Metabolism of Free Hydroxyproline in *Avena* Coleoptiles*

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ABSTRACT: Incubation of *Avena* coleoptiles with [2-^{14}C]hydroxyproline or [5-^{3}H]hydroxyproline resulted in significant incorporation of label into both free and protein-bound proline as well as protein-bound hydroxyproline. No other constituents contained any significant amount of label. The retention of tritium at C-5 and the lack of ^{14}C in glutamate indicate that the conversion of hydroxyproline to proline did not proceed through glutamate. Conversion of hydroxyproline to proline occurred even when protein synthesis was blocked by cycloheximide. Incorporation into proteins of label from [5-^{3}H]hydroxyproline occurs at 20% of the rate of incorporation from [^{14}C]proline. Both sources of label gave the same ratio of labeled proline to hydroxyproline in protein, which indicates that hydroxyproline, at levels which do not inhibit growth, is incorporated into protein only after conversion to free proline.

In plants the widespread occurrence of hydroxyproline, particularly in the cell wall, is well established (Lamport, 1965; Vanetten *et al.*, 1963). Stetten (1949) concluded that proline and not hydroxyproline is the precursor of bound hydroxyproline in animals. Steward and Pollard (1958), Lamport (1965), and Olson (1964) showed the same thing in plants. Recently, Cleland (1963) reported that free hydroxyproline at 10^{-3} M causes almost complete inhibition of auxin-induced growth in oat coleoptiles. His results raised the possibility that this free imino acid may have a regulatory role in development and, if so, that it may be metabolized in this system. With this in mind, we have studied the metabolism of radioactive hydroxyproline in *Avena* coleoptiles *in vivo*. We report here the results of studies in which coleoptiles were treated with noninhibitory levels of hydroxyproline.

Experimental Section

Materials

The plant material consisted of 14-mm sections cut from 25–32-mm coleoptiles of *Avena sativa*, var. Victory. Seedlings were grown and sections were prepared as detailed in Cleland (1960). Leaves were removed from all sections.

[5-^{3}H]Hydroxyproline (550 or 187 mc/mmole) and L-[^{14}C]proline (165 or 200 mc/mmole) were from New England Nuclear Corp.[1] [2-^{14}C]Hydroxyproline mixed isomers (18.5 mc/mmole), hydroxy-L-proline (proline free), and Pronase, B grade, were from California Corp. for Biochemical Research. L-Proline (L-hydroxyproline free) was from Sigma Chemical Co.

Methods

Incubations. Unless otherwise stated, lots of 30 sections were incubated for 22 hr in test tubes with 5 ml of solution. The media contained potassium maleate (2.5 mM, pH 4.7), sucrose (2% w/v), penicillin G (0.1 mM), and imino acid (2×10^{-5} M) as indicated. The test tubes were rotated at 1 rpm on a Rollar drum.

Preparation of Material. The following procedure was used in most experiments. After incubation sections were washed in water, surface moisture was removed and the sections were ground in an all-glass homogenizer with 2 ml of Tris buffer (0.05 M, pH 7.5). Four volumes of 95% ethanol were added and the homogenate was heated for 5 min in boiling water. The cooled homogenate was centrifuged at 2000*g*, the supernatant was removed, and the pellet was washed five times with hot 80% ethanol and then dried with 100% ethanol and ether. The pellet was placed in an ampoule with 4 ml of 6 N HCl and hydrolyzed for 18 hr at 115°. After filtration to remove humin, the filtrate was condensed to near dryness with heat and a nitrogen stream and streaked across one end of a 1.25-in. wide strip of Whatman 1- or 3MM paper. The paper was washed with ethyl acetate–acetic acid–water (10:5:2, v/v). prior to use. The supernatant was also condensed and placed on a paper strip.

In certain experiments the sections were directly hydrolyzed without homogenization. After incubation, washed sections were placed in ampoules with 8 ml of 6 N HCl and hydrolyzed for 18 hr at 115°. The hydrolysate was then treated as detailed above.

