Plant Physiol. (1966) 41, 197–202

PLANT PHYSIOLOGY

Vol. 41 No. 2

February 1966

Role of Oxygen Fixation in Hydroxyproline Biosynthesis by Etiolated Seedlings[1]

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Received May 21, 1965.

Summary. Etiolated maize and soybean seedlings were grown for several days in atmospheres enriched with O^{18}. Hydroxyproline subsequently isolated from the seedlings by column and thin-layer chromatography was labeled with excess O^{18}, but proline was not. Control experiments in which seedlings were grown in H_2O^{18} and unlabeled atmospheres demonstrated that neither proline nor hydroxyproline was labeled with excess O^{18}. It was concluded that oxygen fixation is an essential feature of hydroxyproline biosynthesis in these seedlings, and that the hydroxyl oxygen atom in hydroxyproline is derived from molecular oxygen and not from water; similar results have been reported previously for sycamore cell suspensions.

Within recent years increasing interest has been shown in the oxygenative pathway of biological oxidation, by which O_2 is incorporated into organic material by direct addition of molecular O_2. The most recent reviews appeared in a journal article by Hayaishi (7) and also in book form (8). The enzymes which catalyze oxygenation (i.e., O_2 fixation) reactions are generally called oxygenases and hydroxylases, in accord with recent proposals (18) and recommendations (23), as well as recent usage in the literature [e.g., see reviews by Hayaishi (7)(9)]; specifically, oxygenases catalyze the addition of both atoms of the O_2 molecule to a molecule of substrate, whereas hydroxylases catalyze the addition of 1 atom of O_2, and the second atom probably is reduced to water.

In most investigations of O_2 fixation, tissues and enzyme preparations from microorganisms and animals have been utilized. Very few reports have been published concerning O_2 fixation in higher plant tissues [e.g., see Goldfine and Bloch (5)]. One of these few reports is that of Fritz et al. (3), who incubated etiolated maize seedlings in atmospheres labeled with O^{18} and concluded that a small fraction of the absorbed O_2 gas was incorporated into organic material through the agency of enzymes which catalyze the direct addition of molecular O_2 to organic substrates. Also, Lamport (13), on the basis of results obtained from 1 experiment in which a suspension of sycamore cells was grown in an atmosphere enriched with O^{18}, reported that the oxygen atom of the hydroxyl group in hydroxyproline came from molecular O_2.

The metabolism and role of hydroxyproline has been the subject of increasing interest during recent years. This amino acid was found to be localized in the protein associated with the primary cell wall in sycamore cells (15) and in other species of higher plants (2) (22). Lamport (14) in a recent review stressed the importance of the hydroxyproline-rich protein in primary cell wall structure, and discussed the possible role of hydroxyproline in cell wall extension. Scharpenseel and Wolf (25) observed that the conversion of proline to hydroxyproline in fetal skin preparations depends on a supply of molecular O_2. Fujimoto and Tamiya (4) and Prockop et al. (21) demonstrated with the use of O^{18} that the hydroxyl oxygen atom of hydroxyproline in chick embryo collagen is derived from molecular O_2.

The present investigation was undertaken to demonstrate the incorporation of molecular O_2 into hy-

[1] From the thesis submitted by Ernest R. Stout to the Graduate School of The University of Florida in partial fulfillment of the requirements for the Ph.D. degree; the results reported here were obtained during the tenure (by E. R. Stout) of a National Defense Education Act Fellowship. This investigation was supported in part by the United States Atomic Energy Commission, Contract No. AT-(40-1)-2834, and by a grant (G-21410) from the National Science Foundation. Approved as Journal Series No. 2152 of the Florida Agricultural Experiment Stations.

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droxyproline of etiolated maize and soybean seedlings. Some preliminary work involving the estimation of the amount of hydroxyproline as a function of seedling age was necessary and is reported also. A short communication concerning this investigation has appeared (27).

