magnesium yeast to normal or nearly normal values.

4. A magnesium-rich yeast with normal potassium content was prepared by fermenting directly in 0.2M-magnesium acetate. A large amount of magnesium can be taken up in this way under suitable conditions. The magnesium uptake can exceed 400 m-equiv./kg. of yeast, and no appreciable amount of potassium is lost in the process. The properties of this yeast do not appreciably differ from those of fresh washed yeast.

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Incorporation of ^{18}O from Air into Hydroxyproline by Chick Embryo

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Since the suggestion by Stetten (1949) that most of the hydroxyproline in animal tissues arises from proline rather than from free hydroxyproline, the mechanism of the conversion has been studied by many workers. Although many results support Stetten's findings (Smith & Jackson, 1957; Green & Lowther, 1959), experiments in cell-free systems have so far been unsuccessful, and the mechanism of the hydroxylation of proline to hydroxyproline remains unknown.

It has been confirmed that the hydroxylation of phenylalanine (Mitoma, 1956), steroids (Hayano, Saito & Dorfman, 1958) and some other aromatic compounds (Saito, Hayaishi, Rothberg & Senoh, 1957; Posner, Mitoma, Rothberg & Udenfriend, 1961) are catalysed by oxygenases, i.e. the oxygen atoms of hydroxyl groups are derived directly from atmospheric oxygen. On the other hand, hydroxylations of some heterocyclic compounds such as nicotinic acid are not oxygenation reactions and the oxygen atoms of the hydroxyl groups are derived from water (Hunt, Hughes & Lowenstein, 1958).

This paper presents experimental evidence that the hydroxyl group of hydroxyproline is derived from atmospheric oxygen.

MATERIALS AND METHODS

Materials. The H_2^{18}O was purchased from Research and Development, Weizmann Institute of Science, Rehovoth, Israel; ^{18}O -enriched oxygen gas was prepared from H_2^{18}O by electrolysis.

Incubation of chick embryo with ^{18}O -enriched air. Eight 13-day-old eggs and a balloon (2 l.) filled with ^{18}O -enriched

oxygen were placed in a vessel (9.3 l.). Aq. 10% (w/v) sodium hydroxide (5 ml.) was also placed in the vessel to remove expiratory carbon dioxide. The vessel was flushed with nitrogen for 2 min. and the balloon broken by needle puncture through a rubber stopper. The vessel was incubated for 24 hr. at 37°.

Hydrolysis of embryos. After incubation, embryos were collected and homogenized in ethanol (150 ml.). The precipitate was collected by centrifuging, dried *in vacuo* and hydrolysed by refluxing with 6N-hydrochloric acid (200 ml.) for 24 hr.

Isolation of hydroxyproline. The hydrolysate was evaporated to dryness and the residue dissolved in water (40 ml.). Activated charcoal (2 g.) was added and the mixture stirred for 1 hr. Charcoal was removed by filtration and washed with water (5 ml.), and the filtrate and the washings were combined. Sodium nitrite (8 g. in 15 ml. of water) was added to destroy amino acids, and, after the solution had been left for 1 hr. at room temperature, concentrated hydrochloric acid (60 ml.) was added, and the mixture then refluxed for 1 hr. Most of the sodium chloride separated after concentration, and was washed with 6N-hydrochloric acid. The solution and the washings were combined and evaporated *in vacuo*. The residue, dissolved in water (50 ml.), was passed through an Amberlite IR-120 (H^+ form) column (2 cm.² × 20 cm.) which retained the imino acids together with other cations. The imino acids were eluted with 2N-ammonium hydroxide, the eluate was evaporated and the residue dissolved in water (10 ml.). The solution was passed through another Amberlite IR-120 (H^+ form) column (0.8 cm.² × 40 cm.). The column was washed with water (50 ml.) and with 0.5N-hydrochloric acid (50 ml.), and the hydroxyproline was eluted with 1.5N-hydrochloric acid. The eluate was evaporated to dryness *in vacuo*. The hydroxyproline hydrochloride so obtained was passed through a third column (2 cm.² × 5 cm.) of Amberlite IR-120 (H^+ form) to free the imino acid from hydrochloric acid. The hydroxyproline was eluted with 2N-ammonium hydroxide. The eluate was evaporated, the residue was dis-

solved in the minimum amount of water and the hydroxyproline was precipitated with ethanol. The material was shown to be pure hydroxyproline by its infrared-absorption spectrum.

