THE BIOCHEMICAL ROLE OF ASCORBIC ACID IN CONNECTIVE TISSUE*

Wm. van B. Robertson
College of Medicine, University of Vermont, Burlington, Vt.

Scurvy as described in the 17th and 18th centuries was certainly not due simply to acute vitamin C deficiency but to a multifaceted chronic nutritional deficiency.[1] Nevertheless, even then, abnormalities of the connective tissues were prominent among the lesions described. These included nonhealing ulcers and wounds, falling teeth, bone fractures that did not heal, and fractures and wounds that, years after healing, broke open anew.

During the first half of this century, macro- and micromorphologic studies of scurvy and experimental vitamin C deficiency reinforced the view that the primary defect of scurvy resided in the connective tissues. Aschoff, Holst, Fröhlich, Höjer, and Wolbach are among the names associated with these studies:[2,3] The following conclusions derived from these observations formed the basis for the design of biochemical studies:

(1) The formation and maintenance of normal collagen require ascorbic acid.

(2) A nonfibrous, collagen precursor is formed instead of fibrous collagen during an ascorbic acid deficiency.

(3) Abnormalities of the mucopolysaccharides of ground substance accompany an ascorbic acid deficiency.

(4) The connective tissue lesions of ascorbic acid deficiency are found preeminently in tissues subjected to physical stress.

Maintenance of Collagen

Initial biochemical studies explored the suggestion that ascorbic acid is necessary for the maintenance of established collagen. Contrary to expectation, I found that the concentration of collagen determined chemically in a variety of organs or tissues, including repair tissue, did not decrease in either acute or chronic scurvy.[4,5] Similar data were obtained by other investigators.[6,7] Studies of collagen turnover using isotopes appeared in 1953 also[8] and led to the conclusion that collagen once laid down is metabolically inert. The biochemical evidence appeared to warrant the conclusion that during scurvy collagen was at least as stable as other proteins and that ascorbic acid was not specifically involved in its maintenance. However, it is now clear that although the great bulk of collagen in most tissues is metabolically inert, certain fractions are highly active and that, in some tissues, a large fraction of the collagen is liable to rapid catabolism.[9,10] The disappearance of as much as 75 per cent of newly laid-down collagen from polyvinyl sponge granulomas, when animals were made scorbutic, has recently been reported by Gould.[11] Collagen degradation consequent to ascorbic acid deprivation apparently may occur in collagenous tissues containing an appreciable number of fibroblasts. As will be discussed later, excessive mucopolysaccharides accumulate in these

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cellular tissues during scurvy.[12] The hydrophilic mucopolysaccharides and water inspissate collagen fibers, disaggregating them, thus decreasing the tensile strength of the tissues and probably rendering the collagen more liable to proteolysis.

Two years ago Williams[13] found that carrageenan, a seaweed polysaccharide, when injected intradermally into a normal guinea pig caused disintegration and disappearance of skin collagen, but that collagen was not destroyed to the same extent if the animals were scorbutic. This case of an ascorbic acid requirement for collagen disappearance, inexplicable as it is interesting, confuses the above reasonably satisfactory picture of the relation of ascorbic acid to collagen maintenance.

Synthesis of Collagen

The biochemical demonstration of an ascorbic acid requirement for collagen synthesis, in contrast to maintenance, was readily demonstrable. Any situation such as wounding, granuloma formation, or tumor growth that leads to a rapid and massive formation of new collagen in adequately fed animals provoked the formation of a collagen-poor tissue in guinea pigs deprived of ascorbic acid. I have found the granuloma induced by subcutaneous injection of carrageenan especially satisfactory for these studies since it affords a large amount of tissue within which collagen formation is very sensitive to the availability of ascorbic acid.[14] Although the total mass and protein content of the granuloma that develops in the guinea pig deprived of ascorbic acid is about the same as in the granuloma from the adequately fed animal, the collagen concentration is very much less. Since the "scorbutic" granuloma develops during only a fortnight of vitamin deprivation, it may be obtained in an essentially normal host that is still gaining weight. The hazards of inanition that plague the interpretation of many experiments involving vitamin C deficiency are eliminated. Essentially similar results demonstrating the need for ascorbic acid in collagen synthesis have been obtained by several investigators.[15,16] However, even the universality of this conclusion must be questioned. Gould presents evidence elsewhere in this monograph that some collagen, which he refers to as "growth" collagen, may be synthesized even in the absence of demonstrable ascorbic acid. This finding and the implication of earlier histologic studies that only collagen formation in repair tissue required ascorbic acid prompted a study of the question of whether the need for ascorbic acid is determined by the type of collagen synthesis (repair or nonrepair) or whether the potential rate of collagen synthesis is determining.

