

ASCORBIC ACID AND THE FORMATION OF COLLAGEN*

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The importance of ascorbic acid for the metabolism of connective tissue substances, especially collagen, has been established by numerous investigators, using morphologic criteria (1). However, there have been few biochemical studies of this function of ascorbic acid with the exception of the recent analyses of Elster (2) and Robertson (3), which demonstrated that the maintenance of preformed collagen did not require ascorbic acid. It would appear that a biochemical rôle of ascorbic acid in connective tissue metabolism must be sought in the formation of collagen.

Collagen occurs in all connective tissues of the body, but the amount of new collagen formed in a normal tissue within a given period is rather small and this cannot be separated from older collagen. However, the fibrous tissues formed in wound repair or in response to the presence of a foreign body are sites of rapid collagen formation; these have been used in morphologic investigations of ascorbic acid function (4, 5). The amount of new collagen produced in a healing wound in a short period is insufficient for most biochemical studies, but relatively large quantities of collagen-containing fibrous tissue form rapidly following subcutaneous injection of a dispersible foreign body.

The data presented in this paper describe the chronologic development of collagen in such a tissue and demonstrate that the formation, in contrast to the maintenance, of collagen requires ascorbic acid. Experiments are described which suggest that a collagen precursor with a low hydroxyproline content accumulates during formation of new connective tissue in an ascorbic acid deficiency.

EXPERIMENTAL

Irish moss (*Chondrus crispus*) extract¹ proved to be the most satisfactory stimulus for fibrous tissue formation of several materials tested. It was

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prepared for injection by dispersing 1 gm. in a Waring blender together with 1 gm. of microcrystalline sulfadiazine and 100 ml. of physiological saline at 98°. 10 to 12 ml. of this colloidal solution at about 30–38° were injected subcutaneously in the lower abdominal regions of 250 to 350 gm. male guinea pigs anesthetized with ether. The even spread of the colloid was aided by insufflating 20 ml. of air between skin and underlying tissue immediately preceding injection.

A Macdonald No. 5 scorbutogenic diet plus vitamins A, D, and E (6) was used throughout, except in one experiment in which a commercial guinea pig diet was fed. When a vitamin C-adequate ration was desired, the Macdonald diet was supplemented every other day with 50 mg. of ascorbic acid.

Animals were killed by decapitation, the skin was peeled back from the ventral surface, and the mass of new fibrous tissue was separated from the abdominal and chest walls by blunt dissection. Pockets of blood as well as discrete areas of necrosis were discarded. Portions of the tissue, a tooth, and a costochondral junction were fixed for histologic examination.² The rest of the separated tissue was minced and aliquots were used for the several analyses.

Collagen was determined by a previously reported modification (3) of the method of Lowry *et al.* (7). This method depends upon the characteristic insolubility of collagen in dilute alkali, which dissolves most other tissue proteins, and its ready solubility as gelatin after autoclaving in distilled water. *These properties are characteristic of formed collagen fibers found in animal tissues and define the term collagen as used in this report.*

Development of New Fibrous Tissue in Normal Guinea Pigs—Immediately after injection, the Irish moss could be seen as a large, well delimited bleb, extending from the axillary to the inguinal region on one side. Within 24 hours the Irish moss spread out and the water was absorbed, so that the area was barely palpable. During the next few days a fluctuant swelling was evident over the dependent portions of the abdomen, predominantly on the injected side. 5 days after injection the exposed mass appeared as a gel, which oozed a slightly viscid fluid. It was supported between thin fascial layers of connective tissue. On succeeding days, the tissue seemed to contract and become firm. When removed at 10 days, the tissue separated easily from the skin and abdominal wall. It was pale pink, very soft, and had a mucoidal appearance. At 14 days the tissue had the appearance of a fairly dense fibrous tissue but contained occasional collections of blood, some muscle, and small areas of necrotic material. About 10 gm. of relatively homogeneous tissue could be easily separated from the skin and belly wall of one guinea pig.

