

Vitamin C and copper interactions in guinea-pigs and a study of collagen cross-links

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The purpose of this study was, first to explore metabolic interactions between Cu and ascorbic acid in guinea-pigs, particularly with respect to any possible disadvantages of high ascorbate in the presence of low Cu intakes, and second, to test the hypothesis that variations in ascorbate and/or Cu status might influence collagen cross-linking, either by inducing a change in the cross-links: hydroxyproline ratio, or by inducing a change in the pyridinoline: deoxypyridinoline cross-links ratio. Four matched groups, each of eight male weanling Dunkin–Hartley guinea-pigs, were maintained on purified diets containing either no added Cu, or 150 mg Cu/kg diet, and either 0.1 g or 30 g ascorbic acid/kg diet. They were then killed 8 weeks later, and the following indices were measured: body and organ weights; blood haemoglobin; adrenal ascorbate concentrations; Cu concentrations in plasma, liver and femur; superoxide dismutase (EC 1.15.1.1) activity in whole blood and liver; hydroxyproline, pyridinoline and deoxypyridinoline in femur and in urine. The principal observations were: Cu intake significantly affected blood and tissue Cu concentrations and superoxide dismutase activity; and ascorbic acid intake significantly affected adrenal ascorbate levels and the deoxypyridinoline: pyridinoline cross-links ratio, especially in bone (femur). There was evidence of a significant interaction between ascorbate and Cu with respect to adrenal and plasma Cu concentrations, blood superoxide dismutase activity and body weights. We conclude that interactions between ascorbate and Cu at the functional level were present but modest, and that a new and potentially powerful functional index of ascorbate status may exist within the deoxypyridinoline: pyridinoline collagen cross-link ratio.

Ascorbate: Copper: Guinea-pig: Collagen

Vitamin C (ascorbic acid), which is an essential dietary nutrient for man, higher primates, the guinea-pig and for some other species, is a powerful reducing agent, capable of reducing certain transition metal ions, and thus modifying their biological properties. Its interaction with the copper II (cupric) ion is well known, but the *in vivo* biological consequences of this interaction are less well understood. Previous studies have explored some aspects of this interaction in guinea-pigs (Hitier, 1976; Milne & Omaye, 1980; Smith & Bidlack, 1980; Kassouny *et al.* 1985; DiSilvestro, 1986; Pekiner & Nebioglu, 1994); in rats (Van Campen & Gross, 1968; Johnson & Murphy, 1988; van den Berg *et al.* 1994); in rabbits (Hunt & Carlton, 1965); in chicks (Starcher *et al.* 1964; Carlton & Henderson, 1965; Hill & Starcher, 1965; DiSilvestro & Harris, 1981); in monkeys (Milne *et al.* 1981) and in human subjects (Finley & Cerklewski, 1983; Jacob *et al.* 1987; Milne *et al.* 1988; Pekiner & Nebioglu, 1994). There have also been studies in cultured cells (Harris & Percival, 1991).

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While it is clear from these studies that high concentrations or intakes of ascorbate can reduce tissue concentrations of Cu in a variety of species, there is a less clear consensus and uniformity with respect to possible functional effects of ascorbate overload on Cu-dependent processes *in vivo*. Several early studies on aortic rupture in chicks (Starcher *et al.* 1964; Carlton & Henderson, 1965; Hill & Starcher, 1965) obtained strong evidence for a gross failure of elastin cross-linking, attributed to reduced activity of the key enzyme, lysyl oxidase, in birds that were exposed simultaneously to excessive dietary ascorbate, and to low dietary Cu levels. Studies in mammalian species, however, have not reproduced such a dramatic effect. Nevertheless, in view of the potential importance of this interaction, for human subjects who choose to take daily megadoses of ascorbic acid, there is a need for further studies in relevant animal models such as the guinea-pig, which like humans has an absolute requirement for dietary ascorbate, as well as for Cu. The possibility that high levels of circulating vitamin C may increase the likelihood of Fe acting pro-oxidatively, and thereby influence outcome, has been illustrated by recent studies of human pre-term infants (Silvers *et al.* 1994; Powers *et al.* 1995). Deleterious effects of high ascorbate intakes may thus involve more than one transition metal.

