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Anaerobic Formation of D-Lactate and Partial Purification and Characterization of a Pyruvate Reductase from *Chlamydomonas reinhardtii*[1]

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ABSTRACT

D-Lactate accumulation in *Chlamydomonas reinhardtii* was dependent on anaerobic conditions. As much as 50% of the ^{14}C after 2 minutes of photosynthetic $^{14}CO_2$ fixation moved into D-lactate from sugar phosphates if the cells became anaerobic for short time periods. No lactate accumulated in the dark until the O_2 concentration decreased to less than 0.1%. Lactate was determined to be of the D-configuration using stereospecific lactate dehydrogenases. D-Lactate produced anaerobically by algae grown on 5% CO_2 was only slowly metabolized aerobically in the light or dark, and in the dark, only a trace of the lactate was excreted.

A pyruvate reductase (D-lactate: diphosphopyridine nucleotide oxidoreductase, EC 1.1.1.28) was partially purified 47-fold from *Chlamydomonas*. Because this enzyme catalyzes an essentially irreversible reaction in the direction of pyruvate reduction, it is considered to be a pyruvate reductase. The reductase activity in extracts of *Chlamydomonas* was 30 micromoles per hour per milligram chlorophyll. For the partially purified enzyme, the apparent K_m (pyruvate) was 0.5 millimolar, and the pH optimum was 7.0. Studies with cycloheximide and chloramphenicol indicated that the enzyme was constitutive in aerobic cells. Potassium phosphate stimulated the reductase, and high salt and dithiothreitol were required for stability. The enzyme demonstrated substrate inhibition and was inhibited by ATP. Pyruvate reductase was separated from a hydroxypyruvate reductase by gel filtration chromatography, indicating the presence of separate reductases for these two substrates in *Chlamydomonas*.

The anaerobic production of D-lactate by unicellular green algae was first noted in *Chlorella* by Warburg (31). D-Lactate has also been observed in various other algae (11, 14). Tolbert *et al.* (29) showed that if *Chlamydomonas* were pelleted by rapid centrifugation after 2 min of photosynthesis, 51% of the newly fixed $^{14}CO_2$ was found in lactate in cells which had been grown with air enriched with 5% CO_2 and 26% in cells which had been grown with air levels of CO_2. Despite the evidence for the formation of D-lactate, the metabolic role of this compound in algae is not known.

Studies of anaerobic metabolism in algae have concentrated on quantitative analyses of starch fermentation products formed after several hours of anaerobiosis. The results indicate that various fermentation pathways exist in algae, but most unicellular algae undergo some type of mixed acid fermentation (7, 16). The long-term (6 h) anaerobic starch fermentation products of *Chlamydomonas reinhardtii* include formate, acetate, ethanol, and smaller amounts of CO_2 and H_2. D-Lactate was observed in these long-term studies only at extreme pH values or when the pathway of formate formation was inhibited by hypophosphite (16). However, little is known as to what metabolic changes occur during the initial minutes of anaerobic conditions. The present report demonstrates the rapid accumulation of D-lactate in *C. reinhardtii* during short-term anaerobic experiments of a few seconds to 30 min. Because D-lactate accumulation has not been noted in 6-h periods of anaerobiosis, the possible involvement of D-lactate metabolism during anaerobiosis was also investigated. A preliminary accounting of the studies of D-lactate metabolism in *Chlamydomonas* has been published (12).

D-Lactate is found in yeast (9) and in bacteria. In *E. Coli*, D-lactate serves as an energy source for membrane transport (13). D-Lactate is produced in *E. coli* by a cytosolic D-LDH[2] and is subsequently oxidized by a membrane-bound D-LDH which can donate electrons to a bacterial membrane electron transport chain (13, 26). In unicellular algae, D-lactate can be oxidized slowly by a dehydrogenase, which has generally been named glycolate dehydrogenase (18), and is associated with the mitochondrial membrane (1). The electron acceptor or cofactors for this dehydrogenase are unknown, but there is some evidence that it is coupled to the mitochondrial electron transport system (3, 20). An enzyme activity which reduces pyruvate has been reported for various green algae (10, 12, 14, 24) and in this paper its partial purification and characterization are further reported. This enzyme catalyzes an essentially irreversible reaction in the direction of pyruvate reduction; this irreversibility is also characteristic of the cytosolic D-LDH from *E. coli* (27). To relate the enzyme nomenclature with the *in situ* activity, the soluble enzyme in *Chlamydomonas* will be called pyruvate reductase and the mitochondrial activity as D-lactate or glycolate dehydrogenase.

MATERIALS AND METHODS

Algae. *Chlamydomonas reinhardtii* (Dang.) UTEX 90, *Anabaena variabilis* UTEX B 337, and *Synechococcus leopoliensis* UTEX 625 were from the R.C. Starr collection at the University of Texas, Austin. The F-60 mutant of *C. reinhardtii* was a gift from Dr. R.K. Togasaki, Indiana University.

Wildtype *Chlamydomonas* were grown at 23 to 25°C in a high phosphate medium with $(NH_4)NO_3$ at pH 6.8 (19). The F-60 mutant was grown at pH 7.3 in Tris-acetate-phosphate medium (8). The blue-green algae were grown at pH 8.5 in the medium

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[2] Abbreviations; LDH, lactate dehydrogenase; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Epps, *N*-(2-hydroxyethyl)piperazine-*N'*-2-propane sulfonic acid.

C of Kratz and Myers (15) with the addition of 10 mM sodium *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid. The cells were aerated with either air (0.03% CO_2) or with air enriched with 5% CO_2 in flat Erbach flasks and were continuously illuminated under 125 $\mu E \cdot m^{-2} \cdot s^{-1}$ from cool white fluorescent tubes.

Cells were harvested after 3 to 4 d, near the end of the log phase of growth by centrifuging at 900*g* and 4°C for 5 min. The cells were washed once by resuspension in distilled H_2O and were recentrifuged in tared Corex tubes at 12,000*g* for 10 min. The algal wet weight was recorded and the pelleted cells were resuspended for photosynthetic experiments in 3 mM K-phosphate, pH 7.5, or 25 mM Hepes at pH 7.5. The algal suspensions were stored on ice and all photosynthetic experiments were done within 1 h after harvest. Chl concentration was measured spectrophotometrically after acetone extraction.

