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The Effect of Wilting on Proline Metabolism in Excised Bean Leaves in the Dark[1]

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ABSTRACT

The effects of wilting on the fate of proline and on the rates of nonprotein proline formation and utilization have been determined in excised bean leaves. Wilting did not alter the fate of exogenously added ^{14}C-L-proline (2 mM) in either nonstarved leaves (from plants previously in the light) or starved leaves (from plants previously in the dark). The fate of proline in nonstarved leaves was protein synthesis and in starved leaves was protein synthesis and oxidation to other compounds.

Wilting caused an increase in non-protein proline formation, possibly including release by proteolysis and synthesis from precursors in both starved and nonstarved leaves. Wilting caused a decrease in proline utilization in nonstarved leaves by decreasing protein synthesis. In starved leaves, wilting caused an increase in the rate of proline utilization but this is due to the higher content of proline in wilted leaves compared to the turgid leaves which causes more proline utilization by oxidation. Thus, the primary effects of wilting which lead to the accumulation of proline were to decrease protein synthesis and to increase proline formation. The source of the proline is not known but the increased formation due to wilting is not affected by the carbohydrate content of the leaf. The role of carbohydrates is to prevent the loss of accumulating proline by oxidation.

It has been shown (1, 6, 9, 11) that nonprotein proline accumulates in wilted leaves except when the leaves have a low carbohydrate content (9). The amount of nonprotein proline in a tissue is determined by the relative rates of formation and utilization. Proline formation occurs primarily by proteolysis and by *de novo* synthesis. Proline is utilized mainly in protein synthesis and by oxidation. Since the increase in proline during wilting exceeds the proline released from protein (11) *de novo* synthesis must account for the increase in nonprotein proline.

Also since proline oxidation is inhibited (5, 8) when carbohydrates are present, protein synthesis is responsible for proline utilization when proline accumulates. Thus proline accumulation could be due to an increase in *de novo* proline synthesis or a decrease in protein synthesis or both. This paper demonstrates that in water stress there is an increase in proline synthesis *and* a decrease in protein synthesis. Further, the increased proline synthesis does not require the presence of high levels of carbohydrates.

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MATERIALS AND METHODS

Most of the methods used in these experiments have been described (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 have been described (7). Addition of metabolites by vacuum infiltration, collection of $^{14}CO_2$, extraction, fractionation, chromatography of amino acids, and determination of radioactivity have been described (4, 8, 10). Proline was determined by the method of Chinard (2).

The rate of proline utilization (μmoles/hr·g fresh weight) was calculated from the rate of loss of ^{14}C from nonprotein proline (cpm/hr·g fresh weight) divided by the specific radioactivity of the nonprotein proline (counts/min·μmole). The rate of change in nonprotein proline content (μmoles/hr·g fresh weight) was calculated from the slope of the time course of changes in nonprotein proline content. The rate of proline formation (μmoles/hr·g fresh weight) was calculated from the following formula: rate of change in nonprotein proline = rate of formation minus rate of utilization.

RESULTS

The fate of exogenously added 2 mM proline in turgid (A) and wilted (B) nonstarved leaves is shown in Figure 1. As shown previously (8), proline was incorporated into protein in nonstarved leaves and its oxidation to other amino acids, organic acids, and CO_2 was minimal due to the presence of carbohydrates in the leaves. Wilting did not affect the fate of exogenously added proline qualitatively but did quantitatively. The organic acid fraction is omitted from Figure 1 because it contained 2% or less of the total ^{14}C recovered and the other amino acids are omitted from Figure 1B because they contained 1% or less of the total ^{14}C recovered.

Figure 2 shows the fate of exogenously added 2 mM ^{14}C-proline for turgid (A) and wilted (B) starved leaves. In starved leaves, the oxidation of proline to other amino acids, organic acids, and CO_2 represented a major fate of metabolized proline in addition to the incorporation into protein. Wilting did not alter the fate of added ^{14}C-proline in these leaves but decreased the rate (see below).

