FUNCTION OF ASCORBIC ACID IN COLLAGEN METABOLISM

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Direct involvement of ascorbic acid in collagen synthesis is well known and represents perhaps the most clearly defined biochemical role of the vitamin. The absence of wound healing and occurrence of fractures that fail to repair are classically-recognized features of scurvy that can be attributed to impaired collagen formation arising from lack of vitamin C. The presence of hemorrhage in this disease may conceivably be regarded as another.[1]

In this paper it is my intention to outline the progress that has been made in the understanding of the mode of action of the vitamin in collagen formation since the time this subject was considered at the first conference on vitamin C organized by The New York Academy of Sciences in 1960.[2, 3] It was then mooted [2] that its most likely role was in the formation of collagen hydroxyproline through hydroxylation of proline before the latter was incorporated into peptide linkage. It is now known from studies utilizing isolated collagen-synthesizing systems that ascorbic acid does, as thought possible, participate in this reaction and also in the analogous reaction leading to the formation of collagen hydroxylysine, but that the hydroxylation of both the proline and the lysine occurs only after the pertinent amino acid has been incorporated within the collagen polypeptide chain formed during ribosomal collagen protein synthesis. This paper will briefly summarize the relevant evidence that has accumulated over the past decade leading to this conclusion and will consider whether the collagen lesion as it occurs *in vivo* in scurvy is fully accountable in terms of impaired hydroxylation (of either peptidyl proline and/or peptidyl lysine).

The function of ascorbic acid in collagen synthesis has been reviewed recently by a number of authors.[4-7]

Formation of Collagen Hydroxyproline and Hydroxylysine: Evidence for the Participation of Ascorbic Acid

It was demonstrated some years ago that free hydroxyproline and hydroxylysine were not incorporated into collagen. The presence of these two unusual amino acids in collagen arises in fact through the hydroxylation of particular prolyl and lysyl residues previously incorporated into peptide linkage during the process of ribosomal collagen polypeptide synthesis. Hydroxylation appears to occur primarily while the polypeptide chain is being formulated and is therefore still attached to the ribosome.[8-10, 88, 89]

Evidence that hydroxylation does not precede translation arose initially from isotopic studies utilizing isolated collagen-synthesizing systems that demonstrated that formation of collagen hydroxyproline could be markedly inhibited without, at the same time, preventing collagen protein synthesis. Thus, under appropriate conditions, incorporation of labeled proline into collagen hydroxy-

proline could be inhibited while still achieving incorporation of radioactivity into peptide-bound proline contained in material that, like collagen, was extractable with hot trichloroacetic acid and was collagenase-susceptible. These studies implied the formation of a proline-enriched hydroxyproline-deficient collagen, termed protocollagen, when hydroxylation was impaired.[11] Formation of protocollagen has been demonstrated by isotopic means when hydroxylation is impaired either by inclusion of the chelating agent α,α'-dipyridyl in the incubation medium or through exclusion of oxygen by incubation of tissues under nitrogen.[8] The constituent unhydroxylated polypeptide chains of protocollagen have now been separated by chromatography by procedures similar to those for the separation of the equivalent hydroxylated polypeptide chains of collagen.[12-21]

TABLE 1

INCORPORATION OF [^{14}C]PROLINE INTO THE COLLAGEN PROLINE AND HYDROXYPROLINE OF GRANULOMAS FROM NORMAL AND SCORBUTIC GUINEA PIGS

Specific Activity (dpm/μmole) *					
Normal			Scorbutic		
Proline	Hydroxyproline	Proline/Hydroxyproline	Proline	Hydroxyproline	Proline/Hydroxyproline
790	697	1.13	787	<10	>7.9
745	680	1.10	530	10	5.3
(cpm/flask) †					
644	564	1.1	684	6	114
8994	3888	2.3	212	0	∞
4860	1870	2.6	—	—	—

* Granuloma minces were incubated in a medium containing [^{14}C] proline. Collagen was extracted with hot trichloroacetic acid and precipitated with tannic acid. Proline and hydroxyproline radioactivity were estimated in the precipitate. (From Stone and Meister.[22] By permission of *Nature*, London.)

