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Effects of Ascorbic Acid Deficiency on Kinetics of Drug Hydroxylation in Male Guinea Pigs

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Abstract □ The effects of ascorbic acid (vitamin C) deficiency on components of drug-hydroxylating systems in guinea pig liver were investigated. Although the liver weight–body weight ratio was increased, the concentration of microsomal protein was markedly less in ascorbic acid-deficient guinea pigs. This was reflected in a decrease in aniline and hexobarbital hydroxylation reactions when calculated on a unit of liver weight; however, when analyzed per unit of microsomal protein, ethylmorphine demethylase activity was unaffected. The K_m's for these substrates, as well as the ethyl isocyanide difference spectra, were unchanged, indicating that no qualitative changes had occurred in the enzymes responsible for their metabolism or in the cytochrome P-450. Aniline metabolism per unit protein was depressed by ascorbic acid deficiency, as was the content of cytochromes P-450 and b_5. The return of function by a single injection of ascorbic acid given 1–24 hr. prior to decapitation was not frequently observed. Induction with sodium phenobarbital was not blocked by this dietary deficiency state.

Keyphrases □ Ascorbic acid deficiency—effect on kinetics of drug hydroxylation, guinea pig liver □ Drug hydroxylation kinetics—effect of ascorbic acid deficiency, guinea pig liver

The duration and intensity of action of many drugs are largely determined by the speed at which they are metabolized in the body (1). The rate of such drug metabolism may be altered by drug pretreatment, hormones, or the nutritional status of the animal. Increased rates appear to be due to an increased concentration of microsomal enzyme or to the increased affinity of the enzyme for the substrate. Past studies

Table I—Effect of Ascorbic Acid Deficiency on Body Weight, Liver Weight, and Hepatic Microsomal Protein Content of Guinea Pigs

	Days on Diet	Control	Ascorbic Acid Deficiency	p
Terminal body weight, g. ± *SE*	12	340.1 ± 15.6 (15)	281.8 ± 11.6 (15)	<0.01
	18	389.3 ± 6.8 (4)	302.2 ± 20.8 (4)	<0.05
Liver weight g. ± *SE*	12	13.13 ± 0.79 (15)	13.13 ± 0.59 (15)	>0.05
	18	13.95 ± 1.24 (4)	13.40 ± 1.32 (4)	>0.05
$\frac{\text{Liver weight}}{\text{Body weight}} \times 100$	12	3.85 ± 0.12 (15)	4.68 ± 0.15 (15)	<0.01
	18	3.59 ± 0.18 (4)	4.42 ± 0.21 (4)	<0.05
Protein concentration[a], mg./g. liver ± *SE*	12	22.97 ± 1.03 (6)	16.78 ± 0.79 (6)	<0.01
	18	29.25 ± 2.28 (4)	15.53 ± 0.33 (4)	<0.01

[a] 105,000×*g* microsomal pellet. Number in parentheses is number of animals in group.

Table II—Effect of Ascorbic Acid Deficiency on Activities of Selected Hepatic Enzymes

Enzyme and Substrate	Diet[a]	Substrate Metabolized, μmoles/g. Liver/hr. ± *SE*
Aniline hydroxylase[b] (aniline)	Control	1.645 ± 0.219
	Ascorbic acid deficient	0.871 ± 0.077[c]
Hexobarbital oxidase[b] (hexobarbital)	Control	6.629 ± 0.987
	Ascorbic acid deficient	3.888 ± 0.196[c]
Glucose-6-phosphate dehydrogenase[d] (NADP)	Control	393.6 ± 38.4
	Ascorbic acid deficient	377.5 ± 50.1

[a] Animals on diet 12 days. [b] 9000×*g* supernate. [c] Significantly different from control ($p < 0.05$). [d] 105,000×*g* supernate.

indicated that in ascorbic acid deficiency states, drug hydroxylation by liver microsomes is markedly decreased (1–4), whereas nonsignificant decreases in *N*- and *O*-demethylation and nitro and azo reduction have been noted (2). Since previous studies were conducted at fixed single-substrate concentrations, there is little evidence to indicate whether these effects result from decreased affinity or decreased enzyme concentration. Although there appears to be no reduction in NADPH–cytochrome c reductase or NADH–cytochrome c reductase (2), there is a discrepancy as to whether cytochromes P-450 and b_5 are altered (2, 3). This study will focus on the effects of ascorbic acid (vitamin C) deficiency on the kinetics of drug hydroxylation, the inductive effects of sodium phenobarbital on these kinetics, the rate of recovery of drug hydroxylation activity following injections of ascorbic acid, and the effects of ascorbic acid deficiency on some electron transport components.

