

## **Regulation of Collagen Biosynthesis by Ascorbic Acid: A Review**

SHELDON R. PINNELL, M.D.

*Division of Dermatology, Department of Medicine, Duke University Medical Center,  
Durham, North Carolina*

Received November 12, 1985

---

L-ascorbic acid is an essential cofactor for lysyl hydroxylase and prolyl hydroxylase, enzymes essential for collagen biosynthesis. In addition, L-ascorbic acid preferentially stimulates collagen synthesis in a manner which appears unrelated to the effect of L-ascorbic acid on hydroxylation reactions. This reaction is stereospecific and unrelated to intracellular degradation of collagen. The effect apparently occurs at a transcriptional or translational level, since L-ascorbic acid preferentially stimulates collagen-specific mRNA. In addition, it stimulates lysyl hydroxylase activity but inhibits prolyl hydroxylase activity in human skin fibroblasts in culture.

---

The importance of ascorbic acid in the metabolism of connective tissues was realized as early as the sixteenth century. The explorer Jacques Cartier recognized that dietary intake, particularly of citrus fruits, was essential in preventing scurvy and treating those suffering from the disease. A dramatic example of the effect of ascorbic acid on connective tissue biosynthesis was presented by Jeffries and Martin who showed the marked growth of embryonic chick bones grown in tissue culture media supplemented with ascorbic acid when compared to similar bones grown in media without ascorbic acid [1]. When it became known that ascorbic acid was an essential cofactor in the hydroxylation of proline and lysine to form hydroxyproline and hydroxylysine, amino acids critical to the function of collagen, a reasonable explanation for the essential nature of ascorbic acid in collagen biosynthesis seemed apparent. Hydroxyproline is necessary for collagen helix formation, and in its absence collagen is unable to be properly secreted from fibroblasts. Hydroxylysine is essential in collagen cross-link formation, and in its absence the collagen would be structurally unstable. In this review we detail our own work, which demonstrates that ascorbic acid is a fundamental signal for collagen production which appears to be independent of its cofactor function for hydroxylation of proline and lysine.

### **THE EFFECT OF ASCORBIC ACID ON COLLAGEN PRODUCTION IN HUMAN SKIN FIBROBLASTS IN CULTURE**

The test system for these studies was normal human skin fibroblasts in culture. Under the conditions of these experiments, the cells devote 85 percent of their collagen synthesis to type I collagen and 15 percent to type III collagen. Small amounts of type IV and type V collagen are also produced. In regulation experiments carried out in cell culture, it is desirable to define all media components. In the case of human skin fibroblasts, however, it is necessary to retain small amounts of serum, without which

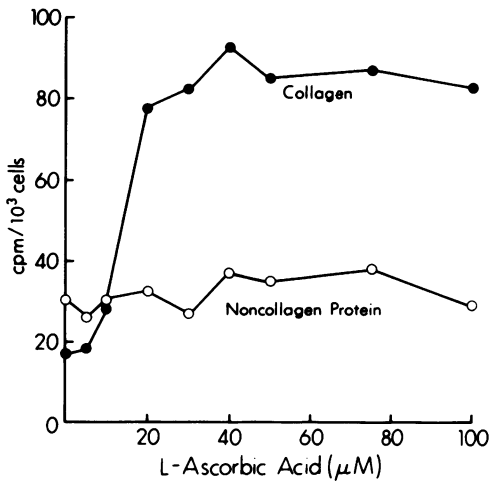


FIG. 1. Effect of L-ascorbic acid on collagen synthesis. Human skin fibroblasts were grown to confluent density and growth arrested in media containing 0.5 percent dialyzed calf serum. Media containing L-ascorbic acid at differing concentrations was added fresh daily for three days. Cultures were labeled during the final six hours with L-[2,3- $^3\text{H}$ ] proline [20  $\mu\text{Ci}/\text{ml}$ ). The radioactivity incorporated into total collagen and noncollagen proteins was determined following digestion with clostridial collagenase.

