Title

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DIFFERENCE IN NITRATE REDUCTION IN "LIGHT" AND "DARK" STATES OF SYNCHRONOUSLY GROWN CHLORELLA PYRENOIDOSA AND RESULTANT METABOLIC CHANGES

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Difference in nitrate reduction in "light" and "dark" stages of synchronously grown Chlorella pyrenoidosa and resultant metabolic changes[1]

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Summary

Using the synchronized cells of Chlorella pyrenoidosa, the incorporation patterns of ^{14}C into various metabolites with and without nitrogen sources were studied under steady-state and non steady-state conditions. From the patterns it was found that the smallest cells which are divided in the dark utilize nitrate and nitrite very little, if at all.

The importance of ammonia for regulation of secondary flow for Chlorella is discussed and the suggested regulatory points are described.

Kinetic studies of the flow of carbon in green cells (1,2,3) indicate regulatory mechanisms controlling the flow of carbon within and out of the the chloroplast and the relation between photosynthetic carbon reduction and biosynthetic metabolism.

Comparison of the patterns of carbon-14 incorporation into various metabolites at various stages of the life cycle of Chlorella pyrenoidosa cells, under steady-state conditions, showed that the smallest cells

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(D-cells[2]), which divided in the dark, incorporated ^{14}C much faster into sucrose and less into amino acids and organic acids than growing cells (L-cells[2]) (4). Some enzymic sites involved in the regulatory mechanism for diversion of carbon flow into secondary products from photosynthetic products were indicated.

We report here experiments with different inorganic nitrogen nutrients. These studies show that the difference of ^{14}C-incorporation patterns between D- and L-cells is to a large degree due to a difference in ability of the cells to utilize nitrate, and to consequent regulatory effects caused by presence or absence of ammonia.

Materials and methods

Synchronization of Chlorella cells

Synchronized cells of Chlorella pyrenoidosa were obtained by successive changes of light (26 hr) and dark (12 hr) using a turbidostat (capacity 800 ml) at 25°C with a stream of air + 4% (v/v) CO_2. Cell density was kept constant at around 0.3-0.4 (v/v) packed cell volume with automatic addition of the medium (Modified Myers Medium). The characterization of synchronized cells has been described elsewhere (4).

Steady-state experiment

The steady-state experiments were performed in three different

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[2] D-cells are the smallest cells which divide in the dark, and L-cells are cells growing under the light (5). For L-cells, the subscript describes in hours of the culture from the start of the illumination, e.g., L_0 cells are the same as D-cells.

media using D- and L_{18-20}-cells; without a nitrogen source (10^{-3} M KH_2PO_4), with nitrate (10^{-3} M KH_2PO_4 + 10^{-3} M KNO_3), and with ammonia (10^{-3} M KH_2PO_4 + 10^{-3} M NH_4Cl).

D-cells were centrifuged down at the end of the dark phase, washed once with 10^{-3} M KH_2PO_4, and kept in the dark in 2% suspension (the packed cell volume was measured by centrifugation 10 min at 2500 rpm). Prior to the experiment, the cell suspension was diluted to 1% in media with or without nitrogen as indicated above. The L-cells were harvested prior to each experiment, washed, and suspended in the respective media.

Fifty ml of 1% suspension was placed in the steady-state apparatus. The pH was kept nearly constant around 6 by adding automatically 0.1 N nitric acid or NH_4OH for media containing NO_3^- and NH_4^+ respectively. When nitrogen sources are omitted from the medium, or in the case of the experiment with nitrate using D-cells, pH adjustment was unnecessary. After 15 min of photosynthesis (20°C) with about 1.5-2% CO_2, $^{14}CO_2$ was added from a small loop into the closed circuit, and at intervals samples (approx. 1 ml) were taken into 4 ml of methanol. At the start of the experiments, CO_2 concentration was about 1.6% with a specific radioactivity of ^{14}C of 8-10 μC/μM. After 30 min, at the end of the experiment, the concentration of CO_2 was 0.4-0.6%.