MATERIALS AND METHODS

Animals and Diet—Male albino guinea pigs (280 ± 46 g.) were fed a pelleted ascorbic acid-deficient diet[1] for 12 or 18 days before sacrifice. The diet consisted of: ground oats, 40.0%; ground bran, 15.0%; alfalfa, 8.0%; sodium chloride, 0.5%; calcium carbonate, 1.0%; and magnesium sulfate, 0.5%. The control animals received daily injections of ascorbic acid (20 mg./kg. i.p.), and the deficient ones received sterile normal saline (1 ml./kg.). Microsomal enzyme induction was accomplished by the injection of sodium phenobarbital (50 mg./kg. i.p.) on the 8th through 11th days of the dietary treatment. Unless otherwise stated, all animals were killed 24-hr. after the last injection.

Preparation of Hepatic Microsomes—The animals were stunned by a sharp blow to the neck and decapitated; the livers were excised, washed in cold 1.15% potassium chloride solution, blotted, weighed, and put into three volumes of cold 1.15% potassium chloride solution packed in ice. Then the livers were cut into small pieces and homogenized with a motor-driven Teflon–glass homogenizer. The homogenate was centrifuged at 9000×*g* for 20 min. in a refrigerated centrifuge[2] to remove nuclei, mitochondria, and unbroken cells. Washed microsomes were prepared by centrifuging this supernate at 105,000×*g* for 60 min., resuspending in 1.15% potassium chloride, and recentrifuging.

Assay of Drug-Metabolizing Activities—Unless otherwise stated, incubation mixtures contained 5 mg. microsomal protein, 2.0 μmoles NADP, 25.0 μmoles glucose-6-phosphate, 25 μmoles magnesium sulfate, 2 units glucose-6-phosphate dehydrogenase, and sufficient 0.1 *M* phosphate buffer (pH 7.4) to make 5.0 ml. When 9000×*g* supernates were used, the glucose-6-phosphate dehydrogenase was omitted from the incubation mixture and the enzyme was equivalent to 250 mg. liver. As drug substrates, the incubation mixtures contained 4.0, 1.0, 0.4, or 0.2 μmoles of hexobarbital; 2.5, 1.0, 0.5, or 0.25 μmoles of aniline; 4.0, 2.0, 1.0, or 0.5 μmoles of zoxazolamine; or 50.0, 5.0, 1.0, or 0.5 μmoles of ethylmorphine. All substrates were incubated at 37° for 20 min. under air in a metabolic incubator[3] at 125 oscillations/min.

The aliphatic hydroxylation of hexobarbital was determined by measuring the disappearance of substrate according to the method of Cooper and Brodie (5). Aromatic hydroxylation of aniline was determined by measuring the formation of *p*-aminophenol, using a modification of the method of Kato and Gillette (6). Aromatic hydroxylation of zoxazolamine was determined by measuring the disappearance of substrate, using the method of Conney *et al.* (7) and Jauchau *et al.* (8). *N*-Demethylation of ethylmorphine was determined by measuring the formation of formaldehyde by the procedure of Nash (9). The NADPH–cytochrome c reductase activity of hepatic microsomes was determined by the method of Phillips and Langdon (10).

Liver microsomal protein was determined by the biuret procedure of Gornall *et al.* (11). Glucose-6-phosphate dehydrogenase activity of the 105,000×*g* supernate was measured by the method of Glock and MacLean (12).

Estimation of Microsomal Cytochromes P-450 and b_5 and Their Reactivity—The cytochrome P-450 and b_5 contents of hepatic microsomes (resuspended in 1.15% potassium chloride containing 0.05 *M* tromethamine buffer at pH 7.4 and at a constant protein concentration of 2.5 mg./ml.) was determined by the method of Omura and Sato (13). Ethyl isocyanide difference spectra of reduced hemoprotein P-450 were measured by the method of Sladek and Mannering (14). Spectral shift studies, using aniline and hexobarbital as substrates for estimating binding affinities (K_s) and the maximum spectral change ($A_{max.}$) were performed by the method of Remmer *et al.* (15), using microsomes diluted to 2 mg. protein/ml. with 0.3 *M* phosphate buffer at pH 7.4. These measurements were made with a dual wave length-splitbeam spectrophotometer[4] using the appropriate mode.

