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Effect of Water Stress on Proline Synthesis from Radioactive Precursors[1]

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ABSTRACT

Barley (*Hordeum vulgare* L. var. Prior) leaves converted more ^{14}C-glutamic acid to free proline when water-stressed than when turgid; neither decreased protein synthesis nor isotope trapping by the enlarged free proline pools found in wilted tissue seemed to account for the result. This apparent stimulation of proline biosynthesis in wilted leaves was not observed when radioactive ornithine or P5C (Δ^1-pyrroline-5-carboxylate, an intermediate following glutamate in proline synthesis) were used as proline precursors unless proline levels were high as a result of previous water stress. We interpret this to mean that any stimulation of proline synthesis by water stress must act on P5C formation rather than its reduction to proline. Experiments showing greater apparent conversion of ^{14}C-glutamate to proline do not unequivocally prove that proline synthesis is stimulated by water stress, as P5C feeding studies show that proline oxidation is inhibited under comparable conditions. This inhibition could account, at least in part, for increased proline labeling, and must be considered an alternate possibility.

Although several plants have been reported to accumulate free proline during periods of water deficit (2, 12, 15, 16, 18) or when subjected to other stress (7, 8), the biochemical changes linking water stress and proline accumulation are not well understood. Barnett and Naylor (2) and Morris *et al.* (13) have observed increased incorporation of ^{14}C-glutamic acid into proline in wilted leaves as compared to turgid leaves of Bermuda grass and turnip. This increase in radiotracer incorporation might reflect a stimulation of proline biosynthesis by water stress, but the data presented did not fully eliminate the possibility that inhibited protein synthesis might account for the results. The Bermuda grass experiment was done after a long period of drought, so that the large amount of free proline already present during ^{14}C-glutamate feeding could have acted as a trapping pool for radioactive proline, accounting for the results in the absence of an actual increase in the rate of conversion of glutamate to proline. In this paper we describe ^{14}C-glutamate feeding experiments designed to avoid these difficulties in interpretation, and to localize the point in the proline biosynthetic pathway at which a stress-induced stimulation would most likely occur. Information on the latter point was obtained from experiments in which radioactive P5C,[3] the intermediate between glutamate and proline, and ornithine, which is converted to proline via P5C, were used as proline precursors.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare* L. var. Prior) was grown in soil or sand; in the latter case, plants were watered daily with half-strength Hoagland solution instead of distilled H_2O. Plants were stressed either by flooding the sand with PEG-4000 (30 g/100 ml, ψ_s about -20 bars) or by excising the experimental leaves (second leaves of 2-week-old plants) and allowing them to dry to 75% of initial fresh weight under the conditions used by Stewart for bean leaves (17). Water status was measured either as RWC (3) or as per cent of initial fresh weight.

Feeding, Sampling, and Extraction. Radioactive compounds were fed to excised leaves in 4 to 5 μl of aqueous solution. Leaves that were excised before wilting were recut before uptake of radioactive precursors. Samples were frozen in liquid N_2 and held at -20 C or extracted immediately in methanol-chloroform-water (12:5:3, v/v/v) as detailed by Bieleski and Turner (4). The aqueous fraction was evaporated and the residue dissolved in 0.5 ml distilled H_2O.

Chromatography and Counting. Aliquots (50 μl) of the redissolved aqueous fraction were chromatographed two-dimensionally on cellulose thin layers as described by Haworth and Heathcote (10). The isobutyric acid-based solvent of Crowley *et al.* (9) was used in the first dimension and *n*-butylalcohol-acetic acid-water (12:3:5, v/v/v) in the second. Plates were dried overnight in a fume hood after development of the first dimension. Radioactive spots were located by autoradiography, removed from the thin layer plate with a razor blade, and either counted directly in 1 ml scintillation fluid (5 g PPO, 0.5 g dimethyl POPOP/liter toluene) or added to 1 ml 80% aqueous ethanol in the scintillation vial, and the dissolved radioactivity counted in 10 ml scintillation fluid containing 8 g butyl PBD, 0.5 g PBBO, 100 ml BBS-3 (Beckman), and 900 ml toluene. Radioactivity of the protein fraction was determined after hydrolysis in sealed tubes of the MCW-insoluble material in 3 N HCl-acetic acid (1:1, v/v) at 121 C for 16 hr. Proline was determined by the acid ninhydrin method as described by Singh (16) except that Dowex 50 (NH_4^+ form) was used to remove basic amino acids (20). ^{14}C-