^{14}C-Labeling of Algae. After harvesting, suspensions of 2% or 5% (w/v) *Chlamydomonas* cells were prepared in 3 mM phosphate buffer, pH 7.5, for photosynthetic experiments. An aliquot of this suspension was placed in a 2-cm diameter glass vial with a flat bottom so that the light path through the solution was 2 cm. This reaction vial was held in a plastic holder in a circulating water bath at 25°C and the contents stirred by a magnetic bar. The samples were preilluminated for 2 min by 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ of light from a projector. After this light adaptation, [^{14}C]$NaHCO_3$ was added to the suspension to a final concentration of 1 mM. When illumination times were longer than 5 min, more [^{14}C]$NaHCO_3$ was added to 1 mM at 5-min intervals to ensure a near saturating level of inorganic carbon for the cells. Aliquots were removed at various time points and CO_2 fixation stopped by one of two methods. In one procedure designed to assay the cellular components and the surrounding medium separately, the cells were immediately separated from the suspension by centrifugation for 5 s in an Eppendorf microfuge. The supernatant was removed with a Pasteur pipette and added to an equal volume of methanol. The pellet was resuspended in 50% methanol to a volume equal to the original volume of the aliquot in order to terminate enzymic reactions and to solubilize the cellular contents. In the other procedure, an aliquot of the photosynthesizing cells was removed and directly added to methanol and mixed, without cell separation from the medium.

To count radioactivity in these samples, 50 μl were added to 0.45 ml of 0.5 N acetic acid and allowed to sit for 3 h to release unfixed ^{14}C. Scintillation fluid (4.5 ml) was then added and the nonvolatile radioactivity was determined in a liquid scintillation spectrometer.

Two-Dimensional Paper Chromatography. Aliquots of the cell and supernatant samples from ^{14}C labeling experiments were chromatographed for product identification (2). The radioactive compounds were located by exposure to Kodak XAR-5 X-ray film for about 3 weeks. To determine the percentage of label in each compound, the spots were cut out of the chromatograms and into approximately 5 mm^2 pieces and placed into scintillation vials. Compounds were eluted off the paper by adding 1.5 ml of water to each vial, followed by gentle mixing for about 2 h. Scintillation fluid was then added and the samples were mixed and the radioactivity counted.

Lactate was identified by cochromatography with a [^{14}C]-lactate standard and with an unlabeled lactate standard. The unlabeled carrier was located on the chromatograms by spraying with a 0.04% solution of bromocresol green in ethanol adjusted to pH 7.0. Lactate was also identified by GC-MS of the trimethylsilyl derivatives of the organic acid fraction from the algal cells. In this case, identification was based upon both the retention index and mass spectrum.

Products of $^{14}CO_2$ Fixation by *Chlamydomonas* under Decreasing O_2 Concentrations. Cells grown with 5% CO_2 were harvested and used to prepare a 5% suspension (w/v) at pH 7.5 in a buffer of 3 mM K-phosphate or 25 mM Hepes (approximately 100–120 μg Chl/ml). An aliquot of this suspension was placed in a Rank Brothers (Bottingham-Shire) O_2 electrode chamber for continuous monitoring of the O_2 concentration. The cells were illuminated with 800 $\mu E \cdot m^{-2} \cdot s^{-1}$ of light from a projector and [^{14}C]-$NaHCO_3$ (0.25 mCi/mmol) was added to a final concentration of 1 mM. The cells were allowed to photosynthesize for 2 to 4 min and then the light was turned off and the chamber covered with aluminum foil. In the dark, the system became anaerobic within 5 to 10 min depending on how high the O_2 concentration had reached during photosynthesis. Throughout the experiment, 200 μl samples were taken and immediately added to 500 μl of methanol to terminate reactions. After each aliquot was removed, the chamber cap was adjusted to prevent formation of air bubbles in the system. The samples were concentrated by evaporation to about 100 μl and analyzed by paper chromatography.

Determination of the Stereospecific Configuration of the Lactate Produced by *C. reinhardtii*. Cells were grown with 5% CO_2, harvested, and resuspended in 3 mM K-phosphate, pH 7.5, for photosynthesis. [^{14}C]$NaHCO_3$ was added to 1 mM at 0 and 15 min. After 30 min, the cells were pelleted by centrifugation at 7700*g* for 5 min at room temperature to produce anaerobic conditions. Reactions were terminated by resuspending the pelleted cells in 5.8 N $HClO_4$. The sample was neutralized with KOH and centrifuged. Bacterial NAD:D-LDH (*Lactobacillus leichmanii*) and porcine heart L-LDH were used to determine the presence of either D- or L-lactate in the resulting supernatant sample according to the method of Gawehn and Bergmeyer (6). The reaction mixture contained 453 mM glycine, 352 mM hydrazine hydrochloride, and 2.47 mM NAD. An aliquot of the supernatant (1.1 ml) was added to 3.3 ml of assay buffer, and 0.5 ml of this was used for each assay. After mixing, the initial absorbance at 340 nm was measured. The reaction was initiated with the addition of D-LDH or L-LDH to a concentration of 60 units/ml (units were μmol of NAD reduced/min at 25°C). After 90 min at 25°C, the *A* at 340 nm was compared with a control which contained no algal sample. No further increase in absorbance was detected after this 90-min time period.

Formation of D-Lactate by the Pyruvate Reductase. An enzyme fraction was prepared from 5% CO_2-grown *Chlamydomonas* by extraction in 50 mM K-phosphate, pH 7.0., containing 3% Triton, 1 mM DTT, and 5 mM EDTA for 40 min, followed by centrifugation. The supernatant was used to prepare a 45 to 80% saturated $(NH_4)_2SO_4$ fraction. The protein was resuspended in 3 mM K-phosphate at pH 6.2 and used for pyruvate reductase assays. No enzyme fraction was added to the blank, and the control assay was run without pyruvate. The assay contained K-phosphate at pH 7.0, 0.12 mM NADH, and 30 μl enzyme fraction. The reaction was initiated with 2 mM pyruvate, incubated for 90 min at 25°C, and then terminated by protein precipitation with 5.8 N $HClO_4$. After neutralization with KOH and centrifugation to pellet the protein and $KClO_4$, the supernatant samples were used as substrates in assays with the stereospecific lactate dehydrogenases, as described above, to determine the configuration of the lactate produced in the pyruvate reductase reaction.

