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Effects of Proline and Carbohydrates on the Metabolism of Exogenous Proline by Excised Bean Leaves in the Dark

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ABSTRACT

Proline was metabolized when vacuum infiltrated into starved bean (*Phaseolus vulgaris* L.) leaves from plants previously in the dark for 48 hours, but an equivalent increase in protein proline was not observed. When ^{14}C-proline was infiltrated into starved leaves, a large percentage of the ^{14}C was recovered in other amino acids, organic acids, and CO_2, in addition to that recovered as protein proline. However, extensive oxidation of proline was observed only if enough proline was added to increase substantially the endogenous concentration of proline. Increasing the endogenous concentration did not affect the amount of proline that was incorporated into protein.

When added to leaves from plants previously in the light of near saturating intensity for more than 16 hr, very little ^{14}C-proline was oxidized, even when a sufficient amount of proline was added to increase the endogenous concentration. Adding sucrose to starved leaves along with the proline slowed down the oxidation of proline. Thus, it appears that some carbohydrate or intermediate of carbohydrate metabolism may inhibit the oxidation of proline in leaves.

Based on kinetics of labeling, the pathway of proline oxidation was by conversion to glutamic acid and subsequent metabolism to intermediates in the Krebs cycle and to CO_2.

Proline is one of the amino acids contained in proteins of all organisms, and plant tissues readily incorporate exogenously added proline into protein. Furthermore, some of the proline that is incorporated into protein is subsequently converted to hydroxyproline (8). More recently, the capability of plant tissues to oxidize and respire proline to CO_2 has been recognized (3, 13). In both reports, the relative amount of proline which was oxidized was small. Further, it has been suggested (6) that the amount of exogenously added proline oxidized by corn root tips was affected by exogenous glucose and by the amount of proline added.

The marked accumulation of proline in plant tissues under various environmental stresses, particularly in plants under water stress (1, 7, 9, 12), prompted further investigation into proline metabolism in plants. Specifically, it has been shown that when wilted leaves are depleted of their carbohydrates, there is a net loss of proline (nonprotein plus protein), indicating that the proline is metabolized. Thus, the purposes of the experiments reported in this paper were to assess the extent to which leaves can oxidize proline and to determine what physiological conditions in the leaves would affect the oxidation of proline.

MATERIALS AND METHODS

Bean plants (*Phaseolus vulgaris* L. var. Tendergreen) were grown in soil in flats in the greenhouse until the primary leaves were fully expanded (about 2 weeks). All experiments were conducted on primary leaves from these plants. Starved leaves were excised from plants which had been kept in the dark for 48 hr. Nonstarved leaves were excised from plants which had been exposed to a light intensity of 2500 ft-c (fluorescent and incandescent) for at least 16 hr in a growth chamber. All solutions, ^{12}C- and ^{14}C-L-proline and sucrose were applied to the leaves by vacuum infiltration. The amount of solution added to each sample of leaves was estimated from the weight of the blotted leaf sample before and after infiltration. Midribs were removed and not included in the leaf sample. A sample of leaves will take up about 0.5 g of solution per g fresh weight. The ^{14}C-L-proline added contained about 1 μc per ml and was uniformly labeled. After infiltration, each sample of leaves was allowed to dry to its original fresh weight under a bank of 100-w incandescent desk lamps positioned about 1 ft above the leaf sample. Drying of the leaf to its original weight required 15 to 30 min, and special care was taken to prevent the leaves from wilting.

Samples of leaves (1 g) were incubated at a constant fresh weight for various periods of time in a humid chamber. The $^{14}CO_2$ was trapped in scintillation vials placed in the flask containing 1 ml of ethanolamine and 2 ml of methyl cellosolve.

At the end of the incubation period, the leaves were immersed in 95% ethanol and subsequently thoroughly extracted with 80% (v/v) ethanol. The ethanol extract was evaporated to dryness, and the resultant residue was redissolved in water after removing lipids with chloroform. The alcohol insoluble residue was dried, then hydrolyzed in a sealed tube containing 1:1 glacial acetic acid: 3 N HCl for 16 to 18 hr at 120 C. The hydrolysate was filtered, evaporated to dryness, then taken up in hot water.

