ELSEVIER

Changes in collagen cross-link ratios in bone and urine of guinea pigs fed graded dietary vitamin C: A functional index of vitamin C status

Harumi Tsuchiya and Christopher J. Bates

Medical Research Council Dunn Nutrition Unit, Downhams Lane, Milton Road, Cambridge CB4 1XJ, UK

Vitamin C is essential for the hydroxylation of lysine in collagen. The resulting hydroxylysine residues, together with the precursor lysine residues, are then incorporated into the stable collagen cross-links: pyridinoline and deoxypyridinoline. A reduction in lysine hydroxylation, resulting from an inadequate supply of vitamin C, might decrease the ratio of the pyridinoline crosslinks to the deoxypyridinoline crosslinks. This hypothesis was tested in a guinea pig model, using a purified diet providing vitamin C in the following amounts: (a) 0.5 mg/day; (b) 1 mg/day; (c) 5 mg/day; (d) 0.25% w/v in the diet (providing 49 mg/day); (e) 1.0% w/v (providing 205 mg/day); or (f) 5.0% w/v (providing 1128 mg/day). Weanling male guinea pigs each received one of these diets for 46 days, after which their tissue vitamin C concentrations, and the concentrations of hydroxyproline, pyridinoline, and deoxypyridinoline in bone, and of creatinine, pyridinoline, and deoxypyridinoline in urine, were measured. The ratio of pyridinoline to deoxypyridinoline in bone collagen and in urinary collagen fragments decreased significantly as vitamin C depletion became more severe. Animals receiving the higher intakes of vitamin C grew more rapidly than did those receiving the lower intakes, but the relationship between vitamin C intake and the collagen crosslinks ratio remained highly significant, even after adjusting for intergroup differences in growth rates. Thus, the ratio between the two collagen crosslinks could provide a specific and sensitive functional index of vitamin C in animals and man. This is an important goal, in view of the need to be able to define optimum intakes of vitamin C. (J. Nutr. Biochem. 9:402–407, 1998) © *Elsevier Science Inc. 1998*

Keywords: guinea pig; vitamin C; collagen; crosslinks; pyridinoline; deoxypyridinoline

Introduction

Vitamin C (L-ascorbic acid) is an essential dietary nutrient for man, higher primates, the guinea pig and a small proportion of other species. It is a powerful reducing agent and is able to prevent and cure scurvy, but most of its essential functions in vivo remain poorly understood. One important role is that of enabling hydroxylation, by molecular oxygen, of specific proline and lysine residues in nascent collagen polypeptide chains to continue, by preventing irreversible oxidative inactivation of the key intracellular collagen hydroxylases.[1,2]

New functional indices of vitamin C status in man are needed to measure human requirements in ways that can be related directly to essential metabolic functions.[3] The requirement for vitamin C in collagen synthesis is an obvious potential source of information, but until recently it has proved difficult to identify a collagen index capable of reflecting vitamin C status in a specific and sensitive manner.[4,5] The development of simplified techniques for measurement of the lysine- and hydroxylysine-derived collagen cross-links—pyridinoline and deoxypyridinoline—in urine and tissues[6–8] have permitted renewed studies in a guinea pig model.[9] The guinea pig not only has an absolute requirement for dietary vitamin C, but is also a useful model for studies of collagen synthesis.[4,5,10]

The purpose of the present study was to confirm and

Address correspondence and reprint requests to Dr. C.J. Bates, MRC Dunn Nutrition Unit, Downhams Lane, Milton Road, Cambridge CB4 1XJ, UK. Harumi Tsuchiya is on study leave from Bunka Women's University, 3-22-1 Yoyogi, Shibuyaku, Tokyo 151, Japan.
Received January 13, 1998; accepted April 30, 1998.

655 Avenue of the Americas, New York, NY 10010
0955-2863/98/$19.00
PII S0955-2863(98)00039-4

extend our previous observations[9] by measuring the ratio of pyridinoline to deoxypyridinoline in bone and urine samples from guinea pigs receiving graded amounts of vitamin C. By examining a wide range of vitamin C intakes, it has been possible to define a physiological intake range over which collagen cross-linking is affected. Measurement of collagen crosslink ratios might therefore provide a functional index of vitamin C status, which could be useful in future studies of human vitamin C requirements.

