HYDROXYLATION OF PROLINE IN PARTICULATE FRACTIONS FROM CARTILAGE

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Repeated observations that labeled hydroxyproline is poorly incorporated into collagen hydroxyproline have prompted the alternate suggestions that either proline is hydroxylated after it is incorporated into an intermediate such as t-RNA-proline, or the hydroxylation occurs after the synthesis of a proline-rich polypeptide precursor of collagen (see Stone and Meister, 1962). Recently, three laboratories (Manner and Gould, 1963; Coronado, et al., 1963, Jackson, et al., 1964) have reported results which suggest that the hydroxylation occurs at the level of t-RNA-proline, but two other laboratories (Peterkofsky and Udenfriend, 1963; Juva and Prockop, 1964) have presented evidence to support the hypothesis that the hydroxylation occurs after synthesis of a polypeptide precursor.

Cartilage fractions pre-labeled anaerobically with proline-C^{14} (Table I) were prepared from embryonic tibias which have previously been shown to synthesize collagen at a rapid rate (Juva and Prockop, 1964). The pre-labeled cartilage fractions were added to the complete hydroxylating system described in Table II, and they were incubated in air at 37° for two hours. In order to obtain a control for the amount of hydroxyproline-C^{14} initially present, one aliquot of each pre-labeled fraction was incubated at 4° with the complete hydroxylating system. After incubation, the samples were centrifuged at 4° at 100,000 x g for one hour, and the sediments were hydrolyzed in about 20 ml of 6 N HCl at 120° for 15 hours. The hydrolysates were evaporated to dryness in vacuo, and

the residues were dissolved in 4.0 ml of water. A 0.2 ml aliquot was taken for assay of total C^{14} (Prockop and Ebert, 1963), and 3.5 ml were taken for assay of hydroxyproline-C^{14} by a modification of the procedure of Prockop, et al., (1961). The final toluene extract was passed through a 0.7 by 5 cm column of silic acid before the colorimetric and C^{14} assays were performed. With this modification interference from proline-C^{14} carried through the procedure was reduced to less than 0.1%, and recoveries of added carrier hydroxyproline as pyrrole ranged from 46 to 55%.

A small increase in total hydroxyproline-C^{14} was observed when a "polysome" fraction was employed as a substrate, but much larger increases were observed with the 15,000 x g and 100,000 x g sediments (Table I). Similar results with increases of two- to six-fold in total hydroxyproline-C^{14} were obtained in eight additional experiments. No significant increase in hydroxyproline-C^{14} was observed when the 100,000 x g supernatant fraction from anaerobically pre-labeled cartilage was dialyzed against cold .25 M sucrose and was then added to the complete hydroxylating system. Also, no increase in hydroxyproline-C^{14} was observed when an aliquot of the 100,000 x g sediment which had been placed in boiling water for 10 minutes was employed as a substrate. Pre-incubation of the 100,000 x g sediment at pH 10 for 30 minutes or with ribonuclease for one hour reduced the amount of hydroxylation, but significant increases were still obtained. When free proline-C^{14} was employed as a substrate, the relative yield of labeled hydroxyproline was considerably less than that observed when the pre-labeled sediments were employed. In six separate experiments from .001 to .02% of the added free proline-C^{14} was recovered as hydroxyproline-C^{14}.

Less than 5% of the C^{14} in the pre-labeled 100,000 x g sediments was extracted when the sediments were incubated at 37° for one hour with solutions buffered to pH 1 or pH 3, and then were centrifuged at 100,000 x g. About one-third the total C^{14} was extracted by incubating at 37°

TABLE I. Conversion of Anaerobically Pre-labeled Fractions and of Proline-C^{14} to Hydroxyproline-C^{14} by the Complete Hydroxylating System*

	---4° incubation----		---37° incubation---	
Labeled precursor	Total C^{14}	Hypro-C^{14}	Total C^{14}	Hypro-C^{14}
	c.p.m.	c.p.m.	c.p.m.	c.p.m.
Experiment 1				
15,000 x g sediment	16,100	441	21,200	809
100,000 x g sediment	39,200	442	32,400	1,440
"polysome" sediment	10,100	162	8,840	201
proline-C^{14}	11,700,000	25	11,700,000	63
Experiment 2				
100,000 x g sediment	56,900	641	41,400	2,480
100,000 x g sediment, boiled 10 minutes			51,000	596
100,000 x g sediment, pretreated pH 10, ½ hr.			43,800	1,440
100,000 x g supernatant	10,400	48	10,000	67
proline-C^{14}			11,700,000	660
Experiment 3				
100,000 x g sediment	13,400	191	19,100	899
100,000 x g sediment, pretreated RNAase			18,100	571

