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ASCORBIC ACID AND DRUG METABOLISM*

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Abstract—Studies *in vitro* with liver microsomes isolated from vitamin C deficient guinea pigs have indicated a significant decrease in over-all drug oxidation, as typified by aniline hydroxylation, aminopyrine *N*-demethylation and *p*-nitroanisole *O*-demethylation. Concommitant with decreased over-all drug oxidation, the quantity of cytochrome P-450 and cytochrome b_5, and the activity of NADPH cytochrome P-450 reductase and NADPH cytochrome *c* reductase also decreased significantly. It was not until the quantity of liver microsomal ascorbic acid had reached 30 per cent of normal values (11 μg/g wet weight compared to 3·5 μg/g wet weight) that a marked decrease in over-all drug oxidation activity, as well as the level and activity of individual electron transport components, occurred. In addition, the decrease in drug enzyme activities was not due to a calorie deficiency in the vitamin C deficient guinea pigs, since studies with fasted animals indicated normal or greater than normal drug enzyme activities. K_m studies with microsomes isolated from normal and vitamin C deficient guinea pigs did not indicate a correlation in the apparent affinity of drug substrates such as aniline, aminopyrine, and *p*-nitroanisole with decreased microsomal enzyme activities in the vitamin C deficient guinea pigs. The K_m value of aniline hydroxylase for aniline was approximately four times higher in normal guinea pig microsomes while the K_m value of *N*-demethylase for aminopyrine and *O*-demethylase for *p*-nitroanisole was in the order of four times higher in the vitamin C deficient guinea pig microsomes. Studies concerned with aniline–cytochrome P-450 binding spectra indicated an atypical altered spectrum with microsomes isolated from vitamin C deficient animals. The usual trough of the spectrum shifted from 390 to 405 nm and the usual peak of the spectrum shifted from 430 to 440 nm. Also, the intensity of the trough and peak was at least 50 per cent lower than normal. Administration *in vivo* of ascorbic acid to vitamin C deficient animals was followed by reversal of over-all drug oxidation activities, quantity of cytochrome P-450, NADPH cytochrome P-450 reductase activity and altered aniline–cytochrome P-450 binding, but these changes required at least 6 days of treatment to return to normal even though normal levels of liver ascorbic acid were established within 3 days. Phenobarbital induction studies indicated that the microsomal protein-synthesizing system responds to such treatment in vitamin C deficient guinea pigs, and the increase in drug enzyme activities and the level and activity of electron transport components are equal to, if not greater than, those observed in normal animals. Alternative pathways involving an ascorbic acid dependent NADH oxidase system, which has been shown to be capable of metabolizing drugs in the absence of a microsomal NADPH P-450 reductase system, have been considered. The significance of vitamin C deficiency in human drug therapy has been discussed.

IT HAS been known for some time that the activity of liver microsomal drug-metabolizing enzymes is influenced by many factors such as age, sex, strain and species, as well as the nutritional state of the animal. In addition, induction of drug enzyme activities has been shown to occur by foreign compounds including drug substrates,

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insecticides, food additives and polycyclic carcinogenic hydrocarbons such as 3-methylcholanthrene.[1,2]

In regard to nutrition, previous studies *in vivo* in vitamin C deficient guinea pigs have suggested a role for ascorbic acid in drug metabolism. Early investigations by Richards *et al.*[3] and Richards[4] indicated that vitamin C deficient guinea pigs were more sensitive to pentobarbital and procaine than animals not deprived of the vitamin. In 1954, Axelrod *et al.*[5] showed a significant increase in the plasma biological half-life of acetanilide, antipyrine and aniline in guinea pigs depleted of ascorbic acid. These studies *in vivo* indicated that the rate of hydroxylation of these compounds was markedly reduced in vitamin C deficiency. In addition, Conney *et al.*[6] have shown that vitamin C deficient guinea pigs, with no obvious signs of scurvy, had a marked sensitivity to the muscle relaxant zoxazolamine. The increased duration of this drug *in vivo* could be explained by a concomitant decrease in its oxidation *in vitro* in vitamin C deficient guinea pig liver microsomes. In 1968, Degkwitz *et al.*[7] reported that scorbutic guinea pigs have a marked reduction in the hydroxylation of coumarin. Recently Kato *et al.*[8] have shown that the hydroxylation of aniline, hexobarbital and zoxazolamine decreased in guinea pigs on a vitamin C free diet for 12 days. However, the *N*-demethylation of aminopyrine, diphenhydramine and meperidine was not affected, nor were there changes in the *O*-demethylation of *p*-nitroanisole and the reduction of *p*-nitrobenzoic acid and *p*-dimethylaminoazobenzene. In addition, these authors found no significant decrease in individual liver microsomal electron transport components, such as cytochrome P-450.

In general, the previous studies, which have been in the main investigations *in vivo*, have shown decreased metabolism of a variety of pharmacological agents in vitamin C deficient animals, although some of these reports are conflicting as to the type of over-all oxidation reactions affected. In addition, very little information is available in regard to the underlying biochemical basis of decreased drug metabolism in vitamin C deficiency. The data presented in this paper are concerned with the type of over-all drug oxidation reactions, such as *N*-demethylation, *O*-demethylation and hydroxylation, affected by ascorbic acid deprivation for various lengths of time as well as the reversal of the impairment in deficient animals. In addition, data are presented on the level and activity of various liver microsomal electron transport components in normal guinea pigs and guinea pigs maintained on a vitamin C deficient diet for 10 and 21 days. Kinetic studies with microsomes isolated from normal and vitamin C deficient guinea pigs were done to determine the apparent Michaelis–Menten affinity constants of various drug enzymes for substrates and cofactors. Studies on the binding characteristics of cytochrome P-450 and drug substrate in normal and vitamin C deficient guinea pig microsomes are given. Furthermore, experiments using phenobarbital to induce the liver microsomal drug-metabolizing system were carried out in normal and vitamin C deficient guinea pigs to examine the effect of vitamin C deficiency on the induction of microsomal protein, electron transport components, and over-all drug oxidation.