The ^{14}C-incorporation patterns were determined by routine procedures using paper chromatography and the semi-automatic counting device (2).

Incorporation of ^{14}C into starch and lipid was measured and calculation of flow rate into secondary products was made as described elsewhere (4). The incorporation of ^{14}C into amino acid residues of protein was measured as follows: Defatted samples were hydrolyzed with

6 N HCl for 30 hr at 110°C in sealed tubes. The tubes were opened and the solutions dried, then taken up into water. An aliquot portion was analyzed by two-dimensional paper chromatography and semi-automatic counting (2).

Comparative studies of effects of nitrogen source (non steady-state)

For concurrent analyses on the effect of various nitrogen sources on the ^{14}C incorporation pattern, photosynthesis was carried out in several 15-ml round-bottom flasks stoppered with serum caps, to which 250 µl of 2% cell suspension in 2×10^{-3} M KH_2PO_4, 200 µl of double concentration of various nitrogen sources in deionized water, and 50 µl of 0.11 M $NaH^{14}CO_3$ solution (about 10 µC/µM) were added successively. These flasks were mounted in a rack which moves in a circular motion (6), allowed to photosynthesize during 15 min, killed by adding 4 ml of methanol, and analyzed as described by paper chromatography and radioautography (2).

Results

Steady-state experiment

Table 1 shows the rate of incorporation of ^{14}C into starch and lipid. These rates are measured as the linear increase with time after an initial slight lag. A slight decrease in rate of ^{14}C incorporation into starch and slight increase into lipid was observed when NH_4Cl was included in the medium. ← Table 1

Table 2 shows the rate of ^{14}C incorporation into certain amino acids (the ones most quickly labeled) in the total protein. Since some free amino acid pools are only partially labeled during the first 30 min

of photosynthesis (7), these data for ^{14}C in bound amino acids after 30 min photosynthesis with $^{14}CO_2$ provide only an approximate indication of the relative rates of carbon entry into protein biosynthesis. Nevertheless, it is clear that protein synthesis proceeds rapidly in L-cells with either nitrate or ammonia as a nitrogen source, or in D-cells with added ammonia but not with added nitrate. ← Table 2

Table 3 shows the steady-state level (C) of secondary products and the flow rates (R). In both types of cells, addition of NH_4Cl caused a decrease of flow of ^{14}C into sucrose and an increase into amino acids and organic acids. With L-cells, addition of nitrate induced effects similar to those of the NH_4Cl. Nitrate addition to D-cells caused very little effect. ← Table 3

Without any nitrogen source, less incorporation into amino acids was detected in both cells. Incorporation into organic acids of the TCA cycle is extremely low. It cannot be determined whether the TCA cycle is operating slowly, if at all, or if the pool sizes in the intermediates are greatly diminished when nitrogen sources are not available.

In the presence of nitrate or ammonia, pool sizes and flow rates for TCA compounds in L-cells can be determined accurately. An increase in ammonia in the cell, either by addition of ammonia or by reduction of nitrate, induces an increase of pool size as well as an increase of flow rate. This suggests that one of the primary effects of ammonia is to increase the carbon flow into the TCA cycle by a mechanism other than increased pool turnover due to increased amino acid synthesis.

Table 4 shows the steady-state level (C) of intermediates of the photosynthetic carbon reduction cycle and related substances. In both cells (D- and L-cells) addition of NH_4Cl caused a slight decrease of incorporation into most of the intermediates. In L-cells, addition of nitrate or NH_4Cl causes a significant decrease in the level of hexose monophosphates but an increase in UDP-glucose. In D-cells, similar effects were seen, but only upon the addition of NH_4Cl. ← Table 4

Study of various nitrogen sources (non steady-state)

