Plant Physiol. (1972) 50, 679–681

Proline Content and Metabolism during Rehydration of Wilted Excised Leaves in the Dark

Received for publication June 22, 1972

CECIL R. STEWART
Department of Botany and Plant Pathology, Iowa State University, Ames, Iowa 50010

ABSTRACT

Excised bean (*Phaseolus vulgaris*) leaves were used to measure changes in proline content and proline metabolism during rehydration in the dark after the leaves had been incubated in the dark 24 hours in a wilted condition.

The increase in nonprotein proline which occurs in wilted leaves stopped immediately upon rehydration, and thereafter levels of proline declined. The rate of decline and the fate of the metabolized proline depended on the amount of carbohydrate present. When the level of carbohydrate was relatively high, the rate of decline in proline content was slow, and the proline was converted mainly to protein proline. When the level of carbohydrate was relatively low, the rate of proline decline was rapid and a large percentage of the proline was oxidized to CO_2 and other amino acids in addition to conversion to protein proline.

The results indicate that nonprotein proline can be oxidized when the level is increased by endogenous synthesis. Proline oxidation has been observed when the levels are increased by adding exogenous proline. Oxidation of endogenous proline is inhibited by carbohydrates in the leaf, as is true of oxidation of exogenous proline.

The accumulation of nonprotein proline in leaves under water stress has been reported by several workers (1, 6, 9, 11). However, little information is available on changes in proline content of leaves when they regain turgidity after a period of water stress. The experimental system of excised leaves, used previously by the author (7, 9, 11), allows measurement of changes in levels of metabolites over a period of time when the leaves are at a constant water content. When excised leaves are wilted, then incubated at a constant water content in the dark, the level of nonprotein proline increased more than protein proline decreases (9, 11). This increase does not occur in turgid leaves. After a period of accumulation, there is a net loss of proline from wilted leaves when they are incubated in the dark. The time at which the loss of proline occurs corresponds to the depletion of leaf carbohydrate. Nonprotein proline does not increase in wilted, starved leaves (9).

When excised bean leaves are wilted until they have lost 25% of their original fresh weight, then incubated at that water content to allow proline accumulation (24 hr), they can be rehydrated to their original fresh weight. Thus, excised leaves can be used to determine the proline content of leaves following rehydration and the fate of metabolized proline.

Previous experiments with bean leaves (8) and corn roots (5) have shown that proline is extensively oxidized by these tissues only if the endogenous level of proline is increased and carbohydrates are depleted. In both studies, the endogenous level was increased by adding exogenous proline. The oxidation was inhibited by carbohydrates in the tissue. Whether or not proline is extensively oxidized as a result of an increase in leaf proline content can be established by determining the fate of metabolized proline in rehydrated leaves. Thus, the changes in proline content of leaves and the metabolic fate of proline during and after rehydration are reported here. These experiments also determine whether or not an expanded pool of proline due to endogenous synthesis was subject to the same fate and control as exogenously added proline.

MATERIALS AND METHODS

Most of the methods used in these experiments have been described previously (7, 8). Fully expanded primary leaves of bean (*Phaseolus vulgaris* L. var. Tendergreen) were used. Starved leaves were from plants which had been in the dark for 48 hr. Nonstarved leaves were from plants which had been in the light (2500 ft-c) for 16 hr or more. Wilting, sampling, and incubation at a constant water content were described previously (7). Addition of metabolites by vacuum infiltration, collection of $^{14}CO_2$, extraction, fractionation, chromatography of amino acids, and determination of radioactivity also have been described (4, 8, 10). Wilted leaves were rehydrated by either placing them between paper towels saturated with water or by vacuum infiltrating them with water. When ^{14}C-proline was added upon rehydration, wilted leaves were simply vacuum infiltrated with a ^{14}C-L-proline solution. Proline was determined by the method of Chinard (3). Sugars (sucrose and reducing sugars) were determined by the reaction of the neutral fraction with 3,5-dinitrosalicylic acid (2) before and after treatment with invertase (7).

RESULTS

The fresh weight of leaves treated in various ways is shown in Figure 1. The wilted leaves lost water (25% of original fresh weight) during the rapid wilt period (45 min), then were incubated for a period of several days at a constant water content. The turgid leaves were floated on water during the 45 min wilt period, then incubated at a constant water content for a period of several days. Samples of leaves after 24 hr in a wilted condition were placed between paper towels saturated with water. They took up water to the extent that they regained their original fresh weight. These rehydrated leaves appeared turgid.