² It is a pleasure to thank Mr. John Boldosser of the Department of Pathology and Oncology for the histologic preparations.

During the 3rd week, no obvious changes in the tissue mass occurred. Thereafter resorption took place slowly and, after 6 weeks, only small amounts of firm fibrous tissue remained. From Table I, which presents the collagen concentration and water content of the tissue at several periods during its development, it is clear that the major portion of the collagen was laid down between the 10th and the 14th day.

Response to Irish Moss Injection in Ascorbic Acid-Deficient Animals—When guinea pigs which had been maintained on a completely adequate diet were injected with the Irish moss extract and ascorbic acid was then withheld, a collagen-poor tissue developed. The original course of events appeared to be the same as in animals maintained on ascorbic acid, except that the large soft mass which appeared during the 1st week did not sub-

TABLE I
Concentration of Collagen and Water in Tissue Developing after Subcutaneous Injection of Irish Moss Extract

The values are given in gm. per 100 gm. of dry, fat-free tissue.

No. of days*	No. of guinea pigs	Collagen	Water
5	4	4.3 ± 0.24†	979 ± 22.3
10	7	4.8 ± 0.42	805 ± 27.3
14	16	11.6 ± 0.64	660 ± 12.1
21	7	13.1 ± 0.74	620 ± 33.2
46	8	14.5 ± 1.14	601 ± 25.2

* After subcutaneous injection of a 1 per cent suspension of Irish moss extract.

† The data are expressed as the mean ± its standard deviation.

sequently decrease in size. At the end of 14 days it was possible to remove a large mass (10 to 20 gm.) of soft granular tissue, which exuded a pale pink, viscous fluid. Necrotic areas and blood-filled cystic spaces were common. The collagen concentration was low (Table II).

In order to ascertain whether a vitamin deficiency already present at the time of injection had a more profound effect on the fibrous tissue development, guinea pigs were deprived of ascorbic acid for 11 days prior to injection of the Irish moss extract as well as during the 14 days of tissue development. The gross appearance of the tissue and its development were similar to that described above for guinea pigs from which the vitamin had been withheld only after injection. The collagen concentration was slightly higher (Table II), probably because other tissue proteins were mobilized more readily during the accompanying inanition (8).

It should be noted that this defect in collagen formation was manifest during the first 14 days of the scorbutigenic régime. At this time the guinea pigs showed essentially no gross or microscopic signs of scurvy.

Apparently this developing collagenous tissue mass has a larger need for ascorbic acid than can be furnished by the stored vitamin of the animals. The magnitude of this requirement was emphasized when one group of animals was inadvertently fed a commercial guinea pig ration which contained enough ascorbic acid for normal growth and development.³ The fibrous tissue removed from these animals after 14 days was soft and had a collagen concentration which was lower than that from ascorbic acid-supplemented animals, although higher than that of the guinea pigs which had received no ascorbic acid (Table II).

TABLE II

Effect of Ascorbic Acid Deficiency on Collagen Formation in Tissue Developing after Subcutaneous Injection of Irish Moss Extract

The values are given in gm. per 100 gm. of dry, fat-free tissue.

	No. of guinea pigs	Collagen	Water
Adequate ascorbic acid*.....	16	11.6 ± 0.64†	660 ± 12.1
No ascorbic acid following injection of Irish moss‡.....	25	2.3 ± 0.14	850 ± 17.3
No ascorbic acid for 11 days preceding injection and none after injection of Irish moss.....	11	2.9 ± 0.28	796 ± 6.8
Commercial guinea pig ration (about 4 mg. ascorbic acid per day).....	8	7.8 ± 0.95	735 ± 15.6

All the tissues were taken 14 days after subcutaneous injection of a 1 per cent suspension of Irish moss extract.

* 50 mg. of ascorbic acid were fed every other day.

† The data are expressed as the mean ± its standard deviation.

‡ The guinea pigs received 50 mg. of ascorbic acid every other day until injection of Irish moss.