The extracts were taken up in water and fractionated into neutral, organic acid, and amino acid fractions using Dowex-50-H$^+$ and Dowex-1-formate as previously described (10). The amount of ^{14}C in various fractions was determined by placing a 0.5-ml aliquot into a scintillation vial and adding 10 ml of scintillation solution. The amino acids were separated by 2-dimensional chromatography and located as previously described (5, 11). The ^{14}C on the chromatogram spot was determined by cutting the spot from the paper, placing it in a scintillation vial, and adding 0.5 ml of water to dissolve the compound from the paper. After a few minutes, 10 ml of scintillation solution was added and allowed to stand 30 min with intermittent shaking. This procedure gave as complete recovery as elution from paper with water. The scintillation solution contained 8 g of 2-(4'-*t*-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxidiazole (Butyl PBD), 0.5 g of 2-(4'-biphenyl)-6-phenyl-benzoxazole (PBBO),

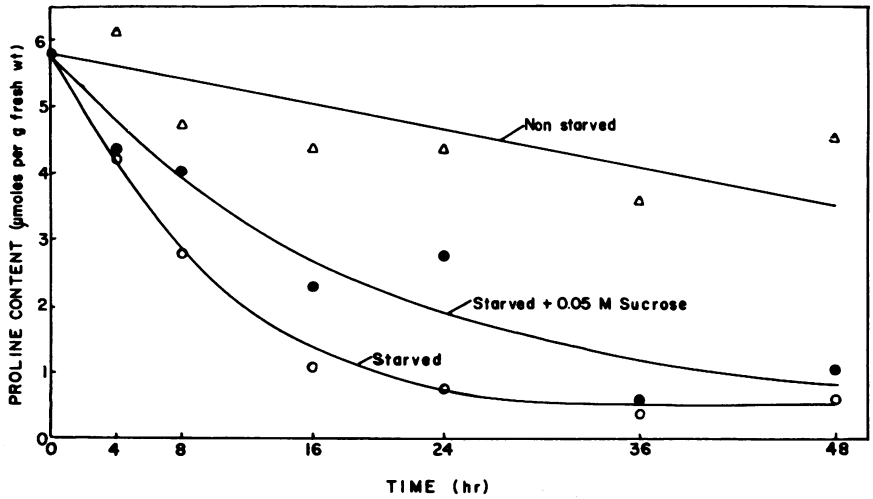

FIG. 1. Effect of incubation time on the proline content of leaves which had been vacuum infiltrated with 10 mM proline.

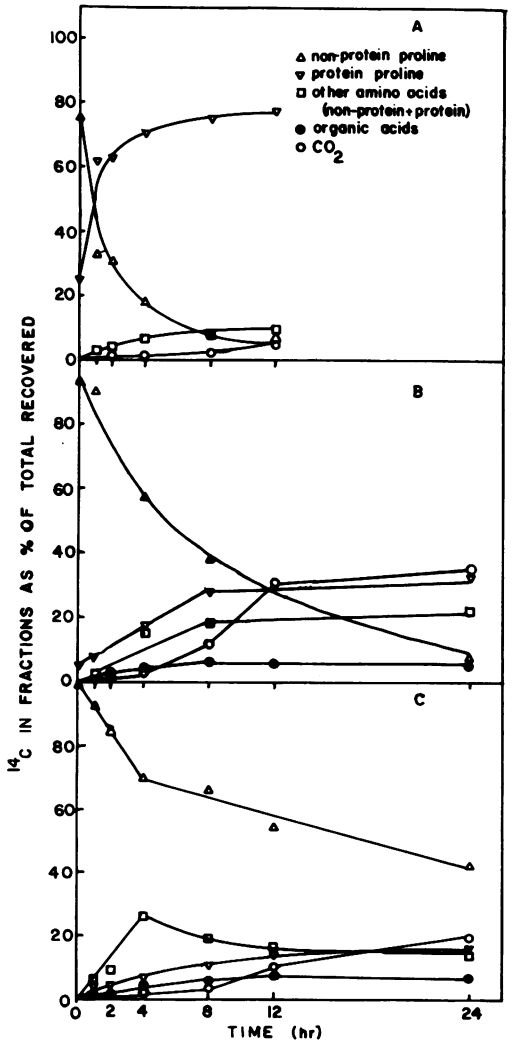

FIG. 2. Effect of incubation time on the distribution of ^{14}C in various fractions in leaves which had been infiltrated with ^{14}C-proline at these concentrations (A: 5 μM; B: 2 mM; C: 10 mM).