Materials and Methods

Plant Material. Maize grain (*Zea mays* L., Wf9 × 38-11, fertile version) and soybean seeds [*Glycine max* (L.) Merr., Hardee variety] were soaked for 6 hours in running tap water, and then were sown in wetted vermiculite, either in aluminum trays (for hydroxyproline estimations) or in 250 ml Erlenmeyer flasks (for O^{18} experiments) with 10 to 12 seedlings per flask. Germination and subsequent growth took place at 25° in the dark with only occasional exposures to white light. Age of seedlings was calculated from the time of immersion in water.

O^{18} Methodology. O_2 gas containing approximately 10 atom percent O^{18} was prepared by electrolysis of O^{18}-enriched water purchased from the Weizmann Institute of Science, Rehovoth, Israel; the exact atom fraction for each electrolyzed sample was determined by diluting an aliquot with nitrogen and analyzing by mass spectrometry. The O_2 gas obtained from electrolysis was passed through a column of soda lime to insure the removal of traces of ozone (1) (10).

Seedlings in 250 ml Erlenmeyer flasks were exposed for several days to labeled O_2 gas mixed with nitrogen (volume ratio, 20:80). A vial containing 5 ml of 40 % KOH with a filter paper wick was placed upright inside each flask. Each flask was equipped with a 1-hole rubber stopper into which a short piece of glass tubing was fitted. To introduce labeled gas mixture into a flask at the beginning of an experiment, the flask was evacuated with a water aspirator and then filled to 1 atm of pressure. O_2 gas absorbed by the respiring seedlings was replaced every 12 hours with pure O_2 gas of the same O^{18} enrichment. The vermiculite was wetted with sufficient water at the start of an experiment so that further additions of water were not needed.

For experiments in which seedlings were grown in water labeled with O^{18}, the gas phase was air containing the normal enrichment of O_2. Labeled water containing 1.52 or 1.33 atom percent O^{18} excess was purchased from Bio-Rad Laboratories, and was added to the Erlenmeyer flasks at the time of sowing.

Hydroxyproline in Amino Acid Mixtures. At harvest, a sample of seedlings which had attained an average size was selected. Maize endosperms and soybean seed coats were removed and discarded, and the fresh weight of the tissue was recorded. For hydroxyproline estimations, 4 to 20 seedlings were taken, depending on the age and species of seedlings. The sample was chopped finely with a razor blade and ground to a slurry in ice-cold water in a mortar with a pestle.

The ground plant material was hydrolyzed in 6 N HCl for 24 hours at about 108° under a water-cooled reflux condenser. The hydrolysate was decolorized with activated carbon, and the acid was removed by evaporation on a rotary evaporator. The residue was dissolved in water, and the solution was passed through a 20 × 1.5-cm column of Amberlite IR-120(H^+), medium-porosity, cation exchange resin. The column was eluted with 2 N NH_4OH, and the eluate was evaporated to dryness on a rotary evaporator. The residue containing the amino acids was dissolved in 0.05 N HCl and was stored at 5°.

Hydroxyproline in these amino acid mixtures was estimated by the colorimetric method of Neuman and Logan (20) as modified by Leach (16). Also, the hydroxyproline content of the cell wall fraction was measured, so that estimates could be made of the percentage of hydroxyproline in the cell wall as compared to the total tissue. To isolate the cell wall, the slurry of plant material obtained by grinding in a mortar with pestle was further ground at 0° for 2 minutes in a motor-driven Teflon tissue homogenizer. The fraction obtained from the ground material by filtration through Miracloth (Chicopee Manufacturing Corporation) was designated as cell wall (22). This fraction was treated as above to obtain amino acid mixtures.

