OXYGEN-18 STUDIES ON THE CONVERSION OF PROLINE TO HYDROXYPROLINE

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Proline has been established as the precursor of hydroxyproline but the mechanism of this conversion is still obscure. Although information concerning enzymes, cofactors, and intermediates will require further advances in in vitro techniques, isotopic studies are possible with currently available in vivo systems. Results of experiments by Ebert and Prockop (1) with tritium-labelled proline were found to be incompatible with a dehydrogenase mechanism for hydroxyproline formation. The present study with oxygen-18 in the intact chick embryo shows conclusively that conversion of proline to hydroxyproline involves an oxygenase type of mechanism.

In the experiments with oxygen-18 enriched water, 1 ml of H_2O^{18} (66 atom % excess)[2], was injected through a hole in the shell into the air sac, the hole sealed with adhesive tape, and the embryos (8 days old) were incubated at 37° until the 15th day. Experiments with O_2^{18} were carried out in a specially designed chamber containing dilute KOH to absorb expired CO_2. The O_2^{18} was formed by electrolysis of H_2O^{18} (10 atom % excess) and was diluted with tank nitrogen and oxygen to make a final atmosphere containing 30% oxygen with 5 atom % excess oxygen-18. The embryos were allowed to grow in this atmosphere at 37°

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[2] H_2O^{18} was obtained from Yeda Research and Development Co., Israel.

from the 9th to the 10th day. The amount of hydroxyproline synthesized during each type of experiment was estimated by measuring the hydroxyproline (2) contents of embryos before and after growth under comparable conditions. The bulk of this hydroxyproline is in collagen.

The estimated increment in hydroxyproline of embryos from the H_2O^{18} experiments was approximately 60 micromoles; these were assayed individually for isotope content. Estimations of the hydroxyproline increment in the O_2^{18} experiments were about 1.5 micromoles per embryo and 8 to 9 were pooled for each isotopic assay. The embryos were homogenized in several volumes of water, an equal volume of conc. HCl was added and the samples placed in an autoclave (124° and 18 pounds pressure) for 3 hours. The hydrolysate was decolorized with charcoal and treated with nitrous acid, according to Hamilton and Ortiz (3) to destroy α-amino acids. The resulting solution containing the imino acids was desalted and the hydroxyproline was separated from the proline on a cation exchange resin (4). The hydroxyproline was further purified by preparative chromatography on Whatman #3 paper using ethanol:water (3:1) and was converted to the hydantoin derivative by the procedure of Fraenkel-Conrat et al. (5). The yield and purity of the hydantoin were determined spectrophotometrically (5) and the major portion was pyrolyzed by the procedure of Rittenberg and Ponticorvo (6) and submitted to mass spectrometric assay.3/

The results of the experiments are summarized in the table. Essentially, no oxygen-18 was found in the hydroxyproline from chick embryos which had been injected with H_2O^{18}. Water isolated from two of these embryos contained 1.73 and 1.48 atoms % excess oxygen-18. Large amounts of oxygen-18 were found in the hydroxyproline isolated from the embryos grown in the presence of O_2^{18}. Water isolated from these embryos did not contain measurable amounts of the isotope. When unlabelled hydroxy-

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3/ Pyrolysis and mass spectrometric assay were carried out by Analytica Corporation, New York City.

Table 1

Incorporation of O_2^{18} and H_2O^{18} into Chick Embryo Hydroxyproline

Sample	Source of Isotope	Atom % Excess Oxygen-18 in Hydroxyproline	
		Estimated*	Found
1	H_2O^{18}	0.40	0.000
2	H_2O^{18}	0.60	0.007
3	H_2O^{18}	0.60	0.048
4	O_2^{18}	>1.25**	0.572
5	O_2^{18}	>1.25**	0.291

* Approximations are based on the incorporation of oxygen-18 from H_2O^{18} (correcting for dilution of about 40 ml of embryonic fluid) or O_2^{18} into the hydroxyl of the newly formed hydroxyproline. Included also are corrections for dilution with the hydroxyproline present at the start of the experiment and by the other oxygen atoms in the hydroxyproline or hydantoin molecule. Sample 1 was isolated as free hydroxyproline; therefore, two additional oxygen atoms in the carboxyl group were available for dilution of the hydroxyl oxygen. All other samples were isolated as hydantoin derivatives with one less carbonyl oxygen. The samples contained a small amount of an impurity (0.2 - 0.3 mg out of 2 - 5 mg) which was derived from the reagents used in the purification and hydantoin preparation.

** Estimations for increments in hydroxyproline in the O_2^{18} experiments are no doubt high since they were based on measurement of hydroxyproline increments in control embryos incubated in a normal atmosphere rather than in the less optimal conditions of the closed incubation chamber.

proline was incubated with H_2O^{18} under conditions comparable to those used for protein hydrolysis only two of the oxygens exchanged. This is consistent with the known lability of the carboxyl oxygens (7) and indicates that the hydroxyl group in hydroxyproline withstands these procedures.

The results indicate that molecular oxygen rather than water is the source of oxygen for the conversion of proline to hydroxyproline. Fujimoto et al. (8) have recently reported preliminary data on incorporation of O_2^{18} into chick embryo hydroxyproline which is in accord with these findings. The report of Ebert and Prockop (1) on the loss of only one of the four tritium atoms of proline 3,4-H^3 during conversion to hydroxyproline, although not in accord with a prior report by Stone and Meister (9), is consistent with an oxygenase mechanism. Thus, conversion of proline to hydroxyproline involves the addition of molecular oxygen, as in aromatic (10, 11) and steroid (12) hydroxylation, rather than dehydrogenation followed by addition of water.

The implications of these findings with respect to cofactor requirements are currently being investigated using the cell-free system recently reported by Peterkofsky and Udenfriend (13).

Complete details of this work will be reported elsewhere.

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