† Collagen was extracted with hot trichloroacetic acid. Extracts were digested with collagenase and then precipitated with tannic acid. Proline and hydroxyproline radioactivity were estimated in the supernatant. (From Gottlieb et al.[23] By permission of *Journal of Biological Chemistry*.)

The participation of ascorbic acid in the hydroxylation of peptide-bound collagen proline was perhaps first clearly indicated by the studies of Meister and Udenfriend and their respective colleagues.[22-24] These workers demonstrated that when minces of granuloma tissue, formed in scorbutic guinea pigs, were incubated in the presence of labeled proline, there was little incorporation of radioactivity into collagen hydroxyproline while incorporation into peptide-bound proline, located in high-molecular weight, hot trichloroacetic acid-extractable, collagenase-susceptible material, remained, in comparison with controls, relatively high (TABLE 1). This pointed to the formation of an unhydroxylated or underhydroxylated collagen in the ascorbate-deficient tissue and implied a role for the vitamin in the hydroxylation of peptidyl proline. Addition

of ascorbic acid to the medium prior to incubation caused a stimulation of incorporation of radioactivity into collagen hydroxyproline.

Jeffrey and Martin,[25] studying collagen synthesis in isolated embryonic chick bone rudiments, also demonstrated the requirement for ascorbic acid in this process and deduced, using puromycin as an inhibitor of protein synthesis, that incorporation of proline into peptide linkage preceded hydroxylation. They concluded that vitamin C participated in the conversion of peptide-bound proline into collagen hydroxyproline since addition of the vitamin to the growth medium whether in the presence or absence of puromycin caused a stimulation in the formation of labeled hydroxyproline in vitamin-C-depleted bones prelabeled with [^{14}C]proline.

Formation of underhydroxylated collagen in response to a lack of vitamin C has also been demonstrated in cell culture studies utilizing ascorbate-deficient growth medium.[19, 26-29] Details of this type of study are given elsewhere in this volume.[30]

Incontrovertible evidence for the participation of ascorbic acid in the hydroxylation of collagen proline has been derived from cell-free studies that proved the existence of a hydroxylase that could utilize as substrate the proline-enriched, hydroxyproline-deficient, collagenlike polypeptide synthesized in the presence of inhibitors such as α,α'-dipyridyl, and was thereby able to catalyze, in the presence of appropriate cofactors, the formation of collagen hydroxyproline. In addition to atmospheric oxygen the cofactors were found to be ferrous ion (removal of which by α,α'-dipyridyl accounted for the inhibitory activity of the latter), a reducing agent such as ascorbic acid and, surprisingly, α-ketoglutarate [31-34] (TABLE 2). The enzyme prolyl hydroxylase has now been isolated in highly purified form and extensively characterized.[10, 35] It is completely free of any activity towards lysyl residues. Its activity may well be of particular importance in controlling collagen biosynthesis.

TABLE 2

DEPENDENCE OF PROLYL HYDROXYLASE ACTIVITY ON α-KETOGLUTARATE, FERROUS ION AND ASCORBATE *

Omission from Complete Hydroxylating System	Tritiated Water Formed (cpm)		
	Chicken Embryo Enzyme	Rat Skin Enzyme	Granuloma Enzyme
None	982	850	600
α-ketoglutarate	40	68	71
Ascorbate	0	47	0
Ferrous ion	153	323	0

* Complete hydroxylating system (volume 2 ml) contained (in μmoles): Tris HCl, pH 7.5, 200; α-ketoglutarate, 0.2; ascorbate, 1.0; ferrous ion as Fe $(NH_4)_2$ $(SO_4)_2$, 0.2; plus [3,4-^{3}H] proline-labeled peptidyl-proline substrate (as 0.5 mg protein), containing 800,000 dpm. Enzyme (as dialyzed 105,000 *g* supernatant of tissue homogenates) added as follows (as mg protein): chicken embryo, 2.0; fetal rat skin, 0.4; guinea pig granuloma 1.4. Hydroxylase activity was estimated as tritium released from the labeled substrate, counted at 8% efficiency (From Hutton et al.[34] By permission of *Archives of Biochemistry and Biophysics.*)

