Biochemical Pharmacology, Vol. 35, No. 4, pp. 637–644, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

ASCORBIC ACID DEFICIENCY AND THE FLAVIN-CONTAINING MONOOXYGENASE*

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(*Received* 20 *March* 1985; *accepted* 19 *July* 1985)

Abstract—Activity of the flavin-containing monooxygenase (FMO) was reduced significantly in ascorbic acid deficient guinea pigs. Reduction in oxidation of dimethylaniline (DMA) and of thiobenzamide was associated with a decrease in the activity of the FMO. In both ascorbate supplemented and deficient guinea pig hepatic 12,000 *g* supernatant fractions, SKF-525A and *n*-octylamine did not inhibit DMA N-oxidation. Phenobarbital pretreatment did not increase the rate of N-oxidation of DMA. In addition, hepatic supernatant fractions thermally treated at 50° were unable to N-oxidize DMA, but 80% of the cytochrome P-450 activity was retained. Also, N-oxidation of DMA was reduced by 53% at pH 7.0, while oxidation of cytochrome P-450 specific substrates was inhibited by only 19%. Kinetic studies of DMA N-oxidation indicate no significant change in the apparent K_m in ascorbate supplemented or deficient animals. The *in vitro* addition of ascorbic acid had no effect on the activity of the FMO. The toxicological implications of the reduction in FMO activity in ascorbic acid deficiency are discussed.

The flavin-containing monooxygenase (FMO; EC 1.14.13.8) catalyzes the oxidation of a large variety of nitrogen- and sulfur-containing drugs and environmental chemicals [1–4]. For the majority of compounds, oxidation leads to less toxic derivatives but, depending on the functional group and substituents near the functional group, oxidation may also result in the formation of reactive intermediates which could lead to toxicological events [5, 6]. In addition, cysteamine is a physiological substrate which is converted to cystamine by the FMO, suggesting a role for this electron transport system in generating disulfide bonds during protein synthesis [7]. The FMO has been localized in the microsomal membrane and the nuclear envelope [8, 9]. The enzyme is present in most mammalian species, the highest quantity in the liver, though it is also present in the kidney and lungs [10].

The cytochrome P-450 electron transport system also oxidizes amine and sulfur compounds [11]. The quantities of the major components of the cytochrome P-450 system are reduced markedly in ascorbic acid deficiency, from 50% for cytochrome P-450 to 85% for cytochrome P-450 reductase [12, 13]. This results in a significant decrease in drug oxidative reactions such as aniline hydroxylation, aminopyrine N-demethylation, *p*-nitroanisole O-demethylation [14, 15] as well as steroid hydroxylation [16].

There is an overlap among substrates for the FMO and cytochrome P-450 system. The FMO catalyzes oxidation of only the nitrogen and sulfur moieties of xenobiotics, whereas the cytochrome P-450 system can also oxidize the carbon substituent [17]. The metabolic products of the substrates will depend on the relative amounts of these two enzymes and the affinity of the compound for each system.

The present study examines the effect of ascorbic acid deficiency on the oxidation of dimethylaniline (DMA) and thiobenzamide by the FMO. The effect of ascorbic acid deficiency on the FMO was differentiated from the cytochrome P-450 system by a variety of physical-chemical measures.

MATERIALS AND METHODS

Materials. Sodium ascorbate, NADPH, NADH, and $NADP^+$ were purchased from the Sigma Chemical Co. (St. Louis, MO). Dimethylaniline and formaldehyde were purchased from the Fisher Scientific Co. (Fair Lawn, NJ). Aniline, thiobenzamide and *n*-octylamine were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Aminopyrine was purchased from K & K Laboratories, Inc. (Plainview, NY). Dithionite and ferricyanide were purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ). *p*-Nitroanisole, *p*-nitrophenol and *p*-aminophenol were purchased from Eastman Kodak (Rochester, NY). Ascorbic acid deficient diet (guinea pig pelleted) was obtained from Nutritional Biochemicals (Cleveland, OH). Male Hartley guinea pigs were purchased from the Michigan Department of Public Health (Lansing, MI). All chemicals used were of reagent grade or better.

