5. R. Snyderman and E. J. Goetzl, *Science* **213**, 830 (1981).
6. Programa del CSIC para el Estudio del Sindrome Toxico. Vol 1 and 2. Consejo Superior de Investigaciones Cientificas. Madrid (1983).
7. Simposium Nacional del Sindrome Toxico. Ministerio de Sanidad y Consumo. Madrid (1982).
8. F. Alonso, M. Sanchez Crespo and J. M. Mato, *Immunology* **45**, 493 (1982).
9. J. A. Salmon and R. J. Flower, *Methods in Enzymology* **86**, 477 (1982).
10. W. R. Mathews, J. Rokoch and R. C. Murphy, *Analyt. Biochem.* **118**, 96 (1981).
11. C. Casals, P. Garcia Barreno and A. M. Municio, *Biochem. J.* **212**, 339 (1983).
12. D. B. Wilson, S. M. Prescott and P. W. Majerus, *J. biol. Chem.* **257**, 3510 (1982).
13. P. Borgeat and B. Samuelsson, *J. biol. Chem.* **254**, 2148 (1979).

Biochemical Pharmacology, Vol. 33, No. 20, pp. 3306–3309, 1984.
Printed in Great Britain.

Increased microsomal oxidation of ethanol by cytochrome P-450 and hydroxyl radical-dependent pathways after chronic ethanol consumption*

(*Received* 16 *December* 1983; *accepted* 26 *April* 1984)

Isolated rat liver microsomes can oxidize a variety of alcohols to their corresponding aldehydes [1, 2]. Recent experiments have suggested that microsomes have the potential to oxidize alcohols by two pathways [3–5]. One pathway involves an interaction of ethanol with cytochrome P-450 and appears to be independent of a significant role for oxygen radicals such as the hydroxyl radical (·OH)† [3, 5]. In the presence of iron, e.g. iron–EDTA, microsomes generate ·OH [6], and alcohols can be oxidized via interaction with ·OH generated during microsomal electron transfer [3–7]. Chronic consumption of ethanol by rats results in a 2- to 3-fold increase in the activity of the microsomal alcohol-oxidizing system [8, 9]. The content of cytochrome P-450 is increased, and a distinct cytochrome P-450 isozyme is induced which proves to be more active than control cytochrome P-450 in catalyzing the oxidation of ethanol [10]. Similar results were reported recently for the rabbit liver system [11, 12]. Liver microsomes from ethanol-fed rats catalyze the oxidation of two typical ·OH scavengers, KTBA and DMSO, at rates which are 2- to 3-fold greater than rates found with control microsomes [13]. These results suggest that the production of ·OH by microsomes may be increased after chronic ethanol consumption, and that this increase may contribute to the increase in ethanol oxidation by microsomes.

The present studies were carried out to evaluate the relative roles of the cytochrome P-450 pathway and the OH·-dependent pathway in catalyzing the oxidation of ethanol by microsomes isolated from rats chronically fed alcohol, and from their pair-fed controls, and to investigate which of the pathways appears to be responsible for the increase in ethanol oxidation by microsomes from ethanol-fed rats.

Materials and methods

Male, Sprague–Dawley rats were fed for 4 weeks a nutritionally adequate liquid diet in which ethanol provided 36% of the total calories. Pair-fed littermates consumed the same diet except that carbohydrate isocalorically replaced ethanol [14]. Prior to the day of sacrifice, the rats received 2 aliquots of diets, one in the morning and one in the evening. Liver microsomes were prepared as previously described, washed once, and suspended in 125 mM KCl [7]. The oxidation of ethanol and DMSO was assayed as previously described [13]. The final concentration of ethanol was 50 mM while DMSO was present at either 33 or 100 mM. When indicated, 0.1 mM EDTA was added to the reaction system. The DMSO binding spectrum was determined by the method of Peterson *et al.* [15] using a Perkin–Elmer model 554-dual beam spectrophotometer. The concentration of DMSO was 140 mM, and microsomal protein was approximately 0.4 mg. The content of cytochrome P-450 [16] and the activity of NADPH-cytochrome *c* reductase [17] were determined by the indicated references.

All values refer to the mean ± standard error of the mean (S.E.M.). Statistical analyses were performed by either the paired or unpaired Student's *t*-test.