and 100 ml of Bio-Solv BBS-3 (Beckman Instruments, Inc.) in toluene to a final volume of 1 liter. Radioactivity was determined with a Beckman DPM-100 liquid scintillation counter. The counting efficiency was >95% with this method, and all fractions gave the same counting efficiency. The $^{14}CO_2$ was counted by adding 10 ml of scintillation solution to the ethanolamine-methyl cellosolve mixture; efficiency was comparable to the aqueous fractions. For the longer incubations, the ethanolamine-methyl cellosolve took up water during the incubation and more (up to 1 ml) methyl cellosolve was added with the scintillation solution to get the mixture into solution. This did not affect the counting efficiency.

Proline was determined by the method of Chinard (2), which has been used previously (9). This method detected the same amount of proline in leaf extracts with or without ornithine and lysine removed.

RESULTS

Figure 1 shows the time course of disappearance of proline from excised bean leaves which had been vacuum infiltrated with 10 mM ^{12}C-L-proline. Proline disappearance is presented for leaves which had been in light prior to infiltration (nonstarved), leaves which had been in dark 48 hr prior to infiltration (starved), and starved leaves which had 50 mM sucrose infiltrated along with proline. The proline (5.7 μmoles/g fresh weight) was metabolized rapidly from the starved leaves with 50% of it being metabolized in about 8 hr. The nonstarved leaves did not metabolize the proline nearly as rapidly, with more than 50% of it remaining after 48 hr. Sucrose (50 mM) slowed down the metabolism of proline from the starved leaves, with about 16 hr required to metabolize 50% of the proline. However, by 36 hr the proline was metabolized to a level about the same as the lowest level reached in the starved leaves, *i.e.*, about 0.5 μmole/g fresh weight.

Metabolism of Exogenous Proline by Starved Leaves. Because proline was metabolized most rapidly in starved leaves and because there was not a corresponding increase in protein proline during the period of rapid proline metabolism, starved leaves were used to investigate the fate of the metabolized proline. It was found that a large percentage of the proline was being oxidized and respired under the conditions of the experiment represented in Figure 1. However, it was found that this was true only if the free proline pool was expanded by adding ^{12}C-proline along with ^{14}C-proline. Figure 2 shows the fate of added proline when infiltrated into the leaves at

three different concentrations; 0.005 mM, 2 mM, and 10 mM (Fig. 2, A, B, and C, respectively). At a concentration of 5 μM, the added proline was so low that the internal proline level of the leaf was not affected, whereas addition of the two higher concentrations represented drastic increases in the level of nonprotein proline in the leaf (endogenous proline = 0.15 μmole/g fresh weight). The data in Figure 2 are expressed as percentage of ^{14}C recovered from the leaf. The total ^{14}C recovered closely agreed with the amount added and was essentially the same per g fresh weight from sample to sample in a particular experiment.

Figure 2A, which shows the results of adding 5 μM proline, indicates that there was a rapid conversion of nonprotein proline to protein proline (77% after 12 hr). Under these conditions, there was a small percentage of ^{14}C recovered in other amino acids (8% after 12 hr) and CO_2 (6% after 12 hr). An even smaller amount was recovered in the organic acid and neutral fractions (less than 2% each).

When 2 mM proline was added to the leaf there was a slower percentage loss of ^{14}C-proline (non-protein) than when the 5 μM proline was added, but 50% of it had been metabolized in about 6 hr. However, a much greater percentage of the ^{14}C was recovered in other amino acids, CO_2, and organic acids than was true when 5 μM proline was added to the leaf. Also, a substantial percentage of the label was recovered in protein proline (27% after 8 hr). When 10 mM proline was infiltrated, the results were similar to those with 2 mM proline. A slower rate of loss of ^{14}C from nonprotein proline was observed, a smaller percentage of ^{14}C was recovered in protein proline, and a higher percentage in other amino acids, CO_2, and organic acids.

