THE CONVERSION OF LYSINE TO HYDROXYLYSINE AND ITS RELATION TO THE BIOSYNTHESIS OF COLLAGEN IN SEVERAL TISSUES OF THE RAT

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(Received for publication, June 10, 1957)

Although hydroxylysine in general comprises only about 1 per cent of the weight of collagen, it deserves considerable attention for two reasons. First, it has been found in no protein other than collagen (1) and might therefore contribute to the properties of this protein. Second, there is a possible parallel in its biosynthesis and properties with hydroxyproline.

It has been shown by Stetten and Schoenheimer (2, 3) that proline is a precursor of hydroxyproline and that exogenous hydroxyproline is not utilized for collagen synthesis. The evidence suggested that the hydroxylation of proline occurs in bound form, perhaps in peptide linkage. This line of investigation was extended to hydroxylysine by Sinex and Van Slyke (4), who showed that hydroxylysine and lysine in skin collagen of the rat had the same specific activity after lysine-Cl4 had been fed. It was our purpose to determine whether free hydroxylysine can be utilized for collagen synthesis or whether it arises only by hydroxylation of lysine in bound form in parallel with hydroxyproline. During the preparation of our data for publication, Sinex and Van Slyke reported (5) that synthetic hydroxylysine labeled with H3 or Cl4 and included in the diet of rats was not incorporated into skin collagen.

We approached the problem by injecting lysine-C14 into rats in the presence of large amounts of the natural isomer of hydroxylysine and examining the specific activities of these amino acids in several collagens relative to a control group which did not receive hydroxylysine. If preformed, unbound hydroxylysine can be incorporated into collagen, the presence of large amounts of the free amino acid would decrease the contribution of lysine-C14 to collagen hydroxylysine. Data were also obtained concerning the lysine and hydroxylysine contents of different collagens from the rat, and the time sequence of lysine hydroxylation was investigated. This evidence bears upon the existence of a non-hydroxylated collagen precursor.
Experiment I—Five 50 gm. male Sprague-Dawley rats were each injected intraperitoneally with 15 mg. of δ-hydroxyl-L-lysine in 1 ml. of water and with an additional 9 mg. every hour for 5 hours. 15 minutes after the first injection of hydroxylysine, 9 μc. of L-lysine-U-C\textsuperscript{14} hydrochloride (25 μc. per mg.) in 0.5 ml. of water were injected. A control group of five animals received only the lysine-U-C\textsuperscript{14}. All the animals were killed by decapitation 1 hour after the last injection of hydroxylysine, the blood was collected in heparinized beakers, and the plasma was prepared for amino acid analysis as described by Stein and Moore (7). The rats were stored at -18° and skin, bone, tail tendon, and molar dentin were obtained and prepared as described below.

Experiment II—Nine 50 gm. male Sprague-Dawley rats were each injected intraperitoneally with 11 μc. of L-lysine-U-C\textsuperscript{14} hydrochloride (80 μc. per mg.) and killed by chloroform inhalation in groups of three, at 1 hour, 6 hours, and 4 days. They were kept at -18° and the skin collagen was prepared as described below.

Skin Collagen—Hair was removed from the backs of the frozen animals with electric clippers and by scraping with a scalpel. A rectangle which comprised the entire back was circumscribed. As the skin began to thaw, it was gently lifted and dissected free from fascia and muscle.

Tail Collagen The skin was stripped from the tail and the tail tendon was removed in several long strands from base to tip. Care was taken to exclude muscle.

Bone Collagen—The long bones were excised and carefully scraped to remove the periosteum. The ends were cut off below the epiphyseal plates and discarded. The shafts were split lengthwise and the marrow and endosteum removed under a dissecting microscope. The cleaned bones were decalcified in 10 per cent ethylenediaminetetraacetic acid, neutralized to pH 7.5 with NaOH. 100 ml. of the decalcifying solution were used for every 300 mg. of bone. The suspension was stirred for 48 hours at 5° and the residue washed with water.

Dentin Collagen—The first and second molars were sectioned transversely in situ just above the gingival attachment. The crowns were washed thoroughly, the pulp was removed by careful dissection, and the chamber cleaned with a strong jet of water. The crowns were crushed in a steel mortar to pass 60 mesh and the dentin and enamel were separated.

This material, the natural isotope isolated from gelatin (6), was kindly provided by Dr. P. B. Hamilton.

In a preliminary experiment it was found that hydroxylysine (synthetic, unresolved) injected intraperitoneally (50 mg. per 50 gm. rat) reached a maximal concentration in the plasma in less than 15 minutes, fell to two-thirds maximal in 1 hour, and was essentially zero in 6 hours.
by flotation (8) in a bromoform-acetone mixture of density 2.70. The
dentin was decalcified in the same manner as the bone.