TABLE 3

COFACTOR REQUIREMENTS FOR THE HYDROXYLATION OF [^{14}C]LYSINE IN PROTOCOLLAGEN *

Incubation Conditions	[^{14}C]Hydroxylysine (dpm)
Substrate alone	500
Complete system	10,600
Complete system minus Fe^{++}	800
Complete system minus α-ketoglutarate	900
Complete system minus ascorbate	500

* Complete system contained 200,000 dpm [^{14}C]lysine-labeled protocollagen as substrate; 50 mg ammoniums-sulphate-precipitated chick embryo extract as enzyme; 0.01 mM $FeSO_4$; 0.5 mM α-ketoglutarate; 1 mM ascorbic acid; 0.05M Tris buffer, pH 7.6; and 0.1M KCl in a volume of 8ml. After incubation at 37°, the incubation mixture was hydrolyzed and the extent of hydroxylation determined by measuring the [^{14}C]hydroxylysine content of the hydrolysates. (From Kivirikko and Prockop.[88])

Evidence similar to that outlined above indicates that the formation of collagen hydroxylysine is comparable to that of collagen hydroxyproline. An enzyme, lysyl hydroxylase, with cofactor requirements identical to those listed above, catalyzes the hydroxylation of specific lysyl residues incorporated into peptide linkage during the process of collagen polypeptide synthesis [36-38] (TABLE 3). The enzyme has been partially purified and as such is free of activity towards prolyl residues in protocollagen.[39-41] Like prolyl hydroxylase,[42-45] lysyl hydroxylase activity is stimulated by bovine serum albumin, catalase, and dithiothreitol.[40, 41] The nature of these stimulations is not understood. It is thought that catalase may act by protecting the enzyme from peroxides formed during the course of the reaction.[45] Dithiothreitol may protect an essential -SH group.[44, 45]

Studies with fibroblast cultures indicate that lysine hydroxylation may be less readily affected by ascorbate deficiency than proline hydroxylation.[46] This could be a reflection of the somewhat lower K_M for ascorbate observed with lysyl hydroxylase in comparison with that found with prolyl hydroxylase.[40] It is interesting that despite this difference the extent of lysine hydroxylation from one collagen to another under normal or pathological situations shows much more variation than the extent of proline hydroxylation.[47, 48] The hydroxylation of lysine in the telopeptide, nonhelical region of the collagen molecule, important in respect to the type of intermolecular crosslink formed, could conceivably be under the control of a separate ascorbate-dependent hydroxylase distinct from the main lysyl hydroxylase.[48, 49]

MODE OF ACTION OF VITAMIN C IN THE HYDROXYLATION OF COLLAGEN PROLINE AND LYSINE

With the requirement for molecular oxygen, prolyl and lysyl hydroxylases could be regarded as mixed-function oxidases operating according to the following equation:

$$R + XH_2 + O_2 \rightarrow ROH + X + H_2O$$

where R is the substrate undergoing hydroxylation (peptidyl proline or lysine), XH_2 the cosubstrate, and ROH the hydroxylated product (peptidyl hydroxyproline or hydroxylysine). With the establishment of ascorbic acid as a cofactor, it was naturally assumed that the vitamin was required as the cosubstrate in the mixed function oxidation, as occurs for example in the hydroxylation of dopamine by the copper-dependent enzyme dopamine-β-hydroxylase.[50] It was thought that α-ketoglutarate might act as an allosteric activator.[34] However, it then became apparent the role of cosubstrate was undertaken by α-ketoglutarate itself, which, during the course of hydroxylation, underwent a stoichiometric decarboxylation to succinate.[51, 52] It was shown that one-half of the oxygen molecule was incorporated into the substrate undergoing hydroxylation while the other half, in contrast to the reaction depicted in the above equation, appeared in succinate rather than water.[53] These enzymes have therefore been classified as dioxygenases.[54]