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[3] Abbreviations: P5C: Δ^1-pyrroline-5-carboxylic acid; PEG: polyethylene glycol; RWC: relative water content; butyl PBD: 2-(4'-*t*-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole; PBBO: 2-(4'-biphenyl)-6-phenylbenzoxazole; BBS-3: Beckman solubilizer.

P5C was generated from ^{14}C-U-L-proline (10 mCi/mmol) using a rat liver mitochondrial preparation and purified by column chromatography (6, 19). All other radioisotopes were obtained from Amersham/Searle. Other reagents, unless otherwise specified, were obtained from Sigma.

RESULTS

Experiments with ^{14}C-Glutamic Acid. Figure 1 shows results from an experiment in which ^{14}C-glutamate was metabolized by wilted and turgid barley second leaves. Stressed leaves showed a rapid increase in radioactivity in total proline largely as the free imino acid, whereas the much slower increase in turgid leaves was due mainly to continued incorporation of radioactivity into protein. In comparable experiments, we have observed that in stressed leaves the proline pool continues to gain radioactivity for at least 12 hr. The effect does not depend on methods of stressing the leaves or of administering the radioactive precursor, as we have obtained similar results with both PEG-wilted and air-dried leaves and with infiltrated and air-dried leaf segments as well.

Concerning the possibility that increased incorporation of ^{14}C-glutamate into proline might reflect reduced incorporation of ^{14}C-proline into protein, Table I shows that the formation of radioactive protein-proline is too slow for this interpretation to be valid. This is true even in tissue wilted 24 hr, long enough for the development of a large proline pool and severely inhibited protein synthesis.

The data of Table I also bear on another possible explanation of the results shown in Figure 1: that the apparent stimulation in proline synthesis might simply reflect isotope trapping by the enlarged free proline pools typical of wilted barley leaves. The essence of trapping is that ^{14}C-proline formed in wilted leaves, mixing with relatively large amounts of unlabeled free proline, is lowered in specific radioactivity, and therefore remains in the proline pool longer. If this happens, two consequences of ^{14}C-glutamate feeding are predicted: proline labeling should be proportional to proline content, and free proline specific radioactivity should be lower in wilted leaves. Table I shows that the first was not always true, and Figure 2 shows that the second was never true in our experiments. Although trapping of radioactive proline probably can occur in leaves with large proline pools, it is unlikely to be the explanation of the increased proline radioactivity shown in Figure 1.

Experiments with Radioactive P5C and Ornithine. In some studies precursors were given to leaves water-stressed for 16 hr which, therefore, contained large pools of free proline. Under these conditions, more ^{14}C from DL-ornithine-1-^{14}C was recovered as proline in wilted leaves than in turgid leaves, and a similar result was obtained when radioactive P5C was fed under the same conditions (Fig. 3). To avoid the possible artifact of isotope trapping, these studies were also done with freshly wilted leaves. Table II (experiment A and the first two lines of experiment B) shows that the apparent stimulation of proline synthesis did not occur in ornithine-feeding experiments with freshly wilted tissue, whether the leaves were wilted by the PEG method or by air drying. There was an increase in proline radioactivity, however, in leaves that had been stressed 8 and 16 hr before ornithine feeding (experiment B). When ^{14}C-P5C was fed to

Table I. *Proline Content and Radioactivity in Free and Protein Proline 2 Hr after Feeding ^{14}C-Glutamic Acid to Turgid and Wilted Barley Leaves*

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Prior treatment[1]	Proline content	Free proline	Protein-proline
	µmol/g dry wt	Radioactivity cpm/leaf	
Turgid	2.3	1,360	9,510
Wilted, 8 hr	17.8	38,200	2,850
Wilted, 16 hr	61.1	48,700	1,490
Wilted, 24 hr	107.0	26,850	710