The choice could not be ascertained by simply determining the effect of an ascorbic acid deficiency on the collagen concentrations of normally growing organs. These concentrations do not change during ascorbic acid deprivation, because before the vitamin stores are depleted inanition supervenes and growth ceases.[4]

One approach used to study the problem was to measure collagen synthesis in the estrogen-stimulated, involuted uteri of ovariectomized guinea pigs. The average amount of new collagen synthesized in the animals receiving ascorbic acid was 70 mg.; in comparable animals deprived of ascorbic acid it was 9 mg. (unpublished data).

Another study made use of proline-C^{14}. Mammalian collagen contains about 14 per cent hydroxyproline, an amino acid that, while not peculiar to collagen and its derivatives, is seldom found in other proteins.[17] It is commonly used for the identification and quantification of collagen in mammalian tissues. Essentially none of the hydroxyproline in collagen can be derived from free hydroxyproline but must arise from hydroxylation of proline.[18,19] Determination of the specific activity of hydroxyproline, isolated from the crude collagen fraction of a tissue after administration of proline-C^{14}, may be used as a measure of collagen synthesis. This technique was applied to skin, bones, and liver from guinea pigs deprived of ascorbic acid for only 11 days (unpublished data). These animals did not show the morphologic defects of scurvy at this time, yet it is clear that the synthesis of skin collagen and bone collagen was impaired in the ascorbic acid-deficient animals and was restored by administration of ascorbic acid (TABLE 1). A similar effect on collagen synthesis in liver could

TABLE 1

ASCORBIC ACID AND NONREPARATIVE COLLAGEN SYNTHESIS

Guinea pigs	Specific activity of hydroxyproline*		
	Bone	Skin	Liver
Normal (adequate diet for 14 days)	140	86	450
Scorbutic (deficient diet for 14 days)	28	4	560
Recovery (deficient diet for 11 days; then 100 mg. ascorbic acid per day)	150	33	560

* Counts per minute per micromole.

Eleven days after beginning the experiment the guinea pigs were injected I.P. with 8 μc. proline-C^{14}. This was repeated at 12-hour intervals until 48 μc. had been injected. Twelve hours after the last dose, the animals were killed and hydroxyproline was isolated from the collagen fraction of each tissue.[27] The recovery guinea pigs received ascorbic acid only during the period of isotope administration.

be demonstrated only in severe vitamin deficiency. It would appear that ascorbic acid is required for maximal collagen synthesis in a variety of tissues and not merely in repair tissue.

This relation of ascorbic acid to maximal collagen synthesis is clearly seen in the carrageenan granuloma, where the potential rate of synthesis is high (FIGURE 1). Although some collagen has been synthesized even in those granulomas with very low ascorbic acid concentrations, the maximum synthesis was not reached until the tissue contained 40 to 50 μg. of ascorbic acid per gram (unpublished data).

Action of Ascorbic Acid in Collagen Synthesis

The elucidation of the biochemical roles of ascorbic acid in collagen synthesis requires a knowledge of the site of action of the vitamin. Does it affect synthesis in the connective tissue only indirectly, by influencing the synthesis or release of a hormone, or by maintaining intact the capillary blood supply; or does ascorbic acid act directly in the collagen-synthesizing tissue?

