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Role of Hydroxyl Radicals in the Iron–Ethylenediaminetetraacetic Acid Mediated Stimulation of Microsomal Oxidation of Ethanol†

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ABSTRACT: The microsomal oxidation of ethanol or 1-butanol was increased by ferrous ammonium sulfate–ethylenediaminetetraacetic acid (1:2) (Fe–EDTA) (3.4–50 μM). The increase was blocked by hydroxyl radical scavenging agents such as dimethyl sulfoxide or mannitol. The activities of aminopyrine demethylase or aniline hydroxylase were not affected by Fe–EDTA. The accumulation of H_2O_2 was decreased in the presence of Fe–EDTA, consistent with an increased utilization of H_2O_2. Other investigators have shown that Fe–EDTA increases the formation of hydroxyl radicals in systems where superoxide radicals are generated. The stimulation by Fe–EDTA appears to represent a pathway involving hydroxyl radicals rather than catalase because (1) stimulation occurred in the presence of azide, which inhibits catalase, (2) stimulation occurred in the presence of 1-butanol, which is not an effective substrate for catalase, and (3) stimulation was blocked by hydroxyl radical scavenging agents, which do not affect catalase-mediated oxidation of ethanol. A possible role for contaminating iron in the H_2O or buffers could be ruled out since similar results were obtained with or without chelex-100 treatment of these solutions. The stimulatory effect by Fe–EDTA required microsomal electron transfer with NADPH, and H_2O_2 could not replace the NADPH-generating system. In the absence of microsomes or catalase, Fe–EDTA also stimulated the coupled oxidation of ethanol during the oxidation of xanthine by xanthine oxidase. These results suggest that during microsomal electron transfer, conditions may be appropriate for a Fenton type or a modified Haber–Weiss type of reaction to occur, leading to the production of hydroxyl radicals.

Recent experiments have implicated a role for hydroxyl radicals (·OH)[1] in the microsomal system for oxidizing primary aliphatic alcohols. Several ·OH scavengers were effective competitive inhibitors of microsomal oxidation of ethanol and 1-butanol (Cederbaum et al., 1977, 1978, 1979). These agents did not inhibit microsomal electron transfer, microsomal drug metabolism, or H_2O_2 plus catalase dependent oxidation of ethanol (Cederbaum et al., 1978). In addition, microsomes metabolized several ·OH scavengers during electron transfer: ethylene was generated from methional or from 2-keto-4-thiomethylbutyric acid, and methane was generated from Me_2SO (Cohen & Cederbaum, 1979, 1980). Hydrocarbon gas production was inhibited by competing ·OH scavengers such as ethanol and 1-butanol (Cohen & Cederbaum, 1979, 1980). These experiments indicated that microsomes generated ·OH, and that the oxidation of alcohols reflected the interaction of the alcohols with ·OH to form the corresponding aldehydes.

By what mechanisms can microsomes generate ·OH? Two H_2O_2-dependent pathways that are often invoked as sources of ·OH in biological systems are the ferrous ion catalyzed decomposition of H_2O_2 [the Fenton reaction (Walling, 1975)]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (1)$$

or the reaction between the superoxide radical ($O_2^{-}\cdot$) and H_2O_2 [the Haber–Weiss reaction (Haber & Weiss, 1934)]:

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

Microsomes generate both H_2O_2 and $O_2^{-}\cdot$ during NADPH oxidation (Aust et al., 1972; Prough & Masters, 1973; Dybing et al., 1976; Strobel & Coon, 1971; Thurman et al., 1972; Hildebrandt & Roots, 1975; Nordblom & Coon, 1977) and thus have the potential for generating ·OH by either reaction 1 or reaction 2. In our experiments with microsomes, a role for H_2O_2 as a precursor for ·OH was evident from the observation that (1) the addition of azide to inhibit catalase and thereby allow H_2O_2 to accumulate increased the oxidation of 1-butanol as well as the metabolism of ·OH scavengers to hydrocarbon gases (Cederbaum et al., 1978; Cohen & Cederbaum, 1979, 1980) and (2) the addition of H_2O_2 in the presence of azide increased the NADPH-dependent oxidation of ethanol and 1-butanol (Cederbaum et al., 1978). However,

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[1] Abbreviations used: ·OH, hydroxyl radical; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Fe–EDTA, ferrous ammonium sulfate and EDTA in a 1:2 molar ratio; SOD, superoxide dismutase.

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it has been concluded that, although feasible thermodynamically (Koppenol & Butler, 1977), the Haber–Weiss reaction does not appear to proceed at any appreciable rate in pure solution (McLune & Fee, 1976; Rigo et al., 1977; Halliwell, 1976; Ferradini et al., 1979).

