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EFFECTS OF DIETARY ASCORBIC ACID SUPPLEMENTATION ON HEPATIC DRUG-METABOLIZING ENZYMES IN THE GUINEA PIG

BRANIMIR IVAN SIKIC, EDWARD G. MIMNAUGH and THEODORE E. GRAM
Laboratory of Toxicology, National Cancer Institute, Bethesda, MD 20014, U.S.A.

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Abstract—Guinea pigs were maintained on four diets which varied only in ascorbic acid (AA), containing 0.3, 1.5, 3.5 and 7.0 mg AA/g of diet. The lowest diet contained sufficient AA to prevent scurvy, while the two diets highest in AA content produced liver saturation of the vitamin after 8 weeks. Several hepatic enzymes were measured after 1, 2, 3, 4 and 8 weeks. There were no significant differences among the four groups in the following parameters of microsomal mixed-function oxidation: cytochrome P-450 content, NADPH cytochrome *c* reductase and aminopyrine *N*-demethylase. Also, there were no differences in native and nucleotide-activated UDP-glucuronyl transferase activities. However, in the post-microsomal supernatant, overall glutathione *S*-aryl transferase activity was significantly lower in the group with the lowest supplementation of ascorbic acid, as compared to the three higher groups. Kinetic parameters of aminopyrine *N*-demethylation were studied in animals maintained for 21 days on a diet deficient in ascorbic acid (< 0.1 mg AA/g), an intermediate control diet (1.5 mg AA/g) and a high-dose AA diet (7.0 mg AA/g). Curvilinear Lineweaver–Burk plots for *N*-demethylation were obtained in all three groups, and apparent K_m and V_{max} calculated separately for both low and high ranges of substrate concentrations. Apparent K_m values did not differ significantly among the three groups. Microsomes from AA-deficient animals had V_{max} values lower than the control and high-dose groups. There was no difference in kinetic parameters between microsomes from animals receiving control and high-dose AA diets.

Most studies of the role of ascorbic acid (AA) in drug metabolism have dealt with the effects of deficiency of the vitamin in the guinea pig [1–13]. After 12–14 days of deficiency, significant decreases in hepatic cytochrome P-450 content and overall microsomal mixed-function oxidation (MFO) *in vitro* are observed, as well as prolonged plasma half-lives and actions of drugs *in vivo*. These effects of scurvy persist and become more marked until death occurs after 28–32 days of AA deficiency, although recovery of health and drug-metabolizing activity occurs within 3–7 days if ascorbic acid is readministered [12, 13]. Deficiency of AA also affects drug conjugation reactions, with decreases in glutathione *S*-aryl transferase and increases in UDP-glucuronyl transferase activities [14]. Mechanisms for these effects of AA deficiency on drug-metabolizing enzymes are not known, although there is evidence that AA may be essential for normal synthesis of the heme component of cytochrome P-450 [8–11].

Because deficiency of AA reduces most parameters of drug metabolism, it has been proposed that increasing levels of ascorbic acid supplementation might increase drug metabolism in a dose–response fashion [15–17]. However, data supporting this suggestion were derived from experiments utilizing weanling guinea pigs in which various diets and routes of administration were employed, so that the amount of ascorbic acid intake was not the only variable present [15–17]. A recent study in humans demonstrated that high doses of ascorbic acid for 10–14 days had no effect on plasma half-lives of antipyrine and on steady-state plasma levels of diphenylhydantoin [18].

The purpose of the present study was to determine whether maintenance of adult guinea pigs on four diets which differed only in their content of ascorbic acid would affect levels of hepatic drug metabolism. Sato and Zannoni report [15–17] increases in aminopyrine *N*-demethylase activity with increasing intake of AA, but no changes in the V_{max} of this enzyme with increasing AA intake, an apparent contradiction. Also, there are suggestions in the literature that the apparent K_m for microsomal aminopyrine *N*-demethylase may be altered in AA deficiency in the absence of changes in V_{max} [12, 13], as well as reports of no effects on K_m [15–17]. Accordingly, we estimated these parameters in microsomes prepared from animals receiving diets deficient in AA, intermediate controls, and supplemented with a large dose of AA for 3 weeks.