Methods and materials

Animals and diets

The purified guinea pig diet, which was prepared in-house, contained the following components (g/kg): sucrose 331; maize starch 50; purified casein (G. Fisk & Co Ltd, Richmond, Surrey, UK) 300; cellulose powder (Solkafloc, Special Diet Services, Witham, Essex, UK) 150; maize oil (S. Black (Import & Export) Ltd, The Colonnade, High Street, Cheshunt, Herts, UK) 73; potassium acetate 25; choline chloride 2; magnesium oxide 5; salt mixture 60; vitamin mixture 2. The salt mixture was that of Greenfield et al.[12] and the vitamin mixture provided the following amounts of essential vitamins (mg/kg diet): retinol 2.4 as retinyl acetate; cholecalciferol 0.0075; α-tocopherol 60; menadione 10; thiamin HCl 16; riboflavin 16; pyridoxine HCl 16; calcium pantothenate 40; nicotinamide 200; pteroylmonoglutamic acid 10; cyanocobalamin 0.05; and biotin 10. All animals were given a small amount of dried hay daily. Groups A, B, and C received the basal diet, with vitamin C solutions given separately, once daily, by dropper. Groups D, E, and F received the basal diet mixed with added vitamin C: see below. The amounts of vitamin C provided per day, per animal were:

Group A: 0.5 mg/day, in a single daily dose, by dropper
Group B: 1.0 mg/day in a single dose, by dropper
Group C: 5.0 mg/day in a single dose, by dropper
Group D: 0.25% w/v in the diet (providing on average 49 mg/day)
Group E: 1.0% w/v in the diet (providing on average 205 mg/day)
Group F: 5.0% w/v in the diet (providing on average 1128 mg/day)

The diets were stored at 4°C for up to 2 weeks before use. All animals had free access to food and water and were housed singly in steel-mesh cages, with a 12-hr light-dark cycle at 19–23°C. There were 5 to 7 animals per group, all male inbred Dunkin-Hartley albino guinea pigs ca. 3 to 4 weeks old, obtained from "Harlan," Wyton, Huntingdon, Cambs, UK. They were distributed among the six groups to ensure similar mean starting body weights per group, and the overall mean initial body weight was 314 ± 27 (SD) g. Animals were maintained on the special diets for 46 days, with daily records taken of body weight and of food eaten. A single 8-hr urine sample in dilute hydrochloric acid was collected from each animal in a metabolism cage on days 43 to 45, and the animals were then killed by CO_2 anesthesia on day 46. Blood (by cardiac puncture) was collected in heparin anticoagulant and was separated to provide plasma. Plasma, together with adrenal glands, spleen, and brain were extracted with metaphosphoric acid (50 g/L, final concentration) for vitamin C assays: see below. Hind limbs were stored at −80°C for the measurement of hydroxyproline and of collagen cross-links in the femurs.

Analyses

Analytical methods were as follows: Vitamin C (L-ascorbic + dehydroascorbic acid) was measured in the diets (to check for possible losses, which proved negligible), and in blood plasma, adrenal glands, spleens, and brains. Diets and organs were immediately homogenized (by Potter-Elvehjem homogenizer) with 10 volumes of cold metaphosphoric acid (50 g/L) and centrifuged. The soluble extracts, after brief storage at −80°C, were analyzed for their vitamin C content by the method of Vuilleumier and Keck,[12] based on ascorbate oxidase (EC 1.10.3.3) treatment, followed by orthophenylene diamine coupling, in a Roche Cobas Bio centrifugal analyzer with a fluorescence attachment.

One femur per animal was dissected free of adventitious tissue and marrow; it was decalcified with EDTA solution as described previously,[9,13] and was dissolved in 9 mL hydrochloric acid (6 mol/L) by heating at 100°C for 24 hr in a glass screw-cap tube with a Teflon-lined cap. The resulting hydrolysates were evaporated at 37°C under a stream of nitrogen, and were re-evaporated several times with water additions to remove any residual hydrochloric acid. They were then redissolved in water, adjusted to neutral pH with dilute sodium hydroxide, and were used for the measurement of hydroxyproline,[14,15] pyridinoline, and deoxypyridinoline (see below). The urine samples were analyzed for creatinine content by the Jaffé reaction (Roche Diagnostics Unimate 5 CREA kit, on the Cobas Bio centrifugal analyzer). Further subsamples, each with an equal amount of creatinine, were then hydrolyzed with hydrochloric acid as described above, and were evaporated, redissolved in water, and used for pyridinoline and deoxypyridinoline analyses.

