Hydroxyproline in *Chlorella* and its Metabolism in Tobacco Leaves*

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Hydroxyproline and other compounds were labeled with C^{14} by *Chlorella pyrenoidosa* supplied with $C^{14}O_2$ in the light. The hydroxyproline recovered from a hydrolysate of the algae was administered through the cut bases of tobacco leaves. The leaves formed little proline from the hydroxyproline, but the C^{14} label was transferred to a variety of other amino acids. Although hydroxyproline is not abundant in plants, it appears to be an active metabolite.

There have been numerous studies on the incorporation of C^{14} -labeled amino acids into plant proteins in recent years. Most of the studies have employed easily metabolizable amino acids, but recently Dougall and Shimbayashi¹⁾ have followed the incorporation of tyrosine, uniformly labeled with C^{14} , into tobacco callus tissue cultures; it was shown that tyrosine is not converted to any other amino acid but is incorporated directly into tobacco tissue protein.

In the experiments with tobacco tissue cultures, the "cell wall residue" fraction had a considerable amount of hydroxyproline, but there was no hydroxyproline in any other fractions, such as the "soluble amino acids", "soluble protein" and "formic acid soluble cell wall material". This localization of hydroxyproline in a specific fraction suggested the present investigation of the metabolism of hydroxyproline in whole tobacco leaves in the dark for extended periods. In addition, the experiments gave some indication of the

occurrence of hydroxyproline in plant proteins.

MATERIALS AND METHODS

Preparation of Labeled Amino Acids.

For preparation of amino acids uniformly labeled with C^{14} , *Chlorella pyrenoidosa* was grown in a medium (McBride, L., private communication) containing per liter: NH_4NO_3 , 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.308 g; KH_2PO_4 , 1.96 g; K_2HPO_4 , 3.43 g; $CaCl_2 \cdot 2H_2O$, 0.073 g; and $KHCO_3$, 1.25 g. Iron chelated by ethylenediamine tetraacetic acid was provided in the ferric form at 5 ppm, and the micro elements were supplied by 0.5 ml per liter of Hoagland and Arnon Solution A₅²⁾ diluted 1/25 from the strength recommended for higher plants. The phosphates were adjusted to pH 6.2, and the remaining nutrients without potassium bicarbonate were autoclaved separately at 120°C for 30 minutes. The potassium bicarbonate was saturated with carbon dioxide, filtered through a fritted glass bacterial filter, and added aseptically to the medium.

The algae were grown in four liters of medium containing 2.5 millicuries C^{14} per liter as potassium bicarbonate. The cultures were stirred magnetically, cooled by a fan, and illuminated by fluorescent lamps. The growth was allowed to continue to maximum population density, and then the algae were collected by centrifugation, washed once with water, and heated to boiling in 75% ethanol. The residue was recovered,

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1) D.K. Dougall and K. Shimbayashi, *Plant Physiol.*, **35**, 396 (1960).

2) D.I. Arnon, *Am. J. Botany*, **25**, 322 (1938).

washed, hydrolyzed with 6 N hydrochloric acid under reflux for 24 hours, and excess acid was removed by vacuum distillation.

The slightly modified Hirs, Moore, and Stein³⁾ method employing acetic and hydrochloric acids for elution was used to separate the amino acids. To separate tyrosine, glutamic acid and aspartic acid, Dowex 1 X10 (200~400 mesh) was used in its acetate form at room temperature in a column 40×2 cm. The concentration of the eluant was increased gradually by feeding 1 N acetic acid into a 250 ml mixing chamber containing 0.1 N acetic acid. The flow rate was 8 ml per hour. The first fraction from the Dowex 1 column, containing various basic and neutral amino acids, was separated on a 2.5×150 cm column of Dowex 50 X4 (H⁺) at room temperature. The concentration of hydrochloric acid was increased gradually after the emergence of glycine, and 5 to 6 four ml fractions of the effluent were collected per hour. The recovery of amino acids appeared to be low with the Dowex 50 column and hydrochloric acid as eluting agent in a preliminary experiment, but the apparent recovery was improved later by strengthening the buffering of the ninhydrin solution to maintain an optimal pH. Each group of effluent fractions corresponding to a peak was pooled and concentrated to dryness, dissolved in a small amount of distilled water, and a sample of the concentrate was developed on a paper chromatogram. The chromatograms were radioautographed to identify each radioactive fraction. The fractions 349 to 367, corresponding to the region of ammonia (and hydroxyproline), were pooled and freed of hydrochloric acid in a rotary evaporator followed by two passages through Dowex 1 X10 with 0.5 N acetic acid as eluting agent.