Isolation of tyrosine. The charcoal was extracted with 20% (w/v) acetic acid containing 5% (w/v) of phenol (100 ml.). After removal of the phenol by extraction with ether, the aqueous solution was evaporated to dryness *in vacuo*. The residue was extracted twice with water (1 ml.), and dissolved in a little hot water. On cooling the solution, tyrosine crystallized. The infrared-absorption spectrum confirmed that the material was tyrosine.

Determination of ^{18}O abundance. To determine the ^{18}O abundance in organic compounds, water and oxygen gas, the preparations were pyrolysed by the method of Rittenberg & Ponticorvo (1956) with the addition of mercuric cyanide to the pyrolysis catalyst (Anbar & Guttman, 1959), and the carbon dioxide produced was introduced into a Process and Instruments model M-60 mass spectrometer.

Determination of amino acids. The hydroxyproline content of the hydrolysates was determined by Leach's (1960) method or by the method of Prokop & Udenfriend (1960). Proline was assayed by the method of Piez, Irreverre & Wolff (1956). Tyrosine was determined by the method of Folin & Ciocalteu (1927).

RESULTS

Exchangeability of oxygen atoms of hydroxyproline. To examine the origin of oxygen atoms, it is necessary to avoid the exchange of the oxygen atoms with water of the medium during the isolation procedure, including the hydrolysis with 6*N*-hydrochloric acid. Rittenberg, Ponticorvo & Borek (1961) found that, though the oxygen atoms of the hydroxyl group of tyrosine and serine did not exchange with those of the medium by heating at 108° with 6*N*-hydrochloric acid, the oxygen atoms of carboxyl groups easily exchanged with those of the medium. We obtained similar results with hydroxyproline. Hydroxyproline (60 mg.) was heated at 110° with 6*N*-hydrochloric acid

(2 ml.) containing H_2^{18}O (0.72 atom % excess) in a sealed glass tube. The solution was evaporated to dryness *in vacuo* over sodium hydroxide pellets. The residual crystals of hydroxyproline hydrochloride were analysed for ^{18}O abundance. It was confirmed that, of three oxygen atoms contained in the molecule, two exchanged completely within 6 hr., and the third remained unexchanged after 72 hr.

Amino acid contents of the developing eggs. For the present purpose it is desirable to employ a system which synthesizes hydroxyproline actively. Various stages of developing whole eggs (without the shells and shell membranes) were hydrolysed and the amounts of hydroxyproline, proline and tyrosine were determined. The results are shown in Table 1. Hydroxyproline increased remarkably, about 40 % a day, in 12–14-day-old eggs, whereas the amounts of tyrosine and proline were nearly constant in 0–14-day-old eggs.

Results of the ^{18}O tracer experiments. Results of the analysis of ^{18}O abundance in hydroxyproline and tyrosine isolated from chick embryos which were incubated in the ^{18}O -containing atmosphere are shown in Table 2. An appreciable amount of ^{18}O

Table 1. *Hydroxyproline, proline and tyrosine content of developing whole eggs*

Whole eggs excluding shells and shell membranes were hydrolysed by refluxing with 6*N*-hydrochloric acid for 24 hr. A portion of the hydrolysate was analysed for tyrosine. Another portion was analysed for hydroxyproline and proline after treatment with activated charcoal.