Statistics—The maximum rate of metabolism ($V_{max.}$) and K_m for aniline hydroxylase, hexobarbital oxidase, and ethylmorphine demethylase were calculated using the computer program of Wilkinson (16), as were the binding constant K_s and its $A_{max.}$ for hexobarbital and aniline.

RESULTS AND DISCUSSION

Body and Liver Weights and Microsomal Protein Concentrations—After 12 and 18 days on the deficient diet, there were significant reductions in terminal body weight and significant increases in liver weight–body weight ratios in the ascorbic acid-deficient animals. The microsomal protein (105,000×*g* pellet) per gram liver in deficient guinea pigs was 75% that of controls at 12 days and 53% that of controls at 18 days on the diet (Table I).

Glucose-6-phosphate dehydrogenase activity of the 105,000×*g* supernatant fraction was not altered by the deficiency state (Table II).

The hydroxylations of hexobarbital and aniline at one substrate concentration by 9000×*g* supernatant fractions were significantly less in the ascorbic acid-deficient guinea pigs (Table II). This finding verified the results obtained by Kato *et al.* (2). The apparent K_m and $V_{max.}$ for aniline hydroxylase, hexobarbital oxidase, and ethylmorphine demethylase are shown in Table III. In ascorbic acid-deficient animals, the apparent $V_{max.}$ for aniline hydroxylation was significantly depressed when calculated as micromoles per gram liver and when measured as metabolism per unit of microsomal protein. This reduction in the rate of aniline hydroxylation is due to a decrease in concentration of microsomal protein and P-450 brought on by the deficiency. Since the apparent K_m for aniline hydroxylation was not changed, it is doubtful that this treatment produced qualitative changes in the composition of the enzyme or

[1] Nutritional Biochemical Corp.
[2] Sorvall RC2-B.
[3] Dubnoff.
[4] Aminco-Chance.

Table III—Effect of Ascorbic Acid Deficiency on the Apparent $V_{max.}$ and K_m of Aniline, Hexobarbital, and Ethylmorphine Metabolism

Substrate	Diet[a]	$V_{max.}$[b], μmoles Metabolized/g. Liver/hr. ± *SE*	K_m, mM ± *SE*	$V_{max.}$[c], nmoles Metabolized/mg. Protein/hr. ± *SE*	K_m, mM ± *SE*
Aniline	Control	1.90 ± 0.21	0.68 ± 0.18	174.61 ± 30.09	0.56 ± 0.14
	Ascorbic acid deficient	0.99 ± 0.12[d]	0.61 ± 0.17	86.20 ± 14.83[d]	0.49 ± 0.13
Ethylmorphine	Control	—	—	300.66 ± 29.05	0.36 ± 0.12
	Ascorbic acid deficient	—	—	247.70 ± 29.85	0.29 ± 0.12
Hexobarbital	Control	—	—	878.52 ± 395.58	2.95 ± 1.29
	Ascorbic acid deficient	—	—	205.42 ± 40.78	0.60 ± 0.16

[a] Animals on diet 18 days. [b] 9000×*g* supernate. [c] 105,000×*g* washed microsomes. [d] Significantly different from control ($p < 0.05$).

Table IV—Effect of Ascorbic Acid Deficiency on Aniline and Hexobarbital Binding to Washed Microsomes

Diet	Aniline		Hexobarbital	
	K_s (mM)	$A_{max.}$ (ΔA/mg. Protein)	K_s (mM)	$A_{max.}$ (ΔA/mg. Protein)
Control	0.325 ± 0.045	0.022 ± 0.001	0.386 ± 0.030	0.050 ± 0.002
Ascorbic acid deficiency	0.315 ± 0.045	0.021 ± 0.001	0.412 ± 0.049	0.034 ± 0.002[a]

[a] Significantly different from control ($p < 0.05$).