the cells eventually become incapable of maintaining their population. In the presence of 0.5 percent dialyzed calf serum, it is possible to maintain cellular density for one week, a period of time in excess of the duration of these experiments. In the standard experimental design, normal human skin fibroblasts were plated at subconfluent density and grown to confluent density in cell culture media containing 20 percent dialyzed calf serum. At confluence, the cells were changed to media containing 0.5 percent dialyzed calf serum and subsequently studied under these conditions in the presence or absence of ascorbic acid. During the last six hours of the study, the cells were pulse labeled with radioactive proline, and collagen and noncollagen protein syntheses were determined following digestion of cellular and media proteins by highly purified clostridial collagenase under conditions in which there is no detectable nonspecific proteolytic activity [2]. In Fig. 1 is shown a dose-response curve for L-ascorbic acid. The cells were stimulated with ascorbic acid for 72 hours; the media and ascorbic acid were prepared fresh daily. Collagen synthesis was markedly stimulated at ascorbate levels greater than 20  $\mu\text{M}$ . In this experiment noncollagen protein synthesis was unchanged. Occasionally noncollagen protein synthesis is slightly stimulated, but usually this effect is minimal. The time course of this reaction is seen in Fig. 2. Stimulation of collagen synthesis continued for three to four days until it reached a plateau. After one corrects proline incorporation for its relative enrichment in collagen, relative collagen synthesis increased in this experiment from 9 percent to more than 30 percent of total protein synthesis. If one measures the intracellular proline pool following ascorbic acid stimulation, no differences were detected between stimulated and control cells [3]. If the length of the radioactive proline pulse was varied between two and six hours, identical results were seen. If one studies the effect of cellular density on the ascorbate effect, no difference was demonstrated between subconfluent and confluent cells [3]. Finally, if one studies the ascorbate effect on collagen biosynthesis at serum concentrations ranging from 0.5 percent to 20 percent, differential stimulation of collagen synthesis by ascorbic acid was found at all serum levels, demonstrating that the effect is not related to relative serum starvation of the cells [4].

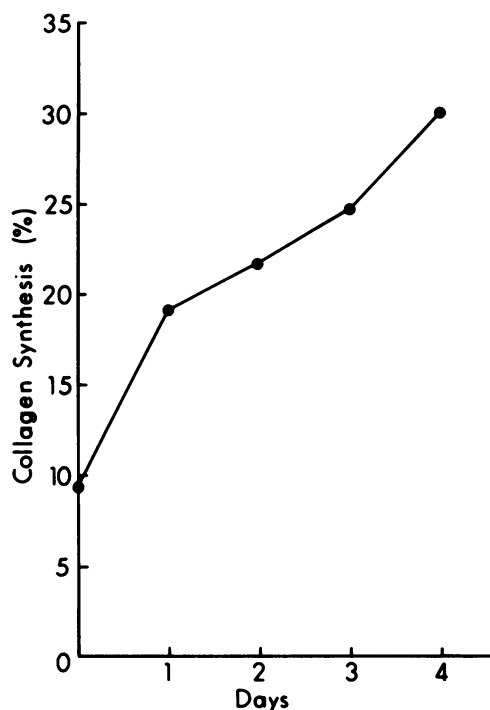


FIG. 2. Time course of L-ascorbate on relative collagen synthesis. Conditions were similar to those described for Fig. 1 except the cells were exposed to L-ascorbate (100  $\mu$ M) for varying times. Collagen synthesis is expressed as percentage of total protein synthesis taking into account the increased proline content of collagen.

#### EFFECT OF ASCORBIC ACID ON HYDROXYPROLINE AND HYDROXYLYSINE CONTENT IN NEWLY SYNTHESIZED COLLAGEN

In an attempt to understand the relationship of ascorbate stimulation to hydroxylation reactions in collagen, we labeled the cells with radioactive proline and lysine in the absence and presence of ascorbic acid [5]. The levels of hydroxyproline and hydroxylysine were determined in small collagen peptides following digestion with clostridial collagenase (refer to Table 1). Hydroxylation of lysine was unchanged whether or not ascorbate was present. This relative insensitivity to ascorbate deprivation may be a result of the high affinity of ascorbic acid for lysyl hydroxylase or may

TABLE 1  
Effect of Ascorbic Acid on Relative Hydroxylation of Proline and Lysine in Soluble Extracellular Collagen

Treatment	% Prolyl Hydroxylation <sup>a</sup>	% Lysyl Hydroxylation <sup>b</sup>
None	34	26
L-ascorbate, 24 hours	47	25
L-ascorbate, 96 hours	46	28

