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Inhibition of Proline Oxidation by Water Stress[1]

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ABSTRACT

The conversion of proline to glutamic acid and hence to other soluble compounds (proline oxidation) proceeds readily in turgid barley (*Hordeum vulgare*) leaves and is stimulated by higher concentrations of proline. This suggests that proline oxidation could function as a control mechanism for maintaining low cellular levels of proline in turgid tissue. In water-stressed tissue, however, proline oxidation is reduced to negligible rates. These results are consistent with the idea that proline accumulation results from inactivation by water stress of normal control mechanisms. It seems likely that inhibition of proline oxidation is necessary in maintaining the high levels of proline found in stressed barley leaves.

Radiotracer experiments have implied that the synthesis of proline from glutamic acid is stimulated by water stress (1, 10). We have confirmed and strengthened these earlier results, and presented evidence that the stimulatory effect of stress is likely to be on P5C[2] formation (6), consistent with the implication of other experiments that loss of feedback control of proline biosynthesis occurs in barley leaves during water stress (4). These studies also produced evidence that proline oxidation may occur rapidly in barley leaves (4) and that water stress may reduce its rate (3, 6). In this paper, we present more detailed information concerning proline oxidation and its response to proline concentration and water stress.

MATERIALS AND METHODS

Growth of barley (*Hordeum vulgare* cv. Prior) plants and methods of wilting, feeding of radioactive precursors, and sample analysis have been described (6). Two-week-old plants were wilted intact by flooding the rooting medium with polyethylene glycol-4000 (30 g/100 ml) 1 hr before beginning the experiment; alternatively, second leaves were excised and wilted to 75% of initial fresh weight in a lighted fume hood. The latter wilting procedure (which also took about 1 hr) was preferred as it gave more reproducible rates of water loss. Radioactive precursors (4- to 5-μl solution/200-mg leaf) were taken up through the cut ends of the leaves. Samples were frozen in liquid N_2 and extracted by the method of Bieleski and Turner (2). Radioactivity was measured by liquid scintillation after two-dimensional TLC of soluble compounds or acid hydrolysis of protein. Proline was determined by the acid ninhydrin method described by Singh *et al.* (13).

Radioactive compounds used were uniformly labeled L-[^{14}C]proline and glutamic acid, and L-[3,4-^{3}H]proline, all obtained from Amersham/Searle. [^{14}C]P5C was prepared from L-[U-^{14}C]proline as detailed previously (5).

RESULTS

Products of Proline Metabolism. In 2-hr experiments with [^{14}C]proline, radioactivity was recovered about 60% as soluble products and 40% as protein. After acid hydrolysis, the protein-bound radioactivity was found to be 85 to 90% proline, with the rest as glutamic and aspartic acids. In the soluble fraction, measurable radioactivity was recovered in glutamate, glutamine, aspartate, γ-amino-butyrate, alanine, and several ninhydrin-negative spots. Traces of ^{14}C were detected in glycine, serine, asparagine, citrulline, and arginine. Although not measured in this experiment, $^{14}CO_2$ has constituted less than 1.5% of the total radioactivity under comparable conditions. Table I (column 1) shows the distribution of radioactivity in these metabolites as a percentage of the total soluble radioactivity. Glutamate was always the most heavily labeled proline metabolite, and in short experiments (10-15 min), was the only labeled compound detected autoradiographically. This suggests that metabolism of proline consists of proline oxidation (conversion to glutamate) followed by further metabolism of glutamate. In further support of this possibility, all compounds labeled in [^{14}C]proline experiments were also labeled when [^{14}C]glutamate was fed (Table I, column 3). When [^{14}C]proline results (column 1) are expressed as a percentage of soluble radioactivity except proline (column 2), the distribution of ^{14}C among the major products (glutamate, glutamine, aspartate, and alanine) is similar to that observed in glutamate feeding experiments.

