Effect of L-3,4-Dehydroproline on Collagen Synthesis and Prolyl Hydroxylase Activity in Mammalian Cell Cultures

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The incorporation of DL-3,4-dehydro[14C]proline into collagen and total protein of 3T3 cells occurred at approximately one-fifth the rate observed for L-[14C]proline. Addition of L-3,4-dehydroproline to the culture medium inhibited markedly the incorporation of [14C]glycine and L-[3H]lysine into the collagen of 3T3 cells, but there was only slight inhibition of the incorporation of the radiolabeled amino acids into total cellular proteins, indicating that the action of L-3,4-dehydroproline is specific for collagen. When 1 mM L-3,4-dehydroproline was added to the culture medium, the [14C]hydroxyproline content was reduced 40% in the cell layer and 70% in the medium. The D isomer of 3,4-dehydroproline did not inhibit [14C]hydroxyproline formation. These findings indicate that L-3,4-dehydroproline reduced the hydroxylation of the susceptible prolyl residues in the collagen molecule and the secretion of collagen from the cell. The reduction in the hydroxyproline content is probably related in part to a reduction in the activity of prolyl hydroxylase; when various mammalian cell cultures were exposed to 0.2 mM L-3,4-dehydroproline, the specific activity of prolyl hydroxylase was reduced markedly, while that of lysyl hydroxylase was not affected. Under these conditions, cell growth and lactic dehydrogenase activity were not affected. The reduction of prolyl hydroxylase activity caused by L-3,4-dehydroproline required protein synthesis. Removal of L-3,4-dehydroproline from the growth medium resulted in a time-dependent increase in the specific activity of prolyl hydroxylase.

Several analogs of proline have been shown to be incorporated into collagenous and noncollagenous protein of various biological systems (1-11). These analogs have proven useful in examining various aspects of the regulation of collagen biosynthesis, such as the effect of a reduced hydroxyproline content on procollagen formation and deposition of collagen in the extracellular matrix. These findings, as well as earlier reports, have stimulated interest in the potential clinical use of the proline analogs to prevent excess collagen deposition during wound healing and to inhibit the development of fibrosis.

Previous studies of Rosenbloom and Prockop (4), using cartilage of chick embryo, demonstrated that DL-3,4-dehydro[14C]proline replaces prolyl residues of collagen and other proteins, and that the dehydroprolyl residues in collagen cannot be hydroxylated. These investigations also demonstrated that DL-3,4-dehydroproline inhibits the incorporation of L-[14C]proline into nondialyzable protein, but has little effect on the incorporation of L-[14C]alanine. Chick tibiae exposed to DL-3,4-dehydroproline in vitro contained a decreased amount of hydroxyproline and hydroxylysine. It was suggested that the dehydroprolyl residues in the collagen molecule prevented the hydroxylation of the otherwise susceptible prolyl and lysyl residues. Autoradiographic studies indicated that dehydroproline-containing collagen was not secreted normally, and that it accumulated intracellularly (4, 11). Since collagen peptides containing dehydroproline appeared to form a stable prolyl hydroxylase-dehydroprolyl collagen complex, it was suggested that this interaction accounted for the decrease in the activity of prolyl hydroxylase.

The purpose of these studies was to investigate the effects of the L isomer of dehydroproline on several aspects of collagen metabolism in mammalian cells grown in culture.

MATERIALS AND METHODS

Confluent cultures of L929 mouse fibroblasts grown in monolayers were kindly supplied by R. Kuttan and G. Cardinale, while the 3T3 mouse fibroblasts were provided by M. Green and S. Pestka of the Roche Institute of Molecular Biology. The WI-38 human diploid lung fibroblasts (CCL-75) were obtained from the American Type Culture Collection. Cultures were grown in 25-cm² or 75-cm² tissue culture dishes at 37° as monolayers in Eagle's minimal essential medium (GIBCO, N. Y.) containing 10% fetal calf serum supplemented...
with 100 \( \mu \)g/ml of ascorbic acid and 9.5 \( \mu \)g/ml of Fe(NO)_3 4HCl. Since Peterkosky (12) has shown that ascorbate deteriorates in culture media, medium containing fresh ascorbic acid was added to cultures at frequent intervals so that the effects of L-3,4-dehydroproline were observed under conditions where ascorbate was not limiting.