<sup>a</sup>Determined as cpm in proline and hydroxyproline isolated on the amino acid analyzer in material digested by clostridial collagenase

<sup>b</sup>Determined as cpm in lysine and hydroxylysine isolated on the amino acid analyzer in material digested by clostridial collagenase

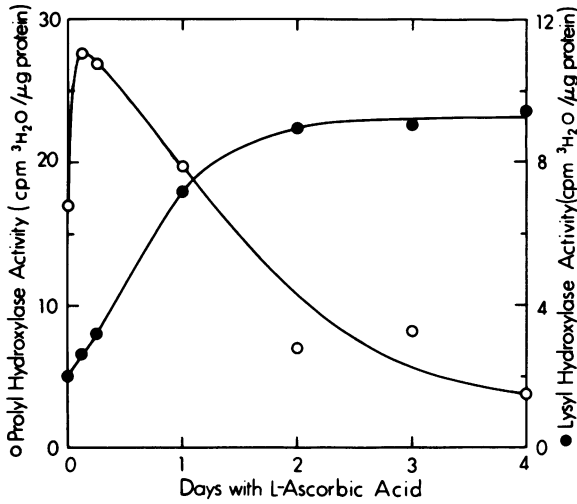


FIG. 3. Time course of L-ascorbate on activities of prolyl and lysyl hydroxylase. Confluent human skin fibroblasts were incubated for four days in media containing 0.5 percent dialyzed calf serum; media was changed daily. Fresh L-ascorbic acid (125  $\mu$ M) was added daily for varying lengths of time before harvest. Cells were harvested following trypsinization, and activities of the enzymes were determined by tritium release assays.

reflect an alternative reducing capacity in the cell. In the absence of ascorbate, hydroxylation of proline was deficient as expected. Hydroxylation of proline was identical whether ascorbic acid had been present 24 or 96 hours. Relative stimulation of collagen by ascorbic acid, however, was continuing over the same time interval. The data suggest a discordance between the ascorbate effect on collagen synthesis and hydroxylation of proline and lysine in the collagen being synthesized in this cell system.

#### EFFECT OF ASCORBIC ACID ON ACTIVITIES OF PROLYL AND LYSYL HYDROXYLASES

The effect of ascorbic acid on levels of prolyl and lysyl hydroxylases was studied in human skin fibroblasts [6]. As shown in Fig. 3, lysyl hydroxylase levels increased for 48 hours but were unchanged with longer intervals of stimulation. Quite surprisingly, levels of prolyl hydroxylase fell markedly over this time course. Since prolyl hydroxylase levels were lowest when collagen synthesis was highest, the two effects were discordant, and in this system prolyl hydroxylase activity was clearly not rate-limiting. This is in contrast to tissue studies in which levels of prolyl hydroxylase ordinarily reflect collagen-synthesizing ability.

#### EFFECT OF ASCORBIC ACID ON INTRACELLULAR DEGRADATION OF COLLAGEN

In recent years it has become apparent that a significant proportion of newly synthesized collagen is degraded before it ever gets out of the cell [7]. Although the reasons for intracellular degradation are unknown, it has been theorized that the mechanism constitutes a quality control step for ridding the organism of defectively synthesized collagen. Since ascorbic acid serves as a cofactor for the hydroxylation of proline, an argument could be formulated that underhydroxylated collagen synthesized in the absence of ascorbic acid is defective and more likely to be degraded while still in the cell. The relative increase in collagen production seen in the presence of