Another separate but related question arises from the need for better biochemical tests for suboptimum *v.* optimum status, with respect to those functionally-critical processes which are dependent on specific micronutrients. Collagen cross-linking is such a process. A severe lack of Cu can impair the activity of the Cu-dependent enzyme lysyl oxidase, responsible for the initiation of cross-linking (Farquharson *et al.* 1989; Robins, 1994). Impairment of cross-linking may then result in loss of tensile strength. Lack of ascorbic acid is well known to affect the hydroxylation of collagen lysyl residues (Kivirikko & Myllyla, 1982; Yeowell *et al.* 1995), and this could, in theory, alter the pattern of collagen cross-links, by altering the ratio of deoxypyridinoline (derived from lysine) to pyridinoline (derived from hydroxylysine) (Robins, 1994).

The dual purpose of the present study was: first to re-examine the interactions of ascorbate and Cu, by dietary modulation in young guinea-pigs, and second, to test the hypothesis that Cu and/or ascorbate status might alter the ratio of deoxypyridinoline collagen-derived cross-links in bone and in urine.

MATERIALS AND METHODS

Animals and diets

The purified guinea-pig diet contained the following components (g/kg): sucrose 331, maize starch 50, ovalbumin 300, cellulose powder 150, maize oil 73, potassium acetate 25, choline chloride 2, magnesium oxide 5, inositol 2, salt mixture 60, vitamins (see later). The salt mixture was based on that of Greenfield *et al.* (1969), except that the CuSO₄ therein was omitted. It contained (g/kg): CaCO₃ 205, CaHPO₄ 325, Na₂HPO₄ 185, KCl 205, MgSO₄ 70, MnSO₄ 4.5, Fe-citrate 4.35, ZnCO₃ 0.75 and KIO₃ 0.025. Water-soluble vitamins, which were mixed with sucrose before addition to the bulk mixture, comprised (mg/kg diet): thiamin hydrochloride 16, riboflavin 16, pyridoxine hydrochloride 16, nicotinamide 200, calcium pantothenate 40, biotin 10, pteroylglutamic acid 10, cyanocobalamin 0.05. Fat-soluble vitamins, which were dissolved in the maize oil before addition to the bulk mixture, comprised (mg/kg diet): retinyl acetate 2.4, α -tocopherol 60, cholecalciferol 0.0075 and menadione 10. To this basic mixture were added the following amounts of Cu (as sulphate) and of L-ascorbic acid (g/kg diet):

diet A: Cu 0.15, L-ascorbic acid 30;
diet B: Cu nil, L-ascorbic acid 0.1;
diet C: Cu nil, L-ascorbic acid 30;
diet D: Cu 0.15, L-ascorbic acid 0.1.

Thus diets A and C had high ascorbate contents, whereas diets B and D had an amount likely to be compatible with adequate growth (Ginter *et al.* 1968; Clarke *et al.* 1977). The recommended intake for adequate growth and health of guinea-pigs is 75–100 mg/kg diet (Clarke *et al.* 1977) but the amount required to approach tissue saturation was found to be of the order of 0.6–1.2 g/kg (Bates *et al.* 1992). The high intake (30 g/kg) was selected in order to maximize the probability of detecting any possible adverse interactions of high ascorbate intakes with low Cu intakes (see pp. 316 and 322).

Diets A and D contained an amount of Cu which was considered to be generous for guinea-pigs (Clarke *et al.* 1977), whereas diets B and C, which had no added Cu, and were estimated to contain no more than 0.002 g Cu/kg diet, were intended to be marginal in Cu.

Diets were prepared in small amounts, stored at +4° until use, and provided freshly each day to minimize deterioration of the labile components, including ascorbate. Spot checks of the dietary ascorbate contents were performed at intervals.

Thirty-two young male inbred Dunkin–Hartley guinea pigs, approximately 2–3 weeks old with a mean body weight of 196 g, were divided into four initially weight-matched groups (four blocks per group), to receive the four diets described earlier, plus autoclaved hay (a small handful daily), and water *ad libitum*. They were housed singly in wire cages at 19–23°, with a 12 h light–dark cycle. After 8 weeks on these regimens, during which they were weighed daily to monitor any possible adverse effects of the diets or divergences between the diet groups, they were killed by CO₂ anaesthesia, and their blood (by cardiac puncture and transfer to a heparinized container), and organs, were removed for analysis. Bone samples used were hindlimb femurs, and the bone marrow was removed before analysis.