MATERIALS AND METHODS

Aniline and *p*-nitroanisole were obtained from Fisher Scientific Company. Aminopyrine was obtained from K & K Laboratories, Inc. Glucose 6-phosphate, NADP, NADPH, cytochrome *c*, ascorbic acid deficient diet (guinea pig), and L-ascorbic acid

were obtained from Nutritional Biochemical Corp. Glucose-6-PO_4 dehydrogenase (Grade II; 0·9 units/mg) was obtained from Sigma Chemical Company. Carbon monoxide was obtained from Matheson Gas Products. Phenobarbital (sodium) was obtained from Amend Drug & Chemical Company. Ascorbyl palmitate was obtained from Hoffmann-La Roche, Inc.

Vitamin C deficient guinea pigs. Groups of male albino guinea pigs (200–250 g) were placed on a vitamin C free diet for either 10 days or 21 days. The animals which were on the diet for 10 days showed no significant weight loss, had no joint hemorrhages, and their liver ascorbic acid level was approximately 6 mg/100 g. The animals on the diet for 21 days also showed no appreciable weight loss and on sacrifice had slight leg joint hemorrhages; their liver vitamin C level was 2·5 mg/100 g.

Groups of normal guinea pigs (200–250 g) consisted of animals which were fed a regular Chow diet or animals on a vitamin C deficient diet supplemented with 50 mg of ascorbic acid/day in their drinking water. There was no appreciable difference in these two groups of animals in that their average weight was 260–280 g and their liver ascorbic acid level was in the order of 20 mg/100 g. In the experiments reported, the control animals were placed on a vitamin C diet supplemented with 50 mg of ascorbic acid/day in their drinking water.

Fasted guinea pigs. Male albino guinea pigs (200–250 g) were fasted for 3–4 days. They received 50 mg of ascorbic acid/day in their drinking water. At the end of the fourth day the animals had lost 20–25 per cent of their body weight and their liver ascorbic acid level was approximately 15 mg/100 g.

Phenobarbital induction. Groups of normal and vitamin C deficient (21 days) male albino guinea pigs were given sodium phenobarbital in their drinking water which contained 1 mg of sodium phenobarbital/ml for 4 days. The guinea pigs drank on the average of 30–40 ml/day. There was no significant difference in the water consumption in the two groups. Preliminary experiments with normal and vitamin C deficient animals indicated that maximal induction occurred between 3·5 and 4 days of phenobarbital administration.

Preparation of guinea pig liver microsomes. Guinea pigs were decapitated, exsanguinated, and their livers were quickly removed and placed on ice. All following procedures were performed at 4°. Homogenates (20%, w/v) were prepared with 1·15% KCl in 0·002 M tris buffer, pH 7·4, with a Potter–Elvehjem homogenizer. The crude homogenate was spun at 15,000 *g* for 15 min. The 15,000 *g* supernatant fraction containing the microsomes was spun at 100,000 *g* for 60 min at 2°. The microsomal pellet was suspended to one-half of the 15,000 *g* supernatant volume with 1·15% KCl in 0·002 M tris, pH 7·4, and the protein concentration was in the order of 8–10 mg/ml. All enzyme assays were performed on freshly prepared microsomes.

p-Nitroanisole O-demethylase activity. Microsomal *p*-nitroanisole *O*-demethylase activity was determined by measuring the product of the reaction, *p*-nitrophenol, at 415 nm as previously described.[9,10] *O*-demethylase activity was calculated from the initial rate of the reaction which was linear with time for at least 15 min. Under the conditions of the assay, 1·0 μg of product, *p*-nitrophenol, gives an optical density reading of 0·082 at 415 nm.

The specific activity of *O*-demethylase is expressed as micromoles of *p*-nitrophenol formed per hour per 100 mg of microsomal protein at 27°.

Aminopyrine N-demethylase activity. Microsomal aminopyrine *N*-demethylase

activity was determined by measuring the formaldehyde formed during the demethylation of aminopyrine to 4-aminoantipyrine. The liberated formaldehyde is measured by the colorimetric procedure of Nash[11] which is based on the Hantzsch reaction. The rate of the reaction was linear with time for at least 60 min and proportional to enzyme concentration. Under the assay conditions, the formation of 1 μg of HCHO gives an optical density reading of 0·088 at 412 nm.

The specific activity of *N*-demethylase activity is defined as micromoles of HCHO formed per hour per 100 mg microsomal protein at 27°.

Aniline hydroxylase activity. Microsomal aniline hydroxylase activity was determined by measuring the product, *p*-aminophenol, after formation of a complex with phenol reagent at 660 nm according to the method of Brodie and Axelrod.[12] The rate of the reaction was linear with time for at least 60 min and proportional to enzyme concentration. Under the assay condition, the formation of 1 μg of *p*-aminophenol gives an optical density reading of 0·100.

The specific activity of aniline hydroxylase activity is expressed as micromoles of *p*-aminophenol formed per hour per 100 mg microsomal protein at 27°.

NADPH-cytochrome c *reductase activity.* Microsomal NADPH-cytochrome *c* reductase activity was based on the absorbance of reduced cytochrome *c* at 550 nm according to the method of Williams and Kamin.[13] The reaction was followed in a Gilford 2000 recording spectrophotometer, and the rate of the reaction was linear with time for at least 20 min.

Under the conditions of the assay the molar extinction coefficient of reduced cytochrome *c* is 6·37 $mM^{-1}cm^{-1}$.[13] The specific activity of NADPH-cytochrome *c* reductase activity is expressed as micromoles of cytochrome *c* reduced per hour per 100 mg microsomal protein at 27°.

Cytochrome b_5. The determination of the quantity of microsomal cytochrome b_5 is based on the method of Omura and Sato.[14] The amount of cytochrome b_5 present in the microsomes is measured after its reduction with sodium dithionite at 426 nm.