The other amino acids in which ^{14}C was recovered were glutamic acid, γ-aminobutyric acid (small amount), aspartic acid, and asparagine (Fig. 3) in order of appearance of label with time. There was negligible ^{14}C recovered in basic amino acids, including ornithine and arginine. In all amino acids except asparagine, the amount of ^{14}C in each increased from zero at zero time to some maximum value at 4 to 8 hr, then declined throughout the remainder of the incubation time, indicating that these amino acids were intermediates in the metabolism of ^{14}C-proline. The ^{14}C in asparagine continued to increase throughout the incubation period, indicating that it was an end product of ^{14}C-proline metabolism. The ^{14}C in the organic acid fraction was contained in succinic, malic, and citric acids (Fig. 3), and they became labeled in that order. The ^{14}C in the succinic and malic acids reached a plateau at 8 to 12 hr which corresponded to the time at which $^{14}CO_2$ was being produced at a maximum rate, indicating that the organic acids were intermediates in the conversion of ^{14}C-proline to $^{14}CO_2$. The ^{14}C in citric acid increased more slowly and was still increasing at 12 hr. Presumably at a later time the amount of ^{14}C in citric acid would decrease as it did in succinic and malic acids. The order of labeling of these amino and organic acids is consistent with proline being oxidized first to glutamic acid which is subsequently metabolized by the reactions of the Krebs cycle which produce CO_2.

The fact that increasing the concentration of added proline to the leaves increases the percentage of proline oxidized to CO_2 and other amino acids and decreases the percentage converted to protein proline is clear from Figure 2. However, it is not clear how much proline is metabolized by these two alternative pathways in each case. The rate of proline conversion to various fractions can be estimated from Figure 2. In Figure 2, A and B, the loss of ^{14}C from nonprotein proline follows first order kinetics. Initially the rate of ^{14}C loss is equal to the utilization of proline for protein synthesis and oxidation. Thus, evaluating the first order rate constants permits calculation of

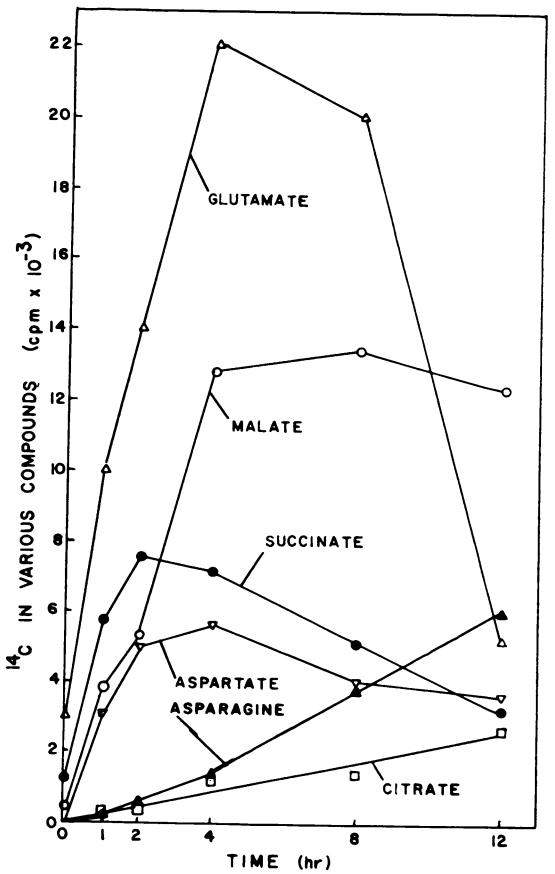

FIG. 3. Effect of incubation time on the amount of ^{14}C recovered in various compounds from leaves after adding 5 μM ^{14}C-proline (specific radioactivity 200 μc/μmole).

the rate of proline utilization. Then simply by partitioning the rate of utilization into protein synthesis and proline oxidation, using the data in Figure 2, A and B, the rates of utilization of proline by these alternative pathways can be estimated. In Figure 2C the rate of loss of nonprotein ^{14}C-proline is linear and the specific radioactivity is constant over the first 4 hr. Thus, the rate of proline utilization can be calculated directly from the slope of the line representing loss of nonprotein ^{14}C-proline. Again, by partitioning this rate of utilization of proline into protein synthesis and oxidation using the data in Figure 2C, the rates of utilization of proline by these two alternative pathways can be estimated. The results of these calculations are shown in Table I, and they indicate that the rate of conversion of nonprotein proline to protein is not greatly affected by the amount of proline added to the leaf, but the amount oxidized is markedly affected. The rate of incorporation into protein varied from 50 to 90 nmoles hr^{-1} g^{-1}, and there was no consistent increase with increasing proline concentrations. This variation is probably within the error of the estimation. However, the rate of proline oxidation varied from 0.001 to 0.43 μmoles hr^{-1} g^{-1} and increased with proline concentration. Thus, there was over 400-fold variation in the calculated rate of proline oxidation whereas the rate of incorporation into protein varied less than 2-fold.

