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Urinary carnitine excretion increases during experimental vitamin C depletion of healthy men

Robert A. Jacob and Frederick S. Pianalto

Western Human Nutrition Research Center, U.S. Department of Agriculture, Agricultural Research Service, Presidio of San Francisco, CA; and California Occupational Safety and Health Administration, West Covina, CA

The requirement for ascorbic acid in the biosynthesis of carnitine may provide an explanation for the muscle weakness of scurvy and the basis for a functional measure of ascorbate status. To determine the relationship between vitamin C nutriture and carnitine status in humans, we measured total plasma and urinary carnitine concentrations in samples taken from two vitamin C depletion/repletion studies performed with healthy men on a metabolic unit. Throughout the 13-week studies, the groups of nine and eight men consumed a vitamin C-deficient diet that was supplemented with ascorbic acid to provide varying intakes of the vitamin from 5 to 605 mg/day. The subjects attained a state of moderate, nonscorbutic vitamin C deficiency during periods of low vitamin C intake, as indicated by plasma and leukocyte ascorbate concentrations. Plasma carnitine and triglyceride concentrations were not affected by the various vitamin C intakes; however, urinary carnitine excretion was increased during periods of ascorbate deficiency and was inversely related to leukocyte ascorbate concentrations. Vitamin C deficiency increases carnitine excretion, but the increased carnitine loss has no effect on carnitine status over a period of nearly 9 weeks. Total plasma carnitine is not a useful functional measure of human vitamin C status. (J. Nutr. Biochem. 8:265–269, 1997) © *Elsevier Science Inc. 1997*

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Introduction

Carnitine is required to transport long-chain fatty acids across the mitochondrial membrane for providing energy via β-oxidation.[1] Ascorbate is a cofactor with iron for two steps in the carnitine biosynthesis pathway: the enzymatic hydroxylations of trimethyllysine and γ-butyrobetaine.

The effect of vitamin C nutriture on carnitine status is of interest for several reasons. The unexplained fatigue and muscle weakness observed in human scurvy may be attributable to the loss of fatty acid-based energy production because of limited carnitine biosynthesis; e.g., tissue carnitine is significantly decreased in ascorbate depleted guinea pigs and human carnitine deficiency results in muscle weakness.[2–4] Current methods for assessing human vitamin C status are limited to plasma and leukocyte ascorbate measures because no suitable functional index of vitamin C status has been identified. Body carnitine concentrations may provide a functional marker of human vitamin C status. Finally, a definitive linkage between vitamin C nutriture and carnitine status would suggest that vitamin C intake may affect energy balance under certain dietary conditions.

Results of guinea pig and human studies do not provide a consistent or clear picture of the vitamin C-carnitine interaction. Generally, guinea pig studies show that ascorbate deficiency results in decreased carnitine concentrations of some tissues, whereas effects on blood carnitine have been mixed.[2–5] Alkonyi et al. found that the carnitine depletion of vitamin C deficient guinea pigs was caused by both impaired conversion of butyrobetaine to carnitine and by increased urinary elimination of carnitine.[4] More recently, Rebouche's experiments indicated that carnitine depletion in scorbutic guinea pigs was not attributable to defective biosynthesis, but to increased urinary excretion resulting from inefficient renal tubular reabsorption of carnitine.[6,7] The results from human studies are even less

Address reprint requests to Dr. Robert A. Jacob at USDA Western Human Nutrition Research Center, P.O. Box 29997, Presidio of San Francisco, CA 94129 USA.
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655 Avenue of the Americas, New York, NY 10010

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Table 1 AA and carnitine concentrations of healthy men during study HNS-7*

Metabolic period		AA intake	Plasma ascorbate	Leukocyte ascorbate	Plasma carnitine	Urinary carnitine
No.	Length (week)	mg/day	μmol/L	nmol/10^8 cells	μmol/L	μmol/day
1	2	65	39.2 ± 3.9[a]	172 ± 14[a]	60.9 ± 3.9[a,b]	580 ± 71[a]
2	4	5	7.4 ± 0.9[b]	95 ± 8[b]	60.0 ± 2.4[a]	568 ± 40[a]
3	3	605	75.5 ± 3.6[c]	232 ± 39[c]	66.0 ± 1.8[b]	581 ± 55[a]
4	4	5	12.5 ± 0.7[d]	53 ± 3[d]	60.7 ± 3.1[a,b]	777 ± 101[a]

*Values are means ± SEM for nine subjects at the end of each metabolic period. Means within vertical columns not sharing the same superscript letter are significantly different by paired *t*-test, $P < 0.05$.

