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M Chojkier, … , R Spanheimer, B Peterkofsky

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Research Article

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Specifically Decreased Collagen Biosynthesis in Scurvy Dissociated from an Effect on Proline Hydroxylation and Correlated with Body Weight Loss

IN VITRO STUDIES IN GUINEA PIG CALVARIAL BONES

MARIO CHOJKIER, ROBERT SPANHEIMER, and BEVERLY PETERKOFSKY, *Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205*

ABSTRACT The question whether ascorbate regulates collagen production solely through its direct role in proline hydroxylation was investigated. Proteins in calvarial bones from control and scorbutic weanling guinea pigs were labeled in short-term cultures with radioactive proline. Proteins were digested with purified bacterial collagenase to distinguish between effects on collagen polypeptide production and hydroxyproline formation. There was a preferential decrease in the absolute rate of collagen biosynthesis beginning after 2 wk of ascorbate deficiency, and this effect was temporally dissociated from decreased proline hydroxylation. There were no significant changes in the absolute rates of collagen degradation or noncollagen protein production. In vitro inhibition of proline hydroxylation in normal bone with α,α'-dipyridyl did not affect the relative rate of collagen synthesis, further dissociating these functions. Ascorbate added to scorbutic bone cultures reversed defective proline hydroxylation but not defective collagen synthesis, suggesting that the latter was an indirect effect of scurvy. There was a linear correlation between the extent of body weight lost during the 3rd and 4th wk of scurvy and the rate of collagen synthesis in scorbutic bone. This correlation also applied to control animals receiving ascorbate, but with weight loss induced by food restriction. These studies establish for the first time that ascorbate deficiency in guinea pigs leads to a specific decrease in collagen polypeptide synthesis and suggest that this decrease results from the reduced food intake and/or weight-loss characteristic of scurvy.

INTRODUCTION

Scurvy in humans has long been known to have a deleterious effect on the healing of skin wounds and bone fractures (1, 2). Impaired collagen deposition due to ascorbic acid deficiency is reportedly the underlying pathogenesis of this condition (3, 4). Ascorbic acid has a well documented role in collagen metabolism as a direct requirement for prolyl (5) and lysyl (6) hydroxylases. It is required for hydroxylation of peptidyl proline in cell culture systems (7, 8), unless an alternate microsomal reducing cofactor is present (9), and it activates prolyl hydroxylase in some cell systems (10). Ascorbate also stimulates the rate of procollagen secretion in some (11–14), but not all (7), cell culture systems, because of its role in the formation of hydroxyproline, which provides stability to the procollagen triple helix (15, 16).

Since guinea pigs lack gulonolactone oxidase (17), they are unable to synthesize ascorbic acid (18), and therefore have been used extensively to evaluate the effect of ascorbate deficiency upon connective tissue (19). Defective collagen metabolism has been reported in repair tissue systems (healing wounds [20], granuloma [21, 22], bone fractures [23]) derived from scorbutic animals. Changes, however, were variable when nonrepair tissues (skin [24], bone [25], lung and liver [26]) were studied. Although there are ample data from such studies showing that the incorporation of radioactive proline into collagen hydroxyproline is decreased in scorbutic tissues, there still is no clear evi-

Dr. Chojkier's present address in Division of Gastroenterology, Department of Medicine, University of California School of Medicine, San Diego, CA 92103. Address reprint requests to Dr. Peterkofsky.

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dence that this decrease represents a specific effect on collagen production relative to total protein synthesis, nor have possible effects on collagen degradation been considered. Furthermore, in most cases, the procedures used did not allow a distinction between effects on proline hydroxylation and collagen polypeptide synthesis. Nevertheless, the involvement of ascorbate directly in the prolyl hydroxylase reaction, its regulation of procollagen secretion, its activation of prolyl hydroxylase, and the related observation that prolyl hydroxylase activity is decreased in scorbutic guinea pig tissues (27) have prompted proposals (11, 19, 27) that the pathology of connective tissue in scurvy is attributable to a defect at the proline hydroxylation step of collagen biosynthesis. The problem of distinguishing between effects on collagen polypeptide synthesis and proline hydroxylation can be overcome with current methodology (28). Using such methods, it has been found that in some cultured cell systems ascorbate increased proline hydroxylation with little or no effect on collagen production (7, 8, 12). Therefore, we initiated studies to clarify the role of ascorbate in collagen metabolism of a normal connective tissue (calvarial bone) of rapidly growing weanling guinea pigs.