Gould[20] adduced evidence for a local action of ascorbic acid when he found

that following an injection of very small amounts of ascorbic acid into one of a pair of sponge granulomas in scorbutic guinea pigs, the hydroxyproline content of the injected sponge was several-fold higher than that of the contralateral sponge.

I have been able to show that *in vitro* addition of ascorbic acid to suspensions of scorbutic carrageenan granuloma incubated with proline-C^{14} increased the specific activity of the isolated collagen hydroxyproline, whereas this was not the case in granulomas from animals that had received ascorbic acid (unpublished data).

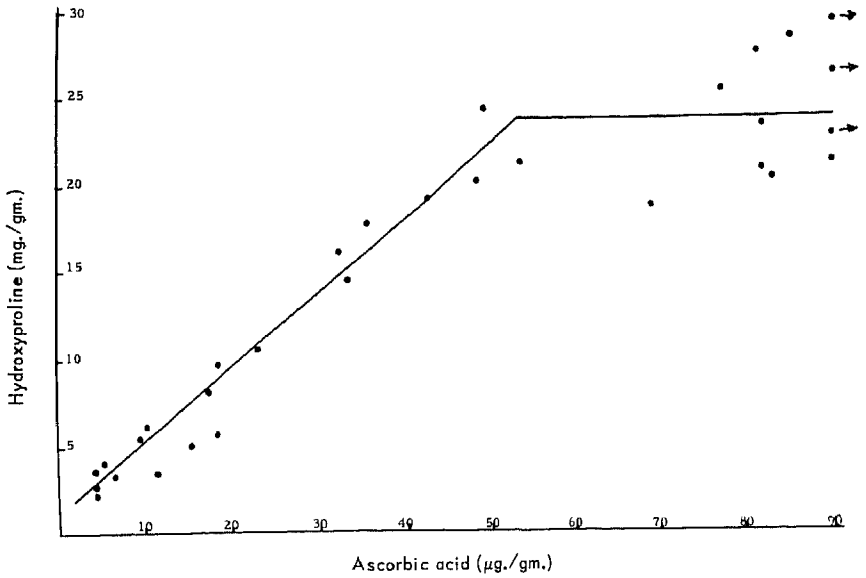

FIGURE 1. The relation of collagen hydroxyproline to ascorbic acid concentration in carrageenan granulomas. Ascorbic acid and collagen hydroxyproline were determined on aliquots of 14-day carrageenan granulomas from guinea pigs that had been given different (0 to 25 mg. per day) but constant ascorbic acid intakes for at least 7 days.

A local action of ascorbic acid on the collagen-forming tissue having been demonstrated, the series of reactions involved in collagen synthesis must be considered in order to specify more explicitly the action of the vitamin. Present evidence indicates that the basic collagen protein, tropocollagen, an asymmetric molecule with a molecular weight of 320,000, is produced intracellularly but aggregates extracellularly, first to fibrils and then to fibers.[21] Tropocollagen is metabolically very active and is soluble in cold neutral salt solutions, but as fibers are formed it becomes metabolically less active and less soluble. All mammalian collagens contain the amino acids hydroxyproline and hydroxylysine. Collagen synthesis requires two reactions in addition to those common to protein synthesis, such as amino acid activation and peptide bond formation. These are hydroxyproline and hydroxylysine synthesis and fiber formation (FIGURE 2).

Fiber formation from the basic collagen molecule takes place *in vitro* non-

enzymatically at body temperature. While many compounds can effect the rate of this reaction, ascorbic acid seems to have no peculiar or profound effect.[22] Jackson and Bentley (personal communication) have studied this conversion in animals with isotopes and have concluded that the rate of conversion of soluble collagen to fibers is unaffected by an ascorbic acid deficiency. The decreased concentration of soluble collagen in the skin of scorbutic guinea pigs[23] also suggests that the defect of scurvy precedes fiber formation.

