The Formation of Collagen Hydroxylysine Studied with Tritiated Lysine*

EDWIN A. POPENOE, ROBERT B. ARONSON, AND DONALD D. VAN SLYKE

From the Division of Biochemistry, Medical Department, Brookhaven National Laboratory, Upton, Long Island, New York 11973

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Hydroxylysine was first isolated from gelatin (2) and probably does not occur in proteins of mammalian origin other than collagen (3). In rats, dietary lysine is an obligatory precursor of the hydroxylysine of collagen (4-7); when 14C-labeled lysine is either fed or given by injection it is incorporated into both the lysine and hydroxylysine of collagen, whereas labeled hydroxylysine is not incorporated into collagen at all (8). Since, very shortly after the injection of 14C-lysine into rats, the specific activity of hydroxylysine of skin collagen is exactly the same as that of collagen lysine, and since it remains the same for as long as 3 months (7), the hydroxylation of lysine must take place before it is incorporated into insoluble collagen. Recent reports from other laboratories (9, 10) make it seem probable that the hydroxylation of proline takes place after the amino acid is incorporated into a peptide chain, but before the peptide chain leaves the ribosome. It is possible that the hydroxylation of lysine takes place at the same time. The studies described in the present report were designed to aid in the understanding of the mechanism of the conversion of lysine to hydroxylysine.

Conceivable mechanisms by which lysine could be hydroxylated are as follows. (a) Direct uptake of an oxygen atom on carbon 5 of the lysine chain

\[
\text{H}_3\text{N}^+\text{CH},\text{CH}_2,\text{CH}_2,\text{CH}_2,\text{CH}_2,\text{CH}(\text{NH}_2)^-\text{COOH}
\]

(b) Oxidation on carbon 5 to a ketone-CO group followed by reduction to -CH(OH) -. (c) Dehydrogenation with formation of a double bond between carbon atoms 5 and 6 followed by uptake of the elements of water. (d) Dehydrogenation with formation of a double bond between carbon atoms 4 and 5 followed by uptake of the elements of water.

To obtain evidence concerning which mechanisms may or may not be involved we have prepared lysine labeled with tritium on carbon atoms 4 and 5, and have isolated hydroxylysine incorporated in vivo from this lysine into the collagen of chick embryos and in vitro into collagen formed in polyvinyl sponges taken from rats. Removal of half of the trichloroacetic acid dissolves the collagen as gelatin. After each extraction, the solids were separated from the extract by centrifugation. After the addition of 1 g of nonradioactive dl-lysine to each 50 ml, the extract was dialyzed against running deionized water, passed through a small column of mixed bed ion exchange resin.

**MATERIALS**

dl-Lysine-4,5-3H was prepared according to Birkofer and Hempel (11). In this preparation, tritium is introduced by catalytic hydrogenation of acetamido-(4-amino-DA-butynyl)malonic acid diethyl ester hydrochloride:

\[
\text{HCl-H}_2\text{N}-\text{CH},\text{CH}_2,\text{CH}_2,\text{CH}_2,\text{CH}(\text{NH}_2)^-\text{COCH}_2
\]

so that the 4 hydrogen atoms on carbon atoms 4 and 5 of the resultant lysine may be assumed to be equally labeled. The hydrogenation with tritium gas was carried out by the New England Nuclear Corporation. The location of all the tritium on carbon atoms 4 and 5 was confirmed by degradation analysis described in the experimental part of this paper.

For the experiments, the dl-lysine-1-14C was mixed with the dl-lysine-4,5-3H so that loss of 3H in the conversion of lysine to hydroxylysine could be measured by the decrease in the 3H to 14C ratio.

**EXPERIMENTAL PROCEDURE**

Incorporation of Labeled Lysine in Vitro into Rat Biopsy Connective Tissue—The studies in vitro followed the experimental procedures of Kao and Boucek (12) whose paper should be consulted for a more detailed description. Polyvinyl sponges (Ivalon, Clay Adams Company, New York City) about 20 x 20 x 8 mm were implanted under the skin of female rats weighing 80 to 100 g. About 3 weeks after implantation, the sponges (filled with connective tissue) were removed and cut into thin slices with a razor blade. Each sponge was placed in a 25-ml Erlenmeyer flask containing, in Experiment 1, Table I, 60 μg of dl-lysine-4,5-3H and 4 μC of dl-lysine-1-14C in 5 ml of Krebs-Henseleit solution. In Experiments 2 and 3, the labeled amino acid was present at a level of 130 μg of 3H and 6 μC of 14C in each flask. A total of eight sponges in eight flasks was used for each experiment.