The collagen cross-links—pyridinoline and deoxypyridinoline—were measured in the acid hydrolysates of the demineralized femur and urine samples by a Metra Biosystems antibody-based ELISA plate assay (Metra Biosystems Ltd, Wheatley, Oxon, UK). These are specific immunoassays for (a) deoxypyridinoline and (b) pyridinoline + deoxypyridinoline, which have been validated against HPLC assays[6–8] and were further validated for guinea pig-derived bone and urine hydrolysates in the authors' laboratory.[9]

Relationships between outcome indices and vitamin C intakes (log-transformed) were tested by linear regression analysis, including multivariate linear regression, using a DataDesk statistical package for a Macintosh personal computer (Data Description, Inc., Ithaca, New York USA). A P-value < 0.05 was deemed to be statistically significant.

Results

Table 1 shows that, as the daily intake of vitamin C increased from 0.5 to 49 mg/day for each animal in Groups A to D, the concentration of vitamin C in plasma, adrenals, spleen, and brain increased progressively. However, between 49 and 1128 mg/day (Groups D to F), there were only minor further increases in tissue vitamin C. *Figure 1* depicts the relationship of brain vitamin C with log (vitamin C intakes), and confirms that a plateau of tissue vitamin C concentration is approached at high intakes. As expected, the trends in plasma and tissue vitamin C concentrations with increasing vitamin C intakes were highly significant (*Table 1*).

Figure 2 shows the growth curves for the six groups. Growth rates increased progressively with increasing vitamin C, and the correlation between growth rate and log (vitamin C intake) was highly significant: $P < 0.0001$. The trend in food intakes with increasing vitamin C was also significant ($P = 0.0015$) but was less strongly so than the trend in growth rates, and the inclusion of growth rate in the model rendered the relationship of food intake with vitamin C intake nonsignificant ($P = 0.92$).

The lower half of *Table 1* shows the trends in collagen cross-link patterns, with increasing vitamin C intake, for bone and urine. The bone concentration of hydroxyproline

Table 1 Vitamin C intakes and status, and bone and urine collagen indices, in guinea pigs

Index	Group[1] A (n = 6)	B (n = 6)	C (n =6)	D (n = 7)	E (n = 5)	F (n = 6)	Pooled CV (%)	P for trend I[2]	P for trend II[3]
	Means								
Vitamin C intake (mg/day)	0.5	1.0	5.0	49	205	1128	—	—	—
Plasma vitamin C (μmol/L)	2.1	6.0	26.1	109	145	169	33.9	<0.0001	<0.0001
Adrenal vitamin C (μmol/g)	0.59	1.26	4.73	10.9	11.4	11.8	13.0	<0.0001	<0.0001
Spleen vitamin C (μmol/g)	0.25	0.40	1.43	2.37	2.48	2.59	12.6	<0.0001	<0.0001
Brain vitamin C (μmol/g)	0.19	0.31	0.84	1.11	1.17	1.21	10.3	<0.0001	<0.0001
Change in body weight[4] (g)	115	120	153	166	177	258	23.8	<0.0001	—
Final body weight (g)	409	429	456	472	487	562	10.2	<0.0001	—
Food intake (g/day), days 23–43	15.2	15.8	20.7	18.0	19.8	22.1	17.7	0.0015	0.92
Bone Collagen Indices									
Hypro/wet weight (μmol/g)	81	79	80	74	76	79	5.2	0.06	0.017
Pyridinoline/hypro (mmol/mol)	0.812	0.770	0.820	0.909	0.906	0.875	9.3	0.019	0.028
Deoxypyridinoline/hypro (mmol/mol)	0.093	0.092	0.075	0.071	0.064	0.070	19.5	0.003	0.021
Pyridinoline/deoxypyridinoline (mol/mol)	8.89	8.91	11.24	14.43	14.78	13.42	22.5	0.003	0.009
Urine Collagen Turnover Indices									
Pyridinoline/creatinine (μmol/mol)	216	231	184	206	251	270	28.4	0.15	0.79
Deoxypyridinoline/creatinine (μmol/mol)	31.2	31.2	20.8	22.7	23.1	24.9	34.6	0.13	0.11
Pyridinoline/deoxypyridinoline (mol/mol)	7.03	7.81	8.92	9.98	11.12	11.01	18.1	0.0002	0.004

Abbreviations: Hypro: hydroxyproline; CV: coefficient of variation.
[1]See "Animals and Diets" for a description of the diet groups and procedures. The most important defining characteristic of each group is the daily vitamin C intake, shown in the first line of data in the table.
[2]Linear regression of each index versus log (vitamin C intake), by univariate regression.
[3]Linear regression of each index versus log (vitamin C intake) after adjustment, by multivariate regression, for intergroup differences in body weight increase.
[4]Change in body weight is the difference in body weight between day 1 and day 46 of the dietary regimen.

per unit wet weight showed a declining trend with increasing vitamin C intake, which failed to reach conventional significance in a univariate regression, but was significant ($P = 0.017$) after adjustment for body weight. The ratio of pyridinoline to hydroxyproline in bone exhibited a small, marginally significant increase with increasing vitamin C intake, whereas the ratio of deoxypyridinoline to hydroxyproline in bone exhibited a larger and more significant decrease with increasing vitamin C intake. The ratio of pyridinoline:deoxypyridinoline showed a progressive increase between Groups A and D, and the overall trend with increasing vitamin C intake was highly significant, both by univariate regression, and by multivariate regression with adjustment for the differences in body weight increments.