Validity of Results with Exogenous Proline. While the results just described give a straightforward description of the fate of exogenously added proline, one can question whether proline taken up from the outside enters the same metabolic compartment(s) as proline formed in the leaf. This can be tested at least partially by simultaneously feeding the leaf [^{14}C]P5C and tritiated proline, and following the disappearance of both isotopes from the proline pool. As P5C is converted to proline quite rapidly (6), this experiment should allow a direct comparison of the metabolism of endogenously formed [^{14}C]proline and exogenously fed [^{3}H]proline. In the experiment shown in Figure 1, [^{14}C]P5C had completely disappeared after 30 min, and 85% of the soluble ^{14}C was recovered as free proline. The similarity of the disappearance curves of ^{14}C and ^{3}H proline provides evidence that exogenous proline is a valid tracer for proline formed in the tissue.

Effect of Proline Concentration on Proline Oxidation. Figure

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[2] P5C: Δ^1-pyrroline-5-carboxylic acid.

Table I. Distribution of radioactivity in various soluble compounds after 2 hr metabolism of L-U-^{14}C-glutamic acid or L-U-^{14}C-proline.

Compound[a]	^{14}C-proline experiment[b] Radioactivity expressed as: % of total soluble ^{14}C	% of total soluble ^{14}C excluding proline	^{14}C-glutamate experiment[c] Radioactivity expressed as: % of total soluble ^{14}C
γ-aminobutyrate	2.6	4.9	1.3
proline	46.1	---	0.4
alanine	0.5	0.8	0.8
aspartic acid	1.7	3.2	2.9
glutamine	7.9	14.6	16.0
glutamate	36.5	69.0	66.0
ninhydrin negative compounds	3.3	6.3	11.2

[a]Other compounds containing traces (less than 0.1% of total) of ^{14}C: arginine, citrulline, glycine, serine, asparagine.

[b]The soluble fraction contained 213,000 dpm and the protein fraction 120,000 dpm.

[c]The soluble fraction contained 97,800 dpm and the protein fraction 16,500 dpm.

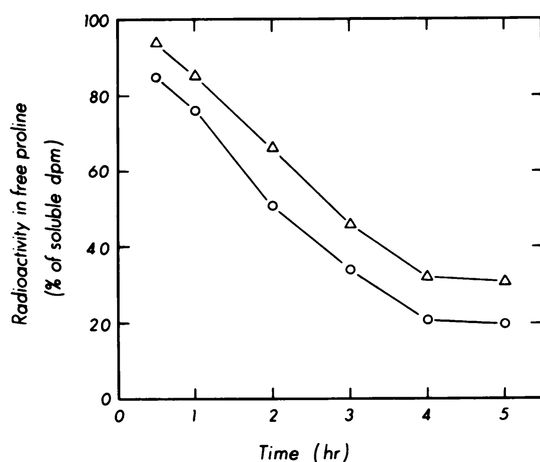

FIG. 1. Disappearance of radioactivity from the free proline pool after feeding tritiated proline and [^{14}C]P5C. Beginning at zero time, excised barley leaves took up 4 μl of a mixture of L-[3,4-^{3}H]proline (10 mCi/mmol) and L-[U-^{14}C]P5C (mCi/mmol). Samples were taken at indicated times. Proline was isolated chromatographically and analyzed for tritium and ^{14}C. The radioactivity of each isotope in proline was expressed as a percentage of the total amount of that isotope in the soluble fraction. At 30 min, absolute radioactivities were 331,000 dpm and 41,700 dpm for tritium and ^{14}C, respectively. Tritium: △——△; ^{14}C: ○——○.

2A shows the results of a 2-hr experiment with various concentrations of tritiated proline in which proline content, and radioactivity in proline, protein, and oxidized products were determined. Rates of proline oxidation and protein incorporation were calculated by dividing the specific radioactivity of proline into the rate of conversion of radiotracer to oxidized products or protein. The calculated rate of proline oxidation was stimulated by increasing concentrations of exogenously fed proline, while incorporation into protein was independent of concentration. Figure 2B shows that the trend continues to higher proline concentrations.

