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STIMULATION OF DRUG METABOLISM BY ASCORBIC ACID IN WEANLING GUINEA-PIGS*

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Abstract—Drug metabolism activities such as *O*-demethylase and *N*-demethylase and liver microsomal electron transport components including cytochrome P-450 and NADPH-cytochrome P-450 reductase were determined in weanling guinea-pigs under vitamin C deficiency as well as with normal and high intake of the vitamin. Animals depleted of the vitamin for only 8 days, with no signs of scurvy or weight loss, had decreased drug enzyme activities. Further depletion of the vitamin resulted in marked reduction of the microsomal drug-metabolizing system. Furthermore, administration of high amounts of ascorbic acid (2–200 mg/day) led to significant increases in liver drug oxidation activities and electron transport components. Studies of K_m values in microsomes from vitamin C-deficient, normal and animals given high supplements of vitamin C showed no difference in the apparent affinity or V_{max} of a typical drug oxidation reaction, *N*-demethylation. Specificity studies indicate that ascorbyl palmitate or D-isoascorbic acid can replace the vitamin, in part, in maintaining drug metabolism.

TO DATE investigations both *in vivo* and *in vitro* from several laboratories have shown that vitamin C deficiency results in decreased metabolism of a variety of pharmacological agents.[1–9] These studies have demonstrated that *O*-demethylation, *N*-demethylation, hydroxylation reactions as well as individual liver microsomal electron transport components such as cytochrome P-450 and NADPH-cytochrome P-450 reductase are decreased in guinea pigs depleted of ascorbic acid. In addition, the decreased drug enzyme activities can be restored to normal levels if the deficient animals are given the vitamin for a period of 6–10 days.[1] However, the previous studies were done in adult guinea pigs (250–400 g) maintained on a deficient diet for a substantial length of time (from 14 to 21 days). The present study has as its aim the determination of liver microsomal over-all drug oxidation activities and electron transport components in weanling guinea pigs (age 2 weeks, weight 90–110 g) maintained on a deficient diet for a short period of time (7–8 days), not approaching the scorbutic state. In addition, it was important to determine what effects increasing amounts of ascorbic acid in the diet would have on the variety of drug-metabolizing activities. Kinetic studies on the apparent Michaelis–Menten affinity constants of a typical drug-metabolizing enzyme, *N*-demethylase, with microsomes isolated from animals on a normal diet, on a diet containing high amounts of ascorbic acid and on a vitamin C-deficient diet are presented. Experiments concerned with the specificity of ascorbic acid compared to analogues of the vitamin, such as D-isoascorbic acid and palmityl ascorbic acid, are given.

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MATERIALS AND METHODS

The following reagents were obtained from Fisher Scientific Co.: *p*-nitroanisole, disodium ethylenediamine tetra-acetate (EDTA), Ciocalteau reagent and thiourea. The following reagents were obtained from Nutritional Biochemical Corp.: glucose 6-phosphate, $NADP^+$, NADPH, nicotinamide, albumin (bovine Fraction V), ascorbic acid-deficient diet (guinea pig), L-ascorbic acid and D-isoascorbic acid. Aminopyrine was obtained from K & K Laboratories, Inc. Glucose 6-phosphate dehydrogenase (grade II) was obtained from Sigma Chemical Co. Carbon monoxide was obtained from Matheson Gas Products. 2,4-Dinitrophenylhydrazine was obtained from Eastman Organic Co. Ascorbyl palmitate was obtained from Hoffmann–La Roche, Inc.

Guinea-pigs. Weanling male albino guinea-pigs, Hartley strain (90–110 g) were placed on an ascorbic acid-deficient diet for 8 or 15 days. The body weights of the animals were not significantly different compared to a control group of animals given a normal chow diet supplemented three times a week with kale. The average weights of animals were: ascorbic acid-deficient diet for 8 days, 141 g; normal chow diet for 8 days, 135 g; ascorbic acid-deficient diet for 15 days, 183 g; normal chow diet for 15 days, 190 g. The concentration of liver ascorbic acid in the 8-day deficient animals was 2·5 mg/100 g as compared to 27 mg/100 g of liver in the control group. Guinea pigs on the vitamin C-deficient diet for 15 days had less than 1·0 mg ascorbic acid/100 g of liver. Upon sacrifice, about half of these animals had developed joint hemorrhages.