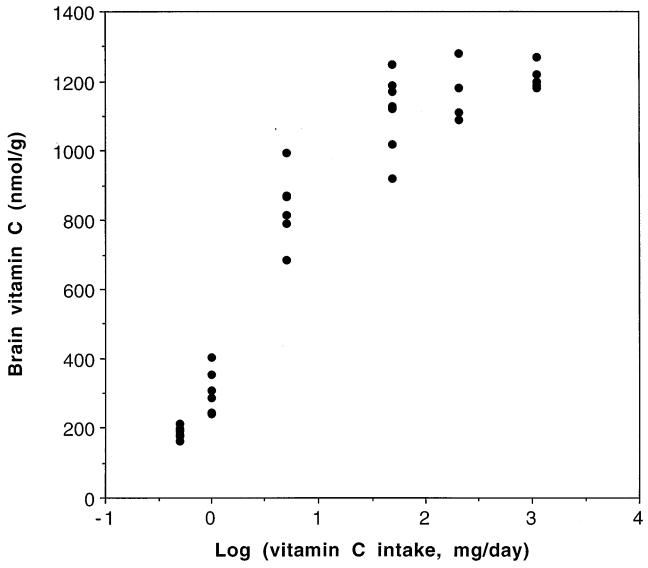

Figure 1 Brain vitamin C concentration plotted against $\log_{10}$ (vitamin C intake). Vitamin C intake has been converted to a log scale, because of the wide range of intakes employed. Values for each animal are shown individually.

The urine collagen cross-link patterns supported a similar conclusion. Whereas neither the ratio of pyridinoline to creatinine nor the ratio of deoxypyridinoline to creatinine in urine was significantly related to vitamin C intake, the ratio of pyridinoline to deoxypyridinoline was highly significantly related to vitamin C intake, both by univariate regression and by multivariate regression with the adjustment for body weight differences.

Introduction of further adjustments to the multivariate regression models, which already contained body weight, for variations in food intake or in bone hydroxyproline:bone weight ratios made no difference to the conclusions. Substitution of final body weight for the increment in body weight likewise made no difference.

The relationship between the pyridinoline/deoxypyridinoline cross-links ratio and log (vitamin C intake) is illustrated graphically in *Figure 3* and *Figure 4*. *Figure 3* confirms that there was a significant increase in the bone collagen crosslink ratio with increasing vitamin C intake, and *Figure 4* shows the same to be true for the urine collagen cross-link ratio. In *Figure 5* it is seen that the collagen cross-link ratio in bone and the ratio in urine were both strongly correlated with each other.