Traces of chelated metal ions are present in biological systems, and it has been proposed that iron can catalyze reaction 2 by the mechanism:

$$Fe^{3+}\text{–chelate} + O_2^{-}\cdot \rightarrow O_2 + Fe^{2+}\text{–chelate} \quad (3)$$

$$Fe^{2+}\text{–chelate} + H_2O_2 \rightarrow Fe^{3+}\text{–chelate} + \cdot OH + OH^- \quad (4)$$

The feasibility of reactions 3 and 4 was demonstrated by the finding that Fe–EDTA increased ·OH generation from the xanthine oxidase reaction as measured either by tryptophan oxidation (McCord & Day, 1978) or by hydroxylation of salicylic acid (Halliwell, 1978 a,b). It was therefore of interest to study the effect of Fe–EDTA on another system potentially dependent on the generation of ·OH, namely, the microsomal alcohol oxidizing system.

Experimental Section

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 1-butanol was assayed at 37 °C as previously described (Cederbaum et al., 1977) with the use of flasks containing 0.6 mL of 15 mM semicarbazide in the center well. Unless otherwise indicated, the final concentration of ethanol or 1-butanol was 51 mM; reaction periods were for 10 min. The basic reaction mixture contained 83 mM potassium phosphate, pH 7.4, 10 mM sodium pyrophosphate, 10 mM $MgCl_2$, 0.3 mM $NADP^+$, 10 mM glucose 6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, and ~5 mg of microsomal protein in a volume of 3.0 mL. When present, the final concentration of azide was 0.5 mM. The reaction was initiated with the NADPH-generating system and was terminated by the addition of trichloroacetic acid (final concentration 5%). The optical density of the semicarbazone complex was determined at 224 nm after an overnight diffusion period. An extinction coefficient of 9.41 mM^{-1} cm^{-1} was used to calculate the concentration of acetaldehyde. The activities of aniline hydroxylase and aminopyrine demethylase and the accumulation of H_2O_2 during NADPH oxidation in the presence of 5 mM azide were determined as previously described (Cederbaum et al., 1978, 1979). Superoxide dismutase (1650 units/mg) was purchased from Worthington Biochemicals.

The oxidation of ethanol by xanthine oxidase (experiments of Table I) was assayed in a reaction system containing 100 mM potassium phosphate, pH 7.4, 1 mM xanthine, xanthine oxidase (12, 20, or 40 milliunits; Boehringer Mannheim 0.4 units/mg), and ethanol (10, 20, or 50 mM) in a final volume of 1 mL. The reaction was terminated after 30 min by the addition of trichloroacetic acid (final concentration 5%), and acetaldehyde was determined by gas chromatography (Dembiec et al., 1976). In view of the low amounts of acetaldehyde formed in the xanthine–xanthine oxidase system, the semicarbazone method could not be accurately utilized.

Chelex treatment of the H_2O, phosphate, pyrophosphate, and KCl solutions was carried out by passing these solutions through Kontes columns (35 × 3 cm) packed with well-washed chelex-100 (Bio-Rad) resin. The concentration of iron in these solutions was determined by the ferrithiocyanate method (Snell & Snell, 1954).

Table I: Effect of Fe-EDTA on the Coupled Oxidation of Ethanol during the Xanthine-Xanthine Oxidase Reaction[a]

expt	concn of Fe-EDTA (μM)	ethanol oxidn (nmol/min) at 10 mM EtOH	20 mM EtOH	50 mM EtOH
A	0	0.55	0.62	0.68
	5	1.07	1.23	1.47
	10	1.17	1.40	1.57
	25	1.40	1.63	1.70
	50	1.70	1.77	2.00
expt	concn of Fe-EDTA (μM)	ethanol oxidn (nmol/min) at 12 milliunits[b]	20 milliunits[b]	40 milliunits[b]
B	0	0.53	0.78	0.53
	10	1.20	1.59	1.43
	25	1.68	1.88	1.79
	50	1.55	1.98	1.91
	100	1.58	1.96	1.88

[a] The reaction system contained 100 mM potassium phosphate buffer, pH 7.4, and 1 mM xanthine. In experiment A, 20 milliunits of xanthine oxidase was used, and the ethanol concentration was either 10, 20, or 50 mM. In experiment B, 50 mM ethanol was used, and the concentration of xanthine oxidase was either 12, 20, or 40 milliunits. [b] Milliunits of xanthine oxidase.

An Fe–EDTA solution was prepared by dissolving ferrous ammonium sulfate (5 mM) in 10 mM EDTA. Under these conditions the iron becomes oxidized to the Fe^{3+} state.

All values refer to the mean ± SEM. Statistical analysis was performed by Student's *t* test. The number of experiments is indicated in the table or figure legends. Levels of significance are indicated in the tables, and $P < 0.05$ was considered to be statistically significant.

Results

Effect of Fe–EDTA on Coupled Oxidation of Ethanol by Xanthine Oxidase. It was shown previously that the coupled oxidation of ethanol during the oxidation of xanthine by xanthine oxidase in the absence of microsomes was inhibited by ·OH scavenging agents (Cohen, 1977). It is known that ·OH is generated during the xanthine oxidase reaction (Beauchamp & Fridovich, 1970). As shown in Table I, experiment A, the addition of Fe–EDTA in concentrations from 5 to 50 μM increased the rate of ethanol oxidation two- to threefold at all concentrations of ethanol tested. A similar stimulation was observed when the concentration of xanthine oxidase was varied from 12 to 40 milliunits (Table I, experiment B). These experiments parallel similar reports that Fe–EDTA increases the coupled oxidation of tryptophan (McCord & Day, 1978) or the hydroxylation of salicylic acid (Halliwell, 1978a,b) by xanthine oxidase. Results with tryptophan and salicylic acid oxidation have been interpreted to indicate that Fe–EDTA stimulates production of ·OH by the xanthine oxidase reaction (McCord & Day, 1978; Halliwell, 1978a,b).

