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Proline Uptake and Utilization by *Chlorella pyrenoidosa*

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ABSTRACT

Conditions for proline uptake and utilization by *Chlorella pyrenoidosa* Chick are described. Proline is taken up by growing cultures during late log phase growth after depletion of glucose from the medium. However, proline uptake by stationary phase cultures requires the presence of glucose in the medium. The results are consistent with the interpretation that some carbohydrate is required for proline uptake but proline uptake is inhibited by the accumulation of intracellular carbohydrates.

Metabolism of proline was by incorporation into protein and oxidation to CO_2 by conversion to glutamic acid and subsequent metabolism of the carbon by the Krebs cycle.

In plants, the major fates of exogenously added proline are incorporation into protein (including subsequent conversion to hydroxyproline) and oxidation via the respiratory pathway (1, 10, 13, 14, 20). The relative amount of proline metabolized by each of these pathways is partially determined by the carbohydrate content (1, 10, 13) and the proline content of the tissue (13). These aspects of proline metabolism are important in understanding why proline accumulates in stressed tissue (2, 4, 5, 8, 11, 15, 16, 19) and the fate of proline during rehydration of wilted tissue (14).

The experiments reported in this paper were done to determine if *Chlorella* would be a useful organism in studying various aspects of the pathway of proline oxidation. The advantages of *Chlorella* are that the material can be grown aseptically, the organism can be manipulated nutritionally and genetically, and there is little or no sampling error. The experiments were specifically designed to determine the conditions under which proline is taken up and metabolized by *Chlorella* and to determine the metabolic fate of exogenous proline in *Chlorella*.

MATERIALS AND METHODS

Chlorella pyrenoidosa Chick. was obtained from the Culture Collection of Algae, Department of Botany, Indiana University, Bloomington. The growth medium of Ellsworth (7) was used with slight modifications. One liter of liquid medium contained 1.23 g of KNO_3, 2.46 g of $MgSO_4 \cdot 7H_2O$, 0.24 g of $Ca(NO_3)_2$, 1.13 g of KH_2PO_4, 0.12 g of K_2HPO_4, 0.077 g of iron EDTA, 0.5 mg of $MnCl_2 \cdot 4H_2O$, 0.05 mg of $ZnCl_2 \cdot 7H_2O$, 0.02 mg of $CuSO_4 \cdot 5H_2O$, 0.01 mg of $NaMoO_4 \cdot 2H_2O$, and 0.04 mg of $CoCl_2 \cdot 6H_2O$. Solid medium contained 1.5% agar. Glucose, when added, ranged from 0.001 to 1%. Subcultures were maintained on slants of the solid medium containing 0.1% glucose, at 15 C, in darkness.

All experiments were done with heterotrophically, dark-grown cells. Liquid cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of medium, shaken on a rotary shaker (170 rpm) at 25 C. Typically, liquid cultures were inoculated from slants and grown until they reached stationary phase; then the experiments were done with cells in their second growth cycle. Usually 5 ml of stationary phase cells were inoculated into 100 ml of medium.

Growth was measured by packed cell volume (9) with a Van Allen thrombocytocrit tube. One milliliter of cell suspension was centrifuged at 1500*g* for 20 min. Glucose in the medium was measured on an aliquot with the 3,5-dinitrosalicylic acid reagent (3) after centrifuging the cells. Proline was determined by the Chinard method (6) on a similar aliquot.

Two labeling experiments were done. The first was a relatively short (5-hr) experiment in which stationary phase cells were centrifuged and resuspended in a fresh medium plus 0.1% glucose. Five-milliliter samples of resuspended cells were pipetted into 50-ml centrifuge tubes. Uniformly labeled ^{14}C-L-proline was added to a concentration of 1 mM (specific radioactivity 0.2 μc/μmole). $^{14}CO_2$ was collected on a 1-cm^2 piece of filter paper suspended from a rubber stopper with a wire and saturated with monoethanolamine. Monoethanolamine was used because it is soluble in scintillation solution and is inexpensive. Although somewhat volatile and toxic, it did not appear to affect the growth of the *Chlorella* under the conditions of the experiments in which it was used. Some $^{14}CO_2$ was undoubtedly trapped in solution in the medium and was not accounted for by the data. At sampling time, the cells were chilled, centrifuged, and resuspended in 95% ethanol. Extraction, fractionation, and determination of radioactivity were done according to methods previously described (13, 17, 18).

The second labeling experiment was conducted through an entire growth cycle in a 250-ml Erlenmeyer flask with a side arm. A 4-ml vessel containing 1 ml of monoethanolamine was attached to the side arm. Five milliliters of stationary cells were inoculated into 100 ml of medium containing 1% glucose and 1 mM ^{14}C-proline (specific radioactivity 0.1 μc/μmole), and the cells were grown through one growth cycle. Five-milliliter samples were removed at intervals during the growth cycle, and simultaneously the monoethanolamine was removed and replaced by fresh. The samples of cells were chilled, centrifuged, extracted, and fractionated as indicated above. Radioactivity in CO_2 and various fractions was determined.