clear. In elderly female hospital patients with low vitamin C status, leukocyte ascorbate did not correlate with plasma carnitine, but correlated positively with urinary carnitine, and 200 mg/day ascorbic acid supplementation of the diet of healthy elderly men resulted in increased carnitine excretion.[8] In contrast, Johnston et al., recently reported an inverse relation between plasma ascorbate and free carnitine in 22 students with mild vitamin C deficiency who were repleted with vitamin C for 3 weeks.[9] The increased plasma carnitine in the ascorbate deficient students was attributed to impaired carnitine transport into tissues because of a rise in butyrobetaine, the immediate biosynthetic precursor to carnitine.

Because ascorbic acid (AA) is required for carnitine biosynthesis, one would hypothesize that vitamin C deficiency results in decreased body carnitine concentrations, and that low carnitine status may represent a functional marker of vitamin C deficiency. To clarify the effect of vitamin C nutriture on carnitine status in humans, we report here the results of plasma and urinary carnitine measurements from two vitamin C depletion/repletion studies performed with healthy men on a metabolic unit.

Methods and materials

Subjects and protocols

Plasma and urine carnitine values are reported for two vitamin C depletion/repletion studies performed in 1984 (HNS-7) and 1989 (HNS-19) on the metabolic unit of the USDA Western Human Nutrition Research Center (WHNRC). The study protocols and Informed Consents were approved by the Institutional Review Committee of the Letterman Army Medical Center, Department of the Army, Presidio of San Francisco, CA, and by the Human Studies Review Committee of the Agricultural Research Service, U.S. Department of Agriculture. In each study, 12 healthy, nonsmoking male volunteer subjects, ages 19 to 43 years, were admitted to the metabolic unit of the USDA, ARS Western Human Nutrition Research Center (WHNRC), after medical and psychological screening. For the duration of the 13-week studies, the subjects lived in and ate all meals in the WHNRC metabolic unit, and were chaperoned at all times when outside the unit.

Because of violations of the study protocol, or for personal reasons, three subjects left the HNS-7 study early and four left the HNS-19 study early. Data from these subjects were not included in this report. A fifth subject left the HNS-19 study after 10.3 weeks, during the final repletion period. This subject was given supplemental tablets of 250 mg of AA and was instructed to take one each day along with his regular diet. The subject remained outside the unit and came back to the unit at the end of the study when a blood sample (but not urine collection) was taken. Data from this subject were included along with the seven subjects who completed the entire study. Hence, complete data are reported for nine subjects for HNS-7 and for eight subjects for HNS-19 (except one urine carnitine measure at the last time point).

Experimental design and diet

The experimental designs are shown in *Tables 1* and *2* for studies HNS-7 and HNS-19, respectively. The subjects were fed a vitamin C deficient diet throughout both studies. The experimental variable was the amount of AA supplemented into the diet, as shown in *Tables 1* and *2*. During periods 3 and 4 of study HNS-19, subjects were split into two groups, receiving 10 or 20 mg AA/day in P3 and 60 or 250 mg/day in P4.

Subjects ate a rotating menu of natural foods, which excluded fruits, vegetables, or their juices in any form. The food was consumed at three meals and an evening snack daily. Details of the 7-day rotating menu for HNS-7[10] and HNS-19[11] have been reported previously. The diets contained a variety of meats (beef, chicken, turkey, tuna), carbohydrates (pasta, rice, bread), and sweet desserts (cookies, cakes, candy, ice cream), as well as cheese

Table 2 AA and carnitine concentrations of healthy men during study HNS-19*

Metabolic period		AA intake	Plasma ascorbate	Leukocyte ascorbate	Plasma carnitine	Urinary carnitine
No.	Length (week)	mg/day	μmol/L	nmol/10^8 cells	μmol/L	μmol/day‡
1	0.6	250†	59.6 ± 3.3[a]	209 ± 9[a]	50.8 ± 4.0[a]	314 ± 48[a]
2	4.6	5	6.8 ± 0.3[b]	117 ± 7[b]	53.6 ± 4.4[a]	564 ± 45[b,c]
3	4	10 or 20	6.2 ± 0.3[b]	100 ± 9[c]	53.2 ± 4.1[a]	641 ± 60[b]
4	4	60 or 250	48.8 ± 7.9[a]	211 ± 9[a]	51.1 ± 5.1[a]	526 ± 44[c]