Effect of Fe–EDTA on Microsomal Oxidation of Ethanol. The addition of Fe–EDTA, in concentrations ranging from 3.4 to 50 μM, resulted in an increase in NADPH-dependent ethanol oxidation (Table II, minus azide). The increase by Fe–EDTA could have reflected either increased generation of H_2O_2 for the peroxidatic activity of catalase or increased generation of ·OH. Experiments were carried out in the presence of 0.50 mM azide (Table II) to distinguish between these possibilities. At this concentration, azide inhibits the peroxidatic activity of microsomal catalase with ethanol by at least 95% (Cederbaum et al., 1978). In the presence of azide, Fe–EDTA also increased the oxidation of ethanol; a

Table II: Effect of Fe-EDTA on Microsomal Oxidation of Ethanol and 1-Butanol[a]

concn of Fe-EDTA (μM)	−azide EtOH oxidn [nmol min^{-1} (mg of protein)$^{-1}$]	−azide effect (%)	+azide EtOH oxidn [nmol min^{-1} (mg of protein)$^{-1}$]	+azide effect (%)	+azide 1-BuOH oxidn [nmol min^{-1} (mg of protein)$^{-1}$]	+azide effect (%)
0	10.17 ± 0.86		9.62 ± 0.60		5.60 ± 0.72	
3.4	12.06 ± 1.35	+19	14.57 ± 0.65	+51[c]	7.31 ± 1.12	+31
8.5	13.69 ± 1.53	+35	17.53 ± 0.74	+82[c]	8.90 ± 0.82	+59[d]
17	17.62 ± 2.13	+73[b]	20.07 ± 0.85	+109[c]	10.62 ± 1.03	+90[c]
50	20.53 ± 2.85	+102[b]	23.15 ± 0.96	+141[c]	11.31 ± 1.16	+102[c]

[a] NADPH-dependent microsomal oxidation of ethanol was assayed as described under Experimental Section in the presence of the indicated concentrations of Fe-EDTA and in the absence ($n = 4$) or presence ($n = 11$) of 0.50 mM azide. Experiments with 1-butanol were carried out in the presence of azide ($n = 5$). [b] $P < 0.02$. [c] $P < 0.001$. [d] $P < 0.01$.

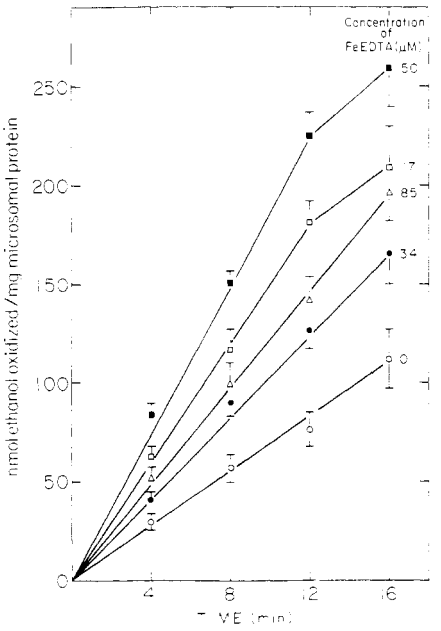

FIGURE 1: Time course of the effect of Fe–EDTA on the microsomal oxidation of ethanol. The rate of ethanol (51 mM) oxidation was determined in the absence or presence of Fe–EDTA. Azide was present at a final concentration of 0.50 mM. Results are from five experiments. (○) 0, (●) 3.4, (△) 8.5, (□) 17, and (■) 50 μM Fe–EDTA.

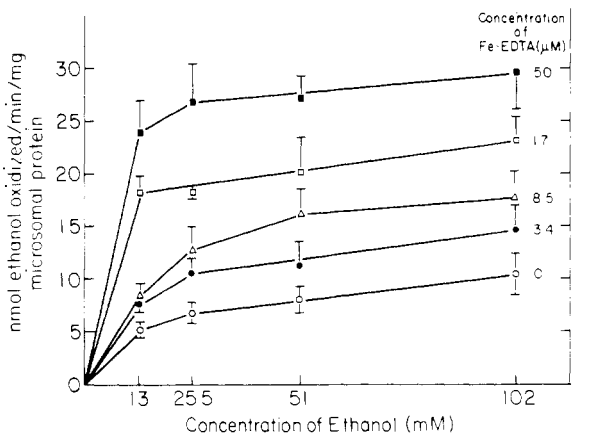

FIGURE 2: Effect of Fe–EDTA on the microsomal oxidation of varying concentrations of ethanol. The concentration of ethanol was varied between 13 and 102 mM. Azide was present, and the reaction period was 10 min. Results are from three experiments. (○) 0, (●) 3.4, (△) 8.5, (□) 17, and (■) 50 μM Fe–EDTA.

significant increase was found at 3.4 μM Fe–EDTA (Table II). Fe–EDTA was a more effective stimulator in the presence of azide, presumably because the inhibition of catalase results in the accumulation of H_2O_2 and increased ethanol oxidation by the ·OH–mediated pathway (Cederbaum et al., 1978).