Cycloheximide and \( \alpha \)-ascorbic acid were obtained from Sigma Chemical Co., St. Louis, Mo., and N-t-butoxycarbonylcarboxylic \( \alpha \)-[carboxyl-\( \text{H}^3 \)]proline (25 mCi/mmol) was obtained from Cal-Atomic, Los Angeles, Calif. Uniformly labeled \( \alpha \)-[carboxyl-\( \text{H}^3 \)]proline (233 mCi/mmol), \( \alpha \)-[carboxyl-\( \text{H}^3 \)]glycine (96 mCi/mmol), and \( L^-\text{H}^2 \)lysine (2.2 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Bacterial collagenase (type II), free of nonspecific proteases, was purchased from Advance Biofactures Inc., Lynbrook, N. Y.

The \( \text{H}^3 \)-hydroxyproline content of cells or dialyzed medium was determined after acid hydrolysis (110°C for 18 hours) by the procedure of Rojkind and Gonzales (13). In this procedure, 20% of the radioactivity of uniformly labeled \( \text{H}^3 \)-hydroxyproline was lost in the oxidation with chloramine T. The results are not corrected for this loss. Recovery of radioactivity after this procedure (13) was between 70 and 80%.

The incorporation of labeled amino acids into collagen or into total protein was determined by a collagenase digestion procedure described earlier (14).

**Synthesis and Resolution of \( \alpha \)-[3,4-Dehydroproline]—The procedure used for the resolution of \( \alpha \)-[3,4-dehydroproline], 1, is outlined in Fig. 1. Pyrrole-2-carboxylic acid was converted in 80% yield to \( \alpha \)-[3,4-dehydroproline] using a modification of the procedure of Corbella et al. (15). Reaction of 1 with \( N^-\text{t-butoxycarbonyl} \) azide using the procedure of Schnabel (16) at pH 8.6 gave \( N^-\text{t-butoxycarbonyl} \)-L-3,4-dehydroproline using a modification of the procedure of Focella et al. (17). Recovery of radioactivity after this procedure (13) was between 70 and 80%.

The incorporation of labeled amino acids into collagen or into total protein was determined by a collagenase digestion procedure described earlier (14).

**Effect of L-3,4-Dehydroproline on Prolyl Hydroxylase and Lysyl Hydroxylase—Replicate confluent monolayer cultures of 3T3, WI-38, and L-1210 (Giemblasts in Eagle's medium were exposed to L-3,4-dehydroproline for various periods of time at 37°C. At the end of the experimental period, 20 \( \mu \)l of cycloheximide were added, and the dishes were chilled. The medium was decanted, and to each dish was added 1 ml trypsin/EDTA solution (GIBCO, N. Y.). Incubation was continued for a time sufficient for detachment of cells from the dish. Cold Eagle's medium was added to stop the action of trypsin, and an aliquot of the uniform cell suspension was counted in a hemocytometer. The cells were harvested by centrifugation at 1000 \( \times \) g for 10 min. The cell pellets were washed twice with 5 ml of cold phosphate-buffere saline (GIBCO, N. Y.) and suspended in 1 ml of cold 50 mm Tris-HCl buffer, pH 7.2, containing 1 \( \times \) 10\(^{-4}\) M EDTA and 1 \( \times \) 10\(^{-4}\) M dithiothreitol (20). The cells were disrupted by sonication for 10 to 20 s in a Biosonic sonic oscillator.

Prolyl hydroxylase activity in the cellular sonicates was determined by the method of Hutton et al. (21), and lysyl hydroxylase activity was determined by the method of Miller (22). The activity of prolyl hydroxylase and lysyl hydroxylase is expressed as counts per min of \( \text{H}^2 \)O released/mg of protein using the assay conditions described; the substrate for both enzymes was prepared from chick embryo collagen. The data on the rate of incorporation of \( \text{H}^3 \)-proline into total protein (and collagen) of 3T3 cells, and the effects of L-3,4-dehydroproline on protein synthesis and collagen metabolism are described in the supplement following this paper.