Isolation of Hydroxyproline from Amino Acids. For O^{18} analyses, it was necessary to separate hydroxyproline from the other amino acids. A number of seedlings was taken which would contain 1 mg of hydroxyproline; the required number of seedlings could be estimated from data regarding the hydroxyproline content of the seedlings (see fig 1). The tissue was frozen and dried exhaustively for 6 hours by means of a vacuum pump evacuating to a pressure of about 0.1 micron. The dried tissue was then powdered in a mortar with a pestle. Amino acid mixtures obtained as described were treated with nitrous acid to deaminate the primary α-amino acids. The nitrous acid was prepared immediately prior to use by mixing 1 part of a solution of 30 % $NaNO_2$ with 2 parts of 9 N HCl. After standing for 1 hour at room temperature, nitrous acid-amino acid mixtures were boiled for 1 hour and then concentrated on a rotary evaporator; the precipated NaCl was removed by filtration. The α-hydroxy acids produced by the nitrous acid treatment were extracted with diethyl ether (6) and discarded, so that only the imino acids (proline and hydroxyproline) remained in the nitrous acid solution.

Since the primary objective was the investigation of O_2 fixation in hydroxyproline biosynthesis, it was desirable to recover as much of the hydroxyproline as possible from the nitrous acid solutions; however, no effort was made to recover proline quantitatively since only an amount (approximately 1 mg) sufficiently large for mass spectrometric analysis was needed. Proline and hydroxyproline in nitrous acid solutions were separated by column chromatography followed by thin-layer chromatography. A partial

separation of the amino acids was made on a 50 × 1 cm column of Dowex 50W-X8(H^+); the column was eluted with 1.5 N HCl (12). Only the 50 to 150 ml fraction was collected, since this portion of the eluate was found to contain 96 % of the hydroxyproline if a mixture containing 1 mg each of hydroxyproline and proline was placed on the column; this fraction contained about 30 % of the proline placed initially on the column. The eluate was evaporated to 1 ml and the proline and hydroxyproline were then separated completely by thin-layer chromatography on a cellulose absorbent, by the method of Myhill and Jackson (19). It was found that recovery of hydroxyproline from thin-layer plates was approximately 100 % for quantities of about 100 μg of hydroxyproline. Therefore, the entire solution containing the imino acids was placed in 10 or more spots on the cellulose absorbent, and each spot was estimated to contain about 100 μg of hydroxyproline. After thin-layer chromatography, hydroxyproline and proline were recovered from the plates and then chromatographed on a 10 × 1 cm column of Amberlite IR-120(H^+) so as to remove soluble components which might have been derived from the cellulose or from traces of chromatographic solvent.

Pyrolysis and Mass Spectrometry. The hydroxyproline and the proline which were recovered from the seedlings were sealed under vacuum in Pyrex tubes and pyrolyzed to CO_2 at 500° to 550° for 90 minutes, according to the method of Lee (17). The atom percent O^{18} in CO_2 was calculated from peak heights of masses 44 and 46 (11), which were measured in a type 21-130 Consolidated Electrodynamics Corporation mass spectrometer. The abundance of O^{18} in reagent proline and hydroxyproline was determined for several samples and found to be 0.204 atom percent.

Results

Hydroxyproline Content of Seedlings. As shown in figure 1, the amount of hydroxyproline in etiolated maize is 8 μg per seedling for 0.25-day seedlings, and increases to 80 μg per seedling for 12-day seedlings. For etiolated soybean, the amount of hydroxyproline ranges from 35 μg per seedling for 0.25-day seedlings to 300 μg per seedling at the fourteenth day after the start of germination. It is also evident from figure 1 that the maximal amount of hydroxyproline in a single soybean seedling (300 μg) is almost 4 times the maximal amount found in a single maize seedling (80 μg).