1-Butanol is a substrate for the microsomal alcohol oxidizing system (Teschke et al., 1974; Lieber, 1975), whereas the peroxidatic activity of catalase with 1-butanol is negligible (Keilin & Hartree, 1945; Tephly et al., 1961). The microsomal oxidation of 1-butanol is inhibited by ·OH scavengers (Cederbaum et al., 1978). If the stimulation of ethanol oxidation by Fe–EDTA involves a catalase-dependent pathway, little effect should be found with 1-butanol as the substrate. However, if the stimulation by Fe–EDTA involves ·OH, the oxidation of 1-butanol should also be increased. As shown in Table II, the oxidation of 1-butanol was increased by Fe–EDTA. The data with azide and 1-butanol show that the stimulation by Fe–EDTA is not mediated by catalase; instead, a mechanism involving ·OH is indicated.

Kinetic experiments characterizing the stimulation of microsomal ethanol oxidation by Fe–EDTA in the presence of azide are shown in Figures 1 and 2. Ethanol oxidation was linear over a 16-min reaction period (Figure 1). Fe–EDTA significantly increased the rate of ethanol oxidation at all time periods tested. There was no lag period in the stimulatory action of Fe–EDTA (Figure 1). A substrate concentration curve is shown in Figure 2. The rate of microsomal ethanol oxidation increased as the concentration of ethanol was increased (Figure 2). Fe–EDTA effectively stimulated ethanol oxidation at all concentrations of ethanol (Figure 2).

Control experiments indicated that Fe–EDTA did not affect the recovery of standard amounts of acetaldehyde. No acetaldehyde was produced in the absence of either ethanol, microsomes, or glucose-6-phosphate dehydrogenase or when boiled microsomes were used; Fe–EDTA had no effect under any of these conditions. When 0.3 mM H_2O_2 was added in place of the NADPH-generating system (in the presence of azide to inhibit catalase), only very small amounts of acetaldehyde were produced from ethanol [<0.5 nmol min^{-1} (mg of microsomal protein).$^{-1}$ Fe–EDTA increased this H_2O_2-dependent ethanol oxidation by less than 0.2 nmol min^{-1} (mg of protein).$^{-1}$ These results should be contrasted with a basal rate (NADPH-generating system in the presence of azide) of 9.62 nmol min^{-1} (mg of protein)$^{-1}$ with an increase to 23.5 nmol min^{-1} (mg of protein)$^{-1}$ upon the addition of 50 μM Fe–EDTA (Table II). It can be concluded that the stimulation of NADPH-dependent microsomal ethanol oxidation by Fe–EDTA is not merely dependent on the provision of H_2O_2 by microsomal electron transfer.

Effect of Hydroxyl Radical Scavengers on the Stimulation of Microsomal Ethanol Oxidation by Fe–EDTA. Previous experiments showed that ·OH scavengers such as Me_2SO or mannitol inhibited microsomal oxidation of ethanol in the presence of azide (Cederbaum et al., 1977, 1978). If the stimulation by Fe–EDTA involved ·OH, Me_2SO or mannitol should inhibit the increased ethanol oxidation evoked by Fe–

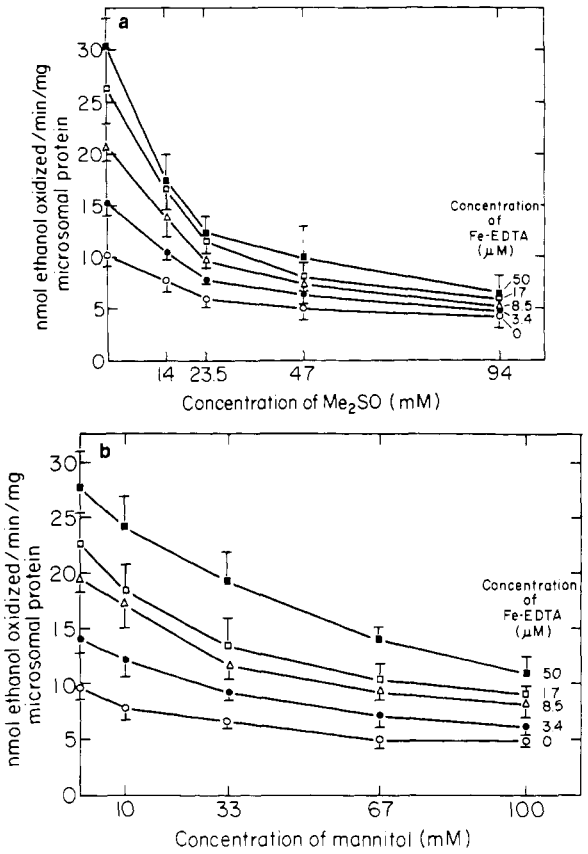

FIGURE 3: Effect of Me_2SO (a) and mannitol (b) on the stimulation of the microsomal oxidation of ethanol by Fe-EDTA. Results are from three experiments. Azide was present at a final concentration of 0.50 mM. (○) 0, (●) 3.4, (△) 8.5, (□) 17, and (■) 50 μM Fe-EDTA.