In other experiments protein was precipitated with

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[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

trichloroacetic acid[2] rather than hot alcohol. After homogenization in Tris buffer, an equal volume of cold 20% TCA was added and the homogenate was left for 2 hr at 4°. After centrifugation, the pellet was washed once with hot (90°) 5% TCA, twice with cold (4°) TCA, and six times with 95% ethanol. The pellet was then dried, hydrolyzed, and treated as detailed above.

Chromatography. The chromatograms were developed in one of the following solvent systems: isopropyl alcohol–formic acid–water (15:2:2, v/v) upflow or downflow; phenol–water (4:1, w/v) downflow; phenol saturated with 0.1 N HCl upflow; isopropyl alcohol–pyridine–acetic acid–water (8:8:1:4) upflow. Since the first two systems gave essentially complete separation of proline and hydroxyproline when used as downflow, they were utilized in most of the separations. Strips with known amino acids were run as controls. Spots were located by spraying with ninhydrin or with a radiochromatogram scanner.

Analyses. After elution from the chromatogram, proline was assayed by the method of Troll and Lindsley (1955) and hydroxyproline was determined by method I of Prockop and Udenfriend (1960) or by the method of Neuman and Logan (1950). To determine ^{14}C, 0.05–0.2 ml of eluate was dried on aluminum planchets and counted in a gas-flow counter. Tritium was measured by combining 0.05–0.2 ml of eluate with 15 ml of either the dioxane-counting mixture of Butler (1961) or the mixture recommended by Loewus (1961) and counting in a liquid scintillation spectrometer. When milligram quantities of proline were counted, 0.5 ml of water, 10 mg of urea, and an equal volume of Cab-O-Sil were added to the counting mixture to prevent precipitation of the proline. These additions did not significantly alter the counting efficiency.

Enzymatic Digests. Aliquots of alcohol- or TCA-washed pellets were digested with Pronase, following the procedure of Nomoto *et al.* (1960). After 18-hr digestion, the samples were boiled, cooled, centrifuged, and an aliquot of the supernatant was chromatogrammed in isopropyl alcohol–pyridine–acetic acid–water (8:8:1:4) or isopropyl alcohol–formic acid–water (15:2:2). Control digests were run with boiled enzyme. The chromatograms were surveyed for radioactivity and sprayed with ninhydrin to locate peptides.

Proline Derivatives. Proline was converted to nitrosoproline by the procedure of Myhill and Jackson (1963) and to proline picrate by the procedure of Bergmann (1935).

Results

When *Avena* coleoptile sections were incubated with 2×10^{-5} M [5-^{3}H]hydroxyproline or [2-^{14}C]hydroxyproline a significant amount of radioactivity was found in the alcohol-insoluble or TCA-insoluble fractions. For instance, after incubation for 22 hr with [5-^{3}H]hydroxyproline, 34% of the radioactivity which had been taken up by the tissue was in the alcohol-insoluble fraction (Table I). Hydrolysis of this fraction and separation of the amino acids revealed that only two amino acids, proline and hydroxyproline, were labeled. The majority (95%) of the label was in proline.

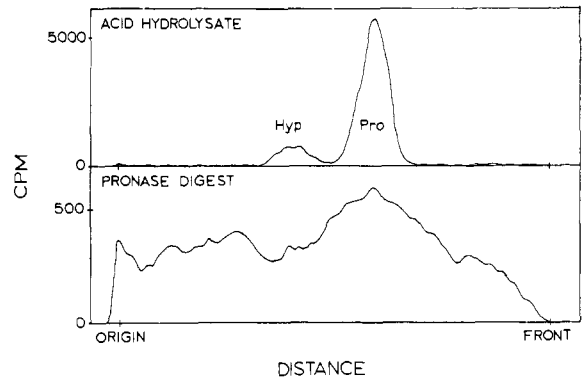

FIGURE 1: Distribution of radioactivity after chromatography of acid or Pronase digests of *Avena* coleoptile protein. Protein precipitated with hot alcohol after incubation of sections for 22 hr with [5-^{3}H]-hydroxyproline. Chromatograms run in isopropanol–formic acid–water.