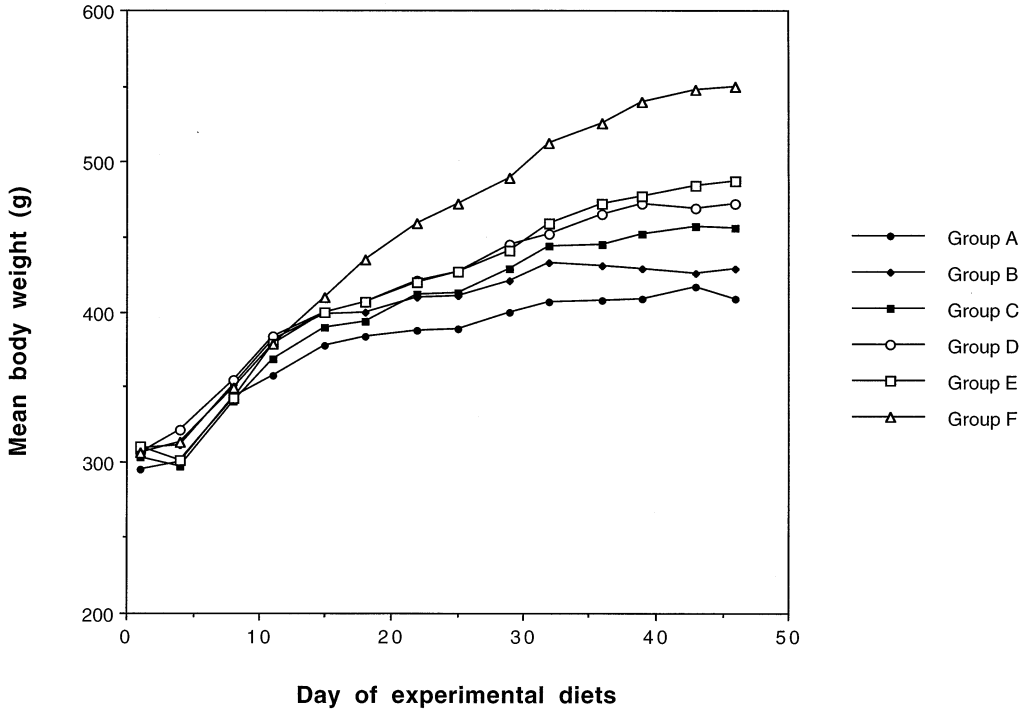

Figure 2 Mean body weight curves for diet Groups A to F.

Discussion

The conclusions of the present study have confirmed and extended those of our previous study.[9] They have confirmed that vitamin C intake in guinea pigs is an important controlling factor in determining the ratio of pyridinoline to deoxypyridinoline collagen cross-links, both in bone and in urine hydrolysates. They have extended it to show that a progressive change in cross-link ratios occurs over the same range of vitamin C intakes, which has a major influence on tissue vitamin C concentrations.

In the present study, the guinea pig growth rates and voluntary food intakes increased progressively with increasing provision of vitamin C. It was therefore necessary to

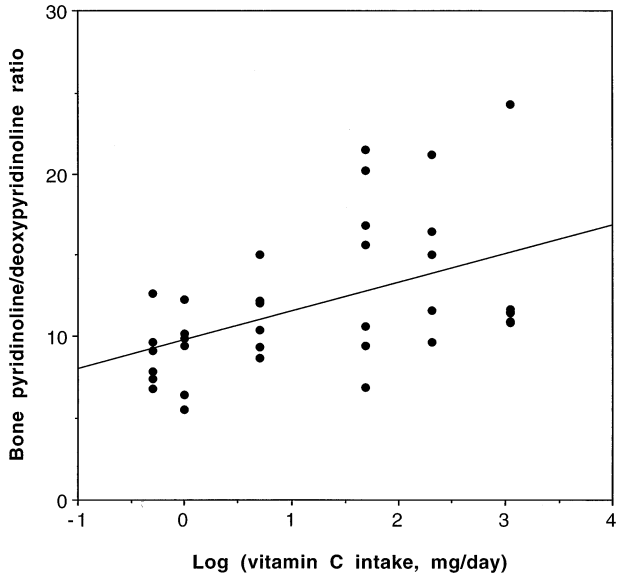

Figure 3 Bone pyridinoline/deoxypyridinoline ratio plotted against $\log_{10}$ (vitamin C intake). Values from each animal in Groups A to F are shown. Linear regression: $y = 9.8 + 1.77$ x, $r = 0.48$, 34 *df*, $P = 0.003$ (see *Table 1*).

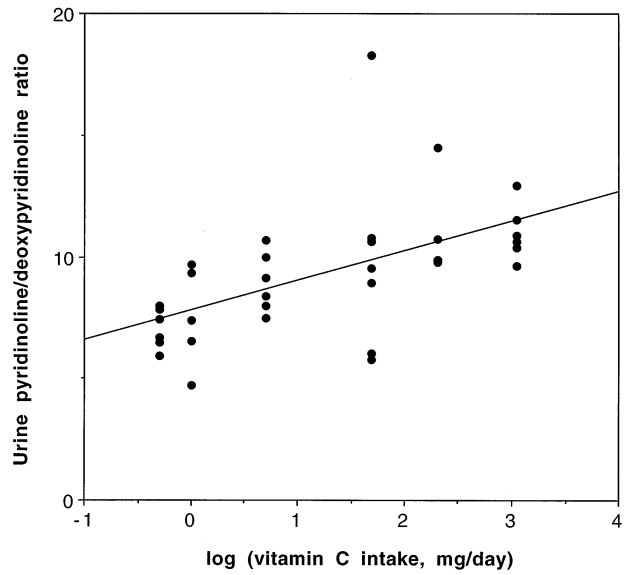

Figure 4 Urine pyridinoline/deoxypyridinoline ratio plotted against $\log_{10}$ (vitamin C intake). Values from each animal in Groups A to F are shown. Linear regression: $y = 7.8 + 1.23$ x, $r = 0.57$, 34 *df*, $P = 0.0003$ (see *Table 1*).