The effect of wilting on the nonprotein proline content during incubation after adding 2 mM proline to nonstarved leaves is shown in Figure 3A. In the turgid leaves, there was a gradual decrease in proline throughout the incubation. In the wilted

leaves, there appeared to be a disappearance of proline during the first 2 hr but after 4 hr there was a steady increase in proline during the remainder of the incubation period. The total increase in proline from hour 4 to 24 is almost 2 μmoles/g fresh weight. This amount of increase is about the same as was observed when nonstarved leaves were wilted and incubated even though the initial proline content was lower (0.1 to 0.2 μmole/g fresh weight) in the absence of added proline.

The effect of wilting on the disappearance of ^{14}C from exogenously added proline is shown in Figure 3B. In turgid leaves, ^{14}C-proline decreased faster than the total nonprotein proline content, indicating that proline was being synthesized. In the wilted leaves, ^{14}C-proline disappeared but at a slower rate than in the turgid leaves and it continued to disappear in the time period during which there was an increase in proline content. The data in Figure 3B are from the same experiment as shown in Figure 1, A and B, but expressed as cpm/g fresh weight to use in calculating the rate of proline utilization.

The effect of wilting on the non-protein proline content during incubation after adding 2 mM proline to starved leaves is shown in Figure 4A. There was a rapid disappearance of the proline from the turgid leaves with the proline content reaching 0.4 μmole/g fresh weight after 12 hr. In the wilted leaves,

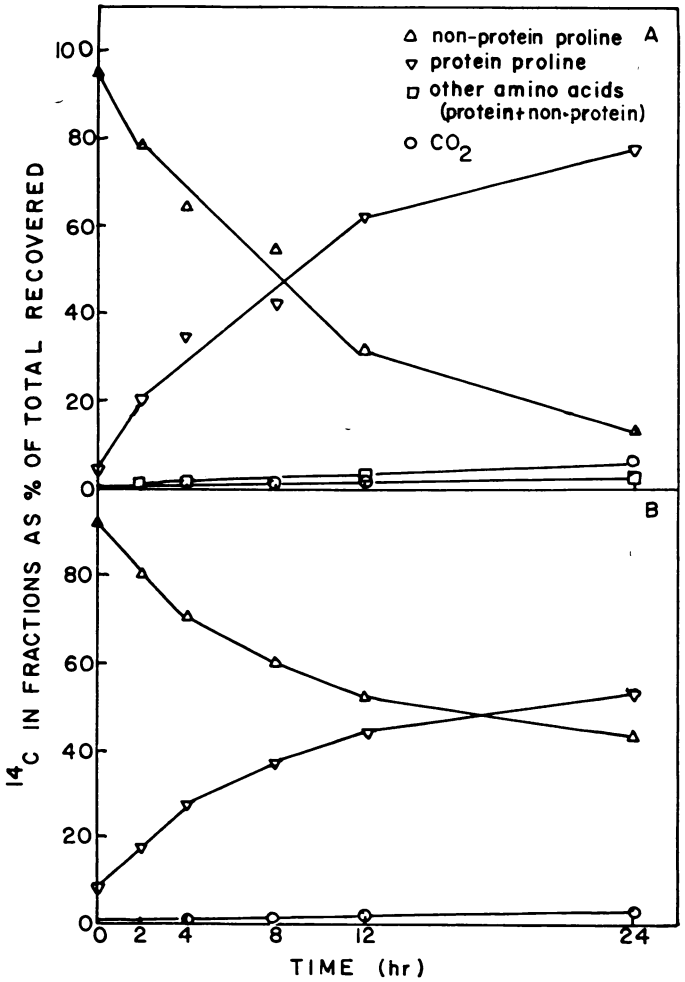

FIG. 1. The percentage of distribution of ^{14}C recovered in fractions at various times after adding 2 mM ^{14}C-proline to excised bean leaves from plants previously in the light for 16 hr. Leaves were incubated in the dark in a turgid (A) and wilted (B) condition.

