

Comparison of vitamin C deficiency with food restriction on collagen cross-link ratios in bone, urine and skin of weanling guinea-pigs

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Mild-to-moderate vitamin C depletion in weanling guinea-pigs affects pyridinoline:deoxypyridinoline (collagen cross-link) ratios in femur shaft and urine, attributed to impairment of hydroxylation of collagen lysine. We investigated: (1) whether the picture at two time points is compatible with progressive accumulation of abnormal collagen; (2) whether any changes are seen in skin, where little deoxypyridinoline occurs; (3) whether total food restriction has similar effects. Male weanling Dunkin–Hartley guinea-pigs were fed diets containing either 0.5 (vitamin C-restricted) or 160.0–320.0 (vitamin C-adequate) mg vitamin C/d. Two groups receiving the vitamin C-adequate diet received it *ad libitum*. Two other groups received the vitamin C-adequate diet in a restricted amount, limited to that which permitted nearly the same growth rate as in the vitamin C-restricted groups. Animals were fed for 4 or 8 weeks; urine was collected, and vitamin C and collagen indices were measured. In the femur shaft, the hydroxyproline content per unit weight was unaffected by vitamin C restriction or by total food restriction. Deoxypyridinoline was increased and the pyridinoline:deoxypyridinoline ratio was decreased in vitamin C-restricted groups, but not in food-restricted groups. Changes in the value of the ratio were greater after 8 than after 4 weeks. Urine indices mirrored bone indices. In skin, the main effect of vitamin C restriction was to reduce hydroxyproline. Here, the cross-link ratios changed less markedly than in bone, and there was less deoxypyridinoline. We conclude that the picture at two time points is compatible with a progressive accumulation of pyridinoline-enriched collagen in vitamin C-deprived animals, that the picture in skin differs from that of bone and urine, and that cross-link changes are not produced by total food restriction.

Vitamin C: Collagen: Cross-links: Guinea-pig

The requirement for vitamin C during hydroxylation of prolyl and lysyl residues in nascent procollagen polypeptide chains is well-established (Kivirikko & Myllyla 1982; Myllyla *et al.* 1984). The enzymes which catalyse these hydroxylation reactions are Fe-containing mixed-function oxidases that require molecular oxygen and 2-oxoglutarate as co-substrates, and a reducing agent, of which L-ascorbic acid is the most efficient, to prevent irreversible denaturation of the enzyme. Many connective tissue lesions characteristic of scurvy are explicable by impaired collagen synthesis during tissue maintenance, repair and remodelling. However, there remain uncertainties about the role or roles of vitamin C. Studies by Peterkofsky, Chojkier and co-workers have emphasized similarities between vitamin C deficiency and inanition for collagen synthesis and its control in guinea-pigs

(Chojkier *et al.* 1983; Spanheimer & Peterkofsky, 1985; Peterkofsky, 1991; Peterkofsky *et al.* 1991). Vitamin C restriction may affect collagen synthesis independently of collagen hydroxylation. Although studies of cultured connective tissue cells suggested that: 'ascorbate's only role is to stimulate hydroxylation; ... ascorbate can act as an inducer of the collagen pathway because most steps are tightly coupled' (Schwarz *et al.* 1987), it remains to be proven whether this is also true in the whole animal or in man.

Paradoxically, neither underhydroxylated collagen nor its degradation products accumulate in skin or urine of scorbutic guinea-pigs (Barnes *et al.* 1970; Barnes & Kodicek, 1972). In skin, collagen with about 10% underhydroxylated proline was produced in rapidly diminishing amounts, as they became scorbutic. The synthesis of collagen fell,

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as is also seen during total food restriction, although food restriction did not result in underhydroxylation of proline.

Less attention has been paid to the hydroxylation of lysine, and to the accumulation of hydroxylysine-derived collagen cross-links, in scorbutic animals. These may provide additional information about vitamin C-restriction effects, because they are metabolically stable and are quantitatively excreted in urine (Robins, 1994). They are not present in the diet; therefore endogenous production is the only source. Each pyridinoline residue is derived from three hydroxylysine residues, whereas deoxypyridinoline is derived from two hydroxylysine and one lysine residue. If lysyl hydroxylase activity is reduced by lack of ascorbate, the value of the deoxypyridinoline cross-links:pyridinoline cross-links ratio increases (Tsuchiya & Bates, 1997; Tsuchiya & Bates, 1998). Unlike hydroxyproline (Schwarz *et al.* 1987), hydroxylysine is apparently not required for the stabilization of the collagen triple helix.