Under the conditions of the assay, the molar extinction coefficient of reduced cytochrome b_5 is 171 $mM^{-1}cm^{-1}$.[14] The specific activity of cytochrome b_5 is expressed as micromoles of cytochrome b_5 per 100 mg liver microsomal protein.

Cytochrome P-450. The quantity of microsomal cytochrome P-450 was determined by the method of Omura and Sato.[15] Cytochrome P-450 is reduced with sodium dithionite and complexed with CO; the latter complex absorbs light at 450 nm. The amount of reduced cytochrome P-450 was proportional to the amount of microsomal protein present. Under the conditions of the assay, the apparent molar extinction coefficient of the reduced P-450–CO complex is 91 $mM^{-1}cm^{-1}$.

The specific activity of cytochrome P-450 is expressed as micromoles of cytochrome P-450 per 100 mg of microsomal protein.

NADPH cytochrome P-450 *reductase.* The determination of microsomal NADPH cytochrome P-450 reductase activity was based on the formation of reduced cytochrome P-450–CO by NADPH. The reaction was followed in a Gilford 2000 recording spectrophotometer at 15°. A 6·0-ml aliquot of microsomes (8 mg protein/ml) was placed in a Erlenmeyer flask, capped and placed in an ice bath. Carbon monoxide (previously passed through a solution of 0·5% sodium dithionite in 0·10 N NaOH) was bubbled through the microsomal suspension with a 20-gauge needle for 5 min. After this time, 3·0 ml of the microsomal suspension was transferred with a syringe to a rubber

capped cuvette (10 mm light path) which had been previously evacuated for 3 min. The sealed cuvette was placed in the spectrophotometer, and the absorbance due to the microsomes was offset to 0 absorbance at 450 nm. Control readings prior to the addition of NADPH were recorded for 10–15 sec. The reaction was initiated by the addition of 0·05 ml of NADPH (final concentration, $8{\cdot}30 \times 10^{-5}$ M) with a syringe and mixed with the microsomal suspension (4–6 sec).

The activity was calculated based on an early rate of 4 sec extrapolated to zero time. The 4-sec rate was used since during this time the velocity of the reaction was, in each case, linear with time (2–3 assays/microsomal preparation) and proportional to enzyme concentration. The rate of the reaction was followed for at least 40 sec and under the assay conditions was found to be linear during this time. In all assays, however, 4-sec rates were used since comparisons could be made with reaction rates at 27°. At this temperature, reaction rates were linear with time for 4 sec, but not thereafter; after 4 sec a slower rate occurred similar to those reported.[16–18] The calculated Q_{10} of the reaction, using 4-sec rates, was 1·91.

Specific activity is defined as micromoles of cytochrome P-450 reduced per hour per 100 mg liver microsomal protein at 27°, using a molar extinction coefficient of 91·0 $mM^{-1}cm^{-1}$.

Aniline-cytochrome P-450 binding spectrum (type II). Aniline (5 mM) was added to a microsomal suspension (2·0 mg protein/ml of 0·05 M tris, pH 7·5). The experimental cuvette (aniline-treated) was scanned from 360 to 500 nm at 5-nm intervals in a Gilford 2000 spectrophotometer. The absorbance at each wavelength minus the absorbance at 500 nm was recorded. The usual type II spectrum was obtained with a trough at 390 nm and a peak at 430 nm.[19] The intensity of the trough and peak was proportional to the concentration of cytochrome P-450 present since aniline was added in excess.

Guinea pig liver ascorbic acid determination. Ascorbic acid was determined in 15,000 *g* supernatant fractions and microsomes by a micro modification of the phenylhydrazine method of Roe and Kuether.[20] Proportional reduction of all reactants were made to give a total fluid volume of 0·92 ml. Under the conditions of the method, 1·0 μg of the phenylhydrazine derivative of dehydroascorbic acid gave an O.D. reading of 0·090 at 540 nm.

Protein was determined with the method of Lowry *et al.*[21] Crystalline bovine serum albumin, Fraction V, was used as a standard.

RESULTS

Drug metabolism in normal and vitamin C deficient guinea pigs. Male albino guinea pigs (200–250 g) were maintained on a vitamin C deficient diet for 10 and 21 days. Normal animals were maintained on a vitamin C deficient diet supplemented with 50 mg of ascorbic acid in their drinking water. All animals were selected from a single group. The activity of over-all drug oxidation reactions as typified by aniline hydroxylation, aminopyrine *N*-demethylation, and *p*-nitroanisole *O*-demethylation and the level and activity of liver microsomal electron transport components such as cytochrome P-450, cytochrome b_5, NADPH cytochrome P-450 reductase and NADPH cytochrome *c* reductase were determined (Table 1). As can be observed, there was no significant decrease in over-all drug oxidation activity or in the level and activity of microsomal electron transport components in the guinea pigs maintained on a vitamin C deficient diet for 10 days. After 10 days on the deficient diet, the amount of ascorbic

acid in the liver was approximately 30 per cent of normal based on the 15,000 *g* supernatant fraction (194 vs. 62 μg/g wet weight) and 50 per cent of normal based on the microsomal fraction (11 vs. 6 μg/g wet weight). In contrast to these results, guinea pigs that were on the vitamin C deficient diet for 21 days showed a marked decrease in both over-all drug hydroxylation activity and electron transport components. Aniline hydroxylase activity decreased 50 per cent; aminopyrine *N*-demethylase activity decreased 56 per cent; and *p*-nitroanisole *O*-demethylase activity decreased 66 per cent. The amount of cytochrome P-450 decreased 40 per cent, and the activity of NADPH cytochrome P-450 reductase decreased at least 85 per cent: not as significantly, however, NADPH cytochrome *c* reductase activity and the level of cytochrome b_5 decreased 33 per cent. It is important to point out that, concomitant with the decrease in over-all drug oxidation activity and microsomal electron transport components, there was a further decrease in the amount of ascorbic acid in the 15,000 *g* supernatant fraction (60 per cent) and microsomal fraction (42 per cent) in the guinea pigs maintained on the vitamin C deficient diet for 21 days as compared to guinea pigs on the diet for 10 days.

