Conversion of Proline to Hydroxyproline and Its Incorporation into Collagen*

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Most, if not all, of the hydroxyproline in collagen appears to be derived from proline (1, 2). It has been proposed by several investigators (2-7), on indirect evidence, that it is a bound form of proline which is hydroxylated and incorporated into collagen. Recent data (8-11) indicate an analogous situation in the hydroxylation of lysine and its incorporation into collagen. The type of compounds which could possibly serve as substrates for the formation of bound hydroxyproline might be (a) those that contain within the same molecule proline destined for hydroxylation as well as the proline destined for collagen-proline, (b) those that contain within the same molecule either proline destined for collagen-hydroxyproline or proline destined for collagen-proline, but not both concurrently. Examples of the first type might be (a1) a precollagen macromolecule with an excess of proline and lacking hydroxyproline, or (a2) a single completed peptide strand of tropocollagen or subunit thereof with an excess of proline and lacking hydroxyproline. Within our present understanding of protein synthesis, examples of the second might be (b1) prolyl adenylate, or (b2) prolyl soluble ribonucleic acid.

To gain more information about the hydroxylation of proline, the specific activities of proline and hydroxyproline from "soluble collagen" (extracted in cold 0.45 M sodium chloride (12)) were compared during a 24-hour period after administration of uniformly labeled L-proline-C14 to guinea pigs. Variables such as the reproducibility of individual specific activity determinations, purity of collagen samples, and adsorption of free labeled proline by isolated collagen were evaluated. The results favor hypothesis (a2) as the bound form of proline which is hydroxylated.

EXPERIMENTAL PROCEDURE

Preparation of Experimental Animals—Sixteen male guinea pigs, ranging in weight from 301 to 368 g and exhibiting weight gain over a 5-day period, were each treated by intracardial injection with 25 μl of uniformly labeled L-proline-C14 (11.8 μc per μ mole, supplied by Nuclear-Chicago Corporation). Two animals were killed at 3-hour intervals during the first 24 hours by exsanguination via intracardiac puncture. The skin of the animal trunks was used as the source of purified "soluble collagen" by the method described by Gross.

Isolation of Proline and Hydroxyproline—Lyophilized, purified collagen samples were each hydrolyzed in sealed tubes with 6 N HCl (1 ml per 10 mg of protein) at 110° for 18 hours. The HCl was removed under vacuum and the residue subsequently made up to 1 ml per 8 mg of protein with distilled water. Samples were kept frozen until analyzed. A 0.2-ml aliquot was streaked along a 12-cm line on a sheet of Whatman No. 1 paper. Marker spots containing 12 μg each of proline and hydroxyproline were placed laterally to the collagen-hydrolysate streak. A descending chromatogram was developed in phenol-water (4:1 weight per volume) until the solvent front had moved 45 cm. The chromatograms were dried for 24 hours in an air-circulating hood. The position of the imino acids of the hydrolysate was determined indirectly, by locating the markers with 0.5% ninhydrin in 95% ethanol on paper strips previously removed from the rest of the chromatogram. Strips containing imino acids from the hydrolysate were eluted with water by descending chromatography. Each of the eluates, concentrated to 0.2 ml, was run on a separate sheet of Whatman No. 1 paper in butanol-acetic acid-water (100: 22: 50 volume per volume) until the solvent front had moved 45 cm. The imino acids were identified and eluted as described above. They were free from other ninhydrin-reaction material. The eluates were made up to 10 ml with water.

Determination of Specific Activities—Duplicate aliquots (each 1 ml) were used for hydroxyproline (13) and proline (14) determinations. For radiochemical determination, 5-ml aliquots were placed in 10-dram vials and taken to dryness on a steam bath under nitrogen. To the dried vial content were added 0.5 ml of water, 6 ml of absolute ethanol, and 10 ml of toluene containing 0.4% diphenyl oxazolone and 0.005% 1, 4-bis-2' (3'phenyl-oxazolyl) benzene. Samples were counted in a liquid phosphor counter. The imino acids in the quantities used for counting (100 to 200 μg) were found to be completely soluble in the scintillation fluid mixture used. The counts of both background and samples were continued for sufficient duration to bring the standard error of the count below ±2% (10).