*Values are means ± SEM for eight subjects at the end of each metabolic period. Means within vertical columns not sharing the same superscript letter are significantly different by paired *t*-test, $P < 0.05$.
†Subjects consumed a supplement of 250 mg AA per day in addition to their free-living diet, for 1 to 2 weeks before entering the study.
‡Mean of 4-day urine pool.

and low-fat milk. As calculated from food nutrient composition tables, the diets provided approximately 54% of energy from carbohydrates, 29% from fat (polyunsaturated/saturated fatty acid ratios of 0.95 and 0.33 for HNS-7 and HNS-19, respectively), and 17% from protein. The diets (plus supplements of folic acid, magnesium, and copper for HNS-7) provided >80% of the RDA of all essential nutrients except vitamin C. The vitamin C content of the diets averaged about 5 mg/day as calculated from food composition tables and about 1.6 mg/day of AA as analyzed by HPLC, the difference presumably due to some oxidized vitamin present in the food and being formed during the composite preparation (diet composites for HNS-19 but not HNS-7 were analyzed for vitamin C and total carnitine). The total carnitine content of the HNS-19 diet averaged 397 μmol/d with the individual daily values (in order of menu rotation) being 424, 122, 602, and 438 μmol/d.

The vitamin C intake was varied by including a drink with each meal which contained an amount of ascorbic acid (USP FCC, Fine Powder, Hoffmann-La Roche Inc., Nutley, NJ) to provide the daily ascorbic acid intakes shown in *Tables 1* and *2*. The ascorbic acid was supplemented into 200 mL of a grape juice drink for HNS-7 and into 100 mL of citrus flavored soda in HNS-19. The ascorbic acid supplemented drinks were prepared the day before serving and kept at 4°C until the aliquots were served at each of the 3 daily meals. Additional aliquots of the supplemented drinks were combined on one day during each metabolic period to provide a sample for checking ascorbic acid content. The aliquots were added to an equal volume of a solution of cold 100 g/L metaphosphoric acid (MPA) with 0.54 mmol/L Na_2EDTA to preserve the vitamin until analysis by HPLC.

Water was consumed ad libitum and consistent amounts of mineral water, noncaloric soda, decaffeinated coffee, and tea were consumed. No tobacco, alcohol, or drugs were allowed, except that aspirin, ibuprofen, or acetaminophen were given as analgesics and physician-prescribed drugs were allowed as needed.

Baseline body weights for each subject were established as the average weight over the first week of the study. Body weights were then maintained within ±3% of the baseline weight by supplementing the basal diet with caloric soda, sucrose, and Polycose® (Ross Laboratories, Columbus OH USA) sweetener for HNS-7 and by altering portion sizes of the diet served for HNS-19.

Specimen collections and analytical methods

Blood was taken by venipuncture after overnight fast using EDTA anticoagulant. For HNS-7, EDTA plasma was deproteinized with an equal volume of 0.61 mol/L trichloroacetic acid and total vitamin C determined by the spectrophotometric procedure using 2, 4-dinitrophenylhydrazine as a derivatizing agent.[12] A mixed leukocyte fraction of polymorphonuclear and mononuclear leukocytes was isolated from EDTA anticoagulated whole blood by a Percoll density gradient procedure and analyzed for AA by HPLC after deproteinization with 0.75 mol/L metaphosphoric acid (MPA).[12]

For HNS-19, EDTA plasma samples were added to an equal volume of a 100 g/L MPA and 0.54 mmol/L Na_2EDTA solution to stabilize ascorbic acid. After centrifugation, the supernatant was stored at −70°C until analysis for AA by the ion-pairing HPLC technique of Kutnink et al.[13] Mononuclear leukocytes (primarily lymphocytes) were isolated from 5 mL of blood with LeucoPREP Cell Separation Tubes (Becton Dickinson, Lincoln Park, NJ USA) as described previously[11] and AA analyzed by ion-pairing HPLC separation and electrochemical detection as described by Kutnink et al.[13] The HNS-19 daily diet composites (four) were homogenized in a blender with distilled-deionized water and aliquots were frozen at −70°C for total carnitine determinations. Separate aliquots of homogenate were mixed with an equal volume of a cold 200 g/L MPA/0.54 mmol/L Na_2EDTA solution to preserve the ascorbic acid, and frozen at −70°C for later determination of AA by the HPLC method of Kutnink et al.,[13] as previously described.[11]

Samples of EDTA plasma were taken for the determination of total carnitine and the samples frozen immediately at −70°C until thawed for analysis. Complete daily urine collections were taken throughout each study with urine collections refrigerated during collection. For HNS-19, 4-day urine pools were prepared by taking a constant volume fraction of each of four daily urine collections and mixing together. Total carnitine in plasma, urine, and diet homogenates was determined by the radioenzymatic method using ^{14}C-acetylCoA and carnitine acetyltransferase.[14]

