

Vitamin C status and collagen cross-link ratios in Gambian children

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Vitamin C (ascorbate) is essential for hydroxylation of prolyl and lysyl residues in nascent collagen, the failure of which leads to connective tissue lesions of scurvy. Of the pyridinium-type cross-links in mature collagen, pyridinoline requires more hydroxylysyl residues than does deoxypyridinoline. Our study tested the hypothesis that pyridinoline:deoxypyridinoline ratios in urinary degradation products may vary with ascorbate status in man. These ratios were compared between British and Gambian prepubertal boys, mean age 8.3 years, and in Gambian boys between two seasons with contrasting ascorbate availability. The mean cross-links ratio in 216 British boys was 4.36 (SD 0.71), significantly greater ($P < 0.0001$) than in sixty-two Gambian boys: 3.83 (SD 0.52). In the Gambians the cross-links ratio was significantly higher in the dry season (with high ascorbate intake and status) than in the rains (with low intake and status). A 7-week controlled intervention was carried out in Gambian boys during the rainy season (the 'hungry' season, when vitamin C-containing foods are virtually unavailable): 100 mg ascorbate/d was given to one group of thirty-two Gambian boys and placebo to another group. The intervention did not, however, significantly alter the cross-link ratio, possibly because the response time and/or intervention–response delay is > 7 weeks. If confirmed, the putative association between ascorbate and collagen cross-link ratios in man could become the basis for a functional test for adequacy of ascorbate status.

Vitamin C: Collagen: Cross-links: Gambian children

Functional status indices can prove useful to investigate human requirements for individual micronutrients. They can also provide a window on status that connects nutrient intakes or tissue concentrations to essential physiological functions and the risk of tissue malfunction during deficiency. For vitamin C, the best-understood molecular lesion that is associated with severe and prolonged deficiency is the failure of collagen synthesis, which leads in turn to a failure of wound healing, to bleeding and to epiphyseal plate lesions in the growing long bones. These lesions are characteristic effects of scurvy. At the enzyme level, failure of hydroxylation of prolyl and lysyl residues in nascent collagen chains (Kivirikko & Myllyla, 1982; Anonymous, 1984; Myllyla *et al.* 1984) leads to a virtual cessation of new collagen synthesis (Barnes & Kodicek, 1972). A more subtle effect, possibly occurring in conditions of less severe vitamin C deficiency, could be impaired hydroxylation of certain collagen lysyl residues. This may result in a shift in collagen cross-link patterns, away from a preponderance of the hydroxylysyl–pyridinoline (here called pyridinoline) cross-link-type, towards the alternative lysyl–pyridinoline (here called deoxypyridinoline) one, which requires one less hydroxylysyl residue since it is replaced by a lysyl residue (Robins, 1994; Bailey *et al.* 1998). Such an alteration in cross-link pattern, potentially the result of a mild dietary vitamin C deficiency, has been demonstrated in guinea-pigs (Tsuchiya & Bates, 1997, 1998, 2003) and in human subjects with the genetic disorder Ehlers–Danlos syndrome type VI. In the latter, a structurally altered lysyl hydroxylase enzyme requires abnormally large amounts of vitamin C in order to achieve partially adequate function (Pinnell *et al.* 1972; Quinn & Krane, 1976; Miller *et al.* 1979; Acil *et al.* 1995).

The hypothesis that variations in vitamin C status may result in variations in ratios of collagen pyridinium-type cross-links has not been tested, to our knowledge, in genetically normal human subjects. In The Gambia in sub-Saharan West Africa, dietary availability of vitamin C changes in an annual cycle over a very wide range between the seasons, because seasonal vitamin C-rich crops, especially mangoes, are available only in the dry season, whereas few vitamin C-rich foods are available during the rainy season (Bates *et al.* 1994). The present study has compared plasma vitamin C and urinary collagen pyridinium-type cross-links between Gambian and British boys, between the rainy and the dry seasons in prepubertal boys in The Gambia, and before and after a vitamin C supplement during the rainy season in the Gambian boys.

Subjects and methods

British population sample

The National Diet and Nutrition Survey of Young People Aged 4–18 Years surveyed a representative sample of British young people during the year 1997. Details of the survey are published in the survey report (Gregory *et al.* 2000). Subjects (n 1193) were selected at random from 132 postcode areas, which were also randomly selected in four seasonal 'waves' over a 12-month period. Because of the random selection procedure, any possible effects of clustering can be ignored. A representative subset provided a blood and urine sample, plus a 7 d weighed diet estimate and anthropometric measurements. Of these, 216

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were boys aged 5 to 11 years, thus matching the Gambian sample (see later).