**RESULTS**

The data on the rate of incorporation of L-3,4-dehydro-\( \text{H}^3 \)-proline into total protein (and collagen) of 3T3 cells, and the effects of L-3,4-dehydroproline on protein synthesis and collagen metabolism are described in the supplement following this paper.

*Some of the data are presented as a miniprint supplement immediately following this paper. Figs. 2 to 5 will be found on p. 509. For the convenience of those who prefer to obtain the supplementary data in a more convenient form, they may be obtained from the authors on request.*

1. Personal communication from Dr. A. Focella, Chemical Research Division, Hoffmann-La Roche Inc., Nutley, N. J. 07110.
2. The resolution of other carboxylic acids with \( R^+ \)- and \( S^- \)-\( \alpha \)-methyl-p-nitrobenzylamine was first accomplished by C. W. Perry, K. Deiteler, W. Tauke, and S. Teitel, Chemical Research Division, Hoffmann-La Roche Inc., Nutley, N. J. 07110. These authors have submitted their findings to Synthesis for publication.

3. Concentrations are expressed in grams/100 ml.

4. The resolution of other amino acids with \( R^+ \)- and \( S^- \)-\( \alpha \)-methyl-p-nitrobenzylamine was first accomplished by C. W. Perry, K. Deiteler, W. Tauke, and S. Teitel, Chemical Research Division, Hoffmann-La Roche Inc., Nutley, N. J. 07110. These authors have submitted their findings to Synthesis for publication.
Effect of Short Term Exposure of Cell Cultures to D-, DL-, and L-3,4-Dehydroproline on Activity of Prolyl and Lysyl Hydroxylase—The finding that L-3,4-dehydroproline inhibited the intracellular [14C]hydroxyproline content could be due to inhibition of the incorporation of L-[14C]proline into collagen and hence a reduction in the [14C]hydroxyproline content and/or reduction in prolyl hydroxylase activity. To clarify this point, L-3,4-dehydroproline was examined for its effect on prolyl hydroxylase activity. Confluent cultures of L929 fibroblasts were exposed to various concentrations of L-, DL-, and D-3,4-dehydroproline, and the cell sonicates were assayed for prolyl hydroxylase and lysyl hydroxylase activity. The results of these experiments are shown in Fig. 6. Exposure of cells to L-3,4-dehydroproline resulted in a concentration-dependent reduction in the activity of prolyl hydroxylase. Under these conditions, a 50% reduction in enzyme activity was obtained at approximately 0.2 mM L-3,4-dehydroproline. Exposure of cells to 0.7 mM DL-3,4-dehydroproline resulted in a 50% reduction in prolyl hydroxylase activity. Exposure of cells to D-3,4-dehydroproline resulted in a negligible change in enzyme activity.

The effect of various concentrations of L- and DL-3,4-dehydroproline on the lysyl hydroxylase activity of L929 cells is shown in Fig. 7. Exposure of L929 cells to L-3,4-dehydroproline had little effect on lysyl hydroxylase activity; at a concentration of 5 mM L-3,4-dehydroproline, lysyl hydroxylase was reduced only 20%. As shown in Fig. 6, prolyl hydroxylase activity was reduced approximately 60% under these conditions.

Time Course of Reduction of Prolyl Hydroxylase Activity by L-3,4-Dehydroproline—Confluent cultures of L929 fibroblasts were exposed to 0.2 mM L-3,4-dehydroproline, and prolyl hydroxylase activity was determined at various times. As shown in Fig. 8, the reduction in prolyl hydroxylase activity was time-dependent; the specific activity of the enzyme decreased linearly with the increase in the time of exposure of cells to L-3,4-dehydroproline.