Analytical methods

Vitamin C (ascorbic + dehydroascorbic acids) was measured in metaphosphoric acid extracts of adrenals by the assay procedure of Vuilleumier & Keck (1989) as described previously (Tsuchiya & Bates, 1994).

Total Cu content of blood plasma and selected internal organs was measured by flame atomic absorption spectrometry. Representative samples were dissolved in 16 M-HNO₃ (BDH 'Spectrosol' grade, BDH, Poole, Dorset), in a CEM laboratory microwave digestion oven (CEM Microwave Technology, Buckingham, Bucks.), and the Cu content of the digests was then measured in a Philips PU 9400 analytical atomic absorption spectrometer (Philips, Cambridge, Cambs.), with a slotted tube atom trap (STAT), and aliquoting device, to optimize the sensitivity. Reagent background was subtracted; the calibration employed Cu(NO₃)₂ (BDH 'Spectrosol' grade standard solution) both as external standards and as internal spikes to check for quenching.

Superoxide dismutase (EC 1.15.1.1) activity was measured in whole blood and in liver homogenate samples stored at –80° until analysed. Whole-blood samples, after thawing, were mixed with 2 volumes of distilled water, centrifuged at 3000 g to remove cell membranes, diluted 1 : 30 in 0.01 M-potassium phosphate buffer pH 7.0, and analysed with a 'Ransod' kit assay (Randox Laboratories Ltd, Ardmore, Crumlin, Co. Antrim) in a Roche Cobas Bio centrifugal analyser (Roche Diagnostic Systems, Welwyn Garden City, Herts.).

This assay depends on the inhibition of formazan dye formation, from its precursor, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT), in the presence of a xanthine-xanthine oxidase (*EC* 1.2.3.2) generator of superoxide, at 37°. Blood haemoglobin concentration was measured by an automated version of the cyanmethaemoglobin assay. Superoxide dismutase in liver was measured, after homogenization of thawed samples in five volumes of 0.01 M-potassium phosphate buffer pH 7.0, containing 0.25 M-sucrose followed by centrifugation at 36 000 rpm (90 000 g) for 1 h, and then dilution 1:128 with 0.01 M-potassium phosphate buffer pH 7.0. This diluted cytosol fraction was then assayed by the 'Ransod' kit assay described earlier. Activity here was related to the cytosol protein content, using an automated Biuret assay, based on a Roche Diagnostics kit: catalogue no. 07.1008.3.

The collagen cross-links deoxypyridinoline and pyridinoline (separately), were measured in acid hydrolysates of demineralized femur shafts and in 8 h urine samples, by Metra Biosystems antibody-based ELISA plate assays (Metra Biosystems (UK) Ltd, Wheatley, Oxon.). The bone samples, stored at -80° were freed of soft tissue, including bone marrow; the mid-shaft segments were isolated, and were decalcified essentially as described by Farquharson *et al.* (1989), except that the removal of mineral, and then the removal of excess EDTA, were performed by dialysis within 14.3 mm Visking dialysis tubing. The non-diffusible residues were hydrolysed for 24 h at 100° in 6 M-HCl. The hydrolysates were then evaporated to dryness under a stream of N₂, redissolved and re-evaporated three times from water, and were then dissolved in water for analysis. Urine samples were collected into HCl over an 8 h period, shortly before killing. A subsample was removed for automated creatinine assay (Roche Diagnostics Unimate 5 CREA); the remainder was hydrolysed in 6 M-HCl, and was evaporated and redissolved as described earlier for bone. The hydroxyproline contents of the acid hydrolysates of bone and urine were measured by the procedure of Ho & Pang (1989), as described previously (Tsuchiya & Bates, 1994). The concentrations of the collagen cross-link moieties deoxypyridinoline and pyridinoline were measured by Metra Biosystems kit assays, which are based on specific immunoassays for (a) deoxypyridinoline and (b) deoxypyridinoline + pyridinoline respectively (Robins, 1994).

Statistical methods

Three-way ANOVA was used to test for significant inter-group differences and to test these differences for effects attributable to Cu intake, vitamin C intake, block effects (four blocks per group, based on initial body weights) and copper × vitamin C interaction respectively. No evidence of non-normal distributions was found. However, because there was a relationship between the variabilities and the magnitudes of the means for some of the variables in Table 2, logarithmic transformation was performed before the statistical analyses of these data. For those group comparisons where a significant interaction term was observed, it is difficult to interpret the main effects, because the main effect of each treatment is dependent on the other treatment value.