Weight and height were measured by standard procedures at a single time-point. A 7 d diary of all food and drink consumed inside and outside the home was kept and checked for accuracy by trained interviewers. The food items were converted to nutrient intakes, including vitamin C, by food tables with 6000 food codes, compiled by the Ministry of Agriculture, Fisheries and Food and the Food Standards Agency.

Single, early-morning, fasting venous heparinised blood samples were collected by phlebotomists and were transported in a cool-box to local hospital laboratories within 4 h of collection. They were separated in a refrigerated centrifuge, and an aliquot of plasma was stabilised with an equal volume of 10% w/v metaphosphoric acid and stored for up to 3 months at -40 to -80°C before analysis of total vitamin C (see later).

A single, first-void, early-morning urine sample was requested from each subject and was posted to the Dunn Nutrition Laboratory in Cambridge (this part of the Dunn Nutrition Laboratory is now part of MRC Nutrition Research). Pyridinium cross-link concentrations and ratios in urinary collagen degradation products, and creatinine, were measured as described later.

Gambian population sample

Seventy-four rural Gambian boys, aged 5 years 8 months to 10 years 5 months at the start of the study, were recruited in the year 2000 from two neighbouring villages, Bajana and Kulikunda, in the West Kiang region. Exclusion criteria comprised: having broken a bone during the previous 6 months, having any infection at the time of recruitment or having recently taken vitamin supplements containing vitamin C. Such vitamin supplements were rarely used, and would only be available by prescription in this community. Informed consent was obtained from a parent or guardian and from each boy before the commencement of the study. The boys were free to withdraw from the study at any time without giving a reason. However, of the seventy-four recruited, only ten withdrew during the entire 9-month period of the study, and of the remaining sixty-four, sixty-two provided nearly complete sample sets. There was no evidence of any significant bias, as indicated by age, anthropometry, initial vitamin C status or supplement assignment, in those who withdrew. Anthropometric measurements (body weight and height) were made by standard procedures (electronic scales and stadiometer) at intervals during the study.

Birth dates, to the nearest month, were established from Road to Health growth charts that are kept by all children in The Gambia, and each boy was matched as closely as possible to another boy of a similar age and stature. One member of each pair was then randomly allocated to the control (placebo) or the vitamin C intervention group, the allocation being maintained as double-blind by confining the knowledge of the code to an independent third party.

The vitamin C-supplemented group received vitamin C powder (100 mg/d; DDSA Pharmaceuticals Ltd, London, UK), dissolved in a proprietary orange-coloured and -flavoured drink, for a 7-week period during the second half of the rainy season (August to October 2000). Double doses of vitamin were given on Fridays and Mondays, in lieu of dosing at weekends. The placebo group received an identical daily drink containing 50 mg citric acid in place of the ascorbic acid.

At three seasonal time-points, about 2.7 ml venous blood was collected by venepuncture early in the morning before vitamin dosing, using serum monovettes (Sarstedt, Leicester, UK). The first time-point was before commencement of the intervention in August (baseline: mid rainy season); the second was at the end of the vitamin C intervention period in October (end-intervention: late rainy season), and the third was during the follow-up study in April 2001 (follow-up: dry season). To stabilise vitamin C in the serum, 0.5 ml aliquots were mixed with 0.5 ml 10% w/v metaphosphoric acid, and the stabilised extracts were stored at -80°C until analysis (see later).

Single early-morning urine samples were collected on each of three consecutive days during the week before the intervention began in August 2000, during each of the seven weeks of the intervention, and again during a 3-week period during the follow-up study in April 2001. The three urine samples that were obtained from each subject during each study week were subsequently pooled to yield a single weekly sample for each subject. This pooling procedure was employed to reduce the high intra-subject variability that can arise if single non-pooled urine samples are used in collagen cross-link studies (Ginty *et al.* 1998). Although separate analysis of individual urine collections is theoretically preferable, the cost and time required to do this were prohibitive. Pyridinium cross-link concentrations and ratios in urinary collagen degradation products, and creatinine, were measured as described later.

For the estimation of vitamin C intakes, dietary recall questionnaires were designed, based on the foods most commonly available in West Kiang. The quantities of staple foods, including grain-based porridges such as millet, sorghum and rice, and the main vitamin C-containing foods, i.e. fruits, vegetables and leaf sauces, were recorded three times a week (Monday, Wednesday, Friday) at each of the three seasonal time-points (August, October and April) with the assistance of Gambian fieldworkers. The records were converted to daily vitamin C intakes by using food table nutrient contents of Gambian foods, compiled by the Databases Section of the MRC Dunn Nutrition Unit.

Biochemical measurements

Total vitamin C (ascorbate + dehydroascorbate) was measured by a fluorescence assay (Vuilleumier & Keck, 1989) using a Roche (Basle, Switzerland) Cobas Bio centrifugal analyser.