TABLE 1. EFFECT OF VITAMIN C DEFICIENCY (10 AND 21 days) ON DRUG ENZYMES AND ELECTRON TRANSPORT COMPONENTS IN GUINEA PIG LIVER MICROSOMES

	Activity*			
	Normal	Vitamin C deficient (10 days)	Vitamin C deficient (21 days)	Decrease (%)
Aniline hydroxylase	1·6 ± 0·2 P < 0·001	1·3 ± 0·1	0·8 ± 0·2 P < 0·001	50
Aminopyrine *N*-demethylase	3·9 ± 0·1 P < 0·001	3·3 ± 0·4	1·7 ± 0·3 P < 0·001	56
p-Nitroanisole *O*-demethylase	3·2 ± 0·4 P < 0·001	3·0 ± 0·2	1·1 ± 0·2 P < 0·001	66
Cytochrome P-450	0·05 ± 0·01 P < 0·01	0·05 ± 0·001	0·03 ± 0·003 P < 0·01	40
NADPH cytochrome P-450 reductase	0·80 ± 0·2	0·87 ± 0·33	< 0·10†	85
NADPH cytochrome *c* reductase	124 ± 21 P < 0·05	167 ± 20	83 ± 11 P < 0·05	33
Cytochrome b_5	0·03 ± 0·004 P > 0·05	0·03 ± 0·003	0·02 ± 0·006 P > 0·05	33
Liver ascorbic acid				
Supernatant fraction 15,000 *g* (μg/g wet weight)	194 ± 29	62 ± 15	25 ± 15	
Microsomal fraction (μg/g wet weight)	11 ± 3·8	6 ± 0·9	3·5 ± 2·0	

* Assay conditions and units of activity are given in Materials and Methods.
† This value is a lower limit of detection. Mean ± S.E. of 10 animals per group.

It should be mentioned that experiments *in vitro* in which attempts were made to restore the activity of aniline hydroxylase, aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase in vitamin C deficient guinea pig microsomes were unsuccessful. Neither the simultaneous addition of ascorbic acid ($2·3 \times 10^{-3}$ M) and

substrate, nor the pretreatment of the vitamin C deficient guinea pig microsomes with the vitamin had any effect in restoring over-all drug oxidation activity.

Although the animals on the vitamin C deficient diet for 21 days did not have appreciable weight loss (at most 5 per cent), it was important to be certain that the observed effects on drug metabolism were not due to a diminished calorie intake by these animals, since it is known that drug metabolism is influenced by the nutritional state of the animal.[22]

Guinea pigs were deprived of food for 3 days and supplemented with 50 mg of ascorbic acid/day in their drinking water. During this time the animals lost 20–25 per cent of their body weight. At the end of 3 days the animals were sacrificed, and overall drug hydroxylation activities and the activity and level of various electron transport components were determined (Table 2). In contrast to the animals maintained on a vitamin C deficient diet, these animals had, in general, an increase in drug metabolism. Aniline hydroxylase and *p*-nitroanisole *O*-demethylase activities increased approximately 2-fold in the starved animals as compared to normal while aminopyrine *N*-demethylase activity was not significantly altered. In addition, there was an increase in the activities of NADPH cytochrome P-450 reductase (7-fold) and NADPH cytochrome *c* reductase (1·6-fold) in the starved guinea pig. It should be mentioned that the quantity of liver ascorbic acid in the 15,000 *g* supernatant fraction and microsomal fraction was in the same order of magnitude in both groups.

TABLE 2. EFFECT OF VITAMIN C DEFICIENCY (21 days) AND STARVATION (3 days) ON THE ACTIVITY OF DRUG ENZYMES AND ELECTRON TRANSPORT COMPONENTS IN GUINEA PIG LIVER MICROSOMES

	Activity*		
	Normal†	Starved‡	Vitamin C deficient†
Aniline hydroxylase	1·6 ± 0·2	3·2 ± 0·5	0·8 ± 0·2
Aminopyrine *N*-demethylase	3·9 ± 0·1	4·8 ± 1·2	1·7 ± 0·3
p-Nitroanisole *O*-demethylase	3·2 ± 0·4	6·2 ± 0·2	1·1 ± 0·2
Cytochrome P-450	0·05 ± 0·01	0·07 ± 0·017	0·03 ± 0·003
NADPH cytochrome P-450 reductase	0·8 ± 0·2	5·4 ± 1·95	< 0·10
NADPH cytochrome *c* reductase	124 ± 21	205 ± 25	83 ± 11
Cytochrome b_5	0·03 ± 0·004	0·03 ± 0·012	0·02 ± 0·006
Liver ascorbic acid			
Supernatant fraction 15,000 *g* (μg/g wet weight)	194 ± 29	144 ± 24	25 ± 15
Microsomal fraction (μg/g wet weight)	11 ± 3·8	13 ± 2·2	3·5 ± 2·0

* Assay conditions and units of activity are given in Materials and Methods.
† Mean ± S.E. of 10 animals per group.
‡ Mean ± S.E. of 8 animals per group.

K_m *determination in liver microsomes from normal and vitamin C deficient guinea pigs.* In general, there was no apparent correlation in the K_m values for various substrates (Table 3) and decreased drug metabolism activity in vitamin C deficient guinea pig liver microsomes (Table 1). For example, the K_m value of aniline hydroxylase for aniline was approximately four times higher using normal guinea pig microsomes as compared to vitamin C deficient guinea pig microsomes. In contrast, the K_m

value of *N*-demethylase for aminopyrine or *O*-demethylase for *p*-nitroanisole was in the order of three to four times higher using vitamin C deficient guinea pig microsomes. Furthermore, the K_m value of NADPH cytochrome *c* reductase for NADPH was in the same order of magnitude with both microsomal preparations. In addition, determination of V_{max} values indicated no significant differences existed in the values between the normal and vitamin C deficient animals. Although some of the decreased activity of aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase in vitamin C deficient guinea pigs may be explained, in part, by a lower affinity of these enzymes for their substrates, the decreased activity in aniline hydroxylase and NADPH cytochrome *c* reductase cannot be due to an alteration in the apparent affinity of these enzymes for their respective substrates.

