

# The Source and State of the Hydroxylysine of Collagen

## II. FAILURE OF FREE HYDROXYLYSINE TO SERVE AS A SOURCE OF THE HYDROXYLYSINE OR LYSINE OF COLLAGEN\*

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The amino acid 5-hydroxylysine, first isolated from gelatin (2),<sup>1</sup> has been found absent in all types of proteins other than collagen that have been examined (3). It is not an essential amino acid for animal nutrition (4), and hence must be formed from other material in the animal body.

Previous papers from this laboratory (5, 6) have shown that lysine is the chief source of the hydroxylysine incorporated into collagen. When uniformly C<sup>14</sup>-labeled lysine was administered to young rats, either with food (5) or by injection (6), both lysine and hydroxylysine in the skin collagen were labeled to an approximately equal extent, indicating that lysine serves as the chief, and probably only significant, source of hydroxylysine in rat skin collagen. The relative rates of labeling of the two amino acids indicated that the hydroxylation of lysine to form hydroxylysine occurs simultaneously, or nearly so, with incorporation into the collagen (6). The results were confirmed by Piez and Likins (7), with regard to rat collagen of skin, tail, bone, and dentin, and by Kao and Boucek (8) with regard to collagen formed in implanted polyvinyl sponges.

The questions remained, whether administered free hydroxylysine can either be incorporated into the hydroxylysine or collagen, or be dehydroxylated, by reversion of the process by which lysine is hydroxylated, and incorporated into the lysine of collagen. With regard to the latter question, the probability of a negative answer is indicated by a feeding experiment by Lindstedt (9). He found that synthetic DL-hydroxylysine ( $\frac{1}{2}$  allo isomer) could not replace an equimolar amount of L-lysine in maintaining the growth of rats. The possibility remained, however, that the hydroxylation of lysine might be to some extent reversible.

The object of the work reported in the present paper has been

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<sup>1</sup> We take occasion to correct a figure in the paper (2) in which the isolation of hydroxylysine in crystalline form, as picrate and monochloride, was first reported. It was stated that hydroxylysine when analyzed for amino nitrogen by the nitrous acid method (3) reacted with 95 per cent of its nitrogen in 3 minutes at 25°, under conditions that give 100 per cent reaction with the  $\alpha$  amino groups of the amino acids. Van Slyke and Plazin (unpublished results) find that hydroxylysine entirely free from lysine reacts with 100 per cent of its nitrogen in 3 minutes at 25°, the  $\epsilon$  amino nitrogen reacting as rapidly as the  $\alpha$ -nitrogen.

to ascertain by isotope analysis whether hydroxylysine administered either orally or by injection to growing rats is incorporated into either the hydroxylysine or the lysine of collagen. Tritium-labeled hydroxylysine has been administered to young rats and the extent of incorporation of the label into the lysine and hydroxylysine of the skin collagen has been measured. The amount of tritium found in either the hydroxylysine or lysine of the collagen was so slight that it does not appear that free hydroxylysine is to a significant extent incorporated into collagen as either hydroxylysine or lysine.

The results are consistent with the deduction of Piez and Likins (7), who observed that the C<sup>14</sup> content of the hydroxylysine isolated from collagen after injection of C<sup>14</sup>-labeled lysine into rats was not altered by accompanying injections of unlabeled hydroxylysine. Piez and Likins reasoned that "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-C<sup>14</sup> to collagen hydroxylysine."

### EXPERIMENTAL

Two experiments were carried out. In each experiment tritium-labeled hydroxylysine was given to four young rats, and the relative extents of incorporation of labeled material into the lysine and hydroxylysine of the skin collagen were measured.

In Experiment I (Table I) the labeled hydroxylysine was administered to rats of 50 to 60 gm. weight by addition to the daily diet for 2 weeks before the rats were killed. The hydroxylysine was a synthetic preparation labeled with tritium attached to carbon 6.

In Experiment II (Table II) the labeled hydroxylysine was given by a single intraperitoneal injection and the rats were killed 4 hours later. The hydroxylysine was a preparation that had been tritiated by the method of Wilzbach (10), and was more radioactive than that used in Experiment I.