It became clear that a number of enzymes could be grouped together as belonging to a newly recognized class of hydroxylase, all requiring as cofactors molecular oxygen, ferrous ion, a 2-keto acid, and a reducing agent, the most effective being ascorbic acid, and all being stimulated by catalase.[55] Thus, in addition to prolyl and lysyl hydroxylases, the enzyme γ-butyrobetaine hydroxylase, from both animal and bacterial origins, that catalyzes the hydroxylation of γ-butyrobetaine to carnitine, oxygenases identified in *Neurospora crassa* that catalyze the oxygenation of thymine to 5-carboxyuracil and the conversion of thymidine to thymine riboside and deoxyuridine to uridine, and the enzyme *p*-hydroxyphenylpyruvic acid oxidase that catalyzes the conversion of *p*-hydroxyphenylpyruvic acid to homogentisic acid have all been shown to display in common those cofactor activity requirements listed above.

With the establishment of α-ketoglutarate as cosubstrate, the role of ascorbic acid as reductant in the reactions catalyzed by prolyl and lysyl hydroxylases was left unexplained. In contrast to the absolute requirement for α-ketoglutarate and ferrous ion, that for ascorbate has been found to be not highly specific.[34, 45, 56, 57] The vitamin, in the isolated cell-free system at any rate, can be replaced by any of its stereoisomers (but not by dehydroascorbic acid), by various reduced pteridines, and by a number of thiol compounds including dithiothreitol and cysteine. The same lack of specificity appears to hold true for the other enzymes in this group. Generally, however, ascorbic acid appears to be the most effective reductant.

Besides its ability to replace ascorbic acid as a reductant, dithiothreitol appears to exert an additional effect since at low concentrations it causes stimulation of enzyme activity even in the presence of saturating levels of ascorbic acid.[45] At the same time, however, dithiothreitol can also cause loss of enzyme activity, probably by disrupting essential disulphide linkages and causing thereby disaggregation of the active enzyme.[35, 44, 57, 58]

A reaction mechanism for prolyl and lysyl hydroxylases and other α-ketoglutarate-dependent dioxygenases is proposed[55] in which oxygen is activated by ferrous ion bound to the enzyme, followed by the formation of an intermediate peroxy compound between substrate and α-ketoglutarate (the initial oxidative attack probably being towards α-ketoglutarate) and then cleavage of the peroxy compound to yield hydroxylated substrate, succinate, and carbon dioxide. The role of the reductant in this scheme is not clear. As proposed by Holme et al.,[59, 60] Fe^{++} remains reduced during the reaction and the reductant may either serve to maintain Fe^{++} in the reduced state or to protect essential

-SH groups. In the scheme proposed by Hurych et al.,[61, 62] iron undergoes a cyclic oxidation and reduction. They have presented evidence for the oxidation of Fe^{++} when added to enzyme and α-ketoglutarate. Oxidation, it is suggested, occurs during activation of oxygen, and reduction is accomplished by the reducing cofactor. In this case ascorbic acid should be utilized in stoichiometric proportions. This has yet to be established. Bhatnagar and Liu [63] also suggest that iron may undergo a cyclic oxidation and reduction and that ascorbate is utilized in the generation of the superoxide radical, which serves to reduce ferric to ferrous ion.

Recent studies in cell culture [57, 58] have introduced a new aspect to the problem of the mode of action of vitamin C in the mechanism of hydroxylation of collagen proline. This subject is dealt with in other contributions to this monograph [30, 64] and will not therefore be described in detail here. It appears that the lack of hydroxylation in cell cultures when ascorbate is absent is due to an actual lack of active enzyme (rather than to a failure of active enzyme to hydroxylate because of the absence of an essential reducing cofactor). Ascorbic acid may be involved in the conversion of an inactive precursor to active enzyme. The question that arises is whether the two activities of ascorbic acid (its activation of enzyme and participation in hydroxylation by active enzyme) are perhaps really two aspects of the same phenomenon. Conceivably, ascorbic acid serves in the cell-free system to maintain enzyme in an active state in a manner related to its ability to cause activation of the inactive enzyme in the whole cell. Possibly reduction of some specific site on the enzyme (Fe^{++}?) not only permits hydroxylation but also permits the retention of an active conformation of enzyme. Dithiothreitol causes inactivation of enzyme both in whole cells [58] and *in vitro* [35, 57] and has been shown to cause disaggregation of the molecule.[35] This can be reversed by ascorbic acid in cell culture.[58] Ascorbic acid also affords protection against the effects of dithiothreitol *in vitro*.[57]