RESULTS

As shown in Table 1, all four groups of animals grew well (increasing from about 200 g to about 400 g in body weight over the 8-week period of the experiment); all four groups were growing at similar rates, at the time of death. Group C, with the combination of high ascorbate and low Cu in the diet, however, grew faster than the other three groups, which is

Table 1. Final body weights, body-weight increases and organ weights as a percentage of body weights, at time of death, for guinea-pigs fed on diets containing different amounts of copper and ascorbic acid

(Mean values with their standard errors for eight guinea-pigs per dietary group)

Group*	Body wt at slaughter (g)		Body wt increase during experiment (g)		Liver: % of body wt		Kidneys: % of body wt		Spleen: % of body wt		Adrenals: % of body wt	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
A	396	27	197	28	4.29	0.20	0.80	0.06	0.15	0.01	0.047	0.005
B	396	13	199	13	3.86	0.10	0.73	0.01	0.19	0.02	0.045	0.002
C	470	8	274	6	4.59	0.10	0.72	0.02	0.26	0.02	0.041	0.002
D	411	20	214	19	4.28	0.12	0.75	0.03	0.18	0.01	0.044	0.002
Cu effect†	NS		NS		NS		NS		0.003		NS	
Vitamin C effect†	NS		NS		0.008		NS		NS		NS	
Block effect†	NS		NS		NS		NS		NS		NS	
Interaction†	0.011		0.009		0.011		NS		0.018		0.05	

* For details of diet groups, see pp. 316–317. Groups B and D received the low-ascorbate diet; groups B and C received the low-Cu diet.

† Significant intergroup differences were detected by three-way ANOVA and are expressed as *P* values (25 degrees of freedom) for Cu effect, ascorbate effect, block effect and Cu × ascorbate interaction term. It should be noted that, for two of the indices (body weight at slaughter and body weight increase during the experiment), there was a significant interaction term, but no significant effect of either nutrient. This observation implies significant nutrient-effects which, however, are variable across the intake range of the other nutrient being studied.

illustrated by the significant Cu × ascorbate interactions for final body weights, and for growth during the experiment. Despite its high growth rate and low adrenal : body-weight ratio, group C paradoxically had the highest spleen : body-weight ratio (Table 1). The spleen : body-weight ratio was significantly affected by Cu intake with a significant interaction term; the liver : body-weight ratio was significantly affected by vitamin C intake, with a significant interaction term (Table 1). Therefore, for all these indices (except kidney : body-weight ratios), there is some evidence of both ascorbate and Cu effects from the ANOVA analyses.

The organ and blood vitamin C and Cu levels, shown in Table 2, very clearly confirm the effects of varying dietary intakes in the concentrations of these nutrients at key sites in the body. Some evidence of interactions between the two nutrients was also available from this dataset: group D, with a higher Cu intake but equal vitamin C intake to group B, had a lower adrenal ascorbate level, suggesting a possible Cu-antagonism at low ascorbate intakes, and group C, with a similar Cu intake to group B, had a lower plasma Cu concentration, which was found to be significant ($P=0.005$) by Student's *t* test. This intergroup difference did not, however, reach significance in the cases of the hepatic and bone Cu concentrations (Table 2).

Those animals with the higher Cu intake also had significantly higher blood haemoglobin levels (Table 3), however, there was no significant effect of vitamin C intake on this index, and no interaction effect.

Superoxide dismutase activity exhibited a clear effect of Cu intake, especially in the liver (Table 3), but in the blood there was also a major influence of ascorbate intakes, such that at low Cu intakes ascorbate tended to suppress its activity, whereas at high Cu intakes it enhanced the enzyme activity: this resulted in a significant interaction term.