METHODS

Materials. Uniformly labeled L-[^{14}C]proline (290 mCi/mmol), L-[2,3-^{3}H]proline (41 mCi/μmol), and L-[4-^{3}H]proline (15.59 mCi/μmol) were obtained from New England Nuclear, Boston, MA; L-[5-^{3}H]proline (27 mCi/μmol) was from Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, NY); and DL-hydroxy-[2-^{14}C]proline (18.87 mCi/mmol) was from Amersham Corp., Arlington Heights, IL. Sources of other chemicals were: sodium ascorbate and α,α'-dipyridyl (2,2'-bipyridine) from Sigma Chemical Co., St. Louis, MO; ascorbate oxidase (cucurbita species) from Boehringer Mannheim Biochemicals, Indianapolis, IN; bovine IgG standard, protein dye reagent and AG50W-X8 resin (100–200 mesh) from Bio-Rad Laboratories, Richmond, CA; M82 resin from Beckman Instruments, Inc., Palo Alto, CA; and DC-6A resin (8% cross-linked) from Dionex Chemical Corp., Sunnyvale, CA. All other chemicals and biochemicals were commercially available analytical-grade reagents. Chromatographically purified *Clostridium histolyticum* collagenase was purchased from Worthington Biochemical Corp., Freehold, NJ, and further purified as described elsewhere (29). Carrier chick embryo protein (28) and serum-free Eagle's minimal essential medium (MEM-0)[1] (7), were prepared as previously described, except that the MEM-0 contained 50 μg/ml gentamicin.

Animals. 1-wk-old female weanling guinea pigs, of an inbred colony (family 2, strain 2, ST2/N), were provided by the National Institutes of Health Laboratory Animal Genetic Center. Animals were housed in individual cages and allowed free access to water until they were killed. Both the scorbutic and control animals received an ascorbic acid-free pellet diet (ICN Pharmaceuticals, Inc., Cleveland, OH), but the controls were supplemented daily with 50 mg ascorbic acid (orally in 1 ml water containing 10 mg sucrose). Control and scorbutic animals gained weight during the first 2 wk, but during the next 2 wk the scorbutics stopped gaining and either remained at a relatively constant weight or began to lose weight at various times thereafter. In the initial study, control animals were pair-fed after 2 wk, without total fasting. The initial weights of the scorbutic and control animals were 129±6 and 126±8 g, respectively, and at the time they were killed, the weights were 186±8 for scorbutics and 191±8 for controls. Scorbutic animals not killed died during the 5th wk. The animal facility was lighted from 6 a.m. to 6 p.m., and the ambient temperature was maintained at 24±1°C.

Radiolabeling of calvarial bones. In an initial 4-wk study, two scorbutic animals and their corresponding pair-fed controls were killed by ether overdose each week for a 4-wk period. Calvarial bones (parietal aspect) were removed and cleaned of adhering tissues, and bone sections were incubated as described elsewhere (30). Quadruplicate bone sections from each animal were labeled for 2 h at 37°C with 9 μCi of L-[4-^{3}H]proline and 2 μCi of L-[^{14}C]proline (0.1 mM). Two sections were incubated in the presence and two in the absence of sodium ascorbate (0.5 mM), in a final volume of 1.0 ml MEM-0. In a second study, animals were killed after 2–3 wk on the experimental diets and identical procedures were used, except that labeling was achieved with 10 μCi of L-[2,3-^{3}H]proline or [5-^{3}H]proline (0.1 mM).

Relative rate of collagen production. After incubation of bone sections, the tubes were chilled to 0°C, the media was separated from bones with a Pasteur pipette, and bones were rinsed with cold 0.11 M NaCl/0.05 M Tris, pH 7.4. Media and washes were pooled and carrier chick embryo protein (1.6 mg) added (28). The tissue was minced and homogenized as described previously (30), and trichloroacetic acid was added to both media and homogenates to yield a final concentration of 100 mg/ml. Samples were centrifuged at 1,000 *g* for 5 min and the precipitates were resuspended in trichloroacetic acid (50 mg/ml) and recentrifuged three times. After tissue and media precipitates were resuspended for the third time, they were pooled, collected by centrifugation, and dissolved by heating in 0.2 N NaOH at 100°C for 5 min (30). The amount of radioactive collagen in the solution was determined by collagenase digestion (29). The collagenase-resistant material was hydrolyzed in 6 N HCl at 120°C, 15 lb pressure, for 3 h, cooled, and a portion used to measure radioactivity. When dual-labeled proline was used, the amounts of ^{14}C radioactivity in collagenase-sensitive and -insensitive proteins were used to calculate the relative rate of collagen production (31). The procedures for experiments in which only tritiated proline was used were essentially the same, except that the bone and medium fractions were combined after homogenization, and proline was present in the NaCl/Tris and trichloroacetic acid solutions to give a final concentration of 10 mM.

Degree of proline hydroxylation in newly synthesized collagen. The extent of proline hydroxylation was determined by a dual-label method by comparing the ^{3}H/^{14}C ratio in collagenase digests to the initial ^{3}H/^{14}C ratio of the radioactive proline (30). The percentage of proline hydroxylation was calculated by the following formula:

$$\frac{1 - (^{3}\text{H}:^{14}\text{C in collagenase digests/initial } ^{3}\text{H}:^{14}\text{C})}{\text{fraction of total tritium in the 4-}trans\text{ position}} \times 100.$$

The fraction of tritium at the 4-*trans* position of the L-[4-

[1] *Abbreviation used in this paper:* MEM-0, serum-free Eagle's minimal essential medium.

^{3}H]proline had been previously determined to be 0.69 for the isotope batch used in these experiments (30).