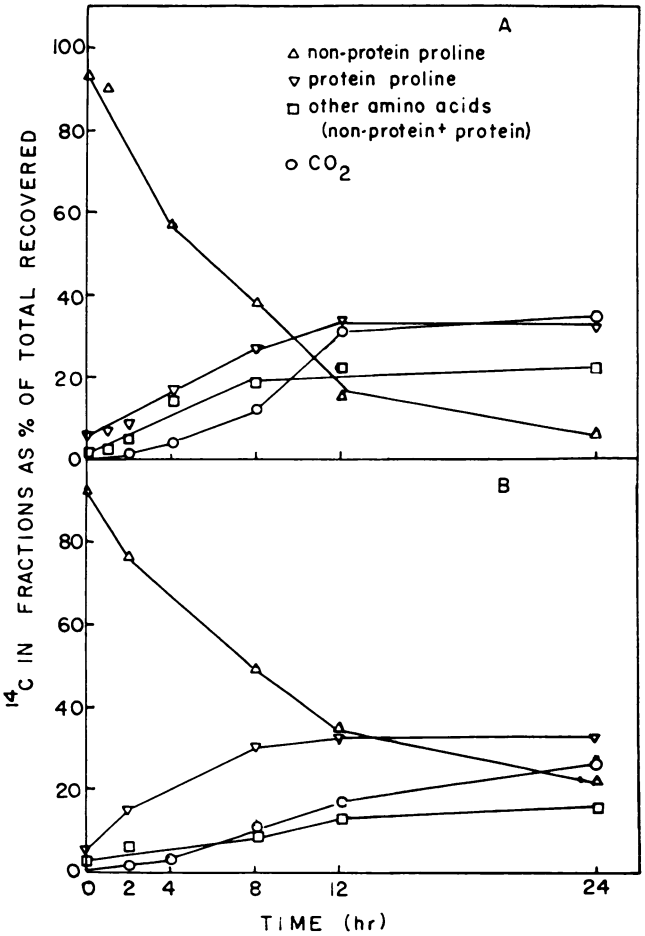

FIG. 2. The percentage of distribution of ^{14}C recovered in fractions at various times after adding 2 mM ^{14}C-proline to excised bean leaves from plants previously in the dark for 48 hr. Leaves were incubated in the dark in a turgid (A) and a wilted (B) condition.

there was an initial decrease in proline for the first 2 hr followed by a gradual increase over the remainder of the incubation period but during the 2- to 24-hr period there was an increase of only 0.35 μmole/g fresh weight. Figure 4B shows the loss of ^{14}C from nonprotein proline in cpm/g fresh weight from wilted and turgid leaves. The ^{14}C disappeared from the nonprotein proline in the turgid leaves which was as expected from the decrease in proline content. In the wilted leaves, ^{14}C was decreasing in the nonprotein proline even though the total nonprotein proline content was increasing slightly. The data in Figure 4B are from the same experiment as shown in Figure 2, A and B, but expressed as cpm/g fresh weight to use in calculating the rate of proline utilization.

The rates of nonprotein proline utilization (oxidation and protein synthesis) and nonprotein proline formation (*de novo* synthesis and release by proteolysis) were calculated from Figures 3 and 4 and are shown in Table I. Wilting caused an increase in proline formation in both starved and nonstarved leaves. The increase in proline formation due to wilting appeared to be greater in the starved leaves than in the nonstarved leaves. Wilting caused a decrease in proline utilization in the nonstarved leaves and an increase in proline utilization in the starved leaves. In the turgid leaves, there was a greater rate of proline formation in the nonstarved leaves compared to the starved leaves. The effect of starvation on the rate of utili-

