Biochemical Pharmacology, Vol. 33, No. 24, pp. 3963–3969, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

ASCORBIC ACID AND ALCOHOL OXIDATION*

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(*Received* 20 *January* 1984; *accepted* 30 *April* 1984)

Abstract—Methanol and ethanol were rapidly metabolized to formaldehyde and acetaldehyde in the presence of ascorbate, 1,10-phenanthroline and either guinea pig hepatic 100,000 *g* supernatant or 12,000 *g* pellet fractions. The specific activity of methanol oxidation was 1720 nmoles formaldehyde formed/min/mg protein in the 100,000 *g* fraction and 790 in the 12,000 *g* pellet fraction. The specific activity of ethanol oxidation was 1590 nmoles acetaldehyde formed/min/mg protein in the 100,000 *g* fraction and 820 in the 12,000 *g* pellet fraction. The activity was enzymatic in that it was linear with time, proportional to protein concentration, and sensitive to temperature. Catalase appeared to be the enzymatic component responsible for the oxidation. In this ascorbate-dependent alcohol oxidation system, oxygen was consumed and H_2O_2 was formed. When purified catalase and ascorbate were used, complex I was detected and methanol was oxidized.

There are three principle enzymatic pathways capable of oxidizing methanol or ethanol to their respective aldehydes [1]. These include cytosolic alcohol dehydrogenase (ADH, EC 1.1.1.1) which utilizes NAD^+, cytosolic and peroxisomal catalase (EC 1.11.1.6) which utilizes hydrogen peroxide, and the more recently described microsomal ethanol-oxidizing system (MEOS) which requires NADPH and O_2.

The effect of ascorbate on alcohol metabolism has been of interest and has been studied in several laboratories [2–4]. Yunice and Lindeman [2] found that blood ethanol concentrations are significantly lower 1 hr after an intraperitoneal injection of ethanol in rats pretreated with 440 mg ascorbate/kg body weight. Tephly and coworkers [3] reported that ascorbate promotes methanol oxidation in mammalian erythrocytes. Pawan [4], however, found that 200 mg of vitamin C, given to male volunteers daily for 10 days prior to a single dose of ethanol, had no effect on the rate of ethanol metabolism.

The present *in vitro* study is concerned with the ability of ascorbate to promote methanol and ethanol oxidation in guinea pig hepatic subcellular fractions. It describes and characterizes the enzymatic system which utilizes the vitamin in the conversion of the alcohols to their aldehydes.

MATERIALS AND METHODS

Reagents. Sodium ascorbate, NAD^+ and NADPH were purchased from the Sigma Chemical Co. St. Louis, MO. 1,10-Phenanthroline and hydrogen peroxide were purchased from the Fisher Scientific Co., Fair Lawn, NJ. Sodium azide was purchased from Eastman Kodak, Rochester, NY. 3-Amino-1,2,4-triazole was purchased from the Aldrich Chemical Co., Milwaukee, WI. Purified beef liver catalase, 65,000 units/mg, was purchased from Boehringer Mannheim, Indianapolis, IN, and glucose oxidase, 1000 oxidase units/g, was purchased from the Nutritional Biochemicals Corp., Cleveland, OH. 95% Ethanol was used to make all ethanol solutions. Other chemicals used were of reagent grade or better.

Isolation of subcellular fractions. Male Hartley guinea pigs (250–350 g) were decapitated, and their livers were removed, placed on ice, and homogenized after mincing in 3 vol. of cold 1 mM sodium phosphate buffer, pH 7.4, with 0.25 M sucrose. A Teflon homogenizer was used to make the homogenate. The homogenate was diluted 1:10 with buffer and filtered through a double layer of surgical gauze. Aliquots were centrifuged at 700 *g* for 10 min. The supernatant fraction was centrifuged at 12,000 *g* for 20 min, and the resulting supernatant centrifuged at 100,000 *g* for 60 min. The 12,000 *g* pellet, and the 100,000 *g* pellet used as the microsomal fraction, were made forty times concentrated based on the initial supernatant volume and contained 16–20 mg protein/ml. The 12,000 *g* and microsomal pellets were washed one time prior to use. In all of the experiments reported, the age of the animals, the preparation of tissue fractions and the length of storage of the preparations were identical except for the preparations used in Fig. 2. Protein was determined by the method of Lowry *et al.* [5] using bovine serum albumin as the standard.

