Renal Handling of Carnitine in Experimental Vitamin C Deficiency

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Experimental vitamin C deficiency is associated with low carnitine concentrations in blood and some tissues, but is not due to a decreased ability of scorbutic animals to synthesize carnitine. The effect of experimental vitamin C deficiency on urinary carnitine excretion in vivo and carnitine transport into renal cortical brush-border membrane vesicles in vitro was investigated in guinea pigs fed normal and vitamin C–deficient diets for 24 days. Excretion of free and total carnitine was approximately fourfold greater in scorbutic animals as compared with normal guinea pigs during the last 6 days of the experimental regimen. The rate of carnitine transport into renal cortical brush-border membrane vesicles prepared from scorbutic animals was approximately 36% lower than the corresponding rate for vesicles prepared from normal animals. However, this effect was not specific, since rates of sodium gradient–dependent transport of glucose, lysine, and taurine (but not alanine) were also lower in vesicles prepared from scorbutic animals, although the magnitude of the decrease was less than for carnitine. The results are consistent with the hypothesis that carnitine depletion in vitamin C deficiency is due to decreased efficiency of carnitine reabsorption.

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A MANIFESTATION of vitamin C deficiency in guinea pigs is carnitine depletion in some tissues and the circulation (see Rebouche[1] for a review). Because ascorbic acid is a cofactor for two enzymes in the pathway of carnitine biosynthesis, it was suggested that carnitine depletion in scurvy could result from a reduced rate of carnitine synthesis. Although some evidence has been reported to support this proposal (see Rebouche[1] for a review), a recent study from this laboratory[2] has shown that when severely scorbutic guinea pigs are provided appropriate precursors (ie, substrates for enzymatic reactions requiring ascorbate), they synthesized carnitine at rates much higher than the rates in normal guinea pigs. Thus, the hypothesis that carnitine depletion in scurvy is due to a reduced rate of carnitine synthesis appears untenable.

Alkonyi et al[3] and Rebouche[2] have shown, with relatively small sample sizes, that the rate of urinary carnitine excretion is elevated in scorbutic guinea pigs as compared with normal animals. These results give rise to a hypothesis: Carnitine depletion in experimental vitamin C deficiency is due to decreased efficiency of carnitine reabsorption. This investigation was designed to extend the findings of Alkonyi et al[3] and Rebouche[2] to a large sample, and to determine if the increased rate of carnitine excretion observed in scorbutic guinea pigs in vivo is correlated with a decreased rate of carnitine transport into renal cortical brush-border membrane vesicles in vitro.

MATERIALS AND METHODS

Materials

Carnitine acetyltransferase and phenyl isothiocyanate (protein-sequencing grade) were obtained from Sigma Chemical (St Louis, MO). [*acetyl*-^{3}H]Acetyl coenzyme A, L-[3-^{3}H]alanine, [2-^{3}H(*N*)]taurine, L-[4,5-^{3}H(*N*)]lysine, and D-[6-^{3}H(*N*)]glucose were obtained from DuPont–New England Nuclear (Boston, MA). L-[*methyl*-^{3}H]Carnitine was synthesized as described previously.[4] All other chemicals were reagent grade and obtained from commercial sources.

Animals and Animal Care

Male Hartley guinea pigs weighing 231 to 318 g (at the start of the diet regimen) were purchased from SASCO (Omaha, NE). Animals were purchased and maintained in groups of six or 10. Animals were housed individually in wire-bottom, stainless steel cages equipped with stationary stainless steel food cups. For the final 6 days of the experimental regimen, some of the guinea pigs were housed in metabolic cages (catalog no. 650-0350; Nalge, Rochester, NY) for continuous collection of urine. All animals were provided free access to food and water, and were maintained on 12-hour light/dark cycles in a temperature-controlled room. All procedures that involved use of experimental animals were approved by the University of Iowa Animal Care and Use Review Committee.

