SEQUENTIAL HYDROXYLATION OF LYSINE AND GLYCOSYLATION OF HYDROXYLYSINE DURING THE BIOSYNTHESIS OF COLLAGEN IN ISOLATED CARTILAGE*

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Recent studies have demonstrated that the hydroxyproline and hydroxylysine in collagen are synthesized by the hydroxylation of proline and lysine after these amino acids have been incorporated into a large polypeptide precursor of collagen called protocollagen (for review see 1). Although other laboratories have presented conflicting data (2-5), several independent studies in our laboratory (6-9) have suggested that most or all of the synthesis of hydroxyproline in collagen occurs after protocollagen polypeptides are released from ribosomal complexes. Since recent reports (10-15) have demonstrated that collagen contains galactose or glucosylgalactose O-glycosidically linked to hydroxylysine, it was of interest to examine the time sequence for the hydroxylation of lysine and the glycosylation of hydroxylysine during collagen biosynthesis.

Cartilagenous tibiae from 10-day-old chick embryos were incubated with ^{14}C-lysine as described in Table I, and the tissues were immediately homogenized in 4.0 ml distilled water containing 1 mM α,α'-dipyridyl

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Table I. Incorporation of ^{14}C-lysine and synthesis of ^{14}C-hydroxylysine in pulse-labeled embryonic cartilage. Tibiae were pre-incubated for 2 hr (16), and then they were pulse-labeled by transferring into 2.5 ml medium containing 60 μc ^{14}C-L-lysine (uniformly labeled; New England Nuclear), 223 μc per μmole. Larger numbers of tibiae were used for brief pulse-labeling periods in order to provide enough ^{14}C for adequate counting.

Pulse time min	Tibiae in sample #	Total ^{14}C-lys dpm x 10^{-4}	Free ^{14}C-hylys recovered: Acid hydrol. dpm x 10^{-3}	Alk. hydrol. dpm x 10^{-3}
1	43	2.62	0.76	
3	20	10.1	7.94	7.36
5	10	8.36	10.8	9.09
10	10	23.4	44.3	20.6
120	5	91.4	191.	79.2

in order to prevent further hydroxylation of protocollagen (6). The homogenates were dialyzed against running tap water for 15 hr, against distilled water for 15 hr, and against 1 mM $CaCl_2$ for 3 hr. The dialyzed samples were incubated at 37° for 15 hr with 30 to 100 μg per ml purified collagenase (Worthington Biochemical Corp.) in 1 mM $CaCl_2$ and 50 mM Tris-HCl buffer, pH 7.6. The samples were heated at 60° for 20 min, and the insoluble proteins were removed by centrifuging at 15,000 x g for 15 min. Aliquots of the collagenase solubilized peptides in the supernatant fractions were then hydrolyzed under either acidic or alkaline conditions. Acid hydrolysis was carried out by adding 4 volumes of 3-times distilled HCl, and heating at 108° for 16 hr in tubes sealed under N_2. Alkaline hydrolysis was carried out by adding solid $Ba(OH)_2$ to a final concentration of 5 N, and heating at 102° for 16 hours in alkali-resistant tubes (Corning 7280) sealed under N_2. Preliminary experiments indicated that the conditions for acid hydrolysis did not produce any destruction of free hydroxylysine, and that the conditions for alkaline hydrolysis completely hydrolyzed the collagenase solubilized peptides, since the yield of ^{14}C-lysine was the same as with acid hydrolysis. The acid hydrolyzed samples were evaporated in a vacuum, and the

^{14}C-hydroxylysine and ^{14}C-lysine in the hydrolysates were separated by ion exchange chromatography on a Beckman Model 116 amino acid analyzer. Aliquots of the column eluates were counted in a liquid scintillation counter. The alkali hydrolyzed samples were desalted on a Dowex-50 column (hydrogen form) before chromatography.

In the tibiae incubated with ^{14}C-lysine for 2 hr, 30 to 35 % of the non-dialyzable ^{14}C was recovered in the collagenase solubilized peptides. Ion exchange chromatography of acid hydrolysates of the collagenase solubilized peptides indicated that 88 to 95 % of the peptide-bound ^{14}C was recovered as ^{14}C-hydroxylysine and ^{14}C-lysine. Acid hydrolysis of pulse-labeled tibiae demonstrated that the synthesis of ^{14}C-hydroxylysine consistently lagged behind the incorporation of ^{14}C-lysine into collagenase solubilized peptides (Table I and Figure 1). The ratio of ^{14}C-hydroxylysine to ^{14}C-hydroxylysine plus ^{14}C-lysine was 2.8 % in 1 min, and the maximal values of 15.9 to 17.3 % were not reached until the tibiae were pulse-labeled for 10 min or more. Similar results were obtained in two additional experiments.