Adsorption by Collagen of Proline-C14—Proline-C14 was added in vitro to unlabeled guinea pig skin. It is assumed that under the conditions used, any proline-C14 bound to isolated collagen will be adsorbed rather than incorporated by peptide linkage. This procedure was used as a measure of the contribution of ad-
sorbed proline-C\textsuperscript{14} to total collagen-bound proline-C\textsuperscript{14} for the animals which had been injected with labeled imino acid.

Three guinea pigs were killed and the skins removed and prepared for extraction with 0.45 m sodium chloride as previously described. Uniformly labeled L-proline-C\textsuperscript{14} was added to the finely divided skin in the cold extracting medium to give concentrations estimated to be 10-fold those in the skin of animals (per unit weight) injected with 25 \(\mu\)c in \(\text{vivo}\). Proline was isolated and its specific activity determined as previously described.

Purity of Collagen-Proline—Soluble collagen samples were incubated with collagencase{	extsuperscript{1} }free from other known proteinase activity, and the specific activity of proline determined in an ultrafiltrate of the incubation mixture. This procedure separates proline derived from collagen from proline derived from any other undigested, contaminating protein.

Several aliquots of purified and lyophilized soluble collagen (from proline-C\textsuperscript{14}-injected animals), coarsely divided, were treated with collagencase. The incubation system consisted of samples (5 to 7 mg) of collagen and 4 mg of collagencase (767 units per mg) in 1 ml of water, and 1 ml of 0.005 M CaCl\textsubscript{2} in Tris buffer pH 7.4, made up to a total of 5 ml. The reactions were run in stopped 25-ml flasks for 2 hours in a Dubnoff shaker at 37\textdegree. Thereafter, the incubation mixture was transferred to a 9-inch long piece of Visking tubing and ultrafiltered in a Toribarn tube (15) by centrifuging at 2000 r.p.m. at 3\textdegree for 18 hours. Thymol was added before ultracentrifugation to prevent bacterial contamination. The ultrafiltrates were hydrolyzed in sealed tubes with 6 N HCl, the proline isolated by paper chromatography, and its specific activity determined as previously described. The purity of the collagencase was substantiated by incubating two 10-mg samples of defatted, dialyzed plasma protein (containing 1000 c.p.m. per mg) obtained from guinea pigs which had received 25 \(\mu\)c of uniformly labeled L-proline-C\textsuperscript{14}. No measurable chemical or radiochemical amounts of proline were found in the ultrafiltrates.

Collagen-nitrogen was determined by a micro-Kjeldahl procedure described by Lang (16).

RESULTS

Purity of Isolated Collagen Samples—Hydroxyproline-nitrogen as the percentage of total nitrogen determined for six purified collagen samples ranged between 5.2 and 7.1\%. This result compares favorably with values of 7.7\% and 8.2\% obtained by Green et al. (2) and Jackson et al. (17) for soluble collagens from other sources.

Reproducibility of Individual Specific Activity Determinations—Six 0.2-ml aliquots from one soluble collagen sample were individually run through the paper chromatographic separations and chemical and radiochemical determinations, as described above. The standard deviations of the mean (expressed as the percentage of deviation from the mean) for the hydroxyproline specific activity and proline specific activity are 6.7\% and 7.8\%, respectively.

Proline Specific Activities of Collagenase-Digested against Acid-Hydrolyzed Collagen—Within the limits of the reproducibility of the method, proline derived from a noncollagenous protein does not have a significant influence on the collagen-proline specific activity determinations. This is indicated by the small differences between the proline specific activities of the same collagen samples which have been either acid-hydrolyzed or collagenase-digested (Table I).