Diets and Experimental Procedures

For the first 4 or 5 days after arrival, all guinea pigs were fed a stock diet (Guinea Pig Chow 5025; Purina Mills, Richmond, IN). After this period of acclimation, the animals were divided into two groups. The control group was fed the stock ration for the remainder of the study. The ascorbate-deficient group received a custom-prepared diet (Purina 5711C-7) formulated by the manufacturer to be identical to the stock ration, except with no added vitamin C. These semipurified diets contained 18% protein, 4% fat, and 16% fiber (manufacturer's analysis). The stock ration contained 1 g/kg vitamin C (from added vitamin C), whereas the ascorbate-deficient diet contained 30 to 40 mg vitamin C/kg diet, derived from natural ingredients (alfalfa, corn meal, soybean meal, etc.). All animals were fed the respective diets for 24 days. For animals housed in metabolic cages for the last 6 days (days 19 to 24 of the experimental regimen), urine was collected for each 24-hour period. Microbial growth was inhibited by addition of 1 mL 1-mol/L sulfuric acid to each collection vessel. All animals were weighed daily from the beginning of the study.

On the morning of day 25, the animals were anesthetized with ether, and blood was obtained by cardiac puncture. Animals were killed by asphyxiation, and liver, heart, and a portion of hindlimb skeletal muscle were removed, weighed, cut into small pieces, frozen in liquid nitrogen, and stored at −80°C for subsequent

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analysis. Kidneys were removed and weighed, and for some animals a small portion was retained for chemical analysis as described for other tissues. Most or all of the kidneys from animals within each group were pooled for preparation of cortical brush-border membrane vesicles, as described previously.[5] Each vesicle preparation was obtained from both kidneys of two to five guinea pigs.

Analytical Methods

Blood was collected into EDTA-treated plastic tubes, and plasma was obtained by centrifugation. Plasma was immediately divided into three aliquots. One aliquot was treated with 1 vol 10% (wt/vol) trichloroacetic acid, and the protein precipitate was removed by centrifugation. The supernatant was stored at −80°C for analysis of ascorbic acid. The second aliquot of plasma was stored untreated at −80°C for analysis of creatinine and free and total carnitine. The third aliquot of plasma and an aliquot of the day-24 urine collection were treated with an equal volume of 0.4 mmol/L methionine sulfone (in 0.1 mol/L HCl) and ultrafiltered by centrifugation in a Pico · Tag Ultracentrifugation Device (Waters, Milford, MA). The ultrafiltrates were stored at −80°C for subsequent amino acid analysis.

Ascorbic acid concentration in plasma and tissues, free (nonesterified) and total (nonesterified and esterified) carnitine concentrations in plasma, urine, and tissues, and creatinine concentration in plasma and urine were quantified as described previously.[2]

Amino acid (aspartic acid, glutamic acid, hydroxyproline, serine, glycine, glutamine, threonine, alanine, arginine, proline, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, tryptophan, lysine, and taurine) concentrations in plasma and urine were measured essentially using the method reported by Cohen and Strydom.[6] Amino acids derivatized with phenyl isothiocyanate were separated on a Pico · Tag Amino Acid Analysis column (Waters) using a Beckman System Gold Solvent Delivery Module (model 126; Beckman Instruments, Palo Alto, CA). The mobile phase consisted of 70 mmol/L sodium acetate, pH 6.5 (A), and acetonitrile (B). A binary gradient program was used: isocratic at 2.5% B, 14 minutes; concave gradient (Beckman program 5) 4.4% B to 5.6% B, 12.5 minutes; convex gradient (Beckman program 2) 5.6% B to 7.7% B, 4 minutes; linear gradient 7.7% B to 16% B, 20 minutes; linear gradient 16% B to 19% B, 10 minutes; linear gradient 19% B to 30% B, 4 minutes; linear gradient 30% B to 60% B, 1 minute; isocratic at 60% B, 3 minutes; linear gradient 60% B to 2.5% B, 1 minute; and isocratic at 2.5% B, 20 minutes. Column temperature was constant at 46°C, and eluent flow rate was 1 mL/min. Amino acid derivatives were quantified by absorption spectrophotometry at 254 nm. Results were indexed to recovery of the internal standard, methionine sulfone.