Differences in hydroxyproline content between maize and soybean seedlings were also evident when hydroxyproline content was expressed in μg per g fresh weight (fig 2). For both maize and soybean, each g of fresh weight contains 140 μg at the 0.25-day stage of development. The amount of hydroxyproline per g of fresh weight of maize tissue then decreases to 50 μg by the fifth day after germination and remains at that level even to the fifteenth day

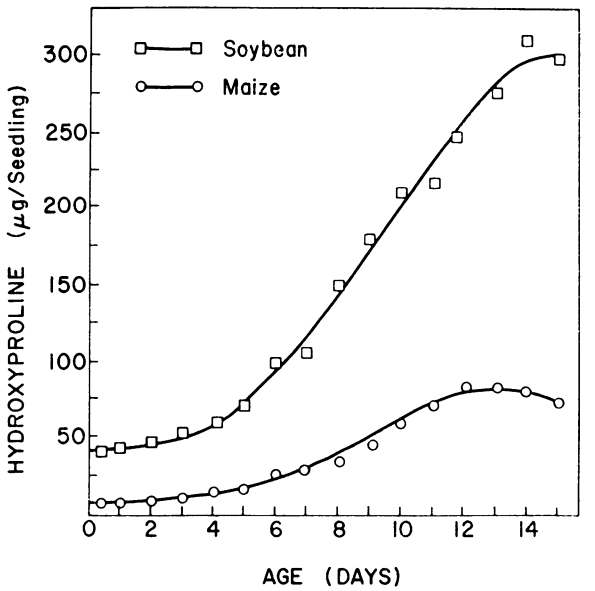

FIG. 1. The hydroxyproline content in μg per seedling of etiolated maize and soybean seedlings, expressed as a function of seedling age.

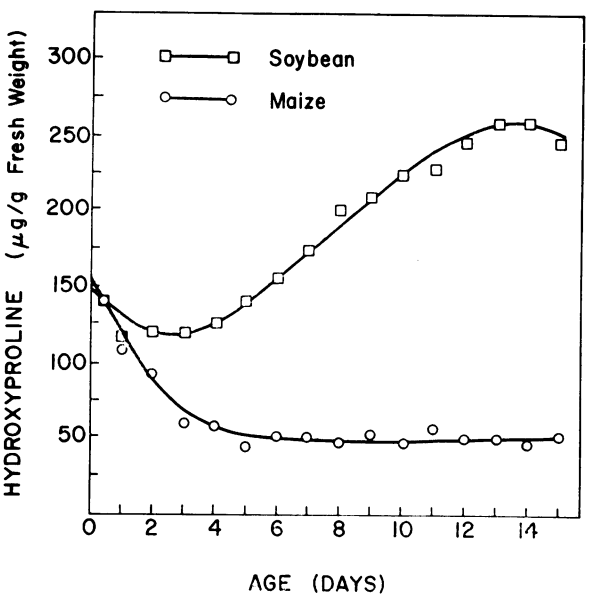

FIG. 2. The hydroxyproline content in μg per g fresh weight of etiolated maize and soybean seedlings, expressed as a function of seedling age.

after germination; on the other hand, the hydroxyproline content of soybean decreases to 125 μg per g fresh weight at the 3-day stage and then increases to 260 μg per g fresh weight by the thirteenth day after the start of germination. Probably the initial decrease in the amount of hydroxyproline in each g of fresh tissue is related to a relatively large uptake of water during the first few days of germination. For each g of fresh weight, the maximal amount of

hydroxyproline in soybean (260 μg/g fr wt) is about 5 times the amount in maize (50 μg/g fr wt).

Seedlings of all ages from 0.25-day to 14-day were examined with respect to the percentage of hydroxyproline in the cell wall fraction. It was found that the bulk of the hydroxyproline was in the cell wall. For maize seedlings, the amount of hydroxyproline in the cell wall fraction was 70 to 83 % of the total in the seedlings, whereas for soybean the amount was 80 to 93 %. The amount of hydroxyproline associated with the cell wall increased with increasing age of the seedlings.

