This is to certify that the

dissertation entitled

ENZYMES OF GLYCOLATE METABOLISM

IN CHLAMYDOMONAS REINHARDTII

presented by

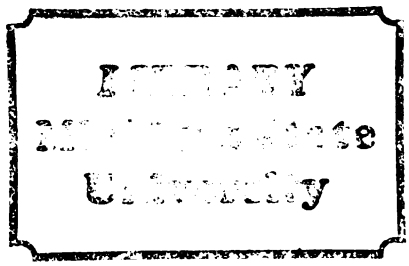
Nicola Leigh Selph

has been accepted towards fulfillment
of the requirements for

PhD degree in Biochemistry

N. E. Tolbert
Major professor

Date December 15, 1982



ENZYMES OF GLYCOLATE METABOLISM IN *CHLAMYDOMONAS REINHARDTII*

By

Nicola Leigh Selph

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1983

ABSTRACT

ENZYMES OF GLYCOLATE METABOLISM IN CHLAMYDOMONAS REINHARDTII

By

Nicola Leigh Selph

Several enzymes of glycolate metabolism (glycolate dehydrogenase, D-lactate dehydrogenase, P-glycolate phosphatase, and NADPH:glyoxylate reductase) were partially purified from the green alga, Chlamydomonas reinhardtii, and some of their properties studied.

Glycolate dehydrogenase was purified at least 25-fold, but lost activity upon further purification. It is known to be a mitochondrial enzyme and does not couple directly to O_2. It was inhibited by 2-hydroxy-3-butynoic acid, indicating a flavin component, as is the case for glycolate oxidase from higher plant peroxisomes. Glycolate dehydrogenase was not stimulated by FMN or FAD in vitro. It could be coupled to some tetrazolium salts.

P-Glycolate phosphatase was purified over 100-fold, but it lost activity upon further purification. Mg^{++} was required for activity. An ammonium sulfate fraction had a pH optimum of 8.7. A factor required for this activity was lost upon purification and the pH optimum shifted to 7.5-8.0.

Chlamydomonas reinhardtii cells, as well as spinach leaves, contained an enzyme which could be detected on native polyacrylamide gels with nitroblue tetrazolium as electron acceptor, and which oxidized

D-lactate and several other α-hydroxy acids, but not glycolate or L-lactate. The activity with D-lactate was different from glycolate dehydrogenase or glycolate oxidase, neither of which were strongly reactive on the acrylamide gels. In spinach leaves, the D-lactate:nitroblue tetrazolium activity was located in the mitochondria, and in both leaves and algae it may exist as two forms. The algae were found to photosynthetically produce much lactate from $^{14}CO_2$.

The algae contained an active NADPH:glyoxylate reductase, and an even more active NADH:hydroxypyruvate reductase. The NADPH:glyoxylate reductase was purified 65-fold, and had a pH optimum at 6.2, a K_m (glyoxylate) of 0.4 mM, and a K_m(NADPH) of 43 μM. It was competitively inhibited by aminooxyacetate with a K_i of 1 mM. It was also inhibited by oxamate, 50 mM bicarbonate, 12.5 mM glycolate, and high concentrations of both its substrates.

Algae grown on high CO_2 had less glycolate dehydrogenase and P-glycolate phosphatase than air grown cells. They had more NADPH: glyoxylate reductase than air-grown algae. Both air-grown and 5% CO_2-grown algae produced lactate. Both of these algal cultures excreted glycolate, and this excretion was greatly increased when the cells were treated with 1 mM aminooxyacetate.

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. N.E. Tolbert, for his aid and attention during the preparation of this dissertation, and for the financial support as a graduate assistant that I received during my many years at Michigan State University.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

AOA	(aminooxy)acetic acid
Bicine	N,N-bis[2-hydroxyethyl]glycine
BSA	bovine serum albumin
Ches	2-[N-cyclohexylamino]ethane sulfonic acid
DCPIP	2,6-dichlorophenol-indophenol
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HBA	2-hydroxy-3-butynoic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPMS	α-hydroxypyridine methane sulfonic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NBT	nitro blue tetrazolium
P_i	inorganic phosphate
PP_i	pyrophosphate
PMS	phenazine methosulfate
ribulose-P_2	ribulose bisphosphate
TEAE	tetraethylaminoethyl
Tris	Tris(hydroxymethyl)aminomethane

INTRODUCTION

Photorespiration occurs in all green plants as an accompaniment to photosynthesis. Some of the newly fixed carbon dioxide is immediately lost by this process which is different from mitochondrial respiration and electron transport. Photorespiration is defined as light-dependent O_2 uptake and concurrent CO_2 release, taking place in a photosynthetically active organism (1,2).

In plants, photorespiration occurs due to the reaction of ribulose-P_2 carboxylase/oxygenase, the principal CO_2-fixing enzyme of photosynthesis, with O_2 instead of CO_2. Ribulose-P_2 reacts with O_2 to produce 2-P-glycolate and 3-P-glycerate (Figure 1). The P-glycolate is dephosphorylated to glycolate by a specific phosphatase present in the chloroplasts. Glycolate is oxidized in the peroxisomes to glyoxylate by glycolate oxidase, which reduces O_2 to H_2O_2. The catalase present converts the H_2O_2 to $\frac{1}{2}O_2$ and H_2O. The glyoxylate is transaminated to form glycine, which is metabolized in mitochondria. Glycine is decarboxylated to CO_2, methylene-tetrahydrofolic acid, and NH_3, with energy conservation by the electron transport system. This reaction is responsible for the CO_2 release of photorespiration (Figure 1).

The methylene-tetrahydrofolic acid reacts with another glycine to form a serine. The serine may return to the peroxisome, where it

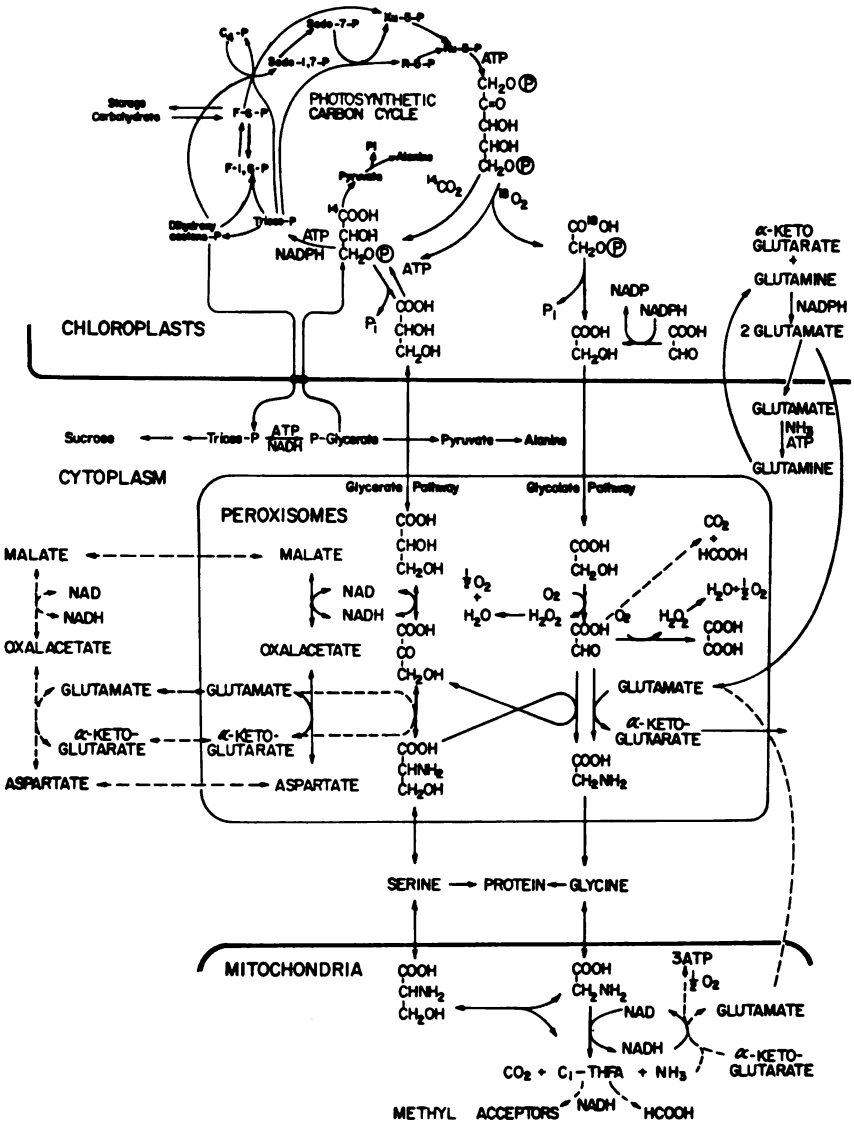


Figure 1. Oxidative photosynthetic carbon cycle in higher plants. This figure is from a review by N.E. Tolbert (2).

is converted to hydroxypyruvate, which is reduced to glycerate, which in turn can be phosphorylated to enter the photosynthetic reductive carbon cycle (1).

Estimations for CO_2 loss during photorespiration range up to 50% of that fixed. One way to estimate photorespiration is by the CO_2 compensation point, which is the level to which the plant will reduce the external CO_2 in a closed system.

The photorespiratory, or oxidative photosynthetic carbon cycle, also occurs in green algae, with some differences (3). The CO_2 compensation point is generally lower than in higher plants (4,5). This may be because the algae excrete part or all of the glycolate rather than metabolize it. The reason for this is not known. The magnitude of glycolate excretion may be very high. A major difference of glycolate metabolism in algae is in its oxidation. Many unicellular green algae do not contain a glycolate oxidase. They appear to have a glycolate dehydrogenase instead, which does not couple directly to O_2. This enzyme is present in low activity, and as currently understood, cannot account for the metabolism of all glycolate formed. A high rate of glycolate metabolism is not necessary, however, since so much glycolate is excreted. The other enzymes of the oxidative photosynthetic carbon cycle have always been assumed to be the same in algae as in higher plants.

The goal of the thesis project was to understand more of the differences in glycolate metabolism between unicellular green algae and the more thoroughly investigated system from higher plants. The alga used was Chlamydomonas reinhardtii.

Chlamydomonas reinhardtii is a member of the division Chlorophycophyta, order Volvocales of the plant kingdom. It has a single chloroplast and a flagellum at one end. It also has a heavy cell wall with a glycoprotein layer and no cellulose (6). It is easy to grow and has been used for many previous studies.

The enzymes of glycolate metabolism from this organism were partially purified and examined. Glycolate dehydrogenase was studied first (Chapter 1). Since this enzyme is also a D-lactate dehydrogenase, this latter activity was also studied (Chapter 2). The enzymes producing glycolate, P-glycolate phosphatase and NADPH:glyoxylate reductase, were also studied for possible clues to glycolate excretion (Chapters 3 and 4). A large increase in glycolate excretion resulting from added aminooxyacetate was also looked at (Chapter 5).

CHAPTER I

GLYCOLATE DEHYDROGENASE

LITERATURE REVIEW

Glycolate is the central product produced during photorespiration, and hence the past usage of the term, "the glycolate pathway." P-Glycolate is biosynthesized from ribulose-P_2 by the reaction with O_2 catalyzed by ribulose-P_2 carboxylase/oxygenase, followed by hydrolysis by P-glycolate phosphatase. The resulting glycolate is oxidized in higher plants by glycolate oxidase. This oxidase is present in peroxisomes, is FMN-dependent, and during the reaction O_2 is taken up with the production of H_2O_2. Many of the first studies on photosynthetic products labeled from $H^{14}CO_3^-$ were done with algae, as well as higher plants, and glycolate was always observed as a product. An equal distribution in the ^{14}C between the two carbon atoms was always observed with plants or algae (7). Glycolate was early shown to be excreted into the medium by algae (8) and attempts to find glycolate oxidase in algae failed, although other enzymes of the glycolate pathway were present (9).

A. Presence of Glycolate Dehydrogenase in Algae

Several groups have studied a glycolate-utilizing system in algae which was first called a glycolate oxidase (10,11). Lord and Merritt (10) made a preparation of Chlorella pyrenoidosa in which they

observed a glycolate-dependent formation of a phenylhydrazone, which could be followed spectrophotometrically. The formation of glyoxylate phenylhydrazone is one assay for glycolate oxidase. They obtained a stoichiometric relationship between glycolate used and glyoxylate formed. Another assay for glycolate oxidase is the reduction of the dye, 2,6-dichlorophenol-indophenol (DCPIP), which can substitute for the O_2 as electron acceptor in an anaerobic assay. Using this assay, Zelitch and Day (11), found activity in Chlamydomonas reinhardtii and Chlorella pyrenoidosa. They showed approximate stoichiometry between dye reduced and glyoxylate formed and demonstrated ^{14}C-glyoxylate production from ^{14}C-glycolate.

In 1969, Nelson and Tolbert (12) reported that Chlamydomonas reinhardtii contained a glycolate:DCPIP reductase that was not directly linked to O_2. They proposed that this was a different enzyme from the previously described higher plant glycolate oxidase. The amount of activity in the cells was affected by the CO_2 level with which the cells were grown. Codd et al. (13) also found that Chlamydomonas reinhardtii, Chlorella pyrenoidosa, and Euglena gracilis have a glycolate:DCPIP reductase that was not affected by, and did not use, O_2.