The two pyridinium collagen cross-links, pyridinoline and deoxypyridinoline, were separated and quantified by an HPLC collagen cross-links assay from Chromsystems (Munich, Germany). First, an aliquot of urine (250 μl) was mixed with 250 μl concentrated HCl plus kit internal standard in a screw-capped glass tube and then hydrolysed at 110°C for 12–16 h. The hydrolysate was mixed with 2.5 ml extraction buffer and applied to a pre-conditioned solid-phase extraction (SPE) cartridge for preliminary purification. After washing with buffer, the cross-links and internal standard were eluted with kit elution buffer. Twenty microlitres of the SPE column eluate, or of a mixed pyridinoline + deoxypyridinoline + internal standard (two levels supplied for calibration), were separated on a 100 mm \times 3 mm, 5 μm particle size, octadecylsilica column (run time 15 min). Fluorescence peak height was measured at 290 nm excitation and 400 nm emission. Preliminary checks in our laboratory indicated 95% (all assay stages) recovery of pyridinoline and 93.5% recovery of deoxypyridinoline, with in intra-day peak height variation of <2% for repeat injections and an inter-day variation of 3.2–5.5% for the

three measured components (two cross-links and internal standard).

Urinary creatinine was measured by an ABx Diagnostics (Montpellier, France) picrate-based assay kit, on a Roche Cobas Bio or Fara centrifugal analyser.

Statistical analyses

Comparisons between British and Gambian indices were analysed by Student's *t* test. Seasonal differences within the Gambian data were analysed by the paired *t* test. For the inter-season analyses, in order to minimise the effects of within-subject variability and analytical error, three weeks of urinary cross-link values were averaged for each subject, within each season. The cross-link ratios (pyridinoline:creatinine, deoxypyridinoline:creatinine, pyridinoline:deoxypyridinoline) exhibited a Gaussian distribution, as indicated by a skewness coefficient <1.0 for all datasets. The response to the vitamin C intervention trial was analysed by an analysis of covariance model with end-intervention (average of weeks 6 to 8) collagen cross-link ratios as the outcome variable fitted to supplementation as a factor and the baseline (week 1) collagen cross-link ratios as a covariable. This tested the null hypothesis that the changes in cross-link ratios, adjusted for baseline values, are identical between the placebo and supplemented groups. The statistical analyses were carried out with Excel (Microsoft Corp., Redmond, WA, USA) and DataDesk (Data Description Inc., Ithaca, NY, USA) statistical packages.

Ethics

Ethical approval for the Gambian study was obtained from Cambridge Local Ethics Committee and the Medical Research Council Gambian Ethics Committee. Ethical approvals for the National Diet and Nutrition Survey and for the additional urinary cross-link measurements after completion of this survey were obtained from the South Thames Multi-centre Research Ethics Committee.

Results

Table 1 shows that, as predicted, the Gambian boys were both shorter and lighter than British boys of the same age, and their BMI was also lower. Overall, dietary vitamin C intakes and plasma (serum) concentrations were lower in the Gambian boys. If broken down by season (see Table 2), in the rainy season Gambian boys had only half the vitamin C intake from food and half the serum vitamin C concentration of their British counterparts, but in the dry season their vitamin C intake from food and their serum level were somewhat higher than those of the British boys. The British boys' plasma vitamin C level did not vary significantly by season: thus the mean value for the summer + autumn (July–December) was within 1% of the mean for winter + spring (January–June). The precise quantitative comparison of vitamin C intakes between the British and Gambian datasets requires cautious interpretation, since the food intake estimation methodologies were somewhat different between the two studies.

The Gambian boys had a significantly higher urinary collagen cross-link pyridinoline:creatinine ratio than the British boys (Table 1). The urinary cross-link deoxypyridinoline:creatinine ratio exhibited a similar pattern, being significantly higher in the Gambian boys than in the Britons. The urinary pyridinoline:

deoxypyridinoline ratio was, in contrast, significantly higher in the British boys than in the Gambians (Table 1).

Table 2 provides a more detailed breakdown of the vitamin C (intake and status) and collagen cross-link patterns in the Gambian boys. Despite an increase in serum vitamin C concentration between mid and late rainy season, attributable to the vitamin C supplement given to half the boys during the controlled intervention trial, there was no significant increase in the urinary pyridinoline:deoxypyridinoline cross-links ratio (by paired *t* test) between mid and late rainy season; indeed the mean ratio was remarkably constant during this period. However, by paired *t* test within individual Gambian boys, the cross-links ratio was significantly greater in the dry season than in the rains. Likewise, both pyridinoline:creatinine and deoxypyridinoline:creatinine were also significantly higher in the Gambian boys at the dry season time-point than they were during the rainy season, although there was no significant difference in either of these ratios between mid and late rains (August *v.* October).