Specific radioactivity of the proline-free pool. The trichloroacetic acid-soluble fraction of bone homogenates was desalted on a 1-ml AG50 column, using 4 ml of 6 N HCl for elution. The eluent was evaporated to dryness under vacuum, the residue was dissolved in water and proline was purified by chromatography on an AG50 column (1 × 15 cm), using 1 N HCl for elution as described earlier (30). The pooled proline peak was evaporated and dissolved in 0.01 N HCl, and the undissolved material was removed by centrifugation at 13,000 *g* for 5 min. Separate portions of the solutions were used for quantitation of proline by ninhydrin reaction on a Durram Instrument Corp. (Sunnyvale, CA) D500 high-pressure amino acid analyzer (DC-6A ion-exchange resin), and for measurement of radioactivity. The specific activity was then calculated as disintegrations per minute per nanomole.

Measurement of collagen degradation. Collagen degradation was measured by analysis of low molecular weight [^{14}C]hydroxyproline in trichloroacetic acid-soluble fractions. This fraction of bones, either hydrolyzed or not, was analyzed by AG50 chromatography and found not to contain radioactivity in the hydroxyproline region. The trichloroacetic acid-soluble fractions of media were hydrolyzed and prepared for amino acid analysis as described above. [^{14}C]Hydroxyproline was eluted with 0.2 M sodium citrate, pH 2.91, at a flow rate of 70 ml/h on a 117 Beckman amino acid analyzer (M82 ion-exchange resin) (32), with a peak at 61–62 min. There were insignificant amounts of radioactivity in the region where 4-*cis*[^{14}C]hydroxyproline would elute (33). To ascertain that neither isotope contaminants (34) nor [^{14}C]proline metabolites (32) would interfere with the analysis of radioactive hydroxyproline, bone sections were incubated in the presence of 1 mM α,α'-dipyridyl to inhibit hydroxyproline formation, and the low molecular weight fraction was analyzed, as suggested elsewhere (32). Amino acid analysis of hydrolyzates from these samples did not reveal any significant amount of radioactivity eluting in the hydroxyproline region. The recovery of internal [^{14}C]hydroxyproline standards, added to unhydroxylated samples, and processed and analyzed as described above was 64%, and data were corrected accordingly.

Degradation of newly synthesized collagen was calculated as the percentage of [^{14}C]hydroxyproline in the low molecular weight fraction compared with the total. The amount of [^{14}C]hydroxyproline in the high molecular weight fraction was calculated by multiplying the total [^{14}C]collagen radioactivity, determined from collagenase digestion, by the fraction hydroxylated, which was determined by the dual-labeled proline method described above. The absolute rate of degradation also was calculated by multiplying the absolute rate of collagen production (picomoles of proline incorporated per microgram of DNA per hour) by the corresponding fraction of collagen degraded.

Ascorbic acid content in liver and bone. Tissues were removed from animals at the time of killing, washed in cold 0.15 M NaCl, and kept in liquid N_2. Portions of liver weighing ~1 g were homogenized at 0°C with 1 vol of twice glass-distilled water, in a glass homogenizer. A 67% (vol/vol) ethanol-soluble fraction was prepared, evaporated under vacuum, and the residue was dissolved in 0.1 M sodium phosphate, pH 5.6. Approximately 0.6 g of bone tissue was thawed and minced, and 1 ml of 10 mM Tris–HCl (pH 7.6), 0.6 mM glutathione was added to the mince. The sample was frozen and thawed at 0°C twice, 3 ml of absolute ethanol was added, and the sample was mixed thoroughly and kept at 0°C for 30 min. The extract was separated from bone by centrifugation (10 min at 1,000 *g*). The bone fragments were rinsed with 1 ml of absolute ethanol and the rinse was added to the initial extract. The pooled extract was evaporated under vacuum at room temperature, and the residue was dissolved in twice glass-distilled water. There was quantitative recovery of ascorbate added to samples and carried through the procedures. Liver samples were assayed by the α,α'-dipyridyl-Fe^{2+} complex method (35) and bone samples were analyzed by this assay as well as by a more sensitive enzymatic radioassay for prolyl hydroxylase reducing cofactor (9). In both cases, absolute specificity for ascorbic acid was achieved by pretreatment of samples with or without ascorbate oxidase (36), at 25°C for 15 min (37). In the colorimetric assay, turbidity of experimental samples was eliminated by adding Triton X-100 (final concentration 1 mg/ml) after color development, which did not affect the values obtained with ascorbic acid standards. In both assays, ascorbic acid content was calculated from the differences between ascorbic acid oxidase-treated and -untreated samples and comparison with internal recovery standards.

Unlabeled collagen and noncollagen protein content in bone. Sections of calvarial bones (frontal aspect) weighing ~100 mg were homogenized, proteins were precipitated with trichloroacetic acid, and the precipitates were prepared for collagenase treatment as described above. Portions of the 0.2-N NaOH solutions were digested with bacterial collagenase (29), the collagenase-sensitive fractions were acid hydrolyzed, and the amino acid composition was determined with a Durram D500 analyzer. The amount of noncollagen protein was determined by a dye-binding method (38) with IgG as a standard.

DNA determination. The DNA content of the 0.2-N NaOH solutions of trichloroacetic acid precipitated material was analyzed by the diphenylamine method (39).

Statistical analysis. All the results are expressed as a mean±SE. When deemed necessary, the *t* test was used to evaluate the differences of the means between groups, accepting $P < 0.05$ as significant (40).