Enzyme Assays. NADH-dependent pyruvate reductase was measured by following the decrease in *A* at 340 nm. Units of activity were μmol of NADH oxidized per min at 25°C. The 1-ml assay mixture contained 50 mM K-phosphate at pH 6.2 or 7.0, 0.12 mM NADH, and 2 mM potassium pyruvate. Enzyme fraction (20–30 μl) was added to initiate the reaction. Assays for hydroxypyruvate and glyoxylate reductase activities used 2 mM hydroxypyruvate or 10 mM glyoxylate as substrates in place of pyruvate. No NADH was oxidized in a control cuvette contain-

ing all components except substrate. The reverse reaction in the direction of lactate oxidation was run at pH 9.2 in 50 mM sodium pyrophosphate or at pH 9.4 in 25 mM Ches with 2.7 mM NAD, enzyme fraction, and 50 mM D- or L-lactate (Li salt). All assays were performed in duplicate or triplicate and average values are reported.

Partial Purification of the Pyruvate Reductase from *Chlamydomonas*. Protein solutions were maintained at 4°C throughout the purification steps. Protein concentrations were determined according to the Lowry procedure.

To prepare cell free extracts, harvested cells were resuspended in 3 volumes (w/v) of 50 mM K-phosphate or 25 mM Mops, pH 7.0, containing 1 mM DTT and 5 mM EDTA. The cells were lysed by two passes through a Yeda press (1,500 p.s.i. of N_2 gas for 5 min). The solution was centrifuged at 15,000*g* for 15 min to remove unbroken cells and membrane material. All of the pyruvate reductase activity was in the resulting supernatant. In an alternate procedure, the harvested cells were resuspended in 3 volumes (w/v) of 0.3% sodium deoxycholate or in 50 mM K-phosphate at pH 7.0, containing 3% Triton X-100, 1 mM DTT, and 5 mM EDTA. The mixture was stirred for 40 min. Cellular debris was removed by centrifugation at 12,000*g* for 10 min and the supernatant decanted and used for subsequent assays. No activity was found in the pellet fraction.

Saturated $(NH_4)_2SO_4$, adjusted to pH 7.0 with KOH, was added to the supernatant from the Yeda press extraction (0.67 ml/ml extract) to provide a final concentration of 40% saturation. After 30 min, the suspension was centrifuged at 17,000*g* for 15 min and the precipitate discarded. A saturated solution of $(NH_4)_2SO_4$ was slowly added to the supernatant to 65% saturation (0.71 ml/ml supernatant). After 60 min, the suspension was centrifuged at 17,000*g* for 15 min and the supernatant was discarded. The pellet was dissolved in 25 mM K-phosphate at pH 7.0, containing 1 mM DTT and 5 mM EDTA. This phosphate buffer was used throughout the purification procedures unless otherwise noted.

The dissolved pellet from the previous step was applied to a Sephadex G-25 column (2.5 × 4.3 cm) which was equilibrated and eluted with the K-phosphate buffer. Fractions containing pyruvate reductase activity eluted in the void volume from the column and were pooled. The pooled fractions were loaded onto a column of Affi-Gel Blue, 100 to 200 mesh (2.0 × 5.0 cm), which had been equilibrated with the K-phosphate buffer. The column was washed with this buffer until the A_{280} of the eluate was less than 0.03. The enzyme was eluted with a linear gradient of 0 to 1 M KCl in 50 mM K-phosphate, pH 7.0, containing 1 mM DTT and 5 mM EDTA. Pyruvate and hydroxypyruvate reductase activities eluted in a broad peak with the bulk of the protein at approximately 0.6 to 0.7 M KCl.

The fractions containing the reductase activities were pooled and concentrated to approximately 25% of the original volume with a 50-ml Amicon concentrator using a Diaflo PM-30 ultrafiltration membrane (43 mm) and 50 p.s.i. of N_2 gas. The concentrated fraction was loaded onto a Sephacryl S-300 (superfine) column (2.5 × 88 cm) which was equilibrated and eluted with the K-phosphate buffer containing 0.2 M KCl. The pyruvate reductase activity eluted after the bulk of the protein and before the main peak of hydroxypyruvate or glyoxylate reductase activity. The fractions containing the pyruvate reductase activity were pooled and used for subsequent characterization.

Materials. [^{14}C]$NaHCO_3$ was from New England Nuclear. Enzyme grade $(NH_4)_2SO_4$ was from the Schwarz/Mann Inc. DEAE-cellulose (DE-52), cellulose phosphate (P-11), and carboxymethyl cellulose (CM-23) were obtained from Whatman Chemical Separation Ltd. Affi-Gel Blue was from BioRad and Sephacryl S-300 (superfine) was from Pharmacia. Matrix Gel Red A and Diaflo PM-30 ultrafiltration membranes (43 mm) were from the Amicon corporation. All other biochemicals, buffers, and enzymes were purchased from Sigma Chemical Company. Common laboratory chemicals were of reagent grade and solutions were prepared in deionized, distilled H_2O.

RESULTS

Anaerobic Formation of D-Lactate. The distribution of ^{14}C among the methanol-soluble, nonvolatile products of *C. reinhardtii* was examined at decreasing O_2 concentrations as monitored in an O_2 electrode (Fig. 1). The O_2 concentration decreased due to O_2 uptake by the algae from dark respiration. [^{14}C]Lactate did not begin to significantly accumulate in the cells until the O_2 concentration was less than 0.9 μM (approximately 0.1%). The amount of lactate subsequently increased with time under anaerobic conditions for at least 20 min. By then, 15% of the fixed ^{14}C was in lactate. In confirming experiments, aliquots of photosynthesizing cells were removed from the light and incubated in 1.5-ml microfuge tubes with a large air space and with frequent stirring to maintain aerobic conditions in the dark for time periods of 10 s to 1 h, before adding them directly to methanol to stop reactions. No [^{14}C]lactate accumulated aerobically in the dark (data not shown).