Rates of transport of radiolabeled solutes into renal cortical brush-border membrane vesicles prepared from control and ascorbate-deficient animals were measured essentially as described previously.[5] Solute concentrations in incubation mixtures were 50 μmol/L, and specific activities were 500 Ci/mol (carnitine and taurine), 200 Ci/mol (lysine), or 100 Ci/mol (glucose and alanine). Brush-border membrane protein concentrations were 1.49 to 2.19 mg/mL (89.4 to 131 μg per assay). All measurements were obtained at 37°C in a total volume of 60 μL. Osmolarity was constant at 300 mOs/L (chloride was the counter ion to sodium and potassium, and osmolarity was adjusted to 300 mOs/L with D-mannitol). Incubation times were 5 seconds (glucose and alanine) or 8 seconds (carnitine, taurine, and lysine). Solute uptake was terminated by addition of 1 mL ice-cold 150-mmol/L KCl and 1 mmol/L HEPES · Tris, pH 7.4, and rapid vacuum filtration and washing on a HAWP 002500 filter (Millipore, Bedford, MA). Transport was measured in the presence of inwardly directed sodium ion or potassium ion gradients (100 mmol/L outside, zero inside). Nonspecific uptake and/or binding was determined in the presence of 5 mmol/L (100-fold excess) unlabeled solute and an inwardly directed potassium ion gradient. Total transport was the difference between transport in the presence of an inwardly directed sodium ion gradient and nonspecific uptake and/or binding. Sodium gradient–dependent transport was the difference between rates measured in the presence of inwardly directed sodium and potassium ion gradients. All measurements were obtained in triplicate.

Specific activities of marker enzymes (alkaline phosphatase, γ-glutamyl transpeptidase, citrate synthetase, succinate dehydrogenase, and ouabain-sensitive [Na^+, K^+]ATPase) were measured as described previously,[5] except that the buffer used in assays of alkaline phosphatase was 50 mmol/L 2-amino-2-methyl-1-propanol, pH 10.5.

Statistical Analyses

Comparisons were made by repeated-measures ANOVA (animal weights and carnitine excretion) using the general linear models procedure.[7] Differences were identified using the least significant difference test. Paired *t* tests were used to compare data for solute transport into renal cortical brush-border membrane vesicles. Unpaired *t* tests were used for all other comparisons. Differences were considered significant at P less than .05.

RESULTS

For guinea pigs in the control group, weight gain was constant throughout the experimental period (Fig 1). On the other hand, ascorbate-deficient animals gained weight through approximately day 18, and then they began a slow but steady decline in weight. These results are typical of experimental vitamin C deficiency in guinea pigs.[2,8,9] Ascorbate concentrations in plasma and tissues (liver, kidney, heart, and skeletal muscle) of guinea pigs fed the vitamin C–deficient diet were markedly lower (<15%) than corresponding concentrations in control animals (Table 1). Carnitine concentrations in plasma and tissues of animals