Preparation of Gelatin—The collagen samples were minced with scissors,
water for about 30 minutes each, and then with several changes of acetone.
Gelatin was prepared by autoclaving the collagen in a sealed tube for 16
hours at 15 pounds pressure with about 20 ml. of water per gm. of collagen.
The gelatin solutions were filtered, concentrated on a rotary evaporator
to one-third their volume, and 10 volumes of acetone were added. The
precipitated gelatin was collected by centrifugation at 5° for 10 minutes
at 25,000 X g, and redissolved in water and lyophilized to give a more
easily handled product.

Before acetone precipitation, the gelatin from duplicate skin samples in
Experiment I and all the samples in Experiment II was dialyzed twice,
each for 24 hours, against 70 volumes of water at 5°. A small amount of
precipitate which formed in the cellophane bags was discarded.

The gelatin samples were hydrolyzed in sealed tubes with 6 N HCl (1
ml. per 10 mg. of protein) for 24 hours at 105°. The hydrolysates were
taken to a syrup once under a vacuum and made up to convenient volumes,
to contain about 10 mg. of protein per ml. They were kept frozen until
analyzed.

Determination of Lysine and Hydroxylysine—In general, the methods
used were modifications of those devised by Moore and Stein (9). Lysine
and hydroxylysine in about 50 mg. of protein were separated by elution
of a 30 X 0.9 cm. column of Dowex 50 X 12, minus 400 mesh, jacketed at
50°. The eluting buffer was 0.3 M sodium citrate, pH 4.80. 1.4 ml. frac-
tions were collected unless otherwise noted, diluted with 2.0 ml. of water,
and 1 ml. was taken for analysis with ninhydrin (10). The remainder was
saved for C14 analysis.

Determination of Proline and Hydroxyproline—The ion exchange separa-
tion previously described (11) for cyclic imino acids was used in a modified
form since only proline and hydroxyproline were present. A 50 X 0.9 cm.
column of Dowex 50X12, minus 400 mesh, jacketed at 50°, was eluted
with a citrate buffer of pH 3.30, 0.25 N in Na+. Under these conditions
hydroxyproline appeared in the effluent at about 35 ml. and proline at 75
ml. The acid hydrolysis resulted in about 2 per cent epimerization of the
hydroxyproline, the allo form appearing about 10 ml. after the natural
isomer (11). The 1 ml. fractions were analyzed with ninhydrin in glacial
acetic acid at room temperature (11) Procedure A).

Radioactivity Measurements—0.5 ml. aliquots from the diluted fractions

* Starting with known, pure solutions of either hydroxy-L-proline or allohydroxy-
proline, heating with 6 N HCl at 105° resulted in about 3 per cent epimerization in
1 day, 6 per cent in 2 days, and 9 per cent in 4 days.
were counted as previously described (12), except that further dilutions were not made and a correction for self-absorption was not necessary since it was found to be constant. As might be expected for a high molecular weight, randomly labeled molecule, the isotope effect which has been observed during the ion exchange chromatography of some amino acids (12) was not measurable with lysine or hydroxylysine. Duplicate or triplicate plates were made for each fraction and counted for sufficient time to provide a statistical counting error less than 5 per cent. The data are expressed in Tables I to III as the average count per minute per micromole of all fractions containing more than 0.25 \( \mu \) mole of lysine or hydroxylysine and in the graphs as counts per minute per fraction.

**Results**

The specific activities of lysine and hydroxylysine in rat plasma (Experiment I) are presented in Table I. At the end of the experimental period,

| Specific Activities and Concentrations of Lysine (Lys) and Hydroxylysine (HOLys) in Rat Plasma 6 Hours after Injection of Lysine-C\(^{14}\) in Presence and Absence of Exogenous Hydroxylysine |
|---|---|---|---|
| | C.p.m. per \( \mu \) mole | \( \mu \) moles per ml. plasma |
| HOLys injected | 15 | 1078 | 1.17 | 0.45 |
| Control | 0 | 1088 | 0 | 0.59 |

the group given hydroxylysine had more than twice as much hydroxylysine as lysine, whereas there was none, measured either with ninhydrin or C\(^{14}\) activity, in the control group. The magnitude of the count in the hydroxylysine was not determined accurately because of its low value, but there is little doubt that it represents labeled hydroxylysine since the radioactivity and ninhydrin color coincide. This is shown in Fig. 1.