Ascorbic acid deficiency, if divorced from the effects of inanition, does not appear to affect activation of amino acids or synthesis of proteins other than collagen. Thus the specific activity of proline in noncollagen proteins after administration of proline-C^{14} is not decreased by vitamin C deprivation (TABLE 2). Similar results have been obtained[24] with glycine-N^{15}. Mitoma[25] has also shown that the synthesis of an adaptive enzyme is unimpaired in the scorbutic guinea pig. These results, of course, should not be construed to preclude the

TABLE 2

ASCORBIC ACID AND THE SYNTHESIS OF NONCOLLAGEN PROTEINS

Guinea pigs	Specific activity of proline*			
	Serum	Bone	Skin	Liver
Normal	2500	1600	1500	2000
Scorbutic	3700	1700	1500	2500
Recovery	3400	1700	1500	3000

* Counts per minute per micromole.

The same guinea pigs were used as in the experiments reported in TABLE 1. After extraction of the collagen with hot 5.5 per cent trichloroacetic acid, proline was isolated from the hydrolyzates of the residual protein.

possibility that ascorbic acid may affect only synthesis of peptide chains in those cells responsible for collagen synthesis.

The *in vivo* conversion of proline to hydroxyproline is intimately connected with collagen synthesis and since proline, but not free hydroxyproline, is incorporated into the collagen molecule, Stetten[18] suggested that proline was hydroxylated to hydroxyproline only after being built into a peptide bond. Some of our results,[14] as well as those of Gould and Woessner,[15] were in apparent agreement with this hypothesis. It was suggested that during an ascorbic acid deficiency the hydroxylation of proline was blocked and a protein accumulated that in some ways resembled collagen but contained no hydroxyproline. Such a protein might correspond to the nonfibrous collagen precursor described by Wolbach and Howe.[26] To test whether, during a deficiency, a protein accumulated that was changed to collagen by administration of ascorbic acid, we administered proline-C^{14} to guinea pigs bearing scorbutic carrageenan granulomas.[27] Large doses of ascorbic acid were given concurrently with the labeled proline to some of the guinea pigs. If there were an accumulation of a proline-rich, hydroxyproline-poor collagen precursor in scorbutic granuloma, the proline would have been built into the precursor before labeled proline was given. We should then have expected the specific activity of the collagen hydroxy-

proline to be low in the scorbutic animals receiving ascorbic acid and proline-C^{14}; however, the specific activity of the hydroxyproline in these recovery animals was even higher than in normals. Specific activity and collagen synthesis were lowest in unsupplemented scorbutic animals. Last year Gould[28] discussed in detail the precursor problem and concluded that although the stage is set for collagen synthesis in scorbutic tissue and collagen rapidly replaces other proteins once ascorbic acid is added, the data indicate that this process does not involve the conversion of an accumulated collagen precursor to collagen.

Not only is there no evidence for the build-up of a collagen precursor in scurvy, but studies of normal collagen synthesis in tissue culture[29] and in granuloma slices[19] have failed to yield evidence for even the transient existence of a protein precursor. Instead it appears that hydroxyproline is derived from proline before the latter is built into a protein molecule.

In order to see whether ascorbic acid was specifically involved in the hydroxylation of proline, we examined the specific activity of nonprotein hydroxyproline,[27] (that is, hydroxyproline soluble in cold trichloroacetic acid) from granulomas of normal and scorbutic guinea pigs after administration of proline-C^{14}. The lower total activity and the specific activity of hydroxyproline from scorbutic granulomas suggested that a primary defect of hydroxylation exists in scorbutic granulomas. However, in so far as some collagen might have been dissolved by the trichloroacetic acid, these results are inconclusive. Mitoma[25] carried out similar experiments using the specific activity of urinary hydroxyproline as a measure of hydroxylation of proline. He obtained no evidence for a specific impairment of hydroxylation of proline during scurvy. More data is needed to settle this important point. The only other evidence bearing on this hydroxylation is in a purely chemical system. Chvapil and Hurych[30] reported that ethylenediaminetetraacetic acid (EDTA), ferrous sulfate, hydrogen peroxide, and ascorbic acid hydroxylated proline to hydroxyproline. We confirmed this by using proline-C^{14}. Allen Price, working in our laboratory, found that lysine was hydroxylated to hydroxylysine by a similar system (unpublished data).

