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Evidence for the Participation of the Reductive Pentose Phosphate Cycle in Photoreduction and the Oxyhydrogen Reaction[1]

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Abstract. The assimilation of ^{14}C-sodium bicarbonate has been measured in *Scenedesmus obliquus* as 1) photosynthesis, 2) photoreduction (light dependent incorporation of carbon dioxide by hydrogen adapted cells under conditions where photosynthesis is inoperative), and 3) the oxyhydrogen reaction (dark assimilation of carbon dioxide by hydrogen adapted cells in an atmosphere of hydrogen and 1 % oxygen). Degradation of the glucose formed in each of these reactions using the *Leuconostoc* technique establishes the participation of the reductive pentose phosphate cycle.

Several distinct reactions have been described whereby certain photosynthetic algae can assimilate CO_2 by mechanisms other than the usual photosynthetic carbon fixation. Hydrogen adapted *Scenedesmus* and *Chlamydomonas* as well as a variety of other algae can assimilate CO_2 in the light by a process which is insensitive to inhibitors of system II of the photosynthetic electron transport chain (2). This process has been called photoreduction (5). Gaffron (4) has also described a CO_2 assimilatory process which takes place entirely in the dark in an atmosphere of H_2 gas and low partical pressures of O_2; this reaction has been termed the oxyhydrogen reaction and it appears to represent a chemosynthetic assimilation of carbon.

A report by Gingras, Goldsby, and Calvin (8) indicated that the kinetics of labeling of soluble compounds by ^{14}C from CO_2 in *Scenedesmus* during photoreduction and the oxyhydrogen reaction were consistent with the operation of the reductive pentose phosphate cycle. However, under the conditions of the photoreduction assay (low light intensity and an atmosphere of H_2), photosynthesis and the oxyhydrogen reaction (using photosynthetically generated O_2) were also taking place (*cf.* ref. 3). The kinetics of labeling under these conditions may well have reflected photosynthetic carbon assimilation. The kinetics of labeling of intermediates during the oxyhydrogen reaction did not support definitely the operation of the reductive pentose phosphate cycle; the carboxylation of a precursor pool of ribulose-1,5-diP without actual turning of the cycle could account for the observed results.

The present communication demonstrates unequivocally that the reductive pentose phosphate cycle operates during the oxyhydrogen reaction and photoreduction in *Scenedesmus.* Under conditions where photosynthesis has been inhibited by more than 99 %, the intramolecular labeling of glucose as determined by degradation of the glucose formed during photoreduction establishes the participation of the reductive pentose phosphate cycle. Similar results have been obtained in experiments on the oxyhydrogen reaction. A preliminary report of these findings has been published (12).

Materials and Methods

Organism. The organism used in this study was *Scenedesmus obliquus,* strain D_3. Cells were cultured in inorganic salts medium (8) at a light intensity of approximately 300 ft-c. A mixture of 5 % CO_2 in air was bubbled through the cultures.

Measurements of Photoreduction and the Oxyhydrogen Reaction. Photoreduction was measured as the light dependent fixation of $^{14}CO_2$ by adapted cells. Cells were incubated in double armed Warburg flasks in a water bath maintained at 25°. The flasks were gassed for 15 minutes with H_2 which had been previously purified by passage through a "deoxo" cartridge. The cells were incubated in the dark overnight. Radioactive bicarbonate was added with a syringe through a serum cap in 1 of the sidearms and tipped into the main vessel. Photoreduction was measured at 1500 ft-c from General Electric Photoflood bulbs. Photosynthesis was prevented by the addition of 3(3,4-dichlorophenyl)-1-1-dimethyl urea (DCMU) in ethyl alcohol to a final concentration of 10 μM. Control experiments showed that this concentration inhibited photosyn-

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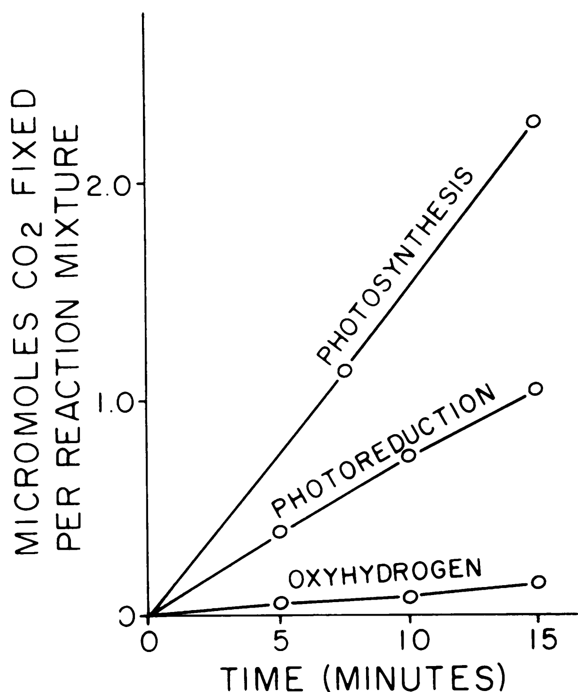