RESULTS

Ascorbate-depletion status. The ascorbic acid content of control livers averaged 161±28 nmol/g of wet tissue. The level decreased by ~90% in scorbutic guinea pigs by the end of the 1st wk, and was virtually undetectable after 4 wk of ascorbic acid deprivation. The levels in calvarial bone showed similar decreases. By 2 wk, it was undetectable in scorbutic bones by the colorimetric assay, but by the enzymatic assay, values obtained after the 3rd wk were 128 and 4.6 nmol/g of wet tissue from control and scorbutic animals, respectively. These results and the fact that the animals that had been on the vitamin C-deficient diet for 2 wk showed typical symptoms, such as loss of appetite, lethargy, and fragile bones, attest to their scorbutic status.

Extent of proline hydroxylation in newly synthesized collagen. Calvarial bones were removed from animals and incubated with [4-^{3}H],[^{14}C]proline, which allowed us to measure the extent of proline hydrox-

ylation and net production[2] of collagen simultaneously (30). Preliminary experiments showed that the rate of incorporation of radioactive proline into collagen and noncollagen proteins was linear for up to 3 h; 2-h incubations were used for this study. As expected, the percentage of proline hydroxylation was normal in control bones throughout the study, whether ascorbate was added in vitro (42.9±0.9%) or not (45.0±1.0%) (Fig. 1 *A*). The extent of proline hydroxylation in bones of scorbutic animals, incubated without ascorbate, was about one-third lower than control values after 2 wk and remained at that level thereafter (Fig. 1 *A*). Hydroxylation reverted to normal (45.4±1.1%; NS), when calvaria from scorbutic animals were incubated with ascorbate in vitro, suggesting that all other factors required for normal proline hydroxylation were present in the tissue.

Relative rate of collagen production. The net production of labeled collagen relative to total labeled proteins in bones of scorbutic animals began to decrease after 2 wk and finally reached a value of ~40% of controls (Fig. 1 *B*). Ascorbate added in vitro did not return the decreased values to the control level (Fig. 2 *B*), unlike its effect on proline hydroxylation (Fig. 2 *A*). Since these results suggested a dissociation between proline hydroxylation and collagen production in bones of scorbutic animals, these parameters were examined in normal bones incubated in the presence of α,α'-dipyridyl (Table I). Although the percentage of proline hydroxylation was decreased to varying degrees in the presence of two different concentrations of the inhibitor, the relative rate of collagen production remained similar to that of fully hydroxylated control samples.

Absolute rate of collagen and noncollagen protein production. Expressing production as a relative rate eliminates consideration of changes in amino acid transport or other factors that would modify the specific activity of the free proline pool. A decrease in the relative rate of collagen production expressed as a percentage, however, may result from either decreased collagen or increased noncollagen protein production. To determine which of these possibilities caused the observed change, the absolute rates of production were determined from the specific activity of [^{14}C]proline in the free amino acid pool of the 4-wk samples from the experiment described above. There were no significant differences between the specific activities of free [^{14}C]proline in control and scorbutic bones at the 4th wk (Table II), suggesting that ascorbate deficiency did not affect free proline transport or metabolism. The absolute rates calculated by using these [^{14}C]proline specific activities showed a specific decrease in collagen production in bone from scorbutic animals after 4 wk (Fig. 2 *B*), whereas noncollagen protein production was unchanged (Fig. 2 *A*).

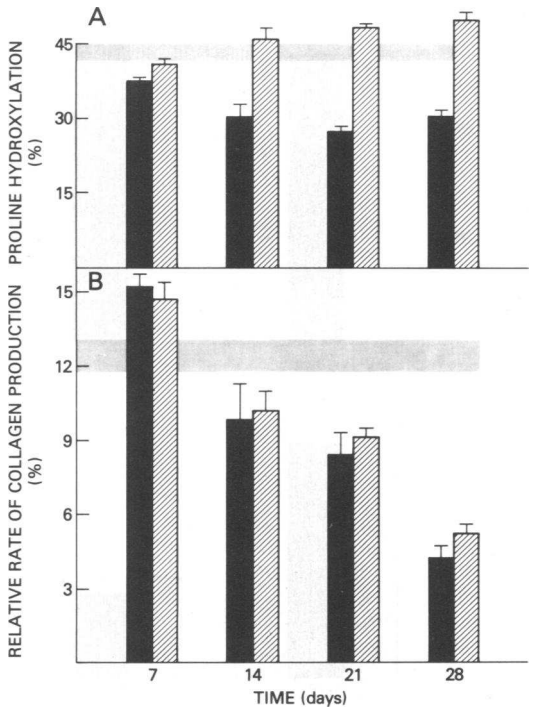

FIGURE 1 Effects of ascorbate deficiency on proline hydroxylation and on the relative rate of collagen production in guinea pig calvarial bone. (*A*) Bone samples were labeled for 2 h at 37°C with 9 μCi of L-[4-^{3}H]proline and 2 μCi of L-[^{14}]C proline (0.1 mM). The extent of proline hydroxylation was determined by measuring changes in the ratio of [^{3}H]/[^{14}C] proline in collagenase digests compared with the initial ratio (30). The horizontal shaded area represents the mean±SE of control values (n = 26). Bone samples from scorbutic animals were incubated with 0.5 mM sodium ascorbate (hatched bars; n = 4; NS) or in its absence (black bars; n = 4; $P < 0.05$). (*B*) The relative rate of collagen production was calculated from the ^{14}C radioactivity in collagenase-sensitive and -insensitive proteins. The horizontal shaded area represents the mean±SE of control values (n = 23). Bone samples from scorbutic animals were incubated as in *A*; $P < 0.05$ in the presence (hatched bars) or absence (black bars) of ascorbate at 21 and 28 d.