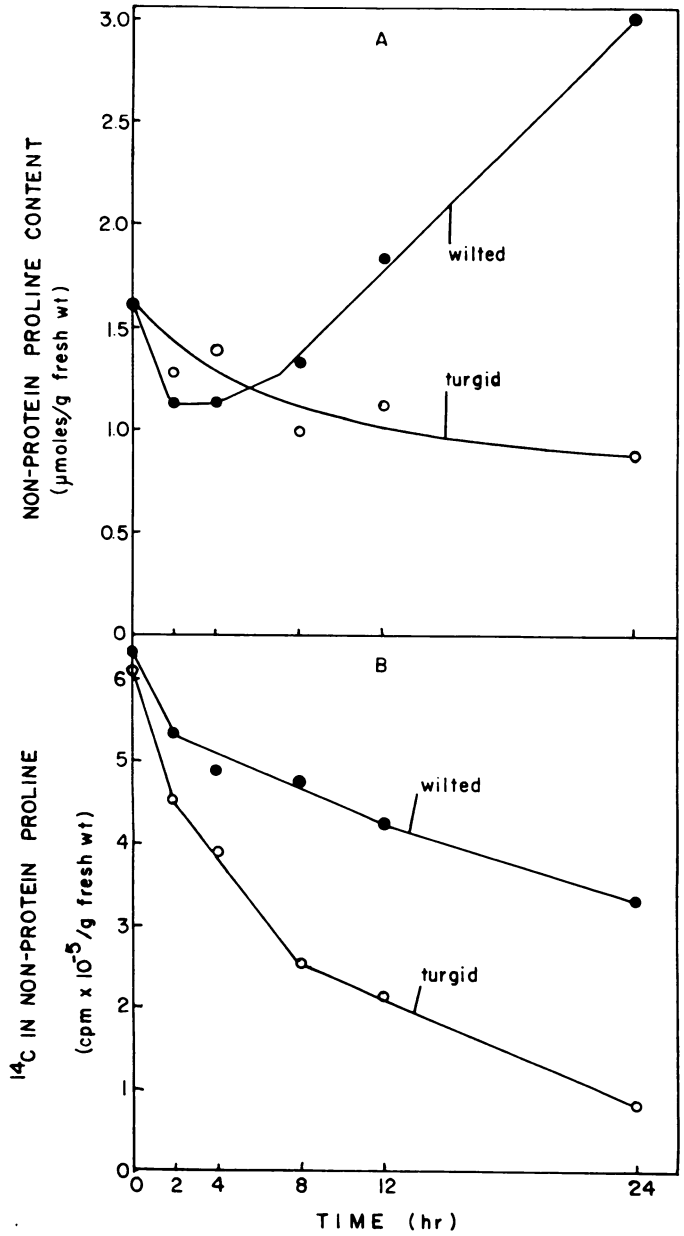

FIG. 3. The non-protein proline content (A) and the ^{14}C in non-protein proline (B) in excised bean leaves at various times after adding 2 mM ^{14}C-proline to leaves from plants previously in the light for 16 hr. Leaves were incubated in the dark in a turgid and wilted condition.

zation of nonprotein proline in turgid leaves was slight even though the fate of the proline is much different under the two different conditions. Calculations of the rates of utilization and formation of nonprotein proline at other times during the incubation gave similar results.

DISCUSSION

The fate of exogenously added proline in starved and nonstarved leaves has been shown previously to be affected by the carbohydrate status of the leaf (8). The results in this paper show that wilting does not alter the fate of the proline, *i.e.*, incorporation into protein in both nonstarved and starved leaves and oxidation in starved leaves (Figs. 1 and 2). The fact that there was an accumulation of proline after 2 mM proline