RESULTS AND DISCUSSION

Proline Uptake. Initially proline uptake and utilization by growing and nongrowing cells were determined. To determine

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uptake by nongrowing cells, proline was added to stationary phase cultures to a final concentration of 1 and 10 mM. Proline was not taken up from the medium by these cells except after a long lag of more than 40 hr (Fig. 2B). Proline was taken up by growing cells. Figure 1 shows the cell growth in a medium initially containing 1% glucose and 10 mM proline. Cell growth showed a typical exponential phase with a doubling time of 12 hr followed by a stationary phase. Glucose disappeared from the medium most rapidly during the most rapid growth, and the decrease in growth rate corresponded to the disappearance of glucose from the medium. However, growth continued for about 30 hr after the glucose was depleted from the medium, suggesting that the cells had stored carbohydrate during the uptake. Proline disappeared from the medium most rapidly after the most rapid disappearance of glucose during late log phase. A later experiment (Fig. 4) with a lower proline concentration indicated that a small amount of proline was taken up during the first few hours of growth, but such uptake was not apparent in these data because it represented such a small fraction of the initial proline in the medium.

As mentioned above, initial experiments showed no proline uptake by stationary phase cells except after a long lag. This suggested that a limiting component of the medium may be involved in proline uptake. Thus, it was of interest to determine the limiting component. Figure 2A shows that when glucose and KNO_3 were added to the medium of stationary phase cells, an additional amount of growth (about 17 μl/ml) approaching that achieved in a typical growth cycle was obtained. With glucose and no KNO_3, a small amount of additional growth was obtained; and with KNO_3, alone, no additional growth was observed. Thus, glucose is the most limiting component and nitrogen is near limiting. The nitrogen limitation may explain why some additional growth was observed with glucose alone since this experiment was done with 1 mM proline in the medium and proline could have supplied the nitrogen. Figure 2B shows the proline disappearance from the medium in this experiment. Proline uptake by nongrowing cells in the absence of glucose did occur but only after the long lag (40 hr) mentioned above. Proline uptake by stationary phase cells in the presence of glucose was very rapid, with most of it being taken up prior to the first sampling before any measurable growth occurred.

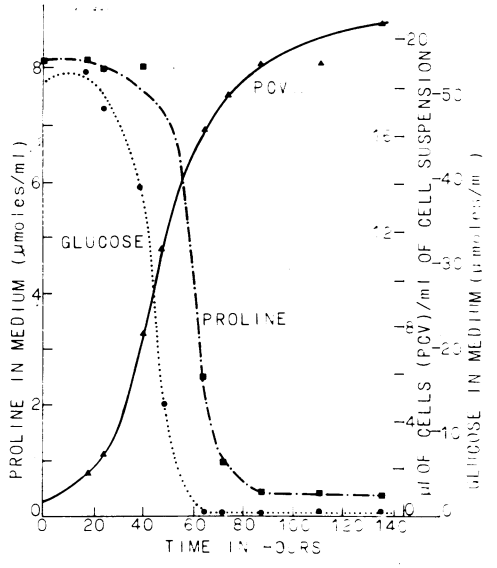

FIG. 1. Relationship of proline uptake and glucose uptake to growth of *Chlorella*.

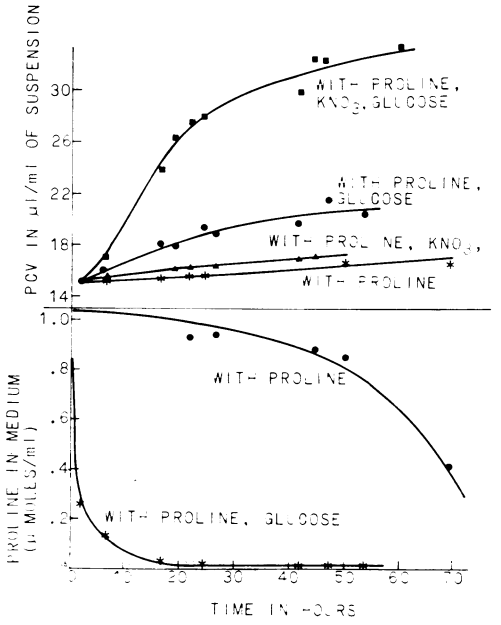

FIG. 2. Growth (A, upper) and medium proline concentration (B, lower) after addition of proline (zero time concentration 1 mM), glucose (zero time concentration 1%), and KNO_3 (zero time concentration 12 mM) to stationary phase *Chlorella* cells.