Results and discussion

Microsomal oxidation of ethanol. Microsomal oxidation of ethanol was doubled after chronic ethanol treatment (Table 1). Table 1 shows that, when EDTA was added, the rate of ethanol oxidation was increased 2-fold with both microsomal preparations. This increase probably reflects the chelation of adventitious iron in the microsomes to produce an iron–EDTA chelate, which catalyzes the production of ·OH [6]. However, the ethanol-oxidizing activity by microsomes from the ethanol-fed rats was still 2-fold greater than the rate for the pair-fed controls. Desferrioxamine, which blocked nearly completely the production of ·OH by microsomes from control chow-fed rats [3], produced 50% inhibition of the rate (in the presence of EDTA) of ethanol oxidation by both microsomal preparations (Table 1). The rate of ethanol oxidation in the presence of EDTA plus desferrioxamine was the same as the rate in the absence of any chelating agent (compare lines 1 and 3 of Table 1), suggesting that desferrioxamine blocks the increase of ethanol oxidation produced by EDTA.

With both microsomal preparations a significant rate of ethanol oxidation persisted in the presence of a concentration of desferrioxamine which nearly completely blocks the production of ·OH ([3]; see below). This desferrioxamine-insensitive cytochrome P-450-mediated rate of ethanol oxidation was nearly 2-fold greater by microsomes from ethanol-fed rats (Table 1). By subtracting the rate of ethanol oxidation in the presence of desferrioxamine (or in the absence of any chelating agent) from the total rate of ethanol oxidation in the presence of EDTA, the ·OH-dependent rate of ethanol oxidation could be calculated. This ·OH-dependent rate of ethanol oxidation, which appears to account for about one-half of the total rate

* This work was supported by USPHS Grants AA03312 and AA03508 (Alcohol Research Center) and Research Career Development Award 2K02-AA-00003 (A.I.C.) from the National Institute on Alcohol Abuse and Alcoholism.

† Abbreviations: ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; DMSO, dimethyl sulfoxide; and KTBA, 2-keto-4-thiomethylbutyric acid.

of ethanol oxidation by both microsomal preparations, increased 65% after chronic ethanol consumption (Table 1). Thus, the ·OH-independent and OH·-dependent rates of ethanol oxidation appeared to be increased after chronic ethanol consumption.

Microsomal oxidation of DMSO. Microsomes from chronic ethanol-fed rats catalyzed the oxidation of 33 mM DMSO to a greater extent than did microsomes from the pair-fed controls (Table 2). The rate of formaldehyde production from DMSO was increased slightly (16% for pair-fed controls; 33% for ethanol-treated) when the DMSO concentration was elevated from 33 to 100 mM. The oxidation of 100 mM DMSO by microsomes was nearly doubled after chronic ethanol consumption (Table 2). The increase in oxidation of DMSO was identical to the increase in the oxidation of ethanol by the ·OH-dependent pathway (about 65%).

Table 2 shows that the rate of DMSO oxidation by both microsomal preparations and at both concentrations of DMSO was decreased by 90% when either EDTA was omitted from the reaction system or when desferrioxamine was added. These results suggest that the increased oxidation of DMSO after chronic alcohol consumption was indeed due to the interaction of this substrate with ·OH and provide some support for the assumption discussed above that the production of ·OH by both microsomal preparations could be blocked by the addition of desferrioxamine or can be stimulated by the addition of EDTA.

Effect of various additions on ethanol oxidation. Scavengers of ·OH such as DMSO or benzoate resulted in an inhibition of ethanol oxidation by both microsomal preparations (Table 3A). EDTA was present at a concentration of 0.1 mM to promote the ·OH-dependent pathway. The extent of inhibition was about the same for both sets of microsomes, which is consistent with the observation that the ·OH-dependent pathway of ethanol oxidation, although of greater activity in the microsomes from the ethanol-fed rats, accounts for about the same percentage of the total ethanol-oxidizing activity of both microsomal preparations. Aniline has been shown to be a particularly good substrate for the ethanol-induced cytochrome P-450 isozyme [12, 18]. Aniline inhibited ethanol oxidation with both microsomal preparations, although it appeared to be somewhat more inhibitory with microsomes from ethanol-fed rats. Recent studies have shown that 2-butanol can be oxidized by ·OH-dependent and -independent pathways in microsomes, but it appears to be an especially effective substrate for the cytochrome P-450-dependent pathway [19]. Similar to results with aniline, 2-butanol inhibited the oxidation of ethanol by both microsomal preparations but appeared to be especially effective with the microsomes from the ethanol-fed rats (Table 3A).