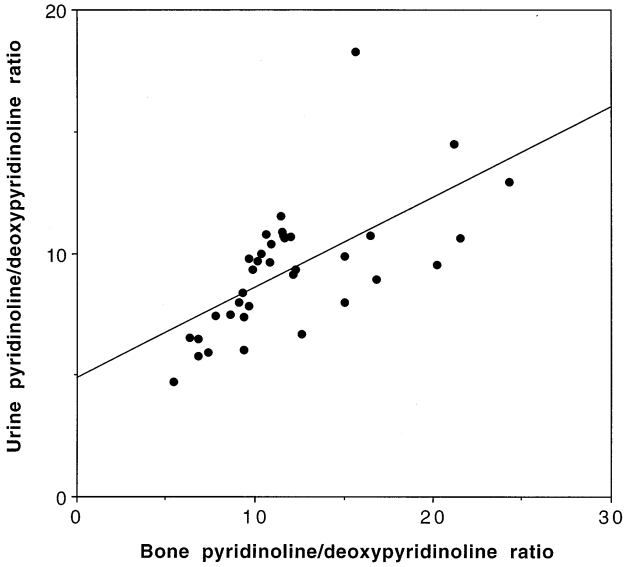

Figure 5 Comparison of bone pyridinoline/deoxypyridinoline ratio with the corresponding urine ratio. Values from each animal in Groups A to F are shown. Linear regression: $y = 4.8 + 0.37$ x, $r = 0.64$, 34 *df*, $P < 0.0001$.

determine whether the changes in collagen cross-link ratios in bone and urine might be a secondary result of the changes in growth or total food intake, rather than a primary result of the differences in vitamin C intakes and status. The multivariate regression model in *Table 1* addressed this question. It showed that after adjustment of the relationship between log (vitamin C intakes) and cross-link ratios in bone and urine for the body weight increment differences, the cross-link relationship remained significant. Therefore, the effect of vitamin C on the cross-link ratios in bone and urine is largely independent of the effect of vitamin C on growth. This conclusion is further supported by observations from our previous study[9] in which the effect of vitamin C on collagen cross-link ratios was not accompanied by parallel growth rate changes.

Guinea pigs require 75 to 150 mg vitamin C/kg diet (equivalent to ca. 1.5–3 mg/day) as a minimum requirement, and 1.5 g/kg diet (equivalent to ca. 30 mg/day) as a recommended amount.[16] The data in *Table 1* and *Figure 1* of the present study suggest that near-maximum tissue concentrations of vitamin C can be attained in weanling male and Dunkin Hartley guinea pigs, by vitamin C intakes around 50 mg/day. This is illustrated for brain vitamin C concentrations in *Figure 1*. Of all the tissue vitamin C indices measured, brain vitamin C was the most strongly related to bone and urine collagen cross-link ratios in the present study. Brain vitamin C is potentially a good long-term status index, since the vitamin C in brain tissue has a half-life of about 20 days[17] and is therefore only slowly affected by dietary vitamin C changes.

Our observations suggest that the collagen cross-link response is likely to be a physiological, as distinct from a pharmacological, response to variations in vitamin C intakes, because it varies over the normal physiological range. It is therefore a promising candidate for the definition of vitamin C dietary requirements and status-adequacy. Because vitamin C clearly affects bone collagen[4,5] and bone mass[11] in guinea pigs, the newly established relation with the urinary collagen cross-link ratio helps to complete the logical sequence from a pathological lesion, via a key biochemical index, to a potential noninvasive probe.

Although there is evidence that the mode of action of vitamin C, with respect to collagen synthesis and the control of collagen metabolism, is complex,[18–21] its functions in the hydroxylation of collagen prolyl and lysyl residues may trigger other interconnected effects at the molecular level. Evidence that the supply of vitamin C may also be important for collagen cross-link ratios in human subjects is supported by recent studies on the human genetically determined connective tissue disease, Ehlers-Danlos syndrome, type VI. This condition is responsive to vitamin C supplements in vivo, and the genetic abnormality is also evident in skin fibroblast cultures.[22–24] Recent studies have revealed increased deoxypyridinoline/pyridinoline ratios, in both the urine[25,27] and the fibroblasts[26] of people with the disease, which are analogous to the changes that we have described in vitamin C-deficient guinea pigs. Both the activity of lysyl hydroxylase and the intracellular concentration of the mRNA for this vitamin C-requiring enzyme were upregulated by vitamin C in cultured Ehlers-Danlos type VI fibroblasts.[28]