In 1970, Nelson and Tolbert (14) further characterized from Chlamydomonas the glycolate:DCPIP reductase, which they called glycolate dehydrogenase. This is the name that will be used throughout this thesis to refer to this activity. Nelson and Tolbert (14) obtained an extract by incubating the algae with 1% Triton X-100 for one hour in the cold. The debris was centrifuged out and a 35-50% ammonium sulfate fraction was prepared. The enzyme could use D-lactate

60% as well as glycolate, but it used L-lactate only at a much lower rate. It could also use glyoxylate, and DL-hydroxybutyrate, but not DL-glycerate, DL-α-phenyl-lactate, P-glycolate, glycine or malate. The pH optimum was at pH 8.5-9.0. The enzyme had a K_m for glycolate of 0.2 mM and 1.5 mM for D-lactate. It was able to couple both to DCPIP and PMS, but not to O_2, ferricyanide, FMN, FAD, NAD^+, $NADP^+$, methylene blue, glutathione, nitrate, or cytochrome c. The enzyme was sensitive to sulfhydryl inhibitors, but not to EDTA. KCN inhibited the algal enzyme, but had no effect on the peroxisomal glycolate oxidase from leaves.

B. Distribution of Glycolate Dehydrogenase

Nelson and Tolbert (14) also looked at the distribution of the enzyme in several species. They found no D-lactate-dependent or CN-sensitive glycolate-dependent DCPIP reduction in the three species of higher plants tested. These plants had an activity that could utilize L-lactate and glycolate. This was the glycolate oxidase. However, in the four species of green algae tested, the glycolate or D-lactate: DCPIP reduction was present and was 100% inhibited by 2 mM KCN. This was the glycolate dehydrogenase. With Euglena results were obtained indicative of the presence of both of these enzymes.

In 1973 a larger survey of the distribution of the two enzymes was published (15). The criteria described above were used to distinguish between the activities of the two enzymes. All the lower land plants, and aquatic angiosperms contained the oxidase. Some green algae contained the oxidase, the rest the dehydrogenase. This difference in enzyme content did not strictly correlate with the

unicellularity or multicellularity of the algae. The distribution seemed most to relate to a phylogenetic scheme based on certain cytological structures visible at mitosis. This hypothesis has not been confirmed because some of the algae with glycolate dehydrogenase had the higher plant type of cytostructure (16).

C. Cellular Location of Glycolate Dehydrogenase

The glycolate oxidase of higher plants is present in leaf peroxisomes (1). It is compartmentalized there with catalase, which can immediately react with the H_2O_2 formed. Stabenau (17) made a sucrose gradient of the homogenate from Chlamydomonas, and got a main peak of catalase which was centrifuged into the bottom of the gradient, whereas the rest of the catalase, the cytochrome oxidase, hydroxypyruvate reductase, malate dehydrogenase and glycolate dehydrogenase all centrifuged together. From the quality of this work it is hard to determine if organelle separation was really obtained, and if these enzymes are really mitochondrial. However, in 1976, glycolate dehydrogenase was located in the outer mitochondrial membrane of Chlamydomonas by electron microscopy with a cytochemical stain (18). D-Lactate dependent activity was also found in the same place. No stain was deposited in the peroxisomes.

Paul and Volcani (19) found the enzyme located in the mitochondria of a thin-walled Chlamydomonas mutant. This enzyme was a glycolate:cytochrome c reductase and was sensitive to antimycin and 2-heptyl-4-hydroxyquinoline-N-oxide, which are mitochondrial electron transport inhibitors. Rotenone had no effect.

Glycolate dehydrogenase has also been found in the mitochondria of diatoms (20,21) and other algae (22). In blue-green algae (prokaryotes) the glycolate dehydrogenase is associated with the thylakoid membranes (23,24,25).

Euglena gracilis contains a glycolate dehydrogenase in the mitochondria (26) sensitive to antimycin A, but insensitive to rotenone. Electron transfer to Euglena cytochrome c was demonstrated. Mitochondria from heterotrophically grown Euglena show a difference spectrum with glycolate (27) with peaks for cytochromes b, c and a, indicating that glycolate donates electrons to the whole transport chain to conserve energy. Similar results were seen in a diatom (21). Some diatom enzymes are atypical in using L-lactate instead of D-lactate (20,28).

Euglena grown autotrophically with air, contain a glycolate oxidizing activity in peroxisomes as well as mitochondria (29).

"Glycolate dehydrogenases" with different properties have been found in E. coli (30) and human liver (31).

D. Regulation of Glycolate Dehydrogenase

It was shown early that Chlamydomonas grown on 5% CO_2 had less glycolate dehydrogenase than air grown cells. These cells also are known to excrete more glycolate (12). Cooksey (32) indicated that nutritional nitrogen limitation was a cause of lower enzyme levels. Nitrogen limitation also caused repression of Euglena glycolate dehydrogenase (27). This suggested that this enzyme is necessary mainly when the growth limiting factor is carbon, either to conserve the needed fixed carbon, or to somehow participate in HCO_3^- uptake.

P-glycolate phosphatase levels also may decrease during growth on high CO_2 (12). This makes sense since P-glycolate should be formed in lower amounts, because high CO_2 should outcompete O_2 for the ribulose-P_2 carboxylase/oxygenase reaction.

P-Glycolate phosphatase is synthesized during regreening of Euglena, even when ribulose-P_2 synthesis is artificially inhibited (33). Euglena grown on 5% CO_2 lose the glycolate dehydrogenase activity in the peroxisomes, but not in the mitochondria (34).

E. Yellow Chlorella mutant

In 1971, Kowallik and Schmid (35) described a yellow Chlorella vulgaris mutant that, along with the wild type Chlorella vulgaris, had glycolate-dependent O_2 uptake. This was demonstratable both with whole cells and with an ammonium sulfate fraction from an extract prepared in a French press. These fractions also had anaerobic DCPIP reduction that was dependent on glycolate. These activities were stimulated by FMN. The rates were in the range of 0.2-20 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$. Especially in the yellow mutant, the O_2 uptake was stimulated by blue light. Chlorella pyrenoidosa and several other green algae did not exhibit this glycolate-dependent O_2 uptake. In fact Chlorella pyrenoidosa was one of the first algae in which glycolate dehydrogenase was discovered (10). Kowallik and Schmid concluded that Chlorella vulgaris, but not Chlorella pyrenoidosa had glycolate oxidase instead of glycolate dehydrogenase. This would be interesting, if true, since these two algae are closely related, and would indicate a transition between two systems for oxidizing glycolate during photorespiration.

MATERIALS AND METHODS

A. Algae

Chlamydomonas reinhardtii, Dangeard (-) strain, was obtained from the Type Culture collection of the University of Texas at Austin as catalog #90. Chlorella vulgaris 211-11h, catalog #263, was from the same source. The yellow Chlorella mutant, 211-11h/20, from the Algal Collection of the Institute of Applied Microbiology, University of Tokyo, catalog #C-425, was the gift of S. Miyachi.

B. Growth of Algae

The green cells were grown at 23-28°C in the medium of Orth, Tolbert and Jiminez (36) in aerated (several ml/min) flat Erbach flasks on a shaker, under lighting of 500 μEinstein$\cdot m^{-1} \cdot s^{-1}$ from cool white fluorescent tubes. The wild type Chlorella were grown on a medium containing 50% less NH_4^+. The yellow Chlorella were grown in the dark at 30°, in a medium containing: 0.81 g KNO_3, 0.47 g NaCl, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.44 g $NaH_2PO_4 \cdot 2H_2O$, 0.36 g $Na_2HPO_4 \cdot 12H_2O$, 0.022 g $CaCl_2 \cdot 6H_2O$, 0.0013 g $ZnSO_4 \cdot 7H_2O$, .0002 g $MnCl_2 \cdot 4H_2O$, 1 drop 5% w/v $FeCl_3$, 7.0 g of glucose in 1 liter of H_2O, pH of 6.0 (Miyachi, personal communication).

Cells were harvested by centrifugation after about 5 days of growth, washed once with distilled water, and centrifuged in weighed Corex tubes at 10,000 rpm, and the wet weight was recorded. They were used immediately.

C. Assay for Glycolate Dehydrogenase

Homogenization procedures will be described in the Results. The assay was the same as one of the assays for glycolate oxidase. The

reduction of 2,6-dichlorophenolindophenol (DCPIP), used as an electron acceptor, was followed spectrophotometrically at 600 nm in 50 mM $NaPP_i$ pH 8.7. The endogenous rate with enzyme but without substrate was subtracted. Because the oxidized dye is colored, and has low solubility, it can only be present in the reaction at less than saturating amounts. The assay for glycolate oxidase was run in an anaerobic Thunberg cuvette with glycolate in the side arm. The cuvette was degassed 10 times with N_2 to remove O_2 to prevent the competing reaction with O_2 to form H_2O_2 which can in turn reoxidize any reduced DCPIP. The glycolate oxidase assay was modified for glycolate dehydrogenase. In 0.9 ml total volume there were 700 µl of 50 mM $NaPP_i$ buffer, pH 8.7, containing 1.87×10^{-4} M DCPIP and 200 µl extract or H_2O. This was incubated at 30° for 15 min, while measuring any endogenous rate, then the reaction was started by addition of 100 µl of 0.125 mM Na glycolate. The rate was followed at 600 nm on a Beckman spectrophotometer. With glycolate dehydrogenase the reaction can be run aerobically because there is no reaction with O_2. The extinction coefficient for DCPIP is 21.5 at pH 8.7 (37).

The enzyme in homogenates was stable on ice for several hours, but the reaction rate in the assay was far from linear, and rapidly decreased. Initial rates were determined. The assay was not entirely satisfactory for homogenates, since apparently something occurred as the reaction proceded to either destabilize the enzyme or to reoxidize the dye. This occurred more in the crude extracts than in partially purified preparations. Although assays in air or N_2 gave the same result, the anaerobic assays were more linear in the crude extracts.

The glycolate dehydrogenase preparation, which was partially purified by ammonium sulfate fractionation appeared to be stable for at least several weeks in the -18° freezer, and to several freeze-thaw cycles. The rates were also linear longer than in crude extracts.

D. Other Methods and Materials

Protein was determined by a modified Lowry assay procedure (38).

Biochemical reagents were purchased from the Sigma Chemical Company, with the exception of the DEAE-cellulose (DE52, microgranular) which was from Whatman, and the ammonium sulfate which was a special enzyme grade from Schwarz/Mann.

RESULTS

A. Glycolate Dehydrogenase-Crude Extract

Following the work of Nelson and Tolbert (14), the principal method used at first to obtain a preparation containing the dehydrogenase was to incubate the cells in a small volume with 1% Triton X-100. This gave on the average 6 nmol·min^{-1}·ml^{-1}, 0.003 units·mg protein^{-1}, or 0.06 units·gram wet weight^{-1}. A unit is defined as 1 μmole DCPIP reduced per min. By the sonication apparatus, with bursts totalling 5 min, an activity of glycolate dehydrogenase as high as 0.6 nmol·min^{-1}·mg protein^{-1} was obtained. Very often, however, this method yielded no activity. No activity was observed in the supernatant medium after incubation of the cells with phospholipase C or after osmotic shock treatment. Since glycolate dehydrogenase may occur in the mitochondrial membrane, an attempt was made to obtain a crude organelle preparation from the cells by grinding them in a

mortar and pestle lined with nylon mesh, followed by a differential centrifugation. The cells were apparently too tough to be opened by this approach. Treatment with cellulase and hemicellulase before the grinding also did not work, because Chlamydomonas cell walls do not contain cellulose (6).

Part of the problem was that the enzyme existed at a low level in the crude extract, and the endogenous rate could be 50% of the enzymic rate. Several methods to partially purify the enzyme were tried. The method of Nelson and Tolbert (14), ammonium sulfate precipitation between 35-50% saturation, gave 2 to 3-fold purification, which is similar to their results, but the yield was only about 20%. After this procedure, the enzyme appeared to be attached to chlorophyllous matter that was of all sizes, and which could not be fractionated with ammonium sulfate. Polyethylene glycol will also precipitate a protein fraction with the enzyme. Purification was not helped by the presence of polyvinylpolypyrolidone, different amounts of Triton X-100, or several concentrations of salts in the extract.

B. Various Extraction Methods for Glycolate Dehydrogenase and P-glycolate Phosphatase

Cells were centrifuged from their culture medium, washed with 20 mM Tris, pH 7.7, centrifuged, and incubated in the various procedures at 3:1 (v:w) for 30 min, with stirring, in the cold. Nearly every procedure caused P-glycolate phosphatase activity (Chapter 3) to be solubilized (Table 1). Whereas KCl or detergent alone or together removed the phosphatase from the cell, salt alone did not remove the dehydrogenase, and it reduced the yield by detergents by 50%. These

TABLE 1

Extraction Methods for Glycolate Dehydrogenase and P-Glycolate Phosphatase from Chlamydomonas reinhardtii Grown with Air

	1% Triton X-100		1% Deoxycholate		1 M KCl	1% SDS	1 mM EDTA	H_2O
		+KCl		+KCl				
Glycolate Dehydrogenase	(100)	51	220	100	0	0	0	0
P-Glycolate Phosphatase	(100)	75	74	100	69	33	13	0

Numbers are percentages of amount extracted by Triton X-100. Approximately 0.018 $\mu mol^{-1} \cdot min^{-1} \cdot mg\ protein^{-1}$ for glycolate dehydrogenase and 0.210 $\mu mole \cdot min^{-1} \cdot mg\ protein^{-1}$ for P-glycolate phosphatase were extracted by Triton X-100.

exploratory extraction procedures suggest different cellular association for the two enzymes and that the phosphatase may be bound ionically, or loosely, and leak out due to any disruption.