Investigations from many laboratories have shown that an obligatory initial step in over-all drug metabolism is the binding of drug substrate with microsomal cytochrome P-450.[19,23] A comparison was made of drug substrate binding with microsomes isolated from normal and vitamin C deficient guinea pigs. For these studies a representative type II substrate, aniline was used since this compound gave reproducible and consistent typical type II spectra with normal guinea pig microsomes (minimum absorption at 390 nm, maximum absorption at 430 nm).

TABLE 3. APPARENT MICHAELIS–MENTEN AFFINITY CONSTANTS (K_m) FOR OVER-ALL HYDROXYLATION REACTIONS*

	Normal	Vitamin C deficient (21 days)
Aniline hydroxylase	$17{\cdot}3 \times 10^{-4}$ M	$4{\cdot}6 \times 10^{-4}$ M
Aminopyrine *N*-demethylase	$1{\cdot}7 \times 10^{-3}$ M	$4{\cdot}0 \times 10^{-3}$ M
p-Nitroanisole *O*-demethylase	$4{\cdot}5 \times 10^{-5}$ M	$15{\cdot}3 \times 10^{-5}$ M
NADPH cytochrome *c* reductase	$3{\cdot}5 \times 10^{-6}$ M	$2{\cdot}8 \times 10^{-6}$ M

* Assay conditions for aniline hydroxylase, aminopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase and NADPH cytochrome *c* reductase, were carried out as described in Materials and Methods. Eight concentrations of each substrate were used in the following range of concentrations: aniline: $5{\cdot}0 \times 10^{-4}$ M–$1{\cdot}6 \times 10^{-4}$ M; aminopyrine: $2{\cdot}7 \times 10^{-3}$ M–$5{\cdot}0 \times 10^{-4}$ M; *p*-nitroanisole: $1{\cdot}8 \times 10^{-3}$ M–$1{\cdot}8 \times 10^{-4}$ M; and NADPH; $5{\cdot}0 \times 10^{-5}$ M–$5{\cdot}0 \times 10^{-6}$ M. K_m values shown are derived from a double reciprocal plot of 1/velocity of product formed vs. 1/substrate concentration.

Aniline–cytochrome P-450 binding spectra in normal and vitamin C deficient guinea pig microsomes. The treatment of normal guinea pig microsomes with aniline resulted in a typical type II spectrum with a trough at 390 and a peak at 430 nm. On the other hand, the treatment of vitamin C deficient guinea pig microsomes with aniline resulted in an altered spectrum (Fig. 1). The trough of the atypical spectrum was at 405 instead of 390 nm for the normal spectrum, and the peak of the atypical spectrum was at 440 instead of 430 nm for the normal spectrum. In addition to a shift in the trough and peak, there were marked differences in the absorption intensity in the atypical vitamin C deficient aniline–cytochrome P-450 spectrum. There was an 80 per cent decrease in the peak and a 60 per cent decrease in the trough (Fig. 1). Also, the general shape of the atypical spectrum was less symmetrical than that obtained with normal guinea pig microsomes. The addition of ascorbic acid ($2{\cdot}3 \times 10^{-3}$ M), either before or after the addition of aniline, to vitamin C deficient guinea pig microsomes had no effect in

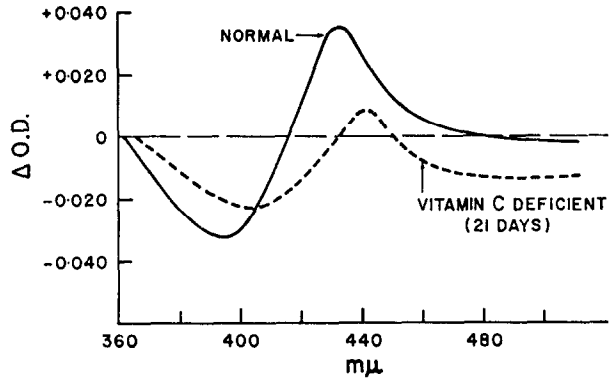

FIG. 1. Aniline–cytochrome P-450 binding spectra (type II) in normal and vitamin C deficient guinea pigs (21 days). Substrate difference spectra were obtained as described in Materials and Methods. The final concentration of aniline was 5 mM and the concentration of microsomal protein was 2 mg/ml. The difference spectra were obtained following the addition of aniline to the microsomes.

restoring the altered spectrum to a typical type II spectrum. It should be mentioned that the microsomes isolated from animals on a vitamin C deficient diet for 10 days, which had normal over-all drug metabolism activity and electron transport components, also showed normal aniline–cytochrome P-450 binding spectra.

Reversal in vivo *of altered drug metabolism activity in vitamin C deficient guinea pigs with ascorbic acid.* Groups of guinea pigs which had been on a vitamin C deficient diet for 21 days were given 50 mg of ascorbic acid/day in their drinking water for 3, 6 and 10 days. At the end of these times the animals were sacrificed, liver microsomes prepared, and the activity of over-all drug oxidation (aniline hydroxylase, aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase) and the quantity and activity of individual electron transport components were determined. As can be seen in Fig. 2, aniline hydroxylase activity required 6 days to return to normal, *O*-demethyl-