In another genetically determined connective tissue disease, Ullrich-Turner syndrome, a lowered ratio of pyridinoline to deoxypyridinoline in urine has been found,[29] suggesting an analogous biochemical lesion. In contrast, in genetically normal children who were suffering from protein-energy malnutrition, the total content of collagen crosslinks in urine increased threefold during a restorative diet, without any change in the pyridinoline to deoxypyridinoline ratio.[6,30] Therefore, it appears that total collagen turnover and the total excretion of collagen cross-links can change dramatically, without any change in the cross-link ratio, provided that the subjects are not genetically abnormal, or vitamin C-deficient.

Age-related changes[31] can affect total collagen synthesis, turnover, and cross-link excretion without changing the cross-link ratio. Bone is the main contributor to urinary collagen cross-links,[6] and it is, of course, possible that some as yet unidentified disease conditions may affect the cross-link ratio in urine by altering the relative contributions from bone and from other nonbony tissues, in the absence of overall changes in lysyl hydroxylation efficiency.

More studies are thus required, of human subjects who are naturally depleted of vitamin C; of those who are generally malnourished; of those who are metabolically compromised in other respects, and of those who suffer from connective tissue diseases, or are genetically abnormal, to determine the specificity and sensitivity of the pyridinoline:deoxypyridinoline ratio index. It will be necessary to demonstrate a consistent response to vitamin C-repletion in naturally vitamin C-depleted subjects, and to confirm that urine samples can yield a sensitive and reliable functional index of vitamin C, suitable for the estimation of human vitamin C requirements.

References

1 Kivirikko, K.I. and Myllyla, R. (1982). Posttranslational enzymes in the biosynthesis of collagen: intracellular enzymes. *Methods Enzymol.* **82,** 245–304

2 Myllyla, R., Majamaa, J., Gunzler, V., Hanauske-Abel, H.M., and Kivirikko, K.I. (1984). Ascorbate is consumed stoichiometrically in the uncoupled reactions catalysed by prolyl 4-hydroxylase and lysyl hydroxylase. *J. Biol. Chem.* **259,** 5403–5405

3 Levine, M., Dhariwal, K.R., Welch, R.W., Wang, Y., and Park, J.B. (1995). Determination of optimal vitamin C requirements in humans. *Am. J. Clin. Nutr.* **62** (suppl), 1347S–1356S

4 Barnes, M.J. and Kodicek, E. (1972). Biological hydroxylations and ascorbic acid with special regard to collagen metabolism. *Vit. Horm.* **30,** 1–43

5 Bates, C.J. (1979). Vitamin C deficiency in guinea pigs: variable sensitivity of collagen at different sites. *Int. J. Vit. Nutr. Res.* **49,** 77–86

6 Robins, S.P. (1994). Biochemical markers for assessing skeletal growth. *Eur. J. Clin. Nutr.* **48** (suppl 1), S199–S209

7 Robins, S.P. (1982). An enzyme-linked immunoassay for the collagen cross-link pyridinoline. *Biochem. J.* **207,** 617–620

8 Robins, S.P., Woitge, H., Hesley, R., Ju, J., Seyedin, S., and Seibel, M.J. (1994). Direct, enzyme-linked immunoassay for urinary deoxypyridinoline as a specific marker for measuring bone resorption. *J. Bone Min. Res.* **9,** 1643–1649

9 Tsuchiya, H. and Bates, C.J. (1997). Vitamin C and copper interactions in guinea pigs and a study of collagen cross-links. *Br. J. Nutr.* **77,** 315–325

10 Kipp, D.E., Grey, C.E., McElvain, M.E., Kimmel, D.B., Robinson, R.G., and Lukert, B.P. (1996). Long-term low ascorbic acid intake reduces bone mass in guinea pigs. *J. Nutr.* **126,** 2044–2049

11 Greenfield, H., Briggs, G.M., Watson, R.H.J., and Yudkin, J. (1969). An improved diet for carbohydrate preference studies with rats: some criticisms of experimental diets. *Proc. Nutr. Soc.* **28,** 43A

12 Vuilleumier, J.P. and Keck, E. (1989). Fluorimetric assay of vitamin C in biological materials using a centrifugal analyser with fluorescence attachment. *J. Micronutr. Anal.* **5,** 25–34

13 Farquharson, C., Duncan, A., and Robins, S.P. (1989). The effects of copper deficiency on the pyridinium crosslinks of mature collagen in the rat skeleton and cardiovascular system. *Proc. Soc. Exp. Biol. Med.* **192,** 166–171