After 12 hours of incubation at 37° under an atmosphere of 95% O2-5% CO2, the slices of sponge connective tissue were removed from the flasks, blotted to remove excess labeled lysine, and pooled. They were homogenized in 0.3 M trichloroacetic acid in a Lourdes Instrument Company homogenizer, then extracted twice with hot 0.3 M trichloroacetic acid, according to Fitch, Harkness, and Harkness (13). The hot 0.3 M trichloroacetic acid dissolves the collagen as gelatin. After each extraction, the solids were separated from the extract by centrifugation. After the addition of 1 g of nonradioactive dl-lysine to each 50 ml, the extract was dialyzed against running deionized water, passed through a small column of mixed bed ion exchange resin.

**REFERENCES**

1. This work was supported by the United States Atomic Energy Commission. A preliminary report has been made (1).
Experimental details are in the text.

<table>
<thead>
<tr>
<th>System and amino acid</th>
<th>Ratio of (^3\text{H}) to (^1\text{C})</th>
<th>(^3\text{H}) remaining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat sponge biopsy</td>
<td></td>
<td></td>
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<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
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<td>(100)</td>
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<tr>
<td>Hydroxylysine</td>
<td>10.9</td>
<td>78</td>
</tr>
<tr>
<td>Allohydroxylysine</td>
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<td>77</td>
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<td>(100)</td>
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<td>(100)</td>
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<td>Hydroxylysine</td>
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<td>Chick embryo</td>
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<tr>
<td>Allohydroxylysine</td>
<td>15.8</td>
<td>69</td>
</tr>
</tbody>
</table>

* The mean of the eight hydroxylysine preparations is 76%; the standard deviation is

\[
\sqrt{\frac{\sum (x - \bar{x})^2}{n(n - 1)}} = \pm 5\%
\]

Standard error of the mean is calculated as

\[
\sqrt{\frac{\sum (x - \bar{x})^2}{n(n - 1)}} = \pm 2\%
\]

(Amberlite MB-1) to remove any remaining adsorbed lysine, and evaporated to dryness.

The resulting gelatin was hydrolyzed by heating it under reflux with 20 ml of 6 N HCl for 20 to 24 hours. Lysine and hydroxylysine were separated from each other and from other amino acids on a column of Amberlite IR-120, 1.9 X 65 cm at pH 4.26 and 30 °C. This is the 50-cm column of Speckman, Stein, and Moore (14), and the resin used was a preparation marketed by Beckman Instruments Company, Spinco Division, Palo Alto, California. The column was operated at a rate of 90 ml per hour and the effluent was collected in 8-ml fractions. The amino acids in the effluent were located by performing colorimetric ninhydrin analyses (15) on suitable aliquots from each tube, and frequently also by determination of radioactivity in aliquots with a Tri-Carb liquid scintillation counter. The fractions containing either hydroxylysine or lysine were pooled for analysis.

Hamilton and Anderson (16) demonstrated that during hydrolysis a part of the hydroxylysine of collagen is converted to allohydroxylysine. The 50-cm column separates these two isomers and in some experiments they were isolated separately. In other experiments they were combined into a single hydroxylysine fraction.

**Incorporation of Labeled Lysine into Chick Embryo Collagen**—For each experiment, 0.1 ml of a solution containing 130 μCi of DL-lysine-4,5-\(^3\text{H}\) and 6 μCi of DL-lysine-1-\(^1\text{C}\) was given by injection onto the chorioallantoic membrane of each of eight 10-day-old embryonated chicken eggs. Incubation of the eggs was continued for 5 hours, then the embryos were removed, combined, and homogenized with a little water in a glass homogenizer. Sufficient trichloroacetic acid was added to the suspension to give a concentration of 0.3%, the collagen was extracted at 90 °C, and the lysine and hydroxylysine were isolated as in the sponge connective tissue experiments described above.