Table 5 shows the effect of various nitrogen sources with the method described as "non steady-state" using D-cells and L_3-cells. As was generally the case with the experiment described earlier (Table 3), addition of ammonia (but not of nitrate) to D-cells, and the addition of ammonia or nitrate to L-cells, caused a decreased flow of ^{14}C into sucrose and an increased flow of ^{14}C into amino acids and into malic and citric acids. Furthermore, the addition of nitrite to L-cells causes these same effects (increased ^{14}C flow into amino acids, citric and malic acids, decreased sucrose synthesis), while the addition of nitrite to D-cells had much less effect. In this experiment ammonia addition produced greater effects than either nitrate or nitrite addition to L-cells. Finally, the addition of NH_2OH to D- or L-cells caused the formation of much ^{14}C-labeled glycolic acid, some increase in ^{14}C-labeled aspartic acids and alanine, and moderate to severe decreases in sucrose, serine, and other compounds. Because of these changes in metabolism, it is not clear whether or not the hydroxylamine was utilized by the D-cells. ← Table 5

Discussion

From the comparison of ^{14}C-incorporation patterns with varying nitrogen sources, it is clear that the main difference between D-cells and L-cells is the difference in utilization of nitrate, together with subsequent changes in metabolism caused by the availability or non-availability of ammonia. Nitrate reduction is known to be stimulated by light (8,9), and there have been proposed three different explanations: (1) Increase of permeability for nitrate by light (10,11), (2) avilability of reducing coenzyme and carbon source (8), (3) elimination of inhibitor(s) by light (12) (activation of nitrate reductase).

In the experiments described here it is clear that light does not appreciably stimulate nitrate utilization of D-cells during 45 min of experiment. Thus, a rapid effect of light can be eliminated as a regulatory mechanism.

Since total amino acid formation without added nitrogen is similar in D-cells and L-cells, endogenous nitrogen sources available within the cells seem to be similar in amount (Table 3). Thus, the difference in effects of added nitrate with the two types of cells seems not to be due to an inhibition of nitrate reduction by endogenous available ammonia.

In higher plants, activity of nitrate reductase shows diurnal rhythm, low in night and high in daytime (9,13). Nitrate reductase is unstable and continuously resynthesized in the light in the presence of nitrate, but not in the dark. On the other hand, nitrite reductase is known as constitutive and stable in higher plants (9) and in algae (14). Nevertheless, in the case of Chlorella, D-cells could not

utilize nitrite (Table 5) during the 15 min of photosynthesis. In any event, the failure of D-cells to utilize nitrate and nitrite may be understood as a part of the long-term regulation of photosynthetic organisms which are subjected to day and night changes.

The effects of added hydroxylamine (Table 5) are probably complicated by some partial interference with electron transport leading to the formation of greatly increased amounts of glycolate and severely diminished formation of sucrose (due to oxidation of hexose monophosphates to glycolate).

Changes in the flow rate of ^{14}C into sucrose and in the steady-state level of sucrose are among the most pronounced effects of altering the nitrogen source. The shift in biosynthesis from sucrose to glutamate and malic and citric acids with addition of ammonia was observed by Hiller (15,16). Perhaps sucrose is functioning as a "buffer" for maintaining the pool sizes of intermediates of the photosynthetic carbon reduction cycle in Chlorella. When more carbon is taken from the cycle at the level of PGA for amino acid synthesis, less is used for sucrose synthesis.

The mechanism by which sucrose is more affected than starch by ammonia is not known. In earlier studies with the same cultures of synchronously grown Chlorella pyrenoidosa (4) we found that the change from post-division metabolism (D-cells) to rapid growth stages (L-cells) was accompanied by decreased sucrose synthesis and increased synthesis of organic acids and amino acids. At the same time, the apparent rate of the conversion of fructose-1,6-diphosphate to fructose-6-phosphate (F6P), a regulated step in the cycle, decreased.

In the present case, when ammonia is added to D- or L-cells, or either ammonia or nitrate is added to L-cells, the level of F6P drops 12 to 20%. However, the level of UDPG rises by about the same degree or more.