Other groups of weanling guinea-pigs were placed on a vitamin C-deficient diet for 8 days and were supplemented once a day with 1·0 ml of varying amounts of the vitamin (2·0, 5·0, 20, 50, 100 or 200 mg/ml), given orally. These groups of animals had normal weight gain (147 g) compared to control guinea pigs (135 g). The ascorbic acid concentration in the livers of the guinea-pigs under the varying regimens of the vitamin varied from 2·0 to 57 mg/100 g.

Guinea pig liver microsomes. At the end of the experimental period *in vivo* the animals were decapitated, exsanguinated and their livers were quickly removed and placed on ice. All the following procedures were carried out at 4°. Homogenates (5% w/v) in 1·15% KCl, 0·002 M Tris buffer, pH 7·4, were made with the livers with a Potter–Elvehjem glass homogenizer. The homogenate was centrifuged at 15,000 *g* for 15 min. The supernatant fraction was centrifuged at 100,000 *g* for 60 min, and the microsomal pellet was resuspended in 1·15% KCl, 0·002 M Tris buffer, pH 7·4, to one-eighth of the original supernatant fraction volume. The microsomal protein concentration was 4–6 mg/ml. All assays were performed on freshly prepared microsomes. However, stability studies with fresh and frozen microsomes at −4° showed no significant loss of drug enzyme activities after 2 days of freezing (less than 5 per cent).

Assays. The quantity of cytochrome P-450 in liver microsomes was measured by the method of Omura and Sato as previously described.[1,10] The quantity of cytochrome P-450 is expressed as μmoles P-450/100 mg of microsomal protein.

The determination of cytochrome P-450 reductase activity in liver microsomes was measured by following the formation of reduced cytochrome P-450-CO with NADPH as previously described.[1,11–13] Specific activity is expressed as μmoles cytochrome P-450 reduced/hr/100 mg of microsomal protein at 27°.

Microsomal *N*-demethylase activity was determined by measuring the formaldehyde (HCHO) formed during the demethylation of aminopyrine to 4-aminoantipyrine. The liberated formaldehyde was measured by the colorimetric procedure of Nash based on the Hantzsch reaction.[1,14] Specific activity is expressed as μmoles HCHO formed/hr/100 mg of microsomal protein at 27°.

The rate of microsomal *O*-demethylation of *p*-nitroanisole was measured spectrophotometrically as previously described.[1,15,16] Specific activity is expressed as μmoles *p*-nitrophenol formed/hr/100 mg of microsomal protein at 27°.

The concentration of ascorbic acid in livers of guinea pigs was determined in 5% (w/v) whole homogenates by the method of Roe and Kuether.[1,17] The concentration of ascorbic acid is expressed as mg ascorbic acid/100 g wet weight of liver.

Liver protein was determined by the method of Lowry *et al.*[18] Crystalline bovine serum albumin, Fraction V was used as a standard.

RESULTS

Effects of increasing amounts of ascorbic acid in vivo *on drug enzyme activities.* The data in Fig. 1 show a significant increase in NADPH-cytochrome P-450 reductase activity in weanling guinea pigs with increasing administration *in vivo* of ascorbic acid. The first point is the activity in 15-day ascorbic acid-deficient animals; 3·4 μmoles cytochrome P-450 reduced/hr/100 mg of microsomal protein at 27°. The second point, representing an activity of 5·3 μmoles cytochrome P-450 reduced, is the amount of reductase in 8-day ascorbic acid-deficient animals. The last point, 10·2 μmoles cytochrome P-450 reduced, is the level found in animals with a liver ascorbic acid concentration of 27 mg/100 g. This activity is 200 per cent higher than the activity in 15-day deficient animals, and 90 per cent higher than that found in 8-day deficient guinea-pigs.