**Determination of Radioactivity**—Both \(^1\text{C}\) and \(^3\text{H}\) in the lysine and hydroxylysine isolated in the biochemical studies were determined by gas phase proportional counting. The \(^1\text{C}\) of the lysine and hydroxylysine in the solutions was determined as \(^1\text{C}\)CO\(_2\) by liberation of the carboxyl carbon with ninhydrin as described by Van Slyke, Dillon, MacFadyen, and Hamilton (17). An aliquot of the solution, 0.20 to 1.0 ml, was transferred to an all-glass reaction vessel (Fig. 1A of Hamilton and Van Slyke (18)), and since the amount of amino acid in the aliquot was almost too small to be detected, 1 ml of a 1% solution of nonradioactive L-lysine monohydrochloride was added to provide enough carboxyl CO\(_2\) for an accurate measurement.\(^1\) Sufficient 6 M H\(_3\)PO\(_4\) was then added to make the H\(_3\)PO\(_4\) concentration 1 M, and ninhydrin was added to make its concentration 20 mg per ml. The procedure used for heating the tubes, measuring the CO\(_2\), and transferring it to counting tubes was the same procedure used in previous work (7). Counting was done in the proportional region in Bernstein-Ballentine counting tubes filled with 90% argon-10% methane (19). Counting was continued until the counts recorded exceeded 30,000 above background.

Tritium in aliquots of the same solutions was counted by Christman's modification (20) of the procedure of Wiltsch, Kaplan, and Brown (21), in which labeled, organic hydrogen is converted to dry H\(_2\) and CH\(_4\) and counted as such in the same proportional counting tubes used for \(^1\text{C}\). We are indebted to Dr. Christman for helpful advice concerning these determinations. Control analyses carried out on DL-lysine-1-\(^1\text{C}\) showed that 65 to 75% of the \(^1\text{C}\) radioactivity of this compound appears in the counting tube under the conditions of the \(^3\text{H}\) analysis. Consequently, a number equal to 0.7 \times \(^1\text{C}\) dpm was subtracted from the observed disintegrations per min to calculate disintegrations per min from \(^3\text{H}\). When the count ratio of \(^3\text{H}\) to \(^1\text{C}\) is about 20:1, as in the present case, variation in the amount of \(^1\text{C}\) contaminating the tritium analysis produces only a trivial variation (about ±0.25%) in the number of counts observed. The tritium does not interfere at all with the determination of \(^1\text{C}\) as \(^1\text{C}O_2\).

For the chemical degradation of DL-lysine-4,5-\(^3\text{H}\), all counting was carried out on 1 to 3 mg of weighed samples in a Tri-Carb liquid scintillation spectrometer. The scintillation mixture contained 5 ml of a 2:1 mixture of ethylene glycol monomethyl ether and ethanolamine, and 10 ml of toluene containing 15 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per liter. To convert counts per min to disintegrations per min.

\(^1\) Since aliquots of the same solution were taken for \(^1\text{C}\) and \(^3\text{H}\) radioactivity measurements, calculation of the \(^3\text{H}\) to \(^1\text{C}\) ratio did not require determination of the concentration of amino acid in the solution. However, in the determination of carboxyl \(^1\text{C}O_2\), adding enough nonradioactive lysine to yield accurately measurable amounts of CO\(_2\) made it possible to assure, from agreement of duplicate CO\(_2\) measurements, that the evolution of carboxyl CO\(_2\) and its transfer from the reaction vessel to the Van Slyke-Neill manometric chamber occurred without loss.
TABLE II

Specific activities of compounds produced in degradation of lysine-4,5-3H

<table>
<thead>
<tr>
<th>Compound</th>
<th>Positions in lysine represented</th>
<th>Specific activity of 3H dpm/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine*</td>
<td>2, 3, 4, 5, 6</td>
<td>234</td>
</tr>
<tr>
<td>Glutaric acid</td>
<td>3, 4, 5</td>
<td>228</td>
</tr>
<tr>
<td>Lysine*</td>
<td>2, 3, 4, 5, 6</td>
<td>1.65 X 10^6</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>4, 5</td>
<td>1.58 X 10^6</td>
</tr>
</tbody>
</table>

* Two different samples of lysine-4,5-3H were prepared for this study as described in the text.

RESULTS AND DISCUSSION

The experimental results obtained are given in Table I. Each ratio value, 3H to 14C, is the ratio of disintegrations per min from the 3H and 14C, respectively, in equal aliquots of the same solution. The results indicate that in both the rat sponge biopsy and in the chick embryos, only 1 of the 4 labeled hydrogen atoms of the incorporated labeled lysine has been removed in the conversion from lysine to hydroxylysine. Thus, of the hydrogen atoms in lysine labeled with tritium on carbon atoms 4 and 5, only the 1 on carbon 5 remains intact during the biosynthesis of 5-hydroxylysine from lysine. Tritium radioactivity was determined as described above in the last paragraph under "Degradation of \( \text{Lysine-4,5-}^{3}\text{H} \)."

