THE HYDROXYLATION OF PROLINE TO HYDROXYPROLINE DURING THE SYNTHESIS OF COLLAGEN IN CHICK EMBRYOS[1]

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Since collagen is essentially the only protein which contains hydroxyproline, and since hydroxyproline as such is not incorporated into collagen (Stetten, 1949), the hydroxylation of proline during the synthesis of collagen has been of considerable interest. Studies with O^{18} have indicated that the source of the oxygen for the hydroxylation is atmospheric oxygen rather than water (Scharpenseel and Wolf, 1959; Prockop, et al, in prep.). Recently, Stone and Meister (1962) used tritiated proline to investigate the synthesis of collagen hydroxyproline in carrageenan granuloma minces. They observed a loss of both hydrogens from the carbon position on which hydroxylation occurs. Concurrently, we have been using similar techniques in chick embryos to study the synthesis of collagen in vivo. Our results indicate that in our system only one hydrogen is lost from the hydroxylated carbon of proline. In addition, the results suggest that no more than one hydrogen atom is lost during the hydroxylation.

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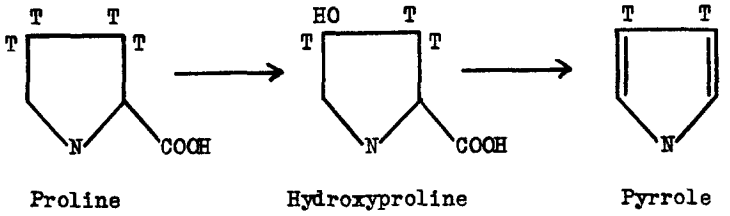
To simplify measurements of tritium retention, a mixture of proline-U-C^{14} (Nuclear Chicago) and proline-3, 4-T (New England Nuclear) was employed. The proline-3, 4-T was prepared by catalytic reduction of 3, 4-dehydroproline and some randomization of the label may occur (Robertson and Witkop, 1962). In the first experiment, 20 microliters of a 0.14 M NaCl solution containing 2.5 microcuries of proline-U-C^{14} and 16 microcuries of proline-3, 4-T was injected into a chorioallantoic vessel of each of two 10-day-old embryonated chicken eggs. In a second experiment, 20 microliters of a similar solution containing 2.5 microcuries of proline-U-C^{14} and 25 microcuries of proline-3, 4-T was employed. The embryos from each experiment were incubated at 37° for one hour, pooled with two untreated embryos, and homogenized in water. The homogenate was precipitated with 5% trichloroacetic acid at 0°, and lipids were extracted from the precipitate with boiling ethanol-ether (1:2). A partially purified collagen solution was then prepared by extracting the residue with 10% trichloroacetic acid at 90° (Fitch, et al, 1955). The collagen solution was dialyzed against water and hydrolyzed in 6 N HCl at 120° for 15 hours. Non-pyrrolidine amino acids in the hydrolysate were degraded by treatment with nitrous acid (Hamilton and Ortiz, 1950), and the proline and hydroxyproline were separated by chromatography on a cation exchange column (AG50, hydrogen form). The proline and hydroxyproline fractions were further purified on paper with 77% ethanol, and the relative amounts of tritium and C^{14} in the imino acids were determined by pulse height analysis in a Packard Tri-Carb liquid scintillation counter.

Since it was prepared by catalytic reduction, the proline-3, 4-T which was employed was assumed to be uniformly labeled in the 3 and 4 positions. To determine whether tritium was retained on the 4 position

Figure I



Proline Hydroxyproline Pyrrole

after hydroxylation, the collagen hydroxyproline was converted to pyrrole (Figure I) by the technique previously reported (Prockop, et al, 1961). Since the hydroxyl group is lost in the conversion, any tritium in the 4 position would be retained. Only 50% of the tritium in the 3 position would be retained, and no more than 50% of the smaller amounts of tritium which might be present in the 2 or 5 positions would be retained. Accordingly, if tritium was present in the 4 position of hydroxyproline, the tritium retention in the pyrrole would approach retention in 2 of 3 positions or 67%. If tritium was not present in the 4 position, the tritium retention would be 50% or less. As shown in Table I, a comparison of the $T:C^{14}$ ratio in the pyrrole and the hydroxyproline indicated 61% and 64% tritium retention in the pyrrole. No tritium isotope effect could be demonstrated, and the $T:C^{14}$ ratio of the pyrrole was the same when the oxidation was performed under conditions which gave yields of 20%, 40%, and 93%.

Table I

	T/C^{14} Ratios			% T Retention	
	collagen pro	collagen hypro	pyrrole from hypro	pyrrole vs. hypro	collagen hypro vs. collagen pro
Expt. I	5.6	4.1	2.5*	61%	73%
Expt. II	7.6	5.6	3.6*	64%	74%

*Corrected for loss of carboxyl carbon.

A comparison of the $T:C^{14}$ ratio of the proline and of the hydroxyproline isolated from collagen indicated a tritium retention of 73% and 74% in the hydroxyproline (Table I). This result suggests that only one hydrogen is lost from proline during the hydroxylation (Figure I). The interpretation of the data, however, is complicated by possible randomization of label in the tritiated proline employed and by the fact that the $T:C^{14}$ ratio of the proline which was isolated from collagen was only 86 and 80% of the $T:C^{14}$ ratio in the injected proline. Comparison of the collagen hydroxyproline with the injected proline, therefore, would indicate a tritium retention of only 62% and 57%. Since collagen hydroxyproline appears to originate from the same proline pools as collagen proline (Stetten, 1949), the estimate of tritium retention based on a comparison of the imino acids isolated from collagen is probably the more valid one; but this aspect of the problem is being investigated further.

It is of interest that definite proof for the loss of a single hydrogen during the hydroxylation of proline would further establish a similarity between this hydroxylation and steroid hydroxylations. In steroid hydroxylations molecular oxygen is also involved and a simple hydroxylation without inversion has been demonstrated (Hayano, et al, 1958; Bergstrom, et al, 1958).

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