Addition in Vitro of Proline-C\textsuperscript{14}—The specific activity of collagen-proline on addition in vitro of proline-C\textsuperscript{14} (see "Experimental Procedure") ranged between 10 and 28 c.p.m. per \(\mu\)mole. The specific activity of collagen-proline after the injection in \(\text{vivo}\) of proline-C\textsuperscript{14} ranged between 600 and 3000 c.p.m. per \(\mu\)mole. It is concluded that the results obtained in \(\text{vivo}\) are not significantly influenced by adsorbed proline, but reflect imino acid incorporated into collagen by peptide linkage.

Intracardial Injection of Proline-C\textsuperscript{14}—The specific activity ratios of hydroxyproline to proline from soluble collagen from skin of guinea pigs treated by injection with 25 \(\mu\)c of uniformly labeled L-proline-C\textsuperscript{14} varied between the limits of 0.94 and 1.20 (Table II).

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\textsuperscript{1} Collagenase prepared as described in Arch. Biochem. Biophys., 83, 245 (1959), was generously donated by Dr. Norman H. Grant, The Wyeth Institute for Medical Research, Radnor, Pennsylvania.
The deviation of the specific activity ratios from unity are random, independent of the time after administration of proline-C\textsuperscript{4}. The “true” values of all the specific activity ratios will be considered as unity. This is consistent with the reproducibility of the individual specific activity determinations and the random nature of the deviations about unity of the specific activity ratios.

**DISCUSSION**

In this discussion it is assumed that the mechanism for the hydroxylation of both proline and lysine is the same, involving a similar bound form of the amino acids which serve as a substrate for hydroxylation.

Several investigators have sought information about the protein- or lysine-containing substrate which is hydroxylated, by comparing the specific activities of collagen-proline or lysine with those of collagen-hydroxyproline or hydroxylysine at varying times after administration of proline-C\textsuperscript{4} or lysine-C\textsuperscript{4} to animals or tissue slices. The results obtained may be divided into three groups: (a) specific activity ratios of OHLys/Lys of less than one as well as unity (8); (b) specific activity ratios of OHPr/Pr of one as well as greater than one (7); (c) specific activity ratios of OHLys/Lys or OHPr/Pr of less than one, progressing with time to values greater than one (2, 10).

First, the specific activity ratios of OHLys/Lys of 0.7 from skin collagen 6 hours after administration of lysine-C\textsuperscript{4} to rats were interpreted by Piez and Likins (8) to show the presence of a “collagen-like” bound lysine which is an intermediate in the conversion to hydroxylysine. The possibility of a noncollagenous protein contaminant containing lysine of higher specific activity than collagen-lysine was considered as an explanation for their results but not ruled out. Interestingly, at the same time that the specific activity ratio of skin collagen OHLys/Lys was 0.7, that of bone collagen was 1.0. The investigators suggest, therefore, that a similar precursor is involved which “is hydroxylated to different degrees, at different rates in different tissues.”

Secondly, Robertson et al. (7) obtained some specific activity ratios of OHPr/Pr from carrageenin granuloma-collagen between 1.0 and 1.18 after three intraperitoneal injections of proline-C\textsuperscript{4} to guinea pigs over a 3-day period. They suggested that the OHPr/Pr specific activity ratios greater than 1.0 indicate that “proline and bound hydroxyproline enter the precollagen molecule as individuals” and that they “militate against the view that collagen synthesis involves the formation of a precollagen of high proline and low hydroxyproline content.” Had data on the reproducibility of individual imino acid specific activity determinations been available, this would have permitted estimation of the significance of specific activity ratio differences within the limits of 0.18.

