Short Communications

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Studies on collagen metabolism with ^{18}O as a tracer

In the study of collagen metabolism, the process of incorporation of [^{14}C]glycine and [^{14}C]proline has been followed by several workers, and it was suggested that collagen soluble in neutral salt solutions was the precursor of insoluble collagen[1-3]. However, it is difficult to obtain pure collagen preparation and to avoid contamination of other proteins which might incorporate these amino acids more rapidly than does collagen. Recently, we found that the hydroxyl group of hydroxyproline in collagen is derived from atmospheric oxygen[4]. Since hydroxyproline is a characteristic component of collagen, ^{18}O incorporated from air into hydroxyproline may be used as a good tracer for the study of collagen metabolism. The present communication describes the results of ^{18}O incorporation from air into hydroxyproline of various fractions in chick embryo.

The incubation of chick embryos in ^{18}O-enriched air was performed in two ways. In Expt. 1 in Table I, incubation was carried out as described in a previous paper[4]. Ten 13-day-old eggs and a balloon (2 l) filled with ^{18}O-enriched O_2 (4.5 atom% excess) were placed in a vessel, 9.3 l in volume. The vessel was flushed with N_2 for 2 min and the balloon broken with a needle puncture through a rubber stopper. The vessel was incubated for 20 h at 37°. After incubation, embryos were collected, chilled immediately, and stored at −20°. 50 embryos from 5 separate incubations were combined and used for the fractionation. In Expt. 2 in Table I, 25 eggs 13 days old were placed in a vessel (9.3 l) in which a NaOH solution (10%, 0.4 l) was also placed. A reservoir filled with ^{18}O-enriched O_2 (7 l of 3.8 atom% excess) and equipped with a device to supply the gas at a constant pressure of 1 atm was connected to the vessel. The vessel was incubated at 37° for 17 h and 50 embryos obtained from two incubations were subjected to fractionation.

The collected embryos were homogenized with 500 ml of water, the homogenate centrifuged at 22500 × *g* for 30 min, and the precipitate was washed with water (100 ml). The supernatant and the washings were combined and deproteinized by adding trichloroacetic acid (36 g). After centrifugation, the supernatant was extracted three times with ether (each 50 ml) to remove trichloroacetic acid and passed through an Amberlite IR-120 column (H^+ form, 6 cm² × 10 cm) to adsorb amino acids. The free amino acid fraction was obtained by eluting the column with 2 N NH_4OH and evaporating it under reduced pressure. The precipitate from the homogenate was suspended in 800 ml cold 0.2 M NaCl solution (pH 7.4), stirred for 16 h at 4°, and centrifuged at 22500 × *g* for 30 min. The extraction was repeated 3 times. To the supernatant solution thus obtained, 4 vol. of ethanol were added and centrifuged. The precipitate was dried *in vacuo* and hydrolyzed with 200 ml of 6 N HCl by refluxing for 24 h and evaporated to dryness under reduced pressure. This was used as the fraction soluble in neutral salts. The residue from the saline extraction, which was

TABLE I

^{18}O ABUNDANCE IN HYDROXYL GROUP OF HYDROXYPROLINE FROM THE FREE AMINO ACID FRACTION, NEUTRAL-SALT-SOLUBLE FRACTION AND THE INSOLUBLE FRACTION

	Expt. 1		*Expt. 2*	
Incubation time (h)	20		17	
^{18}O abundance in air (atom% excess)	Before incubation	3.2		
	After incubation	2.3	(*a*) 3.8	
	Average	(*a*) 2.75		
Fraction	*mg hypro isolated*	*^{18}O abundance in OH-group (atom% excess)*	*mg hypro isolated*	*^{18}O abundance in OH-group (atom% excess)*
Free	11.4	0.538	6.8	0.664
Neutral-salt-soluble	9.1	1.04	4.6	1.15
Insoluble	154	(*b*) 0.730	160	(*b*) 1.00
% incorporation of atmospheric oxygen into OH-group of newly synthesized hydroxyproline		80*		93**

* $\dfrac{b/a}{0.4 \times 20\text{ h}/24\text{ h}} \times 100$.