MATERIALS AND METHODS

Animals. Young adult, male Hartley guinea pigs (Buckberg Laboratories, Tompkins Cove, NY) weighing 400–450 g at the outset were maintained on diets and water *ad lib.*

Diets. During the 8-week experimental period, animals were maintained on one of four pelleted guinea pig diets which differed only in AA content. The diets were formulated by adding ascorbic acid to an AA-deficient diet (ICN Pharmaceuticals, Inc., Cleveland, OH; Cat. No. 100778) before the pelleting process. Weekly ascorbic acid analysis (see below) of the diets revealed the following contents of AA which did not change during the course of the experiment: diet A, 0.3 mg AA/g diet; B, 1.5 mg/g; C, 3.5 mg/g; and D,

7.0 mg/g. The dietary method of administration of AA was chosen because it would provide a more constant tissue level of AA and avoid the trauma and intermittency of injection or oral intubation.

In the experiment measuring kinetic parameters of aminopyrine *N*-demethylation, guinea pigs were maintained for 21 days on diet B (intermediate control), diet D (high-dose AA), or the basic AA-deficient diet which contained less than 0.05 mg AA/g.

Tissue preparation. Six animals in each group were sacrificed by decapitation after 1, 2, 3, 4 and 8 weeks of maintenance on the four diets. Blood was collected for measurement of plasma ascorbic acid content. Livers were excised and microsomes and post-microsomal supernatant prepared as previously described [19].

Ascorbic acid assay. AA content of plasma, tissues and diets was assayed by a modification of the method of Sullivan and Clarke [20], adapted by Zannoni *et al.* [21]. Protein was precipitated by adding 0.25 ml of 40% trichloroacetic acid (TCA) to 1.5 ml of plasma on ice. The samples were centrifuged at 15,000 g for 15 min at 4°, and the following reactants then added to 0.5 ml of the supernatant: 0.05 ml of 85% orthophosphoric acid, 0.05 ml of 8% α,α'-dipyridyl in ethanol, and 0.05 ml of 3% aqueous ferric chloride. The ferrous-dipyridyl chromophore was allowed to develop for 60 min at room temperature and read in a Gilford 2400 spectrophotometer, at 525 nm, using microcuvettes. Standards were determined in triplicate for each set of samples, and experimental samples were determined in duplicate.

Liver AA was determined using 10,000 *g* tissue supernatant, since in prior experiments it was found that this was a valid reflection of whole organ content, and that the AA content of the microsomal fraction was very low. Assay of liver AA was similar to the plasma assay except that protein precipitation was done by adding 2.0 ml of 5% TCA to 0.5 ml of 10,000 *g* liver supernatant.

AA content of the pelleted diets was monitored by homogenization of 5–10 g feed with 10 vol. of glass-distilled water in a Virtis blender, precipitation with 4 vol. of 5% TCA, and analysis as above.

Enzyme assays. Incubations were performed aerobically at 37°, with saturating concentrations of cofactors and substrates (except in the kinetic experiments), and were linear with respect to incubation time of 15 min and to the enzyme concentrations. Final protein concentration for cytochrome P-450 determinations was 3.0 mg/ml and for the other assays ranged from 0.2 to 1.0 mg protein/ml of incubation mixture.

Cytochrome P-450 was estimated by the dithionite difference spectrum according to Omura and Sato [22], using an Aminco–Chance dual wavelength/split beam recording spectrophotometer. NADPH cytochrome *c* reductase activity was measured by the method of Williams and Kamin [23], as modified by Gigon *et al.* [24]. The Nash procedure for formaldehyde [25] was used to determine *N*-demethylation of aminopyrine [26].