*To prepare the pre-labeled cartilage fractions, 12 tibias from 10 day old chick embryos were incubated under nitrogen with 40 μc L-proline-C^{14} (New England Nuclear Corp.) for 30 or 60 minutes at 37° (Juva and Prockop, 1964). The tibias were homogenized in 11 ml of cold .35 M sucrose solution (Bloemendal, et al., 1964), and the homogenate was centrifuged at 1,200 x g at 4° for 10 minutes. The supernatant was either recentrifuged at 1,200 x g (experiments 2 and 3) or at 15,000 x g (experiment 1) at 4° for 10 minutes. Approximately equal aliquots of the supernatants from the second centrifugation were centrifuged at 100,000 x g at 4° for 1 hour. In experiment 1, part of the 15,000 x g supernatant was centrifuged through a sucrose gradient in order to obtain a "polysome" fraction (Bloemendal, et al., 1964). Conditions for the hydroxylation as in Table II. In experiment 3, one of the pre-labeled 100,000 x g sediments was pre-incubated with 1 μg ribonuclease (Worthington Biochem. Corp., 3 x recrystallized) in 1 ml distilled water at 37° for 1 hour, and the sediment was re-isolated by centrifuging. Values for total C^{14} are corrected for background (20 c.p.m.) and aliquots; values for hydroxyproline-C^{14} are observed c.p.m. for pyrrole-C^{14}. Samples were counted in a liquid scintillation counter with counting efficiencies of 47 to 55%.

for one hour with solutions buffered to pH 10 or with 1 M NaCl, pH 7.6. Essentially all the C^{14} extracted under these conditions was in a non-dialyzable form, and even after incubation at pH 13 and 37° for one hour, 85% of the total C^{14} was recovered in a non-dialyzable form.

When the characteristics of the complete hydroxylating system were examined, the yields of hydroxyproline-C^{14} were found to increase with incubation periods up to two hours. No increase in hydroxyproline-C^{14} was observed when the embryonic extract was omitted from the incubation mixture. Creatine phosphate and ATP were not required by the system (Table II), and the essential factor contributed by the embryonic extract was not affected by immersion in boiling water for 10 minutes.

The temperature dependence of the system and the effect of boiling the sediments appear to establish that hydroxylation of the pre-labeled fractions is an enzymatic reaction. Although an embryonic extract was

TABLE II. Hydroxylation of Pre-labeled 100,000 x g Sediments under Various Incubation Conditions*

Hydroxylating system	Hypro-C^{14}		% of total C^{14} recovered as hypro-C^{14}	
	4° incubation	37° incubation	4° incubation	37° incubation
	c.p.m.	c.p.m.		
Experiment a				
complete system	313	1,913	5.0	16.6
minus creatine-P, ATP		1,783		16.5
with 100,000 x g supernatant from embryonic extract		1,465		15.8
Experiment b				
complete system	300	693	5.0	10.8
with boiled embryonic extract		544		10.7

*The complete hydroxylating system in a final volume of 20 ml contained 10 ml of embryonic extract and .25 M sucrose, .05 M tris buffer pH 7.6, .004 M $MgCl_2$, .02 M KCl, .02 M creatine phosphate, and .002 M ATP. The embryonic extract consisted of the 15,000 x g supernatant obtained from 12 embryos homogenized in sucrose solution (Peterkofsky and Udenfriend, 1963). The substrates for the hydroxylation were pre-labeled 100,000 x g sediments from cartilage (see Table I). Because of the heavy precipitate obtained with the boiled embryonic extract, the assays in experiment b were performed on dialyzed extracts obtained by heating the samples with 5% trichloroacetic acid at 90° for 1 hour. Values for hydroxyproline-C^{14} are observed c.p.m. for pyrrole-C^{14}. The % of total C^{14} recovered as hydroxyproline-C^{14} was calculated by correcting the observed c.p.m. for aliquots, counting efficiencies, and loss of the carboxyl carbon in the conversion of hydroxyproline to pyrrole.

required, its insensitivity to boiling indicates that it contributes a stable co-factor rather than an enzyme to the system. The results suggest therefore that both the substrate and the hydroxylating enzyme are closely associated in the same particulate fraction.

The observations made here are difficult to reconcile with any mechanism for collagen synthesis which involves hydroxylation of proline at the level of a typical t-RNA-amino acid complex. The increase in hydroxyproline-C^{14} in the pre-labeled cartilage fractions cannot be explained by a rapid synthesis of t-RNA-proline-C^{14} in the hydroxylating system, since the rate of hydroxylation was considerably greater than it was for free proline-C^{14} in the same system. Only minimal amounts of preformed t-RNA-proline-C^{14} should be present in the 15,000 and 100,000 x g sediments of tissue homogenates, but as much as 14% of the proline-C^{14} in these fractions was hydroxylated. Also, if the substrate consisted of t-RNA-proline-C^{14}, incubation of the sediments at pH 10 should have rapidly destroyed the substrate and released the label as free proline-C^{14}. The substrate was relatively resistant to ribonuclease, but the results do not necessarily exclude the possibility that it consists of polypeptides attached to unusually heavy ribosomes or polysomes (Kretsinger, et al., 1964) through a C-terminal bond with t-RNA. Further studies are now in progress to determine whether the relatively large amounts of unhydroxylated precursor of collagen in the particulate fractions are still attached to ribosomal complexes, or whether they consist of polypeptides or proteins which have already been released from ribosomal sites.

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