FIG. 1. The kinetics of $^{14}CO_2$ fixation by *Scenedesmus* measured as photosynthesis, photoreduction and the oxyhydrogen reaction. Each flask contained 5.3 mg dry weight of cells, corresponding to 160 µg chlorophyll. Radioactive bicarbonate was added at time zero. Photosynthesis and photoreduction were measured at 25° and at a light intensity of 1500 ft-c. DCMU (10 µM final concentration) was added to the photoreduction measurement to prevent photosynthesis. The oxyhydrogen reaction was carried out in the dark in an atmosphere of H_2 and 1 % oxygen. The measurements of photosynthesis and photoreduction are corrected for dark fixation; the oxyhydrogen reaction is corrected for fixation in the absence of O_2.

thetic CO_2 fixation by more than 99 %. The final concentration of ethyl alcohol was 1 % and this had no effect on CO_2 fixation.

The oxyhydrogen reaction was measured by similar techniques, but was carried out in a completely darkened room to exclude any possibility of photoreduction or photosynthesis. Adaptation was carried out overnight as described above. After the adaptation period, radioactive bicarbonate was added and the O_2 tension was brought to 1 % by adding measured amounts of air with a syringe. This O_2 tension was found to be optimal under the conditions described here.

Degradation of Glucose Samples. Glucose was derived from starch by acid hydrolysis. The material was purified by chromatography on Whatman No. 3MM paper in *n*-butyl alcohol/ethyl alcohol/water (52:32:16). After elution from the paper and addition of carrier, the glucose was degraded by fermentation with *Leuconostoc mesenteroides* (6).

Results

The kinetics of CO_2 assimilation by photosynthesis, photoreduction, and the oxyhydrogen reaction are shown in figure 1. All 3 reactions were found to be linear with time. The data are corrected for dark fixation in the absence of O_2 in the case of the oxyhydrogen reaction. In this experiment the rate of CO_2 fixation was 50 µmoles fixed per hour per mg chlorophyll by photosynthesis, 25 µmoles fixed per hour per mg chlorophyll by photoreduction and 3 µmoles per hour per mg chlorophyll by the oxyhydrogen reaction. The kinetics of the oxyhydrogen reaction are compared with the kinetics of ordinary dark fixation in figure 2. Fixation remained essentially linear for 1 hour.

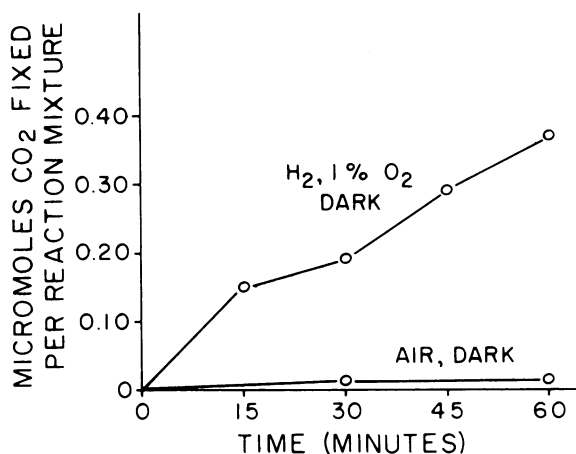

FIG. 2. The kinetics of $^{14}CO_2$ fixation by the oxyhydrogen reaction. Each flask contained 6.0 mg dry weight of cells. Radioactive bicarbonate was added at time zero. The upper curve shows the kinetics of incorporation in an atmosphere of hydrogen and 1 % oxygen; the lower curve was measured in air.

Fractionation of the products of CO_2 assimilation indicated that a substantial fraction of the isotope was being incorporated into polysaccharide during photosynthesis, the oxyhydrogen reaction and photoreduction. For each of these reactions about 20 to 30 % of the isotope incorporated by the cells could be solubilized by acid hydrolysis and recovered as free glucose after chromatography. The intramolecular distributions of isotope from 1 experiment are recorded in table I. In all cases there is substantial spreading of the label from the 3 to 4 positions to the 1 to 2 and 5 to 6 positions. In all cases the glucose molecules are approaching uniform labeling. Marked asymmetric labeling of the 3 and 4 positions (7) can be seen in the case of the oxyhydrogen reaction, especially in the 15 minute sample.