"Native" microsomal UDP-glucuronyl transferase was assayed using *o*-aminophenol as substrate, as previously described [19, 27], in the presence of 25 mM $MgCl_2$ and 5.0 mM UDP-glucuronic acid. Microsomes were also incubated with an optimal concentration of the activator, UDP-*N*-acetylglucosamine (UDP-NAG), 2.0 mM [28, 29], and these values are termed "activated" UDP- glucuronyl transferase.

Glutathione (GSH) *S*-aryl transferase activity in post-microsomal supernatant was measured with 1,2-dichloro-4-nitrobenzene as substrate, according to Grover and Sims [30], using a Gilford 2400 recording spectrophotometer.

Protein concentrations were determined according to Lowry *et al.* [31].

Aminopyrine N-demethylase kinetics. Incubations for the determination of kinetic parameters of aminopyrine *N*-demethylation were performed with a microsomal protein concentration of 1.0 mg/ml, incubation time of 10 min, and the following concentrations of substrate: 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 5.0 and 10.0 mM. A weighted, least-squares fit computer program for statistical analysis of the kinetic data was used, as described by Cleland [32]. In preliminary experiments, it became evident that Lineweaver–Burk plots for aminopyrine *N*-demethylation were non-linear, and that they could be graphically represented by two intersecting straight lines generated by applying the computer program to substrate concentrations of 0.25 to 1.0 mM and 1.0 to 10.0 mM respectively. Thus, separate apparent K_m and V_{max} values were calculated for the lower and higher ranges of substrate concentrations, resulting in a statistically better fit to the actual experimental points than when all substrate concentrations were used to generate only one K_m and V_{max}.

RESULTS

During 8 weeks of maintenance of guinea pigs on diets A, B, C and D, there were no differences among the groups in body weight, liver weight or microsomal protein content.

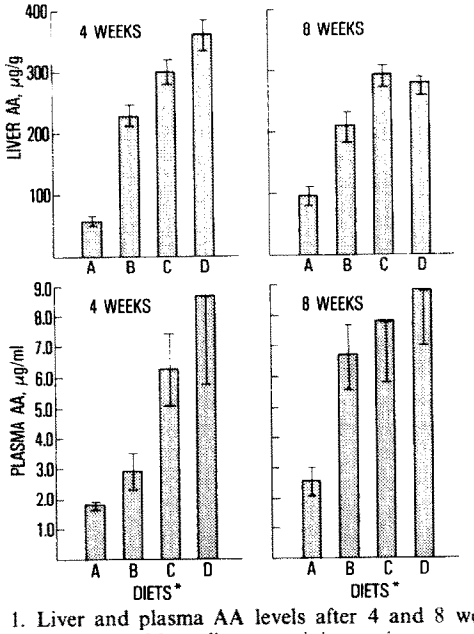

Fig. 1. Liver and plasma AA levels after 4 and 8 weeks of administration of four diets containing various amounts of ascorbic acid. Key: (*) diet A contained 0.3 mg AA/g diet; diet B, 1.5 mg/g; diet C, 3.5 mg/g; and diet D, 7.0 mg/g. Values are expressed as mean ± S. E. M.; N = 6 at each time point.