Enzymatic assays. For the ascorbate-dependent alcohol oxidation, methanol or ethanol was incubated in the presence of a guinea pig hepatic tissue fraction, ascorbate and 1,10-phenanthroline at a pH of 8.5. The total volume was 1.0 ml in a 25-ml flask.

* This work was supported by Grant 23007 from Hoffman-La Roche, Inc., Nutley, NJ. Portions of this work were presented at a meeting of the Federation of American Societies for Experimental Biology, Chicago, IL, April, 1983 [R. L. Susick, Jr. and V. G. Zannoni, *Fedn Proc.* **42**, 1370 (1983)].

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In the case of ethanol, sealed flasks were used. Incubations were carried out in a Dubnoff metabolic shaker at 60 oscillations/min for 5 and 10 min. The alcohol was added to initiate the reaction. The reaction was terminated by the addition of 0.2 ml trichloroacetic acid (TCA) containing 32 mM thiourea. In all experiments, controls were run with boiled protein and the nonenzymatic component was subtracted. At most, the controls were 10% of the enzymatically catalyzed oxidation. Formaldehyde was detected by the colorimetric method of Nash [6]. Acetaldehyde was detected by a modified gas chromatographic method [7]. After the reaction with ethanol was terminated, 0.4 ml of 1.5 mM isopropyl alcohol was injected into the flasks as an internal standard. Each flask was preheated to 60° for 15 min, after which time 5 cc of head space gas was injected into a Varion model 3700 gas chromatograph equipped with a flame ionization detector. The column dimensions were 2 m × 4 mm i.d. and packed with Porapak Q purchased from the Anspec Co. The column temperature was 180°; the detector and injector temperatures were 200°. The carrier gas flow rate was 17 ml/min. Standard curves were plotted as the peak height ratios of acetaldehyde/isopropyl alcohol. The method was sensitive to 10 nmoles of acetaldehyde. ADH was assayed under the conditions of the ascorbate-dependent alcohol oxidation except that glycine buffer, 0.1 M, pH 9.5, and NAD^+ were used. MEOS was also assayed under the conditions of the ascorbate-dependent alcohol oxidation except that sodium phosphate buffer, 0.1 M, pH 7.5, NADPH and guinea pig hepatic microsomes were used. In both cases, acetaldehyde and formaldehyde were measured as described above. Catalase activity was determined by ultraviolet spectrometry according to the method of Chance and Maehly [8]. The disappearance of H_2O_2 (26 mM) was measured at 240 nm following the addition of 7.5 μg of either 100,000 *g* hepatic supernatant fraction or 12,000 *g* pellet protein. These protein samples were given prior exposure to sonication, to rupture the peroxisomes, in order to ensure maximum catalase activity. The activity is reported as the velocity constant with units of sec^{-1}.

H_2O_2 detection. Hydrogen peroxide was detected with a YSI-Clark 2510 oxidase probe and a YSI model 25 oxidase meter. To minimize interfering effects of ascorbate or other reducing agents, an ultrafine cellulose acetate filter membrane was prepared and placed between the probe and the more porous collagen membrane. The cellulose acetate membrane was prepared by pouring a 4% cellulose acetate resin (Eastman Kodak 394–60) in cyclohexanone over still distilled water. Catalase was added to all incubations to ensure that H_2O_2 had formed.

O_2 consumption. Oxygen consumption was detected with a YSI 5331 oxygen probe and a Gilson K-IC oxygraph.

Catalase complex I. A dual-beam, scanning, Varion/Cary 219 spectrophotometer was utilized to detect catalase–H_2O_2 complex I, the catalytically active form of catalase [9, 10]. The loss of absorbance at 405 nm was used as an index of complex I according to the method of Chance [11]. The contribution of complex II, an inactive species [10, 12], absorbing at 435 nm was subtracted from the change in optical density at 405 nm.