If broken down by supplement group, then in the placebo group, serum vitamin C fell from 28.6 (SD 21.5) $\mu\text{mol/l}$ in August to 17.2 (SD 16.3) $\mu\text{mol/l}$ in October, and then rose to 82.9 $\mu\text{mol/l}$ (SD 18.4 $\mu\text{mol/l}$) in April. As a result of the vitamin C supplement, in the supplemented group serum vitamin C rose from 26.6 (SD 18.6) $\mu\text{mol/l}$ in August to 55.3 (SD 22.7) $\mu\text{mol/l}$ in October. It then rose further to 71.8 (SD 19.9) $\mu\text{mol/l}$ in April, as a result of increased vitamin C intake from food such as mangoes in the dry season. Paradoxically, in April, the placebo group which had received no vitamin C supplement during the rainy season had a significantly higher serum vitamin C level than the (previously) supplemented group, after 7 weeks of supplementation during the rains (82.9 *v.* 71.8 $\mu\text{mol/l}$, $P=0.03$).

Table 3 presents the analysis comparing the placebo and supplemented groups in the vitamin C supplementation trial. Neither the urinary pyridinoline:creatinine ratio, nor the urinary deoxypyridinoline:creatinine ratio, nor the urinary pyridinoline:deoxypyridinoline ratio exhibited any significant intervention effects, as judged by the changes in cross-link ratios compared between the two groups, adjusted for baseline values.

Discussion

Although it has been known for more than half a century that severe vitamin C deficiency interferes drastically with connective tissue integrity, and that new collagen synthesis is virtually prevented by a dietary lack of the vitamin in those species that cannot synthesise it *de novo* from precursor sugars, nevertheless the key functional outcomes of sub-optimum vitamin C intakes and status at the molecular level have yet to be elucidated in detail. Collagen pyridinium cross-links are essential for the physical stability of the most common types of collagen fibrillar network, responsible for the overall mechanical strength of most tissues. Their formation is dependent on collagen lysyl hydroxylation (Robins, 1994; Bailey *et al.* 1998), an enzymatic reaction that depends specifically on vitamin C. The amount of total pyridinium cross-links excreted in urine has proved a sensitive and useful diagnostic marker for certain types of metabolic bone disease (Seibel *et al.* 1992; Robins, 1994; Fraser, 1998). The urinary molar ratio of the two pyridinium cross-links, unlike the excretion rate of the individual cross-links, appears to remain remarkably constant in humans over a wide age range, from young children

Table 1. Comparison between British and Gambian population samples: anthropometry, vitamin C intakes and status, and collagen cross-link indices (Mean values and standard deviations)

Population group	Age (months)		Body weight (kg)		Height (m)		BMI (kg/m ²)		Vitamin C intake (mg/d)		Plasma or serum vitamin C (µmol/l)†		Urine pyr:creatinine (nmol/mol)		Urine dpy:creatinine (nmol/mol)		Urine pyr:dpy (mol/mol)		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
NDNS (n 216)†	100.6	19.6	28.8	8.4	1.29	0.107	16.9	2.60	76.4	47.8	62.0	22.3	250.7	60.9	59.1	18.7	4.39	0.71	
Gambia (n 62)†	99.7	15.3	21.1	3.3	1.22	0.084	14.1	1.08	58.3	10.2	47.2	15.0	335.2	61.9	89.1	20.2	3.83	0.52	
Difference 95% confidence interval	0.9		7.7 6.3, 9.1		0.077 0.052, 0.103		2.73 2.35, 3.23		18.1 11.0, 25.1		14.8 9.9, 19.7		84.5 66.9, 102.1		30.0 24.4, 35.7		0.56 0.39, 0.71		

pyr, pyridinoline (= hydroxylysyl-pyridinoline); dpy, deoxypyridinoline (= lysyl-pyridinoline); NDNS, National Diet and Nutrition Survey of Young People Aged 4 to 18 Years (in Great Britain).

†Numbers (n) shown are the maximum numbers in each row. Missing values reduced these to n 208 for vitamin C intake in the NDNS group. The Gambian data are the mean of means (for each subject) for the three time-points in both the wet and dry seasons.

‡In the NDNS, vitamin C status was measured in heparinised plasma; in the Gambian samples it was measured in serum.

§Except for age ($P > 0.05$) and vitamin C intake ($P < 0.01$), all the means were significantly different from each other ($P < 0.0001$) by Student's *t* test.