Collagen degradation. Since the specific decrease in the net production of collagen could have resulted from changes in either biosynthesis or degradation, we measured [^{14}C]hydroxyproline in the low molecular weight (acid-soluble) fraction, which is derived from collagen degradation. The values obtained for the per-

[2] The radioactivity present in high molecular weight protein is considered to reflect net production resulting from incorporation during biosynthesis and loss due to degradation. It can be presented as: Net Production = Biosynthesis − Degradation.

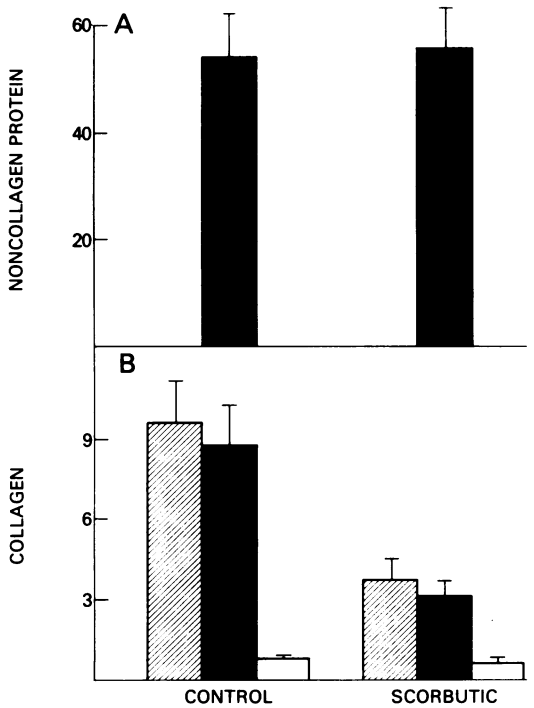

FIGURE 2 Effect of ascorbate deficiency on the absolute rates of protein production in guinea pig calvarial bone. Samples from the 4th wk of the experiment described in Fig. 1 were used. Absolute rates of collagen and noncollagen protein production were calculated from the total incorporation and the specific radioactivities of the [^{14}C]proline-free pools and are expressed as picomoles of proline incorporated per microgram of DNA per hour. (*A*) Rate of noncollagen protein production was calculated from the ^{14}C radioactivity in collagenase-insensitive proteins. Results are mean±SE of control (n = 4) and scorbutic (n = 4; NS) samples. The values were multiplied by 5.4 to correct for the higher content of iminoacids in collagen (31). (*B*) Rate of collagen net production (black bars) was calculated from the ^{14}C radioactivity in collagenase digests. The rate of collagen degradation (white bars) was determined from the relative proportions of [^{14}C]hydroxyproline in the high and low molecular weight fractions and expressed as picomoles of proline incorporated into collagenous proteins that were degraded per microgram of DNA per hour. The absolute rate of collagen biosynthesis (hatched bars) was calculated as the sum of both values. Results are the means±SE of control (n = 4) and scorbutic (n = 4) samples; $P < 0.05$ for biosynthesis and net production.

centage of low molecular weight compared with total [^{14}C]hydroxyproline synthesized in bones of control animals after 3 and 4 wk was ~10% (Table III). Although the percentage of collagen degradation in bones of scorbutic animals was twofold greater than control levels after 4 wk, it represented a relatively small fraction of the total newly synthesized collagen. Therefore, even this twofold change could not appreciably affect the relative rate of collagen production if the rate of synthesis were unchanged. Moreover,

TABLE I
Effect of α,α′-Dipyridyl In Vitro on Bone Collagen Metabolism in Normal Guinea Pig

Experimental* conditions	Proline hydroxylation‡	Relative rate of collagen production§
		%
Sodium ascorbate, 0.5 mM	46.3	12.3
α,α′-dipyridyl, 0.1 mM	30.6	13.1
α,α′-dipyridyl, 0.5 mM	6.8	15.0

* Calvarial bones were removed from 1-wk-old guinea pigs and duplicate sections were labeled with [4-^{3}H],[^{14}C]proline (0.1 mM) during a 2-h incubation in the presence of either sodium ascorbate or α,α′-dipyridyl at the concentrations indicated.
‡ Determined by the dual-labeled proline method in duplicate samples by measuring changes in the ratio of [^{3}H]/[^{14}C] in collagenase digests as described in Methods.
§ Determined in duplicate samples from the ^{14}C radioactivity incorporated into collagenase-sensitive and -insensitive proteins.

when the absolute rates of collagen degradation were determined, there was an insignificant difference between control and scorbutic groups (Table III). The relationship between the absolute rates of collagen net production, collagen degradation, and collagen biosynthesis are depicted graphically in Fig. 2 *B*, and supports our conclusion that the major mechanism for the decreased net production of collagen is decreased biosynthesis, rather than increased degradation.