Guinea pigs. Male Hartley guinea pigs (200–300 g) were pair fed on an ascorbic acid deficient diet for 16–19 days; one group received 1 mg/ml of ascorbic acid in their drinking water daily and the other group received no ascorbic acid in their drinking water. When the animals were killed, the average body weight of the ascorbic acid deficient group was 342 ± 41 g and 382 ± 48 g for the ascorbic acid sup-

* This work was supported in part, by Grant 23007 from Hoffmann-La Roche, Inc., Nutley, NJ.

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plemented group. Guinea pigs on the ascorbic acid deficient regimen had minimal joint hemorrhages, were not frankly scorbutic, and there were no significant differences in their rate of weight gain during the experimental period, compared with animals on the supplemented diet. Phenobarbital-treated guinea pigs were injected with 50 mg/kg phenobarbital in saline i.p. once a day for 4 days prior to being killed.

Preparation of guinea pig tissues. Animals were decapitated and exsanguinated, and their livers were quickly removed and placed on ice. All of the following procedures were carried out at 4°. Homogenates (20%, w/v) were prepared in 0.25 M sucrose and 50 mM sodium phosphate buffer, pH 7.4, with a Potter–Elvehjem glass homogenizer. The homogenates were centrifuged at 1000 *g* for 15 min. The 12,000 *g* fraction was prepared by centrifugation for 30 min. To harvest microsomes the 12,000 *g* supernatant fraction was centrifuged for 60 min at 100,000 *g*. The microsomes were made four times concentrated based on the initial supernatant volume. The 12,000 *g* supernatant fraction and 100,000 *g* microsomal pellet were stored at −20°; the activity remained stable for at least 1 year. Homogenates (20%, w/v) of lungs and kidneys were prepared as described above. The whole homogenates were stored at −20° for no more than 15 days. The 12,000 *g* supernatant fraction was prepared on the day of use according to the procedure given above.

Ascorbic acid and protein determinations. Ascorbic acid concentration was determined by the method of Zannoni *et al.* [18]. Protein was determined by the method of Lowry *et al.* [19], with bovine serum albumin as the standard.

Dimethylaniline N-oxidation. Dimethylaniline N-oxidation was determined by the method of Ziegler and Pettit [8]. Incubations were carried out at 37° in a Dubnoff metabolic shaker using capped 25-ml Erlenmeyer flasks. Standard assay conditions included 25 mM sodium phosphate and 0.1 M glycine buffer, pH 8.4, 3 mM *n*-octylamine, 0.5 mM NADPH, 2 mM dimethylaniline and 9 mg of 12,000 *g* hepatic supernatant protein. The reaction was initiated with the addition of substrate. Aliquots were withdrawn at 3-min intervals; the reaction was terminated with 0.9 M trichloroacetic acid giving a final concentration of 0.3 M. Aliquots were removed, made basic, and extracted three times with peroxide free ether; the aqueous phase was acidified with trichloroacetic acid followed by sodium nitrate to develop the chromagen. A molar extinction coefficient of 8.2 $mM^{-1}\,cm^{-1}$ at 420 nm was used.

Dimethylaniline N-demethylation. Dimethylaniline N-demethylation was determined at 37° by following the formation of formaldehyde using the method of Nash [20]. The standard assay conditions included 0.1 M sodium phosphate buffer, pH 7.4, 0.5 mM NADPH, 2 mM DMA and 2.5 mg of 100,000 *g* microsomal protein. A molar extinction coefficient of 6.9 $mM^{-1}\,cm^{-1}$ at 412 nm was used.

Thiobenzamide S-oxidation. Thiobenzamide S-oxidation was measured as described by Cashman and Hanzlik [21]. Incubations were monitored at 370 nm in a Cary 219 dual beam spectrophotometer at 37°. Standard assay conditions included 25 mM sodium phosphate, 0.1 M glycine buffer, pH 8.4, 1.5 mM *n*-octylamine, an NADPH-generating system and 3 mg of 12,000 *g* hepatic supernatant protein; the final volume was 1 ml. The NADPH-generating system consisted of 0.25 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate and 0.2 units/ml glucose-6-phosphate dehydrogenase. The reactions were initiated by the addition of 5 μl of thiobenzamide, 1.0 mM, in acetonitrile to the sample cuvette and 5 μl of acetonitrile to the reference cuvette. Thiobenzamide *S*-oxide had a molar extinction coefficient of 2930 $M^{-1}\,cm^{-1}$ at 370 nm.