Effects of Carbohydrates on the Metabolism of Exogenous Proline. The data in Figure 1 indicate that sucrose added to starved leaves along with proline inhibited the disappearance of exogenously added proline from the leaf. Figures 4, A and B, show the time course of the distribution of ^{14}C when ^{14}C-

Table I. *Estimation of Rates of Conversion of Nonprotein Proline to Protein and of Oxidation to Other Amino Acids and CO_2 at Different Proline Concentrations*

Endogenous proline level was 0.15 μmole/g fresh wt.

Proline Concn Added to Leaf	Amount Added per g Fresh Wt	Initial Proline Concn in Leaf	Half-time of ^{14}C Loss from Nonprotein Proline	Rate Constant for Initial Rate of Loss of Nonprotein Proline	Initial Rate of Loss of Nonprotein Proline	Rate of Utilization of Nonprotein Proline by	
						Conversion to Protein Proline	Oxidation to Other Amino Acids, Organic Acids, and CO_2
mM	*μmoles*	*μmoles/g*	*hr*	hr^{-1}	*μmole* $hr^{-1}g^{-1}$	*nmoles* $hr^{-1}g^{-1}$	*μmole* $hr^{-1}g^{-1}$
0.005	0.002	0.15	1.5	−0.46	0.07	70	0.001
2	1.5	1.6	6.0	−0.11	0.16	50	0.10
10	7.3	7.4			0.55	90	0.43

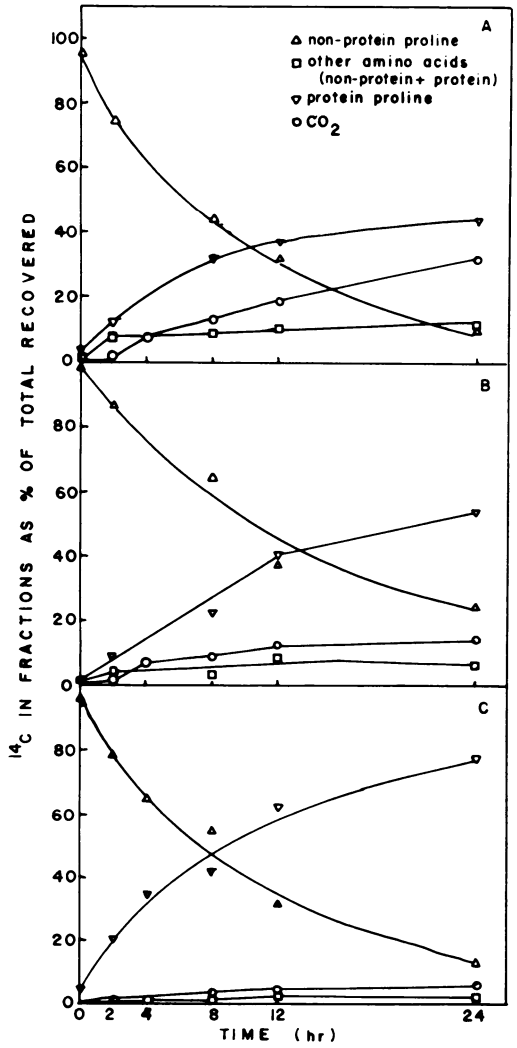

FIG. 4. Effect of incubation time on the distribution of ^{14}C in various fractions in leaves which had been infiltrated with 2 mM ^{14}C-proline under three different conditions (A: 50 mM sucrose added to starved leaves with proline; B: 0.1 M sucrose added to starved leaves with proline; C: nonstarved leaves, *i.e.*, from plants previously in the light for >16 hr).