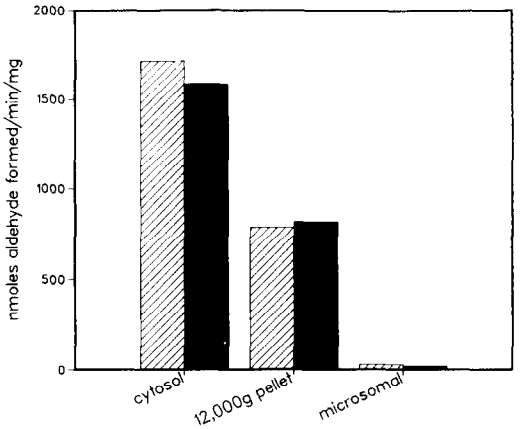

Fig. 1. Subcellular distribution of ascorbate-dependent alcohol oxidation activity. Assay conditions: Tris–HCl buffer, 0.1 M, + 1,10-phenanthroline, 10 mM, pH 8.5; ascorbate, 4 mM; guinea pig hepatic fraction, 15 μg protein; methanol or ethanol, 800 mM; total volume, 1.0 ml, 37°. Key: methanol oxidation, nmoles formaldehyde formed/min/mg protein (▨); ethanol oxidation, nmoles acetaldehyde formed/min/mg (■).

RESULTS

Methanol and ethanol were rapidly metabolized to formaldehyde and acetaldehyde, respectively, in the presence of ascorbate, 1,10-phenanthroline and either guinea pig hepatic 100,000 *g* supernatant or 12,000 *g* fractions (Fig. 1). There was no significant difference in the rates of oxidation of methanol and ethanol. The activity was enzymatic in that the rate of oxidation with each substrate was linear with time, proportional to protein concentration and sensitive to temperature. With each substrate the cytosolic fraction contained the most activity while the microsomal fraction had the least activity.

The apparent K_m for methanol and ethanol in the cytosolic compartment was 0.2 M for methanol and 1.6 M for ethanol. The corresponding maximum velocities were 1910 nmoles formaldehyde/min/mg protein for methanol oxidation and 2321 nmoles acetaldehyde/min/mg protein for ethanol oxidation. The apparent affinity for ascorbate was 1.3×10^{-3} M with either substrate.

The specific activity of the ascorbate-dependent alcohol oxidation was compared to alcohol dehydrogenase and the microsomal ethanol-oxidizing system (Table 1). The ascorbate-dependent oxidation was 280 times more active than MEOS for both alcohols and 140 and 570 times more active than ADH for ethanol and methanol respectively. Subsequent experiments to characterize the ascorbate system were carried out with methanol as the substrate. This was the preferred substrate since the detection of its oxidation could be assayed by a rapid, sensitive, and readily reproducible method. Studies

Table 1. Activities of alcohol dehydrogenase, microsomal ethanol-oxidizing system and the ascorbate-dependent alcohol-oxidizing system with methanol and ethanol*

Substrate	Specific activity† (nmoles CH_2O or CH_3CHO/min/mg protein) Alcohol dehydrogenase	Microsomal ethanol-oxidizing system	Ascorbate-dependent alcohol-oxidizing system
Methanol	3 ± 0.6	6 ± 1	1720 ± 200
Ethanol	11 ± 1.0	6 ± 1	1570 ± 140

* Assay conditions: alcohol dehydrogenase: glycine buffer, 0.1 M, pH 9.5; NAD^+, 1 mg/ml; guinea pig hepatic 10^5g supernatant fraction, 1.0 mg protein; substrate, 12 mM; total volume, 1.0 ml, 37°. Microsomal ethanol-oxidizing system: sodium phosphate buffer, 0.1 M, pH 7.5; NADPH, 2 mM; guinea pig hepatic microsomes, 1.0 mg protein; substrate, 200 mM; total volume, 1.0 ml, 37°. Ascorbate-dependent alcohol-oxidizing system: Tris–HCl buffer, 0.1 M, + 10 mM 1,10-phenanthroline, pH 8.5; ascorbate, 4 mM; guinea pig hepatic 10^5g supernatant fraction, 15 μg protein; substrate, 800 mM; total volume, 1.0 ml, 37°.

† Values are means of at least three experiments.

also indicated that there was no difference in the subcellular distribution, maximum activity, or nature of the enzymatic reaction for either methanol or ethanol.

The specificity for ascorbate was determined and the results are given in Table 2. D-Isoascorbate was as effective as the vitamin; dehydroascorbate, the vitamin's oxidized form, and ascorbyl palmitate, its lipophilic form, were 41 and 32% as effective. Ascorbyl-2-SO_4 which lacks the reducing capability of the vitamin, and gulonolactone, the precursor in the synthesis of ascorbate, were both ineffective. Reduced 2,6-dichlorophenolindophenol, a compound which has the same redox potential as ascorbate, was 41% as active as the vitamin. Cysteine, glutathione and vitamin E had no effect. In addition, there was no detectable activity when 1,10-phenanthroline was incubated without ascorbate.

