Studies on the Role of Ascorbic Acid in Collagen Synthesis

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Although the requirement of ascorbic acid in collagen formation has been well documented (1-5), there is as yet no agreement as to the mechanism involved. Some of the hypotheses that have been proposed are (a) that ascorbic acid is necessary for the formation of collagen fibers from a precollagen (1), (b) that it is required for the formation of hydroxyproline from bound proline in a precollagen peptide (2, 3), (c) that it is involved in the synthesis or the incorporation of "active" hydroxyproline (4), and (d) that a deficiency of ascorbic acid either interferes with the synthesis of new collagen or results in its destruction as it is formed (5).

In this report data are presented to show that, under scurvy conditions, the incorporation into collagen of both proline and hydroxyproline is decreased while the hydroxylation of proline appears to be unaffected. This decreased incorporation of the imino acids is not ascribable to a decreased protein synthesis which is in general the result of inanition, since induction of enzyme synthesis is not impaired under similar conditions."

EXPERIMENTAL

Granuloma tissues were obtained by injecting subcutaneously 5 ml of a 1% carrageenin solution1 into guinea pigs weighing approximately 250 g, as described by van Robertson and Schwartz (3). All animals were fed an ascorbic acid-deficient diet (Nutritional Biochemicals Corporation) throughout the experimental period. The control groups were given injections of 100 mg of sodium ascorbate in 0.5 ml of 0.9% sodium chloride solution on the 8th day. Thereafter they were given orally 50 mg of sodium ascorbate in 10% sucrose solution every day until the 11th day when the granulomas were removed. The control and experimental (scurvy) groups were pair-fed to equalize the caloric intake between the two groups. For the radioaactive experiments, 8 μ of uniformly labeled L-proline-C14 were injected subcutaneously into each guinea pig on the 9th day of granuloma development and subsequently at 12-hour intervals until a total of 24 μ had been administered. Urine was collected from the 9th day immediately before the injection of radioprolne until the experiment was terminated 48 hours later on the 11th day. Urine samples were hydrolyzed overnight in 6 N HCl, and the amino acids were selectively removed from the hydrolysate by the method of Hamilton and Ortiz (6). The procedures for isolating and purifying collagen and the imino acids, and for assaying the latter, have already been described (7, 8).

1 We are indebted to Mr. Leonard Stoloff of Marine Colloids, Inc., New Bedford, Massachusetts, for the generous supply of purified carrageenin.

Microsomal Hydroxylase Induction—Injections of 10 mg of benzpyrene dissolved in 1.0 ml of corn oil (Mazola) were given intraperitoneally to guinea pigs on the 10th day of granuloma development. The animals were killed 24 hours later, and liver granuloma were removed. The liver was assayed for its acetylhydrolase activity as described previously (9).

Tryptophan Pyrrolase Induction—On the 11th day L-tryptophan was administered to guinea pigs in the manner described by Lee (10), and 5 hours later the enzyme activity in the liver was assayed by the method of Mehler et al. (11).

RESULTS

The effect of ascorbic acid deficiency is clearly demonstrated by the lower concentration of hydroxyproline in the granuloma tissue and by the lower specific activity of the isolated gelatin in contrast to those of the control animals (Table I). The data on the specific activities of the gelatin imino acids indicate that the incorporation of proline as well as hydroxyproline is affected by ascorbic acid deficiency. On the other hand, analyses of the urinary imino acids reveal the normal conversion of proline to hydroxyproline.

As indicated above, care was taken to pair-feed the control and the experimental groups so as to eliminate complications arising from inanition in the latter group. Evidence that deficiency in protein synthesis in general does not exist in the experimental group was obtained by the enzyme induction studies. Data presented in Table II show that the scurvy guinea pigs responded as well as the control animals to the two different inductors.

DISCUSSION

Studies by van Robertson and Schwartz (3) of the carrageenin-induced granuloma and by Gould and Woesner (2) of healing skin wounds in scurvy guinea pigs led them to postulate that ascorbic acid was required for the conversion of a protein, rich in proline and glycine, to a collagen precursor rich in hydroxyproline. Recently, van Robertson et al. (4) showed that the tannic acid-precipitable protein in the carrageenin granuloma of the scurvy guinea pig did not contain sufficient proline and glycine to be a collagen precursor. Radioactive studies also revealed that the specific activities of proline and hydroxyproline in normal, scurvy, and recovery granulomas were not consistent with their original hypothesis (4). In view of these findings, they postulated that ascorbic acid may be required for the synthesis of "active" hydroxyproline or its incorporation into collagen. It should be pointed out that the radioactive studies of van Robertson's group were carried out with relatively impure...
collagen. They found that the specific activity of proline in normal and in scorbutic granuloma was approximately the same, whereas the specific activity of hydroxyproline in the former was much higher than that in the latter.