The specific activities of lysine and hydroxylysine in collagen from skin, tail, bone, and dentin are given in Table II. It is apparent that in no case did the presence of exogenous hydroxylysine decrease the specific activity of collagen hydroxylysine relative to lysine. However, the specific activity of hydroxylysine was always lower than lysine in this 6 hour experiment, except in the case of bone. The possibility that the unequal specific activities might be explained by contamination with free lysine-C\(^{14}\) was examined by preparing a duplicate sample of skin collagen (Experiment I) and exhaustively dialyzing the gelatin before acetone precipitation. There was no significant change in the ratio of specific activities, although there was an increase in the mole ratio from 6 to nearly 7. This was probably
the result of precipitation of a non-representative fraction during dialysis. This evidence of protein heterogeneity is considered to be irrelevant to the discussion since the specific activities were unchanged.

![Effluent chromatogram](image-url)

**Fig. 1.** A portion of the effluent chromatogram from the ion exchange separation of the amino acids in 2.56 ml. of plasma from rats injected with lysine-$\mathrm{C}^{14}$ and $\delta$-hydroxy-L-lysine. The 1.8 ml. fractions were analyzed for total micromoles (●) and $\mathrm{C}^{14}$ activity (○).

**Table II**

<table>
<thead>
<tr>
<th></th>
<th>C.p.m. per μmole</th>
<th>N as per cent total N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HOLys</td>
<td>Lys</td>
</tr>
<tr>
<td>Skin (control)</td>
<td>0.133</td>
<td>0.182</td>
</tr>
<tr>
<td>&quot; (HOLys)</td>
<td>0.121</td>
<td>0.161</td>
</tr>
<tr>
<td>Tail (control)</td>
<td>0.087</td>
<td>0.103</td>
</tr>
<tr>
<td>&quot; (HOLys)</td>
<td>0.097</td>
<td>0.109</td>
</tr>
<tr>
<td>Bone (control)</td>
<td>0.073</td>
<td>0.073</td>
</tr>
<tr>
<td>&quot; (HOLys)</td>
<td>0.084</td>
<td>0.085</td>
</tr>
<tr>
<td>Dentin (combined)</td>
<td>0.032</td>
<td>0.037</td>
</tr>
</tbody>
</table>

A typical effluent chromatogram of a collagen sample (skin) from Experiment I is seen in Fig. 2. The ordinates were drawn to make the two lysine peaks coincide. The slightly lower specific activity of the hydroxylysine is readily apparent. The small peak preceding lysine was present in all chromatograms and presumably is ornithine, derived from arginine during hydrolysis.
Also included in Table II are analytical values for lysine and hydroxylysine in the several collagens. Although the totals are about the same, accounting for about 5 per cent of the total N, the ratio differs greatly, ranging from about 6 for skin to nearly 1 for dentin collagen. The hydroxylysine content of dentin collagen, 2.4 per cent by weight, is considerably higher than that found in other mammalian collagens. Proline and hydroxyproline were also measured to see whether their ratio varies in a similar manner. The following values were obtained (mole ratio hydroxyproline-proline): skin 0.80, bone 0.82, tail 0.79, and dentin 0.85. These ratios, which agree with the most recent data for other mammalian col-

Fig. 2. A portion of the effluent chromatogram from the ion exchange separation of the amino acids in a hydrolysate of rat skin collagen 6 hours after the injection of lysine-C<sup>14</sup>. The 1.4 ml. fractions were analyzed for total micromoles (●) and C<sup>14</sup> activity (○).

**TABLE III**

<table>
<thead>
<tr>
<th>Time</th>
<th>Lys</th>
<th>HOLys</th>
<th>Lys/HOLys</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.137</td>
<td>0.099</td>
<td>1.39</td>
</tr>
<tr>
<td>6</td>
<td>0.145</td>
<td>0.106</td>
<td>1.37</td>
</tr>
<tr>
<td>days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.150</td>
<td>0.144</td>
<td>1.04</td>
</tr>
</tbody>
</table>

Specific Activities of Lysine (Lys) and Hydroxylysine (HOLys) in Rat Skin Collagen at Various Times after Injections of Lysine-C<sup>14</sup>

The specific activities are given in counts per minute per micromole.
lagens and gelatins (13), do not differ significantly and provide evidence for the purity of the gelatin samples.

The effect of time on the ratio of the specific activities of lysine to hydroxylysine in skin collagen (Experiment II) is seen in Table III. At 1 hour and 6 hours the ratio is greater than 1, in agreement with Experiment I, but at 4 days the specific activities were nearly equal.