#### *Experiment I*

*Preparation of Hydroxylysine Labeled with Tritium Attached to C-6—DL-Hydroxylysine* was prepared by the method of Touster (11) as modified by Lindstedt (12). However, instead of using KC<sup>14</sup>N, as was done by Lindstedt to label the carbon, we employed tritium gas for reduction of the terminal —CH(OH)·CN group to —CH(OH)—CH<sub>2</sub><sup>3</sup>(NH<sub>2</sub>). Tritium is introduced by the reduction into the —CH<sub>2</sub>·NH<sub>2</sub> group and, by exchange, into the OH group, but in the NH<sub>2</sub> and OH groups is replaced by H<sup>1</sup> in subsequent operations in water solution, leaving H<sup>3</sup> stably bound only in the CH<sub>2</sub> group. The hydroxylysine obtained

was presumably a mixture of equal amounts of DL-*n*- and DL-allohydroxylysine. The specific activity of the preparation was 43.8  $\mu\text{c.}$  per mmole.

**Treatment of Rats**—A rat, weighing 61 gm. at the start, consumed in 2 weeks 250 gm. of the "basal" zein diet of Henderson *et al.* (13), referred to by them as supplement B, to which 2.5 gm. of unlabeled lysine and 0.134 gm. of tritium-labeled synthetic DL-hydroxylysine hydrochloride were added. On this diet, the rat grew 43 gm. in the 2 weeks. The approximate amount of hydroxylysine incorporated is estimated by assuming that 35 per cent of the growth was dry solids, of the solids  $\frac{1}{3}$  was protein, of the protein  $\frac{1}{3}$  was collagen, and of the collagen 0.01 was L-hydroxylysine. The incorporated L-hydroxylysine thus estimated is 17 mg. The amount of L-*n*-hydroxylysine in the food consumed was 33.8 mg.

The conditions of the experiment were similar to those of our previous experiments (5), in which labeled lysine was fed, except that in the present experiment it is the hydroxylysine that is labeled.

**Preparation and Analysis of Skin Collagen**—The preparation of gelatin from skin collagen previously described (6) was followed, with precipitation of nongelatin proteins from the gelatin solution by trichloroacetic acid. The gelatin was not precipitated with acetone. Hydrolysis of gelatin and precipitation of the basic amino acids were carried out as previously described (5). The basic amino acids were chromatographed on Dowex 50 in 0.1 M citrate at pH 5. This procedure, as shown by Hamilton (14) gives good separation of lysine and hydroxylysine from each other and from histidine and arginine. The solutions of lysine and hydroxylysine were desalted with Dowex 50 as previously described (6) and eluted in 6 N HCl, which was removed by concentration in a vacuum and drying over KOH. The dihydrochlorides of lysine and hydroxylysine thus obtained were freed of chloride by treatment with silver sulfate, followed by barium hydroxide to remove  $\text{SO}_4$ , and by  $\text{CO}_2$  to remove barium. To the solution of each amino acid thus obtained exactly enough picric acid was added to form the monopicate, which was crystallized by concentrating the solution. Samples of the picrates were analyzed for tritium by the method of Christman (15), in which the hydrogen is set free by heating with metallic zinc and is counted in the gas counter of Bernstein and Ballantine (16).

### Experiment II

**Preparation of Tritium-Labeled Hydroxylysine**—In order to obtain hydroxylysine of greater specific activity for the injection experiment synthetic DL-hydroxylysine was treated with tritium gas according to the procedure of Wilzbach (10). A gm. of DL-hydroxylysine was exposed to 1.2 curies of tritium gas at room temperature and a pressure of 0.33 atmosphere for 14 days.<sup>2</sup> In order to remove labile tritium the resulting material was dissolved in 5 successive portions of 6 ml. each of distilled water, and each portion of water was removed by evaporation under a vacuum at room temperature.

Chromatography in phosphate buffer by the procedure of Hamilton and Anderson (17) gave a preparation that contained approximately 70 per cent of DL-*n*-hydroxylysine and 30 per cent of the allo isomers.

This material, 10 mg., was rechromatographed and the phos-

<sup>2</sup> The preparation was carried out by Dr. Seymour Rothstein of the New England Nuclear Corporation, Boston, Massachusetts.