Proline Metabolism in Wilted Tissue. Figure 3 compares proline metabolism in wilted and turgid leaves. Figure 3A shows that the disappearance of radioactivity from the proline pool was arrested in wilted tissue, while 3B and 3C show that incorporation of radioactivity into protein was reduced after an initial period, while net incorporation into oxidized products stopped in wilted tissue after 2 hr. A similar reduction of proline oxidation rate in stressed leaves was observed in other experiments regardless of the isotope used (tritiated or [^{14}C]proline), specific radioactivity of the precursor, or method of wilting employed.

Rate of Proline Oxidation in Turgid Leaves. To evaluate the contribution that inhibited proline oxidation might make to proline accumulation, we must determine the rate of proline oxidation in turgid and water-stressed leaves. Figure 3B suggests that the rate in stressed tissue approaches 0; the rate in turgid tissue during a time interval can be estimated by dividing radioactivity recovered in oxidized products (Fig. 3B) by the average specific radioactivity of free proline during the time interval (Table II). For the interval 1 to 3 hr, we have (23,500–11,000 dpm/leaf) ÷ 1,450 dpm/μg = 8.6 μg proline/2 hr · leaf or 4.3 μg proline oxidized/hr · leaf. A similar calculation for the interval 2 to 4 hr gives 4 μg proline/hr · leaf. The increase in the proline pool of wilted leaves was 26 and 42 μg/hr · leaf during the two time intervals. Thus, the normal rate of proline oxidation is about 10% of the maximum proline accumulation rate in water-stressed leaves.

DISCUSSION

As glutamic acid was apparently the first compound to become radioactive in [^{14}C]proline feeding experiments with wilted and turgid barley leaves, and all autoradiographic spots in these experiments were recognizable as glutamate metabolites, our results are consistent with those of Wang (18) in implying that proline metabolism begins with conversion to glutamate. Presumably, this occurs via P5C as has been established in bacteria, mammals, and insects (7, 8, 12, 17). Although our experiments do not provide direct evidence for this, the requisite enzymes, a P5C-forming proline dehydrogenase, and glutamate-forming P5C-dehydrogenase are known to occur in plant tissue (5, 9, 11, 16).

The stimulation of proline oxidation by high proline concentration reported here (Fig. 2) was observed by Stewart (14) in experiments with bean leaves, and can also be inferred from the

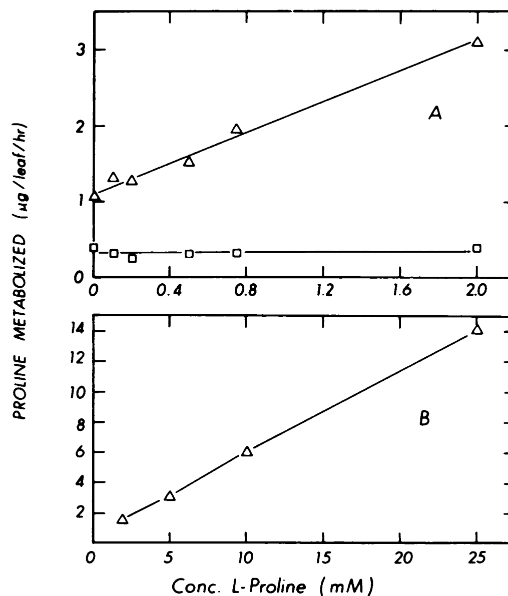

FIG. 2. Effect of concentration on conversion of proline to oxidized products and protein. Excised barley second leaves took up 50 μl of radioactive proline. After 2 hr the radioactivity in oxidized products and protein was determined, and rate of incorporation in μg/leaf · hr calculated as in the text. A: Precursor was 0.5 μCi L-[3,4-^{3}H]proline (43 Ci/mmol) in 50 μl of L-proline at the concentrations noted. Oxidized products: △——△; protein: □——□. B: Precursor was 0.5 μCi L-[U-^{14}C]proline (290 mCi/mmol) in 50 μl of L-proline at the concentrations noted. Oxidized products: △——△.