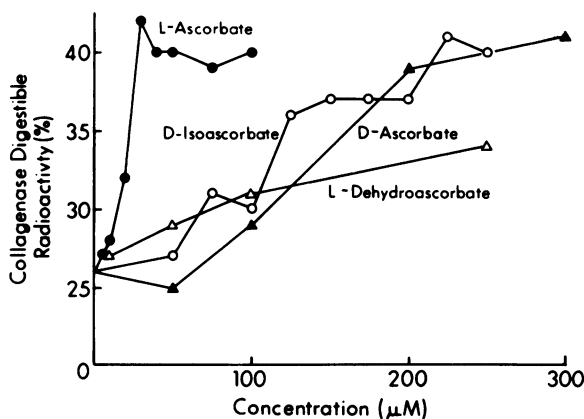


FIG. 4. Dependence of collagen synthesis on concentration of ascorbate and its analogs. Confluent human skin fibroblasts were incubated for 72 hours in Dulbecco's modified Eagle's medium supplemented with 0.5 percent dialyzed calf serum. L-ascorbic acid, D-ascorbic acid, D-isoascorbic acid, or L-dehydroascorbic acid at various concentrations was given for the last 24 hours. Cultures were labeled for the final six hours with L-[2,3- $^3\text{H}$ ] proline 20  $\mu\text{Ci}/\text{ml}/35\text{-mm}$  plate). The radioactivity incorporated into total collagen and noncollagen proteins was determined after digestion with clostridial collagenase.

ascorbic acid could be a result of synthesis of fully hydroxylated collagen, which is less susceptible to intracellular degradation. In order to test this hypothesis we labeled with radioactive proline cells which had been cultured in the absence and presence of L-ascorbic acid, 100  $\mu\text{M}$  [8]. In either case, the intracellular degradation of collagen was 11.2 percent. Since collagen synthesized in the presence of ascorbic acid is known to be hydroxyproline-deficient, we confirmed these results by pulse labeling the cells with lysine and determining intracellular degradation by hydroxylysine analysis. We had previously determined that the collagen synthesized in the absence of ascorbic acid had a hydroxylysine content that was similar to that synthesized in the presence of ascorbic acid [5]. When hydroxylysine was used as the marker for intracellular degradation of collagen, we found that 24.9 percent of the collagen was degraded in the absence of ascorbic acid and 24.5 percent was degraded in the presence of ascorbic acid. We believe that the higher figure we detected while utilizing hydroxylysine as our marker for intracellular degradation of collagen may result from underestimation of degradation when hydroxyproline is used as the marker, caused by preferential degradation of underhydroxylated residues, leading to a low estimate of intracellular degradation. Nonetheless, intracellular degradation of collagen appears to be unaffected by ascorbic acid supplementation in these cells and is insufficient to explain the relative stimulation of collagen synthesis in the presence of ascorbic acid.

#### EFFECT OF ASCORBIC ACID ANALOGS

Several analogs of ascorbic acid have been studied; these include dehydroascorbic acid, D-ascorbic acid, and D-isoascorbic acid [3]. As demonstrated in Fig. 4, each of these analogs was capable of stimulating relative collagen production, but none was effective at the low concentration demonstrated for L-ascorbic acid. In general, a tenfold increase in concentration was required for these compounds. It should be noted that D-ascorbic acid and D-isoascorbic acid are able to substitute for the cofactor effect of ascorbic acid on the actions of lysyl and prolyl hydroxylase at equimolar concentration. Thus a stereospecificity for ascorbic acid is present, although stereoisomers and dehydroascorbic acid were able to support the reaction at tenfold higher concentrations. Reducing agents such as L-lactate and dimethyltetrahydropterine were unable to support the reaction.

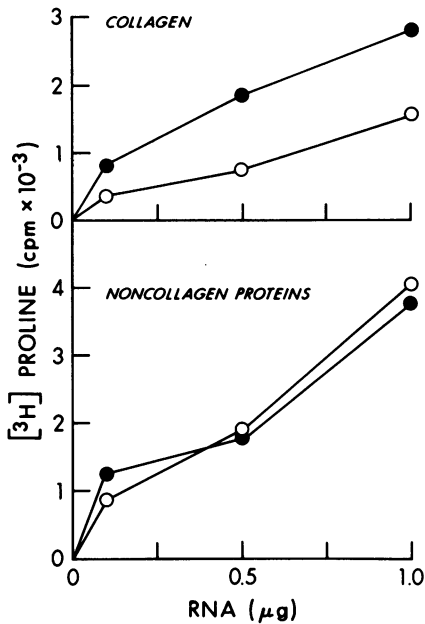


FIG. 5. Effect of ascorbic acid on collagen-specific mRNA. Human skin fibroblasts were grown to confluent density, then growth arrested in media containing 0.5 percent dialyzed calf serum. Media containing 100 mM ascorbic acid was added fresh daily. RNA was extracted from the cells in 6 M guanidine hydrochloride and translated in rabbit reticulocyte lysate in the presence of radioactive proline. Collagen and noncollagen proteins were determined by digestion with purified clostridial collagenase.