Our previous studies (Tsuchiya & Bates, 1997, 1998) have demonstrated that there is an accumulation of deoxypyridinoline-enriched pyridinoline-depleted collagen or its products in bone and urine of vitamin C-deprived guinea-pigs, and that the value of the pyridinoline:deoxypyridinoline ratio changes progressively over a wide range of vitamin C intakes and tissue vitamin C concentrations. The present study has extended these observations to skin, has included inanition (total food-restricted) controls, and has examined the picture at two time points of accumulation of deoxypyridinoline-enriched collagen and/or collagen products.

Animals and analytical methods

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.* (1969) and the vitamin mixture provided the following amounts of essential vitamins (mg/kg diet): retinol (as retinyl acetate) 2.4, cholecalciferol 0.0075, α -tocopherol 60.0, menadione 10.0, thiamin hydrochloride 16.0, riboflavin 16.0, pyridoxine hydrochloride 16.0, calcium pantothenate 40.0, nicotinamide 200.0, pteroylmonoglutamic acid 10.0, cyanocobalamin 0.05, biotin 10.0. All animals were given a small amount of dried hay daily. The treatment schedules are summarized in Fig. 1. Groups A and D received the basal diet, with L-ascorbic acid (vitamin C) given separately (0.5 mg/d per animal, freshly dissolved in dilute sucrose solution and given by a dropper). Groups B, C, E and F received the basal diet mixed with vitamin C (10.0 g/kg diet). Since the average daily food intake was 16–32 g/d, the average daily vitamin C intake in groups B, C, E and F was 160.0–320.0 mg/d.

Groups A, C, D and F received their respective diets *ad libitum*. Groups B and E received a restricted amount of the vitamin C-containing diet, each animal in group B