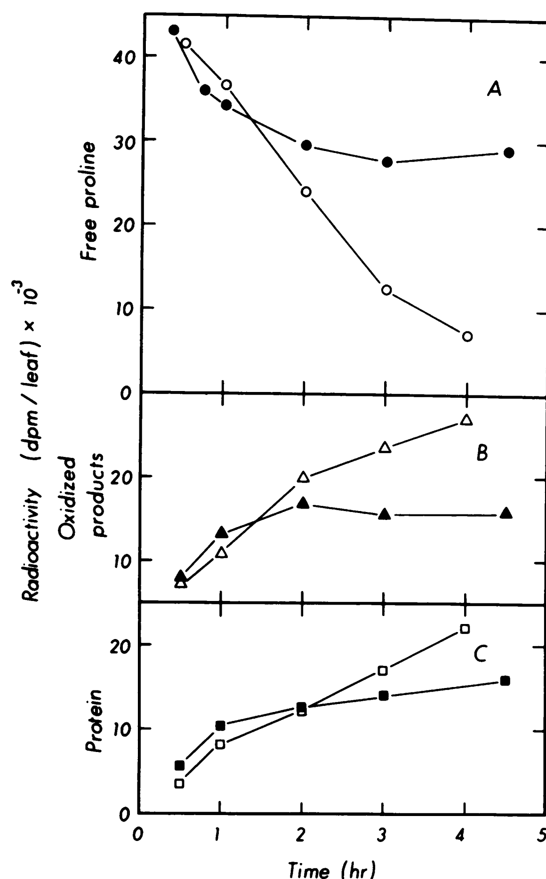

FIG. 3. Loss of radioactivity from proline in water-stressed and turgid barley leaves and its appearance in oxidized compounds and protein. Water stress was imposed by air-drying after excision (see "Materials and Methods"). RWC: turgid leaves = 95 to 100%; wilted leaves = 70% at zero time, 60 to 65% at 5 hr. All leaves took up 4 μl of solution containing 6 × 10^4 dpm L-[$U^{14}C$]proline, 1 mCi/mmol. A: Soluble proline: turgid, ○——○; stressed: ●——●. B: Oxidized products: turgid, △——△; stressed, ▲——▲. C: Protein: turgid, □——□; stressed, ■——■. Oxidized products were taken to be glutamate and its metabolities.

Table II.

Proline content and specific radioactivity of free proline

Treatment time[a] (hr)	Proline content (μg/leaf) Control	Stressed	Specific radioactivity[b] of free proline (dpm/μg)
1	18·5	22·0	2165
2	19·0	36·0	1450
3	17·0	74·0	834
4	18·0	120·0	416

[a]Data from the experiment described in Figure 3.

[b]Control (turgid) leaves.

rapid loss of proline when water-stressed leaves are rehydrated (13, 15). The rates of proline disappearance reported by Singh *et al.* (about 10 μg/leaf · hr[13]) must reflect proline oxidation, as incorporation of proline into protein proceeds much more slowly (Fig. 2A). It seems that the proline-oxidizing system of barley leaves is not saturated by normal proline concentrations, and that oxidation may therefore serve a regulatory function, acting in concert with control of synthesis (4) in maintaining free proline at a low level on turgid tissue. If so, it follows that proline oxidation must be inhibited before the amino acid can accumulate; our results (Fig. 3) provide direct evidence that this is the case in leaves accumulating proline as a result of water stress.

The rate of proline oxidation in turgid leaves calculated from our data is small compared to the rate at which proline accumulated in water-stressed leaves in the same experiment (Table II). Therefore, while inhibition of oxidation may be necessary for proline accumulation, other factors, probably including increased proline synthesis from glutamic acid (6), are required to account for the rates of increase in proline concentration observed in wilted leaves.

At present, we have little information regarding the details of the water stress effect on proline oxidation. P5C dehydrogenase, the enzyme converting P5C to glutamate, was unaffected by lowered water potential in general, but was sensitive to salt (5). Comparable studies with proline dehydrogenase, presumably the first enzyme of proline oxidation in plants, have not been done to our knowledge. The inhibition might result from a compartmentational change that prevents proline from reaching the enzymes of proline oxidation during water stress. Further research is necessary to determine whether enzyme level or compartmentational changes are involved, and how these are triggered by the relatively small water losses that stimulate proline accumulation.

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