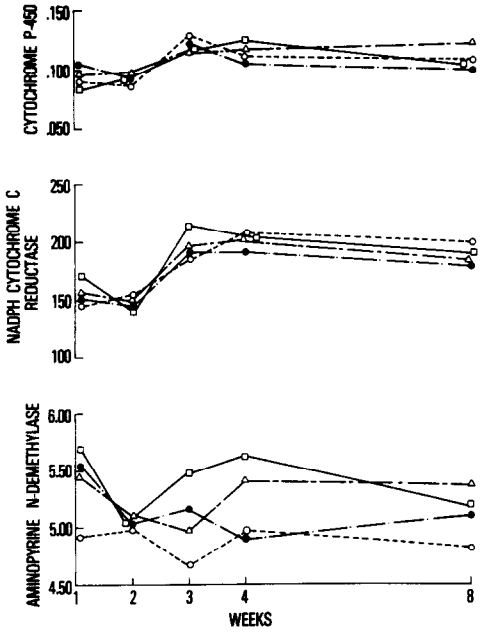

Fig. 2. Parameters of hepatic mixed-function oxidation during 8 weeks of maintenance on four diets containing various amounts of AA. Key: (O---O) diet A, 0.3 mg AA/g; (●-·-·-●) diet B, 1.5 mg/g; (Δ---Δ) diet C, 3.5 mg/g; and (□——□) diet D, 7.0 mg/g. N = 6 at each time point.

Figure 1 presents the liver and plasma AA values of the four groups after 4 and 8 weeks. There was apparent hepatic saturation with AA on diets C and D, as indicated by tissue AA content at 8 weeks. This saturation was not evident in plasma, which thus more closely reflected differences in AA content of the four diets. These adult guinea pigs ingested 20–30 g of feed daily, so that their estimated daily AA intake per animal was as follows: diet A, 6–8 mg AA; diet B, 30–40 mg AA; diet C, 70–100 mg AA; and diet D, 150–200 mg AA.

The effects of these varying intakes of AA on parameters of hepatic microsomal MFO are shown in Fig. 2. There were no statistically significant differences among the four groups in cytochrome P-450 content, NADPH cytochrome *c* reductase activity, or overall MFO activity as measured by *N*-demethylation of aminopyrine.

Furthermore, there were no significant differences among the four groups in either "native" or "activated" UDP-glucuronyl transferase activity after 8 weeks of maintenance on the diets (Table 1).

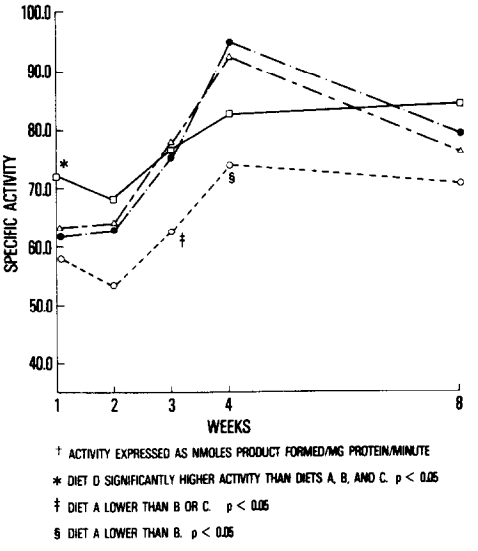

Fig. 3. Glutathione *S*-aryl transferase activities during 8 weeks of maintenance on four diets containing various amounts of AA. Key: (O---O) diet A, 0.3 mg AA/g; (●-·-·-●) diet B, 1.5 mg/g; (Δ---Δ) diet C, 3.5 mg/g; and (□——□) diet D, 7.0 mg/g. N = 6 at each time point.

The only enzyme activity in which differences occurred based on the level of AA intake was glutathione (GSH) *S*-aryl transferase (Fig. 3). Animals receiving diet A, which had the lowest content of ascorbic acid, exhibited consistently lower activities of GSH *S*-aryl transferase at 1, 3 and 4 weeks, amounting to about 25 per cent decreases when compared to the other diets. Except for the first weeks, there were no differences in GSH transferase activity between the intermediate (diet B) and high-dose AA diets, C and D.