In other experiments, samples were removed directly from a suspension of photosynthesizing cells after short times (10 s to 2 min) with [^{14}C]HCO_3 and made anaerobic by rapidly centrifuging them into a tightly packed pellet in the dark. The percentage of the ^{14}C in lactate reached 51% during centrifugation after 10 s of photosynthesis (Table I). The cell suspension that was killed

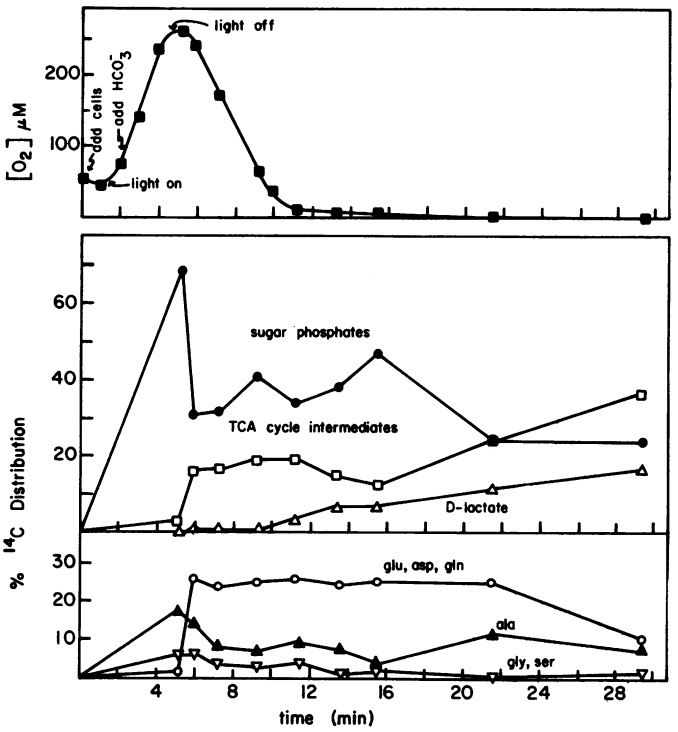

FIG. 1. Distribution of ^{14}C in methanol-soluble, nonvolatile products of *C. reinhardtii* under decreasing oxygen concentrations. Cells grown with 5% CO_2 were harvested and resuspended in 3 mM K-phosphate at pH 7.5 (114 μg Chl/ml). An aliquot was placed in an O_2 electrode chamber at time zero. [^{14}C]$NaHCO_3$ (0.25 mCi/mmol) was added to 1 mM final concentration and, after 5 min, the light was turned off and the chamber was covered with foil. Throughout the experiment, 200-μl samples were taken and immediately added to 500 μl of methanol to terminate reactions. The samples were concentrated and analyzed by paper chromatography.

Table I. *The Effect of Killing Techniques on the ^{14}C Labeled Products of C. reinhardtii*

Cells were allowed to photosynthesize in 3 mM potassium phosphate buffer, pH 7.5, with 1 mM [^{14}C]$NaHCO_3$ for 10 s. Reactions were then terminated by either adding the suspension of cells directly to methanol or by rapidly centrifuging the cells to separate them from the media and then resuspending them in methanol. Samples were concentrated and analyzed by paper chromatography.

Products	Method Used to Terminate Reaction	
	Methanol directly	Centrifugation, then methanol
	% ^{14}C	
Sugar phosphates	74	11
D-Lactate	0.1	51
Tricarboxylic acid cycle intermediates	3.6	15
Aspartate and glutamate	0.5	3.0
Alanine	0.9	7.0
Glycolate, glycine, and serine	0.0	0.0
Other	21	13

by direct addition to methanol without prior centrifugation contained no lactate. The [^{14}C]lactate was apparently derived from the newly labeled ^{14}C-sugar phosphate pools because the label in this fraction decreased dramatically during the centrifugation period.

Although [^{14}C]lactate was formed in both of these short-term experiments, the difference in the amount of [^{14}C]lactate accumulated may be explained in part by the difference in the time period to obtain anaerobic conditions. During the dark period in the experiment using the O_2 electrode, much of the label from the sugar phosphate pools immediately flowed into the tricarboxylic acid cycle intermediates (malate, succinate, citrate, and fumarate) and related amino acids (glutamate, aspartate, and glutamine). When the system became anaerobic, the sugar phosphate pool was further depleted as lactate began to accumulate in the cells. However, by this time in the dark period less ^{14}C-sugar phosphates were available to be converted to pyruvate and lactate via 3-PGA, as 45% of the original ^{14}C fixed was in the tricarboxylic acid cycle intermediates and related amino acids. This carbon could flow back to pyruvate and into lactate from malate by the malic enzyme reaction. Indeed, the percentage of ^{14}C in malate dropped slightly from 9% to 3% in the first 12 min of anaerobic conditions. However, this flow of carbon to lactate appears to be slower than that from the sugar phosphates to lactate via 3-P-glycerate and pyruvate, as evidenced by the large percentages of label seen in lactate in the experiment using rapid centrifugation in which the pelleted cells were presumably anaerobic for only 45 s before the reactions were terminated with methanol.

The anaerobic experiments performed in the O_2 electrode or by centrifugation were done with wildtype *Chlamydomonas* cells which had been grown photoautotrophically with 5% CO_2. Similar experiments were done with cells grown on air levels of CO_2 or in a Tris-acetate phosphate medium in the light, and with the acetate-requiring F-60 mutant of *Chlamydomonas*. In all cases, the cells produced lactate during short times under anaerobic conditions, which was formed from newly fixed ^{14}C, and all cells were shown to have levels of pyruvate reductase activity (30 μmol NADH oxidized/h·mg Chl) comparable to that of the wildtype cells grown photoautotrophically (see below). The blue-green algae, *A. variabilis* and *S. leopoliensis*, however, had only 2 to 4 μmol NADH oxidized/h·mg Chl of the NADH-dependent pyruvate reductase activity and no NADPH-dependent activity. Correspondingly, these blue-green algae accumulated only 1.1% of ^{14}C in lactate during anaerobic conditions.