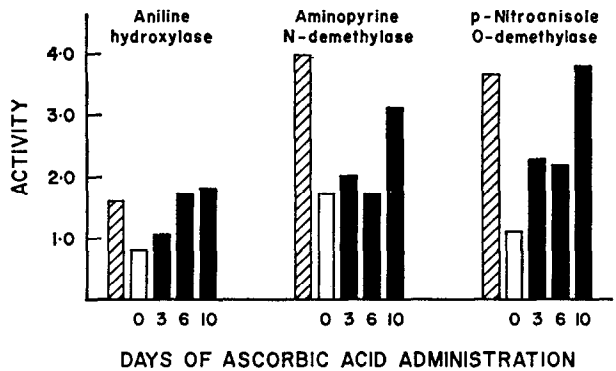

FIG. 2. Reversal of decreased aniline hydroxylase, aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase activities in vitamin C deficient guinea pigs with ascorbic acid. Groups of normal and vitamin C deficient guinea pigs (21 days) were given 50 mg of ascorbic acid in their drinking water for 3, 6 and 10 days as described in Materials and Methods. Aniline hydroxylase, aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase were determined as described in Materials and Methods. Enzyme activity equals the micromoles product formed per hour per 100 mg liver microsomal protein at 27°.

ase activity required 10 days, whereas *N*-demethylase activity had reached only 80 per cent of normal after 10 days. The time for reversal was similar for electron transport components in that cytochrome P-450 required 10 days to reach normal levels as did cytochrome b_5 and NADPH cytochrome P-450 reductase (Fig. 3). NADPH

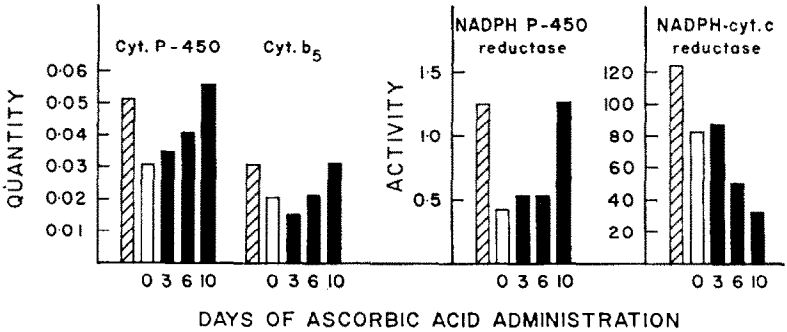

FIG. 3. Reversal of decreased electron transport components in vitamin C deficient guinea pigs with ascorbic acid. Groups of normal and vitamin C deficient guinea pigs (21 days) were given 50 mg of ascorbic acid in their drinking water for 3, 6 and 10 days as described in Materials and Methods. Cytochrome P-450, cytochrome b_5, NADPH cytochrome P-450 reductase and NADPH cytochrome *c* reductase were determined as described in Materials and Methods. The quantity of cytochrome P-450 equals the micromoles per 100 mg liver microsomal protein. The quantity of cytochrome b_5 equals the micromoles per 100 mg liver microsomal protein. NADPH cytochrome P-450 reductase equals the micromoles of cytochrome P-450 reduced per hour per 100 mg liver microsomal protein at 27°. NADPH cytochrome *c* reductase equals the micromoles of cytochrome *c* reduced per hour per 100 mg liver microsomal protein at 27°.

cytochrome *c* reductase activity did not return to normal even after 10 days and, in fact, was maintained at the low 10-day level for at least 16 days. Of significance is the fact that the quantity of liver ascorbic acid in both the 15,000 *g* liver supernatant fraction and microsomal fraction had returned to normal levels within 3 days of ascorbic acid feeding, while over-all drug oxidation activity and electron transport component levels were still depressed (Table 4).

TABLE 4. LIVER ASCORBIC ACID IN VITAMIN C DEFICIENT GUINEA PIGS (21 days) BEFORE AND AFTER ORAL ADMINISTRATION OF ASCORBIC ACID

	Supernatant fraction 15,000 *g* (μg/g wet weight)	Microsomal fraction (μg/g wet weight)
Normal	194	11·0
Vitamin C deficient (21 days)*	25	3·5
Vitamin C deficient + ascorbate (3 days)	258	18·0
Vitamin C deficient + ascorbate (6 days)	241	27·0
Vitamin C deficient + ascorbate (10 days)	227	12·0

* Guinea pigs were on the vitamin C deficient diet for 21 days and groups of 4 were given 50 mg of ascorbic acid/day in their drinking water for the length of time indicated in the table. Ascorbic acid was determined in the liver 15,000 *g* supernatant fraction and microsomal fraction according to the method of Roe and Kuether[20] as described in Materials and Methods.

The aniline–cytochrome P-450 binding spectra with microsomes isolated from vitamin C deficient guinea pigs which were given ascorbic acid for 3, 6 and 10 days is shown in Fig. 4. As with over-all drug metabolism activities, the typical binding spectrum, that is a trough at 390 and a peak at 430 nm, was not obtained in 3 or 6 days, but was obtained after 10 days of ascorbic acid administration.

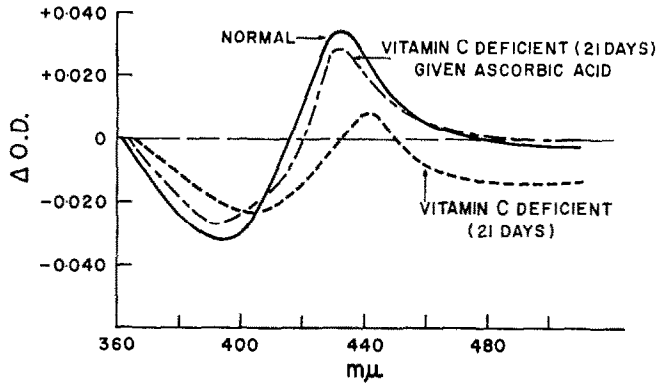

FIG. 4. Reversal of altered aniline–cytochrome P-450 binding spectrum in vitamin C deficient guinea pigs (21 days) given ascorbic acid. Substrate difference spectra were obtained as described in Materials and Methods. The final concentration of aniline was 5 mM, and the concentration of microsomal protein was 2 mg/ml. The difference spectra were obtained following the addition of aniline to the microsomes.