Collagen and noncollagen protein content. The total content of collagen and noncollagen proteins (Table IV), as well as DNA (data not shown) were similar in bones of scorbutic and control animals. The extents of proline hydroxylation (Table IV) and lysine hydroxylation (data not shown) in the bone collagen pool of scorbutic animals were unchanged. These re-

TABLE II
Specific Activity of the Free [^{14}C]Proline Pool in Bone

Experimental group*	Addition of ascorbate in vitro‡	[^{14}C]Proline specific activity§
		dpm/nmol
Scorbutic	−	7,634
	+	7,206
Control	−	7,783
	+	8,261

* Samples from the 4th wk of the experiment described in Fig. 1 were used.
‡ Sodium ascorbate (0.5 mM) was either added (+) or not (−).
§ Proline was purified by ion-exchange chromatography of the acid-soluble fraction of bone homogenates. Separate portions were used for quantitation of proline on an amino acid analyzer and for measurement of radioactivity. Results are averages of duplicate samples.

TABLE III
Absolute and Relative Rates of Collagen Degradation in Control and Scorbutic Bone Cultures

Group	Week	Proline hydroxylation	Collagen degradation	
			Percentage	Absolute
		%		
Control (2)	3	43.5	10.8	0.84
Control (4)	4	43.8	8.7	0.77
Scorbutic (4)	3	27.3	12.7	0.56
Scorbutic (2)	4	31.9	21.8	0.94

Radioactive proline-labeled bone sections from control and scorbutic animals were derived from samples prepared for the experiment described in Fig. 1. Control values for hydroxylation and degradation were obtained from cultures incubated in the presence or absence of ascorbate (0.5 mM), while those for the scorbutic groups are from cultures incubated in the absence of ascorbate. Results are the means of 2 or 4 values, as indicated within the parentheses. The percentage of collagen degradation was calculated from the amount of [^{14}C]hydroxypoline in the low molecular weight fraction relative to the total [^{14}C]hydroxyproline. The absolute rate of degradation was calculated from the percentage of collagen degraded and the absolute rate of net production of collagen. It is expressed as picomoles of proline incorporated into newly synthesized collagen that was degraded per microgram of DNA per hour.

sults indicate that even after 4 wk of deficiency there is no significant turnover of bone collagen.

Influence of weight loss on collagen synthesis. The apparent dissociation between the effects of ascorbate deficiency on proline hydroxylation and collagen synthesis observed at 2 wk prompted us to consider the possibility that ascorbate may influence collagen synthesis via another pathway. One of the characteristic features of scurvy in guinea pigs is the onset of weight loss (41, 42) due to decreased food intake (42) at ~2 wk after initiation of an ascorbate-deficient diet. Examination of the growth patterns of the individual scorbutic animals analyzed in our experiments revealed that only those used at the 4th wk, when the most severe decrease in collagen synthesis was observed, had been rapidly losing weight. Those used at the 3rd wk, where the decrease in collagen synthesis was less striking, had stopped gaining weight but were not yet in the rapid loss stage. Therefore, we examined the relationship between weight loss and collagen synthesis in greater detail.

TABLE IV
Nonradioactive Collagen and Noncollagen Protein Content in Guinea Pig Calvarial Bone

Study period	Experimental group*	Collagen (pro + hyp)‡	Proline hydroxylation	Noncollagen protein§
wk			%	
2	S	209	42.0	20.9
	C	192	42.3	22.2
3	S	193	44.3	23.8
	C	220	44.2	25.1
4	S	142	42.4	25.1
	C	174	43.8	25.5

* S, scorbutic; C, control. In each group $n = 2$ for the 2nd and 3rd wk and $n = 3$ for the 4th wk.

‡ Determined by amino acid analysis of duplicate samples of hydrolyzed collagenase digests of bone and expressed as nanomoles per milligram of wet tissue.

§ Determined by protein assays of duplicate samples of collagenase-insensitive bone tissue and expressed as micrograms per milligram of wet tissue.

Two groups of guinea pigs were placed on the scorbutogenic diet, with part of each group (controls) receiving supplemental ascorbate. All animals were fed ad lib. and gained weight for the first 2 wk. Scorbutic animals either started losing weight at that point or stopped gaining weight with weight loss delayed until varying times during the 3rd and 4th wk. A small percentage of scorbutics continued to gain weight during the 3rd wk before growth stopped. Some controls had their food restricted at varying times to correspond with initiation of weight loss in the scorbutics. At several points during the 3rd and 4th wk, scorbutic animals with different growth patterns were selected, along with appropriate food-restricted controls and controls fed ad lib. The relative rates of collagen synthesis in calvaria bone were determined in vitro, and these were compared with the percentage of weight lost or gained during the 48 h before they were killed (Table V). Several striking observations were made: (*a*) Low relative rates of collagen synthesis, comparable to those observed at 4 wk in the first study, could be found, regardless of the duration of ascorbate deficiency if the animals were rapidly losing weight. (*b*) If scorbutic animals were still gaining weight during the 3rd wk, the relative rate was only slightly depressed compared with control values. (*c*) The relative rates in bones of food-restricted controls were comparable with those in bone from scorbutics exhibiting similar weight losses. Plotting the percentage of weight change in either the scorbutics or food-restricted controls vs. collagen production in bones of these animals compared with the controls fed ad lib., showed a linear correlation with an R value of 0.89 and $P < 0.001$ (Fig. 3).