Cytochrome P-450. Cytochrome P-450 was determined in 12,000 *g* hepatic supernatant fractions from the reduced carbon monoxide difference spectrum according to the method of Omura and Sato [22]. The standard assay conditions included 0.1 ml (1 mg) sodium dithionite solution added only to the experimental cuvette (stock solution, 30 mg of sodium dithionite plus 15 mg of sodium bicarbonate in 3.0 ml of H_2O), 9 mg of 12,000 *g* hepatic supernatant protein, 0.1 mM EDTA and 0.05 M sodium phosphate buffer, pH 7.4, in a total volume of 1 ml [23]. A molar extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ was used.

Cytochrome b_5. The quantity of microsomal cytochrome b_5 was determined from the NADH difference spectrum according to the method of Omura and Sato [22]. The standard assay conditions included 0.4 mM NADH, 3.2 mg of 100,000 *g* microsomal protein, 0.1 mM EDTA and 0.05 M sodium phosphate buffer, pH 7.4. A molar extinction coefficient of 185 $mM^{-1}\,cm^{-1}$ was used.

NADH cytochrome b_5 *reductase.* Microsomal NADH cytochrome b_5 reductase activity was determined as described by Muhara and Sato [24]. The standard assay conditions included 0.1 mM NADH, 1 mM potassium ferricyanide, 0.024 mg of 100,000 *g* microsomal protein and 0.1 M potassium phosphate buffer, pH 7.5, at 27°. A molar extinction coefficient of 1.02 $mM^{-1}\,cm^{-1}$ at 420 nm was used.

p-*Nitroanisole O-demethylation. p*-Nitroanisole O-demethylation in hepatic 12,000 *g* supernatant fraction was determined spectrophotometrically at 415 nm using the modified method of Zannoni [25]. The reaction was started by the addition of 1.7 mM *p*-nitroanisole to 0.25 mM NADPH, 9 mg of 12,000 *g* supernatant protein, and 0.1 M sodium phosphate buffer, pH 7.4, at 37°. A molar extinction coefficient for *p*-nitrophenol of 11.4 $mM^{-1}\,cm^{-1}$ was used at pH 7.4 and 16 $mM^{-1}\,cm^{-1}$ for assays conducted above the pH of 8.4.

Aminopyrine N-demethylation. Aminopyrine N-demethylation was determined at 37° by following the formation of formaldehyde by the method of Nash [20]. The standard assay conditions included 2 mM aminopyrine, 0.5 mM NADPH, 3 mg of 100,000 *g* microsomal protein and 0.1 M sodium phosphate buffer, pH 7.4. A molar extinction coefficient of 6.9 $mM^{-1}\,cm^{-1}$ at 412 nm was used.

Aniline hydroxylation. Aniline hydroxylation was determined by measuring the quantity of *p*-aminophenol formed which, when complexed with phenol, absorbs light at 640 nm [26]. The standard assay conditions included 1.6 mM aniline, 0.5 mM NADPH, 6 mg of 12,000 *g* hepatic supernatant fraction protein and 0.1 M sodium phosphate buffer,

Table 1. Effect of ascorbic acid deficiency on hepatic transport systems

Condition	Dimethylaniline N-oxidation (nmoles/min/mg protein)	Cytochrome P-450 (nmoles/100 mg protein)	Cytochrome b_5 (nmoles/100 mg protein)	Cytochrome b_5 reductase (nmoles/min/μg protein)
Deficient diet plus ascorbate	1.20 ± 0.60 (20)	9.3 ± 3.4 (20)	25.0 ± 5.6 (7)	0.97 ± 0.18 (7)
Deficient diet	0.66 ± 0.30* (20)	4.7 ± 1.6* (20)	22.0 ± 4.6 (7)	1.0 ± 0.15 (7)

Assays were carried out as described under Materials and Methods. Dimethylaniline and cytochrome P-450 were assayed in the 12,000 *g* supernatant fraction and cytochrome b_5 and cytochrome b_5 reductase were assayed in the 100,000 *g* microsomal pellet. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver weight and 1.8 ± 1.3 mg in the deficient animals. The numbers in parentheses equal the number of animals, and the data are means ± S.D.

* $P < 0.001$.

pH 7.4, at 37°. A molar extinction coefficient of 25 mM^{-1} cm^{-1} was used.

Statistical analysis. Data were subjected to statistical analysis using a two-sided Student's *t*-test.

