of NADPH by a mechanism involving the scavenging activity of ethanol for hydroxyl radicals. In recent studies, we utilized methional and 2-keto-4-thiomethylbutyrate, two scavengers that give rise to ethylene gas on reaction with hydroxyl radicals (cf., Cohen, 1977). Both scavengers blocked the microsomal oxidation of ethanol to acetaldehyde and both gave rise to ethylene. These observations, which will be reported separately, offer support for the presumed role of hydroxyl radicals in the microsomal oxidation of alcohols.

Two H_2O_2-dependent pathways that are often invoked as sources of hydroxyl radicals in biologic systems are the ferrous ion-catalyzed decomposition of H_2O_2 (reaction 3, the Fenton reaction) (Walling, 1975)

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

and the reaction between the superoxide radical ($O_2^{-}\cdot$) and H_2O_2 (the Haber-Weiss reaction, reaction 4) (Haber & Weiss, 1934)

$$O_2^{-}\cdot + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH \qquad (4)$$

Although recent reports (Halliwell, 1976; McLune & Fee, 1976; Rigo et al., 1977) indicate that the Haber-Weiss reaction in pure solution is very slow, nonetheless a substantial body of evidence exists that, when superoxide radicals are generated in the presence of H_2O_2, hydroxyl radicals are also generated during the reaction (Cohen, 1977, and references quoted in editors' footnote). In biological systems, reaction 4 may be catalyzed by iron salts or iron chelates which may accept an electron from superoxide (reaction 5)

$$Fe^{3+} + O_2^{-}\cdot \rightarrow Fe^{2+} + O_2 \qquad (5)$$

and subsequently participate in a Fenton-type reaction (Walling et al., 1975; McCord & Day, 1978) or which may participate directly by forming complexes with either superoxide or H_2O_2 and thereby accelerating the formation of hydroxyl radicals (Ilan & Czapski, 1977). Thus, the hydroxyl radicals that arise during microsomal oxidation of NADPH may be derived from modified Fenton or Haber-Weiss reactions in which endogenous iron or iron chelates play a role. In this regard, the possible participation of the microsomal cytochromes should be explored.

The NADPH-oxidation system and the mixed-function oxidase system of endoplasmic reticulum generate superoxide (Aust et al., 1972; Fridovich & Handler, 1961; Sato, 1967; Prough & Masters, 1973). Superoxide production has been found to be associated with NADPH-cytochrome *c* reductase (Prough & Masters, 1973; Aust et al., 1972) and cytochrome P-450 of hepatic microsomes (Dybing et al., 1976; Strobel & Coon, 1971). The possible effect of superoxide dismutase on microsomal oxidation of ethanol is difficult to evaluate. When H_2O_2 is produced via the dismutation of superoxide radicals (reaction 6)

$$O_2^{-}\cdot + O_2^{-}\cdot + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (6)$$

the addition of superoxide dismutase could actually stimulate microsomal oxidation of ethanol by increasing the production of H_2O_2. On the other hand, if the generation of $O_2^{-}\cdot$ were relatively slow, rapid removal of $O_2^{-}\cdot$ by superoxide dismutase would slow reaction 2 and result in decreased production of hydroxyl radicals and, hence, decreased microsomal oxidation of ethanol. Similar considerations have been reported by others (Fong et al., 1973). Actually we have not observed any effect of superoxide dismutase on microsomal ethanol oxidation (unpublished observations). This could reflect the possibility that superoxide dismutase cannot penetrate the microsome to interact at the site(s) of superoxide generation as had been suggested by others, e.g., Panganamala et al. (1976). Studies with superoxide radical scavengers are currently in progress.

In our studies, the inhibitory action of the scavengers might have resulted from their reaction directly with H_2O_2 rather than with the derived hydroxyl radicals. However, mannitol and Me_2SO did not react with H_2O_2 (as determined with the ferrithiocyanate method) while benzoate was reactive only at the highest concentration tested. Therefore, removal of H_2O_2 does not appear to represent the mechanism of action of these agents. We had previously reported that another scavenger, thiourea, also blocked microsomal oxidation of ethanol (Cederbaum et al., 1977). The action of thiourea is complex because it does react directly with H_2O_2. The action of thiourea on microsomal oxidation of alcohols will be the subject of a separate communication.

A common denominator for the inhibitory action of the compounds studied lies in their reactivity with hydroxyl radicals. The specific and competitive nature of the inhibition is consistent with ethanol and the scavengers competing with one another for interaction with hydroxyl radicals. Therefore, we suggest that the catalase-independent pathway for oxidation of alcohols by liver microsomes reflects the interaction of alcohols with hydroxyl radicals that are generated from the microsomal electron transfer pathway. The physical nature of the hydroxyl radical is not defined by these experiments but it is presumed to be bound and not "free" in solution.

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Reduction and Renaturation of Hen Egg Lysozyme Containing Carboxymethylcysteine-6 and -127†

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ABSTRACT: The thermodynamic stability of an enzymatically active derivative (sample LH_1) of hen egg lysozyme containing three, presumably, native disulfide bonds and carboxymethylated cysteines at positions 6 and 127 and exhibiting characteristics of the intact enzyme has been studied with respect to the formation of disulfide bonds. The procedure is to measure the equilibrium between the native and nonnative forms, after reequilibrating the disulfide bonds in the presence of β-mercaptoethanol, by gel filtration on Bio-Gel P-30. The native form has a lower hydrodynamic volume than the nonnative forms. To test whether the equilibrium has been reached, three different disulfide bonded forms of sample LH_1 (native, reduced, and "scrambled") are exposed to the same concentration of β-mercaptoethanol (1.5 mM) at pH 8.0 and 37 °C, which permits the disulfide interchange. All three samples show similar ratios (3:2) of native to nonnative forms after 16 h. This ratio is reached within 5 h in the case of native sample LH_1. Consequently the apparent equilibrium appears to be reached. In these same conditions intact lysozyme shows only the native form. Thus, sample LH_1 is less stable than the intact protein with respect to disulfide bond formation, but more stable than the nonnative isomers of sample LH_1 obtained by reshuffling the disulfide bonds. This latter point has been tested by measuring the proportions of all the partially reduced forms of sample LH_1 by trapping the free sulfhydryl groups present in the equilibrium mixture with iodoacetic acid.

Pancreatic ribonuclease A (RNase A) renatures after reduction and reoxidation, indicating that the free energy associated with a native protein containing a set of disulfide bonds is distinctly lower than that associated with its isomers containing nonnative sets of disulfide bonds (Epstein et al., 1963; Anfinsen, 1967). On the other hand, if the rearrangement of disulfide bonds by sulfhydryl-disulfide interchange is not permitted to occur in the reoxidation system of reduced RNase A, only nonnative species are formed (Venetianer & Straub,

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