Table 4 shows the effect of ascorbate and Cu intakes on the bone collagen indices. Neither nutrient had any measurable effect on total hydroxyproline (and hence total

Table 2. Plasma and tissue vitamin C and copper concentrations at time of death for guinea-pigs fed on diets containing different amounts of copper and ascorbic acid

(Mean values with their standard errors for eight guinea-pigs per dietary group)

Group*	Adrenal vitamin C ($\mu\text{mol/g}$ wet wt)		Plasma copper (nmol/ml)		Hepatic copper (nmol/g wet wt)		Bone copper (nmol/g wet wt)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
A	9.55	0.36	8.47	0.47	212.5	37.3	52.6	4.3
B	2.12	0.16	7.57	0.75	46.4	2.7	43.6	2.2
C	9.94	0.22	4.77	0.38	39.2	2.3	40.5	0.9
D	1.53	0.12	9.29	0.80	240.8	26.7	57.1	1.7
Cu effect†	0.007		0.0002		< 0.0001		< 0.0003	
Vitamin C effect†	< 0.0001		0.006		NS		NS	
Block effect†	0.009		NS		NS		NS	
Interaction†	0.006		0.04		NS		NS	

* For details of diet groups, see pp. 316–317. Groups B and D received the low-ascorbate diet; groups B and C received the low-Cu diet.

† Because there was some evidence that the standard errors varied with the magnitude of the means for these indices, \log_{10} -transformations were performed here before the three-way ANOVA analyses. Significant intergroup differences were detected by three-way ANOVA and are expressed as *P* values (25 degrees of freedom) for Cu effect, ascorbate effect, block effect and Cu \times ascorbate interaction term.

Table 3. Whole-blood haemoglobin (Hb) and superoxide dismutase (EC 1.15.1.1; SOD) concentrations and hepatic SOD activity at time of death, for guinea-pigs fed on diets containing different amounts of copper and ascorbic acid

(Mean values with their standard errors for eight guinea-pigs per dietary group)

Group*	Blood Hb (g/l)		Blood SOD activity (IU/g Hb)		Hepatic SOD activity (IU/mg cytosol protein)	
	Mean	SE	Mean	SE	Mean	SE
A	13.48	0.21	965	23	17.54	0.53
B	12.50	0.36	726	31	6.85	0.47
C	12.25	0.18	587	43	6.02	0.48
D	12.94	0.21	858	23	16.55	0.85
Cu effect†	0.012		< 0.0001		< 0.0001	
Vitamin C effect†	NS		NS		NS	
Block effect†	NS		NS		NS	
Interaction†	NS		0.0004		NS	

* For details of diet groups, see pp. 316–317. Groups B and D received the low-ascorbate diet; groups B and C received the low-Cu diet.

† Significant intergroup differences were detected by three-way ANOVA and are expressed as *P* values (25 degrees of freedom) for Cu effect, ascorbate effect, block effect and Cu \times ascorbate interaction term.

collagen) content per unit of bone weight. Cu intakes also had no significant effect on any of the collagen cross-link concentrations or ratios measured; however vitamin C intakes had a very significant effect on these indices. At higher Cu intakes, the higher vitamin C intake enhanced the pyridinoline:hydroxyproline ratio. At both Cu intakes, the high vitamin C intake depressed both the deoxypyridinoline:hydroxyproline, and the

Table 4. *Hydroxyproline and collagen cross-link concentrations in acid hydrolysates of femur shaft from guinea-pigs fed on diets containing different amounts of copper and ascorbic acid*
(Mean values with their standard errors for eight guinea-pigs per dietary group)

Group*	Hydroxyproline ($\mu\text{mol/g}$ wet wt)		Pyridinoline ($\mu\text{mol/mol}$ hydroxy- proline)		Deoxypyridinoline ($\mu\text{mol/mol}$ hydroxy- proline)		Deoxypyridinoline: total cross-links (mol/mol)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
A	124	8.0	1.04	0.08	0.053	0.004	0.058	0.009
B	126	3.2	0.82	0.08	0.073	0.005	0.104	0.014
C	123	5.4	0.89	0.03	0.046	0.004	0.055	0.004
D	121	4.5	0.82	0.03	0.080	0.005	0.109	0.008
Cu effect†	NS		NS		NS		NS	
Vitamin C effect†	NS		0.05		< 0.0001		0.0005	
Block effect†	NS		NS		NS		NS	
Interaction†	NS		NS		NS		NS	

* For details of diet groups, see pp. 316–317. Groups B and D received the low-ascorbate diet; groups B and C received the low-Cu diet.

† Significant intergroup differences were detected by three-way ANOVA and are expressed as *P* values (25 degrees of freedom) for Cu effect, ascorbate effect, block effect and Cu \times ascorbate interaction term.

deoxypyridinoline : total cross-links ratios. The latter effects of vitamin C were both highly significant ($P < 0.0001$). No significant Cu : vitamin C interaction terms were observed.