RESULTS

As can be observed in Table 1, guinea pigs maintained on an ascorbic acid free diet exhibited a significant decrease (45%) in the hepatic N-oxidation of dimethylaniline, a relatively specific substrate for the flavin-containing monooxygenase [1, 17]. As previously established, the specific quantity of cytochrome P-450 was found to be reduced on the order of 50%. In contrast, the specific quantity of cytochrome b_5, another heme protein, and the specific activity of cytochrome b_5 reductase were found to be unaffected by ascorbic acid deficiency.

The N-oxidation of DMA was stimulated more than 2-fold by 3 mM *n*-octylamine in both normal and ascorbic acid deficient hepatic supernatant preparations (Fig. 1A). In contrast, the oxidation of aniline, a cytochrome P-450 substrate, was inhibited at least 90% (Fig. 1B). In addition, 1 mM SKF-525A, another cytochrome P-450 system inhibitor, inhibited *p*-nitroanisole (*p*-NA) oxidation by at least 60%, but did not affect significantly the N-oxidation of DMA.

The effect of 3 mM *n*-octylamine on the oxidation of aminopyrine, aniline and dimethylaniline in hepatic microsomes was also determined (data not shown). In keeping with the resuts found with the hepatic 12,000 *g* supernatant fractions, the presence of *n*-octylamine inhibited the oxidation of aminopyrine and aniline by at least 90%. The metabolism of DMA to the N-oxide, via the FMO, was stimulated 1.5- to 3-fold while the formation of formaldehyde, via carbon oxidation by cytochrome P-450, was inhibited by 65%.

Phenobarbital pretreatment of guinea pigs increased the specific quantity of cytochrome P-450 and enhanced hydroxylation of aniline in both ascorbate supplemented and deficient animals, whereas N-oxidation of DMA was not enhanced in the presence or absence of *n*-octylamine (Table 2). In fact, in ascorbate supplemented animals the specific activity of DMA N-oxidation was reduced by phenobarbital pretreatment to the level in the deficient

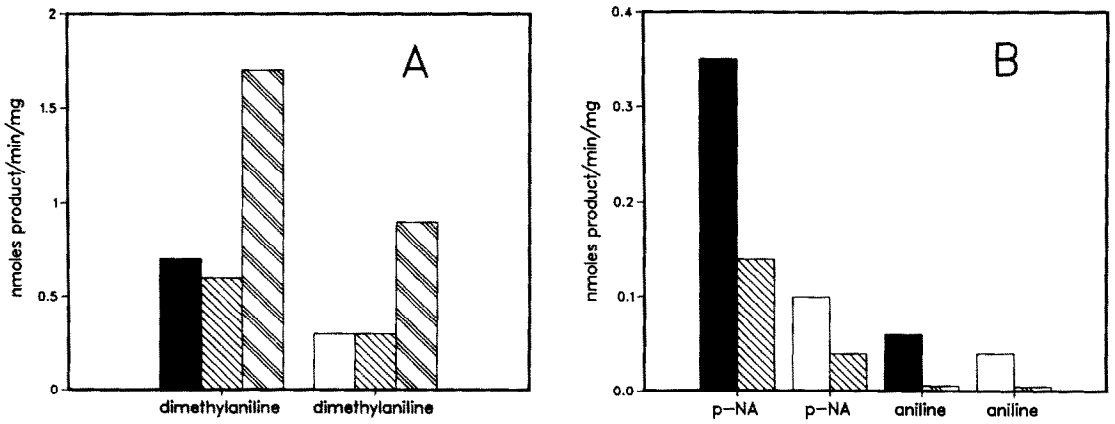

Fig. 1. Effect of cytochrome P-450-MFO inhibitors. Assays were carried out as described in Materials and Methods. Activity equals nmoles product/min/mg 12,000 *g* hepatic supernatant fraction. For DMA the N-oxide product was determined. *p*-Nitroanisole equals *p*-NA. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver and 1.8 ± 1.3 mg in the deficient animals. The data presented in the figure represent a typical experiment. Key: (■) plus ascorbate diet; (□) no ascorbate diet; (▨) SKF-525A, 1 mM; and (▨) *n*-octylamine, 3 mM.