In the scheme of collagen synthesis (FIGURE 2) I have indicated the proline that is hydroxylated and incorporated into the collagen molecule by the noncommittal designation "active." Possible loci of ascorbic acid activity are indicated; all have been discussed except the transfer of tropocollagen out of the cell, for which no data are available.

Ascorbic Acid and Mucopolysaccharides

In addition to affecting collagen metabolism, ascorbic acid deficiency results in changes of the mucopolysaccharides of the ground substance of connective tissue. Histologic descriptions of these changes are conflicting, having been described as a decrease in mucopolysaccharides,[31] an increase in mucopolysaccharides,[32,33] or as a depolymerization of mucopolysaccharides.[34] Two biochemical changes have been reported: one, an accumulation of hyaluronic acid in scorbutic repair tissue, and the other, a decreased incorporation of sulfate into mucopolysaccharides.

A comparison of the normal and scorbutic carrageenan granuloma showed

that there is about five times as much hexosamine-and-uronic-acid–containing mucopolysaccharide in the scorbutic granuloma as in the normal. Isolation of this material from scorbutic granulomas indicated that it is essentially all hyaluronic acid.[12] Similar results have been reported in healing wounds.[16]

A decreased incorporation of sulfate into the mucopolysaccharides of healing tendons in scorbutic guinea pigs was reported by Kodicek and Loewi,[35] while a decreased incorporation of sulfate into the chondroitin sulfate of scorbutic costal cartilage was reported by Reddi and Norström.[36] Friberg[37] reinvestigated this

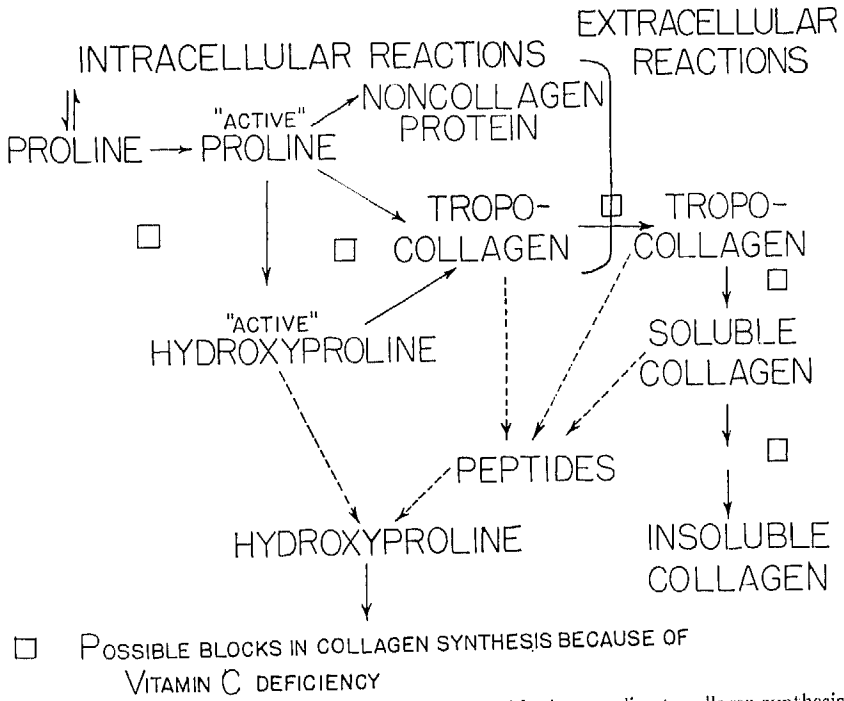

FIGURE 2. Abbreviated scheme relating proline and hydroxyproline to collagen synthesis. Possible sites of ascorbic acid activity are discussed in the text.