TABLE I: Metabolism of [5-^{3}H]Hydroxyproline in *Avena* Coleoptile Tissues.[a]

	10^3 Cpm in		
	Hydroxy-proline	Proline	% in Proline
Administered	2280	0	0
After incubation			
Alcohol soluble	133	93	39
Alcohol insoluble	6.3	112	95

[a] Sections (30, 14 mm) were incubated for 22 hr in 5 ml containing potassium maleate (2.5 mM, pH 4.7), sucrose (2%, w/v), penicillin G (0.1 mM), and [5-^{3}H]-hydroxyproline (20 μc, 2×10^{-5} M).

In order to establish that the labeled proline and hydroxyproline were in protein, the alcohol- and TCA-insoluble fractions were enzymatically digested with Pronase and the resulting peptides were chromatographically separated. Radioactivity was widely distributed on these chromatograms, as would be expected if the amino acids had been in protein (Figure 1).

When the free amino acids in the alcohol-soluble fraction were chromatographically separated, only

[2] Abbreviations used: TCA, trichloroacetic acid; CHA, cycloheximide.

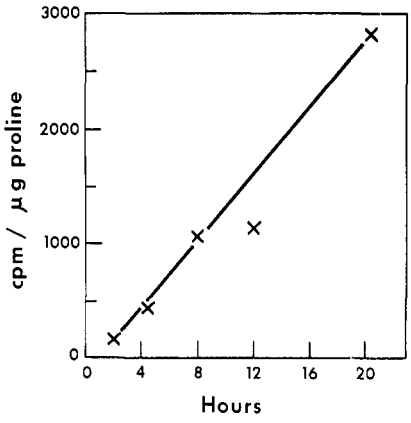

FIGURE 2: Increase in specific activity of proline with incubation time. Each point represents material recovered from incubation of 20 12-mm coleoptiles with 10 μc of [2-^{14}C]hydroxyproline.

proline and hydroxyproline were found to contain significant radioactivity (Table I). In this fraction 39% of the label was in proline.

These results indicated that a sizable conversion of hydroxyproline to proline occurs in *Avena* coleoptile sections. To verify this finding it was necessary to show that proline was absent from the administered hydroxyproline and that the newly formed amino acid was indeed proline. Evidence that the original hydroxyproline solution was free of labeled proline was obtained by chromatographing the hydroxyproline in isopropyl alcohol–formic acid–water, eluting the proline and hydroxyproline regions of the chromatogram, and rechromatographing the proline section in phenol–HCl. After the second chromatography, less than 0.01% of the original label was present in the proline section of the chromatogram (Table II). The proline in the tissues could not have come from proline contaminants in the original hydroxyproline solution.

Verification that the newly formed radioactive amino acid was proline was accomplished in the following manner. First, the labeled proline was chromatographed with authentic unlabeled proline in all four solvent systems. In each case the position of the radioactive material coincided with that of the known proline. Only one radioactive spot was obtained when an aliquot of the suspected proline was chromatographed with known [^{14}C]proline. One aliquot of the suspected proline was mixed with unlabeled proline and recrystallized from 1-propanol to constant specific activity. The mixture was then converted to labeled *N*-nitrosoproline with essentially no loss of counts. A portion of the mixture was also converted to proline picrate (mp 152°) and recrystallized twice from 1-propanol with no loss in radioactivity.

The possibility that the conversion of hydroxyproline to proline occurred as a result of bacterial contamination rather than in the coleoptile tissues was discounted for the following reasons. First, labeled proline could not be detected in the incubation medium even after a 22-hr incubation. Secondly, the addition of 40 μg/ml of streptomycin or 0.1 mM penicillin G had no effect on this conversion. Finally, the specific activity of the proline recovered from whole coleoptiles increased linearly with time (Figure 2); if bacterial contamination was a problem, the increase would unlikely be linear.

The conversion of hydroxyproline to proline might occur at the free amino acid stage or only after incorporation of hydroxyproline into protein. Evidence to favor the former possibility was obtained by use of the protein synthesis inhibitor cycloheximide (Table III). At 3 μg/ml, cycloheximide almost completely inhibited protein synthesis as measured by incorporation of label from [^{14}C]proline or [^{3}H]hydroxyproline into

TABLE II: Evidence That Original [5-^{3}H]Hydroxyproline Does Not Contain Proline.