Thus, it is not clear how the presence of ammonia has caused a decrease in the rate of sucrose synthesis, unless there has been a regulatory (inhibiting) effect on either the enzyme for this reaction between F6P and UDPG or on sucrose phosphatase.

$$\therefore \quad \text{F6P} + \text{UDPG} \longrightarrow \text{UDP} + \text{sucrose phosphate} \longrightarrow \text{sucrose} + P_i$$

Another possibility could be that ammonia increases the utilization of sucrose, but the data in Table 3 show that not only is the sucrose labeling at steady state decreased, but also the rate of flow (R) of ^{14}C into the sucrose pool.

One of the conclusions from the steady-state experiment, and noted earlier by Hiller (16), is that ammonia induces the increase of the inflow of carbon into the TCA cycle. This effect is not due to a toxic effect of ammonia, since nitrate also induces a similar effect when utilized. Calvin and Massini (17) proposed that the TCA cycle is not operating in the light from the transitional changes of metabolites when light is switched off. Our data support the hypothesis, but only when nitrate sources are not available (Table 3). As a result of increased knowledge about the details of the relation between photosynthesis and respiration in green plants (2,3), it appears reasonable to suggest that the TCA cycle is operating mostly for the synthesis of amino acids in the light.

The presence of ammonia (added, or by nitrate reduction) causes the steady-state levels of PGA and PEPA to decline. These changes suggest an *in vivo* activation of pyruvate kinase by ammonia, as has been reported in studies with extracts (18) and partially purified enzymes (19). Also, acetate added to *Chlorella* in the light is readily utilized (7,20), giving an increment of amino acid synthesis over and above that coming from CO_2 assimilation by photosynthesis (7). This is consistent with the pyruvate kinase mediated reaction as the rate-limiting regulated step. We have recently found that upon the addition of ammonia to *Chlorella* photosynthesizing under steady-state conditions, the level of pyruvate increases at least twofold (21).

Such an activation of pyruvate kinase does not explain increased malic acid labeling in the presence of ammonia. This increase in malic acid could be caused either by increased reductive carboxylation of pyruvate or by increased condensation of acetyl CoA with glyoxylate.

Thus, although other regulatory effects of ammonia on *Chlorella* metabolism may exist, the activation of pyruvate kinase is sufficient to explain the effects on amino acid biosynthesis found in these studies.

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Table 1

Rates of synthesis of starch and lipid

(Steady-state experiments)

	μmoles $^{14}C/cm^3$ algae					
	D-cells			L-cells		
	-N	$+KNO_3$	$+NH_4Cl$	-N	$+KNO_3$	$+NH_4Cl$
Starch	9.10	7.55	7.00	10.7	8.40	8.10
Lipid	1.95	1.73	2.15	1.55	1.71	1.98
Total ^{14}C uptake	15.5	13.2	18.0	18.0	17.3	19.0

Table 2

Incorporation of ^{14}C into protein amino acids

(μmoles $^{14}C/cm^3$ algae) after 30 min of photosynthesis

	D-cells			L-cells		
	-N	$+KNO_3$	$+NH_4Cl$	-N	$+KNO_3$	$+NH_4Cl$
Aspartic acid	0.40	0.46	1.76	0.24	2.89	3.49
Serine	0.47	0.35	0.84	0.50	0.93	1.41
Glycine	0.57	0.46	0.95	0.98	1.05	1.12
Glutamic acid	-	0.20	3.09	0.35	2.73	4.60
Alanine	1.88	1.65	3.29	2.34	3.90	4.18
Totals for these amino acids	3.32	3.12	9.93	4.41	11.50	14.80

Table 3

Steady-state levels (C) and flow rates (R) of secondary products

(Steady-state experiments)