Table 2. Comparison of vitamin C intakes and status, and collagen cross-link indices, between seasons, in Gambian boys

Time-point	Vitamin C intake from food only (mg/d)		Serum vitamin C (µmol/l)†		Urine pyr:creatinine (nmol/mol)		Urine dpy:creatinine (nmol/mol)		Urine pyr:dpy (mol/mol)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Mid rains (n 62)	36.9 ^a	5.8	27.6 ^a	19.9	311.4 ^a	68.2	83.8 ^a	19.9	3.81 ^a	0.53
Late rains (n 62)	33.7 ^b	7.2	36.9 ^b	27.5	329.8 ^a	66.3	87.6 ^a	21.2	3.80 ^a	0.53
Dry season (n 62)	104.3 ^c	29.4	77.2 ^c	19.8	364.3 ^b	82.4	95.8 ^b	27.1	3.90 ^b	0.54
Mid-to-late rains difference, 95% CI	-1.0, -5.4		+2.1, +16.5		+2.9, +33.9		-0.3, +7.9		-0.029, +0.053	
Late rains to dry season, 95% CI	+62.8, +78.4		+31.2, +49.3		+18.4, +50.6		+3.1, +13.3		+0.062, +0.143	

pyr, pyridinoline (= hydroxylysyl-pyridinoline); dpy, deoxypyridinoline (= lysyl-pyridinoline); CI, confidence interval.

†Missing values reduced the number (n) to sixty for serum vitamin C.

^{a,b,c}Mean values within columns with different superscript letters are significantly different from each other ($P < 0.01$). Comparisons between the three datasets were made by the paired *t* test. The increase in serum vitamin C between mid and late rains is attributable to the vitamin C supplement given to half the boys. However, the seasonal changes in the collagen cross-link indices were independent of the supplement (i.e. they were observed equally in both the supplemented and unsupplemented groups).

Table 3. Comparisons between placebo and vitamin C-supplemented groups of Gambian boys: vitamin C intakes and status, and collagen cross-link indices between mid and late rains

(Mean values and standard deviations)

Time-point, subgroup	Vitamin C intake from food (mg/d)		Serum vitamin C ($\mu\text{mol/l}$)		Urine pyr:creatinine (mmol/mol)		Urine dpy:creatinine (mmol/mol)		Urine pyr:dpy (mol/mol)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Placebo group, mid rains (<i>n</i> 30)	35.9	6.4	28.6	21.5	304.6	66.8	83.1	19.6	3.78	0.59
Placebo group, late rains (<i>n</i> 30)	34.3	7.6	17.2 ^a	16.3	322.6	59.8	87.3	22.4	3.76	0.58
Difference (late–mid)	–1.6	8.4	–11.4 ^{aa}	18.2	+18.0	61.0	+4.3	18.0	–0.02	0.12
95% CI	–4.7, +1.5		–18.3, –4.4		–4.8, +40.8		–2.4, +11.0		–0.06, +0.02	
Supplemented group, mid rains (<i>n</i> 32)	37.8	5.2	26.6	18.6	317.9	70.0	84.5	20.4	3.84	0.48
Supplemented group, late rains (<i>n</i> 32)	33.1	6.9	55.3 ^b	22.7	336.6	72.2	87.9	20.3	3.83	0.48
Difference (late–mid)	–4.7	8.6	+28.7 ^{bb}	20.5	+18.8	62.4	+3.4	14.4	–0.01	0.20
95% CI	–7.8, +1.6		+21.1, +36.2		–3.7, +41.2		–1.8, +8.6		–0.08, +0.06	
Supplement–placebo difference for the mid-to-late rains increment†, 95% CI	–7.6, +1.5		+27.8, +49.8		–48.8, +25.6		–14.3, +5.6		–0.14, +0.21	

pyr, pyridinoline (= hydroxylysyl–pyridinoline); dpy, deoxypyridinoline (= lysyl–pyridinoline); CI, confidence interval.

^{a,b,aa,bb}Means with different superscript letters (a v. b; aa v. bb) are significantly different from each other ($P < 0.01$) by Student's *t* test. There were no significant differences ($P < 0.05$) between supplemented and placebo groups, for any of the collagen cross-link indices.

† The period of controlled supplementation with vitamin C.

to older adults (Beardsworth *et al.* 1990; Acil *et al.* 1996; Branca *et al.* 2002).

Evidence that optimal vitamin C status may be critical for optimal pyridinium cross-link formation, which in turn may be reflected uniquely by the cross-link ratio pyridinoline:deoxypyridinoline in bone collagen and in the urinary collagen degradation products of bone turnover, has been obtained from two sources. The first was a series of controlled vitamin C intake studies on guinea-pigs, which, like man, are totally dependent on dietary sources of vitamin C. In weanling guinea-pigs, following a wide range of vitamin C intakes and with a correspondingly wide range of tissue vitamin C concentrations, the pyridinoline:deoxypyridinoline cross-link ratio in the long bones and in urinary collagen degradation products were both highly sensitive to the vitamin C supply (Tsuchiya & Bates, 1997, 1998, 2003; Bates, 1999). This dependence of the cross-link ratios on vitamin C was specific, insofar that it was not significantly affected by total food restriction (Tsuchiya & Bates, 2003).