	Cpm	Original Label (%)
(I) Hydroxyproline placed on paper	2950×10^3	100
(II) Proline region, after chromatography of I[a]	20×10^3	1
(III) Proline region, after rechromatography of II[b]	200	0.01

[a] Isopropyl alcohol–formic acid–water (15:2:2, v/v), upflow. [b] Phenol saturated with 0.1 N HCl, upflow.

TABLE III: Conversion of Hydroxyproline to Proline in the Presence of Cycloheximide.[a]

	mμmoles		%
	Control	3 μg/ml of CHA	Inhibition
Hydroxyproline administered	100	100	—
Hydroxyproline taken up	13.2	4.7	64
Proline formed			
Alcohol soluble	3.6	1.3	64
Alcohol insoluble	4.7	0.06	99

[a] Sections (30, 14 mm) were incubated for 22 hr in 5 ml containing potassium maleate (2.5 mM, pH 4.7), sucrose (2% w/v), and [5-^{3}H]hydroxyproline (2×10^{-5}M, 20 μc) with or without cycloheximide (3 μg/ml).

protein but did not prevent the formation of proline from hydroxyproline. The magnitude of the hydroxyproline–proline conversion was depressed by cycloheximide, but this may be due to the inhibition by cycloheximide of the uptake of hydroxyproline into the tissue.

A comparison was made of the relative ability of [^{3}H]hydroxyproline and [^{14}C]proline to be incorporated into protein-bound proline and hydroxyproline in *Avena* coleoptile tissues (Table IV). Results for only a single incubation time of 22 hr are reported here since the incorporation rate from both imino acids was linear with time over this period (*e.g.*, see Figure 2). During this period proline was incorporated into protein five times more rapidly than hydroxyproline. Despite this difference in rate of labeling, the ratio of radioactive proline:hydroxyproline in protein was the same whether the radioactivity was administered as proline or hydroxyproline.

TABLE IV: Comparison of Incorporation into Protein Starting with [^{14}C]Proline and [5-^{3}H]Hydroxyproline.[a]

Source of Label	mμmoles Administered	mμmoles Incorpd into Protein as: Proline	Hydroxyproline	Proline:Hydroxyproline[b]
[^{14}C]Proline	100	25.3	1.31	19.3
[5-^{3}H]Hydroxyproline	100	4.55	0.21	21.7

[a] Sections (30) were incubated for 22 hr in 5 ml containing potassium maleate (2.5 mM, pH 4.7), sucrose (2%, w/v), and [^{14}C]proline (2×10^{-5} M, 1 μc), or [5-^{3}H]hydroxyproline (2×10^{-5} M, 20 μc). Protein precipitated with hot alcohol. [b] Ratio of radioactive proline:hydroxyproline in protein.

Discussion

Evidence has been presented here that hydroxyproline is rapidly converted to proline in *Avena* coleoptile tissues. This conversion differs in two ways from those reported previously from animal (Stetten, 1949; Gianetto and Bouthillier, 1954; Wolf *et al.*, 1956; Mitoma *et al.*, 1958; Jeffrey and Martin, 1966), bacterial (Adams, 1959), or plant systems (Pollard and Steward, 1959). The first difference is in the magnitude of the response. Only a minute fraction of the administered hydroxyproline found its way into proline in the intact rat (Stetten, 1949; Gianetto and Bouthillier, 1954; Wolf *et al.*, 1956), chick embryo (Mitoma *et al.*, 1958), or carrot tissue (Pollard and Steward, 1959). In contrast, over 60% of the hydroxyproline which was taken up by *Avena* coleoptiles was converted to proline.

Secondly, the pathway for proline synthesis seems to be different. In animal (Gianetto and Bouthillier, 1954; Wolf *et al.*, 1956) and bacterial systems (Adams, 1959) the hydroxyproline is first degraded to glutamate and then converted to proline by the normal biosynthetic pathways (Stetten, 1955). In *Avena* coleoptiles, however, the retention of tritium at C-5 and the lack of ^{14}C in free or bound glutamate indicate that glutamate is unlikely to be an intermediate in this conversion. The formation of proline in this tissue may occur *via* dehydration to 3,4-dehydroproline and then reduction to proline.