14 Ho, K-C. and Pang, C.P. (1989). Automated analysis of urinary hydroxyproline. *Clin. Chim. Acta* **185,** 191–196

15 Tsuchiya, H. and Bates, C.J. (1994). Ascorbic acid deficiency in guinea pigs: contrasting effects of tissue ascorbic acid depletion and of associated inanition on status indices related to collagen and vitamin D. *Br. J. Nutr.* **72**, 745–752

16 Clarke, H.E., Coates, M.E., Eva, J.K., Ford, D.J., Milner, C.K., O'Donoghue, P.N., Scott, P.P., and Ward, R.J. (1977). Dietary standards for laboratory animals: Report of the Laboratory Animals Centre Diets Advisory Committee. *Lab. Anim.* **11,** 1–28

17 Pelletier, O. (1969). Turnover rates of D-isoascorbic acid and L-ascorbic acid in guinea pig organs. *Can. J. Physiol. Pharmacol.* **47,** 993–997

18 Berg, R.A., Steinmann, B., Rennard, S.I., and Crystal, R.G. (1983). Ascorbate deficiency results in decreased collagen production: under-hydroxylation of proline leads to increased intracellular degradation. *Arch. Biochem. Biophys.* **226,** 618–686

19 Chojkier, M., Houglum, K., Solis-Herruzo, J., and Brenner, D.A. (1989). Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. *J. Biol. Chem.* **264,** 16957–16962

20 Peterkofsky, B. (1991). Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. *Am. J. Clin. Nutr.* **54,** 1135S–1140S

21 Phillips, C.S., Tajima, S., and Pinnell, S.R. (1992). Ascorbic acid and transforming growth factor-β1 increase collagen biosynthesis via different mechanisms: coordinate regulation of proα1(I) and proα1(III) collagens. *Arch. Biochem. Biophys.* **295,** 397–403

22 Elsas, L.J., Miller, R.L., and Pinnell, S.R. (1978). Inherited human collagen lysyl hydroxylase deficiency: ascorbic acid response. *J. Pediatr.* **92,** 378–384

23 Miller, R.L., Elsas, L.J., and Priest, R.E. (1979). Ascorbate action on normal and mutant human lysyl hydroxylases from cultured dermal fibroblasts. *J. Invest. Dermatol.* **72,** 241–247

24 Dembure, P.P., Janko, A.R., Priest, J.H., and Elsas, L.J. (1987). Ascorbate regulation of collagen biosynthesis in Ehlers-Danlos syndrome, type VI. *Metabolism* **36,** 687–691

25 Pasquali, M., Dembure, P.P., Still, M.J., and Elsas, L.J. (1994). Urinary pyridinium cross-links: a non-invasive diagnostic test for Ehlers-Danlos syndrome type VI. *New Engl. J. Med.* **331,** 132–133

26 Pasquali, M., Still, M.J., Vales, T., Rosen, R.I., Evinger, J.D., Dembure, P.P., Longo, N., and Elsas, L.J. (1997). Abnormal formation of collagen cross-links in skin fibroblasts cultured from patients with Ehlers-Danlos syndrome type VI. *Proc. Assoc. Am. Physicians* **109,** 33–41

27 Acil, Y., Vetter, U., Brenner, R., Muller, P.K., and Brinckmann, J. (1995). Ehlers-Danlos Syndrome type VI: cross-link pattern in tissue and urine sample as a diagnostic marker. *J. Am. Acad. Dermatol.* **33,** 522–524

28 Yeowell, H.N., Walker, L.C., Marshall, M.K., Murad, S. and Pinnell, S.R. (1995). The mRNA and the activity of lysyl hydroxylase are up-regulated by the administration of ascorbate and hydralazine to human skin fibroblasts from a patient with Ehlers-Danlos syndrome type VI. *Arch. Biochem. Biophys.* **321,** 510–516

29 Rauch, F., Seibel, M., Woitge, H., Kruse, K., and Schonau, E. (1995). Increased urinary excretion of collagen crosslinks in girls with Ullrich-Turner syndrome. *Acta Paediatr.* **84,** 66–69

30 Branca, F., Robins, S.P., Ferro-Luzzi, A., and Golden, M.H.N. (1992). Bone turnover in malnourished children. *Lancet* **340,** 1493–1496

31 Acil, Y., Brinckmann, J., Notbohm, H., Muller, P.K., and Batge, B. (1996). Changes with age in the urinary excretion of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP). *Scand. J. Clin. Lab. Invest.* **56,** 275–283