The results with administration *in vivo* of ascorbic acid indicate that although normal levels of ascorbic acid could be achieved within 3 days, there was no reversal of altered drug enzyme activities or atypical binding of drug to cytochrome P-450 until at least 10 days. One possibility is that the time required for reversal in vitamin C deficient guinea pigs depends on the occurrence of newly synthesized microsomal protein. Phenobarbital, a potent drug enzyme inducer which acts by causing a specific increase in microsomal protein, was administered to vitamin C deficient guinea pigs and normal guinea pigs.

Phenobarbital induction in normal and vitamin C deficient guinea pigs. Induction of over-all drug oxidation activities (aniline hydroxylase, aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase) and microsomal electron transport components occurred in both normal and vitamin C deficient guinea pigs (Table 5). The fold increase due to induction (phenobarbital treatment/no treatment) in the vitamin C deficient guinea pigs was in the same order of magnitude, and in some cases greater, than the fold increase in the normal guinea pigs. For example, aniline hydroxylase increased 2·1-fold in the vitamin C deficient animal compared to 1·4-fold in the normal animal, and *p*-nitroanisole *O*-demethylase increased 5·9-fold in the vitamin C deficient animal compared to 3·5-fold in the normal animal. Furthermore, the difference between the level of activity after phenobarbital treatment and basal level (no treatment) in vitamin C deficient and normal guinea pigs was in the same order of magnitude for each enzyme activity. As was found with over-all drug oxidation activities, the individual electron transport components were also induced in vitamin C deficient guinea pigs and the fold increase was equivalent to, or in some cases better than, the normal or vitamin C deficient guinea pigs.

TABLE 5. PHENOBARBITAL INDUCTION OF DRUG ENZYMES AND ELECTRON TRANSPORT COMPONENTS IN NORMAL AND VITAMIN C DEFICIENT GUINEA PIGS

	Activity*					
	Normal†			Vitamin C deficient†		
	No R_x‡	PB R_x§	Fold increase	No R_x‡	PB R_x§	Fold increase
Aniline hydroxylase	1·6 ± 0·2	2·3 ± 0·5	1·4	0·8 ± 0·2	1·7 ± 0·3	2·1
Aminopyrine *N*-demethylase	3·9 ± 0·1	9·6 ± 1·6	2·5	1·7 ± 0·3	7·6 ± 1·7	4·5
p-Nitroanisole *O*-demethylase	3·2 ± 0·4	11·1 ± 2·7	3·5	1·1 ± 0·2	6·5 ± 1·6	5·9
Cytochrome P-450	0·05 ± 0·01	0·11 ± 0·02	2·2	0·03 ± 0·003	0·06 ± 0·01	2·0
NADPH P-450 reductase	0·8 ± 0·2	2·6 ± 0·6	3·3	< 0·10	2·8 ± 1·0	28·0
NADPH cytochrome *c* reductase	124 ± 21	288 ± 49	2·3	83 ± 11	250 ± 49	3·0
Cytochrome b_5	0·03 ± 0·004	0·03 ± 0·006	1·0	0·02 ± 0·006	0·02 ± 0·01	1·0

* Assay conditions, units of activity and phenobarbital treatment are given in Materials and Methods.

† Liver ascorbic acid: normal induced, 195 μg/g wet weight (15,000 *g* supernatant fraction), 19 μg/g wet weight (microsomal fraction); vitamin C deficient induced, 71 μg/g wet weight (15,000 *g* supernatant fraction), 5 μg/g wet weight (microsomal fraction).

‡ Mean±S.E. of 10 animals per group.

§ Mean±S.E. of 7 animals per group.

DISCUSSION

From the studies reported, it is apparent that neither over-all drug oxidation activities nor levels of microsomal electron transport components are decreased significantly in guinea pigs maintained on a vitamin C diet for 10 days, under conditions where the concentration of liver microsomal ascorbic acid was 50 per cent of normal. On the other hand, if guinea pigs were maintained on a vitamin C deficient diet for 21 days where the concentration of liver microsomal ascorbic acid reached approximately 30 per cent of normal, there was a marked decrease in over-all drug oxidation activities as well as the level and activity of individual microsomal electron transport components. The decrease observed in cytochrome P-450 and NADPH cytochrome P-450 reductase activity (40 and 85 per cent respectively) is in contrast to the studies reported by Kato *et al.*[8] These investigators found no appreciable change in electron transport components in their vitamin C deficient guinea pigs, but did find a decrease in the activity of aniline hydroxylase. It should be pointed out, however, that their animals were much larger (400 g) than those employed in this study (200–250 g) and were placed on a vitamin C deficient diet for only 12 days. Our findings are more consistent with the studies of Leber *et al.*[24] who have shown significant decreases in the demethylation of aminopyrine, the hydroxylation of acetanilide, and the quantity of cytochrome P-450 in vitamin C deficient animals. It should be pointed out that our animals on the vitamin C deficient diet for 21 days suffered no appreciable weight loss (at most 5 per cent) and were not frankly scorbutic. Furthermore, experiments with fasted animals, that had lost 25 per cent of their body weight, indicate that the effects observed in vitamin C deficiency cannot be due to decreased calorie intake, since over-all drug oxidation activities and microsomal electron transport components were either normal, or had in fact, increased 2- to 3-fold.

In contrast to the K_m studies where there was no obvious correlation between decreased drug enzyme activities and apparent affinity constants of drug substrates, consistent alteration in the usual aniline–cytochrome P-450 binding spectrum was found with vitamin C deficient guinea pig microsomes. The aniline–cytochrome P-450 binding spectrum was atypical in that the trough of the spectrum appeared at 405 nm instead of 390 nm and the peak occurred at 440 nm instead of 430 nm. There was also a marked decrease in the absorption intensity at these wavelengths. The latter finding can be explained by a concomitant decrease in the quantity of cytochrome P-450 in vitamin C deficient guinea pig microsomes. Furthermore, dilution of microsomes from normal guinea pigs to equivalent cytochrome P-450 levels found in scorbutic guinea pigs' microsomes gave the usual aniline-binding spectra with a trough at 390 nm and a peak at 430 nm. The atypical aniline–cytochrome P-450 spectrum in vitamin C deficient guinea pig microsomes may reflect an alteration in the integrity of the microsomal phospholipid membrane associated with cytochrome P-450 and ascorbic acid may be required and necessary for its maintenance. Attempts to restore over-all drug oxidation activity, NADPH cytochrome P-450 reductase activity, and altered aniline–cytochrome P-450 binding by the addition *in vitro* of ascorbic acid ($2{\cdot}3 \times 10^{-3}$ M) to vitamin C deficient liver microsomes were unsuccessful. This may not be too surprising if the integrity of the microsomal phospholipid membrane had been irreversibly damaged by the depletion of the vitamin. Attempts to replace ascorbic acid with other reducing agents such as glutathione, and 2,6-dichlorophenolindophenol dye were also ineffective. However, ascorbyl palmitate ($2{\cdot}3 \times 10^{-3}$ M), a