The Nature of Impaired Collagen Synthesis *In Vivo* in Ascorbic Acid Deficiency

Studies by the author and his colleagues were undertaken in an attempt to establish if impaired collagen synthesis *in vivo* in ascorbic acid deficiency could be explained satisfactorily in terms of a functioning of vitamin C in the hydroxylation of collagen proline and lysine in accord with the behavior of the vitamin *in vitro*.

There were certain grounds for believing that the lesion *in vivo* might not simply be impaired hydroxylation. Thus, early attempts to separate and identify by amino acid analysis a proline-enriched, hydroxyproline-deficient collagen from the tissues of scorbutic guinea pigs were unsuccessful [65-67] and thereby lent weight to the contention that hydroxylation of proline in the formation of collagen hydroxyproline must precede translation. Isotopic studies by Gould et al.[66] and Robertson et al.[65] also led to the conclusion that the rapid synthesis of collagen in scorbutic guinea pigs following the administration of vitamin C was not due to the hydroxylation of a hydroxyproline-deficient precursor accumulated in scurvy but must involve *de novo* synthesis.

Impaired hydroxylation would be expected to give rise to a fall in hydroxyproline excretion. In scorbutic guinea pigs, however, it was found that hydroxyproline excretion remained normal or even slightly elevated despite impaired

collagen synthesis in the tissues.[7, 68] Similarly the level of diffusible hydroxyproline and hydroxylysine in the skin showed no significant change.[7, 69] A fall in hydroxyproline excretion did not occur until after two weeks of ascorbic acid deprivation, some days after the effect on tissue collagen was first noted. This fall occurred in control animals also and was considered attributable to inanition. An increased excretion of hydroxyproline has been reported in cases of human scurvy.[70, 71]

Although studies with the purified enzyme prolyl hydroxylase have indicated a requirement for ascorbic acid in the hydroxylation of collagen proline, the requirement is not an absolute one and the vitamin can be replaced by other reductants. This raises the possibility that *in vivo* the requirement for a reductant could be met by compounds other than vitamin C and that the impairment of collagen synthesis in scurvy reflects a role of ascorbic acid in collagen metabolism other than one in hydroxylation.

We decided therefore to seek evidence of impaired hydroxylation *in vivo* by isotopic methods, studying the incorporation of labeled proline into collagen proline and hydroxyproline. Since it seemed possible, in view of the failure to detect its gross accumulation in tissues, that formation of protocollagen might be transitory or that its existence was short-lived, we conducted these incorporation studies at varying periods of ascorbic acid deprivation, measuring the incorporation at varying times after administration of the isotope. Incorporation into elastin was studied as well, since this extracellular polymer also contains some hydroxyproline, although much less than collagen. It was anticipated that increasing ascorbic acid deprivation would lead to increasing hydroxylation impairment and therefore incorporation of radioactivity into hydroxyproline relative to that into proline would decrease. Precisely this situation was found in elastin.[69] Incorporation of radioactivity into elastin hydroxyproline became negligible while that into elastin proline was little affected (TABLE 4). The elastin proline/hydroxyproline specific radioactivity ratio thus became increasingly large in contrast to the value of unity in controls (the value of unity, of course, indicating that the degree of hydroxylation in the newly synthesized labeled material was the same as that in the preformed unlabeled protein). The results thus indicated the formation in the scorbutic animals of a polymer increasingly deficient in hydroxyproline.