When the CO_2-grown *Chlamydomonas* were returned to the light after lactate had accumulated anaerobically in the cells, very little turnover of the lactate was observed. The D-lactate remained in the cells and was not excreted as is glycolate. A small amount of the ^{14}C from lactate moved into the sugar phosphates after 1 h. If unlabeled $NaHCO_3$ was added as a chase, the specific activity of the label in lactate was not diluted out after several minutes in the light, as if lactate synthesis or turnover was not occurring aerobically. If the cells were returned to aerobic conditions in the dark after lactate accumulation, only a small amount of the label in lactate flowed into the tricarboxylic acid cycle, and only a trace of lactate, tricarboxylic acid cycle intermediates, and sugar phosphates leaked into the media. Preliminary data with air-grown *Chlamydomonas* indicate that the lactate which accumulated anaerobically in these cells turned over more rapidly than in the CO_2-grown algae. This might be expected since air-grown *Chlamydomonas* have higher levels of glycolate dehydrogenase (and hence D-LDH activity) than do cells grown under high levels of CO_2 (18).

Determination of the Configuration of the Lactate Produced by *Chlamydomonas*. The lactate produced by the algae *in vivo* was oxidized preferentially by the stereospecific D-LDH (Table IIa). It was also shown that the lactate formed during assays of the pyruvate reductase reacted preferentially with the D-LDH (Table IIb). The reason for the lack of apparent absolute specificity of the sterospecific LDHs for the appropriate isomer of

Table II. *Determination of the Stereospecific Configuration of the Lactate Produced by C. reinhardtii (a) and Formation of D-Lactate by the Pyruvate Reductase from Chlamydomonas (b)*

Sample preparation and assays were performed as described in "Materials and Methods."

	Increase in A_{340}		D-LDH Activity / L-LDH Activity
	D-LDH	L-LDH	
			ratio
(a)			
100 μmol D-lactate	1.062	0.260	4.08
100 μmol L-lactate	0.058	1.048	0.06
Algal sample	0.418	0.094	4.45
(b)			
Blank (no enzyme fraction)	0	0	
Control (no pyruvate)	0	0.018	
Product of pyruvate reductase assay	0.150	0.033	4.54

Table III. *Purification Procedure for the Pyruvate Reductase from C. reinhardtii*

Procedures are described in "Materials and Methods."

	Specific Activity	Purification	Yield
	μmol/min·mg protein	*-fold*	%
Yeda press extract	0.015	1.0	100
Pellet from 40–65% saturated $(NH_4)_2SO_4$ precipitation	0.050	3.3	86
Eluate from G-25 column	0.060	4.0	89
Affi-Gel Blue affinity column and concentration through Amicon filter PM-30	0.22	15	66
Eluate from Sephacryl S-300 column	0.71	47	68

lactate (Table IIa) is uncertain. The D-lactate used reportedly contained 0.01% of the L-isomer (Sigma) which does not account for the 20% contamination observed. Also, activity (18%) was observed with the algal samples and the L-LDH. No contamination of the *Chlamydomonas* was observed when aliquots of the culture were inoculated on agar plates supplemented with acetate and algal medium.

Partial Purification of the NADH:Pyruvate Reductase from *Chlamydomonas*. The steps used to partially purify the pyruvate reductase from *C. reinhardtii* are detailed "Materials and Methods" and a summary of the procedure is presented in Table III. After cell breakage through a Yeda press, the crude enzyme extract was used to prepare a $(NH_4)_2SO_4$ cut of 40 to 65% saturation. $(NH_4)_2SO_4$ inhibited the pyruvate reductase activity, but this inhibition was reversed by desalting the enzyme fraction through a Sephadex G-25 column. The enzyme was not stable in $(NH_4)_2SO_4$ and rapid desalting resulted in a higher yield of activity.

Before chromatography on an Affi-Gel Blue affinity column, the pyruvate reductase activity was not stable at 4°C. However, after elution from the dye-ligand column with a KCl gradient, the preparation was stable and retained 70% of the original activity after storage at 4°C in phosphate buffer, pH 7.0, with 0.4 to 0.6 M KCl for 1 month. Dialysis of this fraction against 25 mM K-phosphate buffer at pH 7.0 after elution from the dye-ligand column resulted in a 50% decrease in the pyruvate reductase activity within a few hours, suggesting that the high concentration of salt was necessary to stabilize the pyruvate reductase.

The last step towards purification of the enzyme was gel filtration chromatography on a Sephacryl S-300 column (Fig. 2). KCl (0.2 M) was included in the elution buffer, since high salt was apparently required for enzyme stability. This step was the first in which the pyruvate reductase activity was separated from the main peak of the hydroxypyruvate reductase activity. Previously, Gruber *et al.* (10) concluded that separate reductases for pyruvate and hydroxypyruvate existed in *Chlorella* from studies based on differences in enzyme stability. In *Chlamydomonas*, there are also similar differences in the stability of these two enzymes. For example, 50% of the original hydroxypyruvate reductase activity in a crude extract remained after 52 h of storage at 4°C, whereas the pyruvate reductase activity in the same extract declined to only 13% of the original activity. Consistent with hydroxypyruvate reductase from C_3 plants (28), this enzyme in *Chlamydomonas* catalyzes the reduction of both hydroxypyruvate and glyoxylate; however, the apparent K_m (glyoxylate) for this activity in crude extracts from *Chlamydomonas* was unphysiologically high at 8 mM.

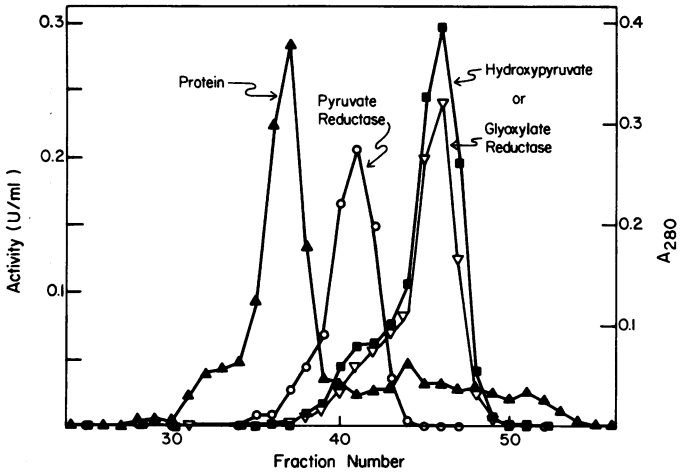

FIG. 2. Separation of the pyruvate reductase from the hydroxypyruvate and glyoxylate reductase activities of *C. reinhardtii* by elution from a sephacryl S-300 column. The elution buffer was 25 mM K-phosphate at pH 7.0 containing 0.2 M KCl, 1 mM DTT, and 5 mM EDTA. When assaying the fractions, 2 mM pyruvate, 2 mM hydroxypyruvate, or 10 mM glyoxylate were used. Protein (▲), pyruvate reductase activity (○), hydroxypyruvate reductase (■), glyoxylate reductase (▽).