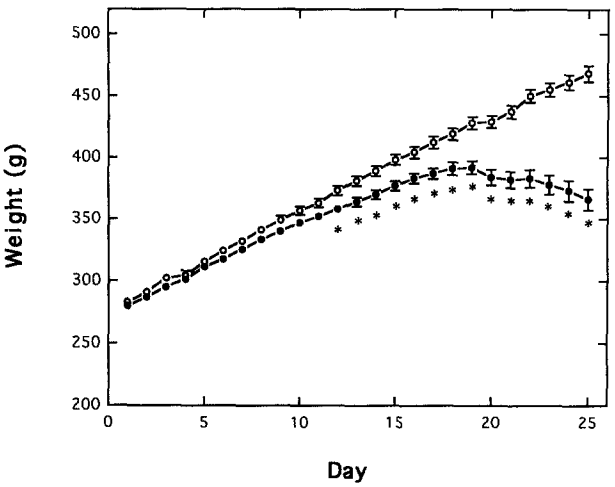

Fig 1. Weight gain or loss during the 24-day experimental regimen. Animal weights were recorded on the morning (beginning) of each day. Each point is the mean of 42 (control, ○) or 50 (ascorbate-deficient, ●) animals; vertical bars indicate the SEM. *Significant difference (P < .05) from control on the same day.

Table 1. Ascorbate Concentrations in Plasma and Tissues

Site	Control Mean ± SD	Control No.	Ascorbate-Deficient Mean ± SD	Ascorbate-Deficient No.
Plasma (μmol/L)	62.8 ± 16.0	37	3.63 ± 1.18*	45
Liver (nmol/g)	2,492 ± 423	37	101 ± 40.7*	45
Kidney (nmol/g)	1,036 ± 125	11	72.8 ± 29.3*	20
Heart (nmol/g)	689 ± 78.4	37	47.8 ± 18.8*	45
Skeletal muscle (nmol/g)	264 ± 55.7	37	37.5 ± 15.9*	44

*Significant difference from control value ($P < .001$).

fed the ascorbate-deficient diet were, on average, lower (15% to 28%) than in control guinea pigs (Table 2).

Rates of both free and total carnitine excretion were significantly elevated (mean, 4.1- and 3.5-fold, respectively) on each of the last 6 days of the experimental regimen in guinea pigs fed the ascorbate-deficient diet as compared with control animals (Fig 2). Carnitine clearance was significantly higher and fractional reabsorption was significantly lower in guinea pigs fed the vitamin C–deficient diet than in control animals (Table 3), despite the lower filtered load of carnitine in vitamin C–deficient animals. Glomerular filtration rate, estimated from creatinine clearance, was not different in the two groups of animals.

Concentrations of some but not all measured amino acids in plasma were different (data not shown). Concentrations of hydroxyproline, glutamine, proline, methionine, and tryptophan were 13% to 27% lower in ascorbate-deficient animals as compared with controls. Concentrations of aspartic acid, glycine, arginine, and leucine were 21% to 27% higher and phenylalanine and taurine concentrations were 49% and 80% higher, respectively, in ascorbate-deficient guinea pigs as compared with control animals (all $P < .05$). Rates of urinary excretion were not different for most amino acids. The exceptions were taurine (3.8-fold), leucine (3.2-fold), and lysine (2.3-fold), which were all increased in ascorbate-deficient as compared with control guinea pigs (all $P < .01$).

The rate of carnitine transport into renal cortical brush-border membrane vesicles prepared from kidneys of ascorbate-deficient guinea pigs was lower than for vesicles prepared from kidneys of control animals (Fig 3). This difference (~36%) was significant for both total (sodium gradient–dependent plus sodium gradient–independent) and sodium gradient–dependent transport of carnitine.

To test specificity of the effect of vitamin C deficiency on renal cortical carnitine transport, rates of transport of glucose, lysine, alanine, and taurine also were measured. Rates of total and sodium gradient–dependent transport for glucose and taurine all were lower in vesicles prepared

Table 2. Carnitine Concentrations in Plasma and Tissues

Site	Control Mean ± SD	Control No.	Ascorbate-Deficient Mean ± SD	Ascorbate-Deficient No.
Plasma (μmol/L)				
Free	42.8 ± 6.57	42	29.0 ± 8.04*	50
Total	57.2 ± 8.66	42	43.4 ± 11.3*	50
Liver (nmol/mg non-collagen protein)				
Free	1.25 ± 0.287	37	0.998 ± 0.307*	45
Total	1.76 ± 0.404	37	1.41 ± 0.389*	45
Heart (nmol/mg non-collagen protein)				
Free	3.62 ± 0.938	37	2.99 ± 0.724*	45
Total	7.54 ± 1.79	37	6.01 ± 1.25*	45
Skeletal muscle (nmol/mg non-collagen protein)				
Free	1.06 ± 0.269	37	0.896 ± 0.256*	44
Total	2.28 ± 0.576	37	1.85 ± 0.448*	44

*Significant difference from control value ($P < .01$).