However, in the case of skin collagen,[69] incorporation into both proline and hydroxyproline showed a rapid fall occurring around the 8th–10th day of ascorbic acid deprivation. The proline/hydroxyproline specific activity ratio showed only a very slight (but nevertheless highly significant: $p < 0.001$) rise (TABLE 4). The results implied the formation, but in rapidly diminishing amounts, of a slightly underhydroxylated collagen in which the level of hydroxylation was reduced by 5–10%. This collagen behaved as normal collagen as regards its distribution between fractions of different solubility and its rate of turnover, and it was concluded therefore that hydroxylation was not sufficiently reduced to impair its function.[7, 69] A similarly, slightly underhydroxylated collagen has since been detected in the scorbutic catfish.[72]

The question that arose was whether the drastic fall in incorporation of radioactivity into skin collagen arose from an actual reduction in collagen protein synthesis or from the formation of a more substantially underhydroxylated collagen that failed to accumulate in the tissues because it was entirely degraded. Degradation would have to be rapid since we could not detect such

TABLE 4

INCORPORATION OF TRITIATED PROLINE *in Vivo* INTO ELASTIN AND COLLAGEN IN CONTROL AND SCORBUTIC GUINEA PIGS *

	Day of Experiment	Number of Animals	Elastin Specific Radioactivity (cpm/μmol)			Collagen Specific Radioactivity (cpm/μmol)			
			Proline	Hydroxy-proline	Pro/Hyp	Proline	Hydroxy-proline	Pro/Hyp	
Ascorbic-Acid-Deficient Group	6	6	222	149	1.5	739	667	1.11	
	8	6	132	62	2.1	357	289	1.24	Mean 1.15
	10	5	142	15	9.5	74	69	1.07	
	12	6	61	3	20.3	21	17	1.24	
	14	6	—	—	—	13	12	1.08	
Control Group	6	6	129	125	1.0	614	583	1.05	
	8	6	111	131	0.9	785	828	0.95	Mean 1.0
	10	4	80	57	1.4	553	563	0.98	
	12	6	35	31	1.1	224	217	1.03	
	14	6	—	—	—	415	417	1.0	

* Each animal received, on the appropriate day of the experiment, 0.1 mCi of L-[G-^{3}H]proline in a single dose. Animals were killed 24 hr later. Aortas were removed and combined within each set of animals before the isolation of elastin. Approximately equal samples of dorsal skin were combined in a like manner within each set of animals before the extraction of collagen with hot trichloroacetic acid. Collagen extracts, after dialysis, were digested with collagenase and the resulting collagen-derived diffusible peptides subjected to hydrolysis. Proline and hydroxyproline in collagen and elastin hydrolysates were separated by ion-exchange chromatography, estimated with ninhydrin, and the radioactivity measured by liquid scintillation counting. Controls were individually pair-fed with animals in the ascorbic-acid-deficient group after day 8. The number of animals refers to the number available at the time of killing from an original total of six. (From Barnes et al.[60] By permission of *Biochemical Journal*.)

a moiety in the tissues even when they were examined as soon as 2 hours after administration of isotope.[10]

An increase in proline radioactivity (measured after hydrolysis) in the diffusible fraction of skin concomitant with the fall in incorporation of radioactivity into collagen suggested degradation of underhydroxylated collagen (protocollagen) might be occurring.[69] We decided to explore this problem further by urinary excretion studies in guinea pigs administered labeled proline following ascorbic acid deprivation for varying periods of time. It was anticipated that if underhydroxylated collagen with an increasing deficiency of hydroxyproline was being formed and degraded in increasing amounts then the excretion of hydroxyproline-containing peptides should be replaced, to an increasing extent, by the excretion of the equivalent hydroxyprolinefree peptides.

TABLE 5

EXCRETION OF FREE GLYCINE AND OF RADIOACTIVITY IN FREE PROLINE AND THE PEPTIDES PROLYLHYDROXYPROLINE AND PROLYLPROLINE IN THE URINE OF SCORBUTIC GUINEA PIGS RELATIVE TO CONTROLS *

Day of Experiment	Glycine	Proline	Prolylhydroxyproline	Prolylproline
6	0.48	3.83	1.34	1.17
8	3.29	2.36	1.17	0.85
10	6.19	1.8	1.43	—
12	0.75	0.84	0.87	1.19
14	0.84	0.79	1.30	1.28

* Animals (six in each group) received L-[G-^{3}H]proline on the days indicated. Urine was collected for 24 hours after administration of the isotope. Urines were combined within each group. The urinary constituents listed were separated by ion-exchange chromatography. Free glycine was estimated with ninhydrin. Radioactivity in free proline, prolylproline, and prolylhydroxyproline was measured by liquid scintillation counting. Results are expressed as a ratio of the excretion in the ascorbic-acid-deficient group relative to that in the appropriate control group for each period of the experiment.