The relative effect of L-3,4-dehydroproline on the activity of prolyl and lysyl hydroxylase was also examined with WI-38 and 3T3 fibroblasts. Exposure of confluent cultures of WI-38 and 3T3 fibroblasts to L-3,4-dehydroproline resulted in a concentration-dependent reduction in prolyl hydroxylase activity, whereas lysyl hydroxylase was only marginally affected (Table I).

Requirement of Protein Synthesis for Reduction of Prolyl Hydroxylase Activity Caused by L-3,4-Dehydroproline—To investigate the requirements for reduction in prolyl hydroxylase activity caused by L-3,4-dehydroproline, replicate confluent cultures of L929 cells were exposed to 0.2 mM L-3,4-dehydroproline. After 6 hours, the specific activity of prolyl hydroxylase was determined in the harvested cells. As shown in Table II, exposure of cells to L-3,4-dehydroproline alone resulted in a 70% reduction of enzyme activity. When cycloheximide was present during this exposure at a concentration which blocks protein synthesis, L-3,4-dehydroproline did not cause a reduction in the activity of prolyl hydroxylase. These findings indicate that protein synthesis is a requirement for the reduction of prolyl hydroxylase activity caused by L-3,4-dehydroproline.

Effect of Long Term Exposure of Cell Cultures to L-3,4-Dehydroproline on Activity of Prolyl Hydroxylase and Lysyl Hydroxylase—Exposure of L929 cells to L-3,4-dehydroproline at concentrations ranging from 0.05 to 0.2 mM for 24 hours resulted in a 70 to 80% reduction in the specific activity of prolyl hydroxylase, whereas lysyl hydroxylase activity did not change significantly (Table III). During this period, cellular protein and the specific activity of lactic dehydrogenase were not affected. Exposure of cells to L-3,4-dehydroproline for 3 days resulted in a 60 to 80% reduction of prolyl hydroxylase activity, whereas lysyl hydroxylase activity was reduced 30%. After 5 days, there was a 10 to 50% reduction in prolyl hydroxylase activity. These results indicate that one of the major effects of L-3,4-dehydroproline is to reduce the activity of prolyl hydroxylase.

Sensitivity of Prolyl Hydroxylase to L-3,4-Dehydroproline during Various Stages of Cell Growth—Studies by Comstock et al. (20) and Peterkofsky (12) demonstrated that the synthesis of total protein and collagen is maximal during the log phase of cell growth, and it is decreased during the late stationary phase.
L-3,4-Dehydroproline Inhibition of Collagen Synthesis

Experiments reported in Table III indicate that as cells approached the stationary phase of growth (Day 5), the reduction of prolyl hydroylsylase activity was considerably less than in log phase cells (Days 1 and 3). For this reason, sensitivity of prolyl hydroxylase to the action of L-3,4-dehydroproline was determined during the various stages of growth of L929 cells (Table IV). During early stages of cell growth (1 to 7 x 10^6 cells/25-cm^2 dish), prolyl hydroxylase activity was reduced maximally by 0.2 mM L-3,4-dehydroproline during a 4-hour exposure. Protein synthesis is also maximal at this stage, suggesting that L-3,4-dehydroproline is being incorporated into cellular protein at a maximal rate. When cells reached a density of approximately 10 x 10^6 cells/dish and protein synthesis was decreased, sensitivity of enzyme activity to L-3,4-dehydroproline was also markedly decreased. Prolyl hydroxylase activity could be reduced in stationary cultures if cells were exposed to L-3,4-dehydroproline for a longer period. For example, when 14 x 10^6 cells/dish were exposed to 0.2 mM L-3,4-dehydroproline for 15 hours, there was a 36% reduction in enzyme activity. These studies demonstrate that the sensitivity of prolyl hydroxylase to inhibition by L-3,4-dehydroproline is related to the stage of growth of the cells and to the rate of protein synthesis.