Statistical analysis

The relation of plasma and urinary carnitine with leukocyte ascorbate and study day were examined by analysis of covariance (ANCOVA), by linear regression of the group means, and by calculation of Pearson product-moment correlation coefficients within individuals.[15] The effect of vitamin C intakes on plasma and urinary carnitine concentrations were assessed by performing paired *t*-tests using values at the end of the metabolic periods. The Mann-Whitney and Wilcoxon Signed Rank nonparametric tests were used where data were not normally distributed.[15] Tests results were considered statistically significant at $P < 0.05$. ANCOVA was performed using SAS for Personal Computers, Version 6 (SAS Institute Inc., Cary, NC USA) and all other statistical tests were performed using SigmaStat version 1.02 statistical software (Jandel Scientific Software, San Rafael, CA USA).

Results

The end of period means ± SEM for plasma and leukocyte ascorbate and plasma and urine carnitine are listed in *Table 1* for HNS-7 and *Table 2* for HNS-19. For HNS-19, because leukocyte ascorbate values were not significantly different between the period 3 (P3) subgroups getting 10 or 20 mg/day of vitamin C (90 ± 10 vs 111 ± 13 nmol/10^8 cells) and between the P4 subgroups getting 60 or 250 mg/day in P4 (197 ± 3 vs 225 ± 15 nmol/10^8 cells) the subgroups were combined for data analysis and to provide the $n = 8$ mean values in *Table 2*.

Plasma carnitine concentrations showed no significant relationship to vitamin C intake. Plasma carnitine increased during the 605 mg/d ascorbic acid repletion period of HNS-7 (*Table 2*), but concentrations after the two depletion periods were not different from baseline and no differences were seen after ascorbate depletion or repletion during HNS-19. Serum triglycerides did not change during HNS-19, the mean ± SEM values near the end of each period P1 to P4 were 1.38 ± 0.31, 1.11 ± 0.20, 0.93 ± 0.09, and 1.16 ± 0.20 mmol/L.

The relationship between urinary carnitine and leukocyte ascorbate for HNS-19 is shown in *Figure 1*. Linear regression of the eight mean values of urinary carnitine versus leukocyte ascorbate for HNS-19 showed a significant inverse relationship, $r = -0.727$, $P < 0.05$ (*Figure 2*). The analogous regression for urinary carnitine vs plasma ascorbate was also significant, $r = -0.746$. Pearson correlation coefficients calculated for urinary carnitine vs leukocyte ascorbate concentrations within individuals averaged −0.58

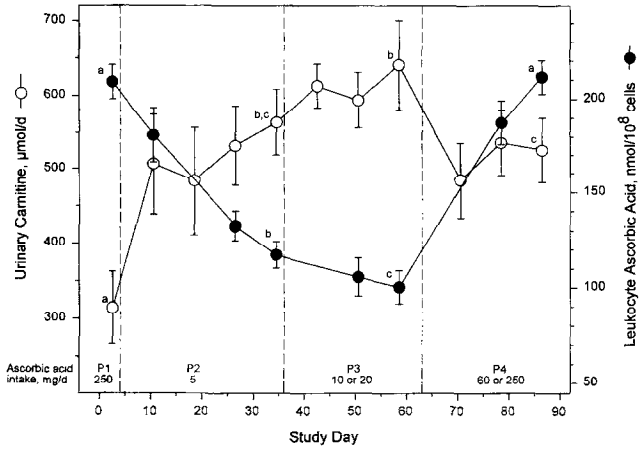

Figure 1 Total urinary carnitine and mononuclear leukocyte ascorbate concentrations of healthy men receiving various intakes of AA during study HNS-19. Ascorbic acid intakes are shown at bottom. Values are means ± SEM for eight subjects. Urine carnitine values are means from analysis of 4-day urine pools. Means not sharing the same superscript letter are significantly different, $P < 0.05$.

and ranged from −0.35 to −0.82 (only one was significant at $P < 0.05$; $n = 8$ data points, except $n = 5$ for subject 1 and $n = 6$ for subject 12). The analogous correlation coefficients for urinary carnitine vs plasma ascorbate concentrations averaged −0.65 and ranged from −0.43 to −0.90 (two were significant at $P < 0.05$; $n = 8$ data points, except $n = 6$ for subject 12).