These studies revealed first that approximately ⅓ of the total hydroxyproline excretion occurred in the form of relatively large, nondiffusible peptides. A similar situation is known to occur in humans.[73] Examination of these peptides by collagenase digestion revealed that there was only a slight underhydroxylation of proline in this fraction from scorbutic animals, similar to that observed in skin collagen. This implied that if a substantially underhydroxylated collagen were formed, its degradation must be different from that of normal collagen, i.e., it must be degraded entirely to a diffusible form.

It was found that the major diffusible hydroxyproline-containing peptide in urine was prolylhydroxyproline. Excretion of radioactivity in this and the peptide prolylproline, however, showed little change in scorbutic animals relative to controls (TABLE 5). This was in accord with the observation that total hydroxyproline excretion and the amount of radioactivity in the total hydroxy-

proline excreted showed little if any change in scorbutic animals. Radioactivity appearing in free proline, however, showed a temporary increase, and at about the same time a very sharp but transitory increase in free glycine excretion was noted; these changes occurring at a time when incorporation of radioactivity into skin collagen was rapidly declining (TABLE 5). These changes were reflected in the plasma concentration of free glycine and proline. Hornig et al.[74] also found a transitory rise in the concentration of free proline in the plasma of scorbutic guinea pigs. It may be argued that these increases could arise from the formation and immediate degradation of protocollagen. However, since there was no indication of an increasing prolylproline excretion relative to that of prolylhydroxyproline, excretion of the latter remaining relatively constant during the above changes and since there was no evidence of substantial and increasing underhydroxylation of collagen proline in the non-diffusible fraction of urine, we conclude that severe underhydroxylation of collagen does not occur in the scorbutic guinea pig.

It seems probable that there is initially in ascorbic acid deficiency a slight fall in hydroxylation of proline, insufficient to impair the functioning of the collagen molecule, with the result that a collagen can be detected in the tissues with a 5–10% reduction in hydroxylation. There then occurs a somewhat greater reduction, causing instability in the molecule, which, as a consequence, undergoes degradation. Degradation of such a molecule may maintain the excretion of prolylhydroxyproline and prolylproline more or less within the normal range and at a normal ratio to each other. It has recently been emphatically demonstrated that the hydroxyproline content of collagen is critical in regard to the stability of the triple helical structure of the molecule and that reduction in the hydroxyproline content gives rise to a molecule in which the triple helix is unstable at body temperature.[15, 20] We consider therefore that in the scorbutic guinea pig a slight additional reduction in hydroxylation beyond the 5–10% detected in the tissues gives rise to a molecule that remains in the form of unassociated α-chains and, lacking the protection of the triple helical structure, is rapidly subjected to the action of preteolytic enzymes.[75]

We also conclude that further reduction in hydroxylation beyond this stage does not occur because of an inhibition of collagen protein synthesis. The increased free glycine excretion and increased amount of radioactivity in free proline in urine is thought to arise from impaired synthesis rather than degradation of protocollagen. Impaired collagen protein synthesis seems the most reasonable explanation for the continuing lack of accumulation of protocollagen in scurvy rather than the continuous formation and degradation of protocollagen, especially in view of the lack of evidence for the occurrence of appreciable underhydroxylation. Impaired hydroxylation by means of anaerobic conditions, use of α,α′-dipyridyl, or ascorbate deficiency in isolated systems *in vitro* appears to cause, at least in short-term incubations, an intracellular accumulation of the un- or underhydroxylated material.[13, 21, 27, 76–78] Similarly, *in vivo* intracellular accumulation of slightly-underhydroxylated α-chains may occur, and this may lead to a feedback inhibition of further synthesis.[27, 69] The electron-microscopical studies of Ross and Benditt [79] and the centrifugation studies of Fernandiz-Madrid and Pita [80] and Harwood et al.[81] demonstrating disaggregation of polyribosomes in the fibroblasts of scorbutic tissues all imply disrupted collagen protein synthesis in scurvy. There is no situation so far known to occur *in vivo* where impaired hydroxylation of collagen proline can be demonstrated without at the same time impaired collagen synthesis or accumulation. Inter-