Table 2. Specificity for ascorbate in methanol oxidation*

	Specific activity (nmoles CH_2O/min/mg protein)
Ascorbate	936
D-Isoascorbate	886
Dehydroascorbate	385
Ascorbyl palmitate	296
2,6-Dichlorophenolindophenol (reduced)	388
Ascorbyl-2-SO_4	ND†
Gulonolactone	ND
Cysteine	ND
Glutathione	ND
Vitamin E	ND

* Assay conditions: Tris–HCl buffer, 0.1 M, + 1,10-phenanthroline, 10 mM pH 8.5; compound tested, 2 mM; methanol, 200 mM; guinea pig hepatic 10^5g supernatant fractions, 15 μg protein; total volume, 1.0 ml, 37°. There was no detectable activity when 1,10-phenanthroline was incubated in the absence of ascorbate or compounds that can replace ascorbate. The data presented in the table represent a typical experiment.

† Not detected, less than 15 nmoles CH_2O/min/mg.

Various chelators were substituted for 1,10-phenanthroline (Table 3). Although the chelating agents were not necessary for methanol oxidation, a number of the chelators caused a 3- to 4-fold increase in activity. Dimercaprol, 8-hydroxyquinoline, α,α'-dipyridyl and bathocuproine could replace 1,10-phenanthroline. On the other hand, diethyldithiocarbamate had no effect, and EDTA completely inhibited the enzymatic oxidation.

Since ascorbate is capable of undergoing autooxidation to form H_2O_2 [13], and the latter can be utilized by catalase to oxidize alcohols [14], it was of interest to determine the role of catalase, if any, in the ascorbate-dependent oxidation. The effects of catalase inhibitors such as sodium azide and 3-amino-1,2,4-triazole, therefore, were examined on the ascorbate-dependent alcohol oxidation (Table 4). Methanol was incubated with hepatic 100,000 *g* supernatant fraction the 12,000 *g* pellet fraction or purified catalase with either ascorbate or a hydrogen peroxide generating system. Methanol was oxidized to formaldehyde in all cases and the reaction was essentially completely inhibited by sodium azide. 3-Amino-1,2,4-triazole also inhibited the oxidation, up to 82%. The inhibition of methanol oxidation utilizing the hydrogen peroxide generating system was found to parallel that observed with ascorbate. In addition, a positive correlation existed between methanol oxidation in the presence of the hydrogen peroxide generating system and that obtained with ascorbate in a number of hepatic cytosolic samples with different specific activities (Fig. 2). The specific activities varied because these samples were prepared from animals of different weights (200–800 g), different ages (1–8 months), and were stored at −20° for various period of time (1 day to 6 months). In view of the above findings, the subcellular distribution of the ascorbate-dependent alcohol oxidation and catalase activity, as measured by H_2O_2 reduction, between the hepatic 100,000 *g* supernatant and 12,000 *g* pellet fractions was examined. The activity of methanol oxidation in the 100,000 *g* supernatant fraction was 1711 nmoles/min/mg and in the 12,000 *g* pellet fraction it was 690 nmoles/min/mg. The ratio of alcohol-oxidizing activity in the

Table 3. Methanol oxidation with various chelators in place of 1,10-phenanthroline*

	Specific activity (nmoles CH_2O/min/mg protein)
No chelator	88
1,10-Phenanthroline	344
Dimercaprol	354
Hydroxyquinoline	302
α,α'-Dipyridyl	258
Bathocuproine	253
Diethyldithiocarbamate	80
EDTA	ND†

* Assay conditions: Tris–HCl buffer, 0.1 M, + chelator, 1 mM, pH 8.5; ascorbate, 2 mM; guinea pig hepatic $10^5 g$ supernatant fraction, 15 μg protein; methanol, 200 mM; total volume, 1.0 ml, 37°.
† Not detected.