Table V—Effect of Ascorbic Acid Deficiency on Content of Microsomal Cytochromes P-450 and b_5 and on Ethyl Isocyanide Binding Spectra

	Diet	
	Control	Ascorbic Acid Deficient
Cytochrome P-450 content (nmoles/mg. protein ± *SE*)	1.676 ± 0.027	1.311 ± 0.079[a]
Cytochrome b_5 (nmoles/mg. protein ± *SE*)	0.984 ± 0.051	1.002 ± 0.063
Ethyl isocyanide spectra (ΔA/mg. protein/ml.)		
Δ430–490 nm.	0.100 ± 0.003	0.122 ± 0.013
Δ455–490 nm.	0.076 ± 0.001	0.087 ± 0.013
Ratio: 430/455	1.32 ± 0.03	1.43 ± 0.06

[a] Significantly different from control ($p < 0.05$).

in its affinity for, or reactivity with, the substrate. This was verified by the apparent dissociation constants (K_s) and maximal spectral shifts ($A_{max.}$) for aniline (Table IV).

The $V_{max.}$ or K_m for ethylmorphine demethylase was not altered in ascorbic acid deficiency; however, hexobarbital oxidase activity per unit of protein appeared to be reduced at the four substrate concentrations used. This, coupled with the fact that the maximal spectral shift ($A_{max.}$) for hexobarbital was significantly depressed (Table IV) in the ascorbic acid-deficient animals, indicates that a qualitative change is produced in this metabolic system. Thus, it appears that oxidative enzymes of hepatic microsomes responsible for aniline and ethylmorphine metabolism depend upon ascorbic acid for their synthesis or stability, whereas hexobarbital oxidase may also be altered in its reactivity with its substrate.

The cytochrome P-450 content per unit of microsomal protein is significantly reduced in the ascorbic acid-deficient guinea pig (Table V). The use of ethyl isocyanide to differentiate cytochrome P-450 from P_1-450 has been proposed (14). The data obtained using this ligand indicate that the two forms of cytochrome P-450 are equally sensitive to ascorbic acid deficiency (Table V). Thus, although total cytochrome P-450 is depressed, it is doubtful that its qualitative properties are altered. From these studies it appears that the limiting factor in ethylmorphine metabolism is the quantity of microsomal enzyme, but the reduction in metabolism of hexobarbital and aniline may be dependent upon both protein and cytochrome P-450 loss in the ascorbic acid-deficient pigs.

Effects of Ascorbic Acid Injections at Timed Intervals on Recovery from An Ascorbic Acid-Deficiency State—Twenty-five guinea pigs were used for this study, all maintained on an ascorbic acid-deficient diet as in the previous experiment. Nine control and 16 deficient animals were injected as described previously.

After 12 days the deficient animals were injected with ascorbic acid (20 mg./kg. i.p.) at 24, 16, 8, 4, and 1 hr. before decapitation. Two animals received no ascorbic acid.

Differences in liver weight–body weight ratios between deficient and control animals were not resolved within the 24-hr. period following one injection of ascorbic acid (Table VI). Although the results were not entirely consistent, the cytochrome b_5 content ap-

Table VI—Recovery of Selected Hepatic Components and Functions following Ascorbic Acid Injections in Deficient Animals

Component Analyzed (Mean ± *SE*)	Deficient	Time of Ascorbic Acid Injection Prior to Decapitation					Controls
		1 hr.	4 hr.	8 hr.	16 hr.	24 hr.	
Protein[a], mg./g. liver	23.0 ± 1.0	21.8 ± 3.0	39.4 ± 5.4	16.0	16.0	24.0	29.0 ± 1.3
$\frac{\text{Liver weight}}{\text{Body weight}} \times 100$	5.20 ± 0.72[b]	4.61 ± 0.11[b]	4.83 ± 0.32[b]	5.44 ± 0.99[b]	5.18 ± 0.33[c]	5.07 ± 0.35[b]	4.06 ± 0.12
Cytochrome P-450[d]	0.46 ± 0.05[c]	0.54 ± 0.05[b]	0.77 ± 0.02	—	—	0.75	1.01 ± 0.06
Cytochrome b_5[d]	0.52 ± 0.03[b]	0.62 ± 0.06	0.76 ± 0.01	—	—	0.70	0.72 ± 0.03
NADPH–cytochrome c reductase[e]	25.18 ± 6.58	18.61 ± 0.64	17.49 ± 1.13	—	—	27.5	22.59 ± 2.28

[a] 105,000×*g* microsomal pellet, mg./g. liver. [b] Significantly different from control ($p < 0.05$). [c] Significantly different from control ($p < 0.01$). [d] nmoles/mg. microsomal protein. [e] nmoles/mg. protein/min.