**DISCUSSION**

The question as to whether free hydroxylysine, present in the plasma, can be incorporated into collagen can be immediately answered in the negative on the evidence of the data in Table II. It might be argued that injected hydroxylysine does not reach the site of collagen synthesis, but since in our experiments the maintenance of a high plasma level served to trap endogenous hydroxylysine (presumably derived from collagen breakdown), it seems likely that there was mixing. Although the evidence is not unequivocal, it is reasonable to interpret the data as indicating that hydroxylysine is not incorporated into collagen from the free state regardless of source. This parallels the evidence with regard to hydroxyproline (3) and extends the demonstration by Sinex and Van Slyke (5) that synthetic hydroxylysine in the diet is not incorporated into collagen.

The fact that the specific activity of hydroxylysine was always less than lysine 6 hours after the injection of lysine-$^{14}C$ (Table II), except for bone, can be interpreted only in terms of heterogeneity if the probability that hydroxylysine arises only from lysine is accepted. And since contamination with free lysine-$^{14}C$ is essentially ruled out, the results would show the presence of a bound form of lysine which is an intermediate in the conversion to hydroxylysine. Since Sinex and Van Slyke (4) found equal specific activities after 14 days, it would appear that the conversion is not complete in 6 hours. This was confirmed by the time study seen in Table III.

Since a certain amount of purification of the collagen is obtained by washing, converting to gelatin, and dialysis, it may reasonably be assumed that the bound lysine intermediate is a collagen-like substance. We cannot completely rule out the possibility of contamination with a metabolically active protein that is unrelated to collagen and not an intermediate. However, this is highly unlikely since the amount required is rather large. For example, the specific activity ratio seen at 6 hours in skin (Table II or III) could have arisen only if 5 per cent of a contaminating protein (the same lysine content being assumed) eight times as active as the collagen were present. In addition, it would have to have a half life considerably less than 4 days.

Further evidence for a collagen-like precursor containing no hydroxy-
lysine may be inferred from the analytical data for lysine and hydroxylysine in Table II. Although the ratio of the two differs greatly, the sum is similar for collagen from the four sources. Possibly a similar precursor is involved which is hydroxylated to different degrees and at different rates in different tissues. Whether proline follows a similar time-course is not, of course, answered by our data, but the possible parallel is self-evident.

It is probable that the degree of incorporation of lysine as represented in Tables II and III is not quantitatively accurate. If the hydroxylation of both proline and lysine is a slow reaction, a whole series of intermediates, ranging from completely unhydroxylated to fully hydroxylated, could be formed. The purification steps might fractionate them to reject the least collagen-like, which in short time periods would be highly radioactive. This could account for the observation (Table III) that the apparent incorporation of lysine-C\(^{14}\) after a single injection is nearly the same at all time periods; that is, from samples taken at 1 hour and to a lesser degree at 6 hours, a highly labeled precursor fraction might have been lost. This would also mean that the specific activity ratios are minimal values.

The idea of a precursor collagen in which the proline and lysine are hydroxylated after the formation of peptide linkages results directly from the work of Stetten and Schoenheimer (2, 3) and is strengthened by the evidence of Sinex and Van Slyke (4, 5). Recently Gould and Woessner (14) have presented preliminary material which they interpret as supporting this mechanism. Our findings, which can be fully explained on this basis, offer further support for the existence of such a precursor.

**SUMMARY**

Collagen from skin, tail tendon, bone, and teeth (dentin) of rats given lysine-C\(^{14}\) has been analyzed for lysine and hydroxylysine with the following findings.

1. After 6 hours the specific activity of hydroxylysine was less than lysine except in bone, where it was equal.
2. In skin the ratio of the specific activities of lysine to hydroxylysine changed from 1.37 at 6 hours to 1.04 at 4 days.
3. The presence of large amounts of exogenous hydroxylysine in the plasma did not alter the degree of conversion of lysine to hydroxylysine in any of the collagens.
4. Exogenous hydroxylysine acted as a trapping agent for the endogenous amino acid, as shown by the presence of C\(^{14}\)-labeled hydroxylysine in the plasma.
5. The ratio of total lysine to hydroxylysine was 6.0 in skin, 3.9 in tail tendon, 1.9 in bone, and 1.1 in dentin, although the sum was essentially the same in all four collagens.
These results are interpreted as indicating that free hydroxylysine cannot be used for collagen synthesis, lysine providing the only source. The conversion apparently takes place in bound form, and may involve the hydroxylation of a collagen-like precursor originally containing no hydroxylysine and perhaps no hydroxyproline. The hydroxylation of lysine proceeds at different rates and degrees in different collagens from the same animal.

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