Response of Collagen-Poor Tissue to Ascorbic Acid—The dependence of collagen formation on ascorbic acid was vividly illustrated when the vitamin was given to guinea pigs from which it had been withheld and in which a collagen-poor tissue had developed. On the 14th day following Irish moss injection, 100 mg. of sodium ascorbate were injected intraperitoneally and on each succeeding day 50 mg. were administered *per os*. As may

³ Dr. M. P. Lamden very kindly assayed several batches of this feed after varying periods of storage. Analyses by both the method of Roe and Oesterling (9) and the method of Robinson and Stotz (10) yielded ascorbic acid concentrations of 25 to 30 mg. per 100 gm. Since the guinea pigs ingested 15 to 20 gm. of diet daily, they received a minimum of 4 mg. of ascorbic acid per day or 2 times the "optimal" daily requirement (11).

be seen in Table III, the collagen concentration of the tissue increased rapidly and on the 3rd day was approximately the same as in adequately maintained animals. This change was reflected in the appearance of the tissue; after 3 days it looked like that which had developed in the adequately fed animal.

Collagen Analyses by Different Methods—In order to test the possibility that tissue which developed during the ascorbic acid deficiency contained an alkali-soluble precursor of collagen, two different "collagen" determinations, based on properties of the protein other than its insolubility in dilute alkali, were carried out; the method of Spencer, Morgulis, and Wilder

TABLE III

Formation of Collagen Following Ascorbic Acid Administration

The values are given in gm. per 100 gm. of dry, fat-free tissue.

Diet after 14 days deficiency	No. of guinea pigs	Collagen	Water
No ascorbic acid following injection of Irish moss	25	2.3 ± 0.14*	850 ± 17.3
Ascorbic acid supplement† for 1 day	4	4.3 ± 0.19	728 ± 7.4
“ “ “ “ 2 days	4	8.0 ± 0.36	627 ± 9.2
“ “ “ “ 3 “	4	11.5 ± 0.87	612 ± 27.8
“ “ “ “ 4 “	17	10.8 ± 0.61	630 ± 10.9
“ “ “ “ 5 “	5	10.7 ± 0.50	620 ± 14.2

All the guinea pigs were fed 50 mg. of ascorbic acid every other day until injected subcutaneously with a 1 per cent suspension of Irish moss extract; no ascorbic acid was given for the next 14 days.

* The data are presented as the mean ± its standard deviation.

† The supplement consisted of 100 mg. of sodium ascorbate intraperitoneally on the 1st day and 50 mg. of ascorbic acid orally on succeeding days.

(12) which depends upon the precipitability by tannic acid of the gelatin, obtained by hydrolysis of collagen, after removal of other proteins by heat coagulation, and the method of Neuman and Logan (13) which is based on the high content (13.5 per cent) of hydroxyproline in collagen and its rare occurrence in other proteins. The tannic acid-precipitable material would include collagen plus gelatin-like precursors and the hydroxyproline would be derived from collagen plus hydroxyproline-containing precursors.

Aqueous suspensions of fresh tissue or of powdered acetone-dried tissue were autoclaved at 50 pounds pressure for 5 hours. After cooling, these were centrifuged sharply. Tannic acid-precipitable nitrogen (8) and hydroxyproline (14) were determined on aliquots of the clear supernatant solution. Table IV presents the results of these analyses and a comparison with collagen concentrations obtained by the alkaline extraction pro-

cedure of Lowry *et al.* (7) on the tissue mass from guinea pigs (1) which received ascorbic acid, (2) which were deprived of ascorbic acid following injection of the Irish moss, and (3) which received ascorbic acid only subsequent to formation of the collagen-poor tissue. The concentrations based on tannic acid-precipitable nitrogen were the same in all three groups, whereas the hydroxyproline values paralleled the collagen values.

TABLE IV

Comparative Assay of "Collagen" by Various Methods

The values are given in gm. per 100 gm. of dry, fat-free tissue.