EDTA. Microsomes were incubated (in the presence of azide) in the absence or presence of 3.4, 8.5, 17, or 50 μM Fe-EDTA and varying concentrations of Me_2SO or mannitol. In the absence of Fe-EDTA, the control rate of ethanol oxidation was inhibited 23, 40, 49, and 61% by Me_2SO concentrations of 14, 23.5, 47, and 94 mM, respectively (Figure 3a). Fe-EDTA stimulated ethanol oxidation 58, 103, 155, and 198% at concentrations of 3.4, 8.5, 17, and 50 μM, respectively (Figure 3a). Me_2SO inhibited ethanol oxidation in the presence of Fe-EDTA; in fact, most of the increase produced by Fe-EDTA was prevented by Me_2SO (Figure 3a). Similarly, in experiments with mannitol, the rate of ethanol oxidation in the absence of Fe-EDTA was inhibited 20, 31, 47, and 52% by mannitol concentrations of 10, 33, 67, and 100 mM, respectively (Figure 3b). Mannitol also effectively inhibited ethanol oxidation in the presence of Fe-EDTA; a major part of the increase produced by Fe-EDTA was prevented by 67 and 100 mM mannitol (Figure 3b).

In the absence of azide, ·OH scavengers such as Me_2SO or mannitol have little effect on the microsomal oxidation of ethanol (Cederbaum et al., 1978); this has been attributed to the predominance of the peroxidatic pathway (catalase) in the absence of azide and to the inability of ·OH scavengers to affect this pathway. In the current study, Me_2SO and mannitol were also studied in the absence of azide (Table III). Me_2SO and mannitol showed only a slight inhibition of ethanol oxidation when Fe-EDTA was absent. However, the increased rate in the presence of Fe-EDTA was more effectively blocked by Me_2SO or mannitol (Table III). These results indicate that the increase in ethanol oxidation produced by Fe-EDTA, even in the absence of azide, involves ·OH.

Table III: Effect of Me_2SO and Mannitol on the Stimulation of Microsomal Oxidation of Ethanol by Fe-EDTA in the Absence of Azide[a]

		rate of ethanol oxidn and effect of scavenger at					
		0 μM Fe-EDTA		17 μM Fe-EDTA		50 μM Fe-EDTA	
addition	concn (mM)	rate	effect (%)	rate	effect (%)	rate	effect (%)
Me_2SO	0	10.09		15.33		21.66	
	14	8.92	−12	12.65	−17	16.28	−25
	23.5	9.11	−10	12.38	−19	12.85	−41
	47	9.00	−11	11.97	−22	10.88	−50
	94	8.86	−12	10.71	−30	10.03	−54
mannitol	0	9.85		14.44		20.52	
	10	9.78	−1	13.93	−4	17.73	−14
	33	8.75	−11	11.46	−21	14.95	−27
	67	8.63	−12	10.71	−26	13.70	−33
	100	8.05	−18	10.04	−30	12.76	−38

[a] Microsomal oxidation of ethanol was assayed as described under Experimental Section in the absence or presence of Fe-EDTA. Azide was not added in these experiments. Rate refers to nmoles of ethanol oxidized per minute per milligram of microsomal protein. Effect refers to the effect of Me_2SO or mannitol under each reaction condition (0, 17, 50 μM Fe-EDTA). Results are the average of three experiments.

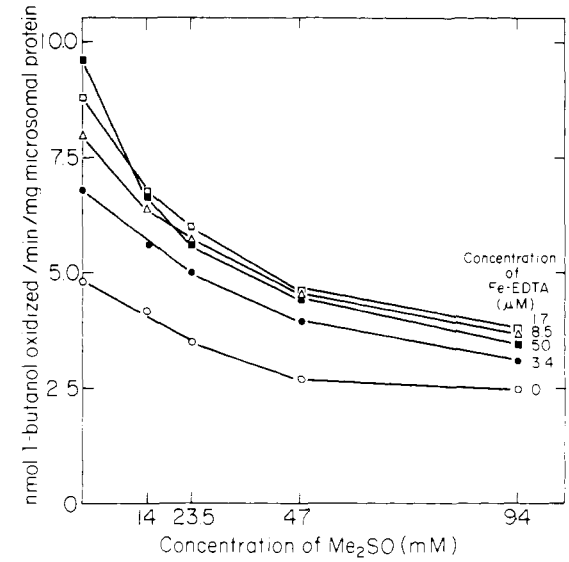

FIGURE 4: Effect of Me_2SO on the stimulation of the microsomal oxidation of 1-butanol by Fe-EDTA. Results are from two experiments. Azide was present at a final concentration of 0.50 mM. (○) 0, (●) 3.4, (△) 8.5, (□) 17, and (■) 50 μM Fe-EDTA.