**Recovery of Prolyl Hydroxylase Activity of L929 Cells after Exposure to L-3,4-Dehydroproline**—Experiments were conducted to determine whether prolyl hydroxylase activity of L929 cells recovers after exposure to L-3,4-dehydroproline (Table V). The medium containing L-3,4-dehydroproline was removed after 24 hours, and the cultures were exposed to fresh medium containing 5 mM l-proline for an additional 4 days. Prolyl hydroxylase activity was measured at various times after removal of L-3,4-dehydroproline. Cells exposed to L-3,4-dehydroproline contained lower prolyl hydroxylase activity (Day 1). However, 2 days after removal of L-3,4-dehydroproline (Day 3), the specific activity of prolyl hydroxylase increased approximately 2-fold, and after 4 days (Day 5), it was approximately 50 to 90% of the control activity. The specific activity of prolyl hydroxylase of cells exposed to L-3,4-dehydroproline (followed

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**TABLE I**

**Effect of various concentrations of L-3,4-dehydroproline on prolyl and lysyl hydroxylase of WI-28 and 3T3 cells**

<table>
<thead>
<tr>
<th>L-3,4-Dehydroproline</th>
<th>Relative specific activity</th>
<th>WI-38</th>
<th>3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prolyl hydroxylase</td>
<td>Lysyl hydroxylase</td>
<td>Prolyl hydroxylase</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>44</td>
<td>82</td>
<td>85</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>44</td>
<td>104</td>
<td>71</td>
</tr>
<tr>
<td>5 mM</td>
<td>39</td>
<td>85</td>
<td>63</td>
</tr>
</tbody>
</table>

---

**TABLE II**

**Requirement of protein synthesis for reduction of prolyl hydroxylase activity by L-3,4-dehydroproline**

Confluent cultures of L929 cells (2 x 10^6 cells/25-cm^2 dish) were exposed to either L-3,4-dehydroproline (0.2 mM), cycloheximide (10 µg/ml), or both for 6 hours. Then, cells were harvested and assayed for prolyl hydroxylase activity as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>Addition to growth medium</th>
<th>Specific activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm × 10^6</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>39.7</td>
<td>0</td>
</tr>
<tr>
<td>L-3,4-Dehydroproline</td>
<td>120</td>
<td>70</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>32.9</td>
<td>17</td>
</tr>
<tr>
<td>L-3,4-Dehydroproline + cycloheximide</td>
<td>42.4</td>
<td>0</td>
</tr>
</tbody>
</table>

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**TABLE III**

**Effect of L-3,4-dehydroproline on growth, lactic dehydrogenase, prolyl hydroxylase, and lysyl hydroxylase activity in L929 cells**

Replicate flasks were seeded with 5.4 x 10^6 cells/25-cm^2 flask in 10 ml of Eagle's medium. Cultures were incubated at 37°C; medium was replaced daily. Cells were harvested by trypsinization and washed twice in phosphate-buffered saline before determining the specific activities of prolyl hydroxylase (PH), lysyl hydroxylase (LH), and lactic dehydrogenase (LDH) as described under “Materials and Methods.” The activity of lactic dehydrogenase is expressed as the change in optical density at 340 nm/mg of protein/min, while the activity of prolyl hydroxylase and lysyl hydroxylase is expressed as counts per min x 10^-9/mg of protein.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>0.2 mM L-3,4-Dehydroproline</th>
<th>0.1 mM L-3,4-Dehydroproline</th>
<th>0.05 mM L-3,4-Dehydroproline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>PH</td>
<td>LH</td>
<td>LDH</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>0</td>
<td>1.5</td>
<td>34.1</td>
<td>1.76</td>
<td>1.5</td>
</tr>
<tr>
<td>1</td>
<td>2.8</td>
<td>28.8</td>
<td>0.65</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>25.1</td>
<td>0.53</td>
<td>6.5</td>
</tr>
<tr>
<td>5</td>
<td>13.0</td>
<td>40.5</td>
<td>0.71</td>
<td>7.9</td>
</tr>
</tbody>
</table>
by exposure to L-proline) was always higher than that of cells exposed only to L-3,4-dehydroproline throughout the course of the experiment. These results indicate that the reduction of prolyl hydroxylase activity caused by L-3,4-dehydroproline is at least partially reversible.