The nonprotein proline content of nonstarved leaves in-

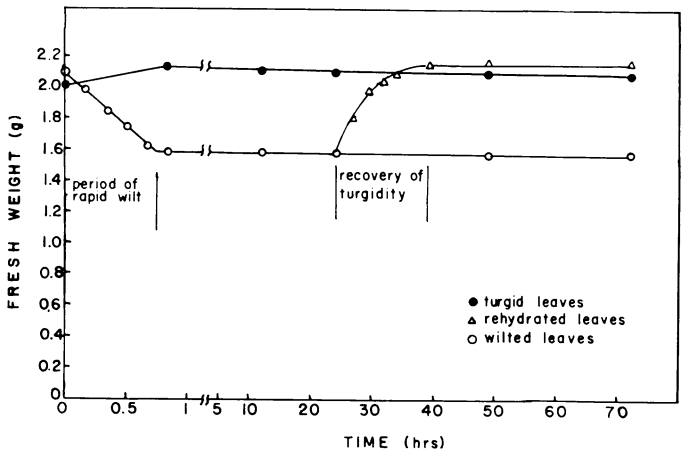

FIG. 1. Fresh weight of turgid, wilted, and rehydrated excised leaves during incubation in the dark in a humid chamber.

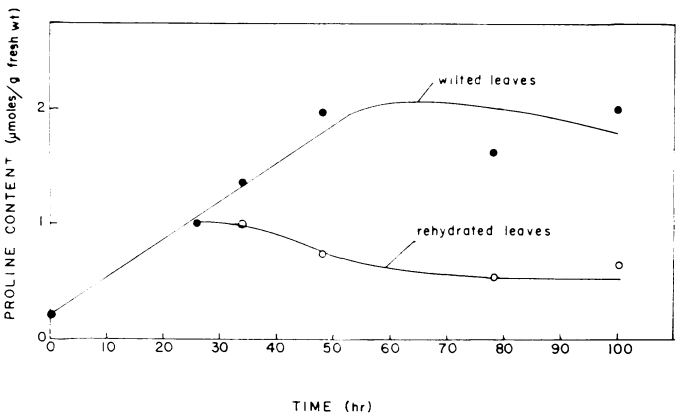

FIG. 2. Nonprotein proline content during incubation in the dark of wilted and rehydrated excised bean leaves from plants in light prior to these treatments.

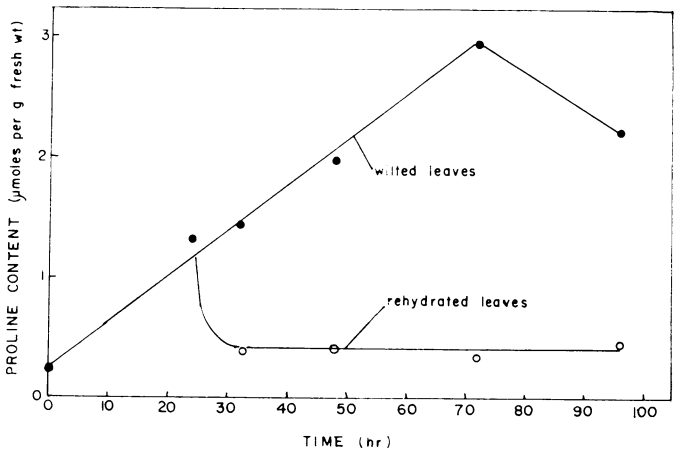

FIG. 3. Nonprotein proline content during incubation in the dark of wilted and rehydrated excised bean leaves from plants previously in the dark 48 hr prior to zero time. Sucrose (0.05 M) was infiltrated into the leaves at zero time.

cubated in a wilted condition is shown in Figure 2. There was a steady increase in nonprotein proline for a period of 48 hr after which it began to level off. The nonprotein proline content of leaves incubated in a wilted condition 24 hr then rehydrated is also shown in Figure 2. No further increase was observed during rehydration and after 12 hr there was a gradual decline. This decline in nonprotein proline continued at a slow rate during the remainder of the incubation period.