Similar experiments conducted with 1-butanol showed that the increased rate of oxidation of 1-butanol evoked by addition of Fe-EDTA was to a large extent blocked by Me_2SO (Figure 4). These results are consistent with a role for ·OH in the stimulatory action of Fe-EDTA on 1-butanol oxidation.

Effect of Fe-EDTA on Microsomal Drug Metabolism. Since NADPH-mediated microsomal oxidation of ethanol involves some of the components which participate in the pathways of microsomal drug metabolism (Lieber & DeCarli, 1970), the effect of Fe-EDTA on aminopyrine demethylation and aniline hydroxylation was determined. The rate [nmol min^{-1} (mg of protein)$^{-1}$] of aminopyrine demethylase activity was 7.23 ± 0.29 in the absence of Fe-EDTA and 7.41 ± 0.30, 7.48 ± 0.27, 7.57 ± 0.30, and 7.84 ± 0.31 in the presence of 3.4, 8.5, 17, and 50 μM Fe-EDTA, respectively (n = 4). The rate of aniline hydroxylase activity was 0.55 ± 0.04 in the absence of Fe-EDTA and 0.55 ± 0.06, 0.57 ± 0.05, 0.54 ±

Table IV: Effect of Fe-EDTA on H_2O_2 Accumulation during Microsomal NADPH Oxidation[a]

concn of Fe-EDTA (μM)	H_2O_2 accumn [nmol min^{-1} (mg of protein)$^{-1}$]	effect of Fe-EDTA (%)
0	3.60 ± 0.69	
3.4	2.88 ± 0.66	−21
8.5	1.68 ± 0.48	−53[b]
17	1.44 ± 0.54	−60[b]
50	1.56 ± 0.39	−57[b]

[a] H_2O_2 accumulation was measured in the presence of 5.0 mM azide. Fe-EDTA was added as indicated. Results are from four experiments. [b] $P < 0.05$.

0.05, and 0.60 ± 0.05 in the presence of 3.4, 8.5, 17, and 50 μM Fe-EDTA, respectively ($n = 3$). These drug-metabolizing activities were previously found to be similarly unaffected by ·OH scavengers (Cederbaum et al., 1978). These results indicate that the actions of Fe-EDTA are relatively specific with regard to effects on microsomal functions.

Effect of Fe-EDTA on Microsomal Accumulation of H_2O_2. In the presence of azide, H_2O_2 accumulates during NADPH-dependent microsomal electron transfer (Hilderbrandt & Roots, 1975; Thurman et al., 1972). H_2O_2 is a precursor for ·OH. If Fe-EDTA stimulates ·OH generation from H_2O_2 (e.g., eq 4), Fe-EDTA should lower the accumulation of H_2O_2. Table IV shows that the accumulation of H_2O_2 was lowered by Fe-EDTA, indicating increased utilization of H_2O_2.

Effect of Chelex Treatment on Microsomal Oxidation of Ethanol. The possibility that iron contaminants in the H_2O or especially in the phosphate–pyrophosphate buffer system promoted alcohol oxidation by microsomes was considered. The H_2O and phosphate, pyrophosphate, and KCl solutions were passed through a chelex-100 resin column to remove "contaminating" Fe, if present. A measurement of the total Fe concentration in these combined solutions even before chelex treatment indicated less than 10^{-6} M Fe (the sensitivity limit for the ferrithiocyanate method). A comparison of the microsomal oxidation of ethanol between the chelex-treated or the regular untreated solutions was made. Rates of ethanol oxidation for the two preparations were identical, 8.48 ± 1.08 nmol min^{-1} (mg of protein)$^{-1}$ for the preparation without chelex treatment and 8.19 ± 1.21 after chelex treatment ($n = 4$; 0.5 mM azide was present). Moreover, Fe-EDTA increased ethanol oxidation to similar extents in both preparations: non-chelex-treated, 33, 77, 126, and 186% stimulation at Fe-EDTA concentrations of 3.4, 8.5, 17, and 50 μM, respectively; chelex-treated, 41, 65, 114, and 190% stimulation ($n = 4$). Previously, it was noted that chelex treatment did not notably alter the metabolism of 2-keto-4-thiomethylbutyric acid (a potent ·OH scavenger) to ethylene by microsomes (Cohen & Cederbaum, 1979). It is therefore unlikely that Fe contaminants in the buffer solutions play a role in these experiments. However, a possible role for adventitious Fe in the microsomes is not excluded.