Apparent kinetic constants of aminopyrine *N*-demethylation were measured after 21 days of maintenance on diet D (high-dose AA), diet B (intermediate, control), and an AA-deficient diet. Lineweaver–Burk plots were non-linear for all three groups, as shown in Fig. 4. Analysis of these data with a computerized, iterative, least-squares method [32] revealed a closer fit when specific activities at substrate concentrations of 0.25 to 1.0 mM were analyzed separately from specific activities at 1.0 to 10 mM aminopyrine. Apparent Michaelis–Menten constants were derived for both lower and higher sets of substrate concentrations, possibly corresponding to a high affinity catalytic site and a lower affinity site. These values are presented in Table 2, along with

Table 1. Native and activated UDP-glucuronyl transferase activities after 8 weeks of maintenance on diets with different AA contents*

Diet	A	B	C	D
Native	1.48 ± 0.24	1.52 ± 0.47	1.06 ± 0.25	1.15 ± 0.52
Activated	5.20 ± 1.43	5.75 ± 1.12	5.43 ± 1.50	4.86 ± 0.72

* Specific activities are expressed as mean ± S. D. in nmoles product formed/mg of protein/min; N = 6. The substrate used was *o*-aminophenol. All incubations contained 25 mM Mg^{2+} and 5 mM UDP-glucuronic acid. Activated liver microsomes contained 2.0 mM UDP-*N*-acetylglucosamine, whereas native microsomal incubations contained no exogenous UDP-*N*-acetylglucosamine.

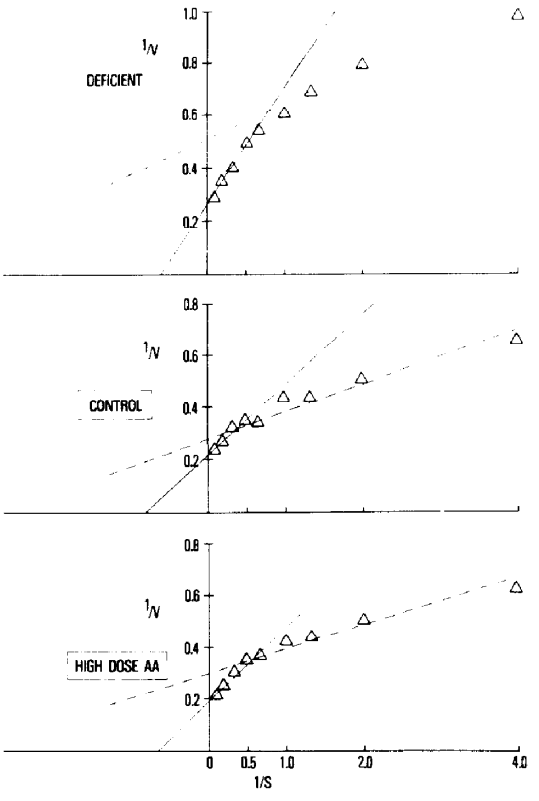

Fig. 4. Lineweaver–Burk plots of microsomal aminopyrine *N*-demethylation after 21 days of maintenance on deficient, intermediate and high-dose AA diets.

plasma and liver ascorbic acid contents for animals in the three groups. There was no significant difference in apparent K_m among the three groups, either at the low or the high substrate concentrations. V_{max} at both sets of substrate concentration of the AA-deficient group was significantly lower than the control and high dose AA groups. However, there was no difference in V_{max} when the control was compared to high-dose AA.

DISCUSSION

Although Sato and Zannoni have suggested that there may be a dose–response relationship between ascorbic acid intake and liver MFO activity, particularly cytochrome P-450 content [15–17], we find no evidence for such an effect in these experiments. The only variable in the four diets used here was AA content, which ranged from low levels which were more than adequate to prevent pathological signs of scurvy (diet A, 0.3 mg AA/g diet) to a tissue-saturating high-dose AA diet (diet D, 7.0 mg AA/g diet). Thus, the decreases of hepatic cytochrome P-450 and overall MFO which accompany AA deficiency [1–13] occur below a fairly low threshold of AA intake, and above this threshold we find no effect of increasing AA intake on hepatic MFO parameters.

Similarly, there was no effect of high levels of AA intake on UDP-glucuronyl transferase activities. In a previous study [14], we found that "native" UDP-glucuronyl transferase was increased in scurvy, whereas UDP-NAG "activated" enzyme levels did not differ from controls, again indicating an effect of deficiency, but no effect of increasing AA above a low threshold level.