** $\dfrac{b/a}{0.4 \times 17\text{ h}/24\text{ h}} \times 100$, where 0.4 is the fraction synthesized in the last 24 h of incubation.

defined as the insoluble fraction, was hydrolyzed by refluxing with 600 ml 6 N HCl for 24 h and evaporated to dryness under reduced pressure.

Hydroxyproline was isolated from each fraction as described in the previous paper[4]. The preparations were confirmed to be pure hydroxyproline by their infrared-absorption spectra.

^{18}O abundance was determined by a mass spectrometer, model M-60, Process and Instrument (Brooklyn, New York, U.S.A.), according to the method of RITTENBERG AND PONTICORVO[5].

In the previous paper[4] it was reported that at least 40–60 % of the hydroxyl group of hydroxyproline was derived from atmospheric oxygen. The method used in Expt. 2 seems to be more effective in avoiding the contamination with natural air. Assuming that the fraction of hydroxyproline newly synthesized during the last 24 h of incubation was 40 % (ref. 4), it was estimated that as much as 93 % of the oxygen atoms of hydroxyl group of newly synthesized hydroxyproline was derived from atmospheric oxygen. In Expt. 1, when the correction for contamination was made by using the average values of ^{18}O abundance in the gas phase before and after the incubation, it was also found that 80 % of the oxygen atoms of the hydroxyl group was derived from air. These results confirm those in the previous paper[4] and suggest that the hydroxylation of proline is catalyzed exclusively by some oxygenase.

The results of analysis of ^{18}O abundance in hydroxyproline of the free amino acid fraction, neutral-salt-soluble fraction and the insoluble fraction are shown in Table I. ^{18}O abundance in hydroxyproline in the neutral-salt-soluble fraction was found to be higher than those in the other two fractions. The results agree with the observation with [^{14}C]amino acids, which suggested that neutral-salt-soluble collagen was the precursor of insoluble collagen[1–3]. In the 13-day-old chick embryos, the

fraction of hydroxyproline synthesized during the last 24 h of incubation was about 40 %. The amount of hydroxyproline in the neutral-salt-soluble fraction was less than 10 % of the total hydroxyproline. Therefore, if the neutral-salt-soluble collagen is a precursor of collagen and the turnover rates of collagen in various organs in chick embryo are the same, all the neutral-salt-soluble collagen would be expected to be synthesized *de novo* during the incubation period. In other words, the ^{18}O abundance in the hydroxyl group of hydroxyproline of the fraction would be the same as that of atmospheric oxygen. In fact, however, the ^{18}O abundance observed was much less than that expected, indicating that not all the soluble collagen was newly synthesized during the experimental period. The following possibilities may be considered to account for the experimental results. (1) The 0.2 M NaCl extracts might have contained some other hydroxyproline-containing fractions besides the real precursor. (2) The soluble fraction might not be a precursor of the insoluble fraction. (3) The turnover rates of collagen in various organs might not be the same.

Among these, the first possibility seems to be the most probable. If this may be assumed to be the only cause, it is estimated from the ^{18}O abundance observed that about 10 % of hydroxyproline in this fraction would have been present in the real precursor of collagen. Before the problem is elucidated more in detail, however, improvements of the method of fractionation of "soluble collagen" are desirable.

Chick embryo has been known to contain a considerable amount of hydroxyproline in free form as compared with other animal tissues. It has been shown by several workers that, in the synthesis of collagen, proline—and not hydroxyproline as such—is incorporated to be subsequently oxidized to hydroxyproline[3,6-8]. The suggestion made by MITOMA *et al.*[9] that at least a part of the hydroxyproline found in collagen in chick embryos might have been derived from free hydroxyproline was denied recently by PROCKOP *et al.*[10] based on the results of their experiments with ^{14}C. Our observation that the ^{18}O abundance in free hydroxyproline was less than that in peptide-bound hydroxyproline also leads us to the conclusion that the hydroxyproline existing in the collagen molecule is not derived from the free hydroxyproline. The free hydroxyproline found in chick embryos is most probably a degradation product of the collagen molecule.

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