Deoxycholate seemed to be a better extraction agent than Triton X-100, since it improved the yield of dehydrogenase more than it reduced the yield of the phosphatase.

Sonication was an effective method for the extraction of the phosphatase, but caused a loss of the dehydrogenase activity (Table 2). In most cases no dehydrogenase activity was found after sonication.

After deciding that deoxycholate did the best job of extraction, deoxycholate was used to prepare all subsequent crude extracts for these two enzymes. The harvested cells were suspended in 3 volumes per gram of cells of several concentrations of Na deoxycholate in H_2O. This was stirred for 30 min at 4°C, and the debris was removed by centrifugation at 12,000 x g in a Sorvall RC2 centrifuge. The supernatant was used as a crude extract. It was normally yellow, or had a slightly green color from older cells. The optimum concentration of deoxycholate was determined to be 0.3% (Figure 2a). The time course of extraction was also determined, with 30 min being the optimum (Figure 2b).

C. Effect of CO_2 Concentration During Algal Growth upon Activities of Glycolate Dehydrogenase and P-Glycolate Phosphatase

It has been reported that Chlamydomonas grown on high CO_2 levels have less glycolate dehydrogenase than algae grown with air (12). High CO_2 is considered to be any level from 1-5% CO_2 added to the

TABLE 2

Disruption Methods for Glycolate Dehydrogenase and P-Glycolate Phosphatase from Chlamydomonas reinhardtii Grown with Air

	Disruption Method		
	Sonication		0.3% Deoxycholate
	in H_2O	in Bicine	
	$nmol \cdot min^{-1} \cdot ml^{-1}$		
Glycolate Dehydrogenase	14	9	47
P-Glycolate Phosphatase	5000	4000	4000

1 g cells (wet weight) in 5 ml of liquid were used. Sonication was in 20 sec. bursts for 3 min. total in a rosette cell cooled by salt-ice bath. Bicine was 50 mM at pH 8.0.

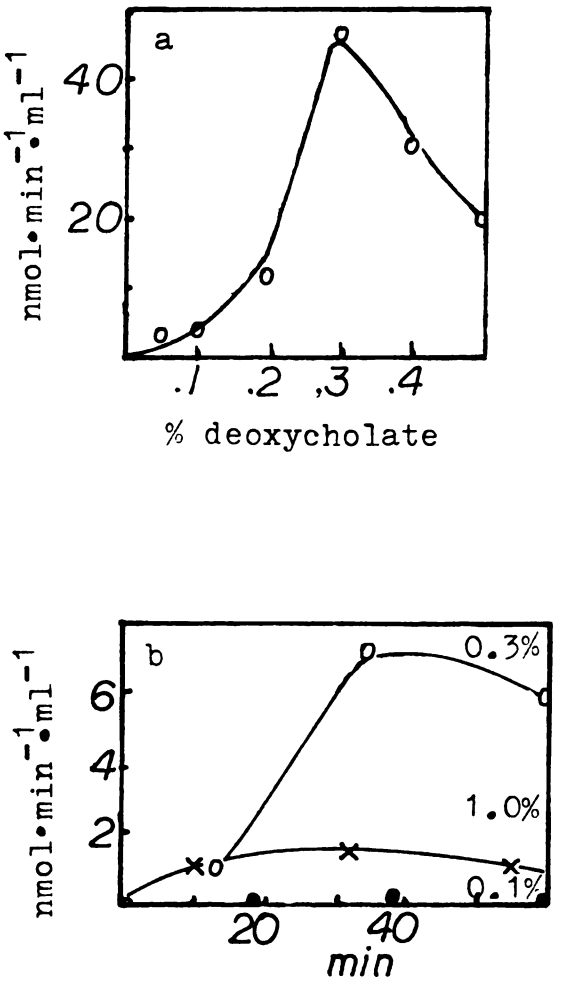


Figure 2

a. Extraction of glycolate dehydrogenase from air-grown Chlamydomonas reinhardtii by different concentrations of deoxycholate.

b. Time course of deoxycholate extraction from air-grown Chlamydomonas reinhardtii by different concentrations of deoxycholate.

● 0.1% deoxycholate; × 1.0% deoxycholate; o 0.3% deoxycholate.

aeration stream, and low CO_2 during growth is the air level (0.03-0.06% CO_2). It was not clear whether the level of CO_2 during growth of Chlamydomonas had an effect upon the amount of P-glycolate phosphatase.

The high CO_2 cells had about half the activity of glycolate dehydrogenase and P-glycolate phosphatase on a per g wet weight, mg protein, or mg chlorophyll basis (Table 3). There was about twice as much material (wet weight) of cells grown on high CO_2 so the total amounts of each enzyme per culture came out about the same for enzyme preparations.

D. Yellow Chlorella Mutant

No evidence for a glycolate-dependent O_2 uptake or glycolate oxidase was found in extracts from Chlorella vulgaris, either the yellow mutant or the wild type. Neither the crude, French press extract nor the ammonium sulfate fraction prepared by the methods of Kowallik and Schmid (37), had any activity for glycolate oxidase (Table 4). However, glycolate-dependent, DCPIP reduction was present, which by the criteria of Frederick, Gruber, and Tolbert (15), one would call glycolate dehydrogenase. The activity was not inhibited by O_2, was inhibited by cyanide, and used D-lactate as well as glycolate much more readily than L-lactate as substrate. The activity was not stimulated by FMN. In these respects the glycolate-oxidizing system in Chlorella vulgaris was similar to the Chlamydomonas reinhardtii enzyme.

It is possible to consider that glycolate dehydrogenase is part of an electron transport chain in the mitochondria, and under the

TABLE 3

Activity of Glycolate Dehydrogenase and P-Glycolate Phosphatase in *Chlamydomonas reinhardtii* Grown with Air or High CO_2

	Glycolate Dehydrogenase		P-Glycolate Phosphatase	
	nmol·g wet weight^{-1}·min^{-1}	nmol·mg protein^{-1}·min^{-1}	μmol·g wet weight^{-1}·min^{-1}	μmol·mg protein^{-1}·min^{-1}
A. Sonicated Cells				
Air	23	0.7	2.5	0.07
High CO_2	0	0	0.9	0.05
B. Detergent treated Cells				
Air	77	6.6	2.0	0.2
High CO_2	50	3.0	1.0	0.05

TABLE 4

Glycolate Oxidation in Extracts of *Chlorella vulgaris* and *Chlamydomonas reinhardtii*

	Substrate				
	Glycolate			D-lactate	L-lactate
	anaerobic	aerobic	1 mM CN^-		
Chlamydomonas reinhardtii	100%	95%	0%	75%	0%
Chlorella vulgaris	100%	125%	2%	117%	3%
Chlorella vulgaris yellow mutant	100%	95%	0%	110%	0%

All assays were described in the Materials and Methods, Chapter 1, for glycolate dehydrogenase. Values are expressed as the percentage of the anaerobic rate with glycolate as substrate.

right circumstances, the electrons removed from glycolate might be transported all the way to O_2. This could account for the differing sensitivities of the enzyme activity to oxygen, and perhaps it would be possible under some circumstances to isolate a particle, perhaps nearly intact mitochondria, that would demonstrate this coupling and be linked to O_2 uptake. In my preparation this was not observed. That Kowallik and Schmid (35) saw a difference in glycolate oxidation between Chlorella vulgaris or its yellow mutant and other algae is interesting but cannot be reproduced.

E. Purification of Glycolate Dehydrogenase

The crude extract was made with 0.3% deoxycholate as described in sections A and B. Next, a 30-45% ammonium sulfate precipitate was collected and suspended in a small volume of water. The enzyme was very stable at this stage and could be stored in the -80°C freezer for months. The suspended material was dialyzed for a few hours against 20 mM Bicine at pH 7.8 and centrifuged to remove any insoluble material. It was then applied to a DEAE-cellulose column that had been equilibrated in the same buffer. Glycolate dehydrogenase activity was eluted with a KCl gradient (Figure 3a). Although the activity was fractionated from a major protein peak, the specific activity as well as total activity decreased (Table 5). Furthermore, the enzyme was no longer stable. When other purification steps were used instead of the DEAE-cellulose column a similar loss of activity occurred. Other purification methods that were tried included: reverse ammonium sulfate fractionation (39) with elution from celite, and elution from native polyacrylamide gel slices. Another method used was sucrose

TABLE 5

Glycolate Dehydrogenase Purification from *Chlamydomonas reinhardtii* Grown with Air

Step	$nmol \cdot min^{-1} \cdot mg\ protein^{-1}$	yield
0.3% Deoxycholate extract	0.9	(100)
30-45% Ammonium Sulfate Fraction	16.3	236
Dialysis	25.1	223
DEAE-cellulose chromatography	4.0	3
Sucrose density gradient	0.6	0.1

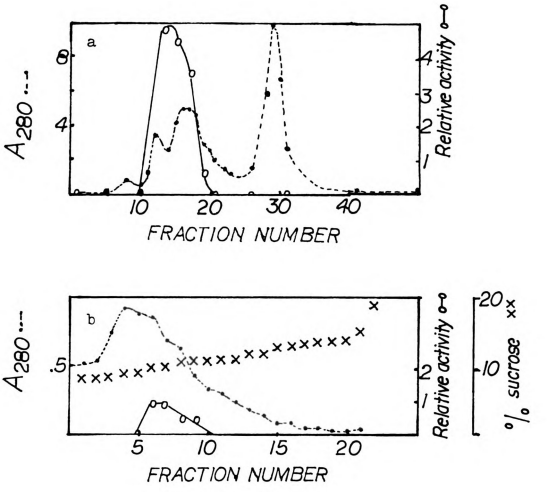


Figure 3

a. DEAE-cellulose fractionation of glycolate dehydrogenase from Chlamydomonas reinhardtii.

o Relative units of glycolate dehydrogenase; ←→ Absorbance at A_{280} (protein level).

b. Sucrose gradient of glycolate dehydrogenase from Chlamydomonas reinhardtii.

o Relative units of glycolate dehydrogenase (the same units as in Figure 2a); ←→ Absorbance at A_{280} (protein level); ×× % sucrose w/w measured on a densitometer.

gradient ultracentrifugation by the method of Martin and Ames (40). The enzyme lost activity even after this mild treatment (Table 5, Figure 3b).

F. Electron Acceptor for Glycolate Dehydrogenase

Various possible cofactors were tried for their effect on the activity of glycolate dehydrogenase. None stimulated the reaction, and several inhibited the reaction. Some of these compounds may be taking a part of the electrons from the reaction or interfering with the DCPIP reduction. Cytochrome c (horse heart) completely prevented any detectable reaction. Since there are reports that cytochrome c may be the natural acceptor (19,20), the assay for glycolate:cytochrome c reductase was tried, but no cytochrome c reduction was observed, with or without O_2 present. The compound, NBT (nitroblue tetrazolium), which will couple to the enzyme on gels in a stain for activity (Chapter 2) inhibited the reaction with DCPIP to a slight extent. $Fe(CN)_6^{3-}$ at 1 mM concentration completely inhibited the reaction of DCPIP. However, the ferricyanide could directly act as an electron acceptor. The reaction mixture contained: 0.7 ml of 1 mM $Fe(CN)_6^{3-}$ in 50 mM KPP_i at pH 8.7 and enzyme plus water in 0.2 ml. The reaction was initiated with 0.1 ml of 12.5 mM glycolate. The extinction coefficient used was 1.0 mM^{-1} at 420 nm in a 1 ml cuvette. A rate of 1.7 $nmol \cdot min^{-1}$ with $Fe(CN)_6^{3-}$ is to be compared with a DCPIP rate of 2.5 $nmol \cdot min^{-1}$.

Among other compounds tested for their effect on the DCPIP assay, antimycin which has been reported to inhibit the reaction of the dehydrogenase (19,26), had no effect in the DCPIP assay. Menadione and rotenone were without effect.

An assay with MTT and PMS as electron acceptors worked well. An analog of NBT, MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium Br) is more soluble in the reduced form. With a 1 ml assay, containing 0.7 ml of a solution of 10 mg MTT per 100 ml of 50 mM $NaPP_i$, pH 8.7, and 0.002 mg $PMS \cdot ml^{-1}$, the reaction was initiated with a final concentration of 10 mM D-lactate or 12.5 mM glycolate. The assay was run at 30°C, and at 570 nm ($E_{mM}^{570} = 0.17$). This reaction was more linear than the DCPIP assay for detecting the activity in the deoxycholate extract. Both the DCPIP assay and the NBT/PMS assay will detect the activity in ammonium sulfate fractions. The ratio of glycolate to D-lactate activity was the same for both assays. The PMS was also necessary for the MTT assay.

Contrary to previous reports (19) the glycolate dehydrogenase did not couple to horse heart cytochrome c, or directly to PMS. It did couple to DCPIP, NBT, $Fe(CN)_6^{3-}$ and MTT/PMS.

The short initial linear rate often observed in assays with this enzyme might be due to product inhibition by glyoxylate. Although it would have been appropriate to trap the glyoxylate, unfortunately phenylhydrazine in the standard assay reduces the DCPIP, and so this compound could not be used in conjunction with the DCPIP assay. Glyoxylate was added to the assay to see if it would cause a qualitative decrease in linearity. It did not. Glyoxylate did cause some inhibition, as expected, which possibly was greater when the glyoxylate was incubated with the extract, than when it was added at the same time as the glycolate. This is not surprising, since glyoxylate is a reactive compound toward sulfhydryl groups.