**DISCUSSION**

Consistent with the *in vitro* findings of Rosenbloom and Prockop (4), radioactivity from [m-3,4-dehydro-14C]proline was found to be incorporated into total protein and collagen of 3T3 cells at one-fifth the rate observed for L-proline. L-3,4-Dehydroproline inhibited markedly the incorporation of labeled proline into total protein, but had little effect on the incorporation of either labeled glycine or lysine. These studies also showed that under these conditions L-3,4-dehydroproline inhibited the incorporation of these amino acids into collagen. Similar results were obtained with an *in vitro* system containing chick embryo membrane-bound polysomes. In this *in vitro* system, the incorporation of glycine into collagen was inhibited approximately 60% by 1 mM L-3,4-dehydroproline, while its incorporation into noncollagenous proteins was inhibited by approximately 10%. These experiments suggest that L-3,4-dehydroproline is a more potent inhibitor of collagen synthesis than noncollagenous protein.

In addition to the inhibition of collagen synthesis, L-3,4-dehydroproline reduced the intracellular hydroxyproline content of cells and markedly retarded the secretion of the collagen molecule. The specificity of the L isomer was shown by the inability of the D isomer to reduce the intracellular hydroxyproline content or the hydroxyproline content of medium proteins. Exposure of various mammalian cell cultures to racemic dehydroproline resulted in a significant reduction in prolyl hydroxylase activity. This effect was due to the L isomer and required protein synthesis. When prolyl hydroxylase activity was reduced by 0.2 mM L-3,4-dehydroproline, the total protein content of the cell was not affected. Removal of the analog from the growth medium resulted in a time-dependent increase in the specific activity of prolyl hydroxylase. These results indicate that the inhibition of prolyl hydroxylase by L-3,4-dehydroproline is reversible.

The mechanism by which dehydroproline reduces the activity of prolyl hydroxylase remains to be determined. L-3,4-Dehydroproline does not inhibit the activity of prolyl hydroxylase when it is added *in vitro*. On the basis of *in vitro* data with collagen peptides, Rosenbloom and Prockop (4) suggested that prolyl hydroxylase remains tightly bound to dehydroproline-containing collagen. Assuming that the enzyme cannot disassociate from this complex, dehydroproline could decrease the level of active enzyme. Evidence for the formation of a dehydroprolyl peptide-enzyme complex has also been reported by McGee et al. (25), who showed that dehydroprolyl bradykinin (the prolyl residue in bradykinin susceptible to hydroxylation is replaced by dehydroproline) is an effective competitive inhibitor of prolyl hydroxylase *in vitro*. An explanation which has not been excluded is that L-3,4-dehydroproline is incorporated into prolyl hydroxylase, resulting in the synthesis of an aberrant, inactive form of the enzyme. Another possible mechanism of action of L-3,4-dehydroproline is that it accelerates the degradation of prolyl hydroxylase. Using an immunological technique (26), it should be possible to determine whether the concentration of prolyl hydroxylase antigen decreases with L-3,4-dehydroproline treatment.

Studies by Rosenbloom and Prockop (4) demonstrated that tibiae pulsed with [14C]hydroxylysine in the presence of DL-3,4-dehydroproline contained approximately one-half the amount of [14C]hydroxylysine found in the control system. The studies reported here with fibroblasts indicate that the exposure of these cells to L-3,4-dehydroproline has only a marginal effect on cellular lysyl hydroxylase. It is possible that a dehydroprolyl collagen prolyl hydroxylase complex cannot interact with lysyl hydroxylysine, due to steric hindrance of the susceptible sites. This could account for the reduction in [14C]hydroxylysine formation observed in the tibiae (4).

The stimulatory effect of 17β-estradiol on collagen synthesis and prolyl hydroxylase activity has been demonstrated in the uterus of the immature rat (27). Preliminary results in this system suggest that L-3,4-dehydroproline inhibits the synthesis and deposition of collagen in the uterus, and that the specific activity and total activity of prolyl hydroxylase in the uterus and lung is reduced significantly in 17β-estradiol-treated and control rats.