problem using paired feeding techniques and showed that ascorbic acid deficiency had no effect on sulfate incorporation into costal cartilage beyond that of inanition. Peyser (personal communication) has studied the effects of acute and chronic scurvy on sulfate in skin. He found no differences in content, uptake, or removal that could be ascribed to ascorbic acid. Hughes and Kodicek[38] found the concentration of galactosamine-containing polysaccharides was much lower in scorbutic than in normal granulomas from pair-fed animals. The significance of this finding is that the galactosamine-containing polysaccharides also contain sulfate and that the study was of a rapidly growing tissue. The possible effect of ascorbic acid deficiency on sulfate metabolism in connective tissue must, I think, still be considered an open question.

Nature of Ascorbic Acid Activity

It is clear from the above that although the biochemical changes brought about by an ascorbic acid deficiency have been characterized to some extent, we are still not able to define how ascorbic acid is involved in chemical reactions in the connective cells. In a purely speculative vein I suggest that the activity of ascorbic acid in connective tissue depends on the formation of monodehydroascorbyl and hydroxyl radicals. The existence of free radicals during either the autoxidation or the enzymatic oxidation of ascorbic acid has been postulated several times, with the most conclusive evidence being based on electron paramagnetic resonance studies.[39] The hydroxylation of proline to hydroxyproline by a system containing ascorbic acid and hydrogen peroxide and presumably involving a free radical mechanism[40] has been referred to earlier. If this model hydroxylation had its counterpart in cells and was a rate-limiting reaction, we could expect collagen synthesis to be decreased during an ascorbic acid deficiency. About 20 years ago we showed that ascorbic acid and hydrogen peroxide depolymerized hyaluronic acid,[41] making it more diffusible. A lack of ascorbic acid in tissues might then be expected to permit accumulation of hyaluronic acid by keeping it polymerized and nondiffusible. Since free hydroxyl radical formation *in vivo* would not necessarily involve ascorbic acid, some collagen synthesis might be expected to take place in the absence of ascorbic acid. However, massive or rapid collagen synthesis would require increasing amounts of ascorbic acid. This describes the observed relation of ascorbic acid to collagen synthesis. If the free radical-forming enediol moiety of ascorbic acid determined acitivity, and if the rest of the molecule determined tissue affinity, vitamin C activity should not require absolute structural specificity. Analogues of L-ascorbic acid, as has been shown, are able to replace the vitamin in preventing scurvy if their concentration in tissues is maintained.[42] Free radicals are notoriously nonspecific in their attack. The ascorbic acid literature and the papers presented in this monograph attest to the variety of processes in which ascorbic acid may play a permissive if not essential or specific role. Although there is no direct evidence for the free radical behavior of ascorbic acid in connective tissue, the hypothesis pulls together several observations, suggests further experiments, and is eventually susceptible to test.

Summary

The present status of biochemical investigations of the functions of ascorbic acid in connective tissue has been discussed. Maintenance of preformed collagen does not generally require ascorbic acid, but a deficiency of the vitamin may result in loss of collagen from tissues that still contain a large number of fibrocytes.

Small amounts of collagen may apparently be synthesized in the absence of demonstrable ascorbic acid, but the rapid synthesis of large amounts of collagen requires ascorbic acid. Ascorbic acid acts at the cellular level to increase collagen synthesis.

There is no evidence for the accumulation of a nonfibrous, hydroxyproline-poor, protein precursor of collagen when ascorbic acid is lacking. Present evidence, although far from conclusive, suggests that ascorbic acid may be

concerned with the conversion of proline to hydroxyproline before synthesis of the peptide chain.

Lack of ascorbic acid leads to accumulation of hyaluronic acid in repair tissue; the evidence for an effect of ascorbic acid on the metabolism of sulfated mucopolysaccharides is equivocal.

It is suggested that the function of ascorbic acid in connective tissue involves free radical activity.

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