DISCUSSION

The study reported here represents the first comprehensive analysis in a normal mammalian tissue of the

TABLE V

The Effect of Weight Change on the Relative Rate of Collagen Production

Experiment	Days on diet	Diet*	Incubations‡	Weight change§	Relative rate of collagen production‖	Percentage of ad lib. control¶
			n		%	
1	18	S	8	−12	7.5±0.5	31
		S	4	−2	14.1±1.2	58
		S	8	+3	18.7±0.9	77
		CR	8	−4	16.3±1.7	67
		C	8	+5	24.4±1.2	100
2	19	S	4	−3	6.4±1.1	34
		S	4	−1	14.9±1.7	78
		CR	4	−1	13.0±1.8	68
		C	4	+10	19.1±0.8	100
1	22	S	8	−10	3.2±0.4	17
		S	4	−5	7.6±0.8	40
		CR	8	−11	4.5±0.2	24
		C	8	+4	18.9±1.3	100
2	25	S	8	−10	6.7±0.7	29
		CR	8	−11	3.3±1.0	14
		C	8	+6	23.0±1.4	100

* 1-wk-old guinea pigs were placed on an ascorbic acid-free diet (day 1) plus (controls) or minus (scorbutic) sodium ascorbate, 50 mg/d orally. All animals were fed ad lib. until weight loss was evident in the scorbutic group (S), at which time the control restricted group (CR) had food restricted to induce weight loss. A third group (C) continued to be fed ad lib.

‡ The number of separate incubations with one-quarter of a parietal bone, so that four incubations represent one animal. All incubations in experiment 2 were carried out in the presence of 0.1 mM ascorbate. Those of experiment 1 were carried out with or without ascorbate, and the results were averaged together since there were no significant differences due to the addition of ascorbate.

§ Weight changes are expressed as the percentage of weight lost or gained during the 48 h before experimentation.

‖ The relative rate of collagen production was determined in vitro as described in Methods using [2,3-^{3}H] or [5-^{3}H]proline to label proteins, and the values are presented as the means±SE.

¶ The relative rate as a percentage of the ad lib. fed control values within each experiment.

effect of ascorbic acid deficiency on the absolute and relative rates of collagen synthesis and degradation and the relationship of proline hydroxylation to these functions. Guinea pig calvarial bone cultures were used, which allowed recovery of the products resulting from degradation of newly synthesized collagen and offered the possibility of determining the reversibility in vitro of ascorbate deficiency-induced effects. Results of these experiments showed that in vivo-induced ascorbate deficiency decreased both proline hydroxylation and the absolute rate of collagen synthesis. They further suggested that the specific decrease in collagen synthesis may not result directly from decreased proline hydroxylation or procollagen secretion. Rather it may result from the imposition of an additional nutritional deficiency or an altered metabolic state caused by the reduced food intake characteristic of scurvy in guinea pigs. The bases for these conclusions are discussed below.

The decreased relative rate of collagen production in scorbutic bone was due specifically to a diminished absolute rate of collagen biosynthesis rather than to increased production of noncollagen protein or increased collagen degradation (Fig. 2). It is possible that there is a pool of completely unhydroxylated collagen that is preferentially degraded in bones from scorbutic animals. In our studies, however, as in others (34), the measurement of low molecular weight hydroxyproline as an index of collagen degradation precluded measuring the degradation of completely unhydroxylated collagen. At any rate, this possibility seems unlikely, since the relative rates of collagen production in normal bones treated in vitro with α,α'-dipyridyl remained unchanged, in spite of the presence of an almost completely unhydroxylated collagen pool.

In our experiments, the specific activity of the free proline pool was used to determine absolute rates of synthesis and degradation. Although some studies have

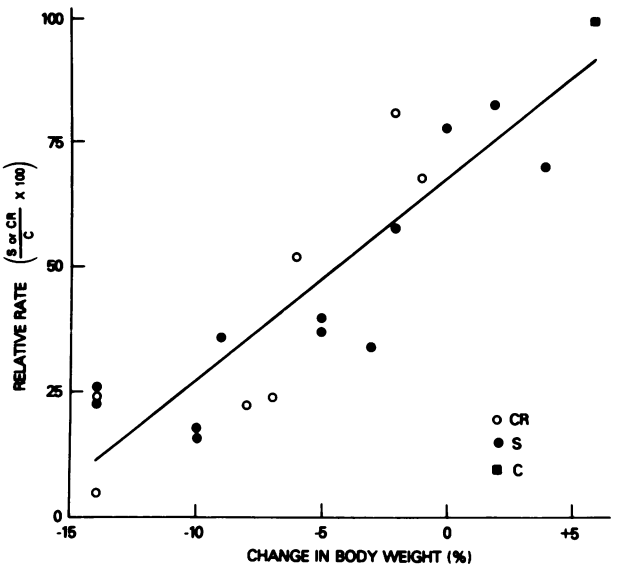

FIGURE 3 Correlation between body weight changes and the relative rate of collagen production in guinea pig calvarial bone. The data are from the experiments shown in Table V and are plotted for individual animals (four incubations) as the relative rate of collagen production expressed as a percentage of the values for the controls fed ad lib. vs. the percentage of weight change in the 48-h period before analysis. Scorbutics (*S*) (●), $n = 12$; food-restricted controls (*CR*; ○), $n = 7$. The controls fed ad lib. are represented by a single point (*C*) (■) at a relative rate of 100% plotted against the average of the weight changes (+6.3%, $n = 7$). ($R = 0.894$; $P < 0.001$).