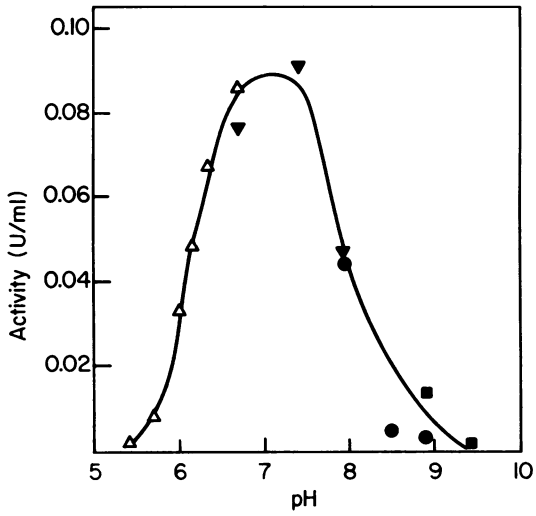

FIG. 3. pH Profile for the partially purified pyruvate reductase from *C. reinhardtii*. Assays were run as described in "Materials and Methods" with 25 mM buffers of either Mes (△), Mops (▼), Epps (●), or Ches (■).

Some hydroxypyruvate reductase activity coeluted with the peak of pyruvate reductase activity (Fig. 2). Since hydroxypyruvate is a good substrate for mammalian L-LDH which forms L-glycerate (17), it is possible that the algal pyruvate reductase could also use hydroxypyruvate. Because the product of the algal pyruvate reductase is D-lactate, the product from hydroxypyruvate reduction should be D-glycerate, which is the isomer found in algae and higher plants. The hydroxypyruvate reductases from higher plants (25) or from *Chlamydomonas* (data not shown) do not catalyze a dehydrogenase reaction with D-or L-lactate as a substrate and, in *Chlamydomonas*, no pyruvate reductase activity was associated with the main peak of hydroxypyruvate reductase activity after gel filtration chromatography (Fig. 2).

Further attempts to purify the pyruvate reductase from *Chlamydomonas* have, as of yet, been unsuccessful. The enzyme does not bind to DEAE-cellulose or DEAE-Sephadex (pH 6.2–8.0), TEAE-cellulose (pH 7.0–8.0), calcium-phosphate gel (pH 6.4 or 7.0), cellulose phosphate (pH 7.0), carboxymethyl cellulose (pH 7.0), or oxamate-agarose or red dye-ligand affinity columns. These various columns were tested with crude algal extracts, with enzyme fraction after the $(NH_4)_2SO_4$ cut and desalting, and with the pooled fractions after elution from the Affi-gel blue column. Elution of the reductase activities from the Affi-gel blue column with NADH or NADPH (1 mM) resulted in poor recoveries, and the activity only slowly eluted from the column in a volume of several hundred ml.

Properties of the Partially Purified Pyruvate Reductase from *Chlamydomonas*. The pH optimum for the activity in the direction of pyruvate reduction was maximal around pH 7.0 (Fig. 3). At pH 7.0, the reductase activity in extracts of *Chlamydomonas* was 30 ± 4 (n = 4) μmol NADH oxidized/h·mg Chl, which is to be compared with the maximal *Chlamydomonas* photosynthetic rate of approximately 120 to 160 μmol CO_2 fixed/h·mg Chl. The reductase activity was dependent on NADH; no activity was observed with equivalent amounts of NADPH.

The activity in the direction of D-lactate oxidation was tested in the pH range between 6.2 and 9.6 and in all cases was extremely low. A maximum rate of only 1 to 3% of the pyruvate

reduction rate was observed at pH 9.4. No oxidation of L-lactate occurred. In *Chlorella*, D-lactate oxidation has aso been reported to be low compared to the reductase reaction and the apparent K_m for D-lactate was 40 mM (10). Apparently, D-lactate oxidation by this enzyme does not occur under physiological conditions in algae and, therefore, it seems appropriate to name the activity as a pyruvate reductase rather than a D-lactate dehydrogenase.

The apparent K_m (pyruvate) for the pyruvate reductase activity in the crude homogenate was 1.1 mM (Fig. 4b); however, after partial purification of the enzyme, this value was 0.5 ± 0.1 mM (Fig. 4a). The higher apparent K_m (pyruvate) for activity in crude extracts suggests that there is an inhibitor(s) of the enzyme in the algal extract. Further evidence for this was that the pyruvate reductase assay was not linear with respect to the volume of crude extract added; activity was inhibited if more than 50 μl of extract were included in the assay. However, after the enzyme had been partially purified, the assay was linear with respect to the volume of enzyme fraction added.

Apparent substrate inhibition occurred at pyruvate concentrations above 2 mM for pyruvate reductase in crude homogenates (Fig. 4b). Similar inhibition was observed with the partially purified enzyme at pH values less than 6.8 (data not shown). Substrate inhibition by pyruvate has been observed for both D- and L-LDHs from other sources and has been attributed to the formation of an abortive ternary complex among enzyme, pyruvate, and NAD (5, 27).

Evidence for the Constitutive Nature of the Reductase. D-Lactate production was observed immediately after *Chlamydomonas* cells became anaerobic (Fig. 1). In the rapid centrifugation experiments, the algae were presumably anaerobic for no more than 45 s in the pellet before they were resuspended in methanol. However, lactate had already accumulated significantly in these cells (Table I). This evidence strongly suggests that the pyruvate reductase was present constitutively in the aerobic cells. However, during harvesting of the algae, the cells had been pelleted during centrifugation after two washings. During this time of temporary anaerobic conditions, synthesis of the pyruvate reductase could have been induced. To evaluate this possibility, *Chlamydomonas* cells in the log phase of growth were incubated with either 1 mM (281 μg/ml) cycloheximide or 2.3 mM (743 μg/ml) chloramphenicol for 30 min prior to harvesting. The cells were then harvested as usual and a crude extract prepared. In the extracts from both cycloheximide-and chloramphenicol-treated cells, the pyruvate reductase activity was the same as that from untreated cells. A recent paper of Roessler and Lien (23) showed that cycloheximide at a level of 15 μg/ml did enter *Chlamydomonas* cells and inhibited protein synthesis by greater than 98% as measured by [^{14}C]arginine incorporation into protein. They also showed that the induction of hydrogenase, which occurs under anaerobic conditions, was inhibited 70% by this level of cycloheximide, although chloroamphenicol at 500 μg/ml was less effective (23). This data indicates that the pyruvate reductase is constitutive in *C. reinhardtii*.