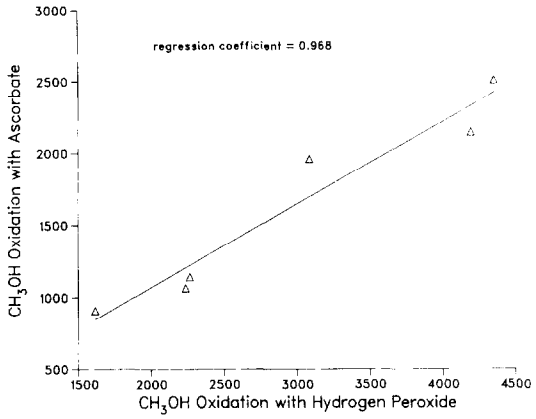

Fig. 2. Methanol oxidation, in samples of guinea pig hepatic 100,000 g supernatant fraction with ascorbate versus an H_2O_2 generating system. Units of activity: nmoles formaldehyde/min/mg protein. Assay conditions: Tris–HCl buffer, 0.1 M, + 1,10-phenanthroline, 10 mM, pH 8.5; ascorbate, 4 mM, or an H_2O_2 generating system, 5.4 mg glucose + 0.1 mg glucose oxidase; guinea pig hepatic 100,000 g supernatant fractions, 15 μg protein; methanol, 800 mM; total volume, 1.0 ml. 37°.

supernatant fraction to that in the 12,000 g pellet fraction is 2.5. The same ratio of distribution existed for catalase activity between these two compartments. Catalase activity in the 100,000 g supernatant fraction was 1.2×10^{-3} sec^{-1} and in the 12,000 g pellet fraction it was 5.3×10^{-4} sec^{-1}. The ratio of activity is 2.3.

Concomitant with these findings, a comparison was made of oxygen consumption, hydrogen peroxide production and the extent of methanol oxidation utilizing either ascorbate or a hydrogen peroxide generating system (Table 5). Hydrogen peroxide production was regulated to be rate limiting and was essentially equivalent under all conditions. As can be seen in Table 5, methanol oxidation was also comparable except under the condition of ascorbate in the absence of 1,10-phenanthroline which did not enzymatically oxidize methanol. Furthermore, oxygen consumption coincided with H_2O_2 production with the generating system. More oxygen, however, was consumed with the ascorbate system than can be accounted for by its incorporation into hydrogen peroxide.

Table 4. Inhibition of the ascorbate-dependent alcohol oxidation*

Assay condition	Inhibitor	Specific activity (nmoles CH_2O/min/mg)	Inhibition (%)
Ascorbate			
$10^5 g$ Supernatant	No inhibitor	1,484	
	Sodium azide (2 mM)	ND†	100
	3-Amino-1,2,4-triazole (100 mM)	547	63
12,000 g Pellet	No inhibitor	760	
	Sodium azide	ND	100
	3-Amino-1,2,4-triazole	258	66
Purified catalase	No inhibitor	45,203	
	Sodium azide	ND	100
	3-Amino-1.2.4-triazole	12,804	72
H_2O_2 generating system			
$10^5 g$ Supernatant	No. inhibitor	867	
	Sodium azide	89	90
	3-Amino-1,2,4-triazole	328	62
12,000 g Pellet	No inhibitor	634	
	Sodium azide	68	89
	3-Amino-1,2,4-triazole	144	77
Purified catalase	No inhibitor	30,009	
	Sodium azide	1,944	94
	3-Amino-1,2,4-triazole	5,468	82

* Assay conditions: Tris–HCl buffer, 0.1 M, + 1,10-phenanthroline, 10 mM, pH 8.5; ascorbate, 4 mM, or H_2O_2 generating system, 5.4 mg glucose + 0.1 mg glucose oxidase; guinea pig hepatic fraction, 15 μg protein, or purified beef liver catalase, 0.4 μg protein; methanol, 800 mM total volume, 1.0 ml, 37°.
† Not detected.

Table 5. Comparison of methanol oxidation, H_2O_2 production, and O_2 consumption with an H_2O_2 generating system and the ascorbate oxidation system*

Assay conditions	Methanol oxidation (nmoles/min/mg)	H_2O_2 production (nmoles/min)	O_2 consumption (nmoles/min)
H_2O_2 generating system			
without 1,10-phenanthroline	218 ± 24 (3)	5.0 ± 1.2 (4)	4.4 ± 0.3 (6)
with 1,10-phenanthroline	223 ± 14 (3)	3.9 ± 0.9 (3)	4.3 ± 0.6 (5)
Ascorbate			
without 1,10-phenanthroline	ND† (3)	5.8 ± 1.9 (4)	10.3 ± 3.4 (7)
with 1,10-phenanthroline	247 ± 22 (3)	6.9 ± 0.4 (4)	23.9 ± 1.0 (6)