The above experiments were repeated in the presence of

Table 1. NADPH-dependent oxidation of ethanol by microsomes from chronic ethanol-fed rats and pair-fed controls*

Reaction condition	Rate of acetaldehyde production (nmoles/min/mg microsomal protein) Pair-fed control	Chronic ethanol	Increase in acetaldehyde production
Control	7.6 ± 2.1	15.0 ± 2.1	7.4 ± 1.9†
+EDTA	16.7 ± 2.4	28.5 ± 3.7	11.8 ± 2.2‡
+EDTA+ desferrioxamine	8.3 ± 0.7	14.7 ± 2.0	6.4 ± 1.5†
·OH-dependent§	8.4 ± 2.1	13.8 ± 1.9	5.4 ± 1.1†

* The production of acetaldehyde from 50 mM ethanol was assayed as described in Materials and Methods. When present, the final concentrations of desferrioxamine and EDTA were 0.25 and 0.1 mM respectively. Results are from six experiments.

† $P < 0.01$.

‡ $P < 0.002$.

§ The ·OH-dependent rate was calculated by subtracting the rate in the presence of desferrioxamine from the rate found in the presence of EDTA.

Table 2. NADPH-dependent oxidation of DMSO by microsomes from chronic ethanol-fed rats and pair-fed controls*

Conc of DMSO (mM)	Reaction condition	Rate of formaldehyde production (nmoles/min/mg microsomal protein) Pair-fed control	Chronic ethanol
33	Control	0.1 ± 0	0.3 ± 0.1
	+ EDTA	3.1 ± 0.8	5.1 ± 1.0
	+EDTA+ desferrioxamine	0.2 ± 0.1	0.3 ± 0.1
100	Control	0.3 ± 0.1	0.5 ± 0.1
	+EDTA	3.6 ± 1.1	6.8 ± 0.3
	+EDTA+ desferrioxamine	0.4 ± 0.1	0.6 ± 0.1

* The production of formaldehyde from either 33 or 100 mM DMSO was assayed as described in Materials and Methods. When present, the final concentrations of desferrioxamine and EDTA were 0.25 and 0.10 mM respectively. Results are from six experiments at the 33 mM DMSO concentration and from three experiments at the 100 mM DMSO concentration.

Table 3. Effect of various additions on the oxidation of ethanol*

Addition	Rate† of acetaldehyde production and effect of addition			
	Pair-fed control		Chronic ethanol	
	Rate	Effect (%)	Rate	Effect (%)
(A) Control (6)	17.3 ± 2.8		28.5 ± 3.7	
DMSO, 33 mM	10.7 ± 1.1	−38	16.0 ± 2.0	−44
Aniline, 5 mM	13.4 ± 2.0	−23	18.5 ± 2.7	−35
Control (3)	20.5 ± 3.7		35.1 ± 3.7	
DMSO, 100 mM	8.7 ± 0.4	−58	14.2 ± 0.9	−60
Benzoate, 33 mM	12.9 ± 1.0	−37	24.9 ± 1.9	−29
2-Butanol, 33 mM	14.8 ± 0.1	−28	17.6 ± 2.2	−50
(B) Control (6)	8.6 ± 0.7		14.7 ± 2.0	
DMSO, 33 mM	7.6 ± 0.5	−12	12.3 ± 1.5	−16
Aniline, 5 mM	6.2 ± 0.3	−28	8.2 ± 0.6	−44
Control (3)	9.4 ± 0.9		17.5 ± 3.1	
DMSO, 100 mM	7.3 ± 0.5	−22	11.6 ± 0.9	−34
Benzoate, 33 mM	8.3 ± 0.3	−12	17.5 ± 1.6	0
2-Butanol, 33 mM	7-1 ± 0.0	−24	7.9 ± 0.8	−55
(C) Control (6)	8.8 ± 2.1		13.8 ± 1.9	
DMSO, 33 mM	3.1 ± 0.6	−65	3.7 ± 0.9	−73
Aniline, 5 mM	7.2 ± 1.8	−18	10.3 ± 2.3	−25
Control (3)	11.1 ± 2.9		17.6 ± 1.1	
DMSO, 100 mM	1.4 ± 0.4	−87	2.6 ± 0.4	−85
Benzoate, 33 mM	4.6 ± 1.4	−59	7.4 ± 0.6	−58
2-Butanol, 33 mM	7.2 ± 0.1	−35	10.0 ± 1.2	−43

* The production of acetaldehyde from 50 mM ethanol was assayed as described in Materials and Methods in the absence and presence of the various additions. Results are from either six or three experiments. Experiment A; total rate of ethanol oxidation in the presence of 0.1 mM EDTA. Experiment B: 0.25 mM desferrioxamine was added to yield the cytochrome P-450-dependent rate of ethanol oxidation. Experiment C: the ·OH-dependent rate of ethanol oxidation was obtained by subtracting the rate in the presence of 0.25 mM desferrioxamine (Experiment B) from the total rate (Experiment A) for each experiment.