The second source of evidence for an effect of vitamin C on collagen pyridinium cross-link ratios arose mainly from studies of the rare human genetic disease, Ehlers–Danlos syndrome type VI. Sufferers have a tendency to sustain fractures, having a low bone mass and abnormal bone structure (Dolan & Arden, 1998). A point mutation in the gene coding the enzyme collagen lysyl hydroxylase (Miller *et al.* 1979) results in much lower than normal levels of hydroxylysine in collagen. This molecular lesion is accompanied by clinical lesions that are attributable to an underlying impairment of collagen structure, characterised by an excess of deoxypyridinoline and a paucity of pyridinoline (Pinnell *et al.* 1972; Elsas *et al.* 1978; Dembure *et al.* 1987; Pasquali *et al.* 1997). Both the clinical abnormalities and the extent of collagen lysyl hydroxylation have been shown to respond favourably to high oral doses of vitamin C (Elsas *et al.* 1978). An altered urinary collagen pyridinium cross-link ratio, with a decreased ratio of

pyridinoline:deoxypyridinoline, is a characteristic feature of this disease (Acil *et al.* 1995).

There is also evidence from other genetic conditions and comparisons that support the relationship between bone quality, collagen lysyl hydroxylation and pyridinium cross-link patterns. A genetic abnormality in which the urinary ratio of pyridinoline:deoxypyridinoline has been reported to be abnormally low is Ullrich–Turner syndrome (Rauch *et al.* 1995). Under-hydroxylation of lysine residues in bone collagen type I telopeptides has also been reported in Bruck syndrome (Bank *et al.* 1999), which is characterised by short stature, fragile bones and osteoporosis. A recent study (Banse & Sims, 2002) found that those bone samples from genetically normal people which had a relatively high pyridinoline:deoxypyridinoline ratio had better bone tissue quality, being stronger and stiffer than those with a lower ratio.

Whereas collagen cross-link ratios appear to be specifically responsive to variations in vitamin C status, they are not responsive to other metabolic insults such as general malnutrition. Thus a study of severely malnourished African children during recovery from protein–energy malnutrition detected major changes in total cross-link excretion rates, but found no significant change in the urinary pyridinoline:deoxypyridinoline ratio (Branca *et al.* 1992).

In the present study, the inter-country comparison of vitamin C intakes, vitamin C status and collagen cross-link ratios appears to provide support for the hypothesis that vitamin C status may influence the hydroxylation of lysine and hence the urinary cross-link ratios, in genetically normal human subjects. For about three-quarters of the year (July to March), vitamin C-rich foods are in short supply in rural Gambian communities (Bates *et al.* 1994), and vitamin C status is then much lower in The Gambia than in Britain. A large difference in vitamin C status, favouring the British boys (whose status and intakes are essentially constant throughout the year), was observed in the present study when

Gambian status was measured in August and October. Only when the Gambian boys' status was measured in April, during the brief 3-month mango season, was it higher than in the British boys. The urinary ratio of pyridinoline:deoxypyridinoline was significantly lower in the Gambian than the British boys (Table 1).

Such an inter-country difference in urinary excretion patterns might, of course, have a genetic origin. A much smaller, but nevertheless statistically significant difference was, however, also observed between the wet and dry season values of the urinary collagen cross-link ratios in the Gambian boys (Table 2). Thus the dry season value for the pyridinoline:deoxypyridinoline ratio, which is the season of higher vitamin C status (even in those Gambian subjects who received the vitamin C supplement during the rains), was significantly higher than the rainy season ratio. When single urine samples were pooled for three days each week, and three weeks' pooled data were then used to characterise the seasonal value for each subject, with the same sixty-two subjects measured at each season the confidence interval was only 2% of the mean value (Table 2). This illustrates the high degree of constancy of the cross-link ratio within individuals over time. In addition to the small but significant change in the cross-link ratio, both the pyridinoline:creatinine ratio and the deoxypyridinoline:creatinine ratio were significantly higher in the dry season than the rainy season. This may result from increased bone growth in the dry season, when food is generally more plentiful and seasonal illness is at a lower prevalence and severity. With sixty-two subjects studied, the minimum detectable ($P < 0.05$) within-subject seasonal change in each cross-link:creatinine ratio was 2.4–5.6% of the mean ratio. The urinary ratios of both pyridinoline:creatinine and deoxypyridinoline:creatinine were significantly lower in the British than the Gambian boys (Table 1), a difference which may result not from differences in collagen turnover but instead from a higher dietary intake of creatinine and its precursors by the British boys, since meat is virtually absent from the rural Gambian diet.

In a study of postmenopausal women in a Western society, urinary pyridinoline concentrations were lower and deoxypyridinoline concentrations were higher in autumn than in spring (Douglas *et al.* 1996). This suggests that some other environmental factors may also modulate urinary pyridinoline:deoxypyridinoline ratios in man, but the nature of these influences in this case is not known.