In the present report, studies were conducted with a more highly purified collagen, as evidenced by the ratio of proline to hydroxyproline of approximately 1:1 (7). The finding that the specific activity of proline as well as of hydroxyproline is lower in the scorbutic granuloma indicates that the functional role of ascorbic acid in collagen synthesis may be more than participation in the hydroxylation of proline. In fact, the data on the urinary hydroxyproline militate against the view that impaired hydroxyproline formation is the primary manifestation of ascorbic acid deficiency. The present data could be accounted for by the assumption of Gross (5) that in scorbutic guinea pigs, destruction and removal of new collagen may be as rapid as its formation. However, there is no direct evidence for this hypothesis. Moreover, Williams (12) has presented evidence that both synthesis and breakdown of connective tissue fibers are inhibited in the absence of ascorbic acid.

The fact that less proline and hydroxyproline is incorporated into scorbutic granuloma than into normal granuloma indicates that the protein synthetic mechanism may be defective in scorbutic guinea pigs. Studies on the formation of microsomal acetanilide hydroxylase and soluble tryptophan pyrrolase with scorbutic guinea pigs revealed no abnormality in their ability to synthesize new liver proteins under the present experimental conditions. The adaptive nature of these enzymes has already been demonstrated (13-15). In scorbutic animals, the fibroblasts remain immature although their proliferative power is not impaired (12). It appears that collagen formation is intimately dependent on the cellular activities of the mature fibroblasts and that ascorbic acid may exert its effect on collagen synthesis indirectly by its participation in the maturation of the cells. The apparent normal conversion of proline to hydroxyproline in scorbutic guinea pigs indicates either that immature fibroblasts can catalyze the formation of hydroxyproline without ascorbic acid or that hydroxyproline is formed at sites other than in fibroblasts and transported there for collagen synthesis.

**SUMMARY**

After the administration of L-proline-C\textsuperscript{14} to nonscorbutic and scorbutic guinea pigs bearing carrageenin granulomas, purified gelatin was isolated and the specific activities of proline and hydroxyproline were determined. Both imino acids showed lower specific activities in the scorbutic granulomas than in the controls. On the other hand, examination of the urinary hydroxyproline indicated that hydroxylation of proline was not affected by ascorbic acid deficiency. Inanition as a cause of the impaired collagen synthesis in the scorbutic animals was ruled out, since the animals in both groups were pair-fed and responded equally to induced enzyme formation.

**REFERENCES**

1. **WOOLBACH, B. D., AND HOWE, P. R., Arch. Pathol. Lab. Med., 1, 1 (1926).**


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**Table I**

<table>
<thead>
<tr>
<th>Group and No. of guinea pigs</th>
<th>Gelatin</th>
<th>Hydroxyproline</th>
<th>Proline</th>
<th>Hydroxyproline</th>
<th>Proline</th>
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<tr>
<td>Control (2)*</td>
<td>1336</td>
<td>1.21</td>
<td>1122</td>
<td>900</td>
<td>3.9</td>
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<tr>
<td>Control (2)*</td>
<td>1766</td>
<td>1.52</td>
<td>1433</td>
<td>1222</td>
<td>5.7</td>
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<tr>
<td>Experimental (2)*</td>
<td>455</td>
<td>0.42</td>
<td>294</td>
<td>336</td>
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<td>Control (1)</td>
<td>1518</td>
<td>1.21</td>
<td>1453</td>
<td>1406</td>
<td>2.2</td>
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<tr>
<td>Experimental (1)</td>
<td>826</td>
<td>0.85</td>
<td>452</td>
<td>615</td>
<td>1.8</td>
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* Granuloma and urine were pooled from each of the 2 guinea pigs before analyses.

**Table II**

<table>
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<th>Preparation</th>
<th>Granuloma hydroxyproline</th>
<th>Acetanilide hydroxylated</th>
<th>Tryptophan metabolized</th>
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<tr>
<td></td>
<td>mg/g (wet)</td>
<td>µmoles/beaker</td>
<td>µmoles kynurenine/ beaker</td>
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<tr>
<td>Control</td>
<td>1.00</td>
<td>0.60</td>
<td>0.14</td>
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<td>Control + benzpyrene</td>
<td>1.83</td>
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<tr>
<td>Control + tryptophan</td>
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<tr>
<td>Experimental</td>
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<td>0.11</td>
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<tr>
<td>Experimental + benzpyrene</td>
<td>0.75</td>
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<tr>
<td>Experimental + tryptophan</td>
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