† Rate refers to nmoles acetaldehyde produced per min per mg microsomal protein.

desferrioxamine to wipe out the ·OH-dependent pathway. The desferrioxamine-resistant rate of ethanol oxidation by both microsomal preparations was not affected by either 33 mM DMSO or benzoate, confirming a lack of a role for ·OH in ethanol oxidation in the presence of desferrioxamine (Table 3B). Some inhibition, which was more noticeable with the ethanol-treated microsomes, was found at 100 mM DMSO. Aniline and 2-butanol inhibited the desferrioxamine-resistant rate of ethanol oxidation, with a greater extent of inhibition being observed with microsomes from the ethanol-fed rats (Table 3B). The greater extent of inhibition of the cytochrome P-450-mediated oxidation of ethanol which occurs in the presence of desferrioxamine by aniline and 2-butanol with microsomes from the ethanol-fed rats may be due to these agents serving as particularly good substrates for the ethanol-inducible cytochrome P-450.

Table 3C shows the effects of the various additions on the ·OH-dependent rate of ethanol oxidation. These data were obtained by subtracting the results for each experiment shown in Table 3B from results in Table 3A. It is clear that the ·OH scavengers are very effective in inhibiting the ·OH-dependent oxidation of ethanol by both microsomal preparations.

DMSO binding spectrum. In agreement with the results of others [12, 20], DMSO produced a reversed type I binding spectrum with microsomes from the ethanol-fed rats (data not shown). No binding spectrum with microsomes from the pair-fed controls could be discerned. The ability of several other ·OH scavengers to produce binding spectra with microsomes was also studied. Neither benzoate (150 mM), mannitol (150 mM) nor *t*-butyl alcohol (180 mM) produced binding spectra with microsomes from ethanol-fed or pair-fed control rats (data not shown). Thus, the alcohol-inducible cytochrome P-450 of *rat liver* appears to display an interaction with DMSO which is not shared with other ·OH scavenging agents.

Content of cytochrome P-450 and activity of NADPH-cytochrome c reductase. To determine if the increase in ethanol oxidation correlates with changes in the microsomal electron transfer system, the content of cytochrome P-450 and the activity of NADPH-cytochrome *c* reductase were measured. The content of cytochrome P-450 (nmoles/mg protein) was 0.754 ± 0.037 for microsomes from pair-fed controls and 1.180 ± 0.096 for microsomes from the ethanol-fed rats (+57%, N = 10). The activity of NADPH–cytochrome *c* reductase (nmoles cytochrome *c* reduced/min/mg microsomal protein) was 152 ± 29 for pair-fed controls and 182 ± 13 for microsomes from ethanol-fed rats (+20%, N = 5).

In summary, based upon experiments with EDTA and desferrioxamine, and the effects of competitive ·OH scavengers, aniline and 2-butanol, it appears that both the ·OH-dependent and cytochrome P-450-dependent pathways were increased in microsomes from ethanol-fed rats and that each pathway seemed to account for about one-half of the total oxidizing activity of the microsomes. The increase in the ·OH-dependent pathway of ethanol oxidation was about the same as the increase in the oxidation of DMSO. When the cytochrome P-450-dependent rate of ethanol oxidation is expressed as a turnover number (nmoles acetaldehyde/min/nmole cytochrome P-450), rates are 10 for the pair-fed control, and 12.7 for chronic ethanol. Thus, the increase in content, as well as the enhanced activity, contributed to the increase in the rate of ethanol oxidation, although the former appears to have made the larger contribution. This probably reflects the possibility that even in the induced microsomes, the ethanol-preferring cytochrome P-450 isozyme(s) accounted for only a small proportion of the total P-450 population. The reductase is an important loci for the production of ·OH [4, 5]; however, the 20% increase in reductase activity does not appear to account fully for the 65% increase in the ·OH-dependent pathway of ethanol oxidation. The increase in content of P-450 is identical to the increase in production of ·OH. Since H_2O_2 is the precursor of ·OH, the increased content of P-450 (and, perhaps, to a lesser extent, of reductase) could result in an increased production of H_2O_2 and, subsequently, of ·OH. Indeed, the rate of microsomal production of H_2O_2 is increased after chronic ethanol consumption [21, 22]. The oxidation of ethanol by ·OH requires the presence of iron or, actually, an appropriate iron–chelate, e.g. iron–EDTA. It is of interest that there are reports that the iron content of the liver cell is increased after chronic ethanol consumption [23, 24]. Perhaps changes in the production of H_2O_2 or the content of iron may contribute to the increase in the ·OH-dependent pathway of ethanol oxidation.