Table VII—Activities of Selected Hepatic Enzymes following Ascorbic Acid Injections in Deficient Animals

Enzyme Analyzed (Mean ± SE)[a]	Deficient	Time of Ascorbic Acid Injection Prior to Decapitation: 1 hr.	4 hr.	8 hr.	16 hr.	24 hr.	Controls
Aniline hydroxylase	1.22 ± 0.19[b]	1.70 ± 0.30	1.74 ± 0.41	1.28 ± 0.12[b]	1.56 ± 0.08[c]	1.79 ± 0.03	2.34 ± 0.12
Ethylmorphine demethylase	—	1.77 ± 0.35	0.96 ± 0.32[c]	—	0.79	1.58	2.53 ± 0.19
Hexobarbital oxidase	1.35 ± 0.26[b]	2.95 ± 0.41	3.36 ± 0.04	1.87	1.76	2.86	3.67 ± 0.15
Zoxazolamine oxidase	—	1.88 ± 0.99	—	0.57	—	0.25	2.23 ± 0.90

[a] μmoles substrate metabolized/g. liver/hr. [b] Significantly different from control ($p < 0.01$). [c] Significantly different from control ($p < 0.05$).

peared to be restored 4 hr. following injection; however, cytochrome P-450 appeared to be depressed for up to 24 hr. NADPH–cytochrome c reductase was not altered by the deficiency state.

The altered drug metabolism may require more than a single injection of ascorbic acid to reverse the depression induced by 12 days of the ascorbic acid-deficient diet (Table VII). When comparing $V_{max.}$ data, it was found that the maximum rate of aniline metabolism per gram liver was not reversed by one injection of ascorbic acid given at 1, 4, 8, 16, or 24 hr. prior to decapitation. The $V_{max.}$ for ethylmorphine demethylase was consistently about 57% that of corresponding control animals, whereas the $V_{max.}$ for zoxazolamine hydroxylase was not different as a result of the deficiency state. The K_m for the reaction was altered only with respect to aniline, where it was significantly less than controls at the 4- and 16-hr. injection periods.

Inductive Effects of Sodium Phenobarbital on Kinetics of Hepatic Microsomal Drug Hydroxylation—Twelve male albino guinea pigs were fed the ascorbic acid-free diet for 12 days. Six of these were injected with ascorbic acid as described previously. On each of the last 4 days of the diet, all animals were injected with sodium phenobarbital (50 mg./kg. i.p.). The animals were killed 24 hr. after the last injection (12th day), and the liver microsomes were prepared and analyzed. The activities of microsomes from deficient guinea pigs were induced to the same level as the controls (Table VIII). Thus, ascorbic acid deficiency does not inhibit induction of aniline hydroxylase by phenobarbital.

Although the apparent $V_{max.}$ and K_m for ethylmorphine demethylase were approximately twice as great in the ascorbic acid.

Table VIII—Effects of Ascorbic Acid Deficiency on Kinetics of Microsomal Drug Hydroxylation in Sodium Phenobarbital[a]-Treated Animals

Substrate	Diet[b]	$V_{max.}$, μmoles Metabolized/g. Liver/hr. ± SE	K_m, mM ± SE
Aniline	Control	10.39 ± 0.94	3.54 ± 0.59
	Ascorbic acid deficient	10.80 ± 0.98	3.54 ± 0.98
Ethylmorphine	Control	21.5 ± 8.0	47.9 ± 22.1
	Ascorbic acid deficient	11.8 ± 3.6	22.6 ± 10.6

[a] Sodium phenobarbital (50 mg./kg./day i.p.) for 4 days prior to decapitation. [b] Animals on diet for 12 days.

treated as in the deficient group, these differences were not statistically significant. Liver weight–body weight ratios in the two groups were not significantly different. These results are in agreement with those of Leber *et al.* (3), who found that phenobarbital induction of aminopyrine demethylase was at least equal in scorbutic animals as in controls whereas greater induction of cytochrome P-450 occurred in scorbutic animals than in controls.

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