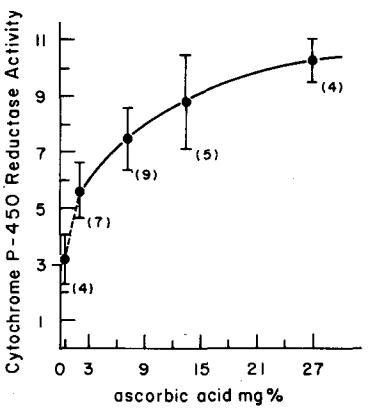

FIG. 1. NADPH-cytochrome P-450 reductase activity in weanling guinea-pigs given varying amounts of ascorbic acid. NADPH-cytochrome P-450 reductase activity was determined in liver microsomes as described in Materials and Methods. Specific activity is expressed as μmoles cytochrome P-450 reduced/hr/100 mg of microsomal protein at 27°. The number of animals in each group is given in the figure. The diets of the various groups containined: (1) less than 1·0 mg ascorbic acid/100 g liver, ascorbic acid-deficient diet for 15 days; (2) 2·3 mg ascorbic acid/100 g liver, ascorbic acid-deficient diet for 8 days; (3) 7·4 mg ascorbic acid/100 g liver, 25 mg/day of ascorbic acid given for 8 days; (4) 13·5 mg ascorbic acid/100 g liver, 75 mg/day of ascorbic acid given for 8 days; and (5) 27·0 mg ascorbic acid/100 g liver chow guinea-pig diet plus greens given for 8 days.

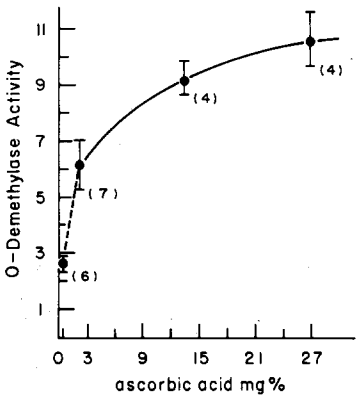

FIG. 2. *p*-Nitroanisole *O*-demethylase activity in weanling guinea-pigs given varying amounts of ascorbic acid. *p*-Nitroanisole *O*-demethylase activity was determined in liver microsomes as described in Materials and Methods. Specific activity is expressed as μmoles *p*-nitrophenol formed/hr/100 mg of microsomal protein at 27°. The number of animals in each group is shown in the figure. The diets of the various groups contained: (1) less than 1·0 mg ascorbic acid/100 g liver, ascorbic acid-deficient diet given for 15 days; (2) 2·7 mg ascorbic acid/100 g liver, ascorbic acid-deficient diet given for 8 days; (3) 13·5 mg ascorbic acid/100 g liver 75 mg/day of ascorbic acid given for 8 days; and (4) 27·0 mg ascorbic acid/100 g liver chow guinea-pig diet plus greens given for 8 days.

The pattern of increase in the quantity of liver microsomal cytochrome P-450 with increasing administration *in vivo* of ascorbic acid was similar to NADPH-cytochrome P-450 reductase but was of smaller magnitude. At a liver ascorbic acid level of 27 mg/100 g, the quantity of cytochrome P-450/100 mg of microsomal protein was 0·068 compared to 0·047 μmole in animals on a deficient diet for 15 days (liver ascorbic acid; $< 1{\cdot}0$ mg/100 g).

The data in Fig. 2 show a substantial increase in *p*-nitroanisole *O*-demethylase activity with increasing concentrations of liver ascorbic acid. The first point represents the activity in 15-day ascorbic acid-deficient guinea-pigs (2·6 μmoles *p*-nitrophenol formed/hr/100 mg of microsomal protein at 27°). The second point, representing 6·2 μmoles product formed, is the activity in liver microsomes of guinea pigs receiving no ascorbic acid for 8 days, a 140 per cent increase over the 15-day deficient animals. The last point in the figure, an activity of 10·5 μmoles product formed, is the level found in guinea pigs with a liver ascorbic acid concentration of 27 mg/100 g. This activity is 300 per cent higher than the level in 15-day deficient animals, and 70 per cent higher than 8-day deficient animals.