Exchange of Oxygen Atoms of Hydroxyproline during Hydrolysis. In experiments in which the origin of oxygen atoms is examined, it is necessary to avoid the exchange of the oxygen atoms with water of the medium. In particular, it was necessary to verify that the hydrolytic method which was employed to isolate hydroxyproline did not result in the exchange of the hydroxyl oxygen atom. Rittenberg et al. (24) found that oxygen atoms of the carboxyl groups of tyrosine and serine exchanged with those of the medium during heating at 108° with 6 N HCl, but the oxygen atoms of the hydroxyl group did not exchange. Fujimoto and Tamiya (4) and Prockop et al. (21) obtained similar results with hydroxyproline. In the present investigation, 20 mg of reagent hydroxyproline were sealed in a glass tube together with 4 ml of 6 N HCl containing 1.15 atom percent excess H_2O^{18}, and the contents of the tube were heated at 108° for 24 hours. The hydroxyproline was recovered and pyrolyzed to CO_2. The results which were obtained confirmed that 2 of the oxygen atoms exchanged completely with water, but the third remained unexchanged. These results are consistent with the view that exchange of the 2 carboxyl oxygen atoms, but not the hydroxyl oxygen atom, occurred during acid hydrolysis.

Incorporation of O^{18} into Hydroxyproline. The results of the analysis of O^{18} abundance in hydroxyproline isolated from maize and soybean seedlings which were incubated in atmospheres enriched with O^{18} are shown in table I; several trial experiments in which similar results were obtained are not tabulated. The data show that appreciable amounts of O^{18} were incorporated into hydroxyproline from atmospheric O_2. Proline isolated from seedlings exposed to atmospheres enriched with O^{18} did not contain an excess of O^{18}. [The higher value in table I for atom percent O^{18} found in maize incubated in $O_2{}^{18}$ for 4 days (0.605) compared to 8 days (0.383) is probably related to different growth conditions which exist in different Erlenmeyer flasks; it has been our experience that the fresh weight increment of seedlings grown in 250 ml Erlenmeyer flasks is not identical in different flasks which receive the same treatments with respect to moisture, temperature, etc. Since it was demonstrated (vide supra) that the oxygen atom of the hydroxyl group of hydroxyproline does not exchange with those of water during the hydrolysis process, whereas the oxygen atoms in carboxyl groups are replaced by those of water, it could be concluded that O^{18} was incorporated into the hydroxyl group of hydroxyproline.

The data (table I) for O^{18} incorporation into hydroxyproline when seedlings were exposed to $O_2{}^{18}$ are lower than might be anticipated. That is, the graphical information presented in figure 1 indicates that roughly one-half of the hydroxyproline in the seedlings can be assumed to be synthesized during the experimental periods of exposure to $O_2{}^{18}$; therefore it appears reasonable to expect that O^{18} enrichments in hydroxyproline might be as high as 1.6 atom percent, or one-half × one-third × 10 atom percent = 1.6 atom percent O^{18} excess, where multiplication by one-third is necessary because only 1 atom in hydroxyproline is labeled. But the observed enrichments (0.383 and 0.605 for maize, and 0.271 and 0.222 for soybean) are much less than 1.6 atom percent O^{18} excess. No single explanation for the measured low enrichments can be offered, but the following possibilities exist. Traces of impurities in the recovered hydroxyproline, particularly impurities containing relatively high proportions of oxygen atoms, would affect the measured O^{18} enrichments. Since the amounts of hydroxyproline which were recovered were 1 mg or less, impurities (e.g., cellulose from the thin-layer plates, or traces of water) in the recovered hydroxyproline might have lowered appreciably the measured O^{18} enrichments. Another possible explan-

Table I. *Incorporation of $O_2{}^{18}$ into Hydroxyproline by Etiolated Maize and Soybean Seedlings*

Atom percent O^{18} excess = atom percent O^{18}–normal abundance of O^{18} (0.204 atom percent).