The result of the 7-week intervention study, summarised in Table 3, was null insofar as no significant differences in cross-link ratios between the placebo and supplemented groups could be detected at any of the weekly sampling time-points after the baseline measurements in August. This was true for all three collagen cross-link ratios shown in Table 3. The 95% confidence intervals for the supplement–placebo comparisons (Table 3) confirmed that no divergence between the supplement and placebo groups was detectable for any of the three cross-link ratio increments. We suggest that a much longer period of intervention and/or larger numbers of subjects and extended urine collections over a longer follow-up period may be needed to produce a significantly positive intervention result. Since urinary collagen peptides reflect collagen structures that may have been synthesised months or even years before, it may be necessary to intervene for much longer periods to change the urinary cross-link ratios to a significant extent. In our previous studies on guinea-pigs and in observations on human subjects with genetic abnormalities of connective tissue metabolism, the difference in lysyl hydroxylase

activity was present for a much greater proportion of the total previous life-span than it was in the present study (i.e. a 7-week intervention in 8-year-old boys).

In conclusion, the present study has shown that the urinary collagen cross-link pyridinoline:deoxypyridinoline ratio remains remarkably constant over time within individuals, so that, whereas the ratio appears to fluctuate from sample to sample and day to day, over longer periods of time the mean value remains very constant within individuals. An overall mean change as small as 1% occurring over an 8-month period was detectable in a sample of about sixty subjects. Both the small seasonal variation in this ratio in Gambian prepubertal boys, and the much larger difference in the cross-link ratio observed between Gambian boys and British boys of similar ages, are consistent with there being a modest (medium- to long-term) vitamin C effect on this ratio. However, the null result of the 7-week intervention study demonstrates the fact that a rapid response to vitamin C intervention is unlikely to be achieved, and indicates that further studies with longer periods of controlled intervention will be needed to determine whether this potential functional index might be specifically responsive to changes in vitamin C status, within the range of values commonly seen in present-day human populations. Future studies of specific regions of collagen may prove informative, especially since there is evidence that different lysyl hydroxylase enzymes may act on the different regions (Royce & Barnes, 1985; Bank *et al.* 1999), and that the ratios of pyridinoline:deoxypyridinoline differ between the N-telopeptide, helix and C-telopeptide sites, with about two-thirds of the deoxypyridinoline cross-links occurring at the N-terminal-to-helix site (Hanson & Eyre, 1996).

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References

- Acil Y, Vetter U, Brenner R, Muller PK & 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.
- Acil Y, Brinckmann J, Notbohm H, Muller PK & Batge B (1996) Changes with age in the urinary excretion of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP). *Scand J Clin Lab Invest* **56**, 275–283.
- Anonymous (1984) Elucidation of the biochemical role of ascorbic acid. *Nutr Rev* **42**, 392–394.
- Bailey AJ, Paul RG & Knott L (1998) Mechanisms of maturation and ageing of collagen. *Mech Ageing Dev* **106**, 1–56.
- Bank RA, Robins SP, Wijmenga C, Breslau-Siderius LJ, Bardoel AF, van der Sluijs HA, Pruijs HE & TeKoppele JM (1999) Defective collagen crosslinking in bone, but not in ligament or cartilage, in Bruck syndrome: implications for a bone-specific telopeptide lysyl