G. Attempts to Stabilize Glycolate Dehydrogenase After Ammonium Sulfate Precipitation

Several attempts were made to stabilize the enzyme activity. There was some indication that a sulfhydryl protectant might help (14). However, such reagents will also reduce the DCPIP, and therefore with the assay. Another possibility was to keep the enzyme under N_2. The results of such experiments were not conclusive, but did not indicate any advantage.

Some lipid may be necessary for stability of the enzyme, but of the various ones tried, none appeared to work. The only approach with some positive results was to add 2% BSA (final concentration).

When the procedure for assaying the enzyme was changed from 25° to 30°, and the enzyme was preincubated at 30°C, no decay of activity in a crude extract was seen over 4 hours. Under these conditions, the enzyme was apparently activated, or at least unharmed.

The enzyme increased in activity when it was dialyzed against buffers probably due to removal of ammonium sulfate. Ammonium sulfate caused an inhibition. Ammonium sulfate appeared to cause an activation in some cases since the rate was more linear, which allowed a better measurement of initial rate. The protein was also more concentrated in the $(NH_4)_2SO_4$ fraction.

Since the activity can be extracted from the cell by detergent, and because of the necessity for preincubation at 30°C, the enzyme may be membrane associated. However, the high ammonium sulfate concentration used in isolation may cause dissociation of an electron acceptor component which competes with the DCPIP, or it may cause better coupling with a necessary component, or have some unclear activating or protective effect.

H. The pH Optimum of Glycolate Dehydrogenase

The pH optimum of glycolate dehydrogenase was at 8.7 as previously reported (14) (Figure 4).

I. Kinetic Properties of Glycolate Dehydrogenase

The enzyme had a low K_m of 45 μM for glycolate (Figure 5a). Oxamate was tested as an inhibitor, because an oxamate affinity column has been used to isolate those lactate dehydrogenases with low K_is for oxamate (41). Oxamate was a competitive inhibitor with a K_i of 3 ± 1 mM (Figure 5b). This is in the range seen with D-lactate dehydrogenases which do not attach to an oxamate column (41).

Another inhibitor tested was HBA (hydroxybutynoate), an inhibitor of flavin containing α-hydroxy acid oxidases (42). It inhibited glycolate oxidase (43,44). HBA also inhibited the glycolate dehydrogenase, and this is indicative of a flavin cofactor (Figure 6).

Another inhibitor tested was aminooxyacetic acid (AOA), due to its effect on glycolate excretion (Chapter 5). AOA did not inhibit glycolate dehydrogenase in a crude extract.

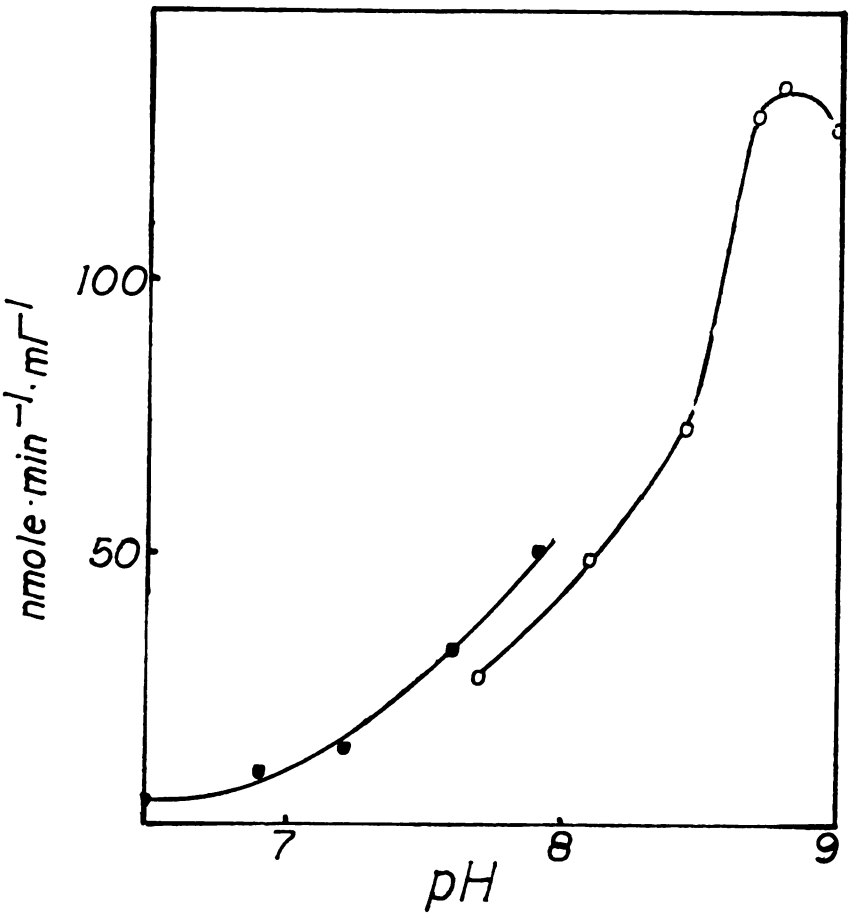


Figure 4

The pH optimum of glycolate dehydrogenase from Chlamydomonas reinhardtii.

Glycolate dehydrogenase activity in 20 mM Hepes ●—● or Bicine o—o buffer.

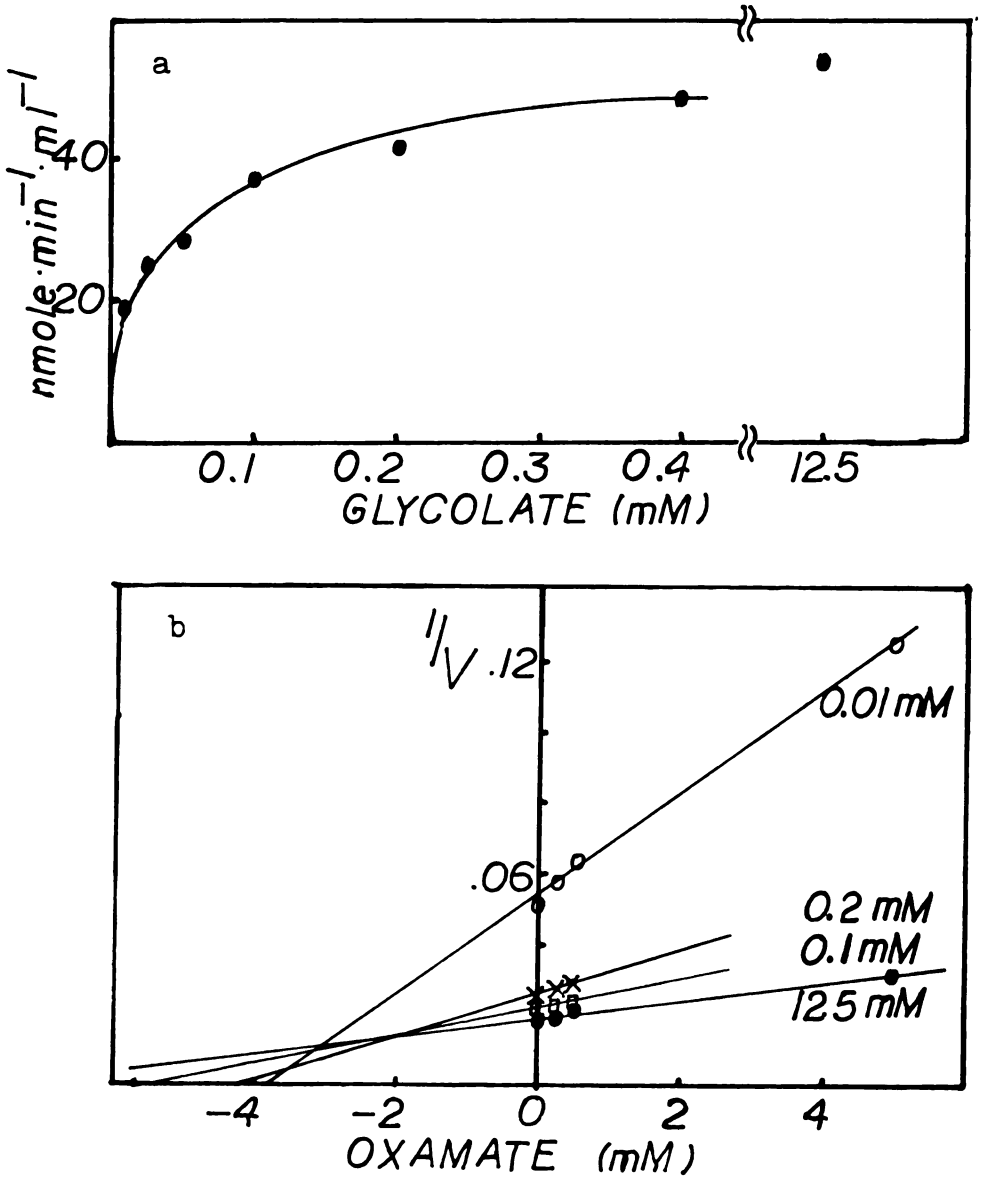


Figure 5

a. Rate of glycolate dehydrogenase versus concentration of glycolate.

b. Dixon plot for inhibition of glycolate dehydrogenase by oxamate.

The concentrations of glycolate in the assay were 0 for 0.01 mM, × for 0.1 mM, □ for 0.2 mM, and ● for 12.5 mM. Oxamate is a competitive inhibitor (data not shown). The K_i was from 2-4 mM.

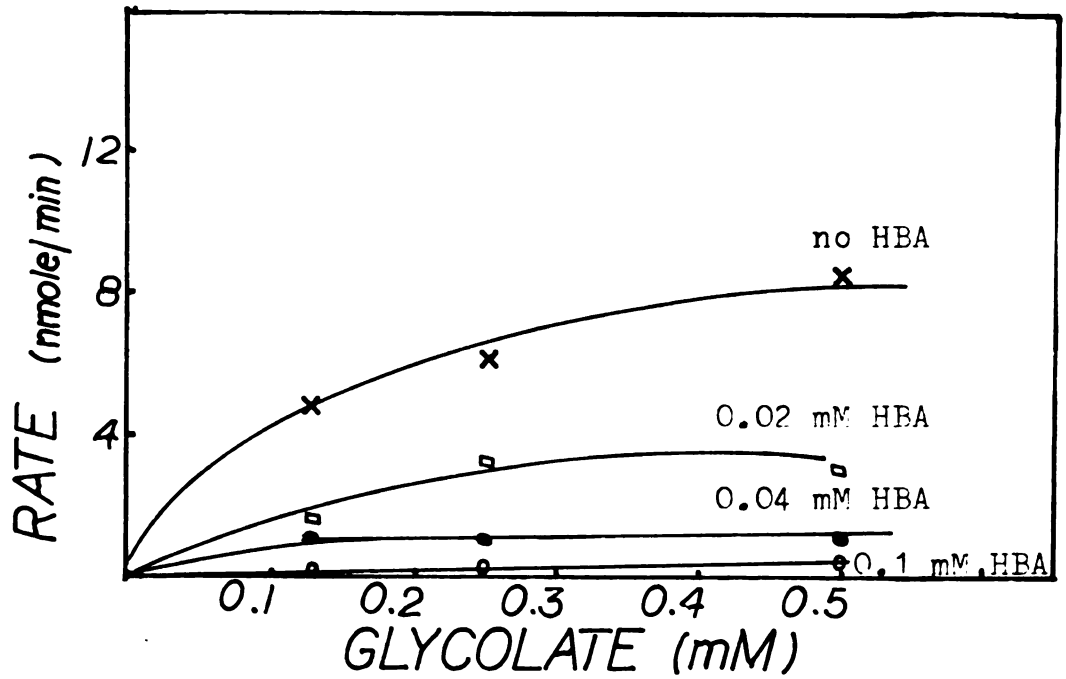


Figure 6

Inhibition of glycolate dehydrogenase from Chlamydomonas reinhardtii by hydroxybutynoate.

× no hydroxybutynoate, ▫ for 0.02 mM hydroxybutynoate, ● for 0.04 mM hydroxybutynoate, and ○ for 0.1 mM hydroxybutynoate.

CHAPTER II

D-LACTATE IN GREEN ALGAE

LITERATURE REVIEW

In 1957 Warburg et al. (45) described the presence of D-lactate in Chlorella. The cells were placed (1v:3V) in a pH 3.8 medium containing only $MgSO_4$ and potassium phosphate buffer and shaken anaerobically for 5 hours. It appears that 1.6 mM D-lactate was produced. They also showed that pyruvate and NADH reacted within two days with a Chlorella extract or an ammonium sulfate fraction of the extract to form D-lactate.

In 1974 Gruber et al. (46) found a NADH:pyruvate reductase in Chlorella extracts that was very labile, which they assumed to be Warburg's lactate dehydrogenase. D-Lactate and NAD^+ were the substrates for the reverse reaction, but not L-lactate. It was possible to separate this activity in the crude extract from glycolate dehydrogenase by differential centrifugation. The glycolate dehydrogenase was found to be particulate. D-Lactate dehydrogenase was also reported to be much more labile (disappearing after one hour) than the glycolate dehydrogenase in Dunaliella. No glycolate-dependent NAD reduction could be demonstrated with these fractions. They ruled out hydroxypyruvate reductase as the responsible enzyme. A preliminary attempt was made to survey in plants the ubiquity of lactate

dehydrogenase in the direction of pyruvate reduction. Several D- and L-lactate dehydrogenases were found. Betsche (47) has described L-lactate dehydrogenases in higher plant leaves.