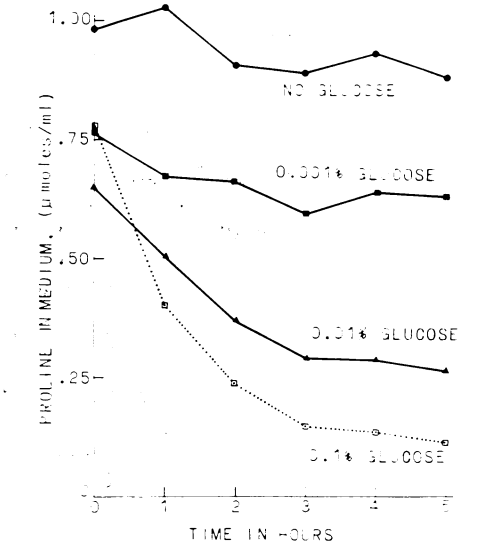

FIG. 3. Proline uptake by stationary phase *Chlorella* cells (20 μl PCV/ml of medium) in media containing various concentrations of glucose (initial proline concentration 1 mM).

Figure 3 shows proline uptake from stationary phase cells which were resuspended in fresh media of differing glucose concentrations and 1 mM proline. The cells were shaken 15 min prior to zero time. Over a 5-hr period during which there was no measurable growth, there was very little proline uptake in the absence of glucose and in 0.001% glucose. In 0.01 and 0.1% glucose there was a rapid rate of uptake, and the rate increased with increasing glucose concentrations. Light, at least in the absence of CO_2 enrichment, would not substitute for this glucose requirement.

The results in Figure 3 indicate that there should have been some proline uptake in the first few hours of the growth cycle as mentioned above in discussing the results in Figure 1. Figure

4 shows results from an experiment similar to that represented by Figure 1, but at lower proline concentration (1 mM). The growth and glucose uptake relationship were the same as in Figure 1; but, as expected, a small amount of proline was taken up during the first 12 hr of the growth cycle. The amount of uptake was small owing to the low cell density. The initial rapid uptake was followed by a lag of 50 hr during which there was very little uptake. After 65 hr, when glucose was depleted from the medium, there was a second period of rapid uptake of proline.

Proline Metabolism. Experiments were conducted to determine the fate of the proline taken up by stationary phase cells in the presence of 0.1% glucose (5 hr) and by growing cells during the growth cycle.

The distribution of ^{14}C in various fractions at intervals after incubating stationary phase cells during a 5-hr period is shown in Figure 5. The cell density was about 20 μl of cells per milliliter of medium (Figs. 1 and 4). The time course of ^{14}C-proline disappearance from the medium is not shown but corresponded to the 0.1% glucose treatment in Figure 3. The amount of radioactivity recovered at each sampling time increased with incubation time. When the ^{14}C in the supernatant was added to the ^{14}C recovered from the cells, the total was the same for all samples and was equal to the amount added (0.5 μc). The initial increase in ^{14}C in the neutral and acidic amino acid fraction was due to the rapid uptake of proline and was mainly proline and some glutamic acid. The decrease in radioactivity after 1 hr was due to metabolism. The ^{14}C in organic acids and basic amino acids increased to a maximum at 1 and 2 hr, respectively, and subsequently declined. The ^{14}C in CO_2 and protein increased throughout the experiment as though they were end products of proline metabolism. The percentage of ^{14}C recovered as CO_2 (43%) represented a greater proportion of the metabolized proline than any previously reported (1, 10, 13, 14, 20).

The ^{14}C in the organic acid fractions was in succinic, malic, and citric acids, and they became labeled in that order. This distribution and time course of label in the organic acids was consistent with metabolism of proline via the Krebs cycle after conversion to glutamic acid. The conversion to basic amino acids was probably to arginine and ornithine, but this fraction was not chromatographed.

The data in Table I show the distribution of ^{14}C in various fractions of *Chlorella* which were supplied ^{14}C-proline (1 mM initially) throughout the growth cycle. This experiment differed from that represented by Figure 5 in that these were growing cells and metabolism of ^{14}C-proline was measured throughout the growth cycle whereas the data in Figure 5 represent short time experiments with stationary phase cells. The pattern of proline uptake was the same as shown in Figure 4. By the end of the experiment, the ^{14}C recovered from the cells was equal to the amount added. During the first 20 hr, ^{14}C-proline was converted to CO_2 and protein as in the previous experiment. From 30 to 80 hr, there was no increase in ^{14}C in CO_2 but a small increase in ^{14}C recovered in protein. This corresponded to a decrease in ^{14}C in free amino acids and no uptake of proline. Thus, the free proline in the cells during rapid growth was incorporated into protein, but additional uptake (Fig. 4) was inhibited. Oxidation was also apparently inhibited because no additional CO_2 was evolved during this time. After growth had ceased, there was another period of rapid uptake. Most of this proline was converted to CO_2. The decrease in percentage recovered in protein from 48 to 96 hr was due to an increase in the total amount recovered without a corresponding increase in the amount recovered in protein.