* Assay conditions: Methanol oxidation: Tris–HCl buffer, 0.1 M, + 1,10-phenanthroline, 10 mM, pH 8.5; ascorbate, 1 mM, or H_2O_2 generating system, 30 mM glucose + 0.02 mg glucose oxidase; guinea pig hepatic $10^5 g$ supernatant fraction, 15 μg protein; methanol, 800 mM; total volume, 1.0 ml, 25°. H_2O_2 production: Tris–HCl buffer, 0.1 M, + 1,10-phenanthroline, 10 mM, pH 8.5; ascorbate, 1 mM, or H_2O_2 generating system, 30 mM glucose + 0.04 mg glucose oxidase; boiled guinea pig hepatic $10^5 g$ supernatant fraction, 30 μg protein; total volume, 2.0 ml, 25°. O_2 consumption: Tris–HCl buffer, 0.1 M, + 1,10-phenanthroline, 10 mM, pH 8.5; ascorbate, 1 mM, or H_2O_2 generating system, 30 mM glucose + 0.03 mg glucose oxidase; boiled guinea pig hepatic $10^5 g$ supernatant fraction, 22.5 μg protein; total volume, 1.5 ml, 25°. Data are means ± S.D. Numbers in parentheses represent number of experiments.

† Not detectable.

It is known that the active oxidizing species of catalase and H_2O_2 is catalase–H_2O_2 complex I [10, 12]. The formation of complex I using purified catalase in the presence of a hydrogen peroxide generating system or ascorbate was ascertained (Table 6). Complex I was detected under each condition and its quantity reduced after the addition of methanol. As can be seen in Table 6, the rate of methanol oxidation was again comparable under all conditions except for ascorbate in the absence of 1,10-phenanthroline which did not enzymatically oxidize methanol. Although ascorbate in the absence of 1,10-phenanthroline was able to produce complex I, and this complex I was reduced in quantity by addition of methanol, no enzymatic oxidation of methanol occurred.

DISCUSSION

An enzymatic system utilizing ascorbate and guinea pig hepatic 100,000 *g* supernatant or 12,000 *g* pellet fractions to oxidize methanol or ethanol has been described. The addition of 1,10-phenanthroline was necessary for maximum activity. The ascorbate-dependent alcohol oxidation is at least 140 times more active than either the NAD^+ alcohol dehydrogenase or the cytochrome P-450 microsomal ethanol-oxidizing system (Table 1). Although the ascorbate system has limited affinity for the alcohols, the maximum velocity of the reactions is extremely high and this may, in fact, compensate for the low substrate affinity.

There is specificity for ascorbate in that other reducing agents such as glutathione, cysteine and vitamin E were without effect (Table 2). Reduced 2,6-dichlorophenolindophenol which has the same redox potential as ascorbate could replace the vitamin. Reducing capability is necessary in that gulonolactone and ascorbyl-2-SO_4, both of which lack a reducing ene-diol moiety, were ineffective. Furthermore, analogs of ascorbate which contain the ene-diol component, such as D-isoascorbate and

Table 6. Comparison of methanol oxidation and catalase complex I formation with an H_2O_2 generating system and the ascorbate oxidation system*

Assay condition	Methanol oxidation (nmoles/min/mg)	Complex I Before MEOH [$\Delta\varepsilon$ (cm^{-1} mM^{-1})]	Complex I After MEOH [$\Delta\varepsilon$ (cm^{-1} mM^{-1})]
H_2O_2 generating system			
without 1,10-phenanthroline	230 ± 44 (3)	26 ± 10 (4)	ND† (4)
with 1,10-phenanthroline	254 ± 53 (3)	69 ± 22 (3)	ND (3)
Ascorbate			
without 1,10-phenanthroline	ND (3)	62 ± 10 (4)	19 ± 16 (4)
with 1,10-phenanthroline	363 ± 40 (3)	67 ± 21 (4)	43 ± 27 (4)

* Assay conditions: Tris–HCl buffer, 0.1 M, + 1,10-phenanthroline, 10 mM, pH 8.5; ascorbate, 1 mM, or H_2O_2 generating system, 30 mM glucose + 0.02 mg glucose oxidase; purified beef liver catalase, 15 μg; methanol, 800 mM; total volume, 1.0 ml, 25°. Data represent means ± S.D. Numbers in parentheses represent number of experiments.