	Expt no.	Age (days) of seedlings at beginning and end of expt	Atom percent O^{18} excess in $O_2{}^{18}$ or H_2O^{18}	Atom percent O^{18} excess in hydroxyproline
			$O_2{}^{18}$ Experiments	
Maize	1	0.25–8	9.98	0.383
	2	3–7	10.42	0.605
Soybean	1	0.25–8	10.36	0.271
	2	3–7	10.42	0.222
			H_2O^{18} Experiments	
Maize		0.25–8	1.52	0.000
Soybean		0.25–8	1.33	0.000

ation for the low O^{18} enrichments in table I involves the different conditions under which seedlings were grown; the data for figure 1 were derived for seedlings grown in trays, whereas those in table I were based on seedlings grown in Erlenmeyer flasks. We have observed on numerous occasions that growth of seedlings in Erlenmeyer flasks, under our conditions, is often much less than growth in open trays, and we have attributed these effects to the production by the seedlings of unknown volatile emanations which are not removed completely by the KOH in the vials which are placed in the Erlenmeyer flasks. Still another possible explanation for the low O^{18} enrichments in hydroxyproline in table I may be related to the development of low O_2 tension in Erlenmeyer flasks. It is possible that at less than normal O_2 tensions, pathways other than that described here may be utilized in hydroxyproline biosynthesis.

Experiments with H_2O^{18}. Although the experiments in which the etiolated seedlings were exposed to atmospheres labeled with O^{18} (see table I) supported the view that a direct incorporation of molecular O_2 into hydroxyproline had occurred, another interpretation was possible. That is, the possibility exists that the observed enrichment in hydroxyproline was an indirect effect caused by the reduction of $O_2{}^{18}$ to water during respiration followed by subsequent incorporation of H_2O^{18} into hydroxyproline. However, it could be calculated readily (knowing the respiratory rates of the seedlings) that the O^{18} in hydroxyproline could not have come from respiratory water, assuming that respiratory water produced during the incubation period mixed completely with the water initially present in the flask.

Even so, the possibility exists that respiratory water may not mix with the rest of the tissue water, but may be compartmentalized within the cells, and then may be used directly in the biosynthesis of hydroxyproline. Therefore, control experiments were carried out in which seedlings were grown in water enriched with O^{18}; the gas phase was air containing normal O_2. The hydroxyproline isolated from seedlings grown under these conditions did not contain an excess of O^{18} (table I). Nor did the isolated proline contain an excess of O^{18}. The lack of incorporation of O^{18} from water into hydroxyproline eliminates the possibility that water may serve as the source of the hydroxyl O_2 atom of hydroxyproline during growth of the seedlings.

Discussion

From the results presented here, it is evident that O_2 fixation has a crucial role in hydroxyproline biosynthesis. The data show that atmospheric O_2 is incorporated directly into hydroxyproline in etiolated maize and soybean seedlings. Also, it may be concluded that the hydroxyl oxygen atom in hydroxyproline is derived from molecular O_2 and not from water. Hydroxyproline is probably produced in these seedlings from proline through the agency of hydroxylating enzymes, but direct evidence from enzyme studies is not presented here. However, even if an hydroxylase is involved in hydroxyproline biosynthesis, it does not necessarily follow that free proline is converted to free hydroxyproline. In fact, the available evidence indicates that free proline is not converted to free hydroxyproline nor is free hydroxyproline incorporated directly into protein (*26*); instead, proline is probably first activated, and then the activated derivative is converted to hydroxyproline. Whether the hydroxylation reaction in higher plant tissues occurs before or after the incorporation of proline into protein is not known.

The localization of hydroxyproline in proteins found in cell walls of several species of higher plants (*2*) (15) (*22*) has been emphasized by Lamport (14), who proposed that an hydroxyproline-rich protein has an important role in cell wall structure and in the ability of the cell wall to extend. The present data also indicate that most of the hydroxyproline in etiolated maize and soybean seedlings is associated with the cell wall.

Acknowledgment

We acknowledge the technical assistance of Mr. S. Pasznik.

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