	D-cells						L-cells					
	-N		$+KNO_3$		$+NH_4Cl$		-N		$+KNO_3$		$+NH_4Cl$	
	C[a]	R[b]	C	R	C	R	C	R	C	R	C	R
Sucrose	52	3.13	47	2.62	26	1.84	60	3.46	22	1.49	10	1.10
Serine	2.0	0.15	2.6	0.17	5.5	0.28	2.0	0.25	4.2	0.25	7.5	0.39
Alanine	1.1	0.063	1.7	0.29	9.5	0.95	0.70	0.23	4.5	0.54	9.8	1.03
Malic acid	>2.1	>0.05	>2.1	>0.05	7.5	0.39	>0.85	>0.029	3.0	0.31	7.0	0.91
Fumaric acid	0.52	0.015	0.40	0.021	1.6	0.087	0.20	0.011	0.80	0.073	1.7	0.21
Aspartic acid	1.0	0.063	1.3	0.072	>4.0	>0.18	>0.37	>0.015	4.0	0.32	6.0	0.55
Citric acid	0.05		0.05		0.4		0.06		0.3	0.03	1.0	
Glutamic acid	>0.4	>0.015	>1.2	>0.030	>10.1	>0.41	>1.5	>0.06	>16	>0.69	>18.7	>0.76
Glutamine	-	-	-	-	>4.0	>0.18	>0.90	>0.035	>2.0	>0.093	>6.3	>0.30

[a] C: μmoles $^{14}C/cm^3$ algae

[b] R: μmoles $^{14}C/min/cm^3$ algae

Table 4

Steady-state levels of intermediates of photosynthetic carbon reduction cycle and related substances

(Steady-state experiments)

	μmoles $^{14}C/cm^3$ algae					
	D-cell			L-cell		
	-N	$+KNO_3$	$+NH_4Cl$	-N	$+KNO_3$	$+NH_4Cl$
PGA	4.4	4.1	3.9	5.1	4.0	4.3
F6P	5.6	5.4	4.8	5.8	4.5	4.6
HMP	13.8	12.4	11.2	17.0	12.9	13.8
UDPG	1.6	1.6	1.9	1.8	2.4	2.6
PEPA	0.31	0.28	0.19	0.26	0.21	0.15
DiP	1.1	1.1	1.1	1.8	1.5	2.1

Table 5

Effect of various nitrogen sources on the ^{14}C incorporation patterns of the secondary products under non steady-state conditions (after 15 min photosynthesis)

	µmoles $^{14}C/cm^3$ algae									
	D-cells					L_3-cells				
	-N	+KNO_3	+NH_4Cl	+KNO_2	+NH_2OH ($10^{-4}M$)	-N	+KNO_3	+NH_4Cl	+KNO_2	+NH_2OH ($10^{-4}M$)
Sucrose	4.29	4.16	1.91	3.69	1.00	5.34	2.29	1.87	2.13	0.57
Serine	2.30	1.83	4.57	2.20	0.33	3.72	5.11	7.19	5.41	0.57
Alanine	1.86	1.13	10.8	1.42	2.46	1.13	3.67	16.7	4.92	2.19
Malic acid	4.75	4.62	10.9	5.08	3.89	3.34	4.54	9.34	4.54	3.48
Aspartic acid	1.70	1.57	5.43	1.75	3.09	1.59	3.16	6.29	2.68	3.97
Citric acid	0.12	0.08	1.11	0.09	0.09	0.07	0.10	0.65	0.15	0.08
Glutamic acid	0.38	0.28	2.39	0.35	0.38	0.94	2.47	5.85	2.48	0.63
Glycolic acid	6.99	8.29	5.95	7.07	23.8	4.63	4.20	4.58	4.58	24.1
Glycine	0.52	0.43	1.34	0.59	0.67	0.77	1.10	1.73	1.23	0.57
Glutamine	-	0.11	0.74	-	-	-	0.33	1.69	0.40	-
Threonine	-	0.07	0.25	-	0.28	0.17	0.28	0.99	0.37	0.11
PEPA	0.16	0.14	0.15	0.17	0.17	0.19	0.16	0.18	0.19	0.11

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