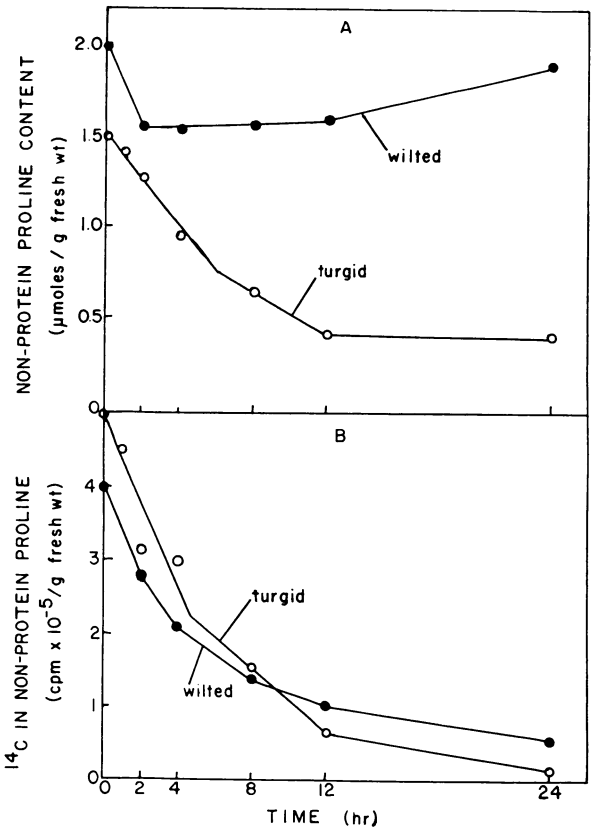

FIG. 4. The non-protein proline content (A) and the ^{14}C in non-protein proline (B) in excised bean leaves at various times after adding 2 mM ^{14}C-proline to leaves from plants previously in the dark for 48 hr. Leaves were incubated in the dark in a turgid and wilted condition.

Table I. *The Rates of Formation and Utilization of Nonprotein Proline in Excised Leaves Incubated under Various Conditions*

The rates are the 8-hr instantaneous rates and were calculated as described under "Materials and Methods."

Condition of Leaves	Proline Utilization	Proline Formation
	μmole/hr·g fresh wt	
Starved, turgid	0.10	0.04
Starved, wilted	0.16	0.16
Nonstarved, turgid	0.09	0.07
Nonstarved, wilted	0.03	0.13

was added to the leaves similar to the accumulation that was observed in wilted leaves without any addition of proline, makes it possible to examine the effect of wilting on proline utilization and formation by measuring changes in proline content and ^{14}C-proline after adding 2 mM proline.

The decrease in proline utilization in nonstarved leaves due to wilting is clearly due to a decrease in the rate of protein synthesis. This effect of low water potential has been observed previously as a decrease in the percentage of polysomes (3) and may represent one of the primary effects of wilting which leads to an accumulation of proline. In starved leaves, the rate of proline utilization was increased by wilting. From previous results (8) it is known that leaves with a higher proline content oxidize proline faster when the carbohydrate content is low. Thus, the increase in proline utilization due to wilting in this experiment is a secondary effect due to the higher proline con-

tent in the wilted leaves resulting from a greater rate of proline formation.

Wilting causes an increase in the rate of proline formation in both starved and nonstarved leaves. The source of the proline cannot be determined from these experiments but it appears that this effect of wilting is not dependent on a large supply of carbohydrate in the leaf. Thus, on the basis of these experiments, the role of carbohydrates in proline accumulation appears to be the prevention of proline oxidation. The source of nitrogen for increased proline formation would be from other amino acids (both protein and nonprotein) because the excised leaf is a closed system. The carbon for increased proline synthesis due to wilting can come from carbohydrates but since the increased proline synthesis is observed in starved leaves the carbon apparently can also come from other amino acids. The possible pathways of proline formation are by proteolysis, synthesis from glutamic acid, and conversion from arginine via ornithine. There are indications (C. R. Stewart, unpublished) that wilting causes a greater synthesis of proline from glutamic acid and arginine.

The results in this paper and others on proline accumulation in wilted leaves support the observation (5) that proline synthesis or a component thereof, is not sensitive to feedback inhibition. If it were, a decrease in proline utilization would cause proline to accumulate and proline synthesis from precursors would be inhibited. Then, the only source of proline would be proteolysis and previous results (11) show that this is not sufficient to account for the amount of non-protein proline that accumulates.

The accumulation of nonprotein proline in wilted leaves can be explained on the basis of two aspects of proline metabolism and on two or three effects of wilting. The aspects of proline metabolism are that the oxidation of proline is inhibited in leaves with a high carbohydrate content and that proline synthesis from precursors is not sensitive to feedback inhibition. The effects of wilting are to decrease protein synthesis and increase proline formation. The latter can be due to either increased proteolysis or increased synthesis from precursors or both.

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