Table 5 shows the effect of vitamin C and Cu intakes on the urine collagen-related indices. Neither nutrient had any significant effect on total hydroxyproline content per unit of creatinine, nor on the pyridinoline : creatinine ratio (although there was a significant block-effect here). Deoxypyridinoline was, by contrast, significantly affected by vitamin C intake, in a similar direction (but smaller extent) to that observed in bone and this was most clearly shown by the ratio of deoxypyridinoline : total cross-links present.

Table 5. *Hydroxyproline and collagen cross-link concentrations in acid hydrolysates of urine samples from guinea-pigs fed on diets containing different amounts of copper and ascorbic acid*
(Mean values with their standard errors for eight guinea-pigs per dietary group)

Group*	Hydroxyproline (mmol/mol creatinine)		Pyridinoline (mmol/mol creatinine)		Deoxypyridinoline (mmol/mol creatinine)		Deoxypyridinoline: total cross-links (mol/mol)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
A	164	8	226	16	27.7	2.4	0.140	0.008
B	173	11	220	20	32.4	4.6	0.178	0.025
C	173	5	214	15	24.3	1.9	0.132	0.010
D	191	6	262	17	39.1	4.3	0.177	0.015
Cu effect†	NS		NS		NS		NS	
Vitamin C effect†	NS		NS		0.016		0.21	
Block effect†	0.008		0.03		NS		NS	
Interaction†	NS		NS		NS		NS	

* For details of diet groups, see pp. 316–317. Groups B and D received the low-ascorbate diet; groups B and C received the low-Cu diet.

† Significant intergroup differences were detected by three-way ANOVA and are expressed as *P* values (25 degrees of freedom) for Cu effect, ascorbate effect, block effect and Cu \times ascorbate interaction term.

DISCUSSION

Several earlier studies have highlighted the deleterious aspects of interactions which occur between vitamin C and Cu metabolism, indicating that very high ascorbate intakes, coupled with a marginal deficiency of Cu, may result in the exacerbation of metabolic Cu deficiency, presumably by converting cupric Cu, at critical active sites, to the metabolically inactive (and less soluble) cuprous form. Such effects were especially severe in early studies on chicks (see p. 316).

In the present study, possibly deleterious interactions between high ascorbate and low Cu intakes were observed, with respect to plasma Cu and blood superoxide dismutase activity (Tables 2 and 3) and perhaps also with respect to increased spleen size (Table 1). Despite these apparently undesirable effects, however, group C with the high ascorbate and low Cu intakes paradoxically had the highest weight gain of all four groups (Table 1), suggesting that this dietary combination need not necessarily be deleterious, if growth is chosen as the dominant outcome index. Further studies need, however, to be undertaken, particularly with respect to the tissue composition of the inter-group body-weight differences (especially fat and water accumulation *v.* lean body mass), and also with respect to the acute-phase reaction, which is well known to increase blood Cu levels, even in the absence of any overall changes in Cu status (Bremner & Beattie, 1995).

In the present study, functional differences consequent upon the differences in Cu status at the two levels of Cu intake used were clearly apparent with respect to superoxide dismutase activity, especially in the liver, and also, although less dramatically so, with respect to blood haemoglobin levels. Haemoglobin production is known to be impaired by Cu deficiency, therefore the lower of the two Cu intake levels in the present study was shown to be somewhat inadequate by two independent criteria.

Importantly, however, there was no evidence for an effect of Cu intakes on the concentration of the collagen cross-links pyridinoline or deoxypyridinoline either in bone collagen or in urine. Previous studies of Cu deficiency in rats (Robins *et al.* 1985; Farquharson *et al.* 1989) showed significant effects on bone collagen cross-links only after a prolonged and severe deficiency, which produced a major decrement in body-weight gain, amongst other deleterious effects. The current consensus, therefore, seems to be that, in laboratory rodents at least, Cu deficiency needs to be both prolonged and severe before the activity of the Cu-dependent enzyme, lysyl oxidase, is impaired sufficiently to cause any significant effects on mature bone collagen cross-linking (Rucker *et al.* 1996).