- hydroxylase on chromosome 17. *Proc Natl Acad Sci USA* **96**, 1054–1058.
- Banse X & Sims TJ (2002) Mechanical properties of adult vertebral cancellous bone: correlation with collagen intermolecular cross-links. *J Bone Min Res* **17**, 1621–1628.
- Barnes MJ & Kodicek E (1972) Biological hydroxylations and ascorbic acid with special regard to collagen metabolism. *Vitam Horm* **30**, 1–43.
- Bates CJ (1999) Collagen crosslink ratios in bone and urine of guinea-pigs fed with graded intakes of vitamin C. *Proc Nutr Soc* **58**, 63A.
- Bates CJ, Prentice AM & Paul AA (1994) Seasonal variations in vitamins A, C, riboflavin and folate intakes and status of pregnant and lactating women in a rural Gambian community. *Eur J Clin Nutr* **48**, 660–668.
- Beardsworth LJ, Eyre DR & Dickson IR (1990) Changes with age in the urinary excretion of lysyl- and hydroxylysylpyridinoline, two new markers of bone collagen turnover. *J Bone Min Res* **5**, 671–676.
- Branca F, Robins SP, Ferro-Luzzi A & Golden MHN (1992) Bone turnover in malnourished children. *Lancet* **340**, 1493–1496.
- Branca F, Spagnoli A, Cianfarani S, Spadoni G, Golden MHN, Boscherini B, Valtuena S & Robins SP (2002) Urinary excretion of pyridinium crosslinks in short children treated with growth hormone. *J Pediatr Endocrinol Metab* **15**, 27–34.
- Dembure PP, Janko AR, Priest RE & Elsas LJ (1987) Ascorbate regulation of collagen biosynthesis in Ehlers–Danlos syndrome. *Metabolism* **36**, 687–691.
- Dolan AL & Arden NK (1998) Assessment of bone in Ehlers Danlos syndrome by ultrasound and densitometry. *Ann Rheum Dis* **57**, 630–633.
- Douglas AS, Miller MH, Reid DM, Hutchison JD, Porter RW & Robins SP (1996) Seasonal differences in biochemical parameters of bone remodeling. *J Clin Pathol* **49**, 284–289.
- Elsas LJ, Miller RL & Pinnell SR (1978) Inherited human collagen lysyl hydroxylase deficiency: ascorbic acid response. *J Pediatr* **92**, 378–384.
- Fraser WD (1998) The collagen crosslinks pyridinoline and deoxy-pyridinoline: a review of their biochemistry, physiology, measurement, and clinical applications. *J Clin Ligand Assay* **21**, 102–110.
- Ginty F, Flynn A & Cashman K (1998) Inter and intra-individual variations in urinary excretion of pyridinium crosslinks of collagen in healthy young adults. *Eur J Clin Nutr* **52**, 71–73.
- Gregory J, Lowe S, Bates C, Prentice A, Jackson L, Smithers G, Wenlock R & Farron M (2000) *National Diet and Nutrition Survey: Young People Aged 4 to 18 Years. Volume 1: Report of the Diet and Nutrition Survey*. London: The Stationery Office.
- Hanson DA & Eyre DR (1996) A specific immunoassay for monitoring human bone resorption: quantitation of type I collagen cross-linked N-telopeptides in urine. *J Bone Min Res* **7**, 1251–1258.
- Kivirikko KI & Myllyla R (1982) Posttranslational enzymes in the biosynthesis of collagen: intracellular enzymes. *Meth Enzymol* **82**, 245–304.
- Miller RL, Elsas LJ & Priest RE (1979) Ascorbate action on normal and mutant human lysyl hydroxylases from cultured dermal fibroblasts. *J Invest Dermatol* **72**, 241–247.
- Myllyla R, Majamaa J, Gunzler V, Hanauske-Abel HM & Kivirikko KI (1984) Ascorbate is consumed stoichiometrically in the uncoupled reactions catalysed by prolyl-4-hydroxylase and lysyl hydroxylase. *J Biol Chem* **259**, 5403–5405.
- Pasquali M, Still MJ, Vales T, Rosen RI, Evinger JD, Dembure PP, Longo N & Elsas LJ (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.
- Pinnell SR, Krane SM, Kenzora JE & Glimcher MJ (1972) A heritable disorder of connective tissue. Hydroxylysine-deficient collagen disease. *N Engl J Med* **286**, 1013–1020.
- Quinn RS & Krane SM (1976) Abnormal properties of collagen lysyl hydroxylase from skin fibroblasts of siblings with hydroxylysine-deficient collagen. *J Clin Invest* **57**, 83–93.
- Rauch F, Seibel MJ, Woitge H, Kruse K & Schonau E (1995) Increased urinary excretion of collagen crosslinks in girls with Ullrich–Turner syndrome. *Acta Paediatr* **84**, 66–69.
- Robins SP (1994) Biochemical markers for assessing skeletal growth. *Eur J Clin Nutr* **48**, Suppl. 1, S199–S209.
- Royce PM & Barnes MJ (1985) Failure of highly purified lysyl hydroxylase to hydroxylate lysyl residues in the non-helical regions of collagen. *Biochem J* **230**, 475–480.
- Seibel MJ, Robins SP & Bilezikian JP (1992) Urinary pyridinium crosslinks of collagen. Specific markers of bone resorption in metabolic bone disease. *Trends Endocrinol Metab* **3**, 263–270.
- Tsuchiya H & Bates CJ (1997) Vitamin C and copper interactions in guinea-pigs and a study of collagen cross-links. *Br J Nutr* **77**, 315–325.
- Tsuchiya H & Bates CJ (1998) 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. *J Nutr Biochem* **9**, 402–407.
- Tsuchiya H & Bates CJ (2003) Comparison of vitamin C deficiency with food restriction on collagen cross-link ratios in bone, urine and skin of weanling guinea-pigs. *Br J Nutr* **89**, 303–310.
- Vuilleumier JP & Keck E (1989) Fluorometric assay of vitamin C in biological materials, using a centrifugal analyser with fluorescence attachment. *J Micronutr Anal* **5**, 25–34.