A control experiment in which the carbohydrate formed during dark fixation under air was isolated and degraded gave no evidence for extensive spreading for the label from the 3 to 4 positions of the

Table I. *Distribution of ^{14}C in Glucose Formed During Photosynthesis, Photoreduction, the Oxyhydrogen Reaction and Dark Fixation by* Scenedesmus

Scenedesmus cells in log phase of growth were harvested, washed twice with 5 mM phosphate buffer (pH 7.0) and resuspended in the same buffer containing 5 mM $MgCl_2$. The cells were incubated in Warburg flasks at a concentration of 7 mg dry weight of cellular material in a total volume of 2 ml. Two to 4 replicates of each reaction mixture were prepared to insure that enough material was available for degradation. For measurements of photoreduction and the oxyhydrogen reaction, the flasks were gassed with purified H_2 gas for 15 minutes and adapted in the dark for at least 8 hours. After adaptation, $NaH^{14}CO_3$ was added with a syringe through a serum cap in the sidearm of the Warburg flask (10 μmoles, 2–10 μcuries/μmole). The flasks were incubated for the indicated times. The reactions were terminated with boiling 95 % ethyl alcohol. The purified sugars in each case contained at least 10^5 cpm. The figures for the C-4 of each degraded sugar represent at least 300 cpm and in most cases represent at least 1000 cpm. In this experiment the amount of CO_2 fixed in 2 minutes of photosynthesis corresponded to 6 minutes of fixation by photoreduction and 30 minutes of the oxyhydrogen reaction.

Source of glucose		C-1	C-2	C-3	C-4	C-5	C-6
Photosynthesis (2 min)	% of C-4	83	80	96	100	40	60
	% of total	18	17	21	22	9	13
Photoreduction (6 min)	% of C-4	52	28	100	100	37	37
	% of total	15	8	28	28	10	10
Oxyhydrogen reaction (15 min)	% of C-4	39	29	67	100	22	22
	% of total	14	10	24	36	8	8
Oxyhydrogen reaction (30 min)	% of C-4	55	45	82	100	[1]	34
	% of total	17	14	26	32	...	11
Dark fixation (60 min)	% of C-4	6	4	88	100	<2	<2
	% of total	3	2	42	48	<1	<1

[1] Sample lost.

glucose (table I). It was not possible to equate the amount of carbon fixed in this experiment to that fixed in the other experiments because the time course of dark fixation reached a plateau after about 30 minutes (fig 2).

Discussion

The evidence presented here strongly indicates the participation of the reductive pentose phosphate cycle in the fixation of CO_2 by *Scenedesmus* through the process of photoreduction. The pattern of labeling of glucose is very similar to photosynthetic glucose labeling and is most easily interpreted as indicating the operation of the cycle. The experiments were carried out under conditions where photosynthesis was totally inhibited. The appearance of radioactivity in carbon atoms 5 to 6 is held to be especially important since the reductive pentose phosphate cycle is considered to be the only pathway under our conditions which will lead to the incorporation of carbon from CO_2 into these atoms.

Since photoreduction can take place in the presence of DCMU, the process provides some of the strongest evidence for the presence of *in vivo* cyclic photophosphorylation, possibly involving a ferredoxin catalyzed cycle (13). The possibility cannot be excluded that hydrogen may act as an internal electron donor; however, no evidence exists to support this suggestion. Hydrogen can presumably reduce TPN in a series of dark enzymatic steps to provide the TPNH for photoreduction.

The oxyhydrogen reaction takes place entirely in the dark. Reduced pyridine nucleotide is presumably generated through enzymatic steps similar to those suggested for the production of reduced pyridine nucleotide in photoreduction. Mitochondrial oxidation of some of the reduced pyridine nucleotide could provide ATP for the process. We would suggest that ATP generated outside the chloroplast might be used inside the chloroplast. The inhibition data of Gingras, Goldsby, and Calvin (8) are consistent with this suggestion.

Both photoreduction and the oxyhydrogen reaction have been shown to be CO_2 fixation reactions involving the participation of the reductive pentose phosphate cycle and leading to the net assimilation of carbon in the cell. The inability of either of these processes to support the growth of *Scenedesmus* must remain largely unexplained.

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