TABLE I

Proportions of lysine and hydroxylysine in skin collagen derived from labeled lysine or hydroxylysine in food

Labeled amino acids in food				Labeled amino acids in skin collagen			
Amino acid	Specific activity of pure amino acid (a)	L-Normal amino acid in 100 gm. of food*	Duration of feeding	Specific activity		Derived from labeled amino acid in food	
				Hydroxylysine (b)	Lysine (c)	Hydroxylysine 100 b/a	Lysine 100 c/a
	$\mu\text{c./mmole}$	mmoles	days	$\mu\text{c./mmole}$	$\mu\text{c./mmole}$	%	%
DL-Hydroxylysine	95.8	0.068	14	$4.10 \times 10^{-3}$	$3.78 \times 10^{-3}$	0.0042	0.0041
L-Lysine†	4.83	6.84	21	0.98	0.98	20.3	20.3

\* Amount of L-*n*-hydroxylysine is calculated as  $\frac{1}{2}$  the DL-isomer mixture.

† The lysine data are from Sinex and Van Slyke (5).

TABLE II

Proportions of lysine and hydroxylysine in skin collagen derived from labeled amino acids intraperitoneally injected

Labeled amino acid injected				Labeled amino acids in skin collagen			
Amino acid	Specific activity (a)	Amount injected per rat		Specific activity		Derived from injected amino acid	
		Total	L-Normal	Hydroxylysine (b)	Lysine (c)	Hydroxylysine 100 c/a	Lysine 100 c/a
	$\mu\text{c./mmole}$	mmole	mmole	$\mu\text{c./mmole}$	$\mu\text{c./mmole}$	%	%
DL-Hydroxylysine*	$9.62 \times 10^3$	0.0103	0.0036	$2.55 \times 10^{-2}$	$2.40 \times 10^{-3}$	$2.63 \times 10^{-4}$	$2.50 \times 10^{-5}$
L-Lysine†	153	0.105	0.105	0.518	0.732	0.34	0.48

\* Preparation with 70 per cent DL-*n*-isomer, 30 per cent DL-allo isomer.

† From data on 4-hour rats of Van Slyke and Sinex (6).

phate buffer removed according to the procedure of Mueller *et al.* (18). Isolation and counting of the sample of monopicate indicated that the specific activity was  $9.62 \times 10^3 \mu\text{c.}$  per mmole of hydroxylysine.

**Treatment of Rats**—Hydroxylysine, 6.68 mg., in four approximately equal portions was injected intraperitoneally into four rats of 60 to 66 gm. weight. Since 70 per cent of the hydroxylysine in the preparation was the DL-*n* form, it is calculated that each rat received 0.59 mg. of L-*n*-hydroxylysine. The rats were killed 4 hours after the injection.

**Preparation and Analysis of Skin Collagen**—The procedure was the same as in Experiment I, except that in Experiment II unlabeled DL-hydroxylysine was added to the gelatin solution to produce a concentration of 0.15 per cent of hydroxylysine in the solution before treatment of the latter with trichloroacetic acid. The addition was made with the thought that the free unlabeled hydroxylysine might displace from the gelatin any of the highly active tritium-labeled hydroxylysine that might have been adsorbed or otherwise nonspecifically bound by the collagen *in vivo* from the injected labeled hydroxylysine (however, the work of Cornwall and Luck (19) appearing after these experiments

were done indicates that addition of the amino acid before dialysis probably had no influence on the results). In order to insure complete removal of the added inactive hydroxylysine, the gelatin solution was dialyzed against 5 successive changes of distilled water instead of the 3 changes used previously (6) and in Experiment I.

Preparation of the picrates of lysine and hydroxylysine from the hydrolyzed collagen, and determination of their activities by Christman's method were as in Experiment I.

#### DISCUSSION

##### *Results of Experiment I. Administration of Labeled Hydroxylysine in Diet*

The results of feeding labeled hydroxylysine are shown in the upper row of figures in Table I. For comparison the results of feeding labeled lysine previously reported (5) are shown in the lower row of figures in the table. The percentages of the lysine and hydroxylysine residues of skin collagen derived from the labeled amino acids in the food are calculated as

$$\frac{\text{specific activity of amino acid isolated}}{\text{specific activity of amino acid fed}} \times 100.$$

The results show that incorporation of fed hydroxylysine into skin collagen was insignificant compared with incorporation of fed lysine. After feeding lysine the activities of both the lysine and the hydroxylysine of the collagen were 20.3 per cent of the activity of the fed lysine, whereas after feeding hydroxylysine the activities of both the lysine and hydroxylysine in the collagen were only 0.004 per cent of the activity of the fed hydroxylysine.