proline (2 mM) was added along with 50 mM and 0.1 M sucrose respectively and are comparable to Figure 2B where no sucrose was infiltrated. From this comparison, it is apparent that sucrose slowed down the disappearance of ^{14}C from nonprotein proline. The presence of sucrose inhibited the oxidation of proline to other amino acids and CO_2 and increased somewhat the conversion of non-protein proline to protein. Figure 4C shows the time course of the distribution of ^{14}C when ^{14}C-proline (2 mM) was added to nonstarved leaves. When the results in Figure 4C are compared with those in Figure 2B, it is clear that in the nonstarved leaves, the disappearance of ^{14}C from nonprotein proline was slowed down, the oxidation of proline to other amino acids and CO_2 was almost stopped, and the conversion of proline to protein was increased, compared to the starved leaves. The ^{14}C recovered in organic acids is omitted from Figure 4 because this fraction accounted for <2% of the ^{14}C in all cases.

The slower net loss of exogenously added proline from starved leaves in the presence of sucrose and the slower loss from nonstarved compared to starved leaves (Fig. 1) could have been due to slower utilization or an increased formation (synthesis) of proline in these treatments. To determine the effect of sucrose and leaf carbohydrate on proline utilization and proline formation, the rates were calculated and are presented in Table II. The rates shown are instantaneous rates for the 8 hr time of incubation, and similar results were obtained at other times. The rate of proline utilization was apparently not greatly affected by sucrose or leaf carbohydrate, but the rate of change in proline content was progressively decreased as the level of carbohydrate increased. Thus, the effect of sugar on the net loss of exogenously added proline from the leaves appears to have been due to a greater synthesis of proline in the presence of carbohydrate. The effect of sugar on the utilization of proline was to bring about a shift in proline utilization by oxidation to protein synthesis (Fig. 4).

DISCUSSION

The observation that increasing the level of free proline in leaves by adding exogenous proline increases the oxidation of proline has been suggested by the data of Oaks *et al.* (6). The fact that the amount of proline converted to protein is not affected by adding a large amount of proline probably reflects the fact that protein synthesis is regulated by other factors which are not affected by adding proline. Whether or not increases in nonprotein proline due to water stress would result in an increase in proline oxidation is not clear from these experiments. However, previous results (9) in which proline disappeared from wilted leaves after a period of accumulation,

Table II. *Rates of Utilization and Formation of Nonprotein Proline in Excised Leaves with Various Carbohydrate Levels*

Carbohydrate Level	Proline Utilization[1]	Rate of Decrease in Proline Content	Proline Formation[2]
	μmole/hr·g fresh wt	*nmoles/hr·g fresh wt*	*nmoles/hr·g fresh wt*
Starved	0.10	60	40
Starved + 50 mM sucrose	0.09	40	50
Starved + 0.1 M sucrose	0.10	20	80
Non-starved	0.09	10	80

[1] Rate of decrease in ^{14}C in nonprotein proline [cpm per hr per g fresh wt ÷ specific radioactivity (cpm/μmole)].

[2] Proline utilization minus rate of decrease in proline content.

and some of the author's unpublished results on the disappearance of proline in rehydrated leaves, indicate that endogenous increases in proline also enhance the utilization of proline via oxidation.

The results from experiments on the effects of sucrose and previous light treatment indicate that the oxidation of proline is prevented when there is sufficient carbohydrate in the leaf. This effect was also suggested by Oaks *et al.* (6). Thus, when proline accumulates in the leaf due to water stress, or some other treatment, it is not oxidized unless the leaf is starved of carbohydrate. The leaf, therefore, can conserve proline even when proline is accumulating to levels that allow it to be oxidized under conditions of carbohydrate starvation. The increased incorporation of proline into protein with increasing carbohydrate indicates that carbon and energy was limiting protein synthesis under the conditions of the experiments. Under these conditions (proline concentration), the increased incorporation of proline into protein was equal to the decreased proline oxidation due to sugars. This result may not be the same under different conditions. The increased proline synthesis with increasing sugars indicates that the supply of carbon and energy was limiting proline synthesis which would be true if the carbon supply was limiting protein synthesis.

Any explanation of the results presented in this paper must await characterization of the enzymatic steps involved in proline oxidation. An L-proline dehydrogenase has been isolated from plants (4) which would be a possible first enzyme, but which has not been shown to function in the proline oxidation observed in these experiments.

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