The nonprotein proline content of starved leaves infiltrated with 50 mM sucrose and incubated in a wilted condition is shown in Figure 3. There was a steady increase in nonprotein proline in wilted leaves over a period of 72 hr followed by a decline. The nonprotein proline content of leaves incubated in a wilted condition for 24 hr then rehydrated is also shown in Figure 3. The nonprotein proline content declined so rapidly during rehydration that, when the first sample was taken, the proline content was almost as low as it was prior to wilting. This 8-hr period was the time required for the leaves to regain their turgidity and approach their original fresh weight. Thus, from Figures 1 and 3 it is evident that by the time the leaves in this experiment were rehydrated, they had metabolized the proline which had accumulated during the wilting period.

The alcohol-soluble sugar (sucrose, glucose, and fructose) content of the 24-hr sample of leaves represented by the data in Figure 2 was 19 mg per g fresh weight, and a corresponding sugar content for the 24-hr sample of leaves represented by the data in Figure 3 was 7 mg per g fresh weight. Starch was not measured, but from previous measurements on comparable leaves, starch content of the 24-hr sample of leaves from plants in the light would have been 2- to 5-fold greater than that of leaves from plants in the dark to which sucrose had been added.

Distribution of ^{14}C in leaves at various times during the 24-hr period following simultaneous rehydration and addition of ^{14}C-proline to wilted leaves is shown in Figure 4 for leaves under different conditions. The data in Figure 4A are from nonstarved leaves which were wilted 24 hr then rehydrated.

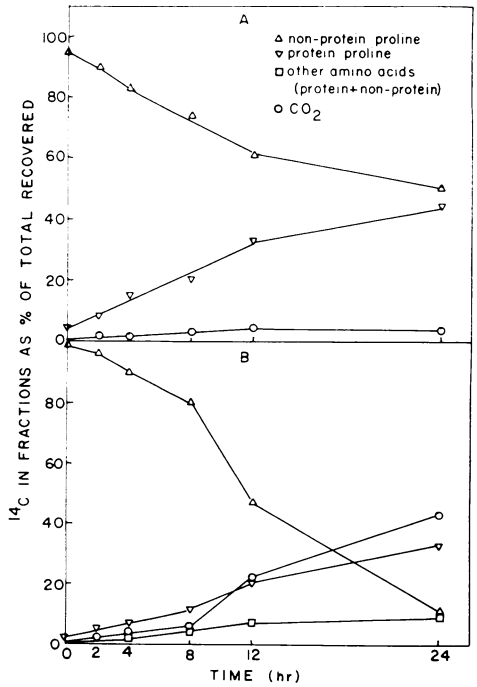

FIG. 4. The percentage of distribution of ^{14}C in fractions at various times after adding ^{14}C-proline to rehydrated excised bean leaves and incubating them in the dark. Prior to zero time, the leaves had been wilted (A, 24 hr; B, 36 hr). Prior to wilting, the leaves were from plants which had been: A: in the light; B: in the dark for 48 hr, then 0.05 M sucrose was infiltrated at the time of wilting.

Time zero was the time of rehydration (by vacuum infiltration) and corresponds to the 24-hr time of Figure 2. ^{14}C-Proline was steadily incorporated into protein throughout the incubation period with 44% of the ^{14}C recovered in protein proline after 24 hr. There was very little conversion of ^{14}C from proline to CO_2 (4% after 24 hr) under these conditions and at all sampling times after adding ^{14}C-proline, other amino acids and organic acids combined accounted for less than 3% of the ^{14}C recovered. Thus, there was very little oxidation of proline by these leaves. The alcohol-soluble sugar content at zero time in this experiment was 12 mg per g fresh weight. The data in Figure 4B are from starved leaves, infiltrated with 50 mM sucrose, incubated in a wilted condition for 36 hr, then rehydrated. Time zero was the time of rehydration and corresponds to the 36-hr time of Figure 3. After a lag of 4 to 8 hr, there was a rapid loss of ^{14}C from nonprotein proline with over 90% of it lost by 24 hr. Some of this proline was converted to protein proline (33% after 24 hr), and a large portion was oxidized to CO_2 (43% after 24 hr) and other amino acids (10% after 24 hr). Organic acids accounted for 3 to 4% of the label, but values are not plotted in Figure 4B. Alcohol-soluble sugar content at zero time in this experiment was 2 mg per g fresh weight.