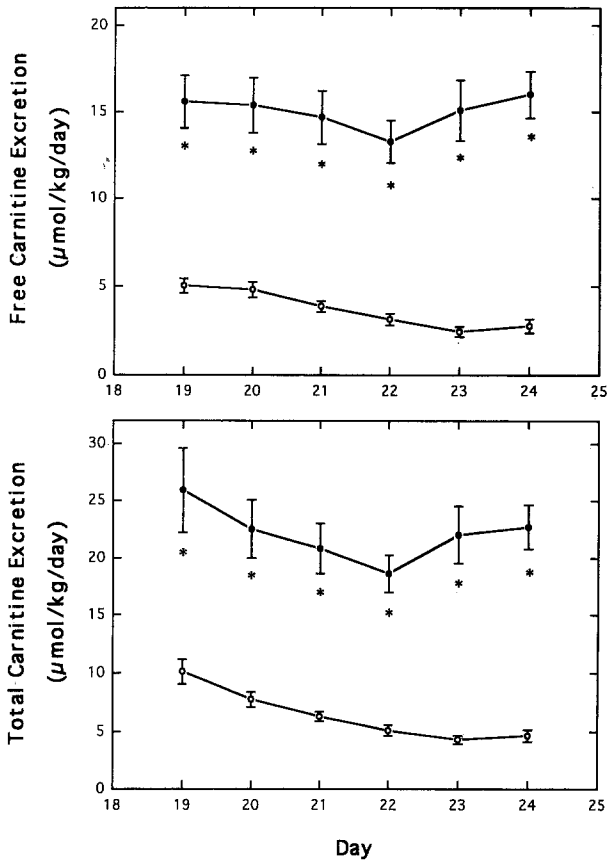

Fig 2. Rates of free and total carnitine excretion on days 19 to 24 of the experimental regimen. (○) Controls (n = 28), (●) ascorbate-deficient (n = 32). Vertical bars indicate the SEM. *Significant difference ($P < .01$) from control on the same day.

To test specificity of the effect of vitamin C deficiency on renal cortical carnitine transport, rates of transport of glucose, lysine, alanine, and taurine also were measured. Rates of total and sodium gradient–dependent transport for glucose and taurine all were lower in vesicles prepared

Table 3. Carnitine Clearance and Reabsorption and Creatinine Clearance

Parameter	Control (n = 28)	Ascorbate-Deficient (n = 32)
Carnitine clearance (μL/min)		
Free	21.6 ± 16.3	149 ± 85.2*
Total	26.6 ± 15.5	138 ± 74.0*
Carnitine reabsorption (%)		
Free	97.6 ± 2.06	81.1 ± 10.9*
Total	97.0 ± 1.99	82.4 ± 9.86*
Creatinine clearance (μL/min)	943 ± 208	858 ± 248

NOTE. Values are the mean ± SD.
*Different from corresponding value in control group ($P < .001$).