In 1974, Kobayashi et al. (48) used several algae to test various growth conditions that resulted in acid production in the medium. D-lactate was one organic acid produced. Unshaken cultures of Chlorella vulgaris produced more acids than shaken cells. The unshaken cells may have become anaerobic. Chlamydomonas produced less lactate than Chlorella. More lactate was produced when glucose was added to the medium than was produced autotrophically. All the algae tested produced D-lactate. The responsible enzyme from Chlorella vulgaris was precipitated with 50% saturated ammonium sulfate and recovered from a DEAE-Sephadex A-50 column by elution with NaCl. It was purified 10-fold over cell extracts. Its pH optimum was at 8 and its K_m (pyruvate) was 0.6 mM, and the K_m (NADH) was 0.17 mM (49).

MATERIALS AND METHODS

The methods used were the same as in Chapter 1, with the addition of polyacrylamide gel electrophoresis. Polyacrylamide gels (7%) with 1.5 cm stacking gels were poured according to the method of Ornstein and Davis (50,51). They were run at 4°C with 2.5 ma per tube. The gels were removed from their tubes and stained according to the method of Grodzinski and Colman (52) with modification to include 25 mM $NaPP_i$ pH 8.7, .016% (w/v) fresh NBT, and substrate (18 mM glycolate, 14 mM lactate or 14 mM other substrates). Gels were incubated in 7 ml of staining solution at 30°C in the dark for 1 hour to overnight.

The acrylamide used was from the Bethesda Research Laboratories, and the N,N,N',N'-tetramethyl ethylene diamine and ammonium persulfate were from the Ames Company. Other materials were as described in Chapter 1.

RESULTS

A. Gels of Crude Extracts

The 0.3% deoxycholate extract of Chlamydomonas was dialyzed against 50% glycerol in 25 mM KPP_i, pH 8.7. A glycolate dependent band was seen at R_f of 0.33 and a D-lactate band at an R_f of 0.33 and a very heavy, double band at R_f of 0.45. L-Lactate gave a band at the R_f of 0.33, the same as for glycolate. Neither FMN or FAD had any effect on these bands when incubated with the activity stain solution.

The "D-lactate bands" (the twin bands at R_f of about 0.45) were much stronger from deoxycholate extracts than from sonicated extracts. A band at this location also developed, but less strongly, with DL-glycerate as the substrate. NAD^+ in the staining solution did not affect the appearance of the band.

B. Ammonium Sulfate Fractions on Polyacrylamide Gels

An ammonium sulfate fraction of the deoxycholate extract manifested two bands with D-lactate at R_f 0.45 (identical to those seen in section A), but DL-glycerate gave only one band at this location. Several other straight chain α-hydroxyacids also gave a strong band at this location (Table 6).

Although CN^- (KCN 1 mM) inhibited the DCPIP assay with glycolate or D-lactate as substrate about 85%, it did not prevent the appearance

TABLE 6

Substrates that Produce an Activity Stain with NBT in the Location of the D-Lactate Band on the Polyacrylamide Gels

Substrate	
D-lactate	++
Glycolate	--
L-lactate	--
DL-α-hydroxy valerate	++
DL-α-hydroxy iso-butyrate	--
DL-α-hydroxy iso-caproate	--
DL-α-hydroxy caproate	++
DL-α-hydroxy caprylate	++
DL-α-hydroxy laurate	++
DL-α-hydroxy palmitate	--
DL-glycerate	++

All positive reactions were very strong.

of the "D-lactate band", when it was included in the medium for the activity stain.

C. DEAE-cellulose Chromatography of Ammonium Sulfate Fractions

A 30-45% ammonium sulfate preparation from Chlamydomonas reinhardtii was put over a DEAE cellulose column, after being suspended in water and dialyzed against the equilibration medium for the column. There were peaks of dehydrogenase with both glycolate and D-lactate, in the original proportions to each other, in the wash eluate and also in the main protein peak, which was eluted with a KCl gradient (Figure 7). The wash fraction was three times as active as the other active fraction. It appeared to have a glycolate dehydrogenase band when visualized on polyacrylamide gels by activity staining, but no "D-lactate band" at R_f of about 0.45. The other active fraction of glycolate dehydrogenase had a prominent "D-lactate band" as well as the glycolate dehydrogenase band. The "D-lactate bands" at R_f 0.45 in this system appeared to predominate in a different fraction than the peak of glycolate and D-lactate:DCPIP spectrophotometric activity, which corresponded to a band of activity on gels at R_f 0.25-0.3.

D. The "D-Lactate Band" in Spinach

Spinach leaves bought from a local market were deveined, and added to 3 v/w of either 50 mM KPP_i pH 8.7 or 0.3% deoxycholate. The PP_i buffer preparation was extracted by high speed homogenization for 30 sec at room temperature. The deoxycholate sample was prepared by one short burst in a Waring Blender and 30 min stirring in the cold. The debris was removed by centrifugation. Samples of the supernatant were

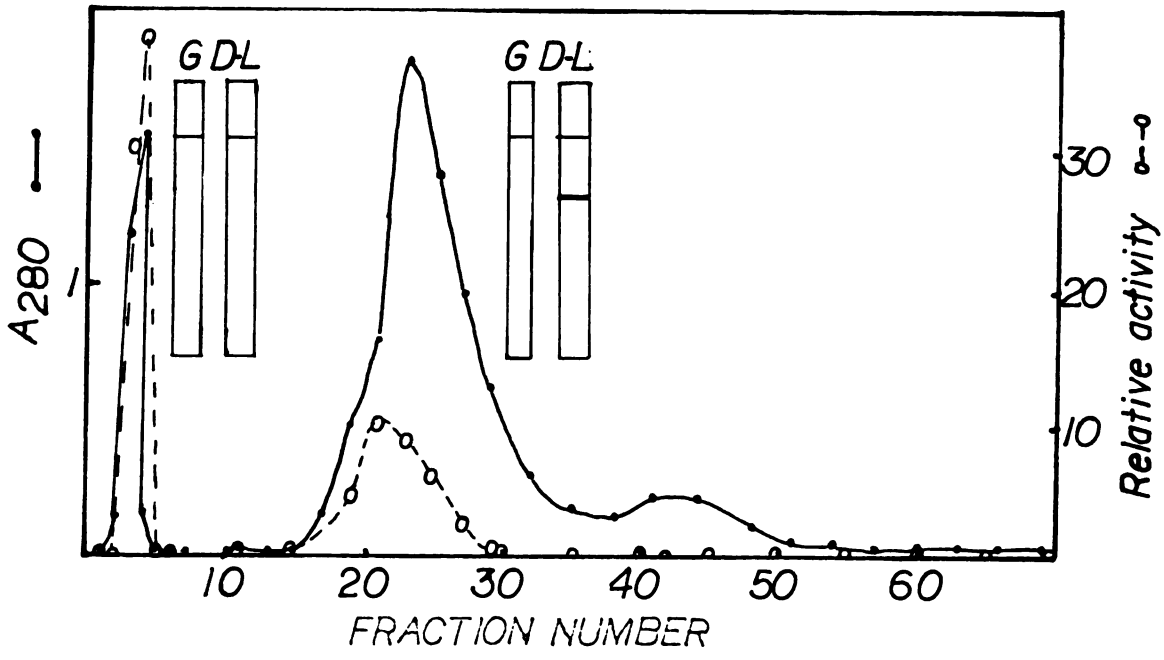


Figure 7

DEAE-cellulose fractionation of glycolate dehydrogenase from Chlamydomonas reinhardtii and polyacrylamide gels of the peak fractions.

The enzyme sample used was a 30-45% ammonium sulfate fraction, dialyzed against 20 mM Bicine pH 7.5. The column was 1.5x6.3 cm, and it was equilibrated in the same buffer. Fractions 1-10 are the void volume. The protein was eluted with a gradient of 0.1 M KCl in 20 mM Bicine. o-o is relative activity of glycolate dehydrogenase with glycolate as the substrate. The activity of the enzyme with D-lactate as the substrate followed exactly the same profile, and the ratio of glycolate to lactate activity was constant and equal across the gradient. •—• is protein concentrations in the gradient fractions (A_{280}). Fractions 4 and 22 were electrophoresed on polyacrylamide gels and stained for activity with glycolate and D-lactate as described in the text. G represents the activity with glycolate, and D-L represents the activity with D-lactate.

applied to polyacrylamide gels, and for both samples one band at R_f of 0.6 was seen which was dependent on D-lactate, DL-glycerate or DL-hydroxycaproate, but not glycolate or L-lactate as substrate. A band for glycolate oxidase was not present.

An ammonium sulfate precipitate (12-45%) of a spinach leaf homogenized in PP_i buffer was made to concentrate the glycolate oxidase. It also contained two enzymic activity bands on the acrylamide gel with D-lactate or DL-glycerate at R_f 0.61. The band for glycolate oxidase was present at a lower R_f, near the top of the gel. As with glycolate dehydrogenase in algae, the "D-lactate band" was much larger than the glycolate oxidase activity with the detection procedures used.

A D-lactate:NBT staining band was also eluted from a DEAE-cellulose column which had a sample from spinach leaf applied to it and which had been developed the same way as for the algal sample in Figure 7. The band was found in an equivalent position to that from the algal sample.

E. Sucrose Gradient Ultracentrifugation of Spinach Subcellular Organelles

Organelles were separated on sucrose density gradients which were prepared in 60 ml cellulose nitrate tubes (53). Storebought spinach leaves (48.5 g) were deveined, cut up with scissors, and mixed with 95 ml of 0.83 M sucrose in 20 mM glycylglycine buffer at pH 7.5 with 0.5 mM EDTA and 5 mM dithiothreitol. After homogenization with one short burst in a Waring blender, the brei was passed through Miracloth, then centrifuged at 270 x g for 10 min. The supernatant

was centrifuged at 10,000 x g for 20 min. The pellet contained the organelles and was resuspended in 5 ml of the grinding buffer and centrifuged again at low speed (270 x g). This was applied to a gradient composed of 5 ml of each of the following: 60%, 52%, 50%, 49%, 48.1%, 46.6%, 45%, 43%, 40%, 35.2%, 30%, w/w sucrose in 20 mM glycylglycine at pH 7.5. The gradient was centrifuged at 25,000 rpm on a Beckman L2 using a 25.2 head. Acceleration was in 5,000 rpm steps, allowing 10 min between each. The gradient was then centrifuged at constant speed for 4 hours.

The gradient was fractionated from the top using an ISCO fractionator with the fractions assayed with organelle specific marker enzymes (Figure 8). The mitochondrial, peroxisomal, and chloroplastic peak fractions were electrophoresed with polyacrylamide gels as before. Only the mitochondrial peak fraction contained a D-lactate dependent:NBT protein band, which was not active with glycolate.

F. Attempts to Assay the D-Lactate Activity Spectrophotometrically

An ammonium sulfate preparation from Chlamydomonas reinhardtii with activities of DCPIP reduction of 30 $nmol \cdot min^{-1} \cdot ml^{-1}$ with glycolate or 21 $nmol \cdot min^{-1} \cdot ml^{-1}$ with D-lactate had only slight rates of activity at 560 nm with NBT under similar assay conditions as for DCPIP. The rate with D-lactate was only 70% of the rate with glycolate, in the presence or absence of PMS. NBT and PMS alone or together inhibited both glycolate and D-lactate activities in the DCPIP assay to the same degree (Table 7).

A mitochondrial sucrose gradient fraction from spinach leaves which had a positive "D-lactate band" (fraction 8, Figure 8) was

Figure 8

Sucrose density gradient centrifugation of spinach organelles.

Chlorophyll was measured by its extinction coefficients at 664 and 647 nm (chlorophyll a+b = 6.31 E_{664} + 18.59 E_{647}). Catalase and cytochrome c oxidase were assayed as in Tolbert (53).