[1] Wilting and feeding methods as described in Figure 1, except for the length of water stress preceding ^{14}C-glutamate feeding

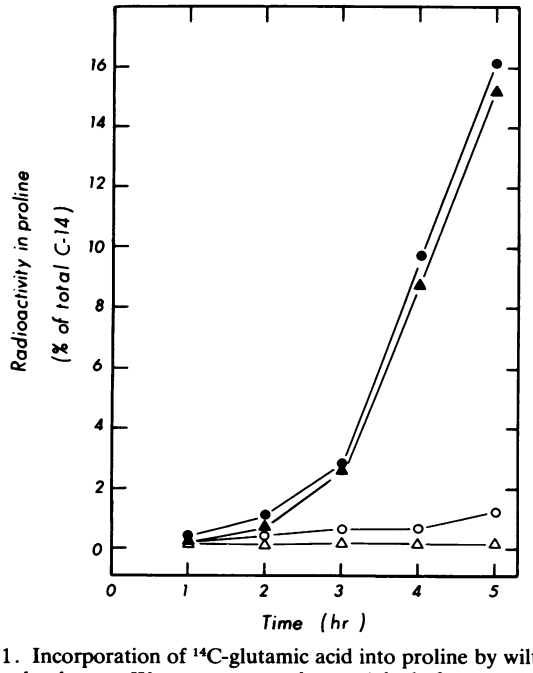

FIG. 1. Incorporation of ^{14}C-glutamic acid into proline by wilted and turgid barley leaves. Water stress was begun 1 hr before zero time by flooding the rooting medium with PEG. RWC: turgid leaves 95 to 100; wilted leaves 75 to 80 at zero time, 70 to 75 at 5 hr. Radioactive glutamate (0.25 µCi in 5 µl water; 10 mCi/mmol) was taken up through the base of wilted and turgid excised leaves. ●▲: water-stressed leaves in all figures. ●,○: total proline (free + protein-proline); ▲, △: free proline.

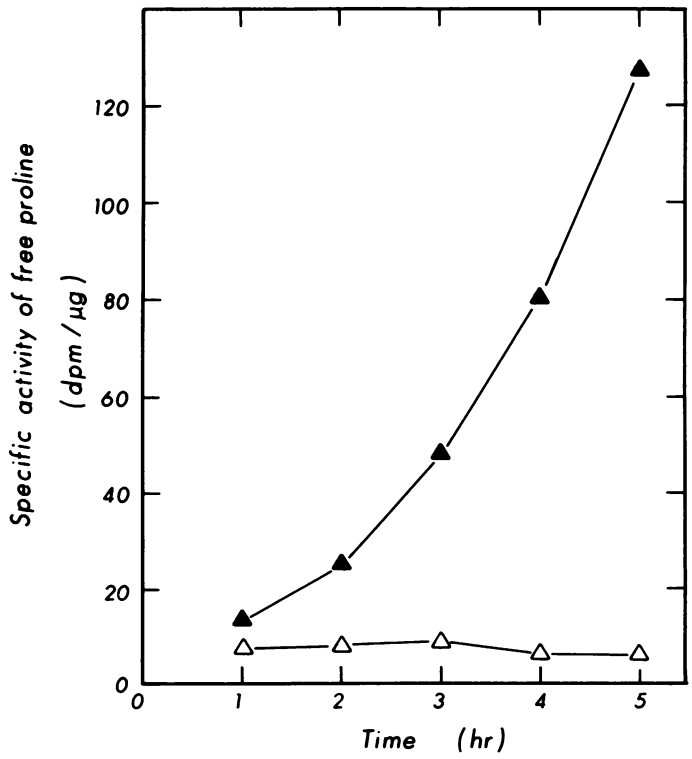

FIG. 2. Specific activity of the free proline pool during metabolism of ^{14}C-glutamic acid. Data from the experiment in Figure 1. ▲——▲: stressed leaves; △——△: turgid leaves.