ference with hydroxylation by means of α,α′-dipyridyl or the use of proline analogues both lead to reduced collagen synthesis or accumulation.[82-85] Studies in guinea pigs with chronic hypovitaminosis C failed to reveal impaired hydroxylation without impaired synthesis. As in acute deficiency, a slightly-underhydroxylated collagen was produced and incorporation of labeled proline into collagen was at the same time markedly reduced.[90] All of these data point to impaired synthesis as a consequence of impaired hydroxylation *in vivo*.

The results with collagen and especially with elastin described above indicate that hydroxylation of peptidyl proline is in fact impaired *in vivo* in scurvy. It has yet to be established, however, whether the hydroxylation of peptidyl lysine is impaired. Studies in cell culture suggest hydroxylation of collagen lysine may be less affected by ascorbate deficiency[16] and preliminary results suggest this may also be so *in vivo*.[90]

It is not yet known whether the hydroxylation of peptidyl proline *in vivo* is impaired through the absence of the vitamin as an essential cofactor participating directly in the hydroxylation mechanism or through lack of active enzyme. As already discussed, these two aspects of the vitamin's action may in any event prove to be different facets of a single mechanism. The studies of Mussini et al.[86] suggest there may be a lack of active enzyme *in vivo* in scorbutic tissues.

Conclusions

Studies in isolated collagen-synthesizing systems have demonstrated that ascorbic acid participates in the synthesis of collagen hydroxyproline and hydroxylysine, both of which are formed by the hydroxylation of particular prolyl and lysyl residues previously incorporated into peptide linkage during the process of ribosomal collagen protein synthesis. The precise mode of action of the vitamin in these hydroxylations has yet to be elucidated.

The need for ascorbic acid as a reductant *in vitro* in these reactions is not highly specific. Because of this lack of specificity, the possibility existed that compounds other than vitamin C might perform this function *in vivo* and that impaired collagen synthesis in scurvy might imply a role for ascorbic acid in collagen metabolism other than one in hydroxylation. There appears to be no evidence, however, for the participation of other reductants in the hydroxylation mechanism *in vivo*. Studies in scorbutic guinea pigs have indicated that hydroxylation at least of peptidyl proline is impaired *in vivo* in ascorbic acid deficiency, and we conclude that this is the primary lesion in collagen synthesis in scurvy. Studies in folic-acid-deficient rats have lent support to the contention that ascorbic acid participates directly in this reaction *in vivo*.[87]

Nevertheless, hydroxylation of collagen proline *in vivo* is only slightly impaired in scorbutic animals. There is no evidence for the occurrence of a substantially underhydroxylated moiety. It is believed that a slight additional reduction beyond an initial 5–10%, which does not appear to impair collagen function, causes a lack of formation of the triple helical structure of collagen, with consequent degradation of the unassociated α-chains. Accumulation of the latter within the cell may also cause a feedback inhibition of further collagen protein synthesis. The continuous absence of accumulation of unhydroxylated material in the tissues of scorbutic guinea pigs is considered attributable to reduced collagen protein synthesis, which is regarded as a secondary feature of impaired hydroxylation.

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DISCUSSION

DR. BARNES: Ascorbate deficiency has no effect on the preformed collagen, that is, the collagen that has been formed before the vitamin deficiency occurred. There is no evidence of increased degradation of this collagen. The amount of soluble collagen is reduced in scorbutic guinea pigs. This fraction of collagen consists of the recently synthesized material and its amount is determined, on the one hand, by the rate of synthesis and, on the other, by the rate of removal from the pool by degradation or conversion to insoluble collagen. The amount is reduced in scurvy because of the lack of synthesis (or retention) of new collagen whilst loss from the pool is occurring as normally. The concentration (but not the amount) of insoluble collagen seems to increase in the scorbutic state, probably because of dehydration of tissues through a reduced intake of water during vitamin deprivation.