† Not detectable.

ascorbyl palmitate, could replace the vitamin. The activity obtained with dehydroascorbate is probably due to its reduction to ascorbate. Evidence for this was obtained in that ascorbate was formed when dehydroascorbate was incubated under our assay conditions. This is in keeping with the possibility that catalytic amounts of ascorbate may be effective in the oxidation of alcohols *in vivo* since it can be regenerated from dehydroascorbate after its oxidation.

The data presented indicate that catalase was the enzyme involved in the ascorbate-dependent alcohol oxidation. For example, the oxidation was sensitive to sodium azide and 3-amino-1,2,4-triazole; purified catalase could be substituted for the hepatic fractions; the degree of methanol oxidation with a hydrogen peroxide generating system paralleled that with ascorbate in various hepatic cytosolic samples; and the specific activity of the ascorbate-dependent alcohol oxidation had the same subcellular compartmental distribution as catalase activity.

The most likely mechanism by which ascorbate promotes the enzymatic oxidation of alcohols in the presence of 1,10-phenanthroline involves the participation of catalase and H_2O_2. In this mechanism, ascorbate is oxidized with the subsequent formation of H_2O_2 which is then utilized by catalase. We have obtained evidence for this by comparing the ascorbate oxidizing system with a hydrogen peroxide generating system and demonstrating that comparable amounts of H_2O_2 and complex I are produced as well as the reduction of complex I upon the addition of methanol (Tables 5 and 6). Importantly, the extent of methanol oxidation was also comparable between the two systems (Table 5). The major difference is that 1,10-phenanthroline was necessary for maximum activity with the ascorbate system while having no effect with the hydrogen peroxide generating system. Furthermore, oxygen consumption was two times greater with ascorbate in the absence of 1,10-phenanthroline and five times greater in the presence of 1,10-phenanthroline compared to oxygen consumption with the hydrogen peroxide generating system.

As an explanation for the limited degree of methanol oxidation with ascorbate in the absence of 1,10-phenanthroline is that the vitamin may be inhibiting catalase. In keeping with this, Orr [15] has shown that ascorbate can inhibit catalase and that the degree of inhibition is increased by catalytic amounts of Cu^{2+}. Orr postulated that ascorbate and Cu^{2+} interact to generate free radicals which are detrimental to the protein. The presence of 1,10-phenanthroline may prevent the inhibition by forming an "inactive" chelate and thus blocking free radical formation. The inhibition of methanol oxidation we observed with EDTA (Table 4) could be due to the formation of hydroxyl radicals, believed to occur when EDTA, ascorbate and iron interact [16]. An additional means by which 1,10-phenanthroline is enhancing alcohol oxidation could lie in its ability to increase oxygen uptake in the presence of ascorbate (Table 5). Others have also demonstrated that 1,10-phenanthroline can increase the autooxidation of the vitamin [17]. This increased oxygen uptake may result in the formation of an alternative "oxidant", perhaps a peroxide derivative of ascorbate, which could be utilized by catalase to oxidize the alcohols. The existence of such an organic peroxide has been postulated by Udenfriend *et al.* and Sippel [19].

The possiblity that the ascorbate-dependent alcohol-oxidizing system could be involved in the *in vivo* metabolism of alcohol warrants investigation. The contribution of catalase in ethanol oxidation has been primarily discounted because ethanol oxidation is virtually unaffected *in vivo* by a fairly specific inhibitor of catalase, 3-amino-1,2,4-triazole [20]. Furthermore, *in vivo* hydrogen peroxide generation (0.05 to 0.10 μmole/g liver/min) is considered too low to allow catalase to make a contribution [21]. More recent studies, however, by Thurman *et al.* [9] with perfused rat liver indicate that, as ethanol concentration is increased, an increasing contribution of the catalase–H_2O_2 system is observed so that at 80 mM ethanol, ethanol metabolism is approximately 50% due to alcohol dehydrogenase and 50% catalase dependent. In addition, stimulation of H_2O_2 production with substrates for peroxisomal flavoproteins such as glycolate, urate and D-amino acids markedly activates ethanol oxidation in perfused kidney and liver [22]. In that ascorbate can generate H_2O_2 and effectively oxidize alcohol via catalase *in vitro*, and that it is a relatively safe, natural, dietary substance, it will be of interest to examine the contribution it can make to the *in vivo* elimination of this most abused drug.

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