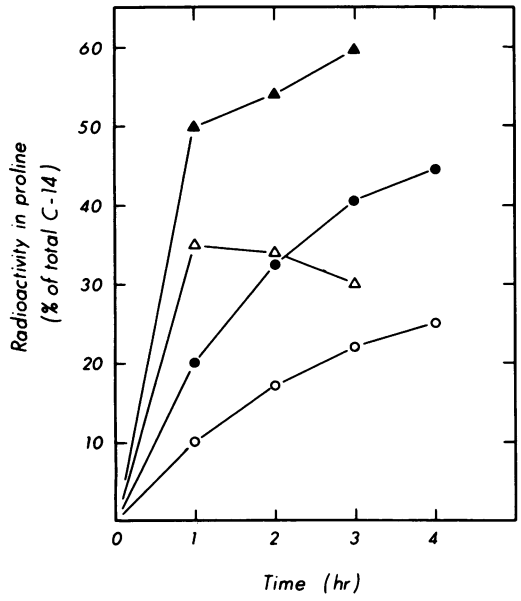

FIG. 3. Incorporation of ^{14}C-ornithine and P5C into proline by water-stressed and turgid barley leaves. Conditions were as in Figure 1 except that water stress was initiated 16 hr before zero time. DL-Ornithine-1-^{14}C (0.25 μCi, 61 mCi/mmol): ●——●: stressed; ○——○: turgid. L-P5C-U-^{14}C (0.2 μCi, 10 mCi/mmol): ▲——▲: stressed; △——△: turgid.

Table II. *Incorporation of Ornithine-^{14}C into Proline by Turgid and Wilted Barley Leaves*

Time wilted before ^{14}C-feeding(hr)	Time metabolizing ^{14}C-ornithine	Radioactivity in proline (% of total recovered) Mean ± standard deviation
Experiment A[1]		
0 (turgid)	2	12.7 ± .14
1	2	11.5 ± 0.6
0 (turgid)	5	17.7 ± 2.1
1	5	15.8 ± 2.4
Experiment B[2]		
0 (turgid)	2	1.9 ± 0.5
1	2	1.4 ± 0.1
8	2	6.8 ± 1.6
16	2	9.3 ± 0.1

1 Wilted by air drying leaves as described in Figure 4. Precursor was L-ornithine-U ^{14}C (261 mCi/mmol, 0.25 µCi/leaf).
2 Wilted by PEG method as described in Figure 1. Precursor was DL-ornithine-1 ^{14}C (58 mCi/mmol, 0.25 µCi/leaf).

freshly wilted leaves (Fig. 4), the precursor was very quickly converted to proline at approximately equal rates in both treatments. The effect of water stress was apparently not on proline synthesis but on proline disappearance, which was more rapid in turgid leaves.

DISCUSSION

The results show that the enhanced labeling of free proline observed previously in ^{14}C-glutamate feeding experiments with other plants also occurs in barley leaves (Fig. 1). It is further shown (Table I and Fig. 2) that this is not a consequence of reduced protein synthesis during the period of water stress, and that labeling of the free proline pool is not proportional to its size nor is its specific radioactivity lower in water-stressed leaves, as would be expected if the increased proline labeling in these leaves were simply due to isotope trapping. If these alternatives are ruled out, the evidence is consistent with a water-stress-induced stimulation of proline biosynthesis. If such a stimulation does occur, studies in which radioactive P5C or ornithine is used as proline precursors should provide evidence as to whether P5C formation or its reduction to proline is the site of the drought effect; if only P5C formation were the point of stimulation, an increased rate of incorporation of ^{14}C-P5C or ornithine into proline would not be expected, while a stimulatory effect at the second step should be readily apparent in such studies. In experiments with P5C and ornithine under conditions comparable to those of Figure 1 (recently wilted leaves), there was no stimulation of apparent proline synthesis from either P5C or ornithine (Table II, experiment A; Fig. 4). This indicates that if proline synthesis is increased by water stress, it is the rate of formation of P5C and not its reduction to proline that is increased. This is consistent with work on *Escherichia coli* (1) and tobacco leaves (14) that implies proline biosynthesis is limited by the rate of formation of P5C. The conversion of P5C to proline, on the other hand, seems to proceed freely in these organisms (1, 14). In leaves that were wilted 8 or 16 hr before feeding the radioactive precursors, an increase in proline radioactivity was observed (Fig. 3; Table II, experiment B). This possibly reflects isotope trapping by the enlarged proline pool found in such leaves, as the incorporation of radioactivity into proline increases with length of the water stress period preceding radiotracer feeding (Table II, experiment B).