suggested that measurement of the aminoacyl-tRNA pool is a more accurate method of determining precursor specific activity, at least for leucine (43, 44), there is much convincing evidence to the contrary. It has been shown that for various rat tissues labeled with several different amino acids, the specific activities in the free amino acid pool and in specific proteins were almost identical (45, 46). These studies also showed that there is rapid equilibration between amino acids in the extracellular fluid and the intracellular pool. To assure equilibration of the medium and internal pools, unlabeled proline was added with the radioisotope in our experiments. The fact that a linear rate of incorporation was attained suggests that equilibration had been reached.

Several of our observations suggested a dissociation between the effects of scurvy on proline hydroxylation and collagen synthesis in guinea pig calvaria. In our initial series of experiments, the maximal decrease in proline hydroxylation was observed after 2 wk on the scorbutogenic diet, whereas there was little effect on collagen production at that time. Furthermore, the addition of ascorbate to short-term cultures of scorbutic bone reverted decreased proline hydroxylation, but not collagen production to control levels. Additional support for this conclusion came from the observation that short-term impairment of proline hydroxylation in vitro with α,α'-dipyridyl did not decrease collagen biosynthesis. Therefore, it seems unlikely that decreased collagen biosynthesis in scorbutic guinea pig bone cultures is an immediate consequence of impaired proline hydroxylation. Analogously, a rapid effect on collagen synthesis because of decreased procollagen secretion also is unlikely. Although the degree of underhydroxylation in collagen of the scorbutic bone was not extensive, it probably was sufficient to inhibit procollagen secretion. A similar reduction of hydroxylation in procollagen of cultured chick embryo bone cells inhibited secretion by 80% without affecting synthesis (12). In bone culture systems, analysis of secretion is difficult because procollagen is rapidly processed and becomes part of the insoluble extracellular matrix (47).

A dissociation between the effect of ascorbate on proline hydroxylation and the relative rate of collagen production has been observed in a number of organ and cell culture systems. The depletion of ascorbate from long-term cultures of fetal rat bone had only a small effect on the relative rate of collagen production (as calculated from data in reference 48). In most studies using cell cultures, ascorbate had no effect on collagen production (7, 8, 13, 49). In both the bone and cell cultures, however, ascorbate was required for normal proline hydroxylation. In a few instances, there was a specific increase in the relative rate of collagen production by ascorbate under well-defined culture conditions, and it has been suggested that this occurs independently of the role of ascorbate in hydroxylation (14, 50). It is not clear whether this modulation of collagen production by ascorbate in cell culture systems occurs by the same mechanism operative in vivo.

The possibility that ascorbate plays a specific role in collagen biosynthesis independent of its role in hydroxylation was considered. The coincidence of weight loss with the reduction in collagen synthesis beginning after 2 wk of ascorbate deficiency suggested that these effects might be related. Our second series of experiments revealed that there was a direct relationship between weight loss and the relative rate of collagen production, regardless of whether such losses were induced by scurvy or by restricting the food intake of ascorbate-supplemented controls. Although relatively few studies have controlled for weight loss, in two cases it was found that incorporation of radioactive proline into hydroxyproline of skin collagen was reduced to varying degrees in food-restricted controls compared to controls fed ad lib., although the effects of scurvy were more severe (25, 41). The more striking effects in scurvy may have resulted from the fact that hy-

droxyproline was used to measure collagen and, in at least one case (41), the fact that the food-restricted controls were not losing weight. It was observed in other studies that pair-fed controls do not lose weight as rapidly as scorbutic guinea pigs (51). This may explain why collagen synthesis in the pair-fed controls of our initial study was higher than in the scorbutic animals. Further evidence that there is a relationship between weight loss and collagen production was the observation of a dramatic decrease in the amount of salt-soluble collagen in skin of guinea pigs that were partially fasted but given vitamin C, as compared with animals fed ad lib. (52). Gross also found that the effect of scurvy on this fraction was more severe (51), which, as in other studies, may have been due to the fact that hydroxyproline was measured as an index of collagen content.

Our findings suggest the hypothesis that vitamin C deficiency leads to decreased food intake and associated weight loss, which either directly causes a decrease in collagen synthesis because of loss of an additional nutrient or this initiates a signal to another regulatory mechanism. In either case, the mechanism would be independent of an effect of proline hydroxylation. The validity of this hypothesis and whether it accounts entirely for the decrease in collagen synthesis in scorbutic bone or whether the same mechanism is responsible for defects in other connective tissues of scorbutic guinea pigs remains to be established.

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