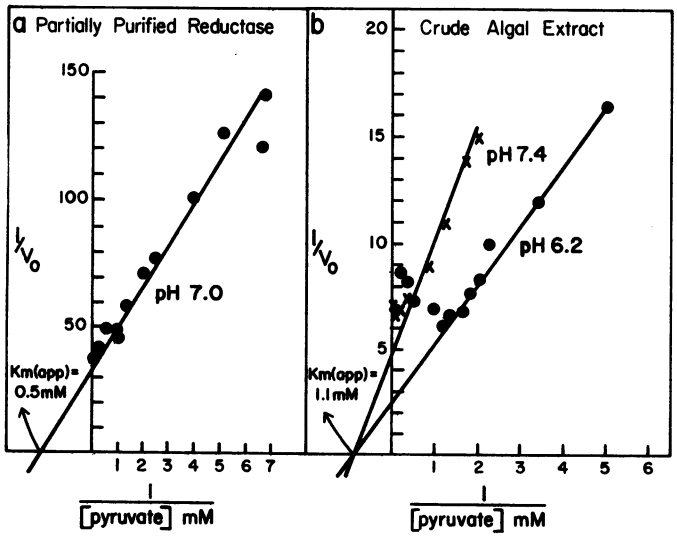

FIG. 4. Double reciprocal activity plots for the pyruvate reductase from *C. reinhardtii*. a, Partially purified enzyme at pH 7.0; b, activity in crude algal extract at pH 6.2 (●) or pH 7.4 (×). All assays were run in 50 mM K-phosphate at 25°C, and the NADH concentration was 0.12 mM.

Inhibition of the Pyruvate Reductase by Various Compounds. ATP inhibits the L-LDH from higher plants (4) or mammalian sources (5). The ATP inhibition was noncompetitive with respect to pyruvate and competitive with respect to NADH. The partially purified pyruvate reductase from *Chlamydomonas* was inhibited by ATP (Table IV) at K_m concentrations of pyruvate and saturating levels of NADH, and this inhibition was more severe at lower levels of NADH.

Although oxamate inhibits LDH from various sources (5), it inhibited the pyruvate reductase activity of *Chlamydomonas* only at concentrations greater than 1 mM (Table IV). Oxalate was also a poor inhibitor of this activity. A slight stimulation by cyanide was noted for the pyruvate reductase activity from *Chlamydomonas*. EDTA was also slightly stimulatory. *o*-Phenanthroline did not significantly inhibit the pyruvate reductase activity (Table IV). The LDH of yeast (9) and the mammalian D-α-hydroxy acid dehydrogenase (30) are zinc-requiring enzymes and are inhibited by oxalate, EDTA, *o*-phenanthroline, and cyanide, all of which reportedly interact with the essential zinc moiety. There is also a report of a zinc-dependent D-LDH in *Euglena gracilis* (21). None of these compounds inhibited the pyruvate reductase reported here, and therefore, it seems unlikely that this enzyme from *Chlamydomonas* requires a zinc moiety.

In *E. coli*, the enzymes involved in the synthesis and oxidation of D-lactate have labile sulfhydryl groups which must be in a reduced state for enzyme activity (13, 26). The pyruvate reductase activity from *Chlamydomonas* was rapidly lost in the ab-

Table IV. *Effects Of Various Compounds on the Pyruvate Reductase Activity from C. reinhardtii*

Assays were conducted using K_m concentrations of 0.5 mM pyruvate and saturating concentrations of 0.12 mM NADH except where indicated (*) only 60 μM NADH was used. The assay buffer was 100 mM K-phosphate at pH 7.0.

Compound	Concn.	Per Cent of Control Activity
	mM	%
ATP	1	73
	1*	57
	10	35
Oxamate	0.05	92
	1	73
	10	19
Oxalate	8	71
	21	9
KCN	1	110
o-Phenanthroline	4	78
EDTA	10	120

sence of DTT or β-mercaptoethanol. This activity could be partially restored with addition of sulfhydryl reagents (data not shown). Gruber *et al.* (10) showed that in crude extracts prepared from *Chlorella pyrenoidosa* without added sulfhydryl reagents, the pyruvate reductase activity decreased by greater than 90% within 90 min at 4°C.

Effect of Potassium Phosphate on the Pyruvate Reductase. Addition of K-phosphate to cell extracts of *Chlamydomonas* resulted in stimulation of the pyruvate reductase activity. If crude extracts were prepared in 25 mM Mops buffer at pH 7.3, only low activity was observed. This activity increased with addition of increasing amounts of K-phosphate up to 0.2 M, after which inhibition was observed (data not shown). The effect of this activation was even greater at lower pH values (Fig. 5). The specificity of this activation has not yet been determined.

DISCUSSION

The pyruvate reductase in *C. reinhardtii* catalyzes D-lactate formation in an irreversible reaction. The lactate only accumulates in the cells during anaerobic conditions, suggesting that the enzyme functions during anaerobic metabolism to reoxidize NADH. A second explanation for anaerobic lactate accumulation may be that if reoxidation of the lactate was coupled to mitochondrial respiration in *Chlamydomonas*, this process would not occur under anaerobic conditions. In *Chlamydomonas*, limited D-lactate or glycolate dehydrogenase activity is associated with the mitochondrial membrane (1, 18). The electron carriers for this dehydrogenase reaction are unknown, but may be coupled to mitochondrial respiration (3, 20). Once lactate was made by *Chlamydomonas* cells grown on 5% CO_2, it was turned over only very slowly in the light or dark. This is consistent