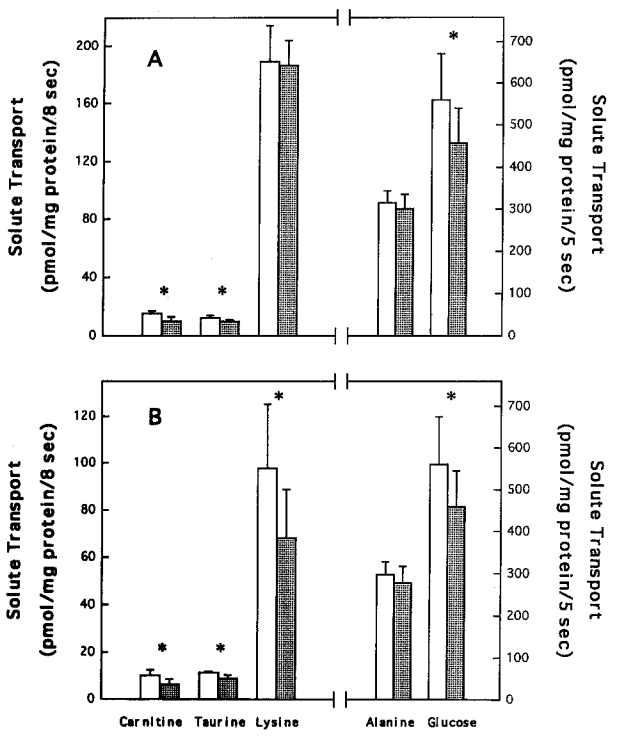

Fig 3. Rates of solute transport into renal cortical brush-border membrane vesicles. (A) Total (sodium gradient–dependent plus sodium gradient–independent) transport. (B) sodium gradient–dependent transport. (□) control guinea pigs, (■) ascorbate-deficient guinea pigs. Vertical bars indicate the SD. *Significant difference ($P < .05$) from control. n = 4 for carnitine, taurine, and lysine; n = 3 for alanine and lysine.

from ascorbate-deficient guinea pigs as compared with control animals (Fig 3). Rates were 20% lower for total taurine transport and 18% lower for glucose transport. Sodium gradient–dependent transport of lysine was 30% lower ($P < .05$) in vesicles from ascorbate-deficient guinea pigs as compared with control animals, but total lysine transport was not different (Fig 3). Alanine transport was not significantly affected by vitamin C deficiency. Virtually all transport of glucose, alanine, and taurine was sodium gradient–dependent, whereas sodium gradient–independent transport accounted for 34% to 35% and 48% to 63% of total transport of carnitine and lysine, respectively.

The quality and consistency of renal cortical brush-border membrane vesicle preparations was assessed by measurement of marker enzyme activities. Two markers for the brush-border membrane, alkaline phosphatase and γ-glutamyl transpeptidase, were enriched approximately 10-fold, relative to the crude cortex homogenate, in preparations from both control and ascorbate-deficient guinea pigs (alkaline phosphatase, 9.92-fold *v* 9.22-fold, and γ-glutamyl transpeptidase, 10.7-fold *v* 9.92-fold, control and ascorbate-deficient, respectively; no significant differences). Mitochondrial marker enzyme specific activities were diminished in brush-border membrane vesicles relative to the crude homogenate. Citrate synthetase specific activity in brush-border membranes was reduced to 4% to 5% and succinate dehydrogenase specific activity was reduced to 7% of that in crude homogenates (no differences in enrichments between preparations from control and ascorbate-deficient animals). Specific activity of ouabain-sensitive [Na^+, K^+]ATPase, a marker for the basolateral membrane, was not enriched in brush-border membrane preparations, and specific activity ratios (membrane to homogenate) were not different in preparations from control and ascorbate-deficient guinea pigs.