Table 2. Effect of phenobarbital on the P-450-MFO and FMO

Treatment	Cytochrome P-450 (nmoles/100 mg protein)	Aniline (nmoles/min/mg protein)	Dimethylaniline (nmoles N-oxide/min/mg protein) +*n*-Octylamine	−*n*-Octylamine
Deficient diet plus ascorbate				
No phenobarbital	9.3 ± 3.4 (20)	0.048 ± 0.013 (7)	1.20 ± 0.60 (20)	0.66 ± 0.17 (4)
With phenobarbital	15.8 ± 3.2 (9)	0.109 ± 0.031 (9)	0.67 ± 0.24 (9)	0.34 ± 0.06 (4)
Deficient diet				
No phenobarbital	4.7 ± 1.6 (20)	0.026 ± 0.009 (7)	0.66 ± 0.30 (20)	0.27 ± 0.10 (4)
With phenobarbital	9.8 ± 1.4 (8)	0.056 ± 0.017 (8)	0.66 ± 0.17 (8)	0.32 ± 0.06 (4)

Assays were carried out as described under Materials and Methods in 12,000 *g* hepatic supernatant fraction. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver weight and 1.8 ± 1.3 mg in the deficient animals. The numbers in parentheses equal the number of animals, and the data are means ± S.D.

Table 3. Hepatic thiobenzamide S-oxidation

Condition	Activity (nmole product/min/mg protein) With *n*-octylamine	Without *n*-octylamine
No treatment		
Deficient diet plus ascorbate	1.13 ± 0.42 (6)	0.55 ± 0.17 (6)
Deficient diet	0.58 ± 0.36* (6)	0.28 ± 0.10* (6)
Phenobarbital treatment		
Deficient diet plus ascorbate	0.80 ± 0.14 (6)	0.74 ± 0.09 (6)
Deficient diet	0.74 ± 0.24 (6)	0.54 ± 0.15 (6)

Assays were carried out at 37° as described under Materials and Methods in 12,000 *g* hepatic supernatant fraction. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver weight and 1.8 ± 1.3 mg in the deficient animals. The numbers in parentheses equal the number of animals, and the data are means ± S.D.

* $P < 0.05$.

Table 4. Thermal inactivation of the cytochrome P-450 system and FMO

Condition	Cytochrome P-450 (nmoles/100 mg)	Aniline (nmoles/min/mg)	Dimethylaniline (nmoles N-oxide/min/mg)
Deficient diet plus ascorbate			
No treatment	10.4 ± 2.8 (3)	0.048 ± 0.009 (3)	1.41 ± 0.79 (3)
50°	10.0 ± 3.9 (3)	0.039 ± 0.009 (3)	ND*
Deficient diet			
No treatment	5.4 ± 1.7 (3)	0.026 ± 0.009 (3)	0.46 ± 0.10 (3)
50°	4.7 ± 0.8 (3)	0.022 ± 0.004 (3)	ND*

Assays were carried out in hepatic 12,000 *g* supernatant fraction as described under Materials and Methods. Both the thermally treated and untreated supernatant fractions contained 0.2 mM butylated hydroxytoluene which stabilizes the cytochrome P-450 system during thermal treatment [27]. The supernatant fraction was heated to 50° for 3.5 min. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver weight and 1.8 ± 1.3 mg in the deficient animals. The numbers in parentheses equal the number of animals, and the data are means ± S.D.

* Non-detectable; limit of assay equals <0.02 nmole/min/mg protein.

Table 5. pH–Activity profiles

	Cytochrome P-450 system pH 7.0	pH 7.4	pH 9.4
	p-Nitroanisole (nmoles/min/mg protein)		
Deficient diet plus ascorbate	0.26 ± 0.09 (3)	0.32 ± 0.13 (3)	0.14 ± 0.09 (3)
Deficient diet	0.10 ± 0.02 (3)	0.11 ± 0.03 (3)	0.05 ± 0.01 (3)
	FMO system pH 7.0	pH 8.4	9.4
	Dimethylaniline (nmoles N-oxide/min/mg protein)		
Deficient diet plus ascorbate	0.38 ± 0.13 (3)	0.81 ± 0.18 (3)	0.66 ± 0.07 (3)
Deficient diet	0.21 ± 0.02 (3)	0.45 ± 0.18 (3)	0.37 ± 0.25 (3)

Assays were carried out as described under Materials and Methods in hepatic 12,000 *g* supernatant fraction. The incubation was carried out in 0.1 M sodium phosphate buffer at pH 7.0 and 0.2 M Tris buffer at pH 9.4. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver weight and 1.8 ± 1.3 mg in the deficient animals. The numbers in parentheses equal the number of animals, and the data are means ± S.D.

guinea pigs. The enhancement of DMA N-oxidation, found with *n*-octylamine, was maintained in both the ascorbate supplemented and deficient phenobarbital-induced guinea pigs.