The finding that both of the carbon-hydrogen bonds at carbon 4 and one at carbon 5 remain intact during the biosynthesis of 5-hydroxylysine from lysine eliminates from consideration two of the four conceivable mechanisms for the hydroxylation mentioned in the introduction. Neither a mechanism involving an intermediate unsaturation between C-4 and C-5 nor one involving 5-ketolysine as an intermediate is possible. Nor can the hydroxylation proceed through a mechanism involving a Walden inversion at C-5.

At least two possible mechanisms, illustrated in Fig. 1, are consistent with our findings. There might be a direct uptake of atmospheric oxygen in a reaction catalyzed by an oxygenase, or there might be a dehydrogenation of the lysine between C-5 and C-6, followed by the addition of the elements of water to form hydroxylysine. Fujimoto and Tamiya (23) have reported that atmospheric \( ^{18} \text{O} \) is not incorporated into hydroxylysine in chick embryos, whereas either \( ^{3} \text{H} \) or \( ^{14} \text{C} \) from labeled water is incorporated. Their results indicate that the hydroxylation probably does not take place through an oxygenase mechanism. If it takes place by hydration of a double bond, our experiments show that it must be a C-5 to C-6 double bond. We have experiments under way at present to test this assumption.

The hydroxylation of lysine and proline show some points of similarity and some differences. Hydroxyproline originates solely from proline, as hydroxylysine does from lysine (24), but to hydroxylate proline the oxygen comes from atmospheric \( ^{18} \text{O} \) (25, 26) while according to Fujimoto and Tamiya (23) the oxygen to hydroxylate lysine comes from the water of the medium. With regard to the hydrogen atoms of proline that are labilized during hydroxylation, the first results were conflicting. The results of Ebert and Prockop (27) who worked with chick embryos and used proline tritiated on carbon atoms 3 and 4, "suggest that only one hydrogen is lost from proline during hydroxylation," but the authors consider the results complicated by possible randomization of the tritium to carbon atoms other than 3 and 4 in the tritiated proline used. Meister, Stone, and Manning (28) working with carrageenin-induced granuloma tissue, report results rather favoring labilization of 2 hydrogen atoms in the hydroxylation, but also reserve decision because of possible randomization of tritium in the proline employed.

FIG. 1. Two possible routes for the formation of hydroxylysine from lysine.

1 We are indebted to Dr. Meister for sending us a copy of his paper in advance of its publication.
Konno and Tetsuoka (29), in a single experiment in vivo with an agar-induced granuloma, found that 6.5 out of a theoretical 7.0 labeled hydrogen atoms were retained in the conversion of uniformly labeled proline-3H to hydroxyproline in rat tissue. All confusion appears to have been resolved by the recent preparation by Fujita, Gottlieb, Peterkofsky, Udenfriend, and Witkop (30) of both cis- and trans-4-3H-L-proline. Enzymatic hydroxylation in vivo with chick embryos led to 94% retention of tritium in cis-4-3H-L-proline and 98% loss of tritium in trans-4-3H-L-proline during the conversion to trans-4-hydroxy-L-proline. Apparently, the hydroxylating enzyme system converts proline to hydroxyproline in a front-side displacement with complete retention of configuration at C-4. 4-Keto-L-proline is certainly eliminated as a possible intermediate. As these authors point out, it would be desirable to study the conversion of lysine to hydroxylysine with selectively tritiated erythro- and threo-5-3H-lysine.

**SUMMARY**

Conversion of lysine to collagen hydroxylysine has been studied in vitro in polyvinyl sponge biopsy tissue obtained from rats and in vivo in chick embryos. After incubation of the tissue with DL-lysine labeled in the carboxyl group with 14C and on carbon atoms 4 and 5 with 3H, lysine and hydroxylysine were isolated from hydrolysates of the collagen. In both systems, the ratio of 3H to 14C in hydroxylysine was three-fourths of the ratio in lysine, indicating that in the conversion only the 1 hydrogen atom replaced by a hydroxyl group is lost. The results eliminate as mechanisms for hydroxylation of lysine either intermediate formation of 5-ketolysine or of an unsaturated double bond between carbon atoms 4 and 5. At least two other mechanisms of hydroxylation are consistent with present results, a direct attack of oxygen on carbon 5 or a dehydrogenation between carbon atoms 5 and 6 followed by addition of the elements of water.

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**REFERENCES**

The Formation of Collagen Hydroxylysine Studied with Tritiated Lysine
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