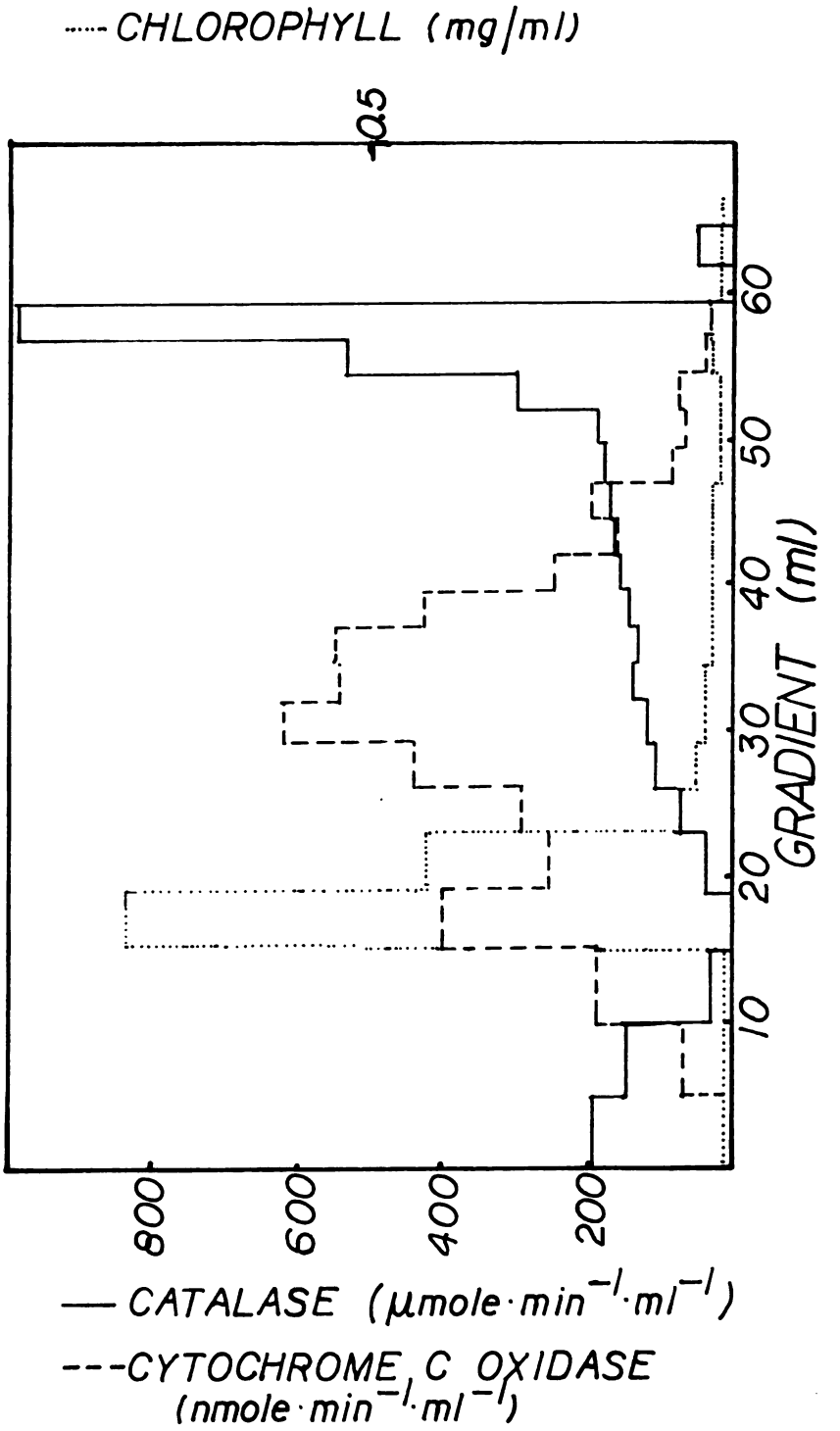

CHLOROPHYLL (mg/ml)
0.5
60
50
40
30
20
10
GRADIENT (ml)
800
600
400
200
CATALASE (μmole·min⁻¹·ml⁻¹)
CYTOCHROME C OXIDASE
(nmole·min⁻¹·ml⁻¹)

Figure 8

TABLE 7

Effect of NBT on DCPIP Assay for Glycolate Dehydrogenase from Chlamydomonas reinhardtii

Substrate	No Addition	NBT	PMS	NBT and PMS
		$nmol \cdot min^{-1} \cdot mg\ protein^{-1}$		
Glycolate	29.4 (100%)	23.4 (80%)	28.3 (96%)	23.3 (79%)
D-lactate	21.0 (71%)	20.7 (70%)	23.5 (80%)	20.2 (69%)

Numbers in parenthesis are the percentages of the glycolate rate with no addition. The concentration of NBT in the assay was 32 $\mu g \cdot ml^{-1}$, the PMS was 25 $\mu g \cdot ml^{-1}$.

assayed by various methods (Table 8). None of the procedures were successful in detecting this activity spectrophotometrically.

G. Lactate Production by Chlamydomonas reinhardtii

Attempts were made with the algae to produce lactate under anaerobic conditions as described by Warburg (45). In one experimental approach cells were prelabelled with large amounts of $H^{14}CO_3^-$ for 60 minutes, collected by centrifugation, resuspended in several media, and then incubated anaerobically in the light and dark with and without NH_4NO_3 for 4 hours. No lactate was detected by two dimensional descending paper chromatography of the cell suspension.

In another set of experiments substantial ^{14}C-labelled lactate was observed by chromatography of whole cells incubated with ^{14}C-HCO_3^- for 2 or 10 minutes. The cells had been resuspended in 3 mM P_i buffer at pH 7.5, at 2% w/v, and placed in a stirred vial in a circulating water bath and pre-illuminated for 2 min, after which $H^{14}CO_3^-$ was added to a 1 mM final concentration. At designated times of 2 to 20 minutes 1 ml aliquots were removed and centrifuged 30 sec in an Eppendorf microfuge. The supernatant and pellets were both stored in 50% methanol (Appendix). Aliquots were chromatographed by two dimensional chromatography (Appendix), and sprayed with 1 N $NaHCO_3$ before drying completely. The chromatograms were exposed to X-ray film for 2 weeks. The lactate spot was identified by its R_f (Figure 9). The dentification was further verified by eluting it with H_2O, and co-chromatographing it with known lactate. The position of the lactate was visualized with bromphenol blue in methanol, and the chromatogram

TABLE 8

Spectrophotometric Methods Tested for D-Lactate Dependent Activity with Spinach Mitochondria

Wavelength	Electron Acceptor
600 nm	0.13 mM DCPIP; ± 0.5 mM KCN and ± PMS
560 nm	2.1 mg NBT/ml; ± PMS
550 nm	1 mg of oxidized cytochrome c (horse heart)·ml^{-1} ± PMS
500 nm	0.2 mg·p-iodonitrotetrazolium violet·ml^{-1}
420 nm	1 mM ferricyanide
388 nm	1.4 mg PMS·ml^{-1}
340 nm	0.25 mg NAD^{+}·ml^{-1}

The fraction exhibited D-lactate dehydrogenase activity on polyacrylamide gels. These fraction assays were run in 1 ml cuvettes with 0.7 ml of 50 mM KPP_i pH 8.7, and the final concentrations of electron acceptor as indicated. Reactions were initiated with 10 mM D-lactate. None of the reactions gave a rate with any protein preparation.

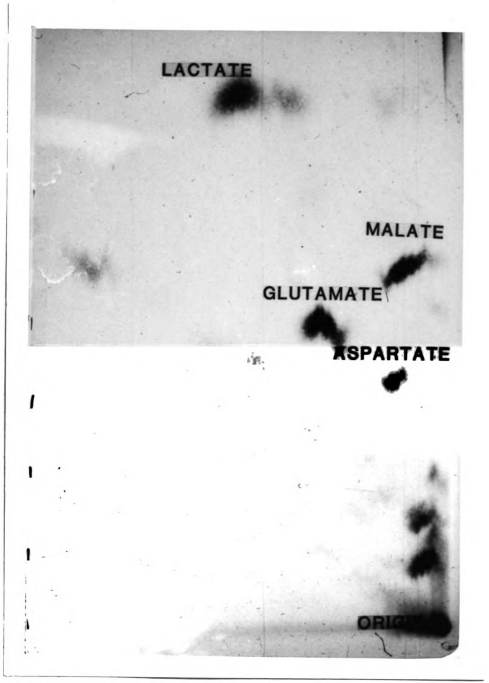


Figure 9

Chromatogram of ^{14}C-labelled products from Chlamydomonas reinhardtii after 10 min of photosynthesis with $NaHCO_3$.

was exposed to X-ray film again to locate the radioactive spot. The percentage ^{14}C in lactate was extremely high after only two minutes of $^{14}CO_2$ fixation (Table 9). The percentage ^{14}C in lactate decreased with time as ^{14}C moved or accumulated into other products of photosynthesis. The lower percentage of ^{14}C in lactate with AOA is not understood (see Appendix), but part of the decrease can be accounted for by a large increase in the percentage ^{14}C in glycolate with AOA treatment.

H. Conclusions

The glycolate dehydrogenase in extracts of Chlamydomonas after electrophoresis on the acrylamide gel was visualized by the NBT activity stain as a band near the top of the gel at an R_f of 0.33. An ammonium sulfate precipitated protein (30-45% saturated) preparation from Chlamydomonas cells that contained glycolate dehydrogenase had a band on polyacrylamide native gels at an R_f of 0.45 with D-lactate but did not react with glycolate as substrate. This band near 0.45 with D-lactate was also present in spinach leaves and appeared to be present in their mitochondria. The algal preparations, when assayed by the standard DCPIP assay used for glycolate dehydrogenase, were about equally active with D-lactate as with glycolate. It has always been assumed that both activities from the algae were due to the same enzyme, since they were not additive, became lost at the same time, and co-purified.

TABLE 9

Lactate Production by *Chlamydomonas reinhardtii* Grown with Air and High CO_2

		Lactate (% of the total 14-C fixed in soluble products in the cells)
Air-grown		
control	2 min	26
	10 min	15
1 mM AOA	2 min	17
	10 min	9
5% CO_2 grown		
control	2 min	51
	10 min	35
1 mM AOA	2 min	0
	10 min	15

CHAPTER III

PHOSPHOGLYCOLATE PHOSPHATASE IN CHLAMYDOMONAS REINHARDTII

LITERATURE REVIEW

The P-glycolate produced by the reaction of ribulose-P_2 with O_2, mediated by the ribulose-P_2 carboxlyase/oxygenase, is dephosphorylated by a specific phosphatase. This enzyme, P-glycolate phosphatase (EC 3.1.3.18), has been characterized from tobacco, spinach and pea leaves. In these leaves it is located in the chloroplasts. It was first reported by Richardson and Tolbert (54) in tobacco leaves. It was purified by ammonium sulfate fractionation, calcium phosphate gel absorption, and a DEAE-cellulose chromatography with batch elution. The protein was purified 100-fold over the crude tobacco sap. The enzyme was specific for P-glycolate. It was also inhibited by EDTA, and stimulated by divalent cations, particularly Mg^{++}. The pH activity profile of the enzyme varied with the metal ion included in the assay. With no cofactor it was most active at pH 6.3, and Co^{++}, Zn^{++}, Mn^{++} and Cu^{++} shifted the optimum downwards. Zn^{++} and Co^{++} gave the best activity with an optimum of pH 5. The stoichiometry was consistent with the hydrolysis of P-glycolate to P_i + glycolate. In 1968 Anderson determined the K_m(P-glycolate) to be 0.075 mM (55).

In 1978 Christeller and Tolbert purified the enzyme 1940-fold from tobacco leaves by acetone fractionation, DEAEcellulose chromatography, Sephadex G-200 chromatography and preparative polyacrylamide

gel electrophoresis (56). The enzyme had an approximate molecular weight of 81 to 86 kilodaltons, and was a tetramer. It had a remarkably low pI of 3.8. The enzyme from spinach was less stable than the tobacco enzyme, and was inactivated by lipase. Citrate and isocitrate stabilized both enzymes. Other properties of the enzyme were characterized (57,58).

In 1979 and 1980 Verin-vergeau et al. reported two isoenzymes of P-glycolate phosphatase in Phaseolus leaves on DEAE-cellulose chromatography (59,60). The isozymes had different pI's by isoelectric focussing. They both had pH optima at pH 6.8, and both were specific for P-glycolate with K_ms of 2 mM and 0.2-0.9 mM. They required divalent metal ions for activity, with Mg^{++} and Co^{++} being the most effective. They were activated by 20 mM ADP. The isozymes were differentially inhibited by various metabolites. One isozyme was inhibited by serine, glycolate, 6-P-gluconate, fructose-6-P, and formate. The other was inhibited by alanine, glutamate, and fructose-6-P. At P-glycolate concentrations greater than 0.4 mM the enzyme was inhibited by glycine, glycolate and glyoxylate.

It was not known if the P-glycolate phosphatase of green algae is similar to that of higher plants since the different metabolic fate of its product, glycolate, is different in the two types of plants. In higher plants the phosphatase is located in the stroma of the chloroplast and the glycolate must exit the chloroplast after P-glycolate hydrolysis. A similar situation must also be true with green algae. The glycolate ultimately ends up outside the cell, but it is not known if the phosphatase is involved in its transport. Since the glycolate

oxidizing systems in plants and algae are not the same, there could be other differences also in the P-glycolate phosphatase.

RESULTS

A. Assay of P-Glycolate Phosphatase

This enzyme was previously assayed in cacodylate buffer (54). Rather than use this poisonous buffer, Tris-maleate was substituted, and it gave the same activity. The assay procedure was developed with P-glycolate phosphatase prepared from pea leaves. The following procedure was used: the reaction was started with 5 mM P-glycolate (tricyclohexylamine salt), and was run in final concentrations of 20 mM Tris brought to pH 6.3 with maleate and 2 mM $MgCl_2$. The reaction was assayed at 30°C in a water bath and was stopped with 0.14% TCA. Phosphate was determined by a modified Fiske-SubbaRow procedure (61). The assay procedure was linear with time and proportional to enzyme concentration.

The previous work in this laboratory by Richardson and Tolbert (54), and by Christeller and Tolbert (56,57), on the enzyme from tobacco had shown that a divalent cation was needed for activity. To see if cation had any effect on the crude algal enzyme in the 0.3% deoxycholate extract, extract that had been treated with 0.25 mM EDTA and then dialyzed, or untreated extract as assayed. The dialyzed extract was fairly active without added Mg^{++}, but it was stimulated 1.7-fold by 5 mM $MgCl_2$. The undialyzed enzyme was stimulated slightly (1.3-fold). The result could be due to no absolute requirement for Mg^{++}, or to not enough EDTA to chelate all divalent cations present, but most likely the naturally occurring cations were too tightly bound

to be removed by dialysis. For the rest of this work 2 mM $MgCl_2$ was added to all reactions, unless otherwise noted.