The effect of ascorbic acid deficiency as well as phenobarbital pretreatment on the metabolism of another FMO substrate, thiobenzamide, was determined in the presence and absence of 1.5 mM *n*-octylamine (Table 3). Consistent with the decrease found in the N-oxidation of DMA, the S-oxidation of this substrate was reduced by 47% in the ascorbic acid deficient guinea pigs; in addition, there was a 2-fold stimulation in the presence of *n*-octylamine. In the ascorbic acid supplemented phenobarbital induced guinea pigs, the S-oxidation of thiobenzamide in the presence of *n*-octylamine approached the level of the deficient animals. This is similar to the findings with DMA N-oxidation (Table 2). In contrast to DMA N-oxidation, the activation by *n*-octylamine was not observed in the phenobarbital induced animals.

The specific quantity of cytochrome P-450 and the oxidation of aniline were reduced by, at most, 18% (which is not statistically significant) when normal and ascorbic acid deficient hepatic 12,000 *g* supernatant fractions were thermally treated at 50° for 3.5 min (Table 4). This is in marked contrast to the effect on DMA N-oxidation, which was inhibited over 95% after thermal treatment.

The pH–activity profiles for the cytochrome P-450 substrate, *p*-nitroanisole, and the FMO substrate, DMA, were measured in 12,000 *g* hepatic super-

Table 6. Hepatic compartmental distribution of DMA N-oxidation and cytochrome P-450

Fraction	Dimethylaniline (nmoles N-oxide/min/mg protein) Deficient diet plus ascorbate	Deficient diet	% Decrease	Cytochrome P-450 (nmoles/100 mg protein) Deficient diet plus ascorbate	Deficient diet	% Decrease
Whole homogenate	1.8	0.65	64			
1,000 *g* Pellet	2.5	0.88	65	17	17	0
12,000 *g* Supernatant	1.4	0.41	70	14	7	51
12,000 *g* Pellet	4.0	2.8	30	39	30	23
Microsomal pellet	9.8	3.2	67	75	35	53

Assays were carried out as described under Materials and Methods. Pellets prior to use were washed with the initial volume of buffer (0.25 M sucrose and 50 mM sodium phosphate, pH 7.4), homogenized, and centrifuged at the appropriate speed. The supernatant fraction was decanted, and the pellet was brought up to the original volume. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver weight and 1.8 ± 1.3 mg in the deficient animals. The quantity of cytochrome P-450 cannot be determined in the whole homogenate. The data presented in the figure represent a typical experiment.

Table 7. DMA N-oxidation in extrahepatic tissues

Fraction	Activity (nmoles N-oxide/min/mg protein)	
	Deficient diet plus ascorbate	Deficient diet
Lung		
Whole homogenate	1.05 ± 0.22 (3)	0.96 ± 0.21 (3)
12,000 *g* Supernatant	0.43 ± 0.19 (3)	0.39 ± 0.09 (3)
Kidney		
Whole homogenate	0.35 ± 0.06 (3)	0.33 ± 0.06 (3)
12,000 *g* Supernatant	0.21 ± 0.07 (3)	0.16 ± 0.03 (3)

Assays were carried out at 37° as described under Materials and Methods. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver and 1.8 ± 1.3 mg in the deficient animals. The numbers in parentheses equal the number of animals, and the data are means ± S.D.

natant fractions (Table 5). At pH 9.4 the oxidation of *p*-nitroanisole was decreased by 56%, whereas the N-oxidation of DMA was decreased by only 18% when compared to their optimal pH activity at pH 7.4 and 8.4 respectively. This is in contrast to the effect at pH 7.0, where the oxidation of *p*-nitroanisole was decreased by 19% while DMA N-oxidation was reduced by 53%. The effect of pH was consistent whether the supernatant fraction was isolated from ascorbate supplemented or deficient guinea pigs.

The decrease in hepatic DMA N-oxidation found in the ascorbic acid deficient guinea pig 12,000 *g* supernatant fractions was maintained in the whole homogenate, the 1000 *g* pellet, the 12,000 *g* pellet, and the microsomal pellet (Table 6). In contrast to the decrease in N-oxidation of DMA in the 1000 *g* pellet, the specific quantity of cytochrome P-450 was not affected.