In the samples pulse-labeled for 3 to 10 min, there was a progressive decrease in the fraction of total ^{14}C-hydroxylysine converted to free ^{14}C-hydroxylysine by alkaline hydrolysis (Table I and Figure 1). In tibiae pulse-labeled for 3 min, essentially all the total ^{14}C-hydroxylysine was released by alkaline hydrolysis. In tibiae pulse-labeled for 10 min, only 46 % of the ^{14}C-hydroxylysine was recovered as free ^{14}C-hydroxylysine. In tibiae pulse-labeled for 120 min, about 42 % of the total ^{14}C-hydroxylysine was recovered. Similar results were obtained in two additional experiments.

Further studies demonstrated that the ^{14}C-hydroxylysine which was not converted to free ^{14}C-hydroxylysine by alkaline hydrolysis was resistant to periodate oxidation (12). Ion exchange chromatography

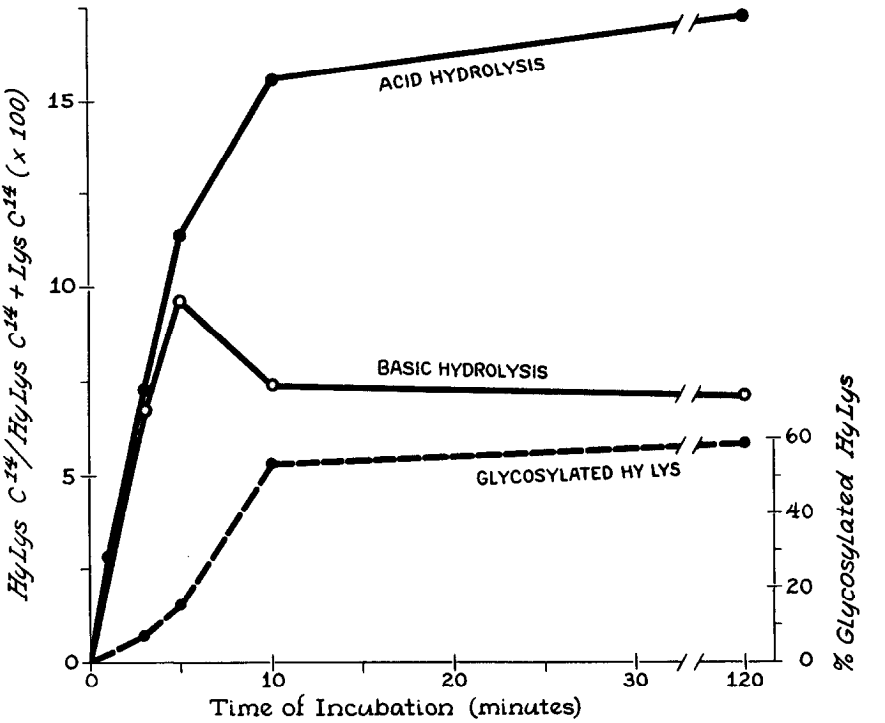

Fig. 1: Incorporation of ^{14}C-lysine and synthesis of ^{14}C-hydroxylysine in pulse-labeled embryonic cartilage. The % of total ^{14}C-hydroxylysine which was glycosylated (broken line) is equivalent to the fraction of total ^{14}C-hydroxylysine not recovered as free ^{14}C-hydroxylysine after alkaline (basic) hydrolysis (see Table 1 and text).

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of alkali hydrolyzed samples indicated that the ^{14}C-hydroxylysine which resisted alkaline hydrolysis was recovered primarily in two peaks corresponding to the chromatographic positions of galactosyl-hydroxylysine and glucosylgalactosyl-hydroxylysine (14). These data indicate that the ^{14}C-hydroxylysine which was not converted to free ^{14}C-hydroxylysine by alkaline hydrolysis corresponded to ^{14}C-hydroxy-lysine which was substituted with galactose or glucosylgalactose (Figure 1).

The time course for the incorporation of ^{14}C-lysine and the synthesis of ^{14}C-hydroxylysine is indistinguishable from that observed

with the incorporation of ^{14}C-proline and the synthesis of ^{14}C-hydroxyproline in the same system (6). Since in this system the time required to synthesize a complete collagen polypeptide of 100,000 Daltons is about 1 min (6), the results suggest that most or all of the hydroxylation of lysine also occurs after the release of protocollagen polypeptides from ribosomal complexes.

Inhibition of protocollagen hydroxylase prevents extrusion of the polypeptides (1), and the effects of puromycin (9) as well as general considerations of the role of hexoses in the extrusion of proteins (17) suggest that the glycosylation of hydroxylysine occurs intracellularly. The biosynthesis of collagen appears therefore to occur in three relatively discrete steps: (a) protocollagen synthesis on ribosomal complexes; (b) hydroxylation of appropriate proline and lysine residues in the protocollagen with most or all of the hydroxylation taking place after the protocollagen is released from ribosomal complexes; and (c) glycosylation of selective hydroxylysine residues before the completed molecules are extruded into the extracellular matrix.

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