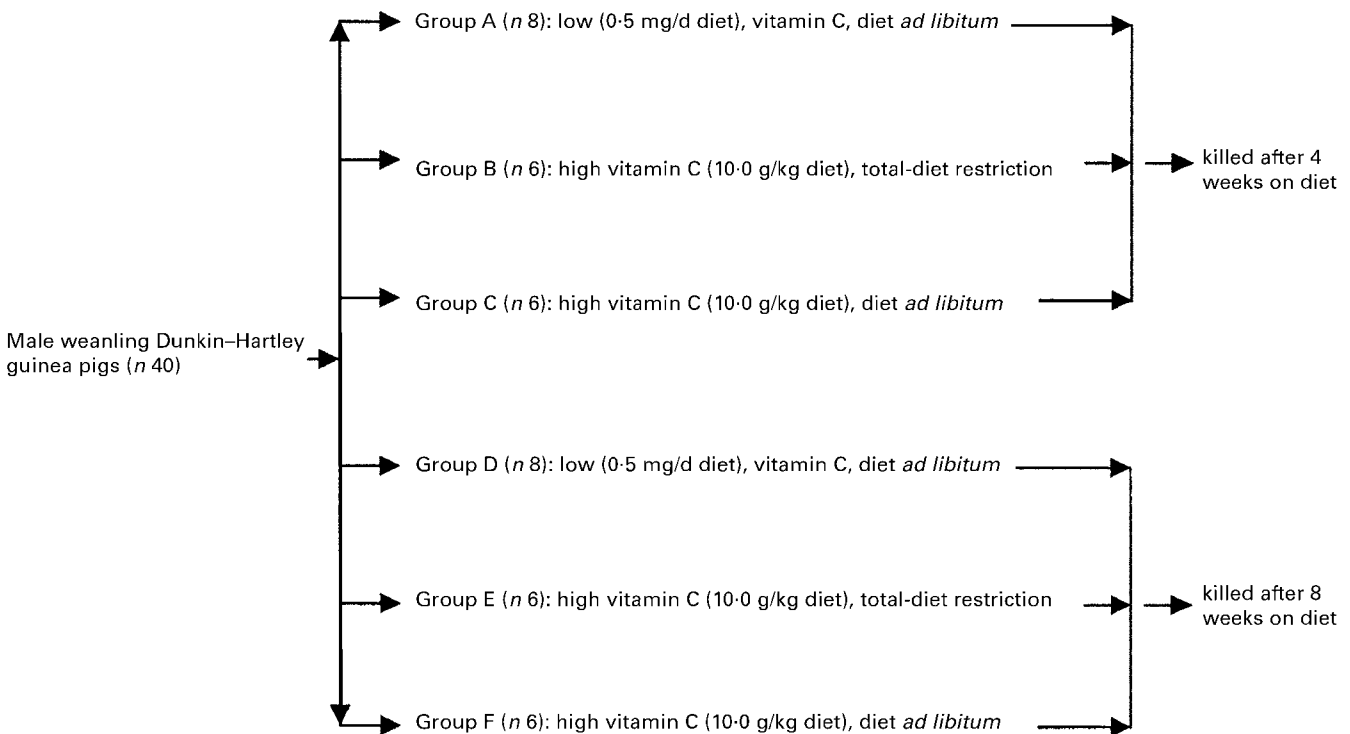


Fig. 1. Treatment groups: summary of schedules.

receiving 85% of the mean intake of all animals in group A on the previous day. Likewise, each animal in group E received 85% of the mean intake of all animals in group D on the previous day. This regimen ensured that the mean growth rates in groups B and E were nearly the same, but slightly less than the mean growth rates in groups A and D. The purpose of the food-restricted groups was solely to investigate the specificity of the vitamin C effects on the collagen cross-link patterns, and not to study the effects of energy restriction for their own sake. Groups A, B and C were fed the diets for 4 weeks before killing; groups D, E and F were fed the diets for 8 weeks before killing.

The diets were stored at 4°C for up to 2 weeks before use, which ensured negligible loss of vitamin C. All animals had free access to water and were housed singly in steel-mesh cages, with a 12 h light–dark cycle at 19–23°C. There were six to eight animals per group, male inbred Dunkin–Hartley albino guinea-pigs (about 3–4 weeks old), obtained from Harlan (Wyton, Huntingdon, Cambs., UK). Within each of the two sets (i.e. groups A, B, C; groups D, E, F), they were distributed to ensure nearly identical mean body weights per group (Table 1). Individual daily records of body weight and of food eaten were kept. A single 8 h urine sample in dilute HCl was collected from each animal in a metabolism cage, 2 or 3 d before killing: the animals were killed by CO₂ anaesthesia. Blood (by cardiac puncture) was collected in heparin anticoagulant and was immediately separated to provide plasma. This, together with both adrenal glands and a portion of liver, was extracted with metaphosphoric acid (50 g/l, final concentration) for vitamin C assays. Dissected femurs and dorsal interscapular skin samples were stored at –20°C for the measurement of hydroxyproline and of collagen cross-links.

Analytical methods

Vitamin C (L-ascorbate + dehydroascorbate) was measured in diets (to check for possible losses) and in blood plasma, adrenal glands and liver samples. Diets and organs were

immediately homogenized (Potter-Elvehjem glass-teflon homogenizer) in 10 vol. cold metaphosphoric acid (50 g/l) and were centrifuged. Vitamin C in the stabilized extracts, after storage at –80°C, was measured by a fluorometric assay (Vuilleumier & Keck, 1989), involving ascorbate oxidase treatment, followed by orthophenylene diamine, in a Roche Cobas Bio centrifugal analyser with a fluorescence attachment (F. Hoffmann La Roche Diagnostica, Basle, Switzerland).

One femur per animal was dissected free of adventitious tissue and marrow; the shaft only, minus the epiphyseal and metaphyseal regions, was decalcified with EDTA solution as described previously (Tsuchiya & Bates, 1997, 1998), and was dissolved in 9 ml 6 M-HCl by heating at 100°C for 24 h in a glass screw-capped container with a Teflon-lined cap. The resulting acid hydrolysates were evaporated at 37°C under a stream of N₂, and re-evaporated several times with water additions to remove all HCl. They were then redissolved in water, adjusted to neutral pH with dilute NaOH, and were used for the measurement of hydroxyproline (Ho & Pang, 1989), and of pyridinoline and deoxypyridinoline (see later). Urine samples were hydrolysed with 6 M-HCl and were evaporated, redissolved in water and used for hydroxyproline, pyridinoline and deoxypyridinoline analyses. Skin samples were scraped free of fur and of adventitious fat and blood vessels, and a measured area (about 76 cm²) from each animal was hydrolysed in 6 M-HCl and was prepared for hydroxyproline and collagen cross-link assays in the same way as for the demineralized femur shaft samples.