The pattern of increase in liver microsomal aminopyrine *N*-demethylase activity was similar to that found with *O*-demethylase activity. The activity in 15-day ascorbic acid-deficient guinea-pigs was 10·0 μmoles HCHO formed/hr/100 mg of microsomal protein at 27°. The activity in 8-day ascorbic acid-deficient guinea-pigs is 11·8 μmoles HCHO formed. Activity in microsomes of guinea pigs with a liver ascorbic acid concentration of 27 mg/100 g was 15·7 μmoles product formed, which is 60 per cent higher than that in 15-day deficient animals, and 35 per cent higher than the activity in animals deprived of the vitamin for 8 days. The increase in *N*-demethylase, although significant ($P < 0{\cdot}001$ compared to 15- and 8-day deficient animals) was not as striking as was found with *O*-demethylase.

The apparent Michaelis–Menten affinity constant of aminopyrine *N*-demethylation was determined with liver microsomes isolated from weanling guinea-pigs 8 and 15 days on a vitamin C-deficient diet, on a vitamin C-deficient diet for 8 days supplemented with 50 or 500 mg ascorbic acid/day, and on microsomes from animals receiving a chow diet. The K_m value for animals on a normal chow diet or 8-day deficient guinea-pigs was $1{\cdot}67 \times 10^{-3}$ M and the K_m value for 15-day deficient animals was $1{\cdot}82 \times 10^{-3}$ M. As was found with microsomes prepared from guinea-pigs on the vitamin C-deficient diet, there was no significant difference in the K_m values when the diets were supplemented with high amounts of ascorbic acid. The K_m value for animals given 50 mg/day of the vitamin was $1{\cdot}57 \times 10^{-3}$ M, while animals receiving 500 mg/day of ascorbic acid had an apparent affinity constant of $1{\cdot}85 \times 10^{-3}$ M. In addition, there were no significant differences in the V_{max} values (less than 8 per cent).

Specificity studies. Studies were carried out to determine if analogues of ascorbic acid, such as D-isoascorbic acid, or a more lipophilic analogue of the vitamin, ascor-

TABLE 1. EFFECT OF ASCORBIC ACID ANALOGUES ON DRUG METABOLISM ACTIVITY*

Treatment†	Liver ascorbic acid (mg/100 g)	P-450	P-450 reductase	*O*-demethylase	*N*-demethylase
Chow (5)	27	0·068 ± 0·002	10·2 ± 0·7	10·5 ± 1·0	15·7 ± 0·4
Ascorbate (75 mg) (5)	14	0·066 ± 0·006	8·9 ± 1·4	9·0 ± 0·5	15·6 ± 0·8
Ascorbate (25 mg) (6)	8	0·060 ± 0·004	7·5 ± 0·8	8·0 ± 0·5	15·9 ± 2·3
Ascorbate (7 mg) (5)	6	0·060 ± 0·004	7·0 ± 0·8	7·5 ± 0·5	14·1 ± 0·9
Deficient diet (8-day) (8)	2·5	0·061 ± 0·003	5·3 ± 0·9	6·2 ± 0·6	11·8 ± 0·8
Deficient diet (15-day) (6)	<1·0	0·047 ± 0·002	3·4 ± 0·4	2·6 ± 0·2	10·0 ± 0·8
D-Isoascorbate (200 mg) (11)	6	0·073 ± 0·012	3·2 ± 0·9	7·2 ± 1·6	19·1 ± 6·0
Ascorbyl Palmitate (50 mg) (6)	9	0·067 ± 0·008	7·5 ± 1·6	8·0 ± 1·8	12·3 ± 1·5

* Activities of cytochrome P-450, P-450 reductase, *O*-demethylase, *N*-demethylase and liver ascorbic acid concentration were determined as described in Materials and Methods. Units of activity: cytochrome P-450, μmoles/100 mg of microsomal protein; P-450 reductase, μmoles P-450 reduced/hr/100 mg of microsomal protein at 27°; *O*-demethylase, μmoles *p*-nitrophenol formed/hr/100 mg of microsomal protein at 27°, *N*-demethylase, μmoles formaldehyde formed/hr/100 mg of microsomal protein at 27°. Mean ± S.E. per group is given. Number in parentheses is number of animals in each group.