more lipophilic analog of ascorbic acid, did restore atypical aniline–cytochrome P-450 binding spectra to the usual type in that the trough and peak of the spectrum were at 390 and 430 nm respectively; however, the absorption intensity at these wavelengths was still decreased as was over-all oxidation activities, both of which could reflect the lower quantity of cytochrome P-450 which was not restored by ascorbyl palmitate.

Reversal of decreased microsomal drug-metabolizing activities in vitamin C deficient animals by the administration *in vivo* of ascorbic acid indicate that, although the quantity of liver ascorbic acid was restored to normal levels within 3 days, over-all drug oxidation activities and levels of electron transport components were still depressed. Furthermore, the atypical aniline–cytochrome P-450 binding spectra was still present at this time. It took from 6 to 10 days of ascorbic acid administration to restore most of the drug-metabolizing activities. *O*-demethylase activity and microsomal electron transport components, such as cytochrome P-450 and NADPH cytochrome P-450 reductase, returned within 6–10 days while aniline hydroxylase activity took 3–6 days. Also, when the level of cytochrome P-450 was restored, aniline–cytochrome P-450 binding spectra also returned to a typical type II spectrum. One possibility to consider is that the time required for reversal may be due to the time needed for resynthesis of liver microsomal protein in the vitamin C deficient animal. Studies by Levin and Kuntzman[25] in the young rat have indicated that there are two rates of synthesis of the hemoprotein associated with cytochrome P-450: a fast phase which has a half-life of 7 hr, and a slow phase which has a half-life of 48 hr. Studies of rates of synthesis of this protein have not been done in young guinea pigs, and it would be of interest to determine if the time required for reversal of drug-metabolizing activities in the vitamin C deficient guinea pigs correlate with the time required for hemoprotein synthesis in this species. Our studies with phenobarbital induction indicate that the protein-synthesizing mechanism in vitamin C deficient guinea pigs is operable and not jeopardized by the depletion of vitamin C. Liver microsomal cytochrome P-450, NADPH cytochrome P-450 reductase, aminopyrine *N*-demethylase, *p*-nitroanisole, *O*-demethylase and aniline hydroxylase were all inducible in vitamin C deficient guinea pigs; in fact, the degree of induction was equal to and in some cases, as with NADPH cytochrome P-450 reductase, greater than that in normal guinea pigs.

It is interesting that under the conditions of vitamin C deficiency, all the over-all drug oxidation activities examined, as well as the level and activity of microsomal electron transport components, were reduced in the order of 50–60 per cent, except for NADPH cytochrome P-450 reductase activity which was well below 85 per cent of normal activity. The possibility should be considered that vitamin C deficient guinea pigs possess auxiliary systems which are capable of metabolizing drugs. Staudinger *et al.*[26] have recently identified a microsomal ascorbic acid dependent NADH oxidase system in rat liver, kidney and adrenals, which, as an oxido-reductive system, was capable of metabolizing such drugs as acetanilide. We have found that normal and vitamin C deficient guinea pig liver microsomes also contain NADH-ascorbic acid oxidase activity in amounts comparable to that found by Staudinger in the rat (0·24 μmoles NADH oxidized/hr/mg microsomal protein in guinea pig compared to 0·40 μmoles in the rat). This enzyme activity in vitamin C deficient guinea pig liver microsomes was found to be approximately 50 per cent of the activity of normal guinea pig

microsomes (0·11 μmoles/hr/mg microsomal protein compared to 0·24 μmoles). This auxiliary NADH microsomal system may be an important pathway for drug metabolism in the absence of a functioning microsomal NADPH P-450 reductase system.

In an attempt to extrapolate the studies *in vitro* presented to the earlier studies *in vivo* in vitamin C deficient guinea pigs, we have found an excellent correlation of decreased microsomal drug-metabolizing activities with the findings *in vivo* of Axelrod *et al.*[5] They determined the biological half-life of aniline, antipyrine and acetanilide in the plasma of normal and vitamin C deficient animals. They also measured the time it took to reverse the increased half-life of the drug in vitamin C deficient animals by repleting the animals with ascorbic acid. The age and weight of the guinea pigs used were in the same order as ours (270 g), and they were on the vitamin C deficient diet for the same length of time (18–20 days). These investigators found that drug metabolism, as measured by the half-life of the drug in the plasma, was increased in the order of 60–70 per cent which is in reasonable agreement with our enzymatic studies *in vitro*. Of importance is the correlation in the reversal *in vivo*; for example, they found that the half-life of aniline required approximately 5–6 days to return to normal, and aminopyrine required 8 days to return to normal.

It is tempting to consider the significance of the studies presented with vitamin C deficient animals to man in that both these species cannot synthesize ascorbic acid. Although the animals were maintained on a vitamin C deficient diet for a relatively long period of time (21 days), they did not show appreciable weight loss, and frank scorbutic symptoms were not apparent. The possibility should be considered that humans, particularly in their growth period, with some deprivation of vitamin C may show sensitivity and possible toxicity to drugs. Of more importance would be the length of time required for a vitamin C deficient human to re-establish normal drug metabolism even after adequate supplements of the vitamin.

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