The ability of DMSO to produce a reversed type I spectral change with microsomes from ethanol-treated rats [12, 20] was confirmed. However, no spectral change was noted with other classical ·OH scavengers, and no significant oxidation of DMSO by a cytochrome P-450, ·OH-independent, pathway was found. These results suggest that, although DMSO does interact with rat liver ethanol-induced microsomes to yield a spectrum, it is not metabolized significantly, at least to formaldehyde*. Perhaps the interaction may be due to solvent-like, hydrophobic interactions between this particular cytochrome P-450 isozyme and DMSO.

* We also did not observe any production of either methane or ethane from DMSO in the presence of desferrioxamine.

† This work is in partial fulfillment of the requirements of the degree of Doctor of Philosophy from the City University of New York.

‡ Send correspondence to: Dr. A. I. Cederbaum, Department of Biochemistry, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

Departments of Biochemistry and Medicine and Alcohol Research and Treatment Center Mount Sinai School of Medicine and Bronx V.A. Medical Center New York, NY, U.S.A.

GRACIELA KRIKUN†
CHARLES S. LIEBER
ARTHUR I. CEDERBAUM‡

REFERENCES

1. R. Teschke, Y. Hasumura and C. S. Lieber, *J. biol. Chem.* **250**, 7397 (1975).
2. R. Teschke, Y. Hasumura and C. S. Lieber, *Archs Biochem. Biophys.* **163**, 404 (1974).
3. A. I. Cederbaum and E. Dicker, *Biochem. J.* **210**, 107 (1983).
4. G. W. Winston and A. I. Cederbaum, *J. biol. Chem.* **258**, 1508 (1983).
5. G. W. Winston and A. I. Cederbaum, *J. biol. Chem.* **258**, 1514 (1983).
6. D. E. Feierman and A. I. Cederbaum, *Biochem. biophys. Res. Commun.* **116**, 765 (1983).
7. A. I. Cederbaum, E. Dicker and G. Cohen, *Biochemistry* **17**, 3058 (1978).
8. C. S. Lieber and L. M. DeCarli, *J. biol. Chem.* **245**, 2505 (1970).
9. C. S. Lieber and L. M. DeCarli, *Science* **162**, 917 (1968).
10. K. Ohnishi and C. S. Lieber, *J. biol. Chem.* **252**, 7124 (1977).
11. D. R. Koop, E. T. Morgan, G. E. Tarr and M. J. Coon, *J. biol. Chem.* **257**, 8472 (1982).
12. E. T. Morgan, D. R. Koop and M. J. Coon, *J. biol. Chem.* **257**, 13951 (1982).
13. S. M. Klein, G. Cohen, C. S. Lieber and A. I. Cederbaum, *Archs Biochem. Biophys.* **223**, 425 (1983).
14. C. S. Lieber and L. M. DeCarli, *Amer. J. clin. Nutr.* **23**, 474 (1970).
15. J. A. Peterson, Y. Ishimura, J. Baron and R. W. Estabrook, in *Oxidases and Related Redox Systems* (Eds. T. E. King, H. S. Mason and M. Morrison), Vol. 2, p. 565. University Park Press, Baltimore (1971).
16. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
17. H. W. Strobel and J. D. Dignam, in *Methods in Enzymology* (Eds. S. Fleisher and L. Packer), II, Part C, pp. 89–96. Academic Press, New York (1978).
18. E. Rubin, P. Bacchin, H. Gang and C. S. Lieber, *Lab. Invest.* **22**, 569 (1970).
19. G. Krikun and A. I. Cederbaum, *Biochemistry*, in press.
20. E. T. Morgan, M. Devine and P. Skett, *Biochem. Pharmac.* **30**, 595 (1981).
21. C. S. Lieber and L. M. DeCarli, *Science* **170**, 78 (1970).
22. R. G. Thurman, *Molec. Pharmac.* **9**, 670 (1973).
23. R. W. Chapman, M. Y. Morgan, R. Bell and S. Sherlock, *Gastroenterology* **84**, 143 (1983).
24. J. Prieto, M. Barry and S. Sherlock, *Gastroenterology* **68**, 525 (1975).