† Chow: animals were fed a chow diet supplemented three times/week with greens for 8 days.

Ascorbate, 75 mg: animals were fed a vitamin C-deficient diet and were given 75 mg ascorbic acid/day, orally, for 8 days.

Ascorbate, 25 mg: animals were fed a vitamin C-deficient diet, and were given 25 mg ascorbic acid/day, orally, for 8 days.

Ascorbate, 7 mg: animals were fed a vitamin C-deficient diet, and were given 7 mg ascorbic acid/day, orally, for 8 days.

Deficient diet, 8-day: animals were fed a vitamin C-deficient diet for 8 days.

Deficient diet, 15-day: animals were fed a vitamin C-deficient diet for 15 days.

D-Isoascorbate, 200 mg: animals were fed a vitamin C-deficient diet, and were given 100 mg D-isoascorbic acid two times/day, orally, for 8 days. It did not interfere with the ascorbic acid determination.

Ascorbyl palmitate, 50 mg: animals were fed a vitamin C-deficient diet, and were given 50 mg ascorbyl palmitate/day, orally, for 8 days. It did not interfere with the ascorbic acid determination.

byl palmitate, would be effective in increasing liver microsomal drug-metabolizing reactions (Table 1). As can be observed in animals on the ascorbic acid-deficient diet for 8 days, cytochrome P-450 reductase, *O*-demethylase and *N*-demethylase activities were significantly lower than in animals on a chow diet, or on a deficient diet receiving supplements of ascorbic acid with a liver ascorbic acid concentration of 14 mg/100 g ($P < 0.001$). NADPH-cytochrome P-450 reductase was 48 per cent lower; *O*-demethylase activity, 41 per cent; and *N*-demethylase activity, 25 per cent. However, there was no significant decrease in the level of cytochrome P-450 ($P < 0.4$). Fifteen-day deficiency caused an additional significant decrease in all the activities compared to controls ($P < 0.001$). Cytochrome P-450 was 31 per cent lower; P-450 reductase, 67 per cent; *O*-demethylase, 75 per cent; and *N*-demethylase, 36 per cent. D-Isoascorbic acid could replace the effect of the vitamin on some of the drug oxidation reactions. There was no significant difference in groups of animals receiving D-isoascorbic acid (liver ascorbate, 6 mg/100 g) and groups of animals receiving ascorbic acid with a liver ascorbic acid concentration of 6 mg/100 g for *O*-demethylation ($P = 0.5$), and *N*-demethylation ($P > 0.1$). However, P-450 reductase, in fact, was found to be lower than the control group (3·2 μmoles P-450 reduced compared to 5·3 μmoles P-450 reduced). Similarly, ascorbyl palmitate (liver ascorbic acid, 9 mg/100 g) could replace ascorbic acid compared to animals receiving ascorbic acid with a liver ascorbic acid concentration of 8 mg/100 g. There was no significant difference in P-450 reductase ($P > 0.5$), *O*-demethylase ($P > 0.5$), or *N*-demethylase activities ($P = 0.02$). It should be pointed out that there was a significant increase in the activities of P-450 reductase, *O*-demethylase and *N*-demethylase in animals on a deficient diet given supplements of ascorbic acid with liver ascorbic acid concentrations of 6 or 8 mg/100 g compared to animals on a deficient diet for 8 days ($P < 0.005$).