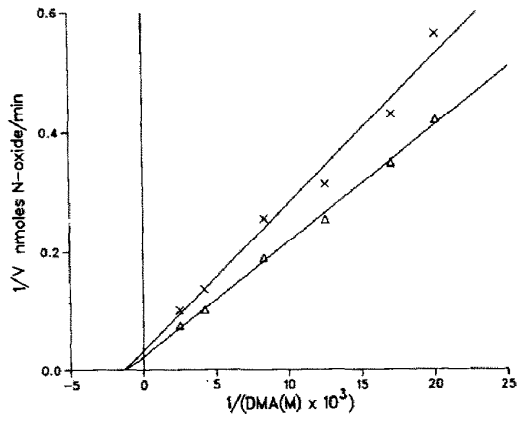

Fig. 2. Lineweaver–Burk plot of DMA N-oxidation. Each data point was determined individually with 12,000 *g* hepatic supernatant fraction, 3 mM *n*-octylamine, 0.1 M glycine, 25 mM sodium phosphate buffer, pH 8.4, incubated at 37° for 25 and 50 min. The quantity of DMA present is indicated in the figure. The average hepatic level of ascorbic acid in supplemented animals was 29 ± 13 mg/100 g wet liver and 1.8 ± 1.3 mg in the deficient animals. The data presented in the figure represent the mean of four individual experiments. Key: (△) plus ascorbate diet; and (×) no ascorbate diet.

The N-oxidation of DMA in the kidney and lung was not affected significantly by ascorbic acid deficiency (Table 7). The specific activity of DMA N-oxidation in ascorbate supplemented guinea pig lung 12,000 *g* supernatant fraction was 36% of the hepatic activity, while the deficient guinea pig lung supernatant fraction was 60% of the hepatic activity (Table 1).

The apparent Michaelis–Menten affinity constant (K_m) for DMA N-oxidation was 8.2×10^{-4} vs 7.4×10^{-4} M at pH 8.2 in the hepatic 12,000 *g* supernatant fraction of the ascorbate supplemented and deficient guinea pigs respectively (Fig. 2). Furthermore, ascorbic acid (1 or 5 mM) added, *in vitro*, had no effect on the oxidation of DMA or thiobenzamide. In addition, when 12,000 *g* hepatic supernatant fractions from ascorbate supplemented and ascorbic acid deficient guinea pigs were combined, the resulting specific activity of DMA N-oxidation was additive.

DISCUSSION

The participation of ascorbic acid in drug metabolism is relatively selective, in that it affects specific enzyme systems, and is not a general membrane effect. The N-oxidation of dimethylaniline (DMA), a substrate of the flavin-monooxygenase (FMO), is reduced significantly in ascorbic acid deficiency. The cytochrome P-450 monooxygenase electron transport system is also jeopardized, but only the quantity of three specific hepatic microsomal cytochrome P-450 polypeptide hemoproteins are decreased [28]. In addition, the present study indicates that the specific quantity of cytochrome b_5, another membrane heme protein, and the specific activity of cytochrome b_5 reductase were not affected by ascorbic acid deficiency (Table 1).

The observed decrease in oxidation by the FMO in ascorbic acid deficiency was differentiated from

the decrease in the cytochrome P-450 system by the use of specific inhibitors, phenobarbital pretreatment, thermal treatment, and pH–activity profiles. SKF-525A, a potent inhibitor of the cytochrome P-450 system, did not significantly affect the N-oxidation of DMA. *n*-Octylamine, another inhibitor of the cytochrome P-450 system increased the rate of N-oxidation of DMA. In keeping with this, *n*-octylamine has been reported previously to activate the FMO in hog, hamster and guinea pig [29]. It acts, presumably, at a regulatory site distinct from the catalytic site [30]. The decrease in DMA N-oxidation found in ascorbic acid deficiency was maintained in the presence of either SKF-525A or *n*-octylamine.