Age (days)	Wet wt. of egg contents (g.)	Hydroxy- proline (mg.)	Proline (mg.)	Tyrosine (mg.)
0	52.6	0.27	310	356
12	49.5	2.46	303	320
13	52.3	5.99	306	361
14	51.0	10.3	312	346

Table 2. *Incorporation of atmospheric oxygen into hydroxyproline and tyrosine by chick embryos*

Eight 13-day-old eggs were incubated in ^{18}O -enriched air for 24 hr. at 37°. Hydroxyproline and tyrosine were separated and analysed for ^{18}O content. Values of (c) and (e) were calculated as follows: (c) = $100 \times 3 \times (b)/(a)$; (e) = $100 \times (c)/(d)$, where $3 \times (b)$ corresponds to the ^{18}O abundance in the hydroxyl group. —, Not measured.

	Expt. 1	Expt. 2		Expt. 3	
^{18}O abundance in O_2 in balloon (a) (atoms % excess)	1.41	4.11		4.50	
	Hydroxyproline	Hydroxyproline	Tyrosine	Hydroxyproline	Tyrosine
^{18}O abundance in amino acid (b) (atom % excess)	0.068	0.195	0.013	0.344	0.027
Oxygen derived from air into OH group (c) (%)	14.5	14.2	0.95	23.0	1.8
Increase of amino acid during experimental period (d) (%)	—	Approx. 33	—	Approx. 38	—
Oxygen derived from air into OH group of newly synthesized amino acid (e) (%)	—	Approx. 43	—	Approx. 61	—

was incorporated into hydroxyproline from atmospheric oxygen, but little into tyrosine. As shown above, the oxygen atoms of the hydroxyl groups of hydroxyproline and tyrosine are not exchangeable with those of water during refluxing with 6N-hydrochloric acid for 24 hr., but the oxygen atoms of the carboxyl groups are replaced by those of the medium. The results indicate, therefore, that ¹⁸O was incorporated into the hydroxyl group of hydroxyproline.

Considering the increment of hydroxyproline during the experimental period as assayed on the hydrolysate before and after the incubation, 40–60% of the hydroxyl groups of newly synthesized hydroxyproline was derived from atmospheric oxygen. Though the hydroxylation of phenylalanine to tyrosine is known to be an oxygenation reaction, the amount of ¹⁸O incorporated into tyrosine was negligibly small. As shown above, the amount of tyrosine in eggs was almost constant during the development. It is concluded, therefore, that the synthesis of tyrosine from phenylalanine did not occur in developing eggs, so that there was no incorporation of ¹⁸O into the tyrosine fraction.

DISCUSSION

About 40–60% of the hydroxyl groups of hydroxyproline was derived from atmospheric oxygen. The ¹⁸O content of oxygen gas introduced into the vessel could be lowered by contamination with air or by exchange reactions catalysed by the living organism, although the latter mechanism is not yet known. In one experiment (Expt. 3 in Table 2), the ¹⁸O abundance of the air in the vessel after the incubation was only 1.8 atoms % excess. Consequently the value of 40–60% given above is too low.

Atmospheric oxygen is incorporated into water by the embryos by reactions involving respiration. It was calculated, however, that even if all of the atmospheric oxygen in the vessel were incorporated into the water in the eggs, ¹⁸O abundance in the water could reach only 0.036 atom % excess in Expts. 2 and 3 in Table 2. Therefore the possibility that incorporation of ¹⁸O was effected by way of water was eliminated. However, a possibility still

remains that the water derived from atmospheric oxygen is retained by some structure, as a kind of 'caged water', which is then used for hydroxylation without mixing with the rest of the body water.

The evidence produced here suggests that the hydroxylation of proline is catalysed by an oxygenase, although the enzyme has not yet been isolated.

SUMMARY

1. The incorporation of atmospheric oxygen into the hydroxyl group of hydroxyproline by developing chick embryos was confirmed with ¹⁸O as a tracer. The hydroxyl group of tyrosine was not derived from air under these conditions.

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