Effect of Superoxide Dismutase on Stimulation of Microsomal Ethanol Oxidation by Fe-EDTA. McCord & Day (1978) had previously shown that superoxide dismutase inhibited the stimulation by Fe-EDTA of the coupled oxidation of tryptophan by xanthine oxidase; these results showed that the superoxide radical played an essential role in the observed stimulation by Fe-EDTA. Similar experiments were carried out in the microsomal system (Table V). Results with superoxide dismutase were complex. In the presence of either 66 or 165 units of superoxide dismutase (13 or 33 μg/mL), less stimulation by Fe-EDTA was seen (Table V, effect column = % increase). These results suggest that superoxide plays some role in the stimulatory action of Fe-EDTA in the microsomal system as well. However, superoxide dismutase also produced some stimulation (35 and 33%) of ethanol oxidation in the absence of Fe-EDTA (Table V, rate columns). Previously, Lai et al. (1979) observed some stimulation of lipid peroxidation by superoxide dismutase in studies with purified NADPH–cytochrome *c* reductase; they attributed the stimulation to increased production of H_2O_2, which served as a precursor for ·OH. A similar mechanism may have been operative in the microsomal system, or, alternatively, superoxide dismutase may have prevented the loss of the hydroxyalkyl radical (the primary product or intermediate on reaction of ·OH with ethanol) or the loss of ·OH through a radical–radical interaction with superoxide. It should be noted that in contrast to the stimulation by superoxide dismutase observed with microsomes in the presence of azide, no effect of superoxide dismutase is observed in the absence of azide (Cederbaum et al., 1978). In addition, part of the increase in ethanol oxidation evoked by Fe-EDTA was not prevented by superoxide dismutase. Whether this lack of complete prevention by superoxide dismutase reflects poor permeability into the microsomes or poor competition with Fe-EDTA for O_2^-· (McCord & Day, 1978) is not known.

Discussion

Current evidence indicates that the microsomal alcohol-oxidizing system reflects the interaction of alcohols with ·OH generated by microsomal electron transfer (Cederbaum et al., 1977, 1978; Cohen & Cederbaum, 1980). Results reported here show that externally added Fe-EDTA increases the microsomal oxidation of ethanol in much the same manner as that found with the model xanthine–xanthine oxidase system (a ·OH-generating system). The stimulation by Fe-EDTA also shows specificity in that concentrations which double and triple microsomal oxidation of alcohols are without effect on microsomal drug metabolism. Microsomal H_2O_2 levels are decreased in the presence of Fe-EDTA, indicating an increased

Table V: Effect of Superoxide Dismutase on the Stimulation of Microsomal Oxidation of Ethanol by Fe-EDTA[a]

concn of Fe-EDTA (μM)	rate of ethanol oxidn and effect of Fe-EDTA at					
	0 units SOD[d]		66 units SOD[d]		165 units SOD[d]	
	rate[b]	effect[c]	rate[b]	effect[c]	rate[b]	effect[c]
0	8.24 ± 0.31		11.11 ± 0.72		10.94 ± 1.24	
3.4	12.31 ± 1.61	+49	14.74 ± 0.84	+33	16.18 ± 2.29	+48
8.5	15.87 ± 2.08	+93	19.37 ± 1.17	+74	18.39 ± 2.48	+68
17.0	24.72 ± 2.34	+200	22.01 ± 1.17	+98	20.06 ± 2.80	+83
50.0	32.19 ± 4.65	+291	27.78 ± 2.49	+150	26.12 ± 4.57	+139

[a] Ethanol oxidation was assayed in the presence of the indicated concentrations of Fe-EDTA and superoxide dismutase. Experiments were carried out in the presence of 0.5 mM azide. Results are from five experiments. [b] Rate refers to nanomoles of ethanol oxidized per minute per milligram of microsomal protein. [c] Effect refers to percent increase by Fe-EDTA. [d] Units of superoxide dismutase.

utilization of H_2O_2. A role for H_2O_2 as the precursor of ·OH generated by microsomes has previously been suggested (Cederbaum et al., 1978; Cohen & Cederbaum, 1980). In other experiments, we observed that Fe–EDTA increases the microsomal generation of ethylene gas from 2-keto-4-thiomethylbutyric acid in a reaction sensitive to inhibition by ·OH scavengers such as Me_2SO or mannitol (unpublished experiments). These latter observations suggest, in a more direct manner, that Fe–EDTA increases ·OH generation by microsomes.

Although the exact mechanism(s) whereby Fe–EDTA increases ·OH generation is not clear, the fact that microsomal electron transfer is required for the stimulatory effect of Fe–EDTA suggests that reduction of Fe^{3+}–EDTA to Fe^{2+}–EDTA may be a necessary condition. It is possible that Fe–EDTA may catalyze a Fenton type (eq 8) or modified Haber–Weiss type (eq 5, 7, and 8) of reaction, i.e.

$$\text{NADPH} + \text{microsomes} + O_2 \rightarrow O_2^{-}\cdot \quad (5)$$

$$O_2^{-}\cdot + O_2^{-}\cdot \xrightarrow{2H^+} H_2O_2 + O_2 \quad (6)$$

$$Fe^{3+}\text{–EDTA} + O_2^{-}\cdot \rightarrow Fe^{2+}\text{–EDTA} + O_2 \quad (7)$$

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + \cdot OH + OH^{-} \quad (8)$$

$$\cdot OH + \text{ethanol (1-butanol)} \rightarrow \text{acetaldehyde (1-butyraldehyde)} + H_2O \quad (9)^2$$

We do not exclude other possible pathways for the reduction of Fe–EDTA, such as by reaction with reduced components of the microsomal electron transfer chain.