The collagen cross-links, pyridinoline and deoxypyridinoline, were measured in the evaporated acid hydrolysates of the demineralized femur shaft, urine and skin samples by an antibody-based ELISA plate assay (Metra Biosystems (UK) Ltd, Wheatley, Oxon., UK). They were specific immuno-assays for: (1) deoxypyridinoline; (2) pyridinoline + deoxypyridinoline, which have been validated against HPLC assays (Robins, 1982, 1994; Robins *et al.* 1994) and further validated for guinea-pig-derived bone and urine hydrolysates in the authors' laboratory (Tsuchiya & Bates, 1997).

Table 1. Body weights and food intakes of guinea-pigs fed vitamin C-restricted or -adequate diets *ad libitum* or in restricted quantities* (Mean values and standard deviations)

	Treatment group†											
	A (n 8)		B (n 6)		C (n 6)		D (n 8)		E (n 6)		F (n 6)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Body weight (g)												
Initial	250 ^a	16.5	251 ^a	19.8	251 ^a	12.3	274 ^c	15.5	275 ^c	21.7	274 ^c	17.4
Final	388 ^a	40.6	381 ^a	25.7	429 ^a	25.6	459 ^c	83.1	449 ^c	38.3	622 ^d	79.3
Change	137 ^a	31.8	130 ^a	23.8	178 ^b	22.0	185 ^c	69.8	174 ^c	24.6	347 ^d	89.0
Food intake (g/d)												
Entire period	19.5		16.5		22.5		20.0		17.1		22.5	
Last 10 d	19.8		16.5		27.2		19.8		16.1		31.9	

^{a,b,c,d}Mean values within a row for each set of three groups of animals (A, B, C and D, E, F) with unlike superscript letters were significantly different (ANOVA followed by the Scheffé test): *P* < 0.05.

* For details of diets and procedures, see p. 304.

† For descriptions of groups, see Fig. 1. Groups A and D received the diet with no added ascorbic acid, and received 0.5 mg ascorbic acid in dilute sucrose solution/d by a dropper. Groups B, C, E and F received the diet containing 10.0 g ascorbic acid/kg diet. Groups C and F received this diet *ad libitum*; groups B and E had their intakes restricted by group-matching to groups A and D respectively, and received on average 82% of the voluntary intake of groups A and D.

Relationships between outcome indices were tested by ANOVA, followed by the Scheffé test, with a DataDesk statistical package (Data Description, Inc., Ithaca, NY, USA). $P < 0.05$ was deemed to be statistically significant.

Results

Table 1 confirms that, within each of the two sets, the three groups of animals were well matched in terms of initial body weight. Over the 4-week feeding period of groups A, B and C, the vitamin C-restricted group A grew, on average, at 77% of the rate of the control group C. Over the 8-week feeding period of groups D, E and F, the vitamin C-restricted group D grew, on average, at 53% of the rate of the control group F. The food-restricted groups B and E grew at about 94–95% of the rates of the vitamin C-restricted groups A and D. The observed differences in food intakes mirrored these differences in growth, especially during the latter part of the study. All animals, including those in the vitamin C-restricted groups, remained apparently healthy during the entire period, and none exhibited any overt pathological symptoms of scurvy.

Table 2 shows that whereas groups A and D had similar very low concentrations of ascorbate in their plasma and tissues when killed after 4 or 8 weeks on the diets, groups B, C, E and F all had much higher concentrations of the vitamin. The plasma ascorbate:tissue ascorbate concentration ratios between the vitamin C-restricted and control groups varied between 10- and 50-fold, but these ratios were little affected either by the duration of feeding or by the presence or absence of food restriction.

