HYDROXYLATION OF LYSINE IN A POLYPEPTIDE PRECURSOR OF COLLAGEN

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Observations in several laboratories (see Lukens, 1965) suggest that the hydroxyproline in collagen is synthesized by the hydroxylation of proline after proline is incorporated into a polypeptide form. We have reported (Prockop and Juva, 1965; Juva and Prockop, 1965 a and b) the partial isolation and characterization of "protocollagen", a polypeptide precursor of collagen which serves as a substrate for the enzymatic synthesis of protein-bound hydroxyproline. Hydroxylysine is similar to hydroxyproline in that essentially all the hydroxylysine in animal tissues is found in collagen, and in that free hydroxylysine is poorly incorporated into collagen (see Sinex et al., 1959). We now report results which suggest that hydroxylysine is synthesized by an oxygenase mechanism similar to the one which has been demonstrated for hydroxylation of proline (Fujimoto and Tamiya, 1962; Prockop et al., 1964), and that the hydroxylation probably occurs in the same polypeptide precursor of collagen which is a substrate for the hydroxylation of proline.

Tibiae which consisted primarily of cartilage were removed from 10-day old chick embryos by microscopic dissection under sterile conditions. In experiments on the synthesis of hydroxylysine by the whole tissue, two tibiae were incubated with 5 μcuries L-lysine-C^{14}, 220 μcuries per μmole (New England Nuclear Corp.), in 2.5 ml of simple medium containing glucose, inorganic salts, and bicarbonate-phosphate buffer (Prockop and Juva, 1965). After incubation at 37° for 1 hour, the tibiae were homogenized in distilled water, and the homogenates were dialyzed, hydrolyzed, and evaporated in vacuo.

Aliquots of samples were chromatographed on Whatman #3 paper in a descending system with phenol-water, pH 4 to 5. The observed R_f values for L-lysine and δ-DL-allo-hydroxylysine (Nutritional Biochemical Corp.) were 0.46 and 0.19, respectively. In order to improve the separation, the development of the chromatograms was not interrupted until about 8 hours after the solvent front reached the end of the paper. The chromatograms were cut into one-half inch strips, and the strips were counted with 20 ml toluene and 1 ml phosphor solution in a liquid scintillation counter.

Incubation of the tibiae under nitrogen for 1 hour reduced the total lysine-C^{14} incorporation by about one-third, but the presence of the ferrous chelator, α,α'-dipyridyl, did not affect the lysine-C^{14} incorporation (Table I). When tibiae were incubated with lysine-C^{14} under control conditions, 15 to 18% of the non-dialyzable C^{14} in the tissue was recovered as hydroxylysine-C^{14} (Figure 1A). When samples were incubated with α,α'-dipyridyl, less than 0.5% of the non-dialyzable C^{14} was recovered as hydroxylysine-C^{14} (Figure 1B). When samples were incubated under nitrogen, less than 6% of the non-dialyzable C^{14} was recovered as hydroxylysine-C^{14} (not shown).

In order to prepare lysine-labeled protocollagen, tibiae were pre-incubated at 37° with α,α'-dipyridyl, 1 x 10^{-3} M, for 1 hour, and then 30 μcuries lysine-C^{14} were added for an incubation period of 1 hour. The tissues were homogenized in 18 ml water and the homogenate was stored a 4° overnite. The sample was then centrifuged at 100,000 x g for 1 hour, and the pellet and "soluble fraction" were recovered. The soluble fraction was dialyzed twice against 4 liters of 1 M KCl, and then the pellet and soluble fraction were each incubated for 1 hour at 37° in the hydroxylating system previously used for the hydroxylation of proline-labeled protocollagen (Figure 1). After the incubation, the samples were extracted twice at 90° for 30 minutes with 5% trichloracetic acid (TCA). The TCA extracts were dialyzed, hydrolyzed, evaporated, and chromatographed as described above. After incubation with the hydroxylating system, the hydroxylysine-C^{14} in the pellet fraction in-