DISCUSSION

The present studies include the effect of ascorbic acid on drug metabolism in weanling animals maintained for a short period of time on a vitamin C-deficient diet; these animals were used, since they are in their rapid growth period and are more susceptible to a vitamin deficiency. Importantly, these studies indicate that deficiency not only results in decreased drug metabolism, but also the activity of the microsomal drug oxidation system increases over basal levels when the animals were given high supplements of the vitamin. NADPH-cytochrome P-450 reductase, *N*-demethylase and *O*-demethylase activities increased as much as 200 per cent, while the increase in the quantity of cytochrome P-450 was smaller but nevertheless significant over control values. This indicates that the cytochrome is not rate limiting with respect to the effect of ascorbic acid. On the other hand, cytochrome P-450 reductase activity markedly increased and showed a greater dependence on the liver concentration of ascorbic acid, since the increases observed correlated well with the amount of ascorbic acid present in the liver.

Specificity studies with analogues of ascorbic acid indicate that the stimulation of drug enzyme activities occurs with such compounds as D-isoascorbic acid or ascorbyl palmitate. However, much higher amounts of D-isoascorbic acid were required than the vitamin, which can be explained, in part, by the difficulty in obtaining equivalent liver concentrations of the D-isomer compared to vitamin C, since the former is rapidly excreted by the kidney.[19] Recently Degkwitz and Kim[20] found similar results

with the D-isomer of the vitamin. On the other hand, ascorbyl palmitate, a more lipophilic compound than the vitamin, was as effective as ascorbic acid on a molar basis.

The precise mechanism of how ascorbic acid either maintains or increases the drug-metabolizing system warrants further investigation. Determination of kinetic constants, K_m or V_{max}, under conditions of decreased, or for that matter increased over-all drug oxidation activities shows no alteration in the apparent affinity of a drug substrate such as aminopyrine. Previous studies by Zannoni *et al.*[1], Gundermann *et al.*[21] and Avenia,[8] on the other hand, have indicated that the quality as well as the quantity of the cytochrome P-450 is altered in vitamin C-deficient microsomes. Both type I and type II substrate binding spectra are atypical with microsomes isolated from vitamin C-deficient animals, in that the peak, trough and magnitude of absorption are altered. Changes in cytochrome P-450 substrate binding spectra may indicate an alteration in the structure of the hemeprotein. For that matter, the atypical binding spectra in vitamin C-deficient guinea-pig microsomes may reflect an alteration in the phospholipid membrane associated with cytochrome P-450. On the other hand, protein synthesis involved in the electron transport system does not appear to be affected in vitamin C deficiency, in that induction does occur in deficient animals given phenobarbital to the same extent as normal animals.[1,2] Further studies on the kinetics of drug-metabolizing reactions, alteration of physical and chemical properties of the various electron transport components, as well as binding properties of substrates to cytochrome P-450 with purified individual enzyme components involved in the partial reactions of the electron transport system are under investigation.

The role of vitamin C in the metabolism of drugs in man may be of clinical importance especially in the young. In this regard the recommended amount of the vitamin, 70–75 mg/day,[22] may not be adequate for optimum activity of the microsomal drug enzymes, if an increased formation of metabolites followed by subsequent conjugation reactions is necessary to ensure efficient detoxification. For example, the daily requirement of the vitamin for guinea pigs, i.e. to prevent scurvy, is 1–2 mg/day.[23] This amount of the vitamin gives rise to a liver ascorbic acid concentration in the order of 5 mg/100 g of liver and is not sufficient for optimal microsomal drug enzyme activity. On the other hand, other species, such as the mouse and rat, which can synthesize the vitamin, have liver ascorbic acid concentrations in the order of 25 mg/100 g of liver.[23] This concentration of ascorbic acid gives maximum drug enzyme activity in the guinea-pig and can be attained in guinea-pigs, a species which, like man, cannot synthesize the vitamin, only when high supplements of the vitamin are given (about 50 times higher than the recommended daily requirement). It would be of interest to determine if increased intake of ascorbic acid in man, above the daily recommended requirements, would decrease the biological plasma half-life of a variety of commonly used drugs.

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