The results with glutathione *S*-aryl transferase are different in that there was an approximately 25 per cent decrease in activity in the diet A group as compared to the other three groups. Deficiency of AA produces 30–50 per cent decreases in GSH transferase activity [14]. Optimal levels of GSH transferase activity appear to require higher levels of AA supplementation in the guinea pig than either MFO or glucuronyl transferase. Experiments are currently underway in our laboratory to investigate the relationship between GSH transferase, endogenous levels of the cofactor GSH, and dietary levels of AA, and the implications these might have on the toxicity of drugs which are excreted partly via glutathione conjugation.

The discrepancies between our results and those of Zannoni's group may be due to a number of reasons. We used pelleted diets varying only in AA content, while they compared animals undergoing depletion (8 and 15 days on a deficient diet) to animals on a chow plus greens diet, and others to which AA was administered via drinking water or by an oral bolus [15–17]. In addition, we worked with young adult guinea pigs rather than weanlings.

Our kinetic data on microsomal aminopyrine *N*-demethylase are in contrast to previously published studies in guinea pigs [16, 17] in that our Lineweaver–Burk plots are consistently non-linear in control, deficient, and high-dose AA groups. However, kinetic studies with aminopyrine demethylation in other species, notably rats, rabbits, and goats, also

Table 2. Effects of dietary ascorbic acid deficiency and high-dose supplementation on kinetic parameters of microsomal aminopyrine *N*-demethylation

	Plasma AA (μg/ml)	Liver AA (μg/g)	Substrate concn* (0.25–1.0 mM)		Substrate concn* (1.0–10.0 mM)	
			K_m (mM)	V_{max}†	K_m (mM)	V_{max}†
Deficient	1.4 ± 0.3	27 ± 7	0.28 ± 0.10	1.98 ± 0.70	1.53 ± 0.41	3.68 ± 1.02
Control	3.4 ± 1.6	246 ± 60	0.40 ± 0.14	3.66 ± 0.73‡	1.23 ± 0.30	4.56 ± 0.87§
High-dose AA	6.6 ± 2.2	272 ± 32	0.31 ± 0.09	3.28 ± 1.12	1.51 ± 0.28	5.28 ± 1.39‡

* N = 10 for aminopyrine concentrations 0.5, 1.0, 2.0, 3.0, 5.0 and 10.0 mM. N = 4 for aminopyrine concentrations 0.25, 0.75 and 1.5 mM.

† Formaldehyde [nmoles formed/mg of protein/min (mean ± S. D.)].

‡ $P < 0.05$, control or high-dose AA vs deficient.

§ $P = 0.05$, control vs deficient.

demonstrated non-linear kinetics [33, 34]. The latter authors speculate that two enzymes may be involved in the reaction, or that product or substrate inhibition may occur at higher concentrations of substrate. The fact that the kinetics of 4-monomethyl-aminoantipyrine demethylation are also non-linear favors the involvement of two separate enzymes or catalytic sites on the same enzyme [34]. Thus, our kinetic data may indicate the presence of a high affinity binding site operational at lower substrate concentrations and a lower affinity binding site at higher substrate concentrations. The presence of multiple forms or isozymes of cytochrome P-450 is now well-recognized [35–37], and these differ in substrate specificities, cyanide affinity and other physicochemical properties. It is conceivable that the non-linearity and our kinetic plots represents binding by two or more forms of cytochrome P-450. It is of interest that if one derives separate apparent V_{max} values for the set of lower substrate concentrations and the set of higher substrate concentrations, both of these V_{max} values are reduced in AA-deficient animals. Thus, the effects of deficiency of AA are not specific to one or the other of the hypothesized "binding sites" for aminopyrine. As in the 8-week diet experiment, our kinetic data showed that AA deficiency may produce changes compared to an intermediate level of AA intake, but there were no significant differences between animals receiving intermediate and high doses of AA.

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