The results presented in Table 3 show that neither vitamin C restriction nor food restriction significantly affected the concentration of hydroxyproline per unit wet weight of bone. Neither the total cross-links:hydroxyproline ratios nor the pyridinoline:hydroxyproline ratios in femur shaft differed significantly between groups (Table 3). If the model was adjusted for final body weight, the pyridinoline:hydroxyproline ratio was significantly lower ($P < 0.05$) in group D than in group F (results not shown). The deoxypyridinoline:hydroxyproline ratios were significantly higher ($P < 0.05$) in the vitamin C-restricted groups (A and D) than in any of the other groups at both time points (Table 3). The values for the food-restricted groups (B and E) did not, however, differ significantly from the control groups fed *ad libitum* (C and F) at either time point. The conclusions were similar for the pyridinoline:deoxypyridinoline ratio (Table 3), except that this ratio was reduced, instead of being increased, in the two vitamin C-restricted groups.

The inter-group comparisons for the urine indices, as shown in Table 4, were very similar to the inter-group comparisons for the bone indices, especially at the 8-week time point. Again, the total cross-links:hydroxyproline ratio and the pyridinoline:hydroxyproline ratio did not differ significantly between groups. Both the deoxypyridinoline:hydroxyproline and the pyridinoline:deoxypyridinoline ratios were significantly shifted towards deoxypyridinoline accumulation in the vitamin C-restricted group (D), but not in the food-restricted group (E). At the 4-week time point, both the vitamin C-restricted group A

and the food-restricted group B were significantly different from the control group C fed *ad libitum* with respect to both the deoxypyridinoline:hydroxyproline ratio and the pyridinoline:deoxypyridinoline ratio. When adjusted for final body weight, the pyridinoline:hydroxyproline ratio was significantly lower ($P < 0.05$) in group A than in group C (results not shown).

The pattern of indices in skin differed from those of bone and urine (Table 5). Here, the largest inter-group differences were in hydroxyproline concentration per unit of tissue, especially when expressed per unit area. This index was significantly reduced in the two vitamin C-restricted groups A and D, but not in the food-restricted groups B and E. Comparison between the growth rates shown in Table 1 and the skin hydroxyproline contents per unit area shown in Table 5 suggests that in the vitamin C-restricted groups A and D, the positive changes in body weight and surface area were accompanied by approximately proportionate negative changes in collagen concentration per unit area. Clearly, this was not the case for the other four groups, where the collagen (hydroxyproline) content per unit area of skin increased markedly with age.

At the 4-week time point, there were no significant differences between groups for any of the cross-link ratios in skin (Table 5). At the 8-week time point, however, there was a modest shift in the deoxypyridinoline:hydroxyproline ratio and in the pyridinoline:deoxypyridinoline ratio in the vitamin C-restricted groups that was in the same direction to that in bone and urine. However, the food-restricted group had values intermediate between those of the vitamin C-restricted and the control groups fed *ad libitum*. The proportion of deoxypyridinoline was very low in all six groups, and even in the vitamin C-restricted group at the 8-week time point deoxypyridinoline contributed only 5% total (measurable) cross-links.

Discussion

The present study has confirmed and extended the observations of our previous studies (Tsuchiya & Bates, 1997, 1998). Although a vitamin C intake of only 0.5 mg/d restricted the growth rate of 250 g weanling male guinea-pigs, the animals remained healthy and free from scurvy for the 8 weeks of the study. The growth rate of the 8-week vitamin C-restricted group was about half that of the control group fed *ad libitum*, and their voluntary food intake was about two-thirds that of the control value. All four non-vitamin C-restricted groups received a generous amount of vitamin C compared with the minimum amount that is normally required by guinea-pigs (Clarke *et al.* 1977). We were able, thereby, to ensure high tissue vitamin C concentrations in all groups that were not vitamin C-restricted, including those that were total-food-restricted, as shown in Table 2. The vitamin C concentrations and inter-group differences observed in plasma, liver and adrenal glands (Table 2) are believed to provide reliable surrogate comparisons for the vitamin C concentrations at the sites of collagen synthesis in bone and skin. These tissues do not easily lend themselves to vitamin C analysis; however, there is indirect evidence that they are affected by vitamin C intakes in the same

Table 2. Ascorbate concentrations in plasma and tissues of guinea-pigs fed vitamin C-restricted or -adequate diets *ad libitum* or in restricted quantities*
(Mean values and standard deviations)