Phenobarbital pretreatment did not induce DMA N-oxidation and, in fact, DMA N-oxidation in the ascorbic acid supplemented guinea pigs was reduced to the level of the deficient animals. On the other hand, the cytochrome P-450 system was induced on the order of 2-fold in both the ascorbic acid supplemented and deficient guinea pigs. Phenobarbital pretreatment has been shown previously to reduce significantly the specific content of the hepatic FMO, and this effect has been explained as due, in part, to an increase in the quantity of other non-FMO endoplasmic reticulum proteins such as cytochrome P-450 [10]. However, since the reduction in specific activity occurred only in the ascorbate supplemented guinea pigs, and the induction of cytochrome P-450 occurred in both the ascorbate supplemented and deficient animals, it is difficult to attribute the effect of phenobarbital to this.

Since the FMO is highly sensitive to thermal inactivation [27], it was possible to selectively denature the FMO without inactivating cytochrome P-450. N-oxidation of DMA was not detected after thermal treatment of both ascorbic acid supplemented and deficient hepatic preparations. In contrast, the specific quantity of cytochrome P-450 was unaffected and the oxidation of cytochrome P-450 substrates was reduced by less than 20%. The reduction in N-oxidation of DMA in ascorbate deficiency via the FMO was further clarified by using pH–activity profiles. The N-oxidation of DMA was reduced by 53% at pH 7.0 in both ascorbate supplemented and deficient preparations, while the rate of oxidation of cytochrome P-450 substrates was not significantly affected at this pH.

The sulfoxidation of thiobenzamide, another substrate for the FMO, was also reduced in ascorbic acid deficiency. However, compared to the N-oxidation of DMA, it was a less specific substrate in that it is S-oxidized also by the cytochrome P-450 system [31]. In ascorbate supplemented phenobarbital-pretreated guinea pigs, as was found with the N-oxidation of DMA, the oxidation of thiobenzamide in the presence of *n*-octylamine was reduced to the level of the deficient animals. The activation of metabolism by *n*-octylamine, however, was not found with thiobenzamide in the phenobarbital-treated guinea pigs. This may be due to the induction of the cytochrome P-450 system which contributes to the oxidation of this substrate in the absence of *n*-octylamine.

Compartmental distribution of the N-oxidation of DMA indicated that it was diminished in the hepatic 1000 *g* pellet isolated from the ascorbate deficient guinea pigs, whereas the quantity of cytochrome P-450 was unaffected (Table 6). This would result in an imbalance in the relative amounts of cytochrome P-450 and FMO in the deficient animals, with possibly toxicological consequences. For example, the *in vivo* administration of corticosteroids or vitamin E depletion causes an increase in N-oxygenation of aromatic amines accompanied by a decrease in their N-demethylation, via cytochrome P-450, which results in an enhanced tumor-inducing capacity of the amines [32]. In addition, Chiele and Malvaldi [6] have demonstrated that *in vivo* liver necrosis which results from the metabolism of thioacetamide is inhibited by preadministration of methimazole, an inhibitor of the FMO. Furthermore, an alteration in the liver to lung FMO activity ratio occurs in ascorbic acid deficiency (Tables 1 and 7). This alteration may enhance the susceptibility of deficient guinea pigs to pulmonary toxic compounds that are metabolized by the FMO, such as α-naphthylthiourea [33]. The fact that ascorbic acid does not affect FMO in lung and kidney may reflect differences in the structures of the enzyme in these two tissues. In keeping with this, Williams *et al.* [34] have purified rabbit lung FMO and found it to be immunochemically and catalytically distinct from the liver enzyme.

Our investigation of the effect of ascorbic acid on FMO indicates that there is no significant difference in the apparent Michaelis–Menten affinity constant for DMA N-oxidation. Furthermore, the addition of ascorbic acid *in vitro* had no effect on the oxidation of DMA or thiobenzamide. Also, when hepatic supernatant fractions from ascorbic acid supplemented guinea pigs are combined with deficient guinea pig fractions, the resultant N-oxidation of DMA is additive. It has been suggested that the FMO enzyme exists as aggregates of several monomeric units and that the active enzyme is an octamer which is in equilibrium with tetrameric forms [1, 35]. One possible explanation for the decrease in FMO activity found in ascorbic acid deficiency is that an alteration occurs in the equilibrium between monomeric forms, which could lead to decreased FMO activity. Another is that the FMO can be controlled by levels of steroids [36], and it is known that ascorbic acid deficiency leads to an alteration in steroid metabolism [16]. However, the exact biochemical mechanism and toxicological consequence of the reduction in FMO activity in ascorbic acid deficiency are heretofore unknown.

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