Thirdly, Van Slyke and Sinex observed specific activity ratios of OHLys/Lys from skin collagen of 0.68, 0.71, and 1.1, at 1 hour, 3 hours, and 2 weeks, respectively, after intraperitoneal injection of lysine-C\textsuperscript{4} to rats. The deviations of the activity ratio on both sides of unity were considered to be attributable to hydroxylysine free protein contaminants of their isolated collagen. Green and Lowther (2), in the discussion section of their studies in vitro, reported specific activity ratios of OHPr/Pr at 10 minutes after proline-C\textsuperscript{4} incubation of 0.5 to 0.7, proceeding upward in a regular progression by 4 hours to 1.6. The specific activity ratios of OHPr/Pr at time intervals of 1 hour and less, calculated (by E. H.) from their raw data (Fig. 3 in reference (2)) were found to be 0.78 at 10 minutes, 1.38 at 20 minutes, 0.83 at 30 minutes, 1.1 at 45 minutes, inconsistent with the discussion steady upward progression. No estimation of the experimental error associated with the 10 minute-1 hour determinations is available. However, the 4-hour OHPr/Pr specific activity ratio of 1.6 is very adequately substantiated by a reported standard error for it of ± 0.1. No explanation for the difference between the latter activity ratio of 1.6 and the results of this study is readily apparent. Tentative explanations for the specific activity ratios obtained by Green et al. (2) were suggested to be “difficult to reconcile with any scheme of collagen synthesis involving hydroxylation of proline after completion of the collagen peptide chain.”

In the studies reported at present, contamination of isolated collagen by protein not metabolically related to collagen but containing proline was ruled out. The reproducibility of an individual imino acid specific activity determination was evaluated. The possibility that the purified collagen samples were associated with significant amounts of free proline-C\textsuperscript{4} was excluded. The consistent OHPr/Pr specific activity ratios of unity, independent of time after administration of proline-C\textsuperscript{4}, best fit the hypothesis of hydroxylation proceeding in an intermediate which contains within the same molecule both proline destined for hydroxylation and proline destined for collagen-proline. This bound form of proline very likely represents a molecular species on the pathway of collagen synthesis dissimilar in physical properties (solubility characteristics) to the finished product, namely tropocollagen. If hydroxylation were to occur in a macromolecular intermediate, for example, a fully formed triple stranded collagen molecule with an excess of proline and lacking hydroxyproline, one might expect that the unhydroxylated intermediate would be associated with collagen of mature OHPr/Pr, even after extensive purification. In the latter case, at early time periods after administration of proline-C\textsuperscript{4}, one would have obtained OHPr/Pr specific activity ratios of less than one.

Another possible intermediate which could serve as substrate for hydroxylation is one containing within the same molecule either proline destined for collagen-hydroxyproline or proline destined for collagen-proline, but not both concurrently. In such a case, it would be fortuitous that the rates of accumulation of intermediates between free proline and collagen-proline, on one hand, and free proline and collagen-hydroxyproline, on the other hand, were the same. Specific activity ratios of OHPr/Pr more or less than one, rather than unity, would be most likely.

Studies in which cell-free systems of connective tissue origin are used and in which proline-C\textsuperscript{4} is traced through the intermediates associated with its incorporation into microsomal protein may be the solution to identify definitively the form of proline which is hydroxylated.

**SUMMARY**

1. The specific activities of proline and hydroxyproline from 0.45 m sodium chloride-soluble skin collagen were compared at 3-hour intervals for 24 hours after injection of labeled proline-C\textsuperscript{4} into guinea pigs.

2. The specific activity ratios, collagen-hydroxyproline to collagen-proline ranged between 0.94 and 1.20. The “true” values were considered as 1.00, consistent with the reproducibility of individual imino acid specific activity determinations.
and the randomness with time of the deviations of the specific activity ratios about 1.00.
3. The possibility of contamination of the purified collagen samples by protein not metabolically related to collagen which contains proline with a specific activity different from collagen-proline was ruled out.
4. The amount of free proline-$^{14}C$ which is adsorbed on collagen, influencing the specific activity of peptide-incorporated proline, was found to be negligible.
5. The data are most consistent with the view that hydroxylation occurs in a bound form of proline, not of macromolecular dimensions, but which contains within the same molecule both proline destined for hydroxylation and proline destined for collagen-proline.

REFERENCES
Conversion of Proline to Hydroxyproline and Its Incorporation into Collagen
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