In contrast to the lack of any detectable effect of Cu, even moderate changes in vitamin C status in the present study exerted a highly significant, indeed a dramatic, effect on the ratio of the two cross-links pyridinoline and deoxypyridinoline, at least in bone, without affecting the total number of cross-links present, per unit of hydroxyproline. This effect, namely a relative increase in deoxypyridinoline content and a corresponding decrease in pyridinoline content, in the animals with a low vitamin C intake, is clearly consistent with the known facts: (a) that pyridinoline cross-links are more dependent on hydroxylysyl residues in the collagen molecule than are deoxypyridinoline cross-links, and (b) that ascorbic acid is an essential cofactor in the post-translational conversion of collagen lysyl to hydroxylysyl residues (Kivirikko & Myllyla, 1982; Robins, 1994). The observations from the present study are thus consistent with the hypothesis that a relative deficiency of ascorbic acid, at critical collagen-forming sites such as bone, results in a relative deficiency in collagen hydroxylysyl residues, which in turn tips the balance towards the formation of deoxypyridinoline cross-links, in preference to the alternative pyridinoline cross-links. The question needs to be asked: whether the observed change in pyridinoline : deoxypyridino-

line cross-links ratio at low tissue ascorbate levels has any significance for the physical or metabolic robustness of the collagen cross-link network, in bone and elsewhere?

The contrast between the low and high ascorbate intake groups was very marked for bone collagen, and was detectable, but less marked, for the urinary collagen-derived cross-link moieties. This presumably arises because the urinary products are derived from a mixture of tissues, some of which are less severely affected by variations in ascorbate availability than the shaft of the femur. A previous study (Bates, 1979) indicated that bone collagen is more sensitive to the overall effects of vitamin C deficiency (on total hydroxyproline synthesis), than is the collagen at many other sites in the body. The urinary cross-link analyses in the present study were performed on acid hydrolysates of urine, and thus included the peptide-bound cross-links, as well as the free cross-links which are normally measured in clinical studies of bone turnover (Robins, 1994).

The relative sensitivity of lysyl hydroxylase to vitamin C deficiency, in different experimental models, is a complex issue. Studies in a 3T6 fibroblast model in tissue culture (Bates *et al.* 1972) revealed little or no evidence of any effect of ascorbate depletion on collagen lysyl hydroxylation, despite a major effect, in the same model, on collagen prolyl hydroxylation. Since ascorbate appears to play a rather subtle protective role in the maintenance of full activity of mixed-function oxidase enzymes such as collagen prolyl and lysyl hydroxylases (Kivirikko & Myllyla, 1980), it seems possible that other redox effectors may modulate the ascorbate response, perhaps differently in different systems. Another unexpected aspect of the present study was the magnitude of the ascorbate effect between treatment groups, none of which was severely deficient in vitamin C. Previous guinea-pig-based studies by the same authors (Bates, 1979; Tsuchiya & Bates, 1994) used diets with much lower concentrations of ascorbate, resulting in a much greater degree of tissue ascorbate depletion, before growth and health indices were severely impaired.

The present study needs to be followed by a more detailed examination of the collagen cross-link response patterns, to include a wider range of ascorbate intakes in the guinea-pig and in other models, and also to include a wider variety of tissue sites where collagen biosynthesis is known to occur. The specificity of the ascorbate-effect also needs to be examined in greater detail: for instance it is conceivable that either severe inanition or deficiencies of other nutrients important for collagen synthesis, such as Fe, O, Zn, Mn, etc., might exert effects on the cross-link ratios. A recent study has described an effect of age on pyridinoline cross-links in two guinea-pig tissues (Kim *et al.* 1994) thus raising the possibility that cross-link ratios may also change with age.

The observation that collagen cross-link ratios may be sensitive to low ascorbate intakes, even within ranges which are adequate for normal growth and for general health, is potentially relevant to the ongoing search for functional indices of marginal micronutrient deficiencies, particularly those which can be used to define 'optimum' functional status in humans. Such functional indices include plasma homocysteine in relation to folate, vitamin B₁₂ and vitamin B₆ status; plasma methyl malonate in relation to vitamin B₁₂ status; plasma Gla-proteins in relation to vitamin K status, etc. If a collagen-derived cross-link ratio, at an accessible site such as plasma or urine can be developed as a functional index of vitamin C status, then the concept of functionally 'adequate' vitamin C status and optimum intakes may thereby become more readily definable.

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