The previous work on tobacco enzyme had noted that the enzyme had an acidic pH optimum, somewhere around pH 6.3, although this varied due to the particular cation used. In my work with Chlamydomonas, a pH optimum of around 8 was seen in the crude deoxycholate extract. The pH had to be measured after the reaction for each point, since the P-glycolate (tricyclohexylamine salt), even though it was neutralized, had some buffering capacity, and affected the pH. The difference in pH optimum of 8 for the algal enzyme and 6.3 for the tobacco enzyme could be due either to a difference in the enzyme or to cations tightly bound to the enzyme. Therefore, the algal enzyme was assayed in 20 mM Bicine at pH 8.0 in the crude extract.

B. Extraction of P-Glycolate Phosphatase from Chlamydomonas Cells

Extraction in 0.3% deoxycholate was at least as efficient as several other methods of solubilizing this activity (Table 1). The time course of solubilization was also determined for 0.3% deoxycholate, 1% Triton X-100, 1 M KCl, and 1% Triton X-100 + 1 M KCl. In all these cases the majority of the activity was liberated by 10 minutes, and no more activity was released after 30 minutes.

C. Purification of P-Glycolate Phosphatase from Chlamydomonas reinhardtii

Glycolate dehydrogenase and P-glycolate phosphatase were differentially fractionated with ammonium sulfate. Procedures involving acetone precipitation, which can be used to purify the tobacco enzyme (56), inactivated the algal enzyme. The optimum method was found to

be as follows: the cold 0.3% deoxycholate extract of Chlamydomonas cells was brought to 30% saturation in ammonium sulfate by the addition of the appropriate volume of 100% saturated ammonium sulfate previously adjusted to a neutral pH. This was stirred for 15-30 min in the cold, and then allowed to sit for 15-30 min before centrifuging the precipitate, which was discarded. The supernatant was brought to 45% saturation with ammonium sulfate in the same manner. The precipitate was used for glycolate dehydrogenase preparations. It contained 88% of the glycolate dehydrogenase and only 3% of the P-glycolate phosphatase. The supernatant was brought to 60% saturation in ammonium sulfate and centrifuged. The precipitate contained 96% of the P-glycolate phosphatase and 5% of the glycolate dehydrogenase. The pellets were resuspended in distilled water.

The P-glycolate phosphatase fraction was desalted by passing it over a short G-50 Sephadex column with 10 mM Tris-citrate, pH 7.2, in an effort to retain any factor which might be dialyzed out (next section). Despite this precaution, 26% of the activity was lost in this step, which was similar to the amount lost in dialysis (Table 10).

The enzyme was then passed over a DEAE-cellulose column equilibrated in the same buffer. The column was washed extensively, and the activity eluted with a gradient of the buffer from 10 to 250 mM Tris with the pH adjusted to 7.2 with citrate (Figure 10). Two isozymes were never observed. Since this procedure did not separate the activity from the main protein peak, a shallower gradient of 10-35 mM Tris-citrate was used (Figure 11). Although the latter gradient did

TABLE 10

Purification of P-Glycolate Phosphatase
from Chlamydomonas reinhardtii

Step	$\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein	yield %
(whole cells)	(0.012)	---
0.3% Deoxycholate extract	0.022	(100)
45-60% Ammonium sulfate precipitate	0.8	100
G-50 Sephadex	0.6	64
DEAE-cellulose	2.8	8

The specific activity in the "whole cells" is the value obtained from a sonicated extract.

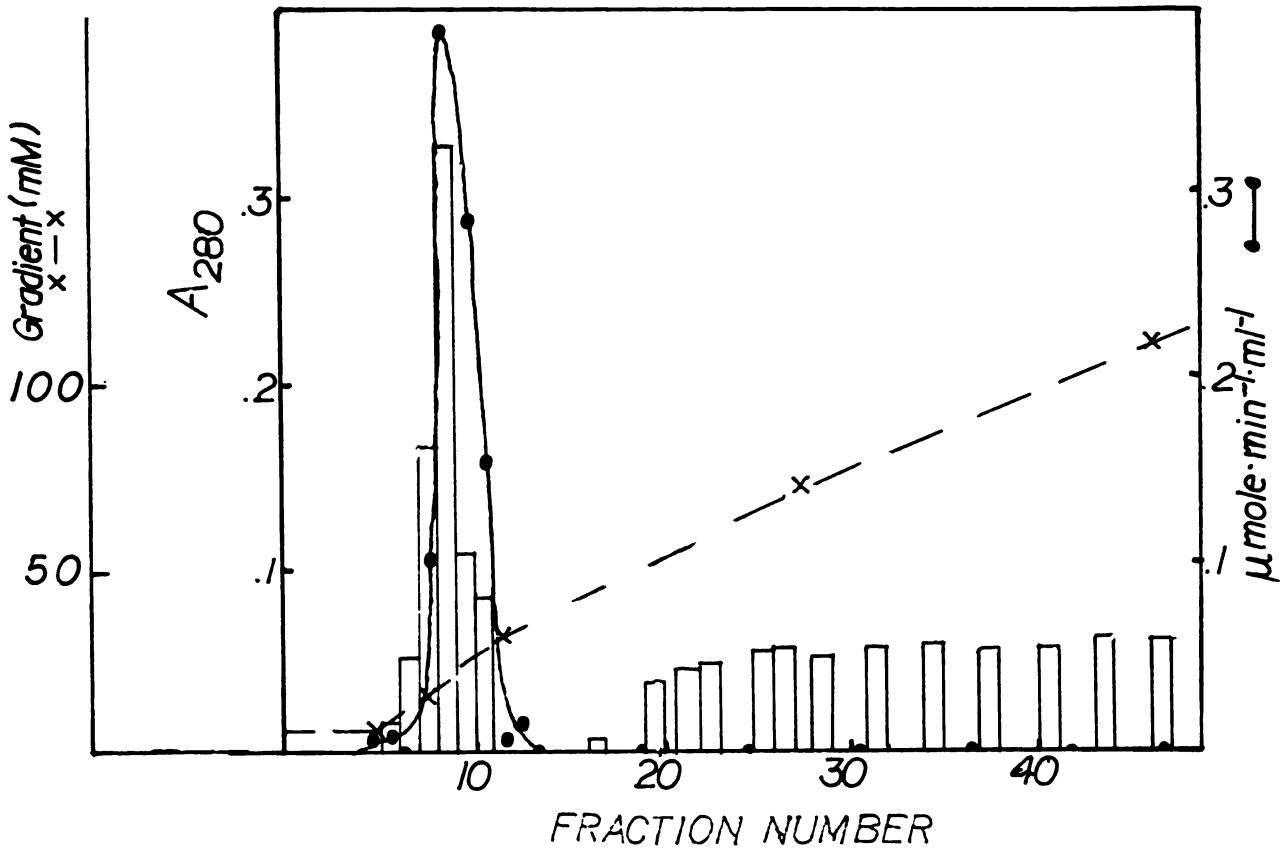


Figure 10

DEAE-cellulose fractionation of P-glycolate phosphatase from Chlamydomonas reinhardtii.

The column was 1.2x2.5 cm and was equilibrated with 10 mM Tris-citrate pH 7.2. The sample was an ammonium sulfate fraction re-suspended in water and was diluted before application to the column. The protein was eluted with a gradient of 10-250 mM Tris-citrate. ●—● activity of P-glycolate phosphatase; bars represent the amount of protein (A_{280}); and x—x the concentration of buffer in the fractions. The end of the gradient is not shown. Salt was measured on a conductivity meter and compared with standard concentrations.

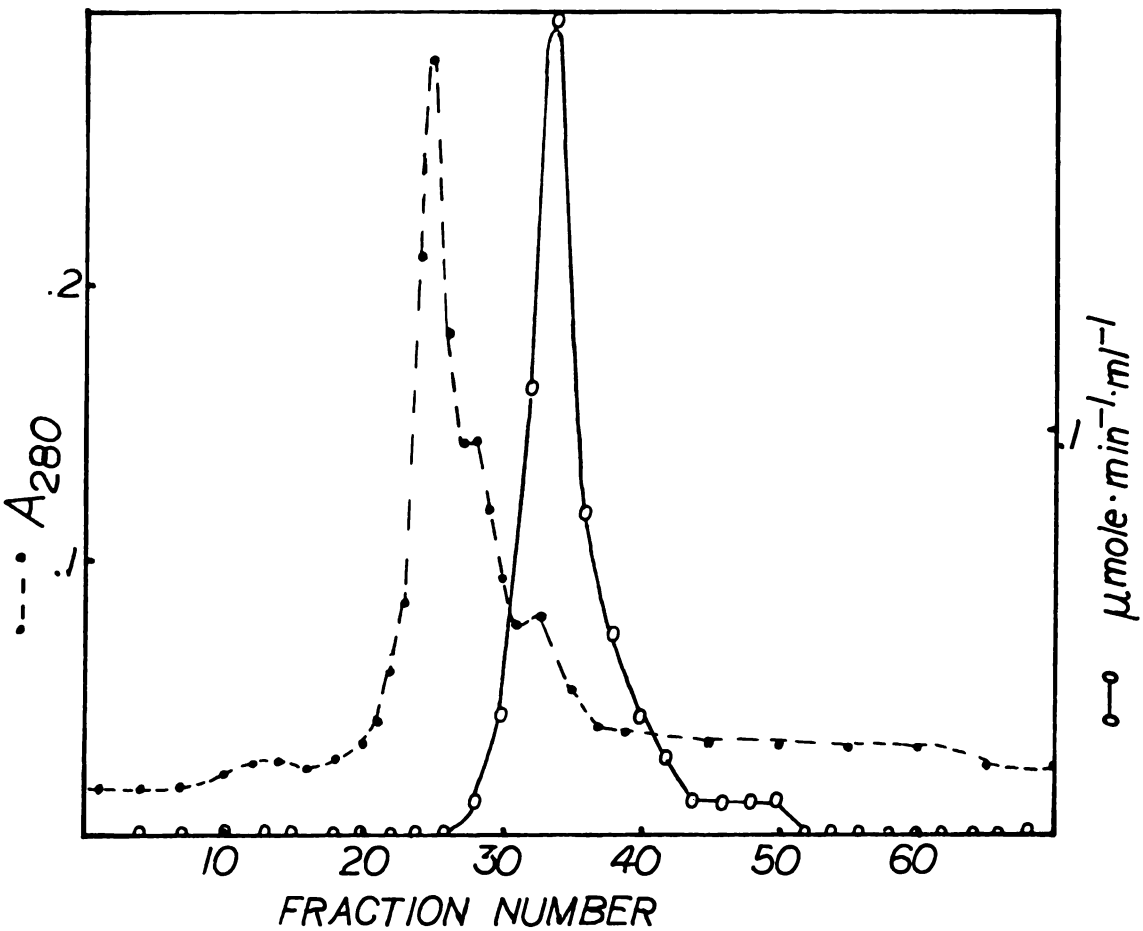


Figure 11

Revised DEAE-cellulose fractionation of P-glycolate phosphatase from Chlamydomonas reinhardtii.

This column was identical to that in Figure 10, except that it was developed with a gradient of 10-35 mM Tris-citrate. o—o P-glycolate phosphatase activity, •–• protein concentration (A_{280}).

separate the phosphatase with a 4-fold purification, most of the activity was lost (Table 10).

Other methods were tried to purify this phosphatase without success. This included having 2 mM $MgCl_2$ present throughout the DEAE-column chromatography. Sephadex G-200 chromatography caused a 99% loss in activity from the previous step. The enzyme activity does not bind to carboxymethyl-cellulose column with 10 mM Bicine at pH 8.0 with a 60% loss of activity. Hydroxylapatite chromatography in Hepes buffer also did not work. A similar failure to purify the P-glycolate phosphatase due to loss of activity has been experienced with the enzyme from tobacco leaves (56).

D. Search for Isozymes of P-Glycolate Phosphatase from Chlamydomonas reinhardtii

It has been reported that there are two isozymes of P-glycolate phosphatase in pea leaves (59). These forms had been separated both by DEAE-cellulose chromatography and by polyacrylamide gel electrophoresis. DEAEcellulose chromatography in a Tris-citrate buffer from 10 to 250 mM was tried on $(NH_4)_2SO_4$ fractions from Chlamydomonas extracted by both sonciation and detergent treatment. Both times one major peak of activity was found halfway into the gradient and sometimes a tiny amount of activity was seen at the end of the gradient, that had disappeared by the time the fractions were reassayed (Figure 10). If there were two isoenzymic forms, one was present in vanishingly small amounts. Polyacrylamide gels (7%) were run on fractions from both column experiments, and on the material applied to the columns, as well as on the main peak. The material from the possible

second peak was also used, but no activity was seen on the gradients. The gels were stained for protein by Coomassie blue, and for phosphatase activity by lead precipitation of the liberated phosphate from P-glycolate (56). In all cases only one band at an R_f of 0.4 was visualized by the activity stain. The phosphatase was not the major protein band in those gels run on the DEAE-cellulose pools.