	Treatment group											
	A (n 8)		B (n 6)		C (n 6)		D (n 8)		E (n 6)		F (n 6)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Plasma (µmol/l)	4.1 ^a	1.3	193 ^b	20	205 ^b	28	4.0 ^a	1.0	173 ^b	28	197 ^b	35
Liver (mmol/g)	0.049 ^a	0.007	1.50 ^b	0.15	1.72 ^b	0.31	0.048 ^a	0.010	1.47 ^b	0.25	1.68 ^b	0.16
Adrenal glands (mmol/g)	0.57 ^a	0.07	10.9 ^b	2.3	11.1 ^b	1.7	0.48 ^a	0.08	10.5 ^b	0.42	10.9 ^b	1.47

^{a,b}Mean values within a row for each set of three groups of animals (A, B, C and D, E, F) with unlike superscript letters were significantly different (ANOVA followed by the Scheffé test); *P*<0.05.

* For details of diets and procedures, see p. 304.

† For description of groups, see Fig. 1. Groups A and D received the diet with no added ascorbic acid, and received 0.5 mg ascorbic acid in dilute sucrose solution/d by a dropper. Groups B, C, E and F received the diet containing 10.0 g ascorbic acid/kg diet. Groups C and F received this diet *ad libitum*; groups B and E had their intakes restricted by group-matching to groups A and D respectively, and received on average 82% of the voluntary intake of groups A and D.

Table 3. Collagen indices in bone of guinea-pigs fed vitamin C-restricted or -adequate diets *ad libitum* or in restricted quantities*
(Mean values and standard deviations)

	Treatment group											
	A (n 8)		B (n 6)		C (n 6)		D (n 8)		E (n 6)		F (n 6)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hydroxyproline (µmol/g wet wt)	83.2 ^a	8.7	91.4 ^a	11.2	87.6 ^a	8.6	87.5 ^b	7.0	90.9 ^b	7.9	91.2 ^b	6.3
Total cross-links:hydroxyproline ratio (µmol/mol)	735 ^a	91	663 ^a	38	699 ^a	69	825 ^b	118	806 ^b	63	842 ^b	61
Pyridinoline:hydroxyproline ratio (µmol/mol)	620 ^a	70	598 ^a	40	630 ^a	71	646 ^b	118	732 ^b	61	761 ^b	62
Deoxypyridinoline:hydroxyproline ratio (µmol/mol)	115 ^a	25	66 ^b	4	70 ^b	11	179 ^a	35	74 ^b	6	81 ^b	8
Pyridinoline:deoxypyridinoline ratio (mol/mol)	5.55 ^a	0.96	9.11 ^b	1.01	9.26 ^b	1.96	3.70 ^a	0.89	9.87 ^b	0.85	9.52 ^b	1.31

^{a,b}Mean values within a row for each set of three groups of animals (A, B, C and D, E, F) with unlike superscript letters were significantly different (ANOVA followed by the Scheffé test); *P*<0.05.

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Table 4. Collagen indices in urine of guinea-pigs fed vitamin C-restricted or -adequate diets *ad libitum* or in restricted quantities*
(Mean values and standard deviations)

	Treatment group†											
	A (n 8)		B (n 6)		C (n 6)		D (n 8)		E (n 6)		F (n 6)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total cross-links:hydroxyproline ratio (μmol/mol)	1000 ^a	160	1260 ^a	290	1220 ^a	200	1240 ^b	180	1260 ^b	160	1150 ^b	350
Pyridinoline:hydroxyproline ratio (μmol/mol)	800 ^a	120	1030 ^a	290	1060 ^a	200	870 ^b	180	1060 ^b	180	990 ^b	300
Deoxyypyridinoline:hydroxyproline ratio (μmol/mol)	206 ^a	34	236 ^a	60	162 ^b	11	372 ^a	67	203 ^b	27	165 ^b	48
Pyridinoline:deoxyypyridinoline ratio (mol/mol)	3.89 ^a	0.29	4.60 ^a	1.55	6.63 ^b	1.32	2.41 ^a	0.69	5.40 ^b	0.60	5.97 ^b	0.62

^{a, b}Mean values within a row for each set of three groups of animals (A, B, C and D, E, F) with unlike superscript letters were significantly different (ANOVA followed by the Scheffé test); $P < 0.05$.

* For details of diets and procedures, see p. 304.