The concentrated amino acid solution recovered from fractions 349 to 367 gave little violet color with ninhydrin. An aliquot of the solution was analyzed by paper chromatography and radioautography, and another aliquot was added to a standard amino acid mixture containing 1 μ mole of most amino acids and 5 μ moles of hydroxyproline. The mixture was separated⁴⁾ with Dowex 50 X8 at 30°C and the unknown amino acid mixture proved to be mainly hydroxyproline together with some glutamic acid and glycine.

The amino acid solution used for the tobacco leaf

experiments had the following C¹⁴ distribution upon passage through the Dowex 50 column: 74.0% of the total C¹⁴ added to the column was recovered as hydroxyproline, 3.2% as glutamic acid, 6.0% as glycine, and 7.6% as an unknown compound (91% total recovery). The 75,000 c.p.m. added to each tobacco leaf thus contained approximately 55,500 c.p.m. as hydroxyproline, 2,400 c.p.m. as glutamic acid, 4,500 c.p.m. as glycine, and 5,700 c.p.m. as an unknown compound.

Administration of C¹⁴-Hydroxyproline to Tobacco Leaves and Analysis.

Young leaves weighing about 5 g were picked at about 9:00 a.m. from tobacco plants (*Nicotiana tabacum* var. *Wisconsin Havana* 38), and the cut leaf bases were immediately recut under water. The cut base of each of three leaves was immersed in 1 ml of C¹⁴-hydroxyproline solution in a small beaker; each ml of the amino acid solution gave 75,000 c.p.m. with a thin window Geiger counter as indicated earlier. The leaves were kept in a dark room for 1.5 hours, 3 hours, and 10 hours after the treatment was started, and during the experiment small amounts of water were added to the beakers as needed. To stimulate transpiration, air was blown across the leaves with a fan. After each experimental period, a leaf was ground in a small amount of 6 N hydrochloric acid with mortar and pestle, and then was transferred into a 500 ml round bottom flask with 50 ml of 6 N hydrochloric acid. After refluxing for 24 hours, the hydrolysate was filtered to remove humin, transferred into another round bottom flask, connected to a rotating vacuum evaporator and the hydrochloric acid was removed at 50~60°C. Water was added and evaporated twice, and each of the final residues was dissolved in 10 ml of water.

The acidic and neutral amino acids from 1 ml of hydrolysate were separated⁴⁾ on Dowex 50 X 8 (200~400 mesh, sodium form) in a 150×0.9 cm column operated at 50°C with 0.2 N sodium citrate buffer plus redistilled thiodiglycol, pH 3.25, as eluant until alanine was displaced (about 160 fractions); then the buffer and temperature were changed to 0.2 N sodium citrate buffer plus redistilled thiodiglycol, pH 4.25, and 60°C. The effluents were collected in 2 ml fractions at a rate of 12 ml per hour; the flow rate was regulated by a proportioning pump. To separate aspartic acid and hydroxyproline properly, another column was operated at 30°C.

3) C.H.W. Hirs, S. Moore and W.H. Stein, *J. Am. Chem. Soc.*, **76**, 6063 (1954).

4) S. Moore, D.H. Spackman and W.H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

The basic amino acids were separated with Amberlite 120, type 3, in a 15×0.9 cm column operated at 50°C with 0.35 N sodium citrate buffer, pH 5.28; 1 ml of hydrolysate was added to the column, and 2 ml fractions were collected at a rate of 12 ml per hour. The Amberlite resin was fractionated into various particle sizes by a hydraulic method⁹, and the fraction of mean particle size 32 microns was used (Table I).

TABLE I. RECOVERY OF AMINO ACIDS ON A DOWEX 50 COLUMN FROM A MIXTURE CONTAINING ONE MICROMOLE OF EACH AMINO ACID

Amino acid	Micromoles recovered, leucine color standard	Average	Color yield factor
Lysine	1.05, 1.06, 1.11	1.07	0.935
Histidine	1.06, 0.94, 1.01	1.00	1.00
Ammonia	1.06, 1.06, 1.16	1.09	0.917
Arginine	0.97, 1.02, 1.05	1.01	0.990
Aspartic acid	0.95, 0.91, 1.135	0.93	1.075
Threonine	0.92, 0.91, 0.92	0.915	1.093
Serine	0.95, 0.93, 0.92	0.93	1.075
Glutamic acid	0.93, 0.91	0.92	1.087
Proline	0.19, 0.21, 0.22	0.21	4.762
Glycine	0.93, 0.96, 0.90	0.93	1.075
Alanine	0.97, 0.93, 0.99	0.97	1.031
Cystine	0.52, 0.52, 0.46	0.50	2.000
Valine	0.93, 0.99, 0.89	0.94	1.064
Methionine	0.96, 0.95, 0.89	0.92	1.087
<i>i</i> -Leucine	1.01, 0.95, 0.96	0.97	1.031
Leucine	1.01, 1.00, 0.97	0.99	1.010
Tyrosine	0.98, 0.95, 1.03	0.99	1.010
Phenylalanine	0.92, 0.93, 0.93	0.93	1.075
β -Alanine	0.40, 0.44	0.42	2.381

Hydroxyproline 0.433, 0.424 μ moles recovered (leucine standard), 5.00 μ moles added
11.50

The ninhydrin method of Moore and Stein⁹ was employed; the reagent was stored under N₂ and consisted of 10 g ninhydrin, 1.5 g hydrindantin, 375 ml methyl cellosolve freed of peroxide and 125 ml pH 5.5, 4 N acetate buffer.