Discussion

Few studies of the effects of vitamin C status on human carnitine metabolism have been published; the present report is the first to analyze carnitine excretion during experimental vitamin C depletion and repletion using 24-hr urine collections. Our results are similar to those of Davies et al.[8] in that plasma carnitine was not sensitive to moderate vitamin C deficiency short of scurvy. The lower limit of normal for leukocyte ascorbate is 114 nmol/10^8 cells for mixed leukocytes (used in HNS-7) and 142 nmol/10^8 cells for mononuclear cells (used in HNS-19).[16] This compares with mean ascorbate depletion values of 53 and 100 for HNS-7 and -19, as shown in *Tables 1* and *2*, respectively. Decreased leukocyte ascorbate concentrations more accurately reflect whole body and tissue ascorbate depletion than do plasma ascorbate values.[17] Our results, however, are not consistent with the recent findings of Johnston et al., where a significant inverse correlation of plasma-free carnitine with plasma vitamin C was observed in moderately vitamin C-deficient subjects who were repleted with vitamin C under controlled conditions (urinary carnitine was not measured in the study).[9] A convincing explanation for these differences is not apparent.

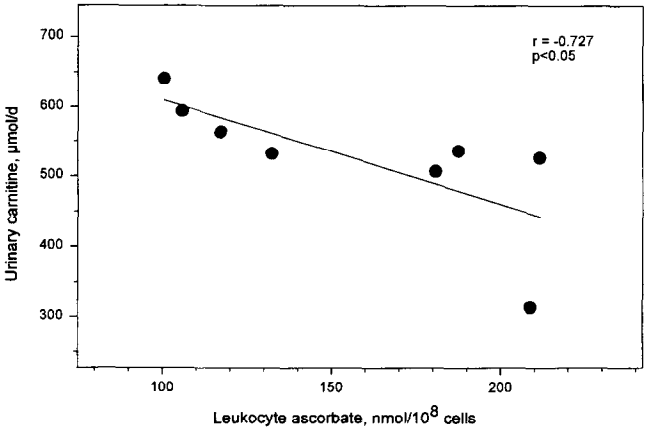

Figure 2 Linear regression of total urinary carnitine and mononuclear leukocyte ascorbate concentrations of healthy men receiving various intakes of AA from 5 to 250 mg/day during study HNS-19. Regression points are means ± SEM for eight subjects at the times shown for the leukocyte values in *Figure 1*. Urine carnitine values are means from analysis of 4-day urine pools.

The present results indicate that urinary carnitine excretion is increased during vitamin C deficiency. In study HNS-7 this is not seen in the first ascorbate depletion period P2 (*Table 1*), but is seen in the second depletion period P4 where leukocyte ascorbate drops to lower values compared with the first depletion period (the urinary carnitine increase in P4 was not statistically significant compared with P3, $P = 0.08$, because of large variances and small n values, but urine carnitine increased in eight of the nine subjects). In study HNS-19 the increase in urinary carnitine is seen during the 9-week depletion periods P2 and P3 as leukocyte ascorbate drops to deficient values, and the reverse pattern appears during the P4 repletion period (*Figure 1*). The initial rise in carnitine excretion may be partially attributable to increased carnitine in the experimental diet compared with the subjects' free-living diets; however, the *Figure 2* inverse correlation is similar with the baseline point deleted ($r = -0.789$). These results are at odds with those of Davies et al.,[8] who found a positive correlation of the urinary carnitine/creatinine ratio with leukocyte ascorbate in elderly female hospital patients with poor ascorbate status (although plasma carnitine was not related to ascorbate status). The present results, however, support the findings in guinea pigs that tissue carnitine depletion in vitamin C deficiency is attributable to decreased efficiency of renal reabsorption of carnitine rather than compromised biosynthesis.[4,7]

In ascorbate deficient guinea pigs, increased plasma triglycerides accompanying decreased tissue carnitine has been attributed to altered lipid metabolism due to limited transport of long-chain fatty acids into mitochondria.[3] The lack of change in plasma carnitine and serum triglycerides throughout HNS-19 suggests that the increased carnitine excretion over the 9 weeks of vitamin C depletion had no significant effect on functional carnitine status. Carnitine body status and function, however, may well have been compromised over a longer period of vitamin C deficiency.

In conclusion, urinary carnitine excretion increases in healthy men after tissue ascorbate depletion, but the increased carnitine excretion has no substantial effect on carnitine status over a period of 9 weeks. These results support previous work from guinea pig studies that show vitamin C deficiency increases carnitine excretion and

indicate that plasma carnitine would not be a useful functional measure of human vitamin C status.

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