† For description of groups, see Fig. 1. Groups A and D received the diets with no added ascorbic acid, and received 0.5 mg ascorbic acid in dilute sucrose solution/d by a dropper. Groups B, C, E and F received the diet containing 10.0 g ascorbic acid/kg diet. Groups C and F received this diet *ad libitum*; groups B and E had their intakes restricted by group-matching to groups A and D respectively, and received on average 82% of the voluntary intakes of groups A and D.

Table 5. Collagen indices in skin of guinea-pigs fed vitamin C-restricted or -adequate diets *ad libitum* or in restricted quantities*
(Mean values and standard deviations)

	Treatment group†											
	A (n 8)		B (n 6)		C (n 6)		D (n 8)		E (n 6)		F (n 6)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hydroxyproline concentration (μmol/mm ²)	0.172 ^a	0.021	0.256 ^b	0.046	0.265 ^b	0.051	0.182 ^a	0.068	0.348 ^b	0.058	0.373 ^b	0.086
Hydroxyproline concentration (μmol/g wet wt)	168 ^a	19.2	210 ^b	26.1	176 ^a	20.3	173 ^a	37.9	263 ^b	22.7	224 ^b	35.0
Pyridinoline:hydroxyproline ratio (μmol/mol)	8.71 ^a	1.64	8.96 ^a	1.89	8.19 ^a	1.65	9.40 ^a	2.01	7.63 ^a	1.11	7.90 ^a	2.32
Deoxyypyridinoline:hydroxyproline ratio (μmol/mol)	0.59 ^a	0.15	0.59 ^a	0.16	0.41 ^a	0.14	0.51 ^a	0.15	0.31 ^b	0.13	0.24 ^b	0.09
Pyridinoline:deoxyypyridinoline ratio (mol/mol)	16.7 ^a	2.63	16.3 ^a	3.81	18.9 ^a	5.89	17.7 ^a	5.42	19.8 ^{ab}	3.20	25.9 ^b	6.00

^{a, b}Mean values within a row for each set of three groups of animals (A, B, C and D, E, F) with unlike superscript letters were significantly different (ANOVA followed by the Scheffé test); $P < 0.05$.

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way as the tissues analysed and presented in Table 2. We predict that the vitamin C concentrations at the collagen-synthesizing sites determine the extent of hydroxylysine formation, which in turn determines the inter-group differences in cross-link ratios.

As in our first study (Tsuchiya & Bates, 1997), the concentration of hydroxyproline per unit wet weight of femur did not differ significantly between feed groups. In our second study (Tsuchiya & Bates, 1998), a small and unexpected increase in femur shaft hydroxyproline per unit wet weight was seen when the vitamin C content of the diet was decreased. Taking all three studies together, it appears that femur shaft collagen, unlike dorsal skin collagen, does not fall precipitously in animals that are deprived of vitamin C, nor is it significantly affected in animals whose growth is retarded by total food restriction. This implies either that the accumulation of hydroxyproline in the femur shaft is able to keep pace with overall growth, or that the rate of growth is dictated by the rate of synthesis of collagen here.

In contrast to hydroxyproline and to the total cross-link content of femur shaft, deoxypyridinoline and the pyridinoline:deoxypyridinoline ratio both changed markedly in the animals deprived of vitamin C. This had also been observed in our previous studies (Tsuchiya & Bates, 1997, 1998). In the present study, it was clear that the inter-group differences in cross-link ratios increased progressively with time in the vitamin C-deprived groups, and that these differences were not evident in the energy-restricted groups, whose growth rates were similar to those of the vitamin C-restricted groups. The changes in cross-link ratios, therefore, seem to be specific for vitamin C restriction, and are not a consequence of reduced growth in the vitamin C-restricted animals. In our vitamin C-restricted animals, the pyridinoline:hydroxyproline ratio was either unchanged or was diminished in femur shaft and in urine, whereas in another study (Kim *et al.* 1998), this ratio was either unchanged or was increased in costal cartilage of scorbutic animals. In view of these discrepancies between different studies and/or tissues, we consider the pyridinoline:hydroxyproline index to be less useful than the deoxypyridinoline:hydroxyproline ratio or of pyridinoline:deoxypyridinoline.

A reduction in formation of collagen, specifically in the telopeptide hydroxylysine residues, might cause a shift away from hydroxylysine production and towards allysine production in tissues such as cartilage and bone (Robins, 1988). This, in turn, could result in the formation of cross-links other than the pyridinium type, as reported for skin. This possibility has yet to be investigated in the context of vitamin C deficiency, but if it were to occur, it might result in impaired stability of the collagen cross-links in both bone and cartilage (Bailey *et al.* 1998).