	No. of guinea pigs	Method of Lowry <i>et al.</i> (7)	Method of Spencer <i>et al.</i> (12)	Method of Neuman and Logan (14)
Adequate ascorbic acid	7	10.7 ± 0.65	18.6 ± 1.38*	
	7	11.6 ± 0.57		16.5 ± 0.62†
Deficient for 14 days (no ascorbic acid following injection of Irish moss)	10	2.5 ± 0.30	16.1 ± 0.89	
	8	2.7 ± 0.13		3.3 ± 0.97
Deficient for 14 days; then ascorbic acid supplement for 4 days	7	9.0 ± 0.95	18.6 ± 1.67	
	7	10.6 ± 1.26		13.9 ± 0.93

* Tannic acid-precipitable nitrogen ÷ 0.186.

† Hydroxyproline (not corrected for tyrosine) ÷ 0.135.

DISCUSSION

The analytical results presented above clearly confirm numerous histologic studies which have demonstrated the necessity of ascorbic acid for collagen formation. Considerably more collagen formed in animals receiving adequate ascorbic acid than in those deprived of the vitamin. This is true whether one expresses the data on a fresh or dry weight basis or on a total collagen basis. Indeed it is our impression from histologic examination of the tissue that most of the collagen present in the new tissue mass from guinea pigs deprived of ascorbic acid was preexisting fascial collagen. This impression is strengthened by the observation that similar concentrations of collagen were found in the tissue from adequately maintained animals 5 and 10 days after injection of the Irish moss (Table I) and from animals rendered partially scorbutic before injection (Table II).

Previous work had indicated that collagen formation, as in wound repair, increases the need for ascorbic acid (15). Impaired collagen development in new tissue of animals eating a commercial guinea pig ration, as reported above, emphasizes the increased nutritional vitamin C requirement of the organism during massive collagen formation. The occurrence of this impaired collagen formation during the first 14 days of a scorbutogenic régime demonstrates the inadequacy of stored ascorbic acid to meet this demand.

What is the specific rôle of ascorbic acid in collagen formation? Some 25 years ago Wolbach and Howe (16), on the basis of their studies in wound healing, suggested that vitamin C was necessary for the gelation of an intercellular fluid matrix secreted by the fibroblasts. In contemporary terminology we might say that ascorbic acid was necessary for the formation of collagen fibers from a precollagen. Although Wolbach and Howe stressed the intercellular aspect of this phenomenon, they pointed out that the fibroblasts might play a part in the gelation as well as in the secretion of the intercellular material. Their hypothesis would be strengthened by the chemical demonstration of an accumulation of precollagen in connective tissue developing during ascorbic acid deficiency.

The attempts made in the present study to demonstrate a precollagen led to equivocal results. The similar concentrations of "collagen," as determined by tannic acid precipitation, in the tissue from normal, deficient, and recovered guinea pigs suggest the presence of a collagen precursor. On the other hand, the observation that the hydroxyproline concentration was lower in the tissue from deficient animals and was parallel to the collagen fiber concentration might be taken as evidence for the absence of a precollagen. Although the explanation for these discordant results is not presently available, two possibilities must be considered, (1) that the tannic acid method of Spencer *et al.* (12) is not sufficiently specific for collagen-like compounds or (2) that precollagen has a low hydroxyproline content (*cf.* (17-19)). Should the latter prove true, it would suggest that the function of ascorbic acid in collagen fiber formation is related either to the synthesis of hydroxyproline or to its introduction in the collagen macromolecule.

SUMMARY

A method is described for inducing, within a short period, the development of a tissue containing relatively large amounts of collagen.

Ascorbic acid was needed for collagen formation in this tissue.

The rapid formation of a large collagen-containing tissue increased the nutritional ascorbic acid requirement of guinea pigs.

Attempts were made to demonstrate a precollagen in the tissue from ascorbic acid-deficient guinea pigs. The results were not unequivocal but suggest that, if a precollagen is present, it must have a low hydroxyproline content.

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