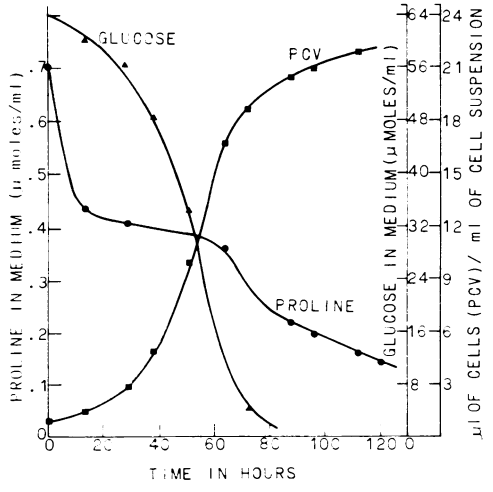

FIG. 4. Relationships of proline uptake and glucose uptake to growth of *Chlorella* using low initial proline concentration (1 mM).

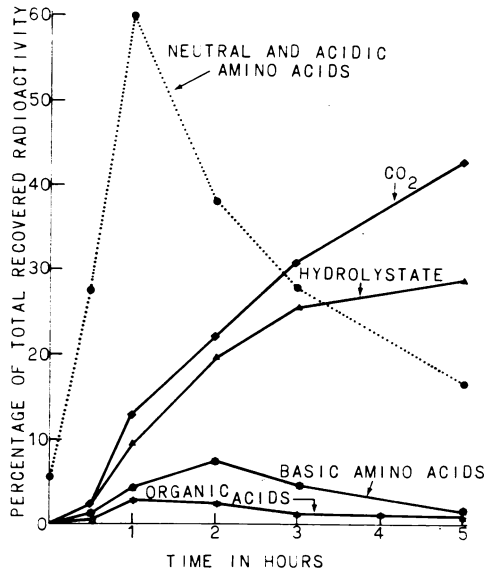

FIG. 5. Percentages of ^{14}C recovered in various fractions from *Chlorella* after incubation for various times with uniformly labeled proline (1 mM).

Table I. *^{14}C Found in Various Fractions after Incubation of Chlorella Cells with Uniformly Labeled 1 mM Proline*

Time	CO_2	Free Amino Acids	Protein Hydrolysate	Organic Acids	Neutral Sugars
hr			%		
0	0	91.7	4.5	2.4	1.4
8.0	10.8	50.1	34.7	4.0	0.5
23.5	56.6	15.0	27.3	0.8	0.3
32.0	45.5	22.4	31.5	0.5	0.2
48.0	43.3	6.9	49.4	0.2	0.2
56.0	45.5	13.0	41.0	0.4	0.2
72.5	43.3	13.3	42.9	0.5	0.2
80.0	46.6	12.3	40.4	0.6	0.2
96.0	64.2	11.4	23.6	0.5	0.3

INTERPRETATIONS

A working model for interpreting the results presented on proline uptake can be based on the assumptions that glucose

is required for rapid proline uptake and that the accumulation of carbohydrates in the cells inhibits proline uptake. According to this model, the initial rapid proline uptake (Fig. 4) was possible because ample glucose was available but the cells were sufficiently starved of carbohydrate to allow proline uptake. The slow rate of proline uptake between 20 and 60 hr was due to the inhibition of proline uptake by accumulated carbohydrates in the cells. The second period of rapid proline uptake (70 to 120 hr) was possible because the carbohydrate level in the cells was declining owing to depletion of glucose from the medium. The results in Figures 1 through 3 and in the labeling experiments are consistent with the model.

If this model is correct, measurable accumulations of carbohydrate in the cells during the period 20 to 60 hr would be predicted. In a preliminary experiment, carbohydrates were extracted from cells with perchloric acid and determined with α-naphthol as described previously (12). The cells at 35 hr (Fig. 4) contained 33 μmoles of glucose equivalent per milliliter PCV,[2] whereas the cells at 0 and 60 hr contained 19 μmoles/ml PCV. A further prediction from this model is that preincubating cells with glucose would eliminate the initial rapid proline uptake. A preliminary experiment to test this prediction indicated that preincubation with glucose did indeed inhibit proline uptake.

The results from the labeling experiments are consistent with proline being oxidized to CO_2 by conversion to glutamic acid, then oxidized by the reactions of the Krebs cycle, as has been shown for higher plants (1, 10, 13, 20). It is not possible to say whether or not this oxidation is subject to control by proline and carbohydrate content as was shown for corn root tips and bean leaves (1, 10, 13).

[2] Abbreviation: PCV: packed cell volume.

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