The P5C feeding study is of interest in that this compound was converted very quickly to proline so that after the first 30 min, the data describe proline disappearance rather than its synthesis. It may be significant that proline disappeared rapidly from turgid leaves but more slowly from stressed leaves (Fig. 4). As barley leaves can oxidize proline (5), inhibition of this process by water stress offers an alternate explanation for the increased radioactivity found in proline after ^{14}C-glutamate feeding (Fig. 1).

Huber (11) has suggested that ABA and salinity-induced proline accumulation in *Pennisetum* follows in part from a stimulation of P5C reduction consequent on increased synthesis of the enzyme P5C reductase during stress. This implies that it is P5C reductase content that limits proline levels in control tissue; our results, on the other hand, imply that P5C formation normally

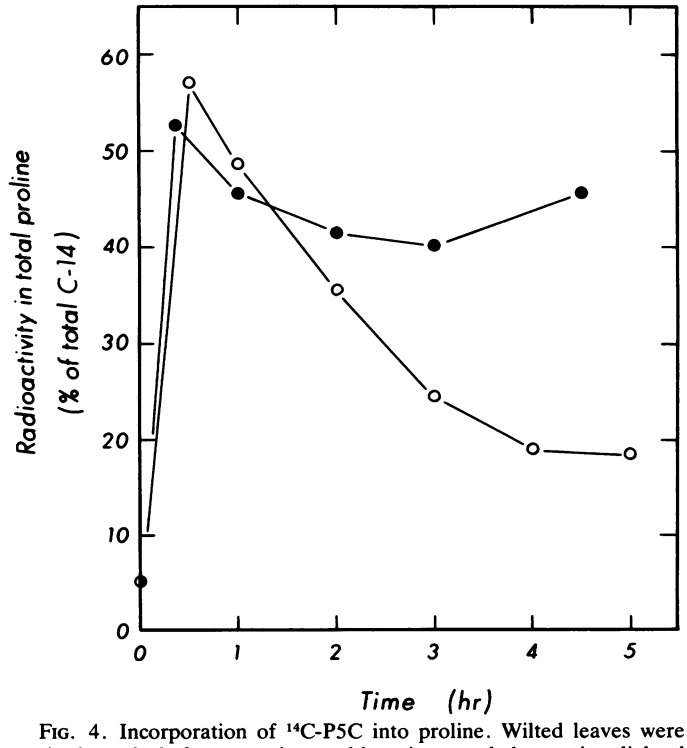

FIG. 4. Incorporation of ^{14}C-P5C into proline. Wilted leaves were excised at 1 hr before zero time and kept in a gentle breeze in a lighted hood. Leaves that had reached 75% of initial fresh weight at the end of 1 hr were allowed to take up 5 μl ^{14}C-P5C (0.1 μCi; 1 mCi/mmol). RWC by the end of the experiment was 65 to 67%. Turgid leaves were excised at zero time and received the same amount of ^{14}C-P5C. ●——●: stressed; ○——○: turgid.

limits proline synthesis. Unstressed *Pennisetum* tissue appears to contain a level of P5C reductase activity (capable of forming 13 μmol proline/hr·g fresh weight when supplied with NADH and P5C) that is sufficient to account for the amount of proline accumulated by the plants (12 μmol/g fresh weight, presuming dry weight was 10% of fresh weight). Thus, enough P5C reductase could be extracted from control tissue to account for the observed proline accumulation (a 10-fold increase over controls) in stressed tissue in 1 hr. Although the duration of the experiments was not given, it seems unlikely that P5C reductase activity limits proline synthesis in *Pennisetum*. It is possible that the increased enzyme content of stressed tissue (while interesting in itself) is not a causal factor in proline accumulation. Nevertheless, the possibility is raised that plants may differ in the biochemical mechanism through which they accumulate proline; alternatively, salt and ABA treatment may differ from desiccation in the sequence of events leading to proline accumulation.

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