A one ml aliquot of each effluent fraction from each amino acid peak was mixed with 0.5 ml of 2% gelatin solution in 0.3 N sodium hydroxide, and then 1 ml of the mixture was plated on a 1 inch diameter flat copper planchet. The planchets were dried over night at room temperature and were counted with a thin window Geiger counter.

5) P.B. Hamilton, *Anal. Chem.*, **30**, 914 (1958).

6) S. Moore and W.H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

RESULTS AND DISCUSSION

Hydroxyproline in *Chlorella pyrenoidosa*.

The use of *Chlorella pyrenoidosa* for the biological preparation of C¹⁴-labeled compounds has been rather widely adopted, because the organism utilizes C¹⁴ efficiently, and can be grown conveniently on a large scale⁷. Although hydroxyproline has been found in a few plants⁸⁻¹¹ it has not been reported in earlier analyses of *Chlorella pyrenoidosa*^{7,11}; it was clearly present, though not abundant, in the hydrolysate we recovered from the organism. Its low concentration and low color yield with ninhydrin probably account for the fact that others have overlooked it. The procedure used in our experiments for this separation of amino acids involved only slight modifications

TABLE II. AMINO ACID CONTENT OF HYDROLYSATE OF *Chlorella pyrenoidosa*

Amino acid	μ mole recovered from column	Mole percentage composition calculated from recovered amino acids
Aspartic acid	276.5	9.34
Glutamic acid	256.6	8.67
Tyrosine	74.0	2.50
Serine	77.8	2.63
Threonine	78.0	2.64
Glycine	226.6	7.69
Ammonia	520.2	17.58
Alanine	327.5	11.08
Valine	156.6	5.30
Proline	183.2	6.20
Methionine	39.8	1.35
<i>i</i> -Leucine	90.7	3.06
Leucine	234.9	7.95
Cystine	2.5	0.08
Lysine	142.4	4.82
Histidine	32.0	1.08
Arginine	123.6	4.18
Phenylalanine	113.7	3.85

7) L. Schieler, L.E. McClure and M.S. Dunn, *J. Biol. Chem.*, **203**, 1039 (1953).

8) G. Leaf, I.C. Gardner and G. Bond, *Biochem. J.*, **72**, 662 (1959).

9) F.C. Steward, J.F. Thompson and J.K. Pollard, *J. Exptl. Botany*, **9**, 1 (1958).

10) F.C. Steward and J.K. Pollard, *Nature*, **182**, 828 (1958).

11) L. Schieler, L.E. McClure and M.S. Dunn, *Food Research*, **18**, 377 (1953).

of the method of Hirs, et al.³). In their experiments with a hydrolysate of human serum albumin no hydroxyproline appeared, so others following the method may not have looked specifically for hydroxyproline. Hydroxyproline appears to be much less abundant than proline in living materials; although low in concentration, hydroxyproline may be widespread in higher plants.

Table II indicates the mole percentage composition of the amino acids in an acid hydrolysate of *Chlorella pyrenoidosa*. Alanine, aspartic acid, glutamic acid, leucine and glycine were particularly abundant, and proline, valine, lysine, arginine and phenylalanine also were present in high concentrations. Cystine, histidine and methionine were least abundant.

Amino Acid Composition of the Hydrolysates of Tobacco Leaves.

Table III indicates the reasonably constant amino acid composition of tobacco leaves after

TABLE III. AMINO ACID CONTENT OF HYDROLYSATES OF TOBACCO LEAVES

Amino acid	Period of exposure to C ¹⁴ -hydroxyproline		
	1.5 hr., mole %	3.0 hr., mole %	10 hr., mole %
Aspartic acid	6.86	7.35	8.21
Threonine	3.38	3.35	4.21
Serine	3.36	3.35	5.00
Glutamic acid	11.53	11.21	12.19
Proline	6.55	4.86	7.76
Glycine	8.15	7.80	7.82
Valine	5.70	6.05	5.55
Methionine	1.29	1.36	1.26
iso-Leucine	3.82	4.43	3.65
Leucine	6.56	7.99	6.51
Tyrosine	1.88	1.82	1.88
Phenylalanine	2.77	3.27	3.04
Lysine	5.36	5.79	4.73
Histidine	1.34	1.42	1.31
Ammonia	18.80	17.04	16.70
Arginine	3.40	3.71	3.25
Cystic acid	0.19	0.88	0.84
Total	99.29	99.67	101.00