Proline is the normal precursor for protein-bound hydroxyproline in plants as well as animals (Lamport, 1965; Olson, 1964). In most systems, free hydroxyproline is not directly incorporated into proteins (Stetten 1949; Lamport, 1965). In fact, only in chick embryo (Mitoma *et al.*, 1958) and *Streptomyces antibioticus* (Katz *et al.*, 1962) has reasonable evidence been obtained for a direct incorporation of hydroxyproline into peptides.

Radioactive hydroxyproline was found in *Avena* coleoptile protein following incubation with radioactive hydroxyproline. This might have arisen *via* the normal hydroxyproline-forming pathways from the newly formed radioactive proline. Alternatively, a portion of the hydroxyproline might have been directly incorporated into the protein. In the former case the ratio of radioactive proline:hydroxyproline in protein should be the same regardless of whether the label was presented as proline or hydroxyproline. In the latter case incubation with hydroxyproline should result in a lowering of the proline:hydroxyproline ratio since in addition to the proline and hydroxyproline formed by the normal pathway there would be some hydroxyproline which arose *via* direct incorporation. It has been shown here (Table IV) that the proline:hydroxyproline ratio is unaffected by the source of the label. This indicates that no significant direct incorporation of hydroxyproline occurs when *Avena* coleoptile sections are incubated with low levels of hydroxyproline. However, these results do not eliminate the possibility that some direct incorporation of hydroxyproline may occur at higher, growth-inhibiting levels of hydroxyproline.

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Model Reactions for Coupling Oxidation to Phosphorylation*

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ABSTRACT: The oxidation-linked phosphorylation in *N,N*-dimethylacetamide solution is further examined along with some new experimental data. Phosphorylation occurred when a ferrohemochrome solution was oxidized by air in *N,N*-dimethylacetamide solution containing imidazole, adenosine 5′-monophosphate, and/or inorganic orthophosphate. When inorganic orthophosphate was absent, P^1,P^2-diadenosyl pyrophosphate could be produced at 10% yield. Phosphorylation was also observed when a *N,N*-dimethylacetamide solution of porphyrin plus imidazole plus adenosine 5′-monophosphate and/or inorganic orthophosphate was photoreduced under nitrogen and then reoxidized by exposure to air. Controlled experiments show that phosphorylation took place in the subsequent oxidation instead of the prior photoreduction step. Kinetic measurements show the existence of an intermediate capable of phosphorylating adenosine 5′-diphosphate to adenosine 5′-triphosphate. By using ^{32}P-labeled inorganic orthophosphate and paper chromatography, it was shown that this intermediate has the same R_F value as 1-phosphoimidazole. A possible oxidation mechanism leading to the formation of 1-phosphoimidazole, which can subsequently phosphorylate adenosine 5′-monophosphate or adenosine 5′-diphosphate, is suggested.

The elucidation of the molecular mechanisms involved in photo- and oxidative phosphorylation is a very difficult problem both because of the great structural complexity of the particulate systems wherein these reactions occur and because of our very limited knowledge concerning the type of chemical mechanisms capable of coupling oxidation to phosphorylation. During the last few years, attempts have been made in our laboratory to find oxidation reactions which cause inorganic phosphate to condense with AMP[1] and ADP, respectively, to form ADP and ATP. Initially it was established that both 1-phosphoimidazole and acetylimidazole plus orthophosphate would phosphorylate AMP and ADP at respectable rates in polar organic solvents (Brinigar and Wang, 1964a;

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[1] Abbreviations used: AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates; APPA, P^1,P^2-di(adenosyl-5-) pyrophosphate; DMAC, *N,N*-dimethylacetamide; IMP, inosine monophosphate; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, (*p*-bis[2-(5-phenyloxazolyl)]benzene; TPP, $\alpha,\beta,\gamma,\delta$-tetraphenylporphine; PP, inorganic pyrophosphate; PPP, inorganic triphosphate; AP_4, adenosine tetraphosphate.