The fact that after feeding labeled hydroxylysine the specific activities of lysine and hydroxylysine in the collagen were equal could be interpreted to indicate that about  $\frac{2}{3}$  of the incorporated hydroxylysine was reduced to lysine during the process of incorporation, a reversal of the hydroxylation of lysine that was noted after either feeding (5) or injecting (6) labeled lysine. However, the results of injecting hydroxylysine in Experiment II do not support such an interpretation. It appears more probable that a small part of the fed hydroxylysine was reduced to lysine by bacterial action in the intestine, and that this lysine was the source of the small amounts of labeled lysine and hydroxylysine that were incorporated into the collagen.

Correction for the effect of the greater amount of labeled lysine fed does not alter the significance of the difference in incorporation between the two amino acids. The amount of L-hydroxylysine in the hydroxylysine feeding experiment was only  $\frac{1}{100}$  of the amount of L-lysine in the lysine feeding experiment, but was equivalent, as a source of collagen hydroxylysine, to  $\frac{1}{11}$  the amount of lysine fed, because only  $\frac{1}{3}$  of incorporated lysine is changed to hydroxylysine (5, 6). If hydroxylysine were incorporated to the same extent as lysine per unit of each present, the percentage of labeled collagen hydroxylysine derived from fed hydroxylysine would therefore be  $\frac{1}{11}$  the percentage derived from fed lysine. However, the percentage, 0.004, in the hydroxylysine feeding (upper row, Table I) is only  $\frac{1}{5000}$  the percentage, 20.5, in the lysine feeding (lower row, Table I).

##### *Results of Experiment II. Intraperitoneal Injection of Labeled Hydroxylysine*

In Table II the results of labeled hydroxylysine injection in Experiment II are given in the upper row of figures. In the lower row are given for comparison the results of a previous experiment (6) in which labeled lysine was injected. In both experiments the rats were killed 4 hours after the injections.

The incorporation of labeled injected hydroxylysine into the collagen was quantitatively insignificant compared with the incorporation of labeled lysine. Correcting for the difference in amounts of labeled L-lysine and L-hydroxylysine injected, as in the discussion of Experiment I, one would expect the percentage of collagen hydroxylysine derived from injected hydroxylysine to be  $\frac{1}{12}$  as great as the percentage of collagen lysine derived from injected lysine, if the same proportion of injected hydroxylysine as of lysine were incorporated. Actually after injection of the labeled hydroxylysine the percentage of collagen hydroxylysine derived from the injected material was only  $\frac{1}{1300}$  as great as after injection of labeled lysine.

The results (upper row, Table II) indicate that a slight but measurable amount of the injected hydroxylysine was taken up directly by the skin collagen. This conclusion is indicated by the fact that the specific activity of the hydroxylysine isolated from the collagen was about 10 times the specific activity of the isolated lysine. This activity ratio excludes the possibility that the labeled hydroxylysine found in the collagen was formed, as suggested for fed hydroxylysine in Experiment I, via intestinal reduction to lysine and incorporation of the resultant lysine accompanied by its partial hydroxylation. It is possible that the small amount of labeled hydroxylysine found in the collagen was taken up by a nonspecific adsorption or combination of the amino acid with the collagen such as that studied *in vitro* by Cornwall and Luck (19). These authors have shown that the proteins, histone and insulin, in solution can form combinations with small amounts of phenylalanine and lysine that are not dissociated by precipitation and dialysis. Whatever the mechanism of the observed combination of collagen with injected hydroxylysine, the proportion combined is too slight to indicate that direct incorporation of free hydroxylysine can serve as a physiologically significant source of collagen hydroxylysine.

#### SUMMARY

Feeding or injecting radioactive hydroxylysine into young rats did not lead to the incorporation of significant amounts into the hydroxylysine of the skin collagen. Slight amounts of radioactive hydroxylysine and lysine were found in the collagen hydrolysates, but the amounts were so small, compared with those obtained after similar administration of radioactive lysine, that they excluded free hydroxylysine as a physiologically significant source of either hydroxylysine or lysine in the collagen.

The results reinforce the conclusion reached by the authors from experiments with labeled lysine (5, 6), that the hydroxylysine of collagen in the rat originates from hydroxylation of lysine during formation of the collagen.

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