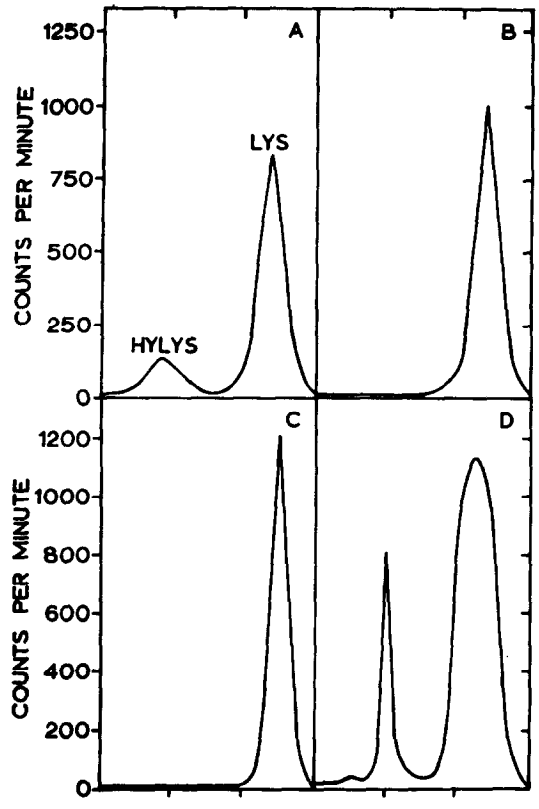

Figure 1: Radiochromatograms of whole tibiae and of hydroxylated "soluble fraction". (A) Hydrolysate of tibiae incubated with Lysine-C^{14} under control conditions as described in text. (B) Hydrolysate of tibiae incubated with lysine-C^{14} in the presence of α,α'-dipyridyl, 1 x 10^{-3} M. (C) Control sample for incubation of lysine-labeled soluble fraction with hydroxylating system. (D) Test sample for incubation of lysine-labeled soluble fraction with hydroxylating system.

In (C) the soluble fraction was added to the hydroxylating system at 4^{o} and the sample was immediately precipitated by adding TCA. In (D) the soluble fraction was incubated at 37^{o} for 1 hour with the hydroxylating system. The hydroxylating system consisted of 6 ml of a soluble protein preparation from chick embryos; 0.05 M tris buffer, pH 7.6; 0.5 M KCl; $FeSO_4$, 5 x 10^{-4} M; ascorbic acid, 1.5 x 10^{-5} M; and EDTA, 5 x 10^{-8} M. The soluble protein preparation from chick embryos was prepared by homogenizing 11-day old chick embryos briefly in a Servall macrohomogenizer, centrifuging the homogenate at 15,000 x g for 15 min., and then re-centrifuging the supernate at 100,000 x g for 1 hour.

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creased from less than 1% of the total C^{14} to 7% (not shown). With the soluble fraction the hydroxylysine-C^{14} increased from less than 1% of the total C^{14} (Figure 1C) to 24% (Figure 1D).

The synthesis of hydroxylysine in embryonic cartilage was inhibited by anaerobiosis and α,α'-dipyridyl, two conditions which also inhibited the synthesis of hydroxyproline (Prockop and Juva, 1965; Juva and Prockop, 1965b). Inhibition of hydroxylysine synthesis by anaerobic conditions is inconsistent with the report (Fujimoto and Tamiya, 1963) that atmospheric oxygen is not involved in the hydroxylation, but studies (Popenoe et al., 1965) with specifically-labeled lysine-H^3 have not excluded an oxygenase mechanism, and they are consistent with the present finding.

Table I. Effect of Anaerobic Conditions and α,α'-Dipyridyl on the Incorporation of Lysine-C^{14}.

Additions to medium	Atmosphere	Total C^{14} cpm x 10^{-4}
None	air	6.1
None	N_2	4.3
α,α'-dipyridyl, 1 x 10^{-3} M	air	6.3

The substrate for the synthesis of hydroxylysine was not fully characterized, but it was prepared under the same conditions as the polypeptide substrate for the hydroxylation of proline (Prockop and Juva, 1965). The lysine-labeled preparations were obtained from both soluble and particulate fractions of cartilage in which the hydroxylation was inhibited. The hydroxylysine-C^{14}-containing material recovered after the hydroxylation was similar to protocollagen and collagen in that it was extractable in a non-dialyzable form with hot TCA. The following observations have been made with proline-labeled protocollagen (Juva and Prockop, 1965 a and b; Juva et al., 1965): (a) a 20-fold purified preparation of the hydroxylating enzyme required ferrous iron, potassium, and one or more unidentified cofactors; (b) synthetic polytripeptides of the structure $(gly\text{-}pro\text{-}pro)_n$ and with molecular weights of 4,000 or greater were hydroxylated under the same conditions as proline-labeled

protocollagen; (c) protocollagen preparations did not contain significant amounts of RNA, and they were hydrolyzed by a highly-specific collagenase; (d) partially purified protocollagen had a molecular weight of greater than 20,000, and it readily formed large, insoluble aggregates which were also hydroxylated; (e) autoradiographic studies indicated that when the hydroxylation of proline was inhibited, labeled protocollagen accumulated within the cells synthesizing collagen. Our results suggest that hydroxylysine is synthesized under similar conditions as hydroxyproline.

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