The observed urinary cross-link patterns in the present study have confirmed the conclusions of our previous studies (Tsuchiya & Bates, 1997, 1998) and they clearly reflect the similar patterns seen in the femur shaft. This accords with the well-established fact (Robins, 1994) that urinary collagen breakdown products are derived predominantly from bone turnover, as distinct from that of collagen at other sites in the body. After 8 weeks of feeding, the inter-group differences clearly confirmed the specificity

of the vitamin C restriction response, since they were not shared by the energy-restricted group.

The picture in skin was different from that in bone and urine. Here, the major effect of vitamin C deficiency (not shared by the energy-restricted group) was a large reduction in the total amount of collagen (i.e. of hydroxyproline) per unit area of skin, implying that skin collagen synthesis was failing to keep pace with bone growth and bone collagen. Previous studies have shown that there is only minimal accumulation of proline-rich, hydroxyproline-poor collagen in the skin of scorbutic guinea-pigs, and no evidence of massively increased collagen turnover in this situation (Barnes *et al.* 1970; Barnes & Kodicek, 1972). Therefore, it seems likely that collagen synthesis is largely switched off in scurvy (Barnes & Kodicek, 1972), probably at the level of transcription or translation. The fact that both pyridinoline and deoxypyridinoline were detectable in guinea-pig skin (albeit at a much lower pyridinoline:hydroxyproline and deoxypyridinoline:hydroxyproline ratio than in femur or urine) was unexpected in view of reports that these cross-links are generally absent from skin (Bailey *et al.* 1998). However, it is likely that these cross-links arise from fascia rather than from dermal collagen. In skin, there was a small increase in the percentage of deoxypyridinoline in the vitamin C-restricted group, but after 8 weeks of the diet, its concentration as a percentage of the total pyridinium-type cross-links was only 5%. In terms of numbers of cross-links per collagen molecule, the contribution of the pyridinium cross-links is almost negligible. For skin, it will be particularly pertinent to determine whether changes in lysine hydroxylation affect the mature histidine-derived cross-links that are of major importance in dermal collagen.

An important conclusion from our present study is that the changes in collagen cross-link ratios, presumably resulting from changes in the hydroxylysine formation when dietary ascorbate is inadequate, are not shared by total food-restriction. This observation helps to distinguish those aspects of collagen synthesis and growth that are shared by inanition from those which are not. Adjustment for differences in body weight between the groups did not reduce the magnitude or significance of the vitamin C-related inter-group differences in cross-link ratios; indeed, at two sites the significance of the relationship for the pyridinoline:hydroxyproline ratio was actually enhanced by this adjustment.

Studies of patients with Ehlers–Danlos syndrome type VI have identified a lesion in hydroxylysine formation that shares certain characteristics with vitamin C deficiency. This lesion was responsive to vitamin C intakes above normal human requirements (Pinnell *et al.* 1972; Elsas *et al.* 1978; Dembure *et al.* 1987; Pasquali *et al.* 1994, 1997). In contrast, in children suffering from protein–energy malnutrition, whose growth rates are severely reduced but who are not generally vitamin C-deficient, a restorative diet that increased the total urinary cross-link excretion rate 3-fold did not alter the pyridinoline:deoxypyridinoline ratio (Branca *et al.* 1992). Ageing also affected the rate of collagen cross-links excretion, apparently without affecting the ratio of the cross-links (Acil *et al.* 1996). These contrasts help to

confirm the specificity of the vitamin C deficiency-effect on the cross-link ratios.

It remains to be determined whether the increased formation of deoxypyridinoline in vitamin C-restricted guinea-pigs has any parallel in human subjects, and whether the compositional changes have any functional sequelae. There may be opportunities for development of functional indicators of nutritional status (e.g. vitamin C inadequacy) in man. As noted previously (Tsuchiya & Bates, 1998), the high sensitivity of bone and urinary collagen cross-link ratios to moderate degrees of vitamin C-restriction in guinea-pigs helps to complete the sequence from a pathological lesion (scurvy), via a defined biochemical reaction (altered cross-link ratios), to a potential non-invasive biochemical probe of vitamin C status.

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