#### EFFECT OF ASCORBIC ACID ON COLLAGEN-SPECIFIC mRNA

In order to understand the level of regulation of ascorbic acid on collagen synthesis, mRNA was isolated from ascorbate-stimulated and control cells [9]. The mRNA was translated in a cell-free translation system and the resultant synthesized protein labeled with tritiated proline. Collagen and noncollagen protein were measured by digestion with highly purified bacterial collagenase. As shown in Fig. 5, mRNA for noncollagen protein was unchanged whether or not ascorbic acid was present. mRNA specific for collagen, however, was stimulated twofold in the presence of ascorbic acid. The data demonstrate that collagen-specific mRNA is stimulated in the presence of ascorbic acid, but whether or not this specific effect related to stimulated transcription, stability of mRNA, or mRNA processing is not distinguished in this experiment.

TABLE 2  
Effect of Ascorbate on Relative Collagen Synthesis in Skin Fibroblasts  
from Donors of Varying Age

Donor Age	% Collagen <sup>a</sup>	
	- Ascorbate	+ Ascorbate (100 μM)
Fetal	21.7	54.4
Newborn	11.1	35.7
11 years	6.3	13.2
26 years	6.3	20.8
35 years	7.9	24.7

<sup>a</sup>Expressed as proportion of total protein synthesis devoted to collagen. Data has been corrected to take into account the increased proline content of collagen.

## EFFECT OF AGE ON ASCORBATE STIMULATION OF COLLAGEN

The effect of ascorbic acid on collagen synthesis was studied in fibroblasts derived from normal persons of varying age (Table 2). In all cells from ages fetal to adult, collagen synthesis was stimulated by ascorbic acid. Strikingly different results were demonstrated according to the age of the fibroblast donor, however. Fetal fibroblasts devoted 21.7 percent of their collagen synthesis to collagen production, which could be stimulated by ascorbic acid to 54.4 percent. In contrast, newborn cells and adult cells devoted correspondingly less of their protein synthesis to collagen production, but each could be stimulated appropriately. These results suggest that a differential threshold for relative collagen synthesis is set in fetal and newborn cells when compared to older cells. Although these effects are most probably related to developmental events, they require further study to see if they correlate to any aging effect.

## REFERENCES

1. Jeffrey JJ, Martin GR: The role of ascorbic acid in the biosynthesis of collagen. I. Ascorbic acid requirement by embryonic chick tibia in tissue culture. *Biochim Biophys Acta* 121:269–280, 1966
2. Freiburger H, Grove D, Sivarajah A, et al: Procollagen I synthesis in human skin fibroblasts: Effect of culture conditions on biosynthesis. *J Invest Dermatol* 75:425–430, 1980
3. Murad S, Tajima S, Johnson GR, et al: Collagen synthesis in cultured human skin fibroblasts: Effect of ascorbic acid and its analogs. *J Invest Dermatol* 81:158–162, 1983
4. Murad S, Sivarajah A, Pinnell SR: Serum stimulation of lysyl hydroxylase activity in cultured human skin fibroblasts. *Conn Tiss Res* 13:181–186, 1985
5. Murad S, Grove D, Lindberg KA, et al: Regulation of collagen synthesis by ascorbic acid. *Proc Natl Acad Sci USA* 78:2879–2882, 1981
6. Murad S, Sivarajah A, Pinnell SR: Regulation of prolyl and lysyl hydroxylase activities in cultured human skin fibroblasts by ascorbic acid. *Biochem Biophys Res Commun* 101:868–875, 1981
7. Bienkowski RS: Collagen degradation in human lung fibroblast: extent of degradation, role lysosomal proteinases, and evaluation of an alternate hypothesis. *J Cell Physiol* 121:152–158, 1984
8. Geesin J, Murad S, Pinnell SR: Stimulated collagen production in human skin fibroblasts by ascorbic acid is unrelated to intracellular degradation. *J Invest Dermatol* 84:296, 1985
9. Tajima S, Pinnell SR: Regulation of collagen synthesis by ascorbic acid. Ascorbic acid increases type I procollagen mRNA. *Biochem Biophys Res Commun* 106:632–637, 1982