The hydroxyproline residue of collagen appears to be required for stabilization of the triple helical configuration of the molecule (28-30). Underhydroxylated collagen has a lower melting temperature than its hydroxylated counterpart, and at body temperature it assumes a random coil configuration and can be digested by tissue proteases. Normally hydroxylated collagen in the triple helical form is resistant to nonspecific proteolysis. *Uroto* and Prockop (11) tested several proline analogs, including DL-3,4-dehydroproline, and demonstrated that collagen containing these analogs was far less resistant to pepsin digestion than normal collagen. Their data also suggested that the presence of proline analogs may interfere with the formation of the triple helix.

The results presented in this report indicate that L-3,4-dehydroproline is a potent inhibitor of collagen synthesis and growth medium containing L-3,4-dehydroproline was removed and replaced with Eagle's medium containing 5 mM L-proline. The values in parentheses show the specific activity of prolyl hydroxylase when L-3,4-dehydroproline was present in the growth medium throughout the duration of the experiment.

**Table V**

<table>
<thead>
<tr>
<th>Growth day</th>
<th>Prolyl hydroxylase activity</th>
<th>Concentration of L-3,4-dehydroproline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>0</td>
<td>34.1</td>
<td>34.1</td>
</tr>
<tr>
<td>1</td>
<td>28.8</td>
<td>5.9</td>
</tr>
<tr>
<td>3</td>
<td>25.0</td>
<td>13.6</td>
</tr>
<tr>
<td>5</td>
<td>40.4</td>
<td>31.7</td>
</tr>
</tbody>
</table>


* R. A. Salvador, I. Tsai, R. J. Marcel, and S. S. Kerwar, unpublished observations.
secretion in mammalian cell cultures. Its inhibitory effect is due in part to a reduction of prolyl hydroxylase activity, but the mechanism of this reduction is not known. It is also likely that collagen which contains dehydroproline in place of proline is digested rather than secreted from cells. Whether L-3,4-dehydroproline will have potential in the prevention of excessive collagen deposition largely depends on the toxicity of the compound. A recent review has discussed this aspect in detail (31).

Acknowledgments—The authors would like to thank Drs. G. Cardinale, S. Udenfriend, and H. Weissbach for valuable discussion and critical reading of this manuscript. They would also like to thank Janet Hansen for her excellent secretarial assistance.

REFERENCES
Inhibition of Collagen Synthesis

To determine the effect of 4-5,6-dehydroproline on collagen synthesis and prolyl hydroxylase activity in normal cell cultures,

Materials and Methods

Collagen synthesis and prolyl hydroxylase activity were measured in the presence of 4-5,6-dehydroproline and compared to control cultures. The results are presented in Table 1.

Figure 1: Effect of 4-5,6-dehydroproline on the incorporation of L-proline into total protein and collagen. At various time periods, cultures were harvested and total protein and collagen were extracted. The incorporation of L-proline was measured by acid precipitation of insoluble protein and subsequent measurement of radioactivity.

Figure 2: Effect of 4-5,6-dehydroproline on the incorporation of L-proline into type I collagen. Type I collagen was isolated from the cultures and its radioactivity was measured.

Figure 3: Effect of 4-5,6-dehydroproline on the incorporation of L-proline into type III collagen. Type III collagen was isolated from the cultures and its radioactivity was measured.

Figure 4: Effect of 4-5,6-dehydroproline on the incorporation of L-proline into type IV collagen. Type IV collagen was isolated from the cultures and its radioactivity was measured.

The results indicate that 4-5,6-dehydroproline inhibits the synthesis of collagen, as well as the activity of prolyl hydroxylase, in normal cell cultures.

Discussion

The inhibition of collagen synthesis by 4-5,6-dehydroproline suggests a potential role for this metabolite in the regulation of collagen biosynthesis. Further studies are needed to understand the mechanisms underlying this effect.

Conclusion

4-5,6-dehydroproline inhibits collagen synthesis and prolyl hydroxylase activity in normal cell cultures, providing insights into the potential regulatory role of this metabolite in collagen biosynthesis.
Effect of L-3,4-dehydroproline on collagen synthesis and prolyl hydroxylase activity in mammalian cell cultures.

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