DISCUSSION

The fact that excised leaves can be rehydrated after incubation in a wilted condition makes it possible to measure changes in composition of leaves during rehydration. Results from experiments reported in this paper with excised leaves indicate that the accumulation of non-protein proline caused by wilting is stopped when leaves are rehydrated. The nonprotein proline content of leaves during rehydration declines, but the rate of decrease and the fate of the nonprotein proline depends on the carbohydrate status of the leaves. If the leaves have high levels of carbohydrates during rehydration, the rate of loss of nonprotein proline is slow (Fig. 2), and this proline is converted to protein (Fig. 4A). If the leaves have low levels of carbohydrate, there is a rapid loss of non-protein proline (Fig. 3), and it is converted both to protein proline and to other amino acids, organic acids, and CO_2 (Fig. 4B). Conversion to other amino acids, organic acids, and CO_2 is a result of proline oxidation (5, 8).

These results are expected on the basis of previous results from experiments in which the endogenous level of nonprotein proline was increased by adding exogenous proline (8). In those experiments, increasing the endogenous level of nonprotein proline of starved leaves resulted in an increase in the percentage of the added proline being oxidized. Adding proline to nonstarved leaves did not result in extensive oxidation and adding sucrose to starved leaves also inhibited proline oxidation. Thus, an important conclusion from the results in this paper is that when the level of nonprotein proline is increased by endogenous synthesis, its metabolic fate is the same as when the nonprotein proline is increased by adding exogenous proline. The presence of carbohydrates in leaves is necessary for proline to accumulate, because in the absence of carbohydrates and as proline levels increase, the oxidation of proline would increase and large accumulations of proline would therefore not be observed.

From these results, the fate of proline which would accumulate in leaves on plants under moderate, temporary water stress can be predicted. This stress would occur under conditions of high light and of transpiration in excess of water uptake. Thus, proline would accumulate while photosynthesis proceeds and carbohydrate would be present to prevent proline oxidation. As the plant recovers due to decreased transpiration in the dark, proline accumulation would cease and proline would only be incorporated into protein as long as carbohydrate is present. If some of the accumulated proline was still present when carbohydrates in the leaf had become depleted by translocation and respiration, then proline would be metabolized by oxidation in addition to protein synthesis.

Acknowledgment—The support of the Iowa State University Research Foundation is appreciated.

LITERATURE CITED

1. Barnett, N. M. and A. W. Naylor. 1966. Amino acid and protein metabolism in Bermuda grass during water stress. Plant Physiol. 41: 1222–1230.
2. Bernfeld, P. 1955. Amylases, alpha and beta. *In:* S. P. Colowick and N. O. Kaplan, eds., Methods in Enzymology, Vol. 1. Academic Press, Inc., New York. pp. 149–150.
3. Chinard, F. P. 1952. Photometric estimation of proline and ornithine. J. Biol. Chem. 199: 91–95.
4. Morris, C. J. and J. F. Thompson. 1965. Conversion of m-carboxyphenylalanine to m-carboxyphenylglycine in Wedgewood iris leaves. Arch. Biochem. Biophys. 110: 506–510.
5. Oaks, A., D. J. Mitchell, R. A. Barnard, and F. J. Johnson. 1970. The regulation of proline biosynthesis in maize roots. Can. J. Bot. 48: 2249–2258.
6. Routley, D. G. 1966. Proline accumulation in wilted ladino clover leaves. Crop Sci. 6: 358–361.
7. Stewart, C. R. 1971. Effect of wilting on carbohydrates during incubation of excised bean leaves in the dark. Plant Physiol. 48: 792–794.
8. Stewart, C. R. 1972. Effects of proline and carbohydrates on the metabolism of exogenous proline by excised bean leaves in the dark. Plant Physiol. 50: 551–555.
9. Stewart, C. R., C. J. Morris, and J. F. Thompson. 1966. Changes in amino acid content of excised leaves during incubation. II. Role of sugar in the accumulation of proline in wilted leaves. Plant Physiol. 41: 1585–1590.
10. Thompson, J. F. and C. J. Morris. 1959. The determination of amino acids by paper chromatography. Anal. Chem. 31: 1031–1037.
11. Thompson, J. F., C. R. Stewart, and C. J. Morris. 1966. Changes in amino acid content of excised leaves during incubation. I. The effect of water content of leaves and atmospheric oxygen level. Plant Physiol. 41: 1578–1584.