3 periods of exposure to C¹⁴-hydroxyproline. Aspartic acid, threonine, serine, phenylalanine, cystic acid and proline showed the only marked increases with time. The concentration of ammonia decreased gradually, as did that of a few of the amino acids. Because of its extremely faint color with ninhydrin, hydroxyproline was not prominent but was demonstrable in the effluent from the column operated at 30°C. Cystine was not detected in any experiment. Fractions 45~55 yielded a red color with ninhydrin; this unknown compound(s) appearing before aspartic acid had slight radioactivity.

Metabolism of Hydroxyproline.

About 50% of the hydroxyproline added (55,500 c.p.m. initially) to tobacco leaves was metabolized within 1.5 hours, and about 80% within 3 hours (Table IV). The radioactivity of threonine and glutamic acid (2,400 c.p.m. initially present in solution as a contaminant) decreased with time, and a slower decrease was noted for glycine, valine and the unknown compound. Contrary to this, the radioactivity of proline increased actively. Accumulation of C¹⁴ during the first 1.5 hours was particularly marked into threonine. It is quite possible that the C¹⁴ in threonine could have arisen chiefly from labeled glycine and glutamic acid which contaminated the solution of hydroxyproline.

The metabolism of hydroxyproline in higher plants has not been studied thoroughly, probably because hydroxyproline often escapes notice as a result of its low color yield with ninhydrin, and because of the general belief that it is not a significant metabolite in plants. However, hydroxyproline was demonstrated in tobacco leaves by Kunitake et al.¹²). The leaves they exposed to C¹⁴O₂ in the dark for 15 minutes incorporated C¹⁴ into hydroxyproline; although the level of C¹⁴ in hydroxy-

¹²) G. Kunitake, C. Stitt and P. Saltman, *Plant Physiol.*, **34**, 123 (1959).

TABLE IV. C^{14} IN AMINO ACIDS OF TOBACCO LEAVES AFTER EXPOSURE TO C^{14} -HYDROXYPROLINE FOR 1.5, 3 AND 10 HOURS

Amino acid	1.5 hrs.		3.0 hrs.		10 hrs.	
	c.p.m./whole leaf	c.p.m./ μ mole amino acid	c.p.m./whole leaf	c.p.m./ μ mole amino acid	c.p.m./whole leaf	c.p.m./ μ mole amino acid
Hydroxyproline	27,270	—	11,550	—	13,800	—
Threonine	4,740	101.6	3,150	57.8	840	12.7
Glutamic acid	1,620	10.1	720	4.4	360	2.3
Proline	570	6.3	1,290	18.4	3,450	33.5
Glycine	780	6.4	600	4.4	570	5.5
Valine	2,040	25.9	1,650	19.0	1,860	25.4
Leucine	—	—	420	3.6	—	—
Unknown compd.*	1,140	—	960	—	720	—

* The unknown compound gave a red color with ninhydrin.

proline was low, it was comparable to that of many other amino acids in the leaves.

The present experiment suggested that hydroxyproline was relatively rapidly metabolized in the dark. Much of the radioactivity which was added to the tobacco leaves remained in the leaf hydrolysate, even after 10 hours exposure to labeled hydroxyproline. The incorporation of C^{14} into other amino acids than those supplied occurred quickly, and the decrease in the C^{14} of the amino acids after 1.5 hours was probably because of their respiratory oxidation to carbon dioxide. Although the increase in radioactivity of proline might have resulted from the conversion of the labeled glutamic acid contaminant to proline, it seems likely that proline arose in part from hydroxyproline. The decrease in radioactivity in hydroxyproline was far greater than the increase in radioactivity of proline. During the first 1.5 hours, the C^{14} in hydroxyproline decreased by over 28,000 c.p.m. while proline accumulated 570 c.p.m. The amino acids recovered account for but a minor part of the total C^{14} lost from hydroxyproline.

The amount of aspartic acid in the tobacco leaves increased with time; however, the original content of aspartic acid may have varied somewhat among individual leaves. Aspartic acid and malic acid are the prominent early products of $C^{14}O_2$ fixation in the dark, and it can be assumed that in our experiment the radioactivity which was incorporated initially into aspartic acid might later have been transferred to other compounds.

The amount of hydroxyproline is generally small compared to the amount of proline in higher plants, but hydroxyproline may be the more active metabolite. Proline may serve chiefly as an end product in amino acid metabolism in the tobacco leaf, whereas our data on the rapid loss of C^{14} -hydroxyproline from the leaves suggests that this amino acid fills an active metabolic role.

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