DISCUSSION

Carnitine homeostasis in mammals is maintained by dietary carnitine intake, a modest rate of endogenous carnitine biosynthesis, and efficient conservation of carnitine by the kidney.[10] Several observations point to changes in the ability of the kidney to conserve carnitine as the factor responsible for carnitine depletion in experimental scurvy: (1) the carnitine content of the diet of guinea pigs is negligible, (2) the capacity to synthesize carnitine is not compromised in scurvy, and (3) the rate of urinary excretion of carnitine is substantially increased in scurvy. In this investigation, we have demonstrated unequivocally that the rate of carnitine excretion is increased by approximately fourfold in moderate to severe scurvy in guinea pigs. In normal guinea pigs, the rate of carnitine excretion is approximately 3% of filtered load; 97% of carnitine is reabsorbed (Table 3). The fourfold increase in the rate of carnitine excretion in scorbutic guinea pigs corresponds to a 15% decrease (as percent of filtered load) in the rate of carnitine reabsorption. The difference in the rate of carnitine reabsorption in vivo (15%) is directly comparable to the difference in the rate of carnitine transport into renal brush-border membrane vesicles in vitro. The difference between 15% in vivo and 36% in vitro likely reflects the dissimilarity in filtered load of carnitine (primarily the difference in plasma carnitine concentration) between scorbutic and normal guinea pigs. These results suggest that mechanisms responsible for reabsorption of carnitine in guinea pigs are altered by vitamin C deficiency.

The effect of vitamin C deficiency on carnitine transport into renal cortical brush-border membrane vesicles was not entirely specific: rates of sodium gradient–dependent glucose, taurine, and lysine transport also were significantly decreased in preparations from scorbutic guinea pigs. However, the effects on transport of these solutes were not as pronounced as those on carnitine transport. Rates of both lysine and taurine excretion were increased in vivo by 2.3- and 3.8-fold, respectively. On the other hand, the rate of alanine transport into renal cortical brush-border membrane vesicles in vitro was not significantly affected by vitamin C deficiency, nor was the rate of alanine excretion different in vivo.

Normal rates of carnitine excretion and the efficiency of carnitine reabsorption are affected by a number of physiological variables. For example, in humans, dietary macronutrient content influences the rate of carnitine excretion but apparently not the intrinsic mechanisms responsible for carnitine reabsorption.[11] Diets low in protein decrease the

rate of carnitine excretion by decreasing the glomerular filtration rate, whereas diets high in fat increase the rate of carnitine excretion (compared with diets high in carbohydrate and low in fat) by increasing the filtered load of carnitine, at least over the short term. Dietary carnitine also affects the rate of carnitine excretion, and does so in part by decreasing the efficiency of carnitine reabsorption in the absence of changes in filtered load or glomerular filtration rate.[12] In rats, the rate of carnitine transport (but not rates of glucose, alanine, lysine, or glutamate transport) into renal cortical brush-border membrane vesicles was markedly reduced when rats were fed a diet containing 1% L-carnitine as compared with a diet devoid of carnitine,[5] suggesting that dietary carnitine specifically downregulates reabsorption of carnitine.

The mechanism by which vitamin C deficiency increases the rate of carnitine excretion and decreases the rate of carnitine transport into renal cortical brush-border membrane vesicles is not known. Vitamin C deficiency is associated with a decreased rate of collagen synthesis in guinea pigs. Although it was thought that this effect resulted from decreased hydroxylation of procollagen, more recent data suggest that collagen synthesis in vitamin C deficiency is due to inhibition of the action of insulin-like growth factors by increased circulating concentrations of insulin-like growth factor–binding proteins-1 and -2.[9,13]

Food consumption is lower in scorbutic guinea pigs than in normal animals. Vitamin C–sufficient guinea pigs fasted or food-restricted (by pair-feeding to ascorbate-deficient guinea pigs) also have increased circulating concentrations of insulin-like growth factor–binding proteins, which inhibit collagen synthesis in cultured chick embryo chondrocytes and human fibroblasts.[9,13] Thus, decreased collagen synthesis in experimental scurvy may in fact be due to decreased food intake and not to vitamin C deficiency per se. In our previous study,[2] we observed no difference in the rate of carnitine excretion by animals pair-fed to scorbutic animals versus control guinea pigs (thus, no pair-fed group was included in this study). Therefore, the mechanisms underlying decreased collagen synthesis and increased carnitine excretion are likely to be different. The lack of specificity of effect on transport of solutes into renal cortical brush-border membrane vesicles suggests that vitamin C deficiency may alter the membrane environment in which the respective transporters function.

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