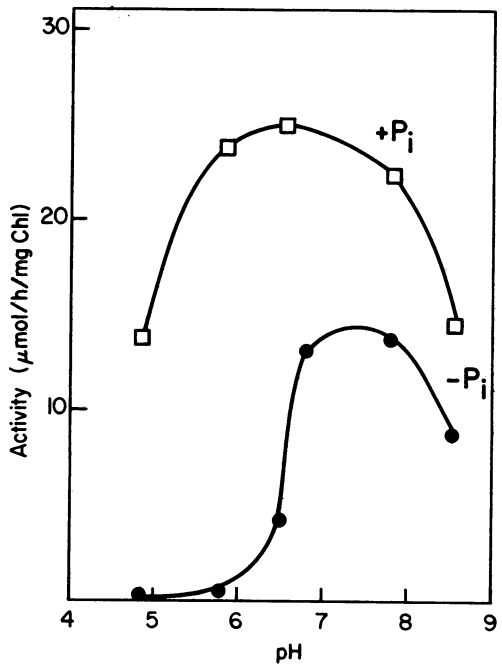

FIG. 5. Activation of the pyruvate reductase in crude extracts of *C. reinhardtii* by K-phosphate. The crude extract was prepared by breaking the cells in 3 volumes (w/v) of 50 mM Mops buffer, pH 7.0, containing 1 mM DTT and 5 mM EDTA. The suspension was centrifuged at 35,000 *g* for 15 min, and an aliquot of the resulting supernatant was added to 50 mM buffers: acetic acid (pH 4.9); Mes (pH 5.8, 6.4, 6.7); Mops (pH 7.8); Epps (pH 8.5). Phosphate, when included in the assays, was adjusted to the appropriate pH with KOH and added to give an approximate concentration of HPO_4^{2-} of 5 mM per assay. Endogenous phosphate was determined to be 40.2 μM P_i per assay.

with the pyruvate reductase being an essentially irreversible enzyme. Also, the algal glycolate or D-LDH is present only in trace levels in *Chlamydomonas* grown with high CO_2 (18). Air-grown cells have higher levels of the glycolate dehydrogenase or D-LDH and would be expected to have a more rapid rate of metabolism of the accumulated D-lactate. Preliminary experiments indicated that this was the case (DW Husic and NE Tolbert, unpublished data). The cellular effects of the accumulation of this lactate are unknown.

In *E. coli*, D-lactate is produced by a cytosolic D-LDH (13, 26) which, like the pyruvate reductase in *Chlamydomonas*, catalyzes an irreversible reaction in the direction of pyruvate reduction (27). The lactate is then oxidized by a membrane-bound D-LDH coupled to the bacterial electron transport system to provide energy for the uptake of nutrients (13).

In higher plants, accumulation of L-lactate is limited by aerobic conditions which promote NADH oxidation by the mitochondria. In the light, high levels of ATP and P-enolpyruvate may inhibit glycolysis and have been shown to inhibit the L-LDH of higher plants (4). Under anaerobic conditions (*i.e.* in germinating seeds and roots), L-lactate production can occur, subsequently causing a decrease in cellular pH (22). Since low cellular pH values are undesirable, overproduction of lactate under these conditions must also be controlled. This is accomplished in two ways (4). At slightly acidic pH values, NADH and ATP are potent and cooperative inhibitors of the L-LDH whereas at alkaline pH, ATP only slightly inhibits the enzyme and the inhibition is competitive with respect to NADH. Second, under anaerobic conditions, the decrease in pH approaches the pH optimum of pyruvate decarboxylase which decreases the amount of pyruvate available to be converted to lactate. Once the pyruvate decarboxylase is activated, the cells begin to produce ethanol instead of lactate (22). Because of this control mechanism and the ability to regenerate NAD without acid production, plants are able to tolerate anaerobic conditions for much longer time periods than animal cells.

It is not known if this same sort of control mechanism exists in unicellular green algae, but some differences have already been noted. Primarily, the D-isomer of lactate is formed as compared to L-lactate in higher plants. D-Lactate is found in other lower forms of life such as yeast and bacteria, suggesting an evolutionary difference for the formation of either D- or L-lactate in organisms. In *Chlamydomonas*, the main products of long term anaerobic fermentation are formate and acetate (7, 16) rather than ethanol which is produced in anaerobic tissue of many higher plants. Finally, the pyruvate reductase reported here catalyzes an essentially irreversible reaction in the direction of pyruvate reduction; whereas the L-LDH of higher plants catalyzes reversible reactions (4).

In long term studies (1–6 h) of the anaerobic fermentation products of starch in *Chlamydomonas*, significant D-lactate accumulation has not been previously observed (7, 16). The lactate we observed in *Chlamydomonas* cells was labeled with newly fixed ^{14}C from the sugar phosphate pools during short time periods under anaerobic conditions. It is suggested that during the initial minutes of anaerobic conditions, the glycolytic pathway is active to produce energy for the cell. Reducing equivalents (NADH) formed in the glycolytic reactions could be reoxidized by the pyruvate reductase. ATP is required to activate the glycosyl units of starch for further metabolism to occur (16). After initiation of starch fermentation, the subsequent metabolism is apparently switched from D-lactate formation to formate formation as was observed in the long-term anaerobic experiments. D-Lactate formation was only observed in long-term fermentation experiments when starch degradation to formate was blocked with hypophosphite, an inhibitor of the pyruvate-formate lyase reaction (16). How the flow of carbon may be switched from

initial D-lactate formation to the formate pathway with the onset of starch breakdown is unknown.

Since the pyruvate reductase activity is apparently constitutive in the algal cells, it is interesting to speculate on its regulation *in vivo*. Under aerobic conditions, NADH might be limiting and ATP levels high. It was shown that the enzyme was inhibited by ATP. Once the cells become anaerobic, the ATP levels should drop and NADH would become more readily available for pyruvate reduction as it can no longer be oxidized by the mitochondria. Another consideration is that during aerobic conditions, the high K_m (pyruvate) of the reductase may channel pyruvate towards other pathways. It is possible that, under anaerobic conditions, pyruvate levels may begin to increase and the pyruvate reductase would then become a significant reaction and lead to the observed accumulation of D-lactate. [^{14}C]Pyruvate is never a significant product of the photosynthetic labeling experiments (0–2% of the acid stable counts), and in the anaerobic experiments, pyruvate accounts for only 1 to 3% of the fixed ^{14}C. Finally, phosphate, as well as the redox state of possible essential sulfhydryls on the pyruvate reductase, may also play a role in the regulation of this activity.

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