Since microsomes are contaminated with catalase, microsomal oxidation of ethanol in the absence of azide can be attributed, at least in part, to the peroxidatic activity of catalase (Thurman et al., 1972; Thurman, 1973; Isselbacher & Carter, 1973). An iron chelate, Fe–ADP, has been shown to double the rate of the microsomal oxidation of ethanol (Isselbacher & Carter, 1973). This increase was attributed to an increase in H_2O_2 generation since the activity of NADPH oxidase was also doubled. The results reported in the present paper indicate that the stimulation by Fe–EDTA cannot be described by a catalase-dependent mechanism for the following reasons: (1) Fe–EDTA stimulates microsomal ethanol oxidation in the presence of concentrations of azide which nearly totally inhibit catalase activity; (2) the simulation by Fe–EDTA is blocked by ·OH scavengers, such as Me_2SO and mannitol, which do not inhibit catalase-dependent oxidation of ethanol; (3) Fe–EDTA stimulates microsomal oxidation of 1-butanol (which is not an effective substrate for the peroxidatic activity of catalase), and the latter rate is inhibited by Me_2SO; (4) the accumulation of H_2O_2 in the presence of azide is decreased, not increased, by Fe–EDTA. These results are best interpreted by a mechanism involving ·OH and not catalase.

In the absence of azide, the stimulation by Fe–EDTA appeared to involve ·OH because it was nearly totally prevented by the ·OH scavengers Me_2SO and mannitol (Table III). However, as noted earlier, in the absence of azide, Me_2SO and mannitol had little effect on control rates of ethanol oxidation in the absence of Fe–EDTA. An explanation of the events that take place in the absence of azide can be found in the equations:

$$\text{microsomes} + \text{NADPH} \rightarrow O_2^{-}\cdot \rightarrow H_2O_2 \quad (10)$$

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} \quad (11)$$

In view of the rapid interaction of catalase with H_2O_2 to form the catalase compound **1**, there may be little utilization of H_2O_2 to form ·OH. However, in the presence of an iron catalyst (Fe–EDTA), H_2O_2 may be capable of generating ·OH as well as interacting with catalase:

$$Fe^{3+}\text{–EDTA} + O_2^{-}\cdot \rightarrow Fe^{2+}\text{–EDTA} + O_2 \quad (12)$$

$$\cdot OH \xleftarrow[(a)]{Fe^{2+}\text{–EDTA}} H_2O_2 \xrightarrow[(b)]{\text{catalase}} \text{compound } \mathbf{1} \quad (13)$$

Consequently, ·OH scavengers such as Me_2SO or mannitol inhibit ethanol oxidation in the presence of Fe–EDTA (path 13a) but not in the absence of Fe–EDTA (path 13b). A similar argument had been presented previously to explain the increased effectiveness of ·OH scavengers in blocking ethanol oxidation (in the absence of Fe–EDTA) after addition of azide to microsomes (Cederbaum et al., 1978).

These results indicate that the iron-catalyzed oxidation of alcohols is blocked by competing ·OH scavengers in much the same manner as the non-catalase alcohol oxidizing pathway in the absence of external iron. During microsomal electron transfer in the presence of Fe–EDTA, conditions may be appropriate for a Fenton type or modified Haber–Weiss type of reaction to occur. Iron chelates have previously been utilized in studies concerning lipid peroxidation (Fong, et al., 1973) and electron spin resonance detection of ·OH production by microsomes (Lai & Piette, 1977, 1978). The sensitivity of the iron-stimulated pathway, in part, to superoxide dismutase indicates that this pathway occurs, in part, in solution. The lack of inhibition by superoxide dismutase of the control microsomal pathway (absence of Fe–EDTA) may suggest that free radical intermediates, superoxide, ·OH, or equivalent species, are not free in solution but rather are bound. It remains to be seen whether or not the concept of a bound intermediate is consistent with results indicating that the microsomal alcohol-oxidizing pathway shows stereospecificity (Gang et al., 1973; Corrall et al., 1975). Since alcohols are metabolized by microsomes in the absence of Fe–EDTA and ·OH is generated during microsomal electron transfer in the absence of Fe–EDTA (Cohen & Cederbaum, 1979), the question can be asked: what is the endogenous catalyst present in microsomes? The total contaminating iron concentration of the solutions used in these experiments was less than 10^{-6} M, and, furthermore, chelex treatment of the H_2O and buffers did not change the results. A possible role for adventitious iron in the microsomes or catalysis by heme proteins such as cytochrome P-450 remain distinct possibilities. These experiments may be important in understanding the mechanism responsible for the increase in microsomal oxidation of alcohols after chronic ethanol consumption since a "unique" species of cytochrome P-450 appears to be induced after chronic ethanol consumption (Teschke et al., 1974; Ohnishi & Lieber, 1977).

[2] The initial product of the reaction of ethanol with ·OH is a hydroxyalkyl radical (CH_3·CHOH). Acetaldehyde can be formed subsequently by dismutation or by reactions of the free radical with a source of ferric ions (perhaps Fe^{3+}–EDTA, Fe^{3+}–cytochrome P-450, or some endogenous microsomal Fe^{3+}–chelate).

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