E. pH Profile of Activity of P-Glycolate Phosphatase from *Chlamydomonas reinhardtii*

The deoxycholate extract of *Chlamydomonas* cells grown with air had a pH optimum at pH 8, which was not the same as that for the enzyme from pea, spinach, or tobacco leaves (see section A). The ammonium sulfate precipitate had a pH optimum of 8.7, which was the same as that for glycolate dehydrogenase. When the $(NH_4)_2SO_4$ fraction was dialyzed against 2 mM $MgCl_2$, the activity at pH 8.7 was about 60% lost (Figure 12) and a broad pH profile was obtained for the remaining activity with an apparent peak between pH 7 and 8. This change during dialysis occurred in the presence of added $MgCl_2$. In the absence of $MgCl_2$ during dialysis, the same pH shift occurred (Figure 13), without much loss of maximum activity. The maximum activity was, however, less without $MgCl_2$ and in the range seen in the sample dialyzed with $MgCl_2$. The pH profile of the low remaining enzyme activity after DEAE chromatography was also assayed with Mg^{++}. Activity extended over a broad range between 6.5 to 8.7 (Figure 14), and was similar in both dialyzed and undialyzed samples. Whatever factor or reason for the activity maximum to peak in the basic range seems to have been lost from the enzyme either by dialysis or chromatography on DEAE-cellulose.

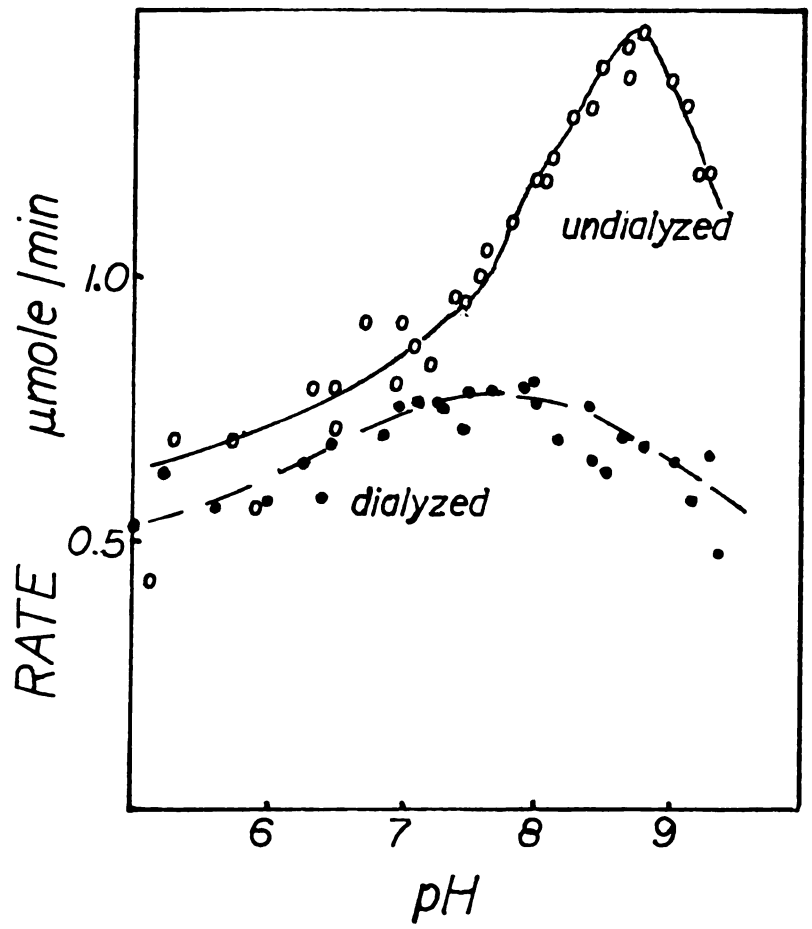


Figure 12

The pH profile of the ammonium sulfate fraction of P-glycolate phosphatase from Chlamydomonas reinhardtii before and after dialysis assayed with 5 mM $MgCl_2$.

The buffers used were 50 mM succinate, Bis-tris, Hepes, Bicine, and Ches.

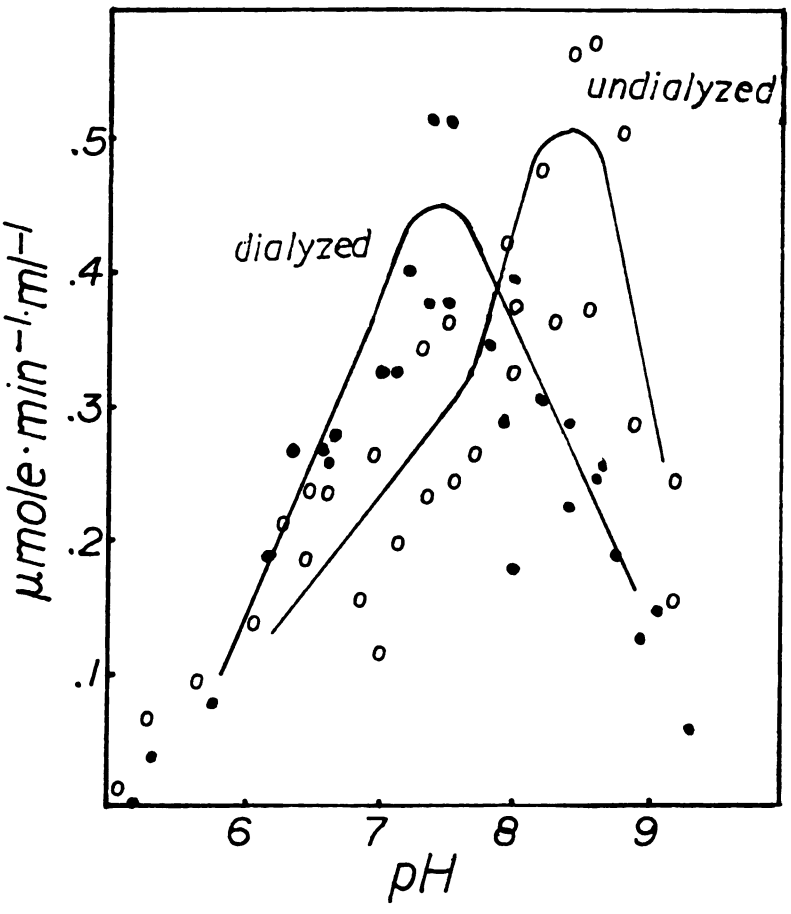


Figure 13

The pH profile of the ammonium sulfate fraction of P-glycolate phosphatase from Chlamydomonas reinhardtii before and after dialysis assayed without $MgCl_2$.

Conditions are the same as in Figure 12.

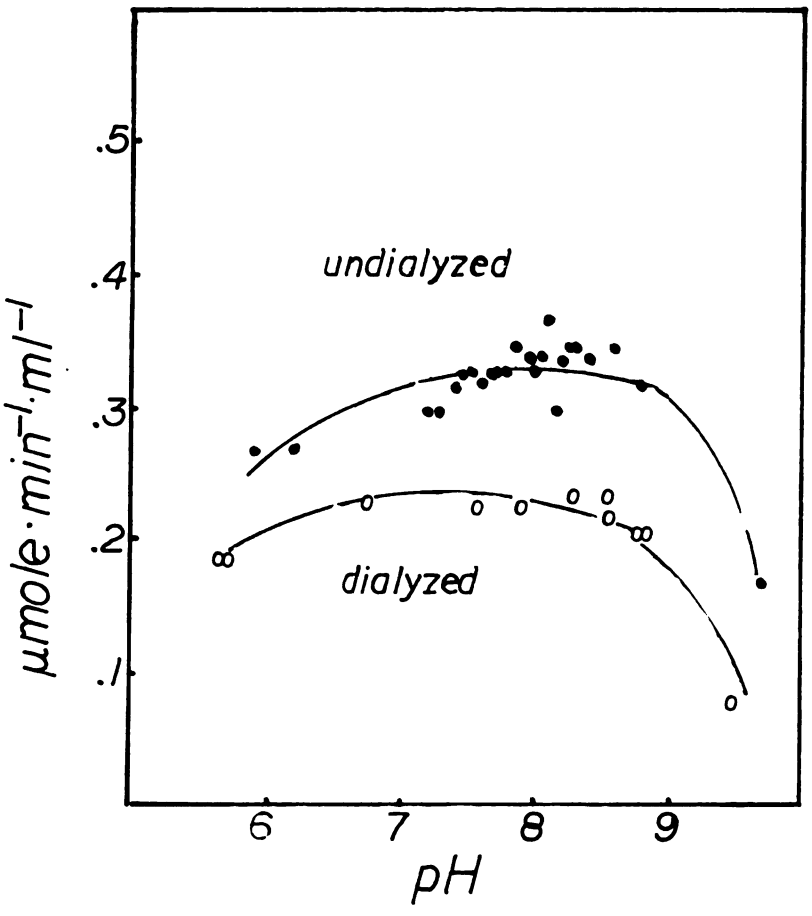


Figure 14

The pH profile of fractions from the DEAE-cellulose fractionation of P-glycolate phosphatase from Chlamydomonas reinhardtii before and after dialysis with 5 mM $MgCl_2$.

Conditions are the same as in Figure 12.

Although citrate shifted the pH optimum of the higher plant enzyme (56), added 1 mM citrate had no effect on the ammonium sulfate fraction after dialysis with Mg^{++}.

The ammonium sulfate fraction containing the P-glycolate phosphatase from high CO_2 grown cells was also tested. There was no difference in the enzyme purification characteristics from the air grown cells, and the pH profiles were the same.

F. Substrate Specificity of P-Glycolate Phosphatase

There existed the possibility that what was being assayed was not a specific P-glycolate phosphatase as exists in higher plants, but alkaline or acid phosphatases with residual activity with P-glycolate. However, the activity of the phosphatase was specific for P-glycolate at pH 8 (Table 11). No acid phosphatase would be detectable, because the assays were run above that pH range.

In Figure 15 are the pH profiles for the various phosphatases present in the ammonium sulfate fraction from air grown Chlamydomonas. The peak of P-glycolate phosphatase activity between pH 8 and 9 was not due to alkaline phosphatase, which utilizes p-nitrophenyl-P (Figure 15a). There was an acid phosphatase which corresponded to a D-3-P-glycerate phosphatase (Figure 15b), but it had its maximum activity at or below pH 5 and could not contribute to P-glycolate hydrolysis above pH 7. The activity with ATP was negligible across the pH range. A peak of activity with pyrophosphate was seen at pH 7.5 which disappeared upon dialysis and was dependent on Mg^{++}. The P-glycerate activity was also dependent on Mg^{++}, but neither pyrophosphate, ATP, o-carboxyphenyl-P or p-nitrophenyl-P were sensitive to Mg^{++} or dialysis.

TABLE 11

Substrate Specificity of P-Glycolate Phosphatase from Chlamydomonas reinhardtii

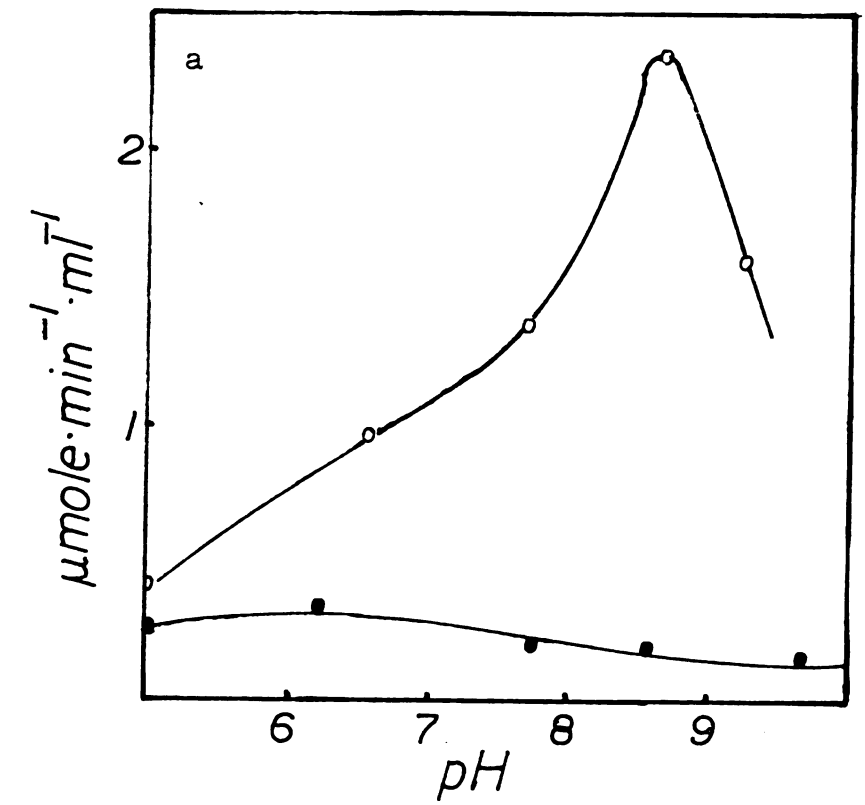
Substrate	Relative Activity %
P-Glycolate	100
D-3-P-glycerate	0
β-Glycerol-P	0
Fructose-6-P	0.8
6-P-gluconate	0
o-carboxyphenyl-P	4.4
ADP	0
p-nitrophenyl-P	2.6
Potassium Pyrophosphate	1.0
ATP	0

Assayed with 0.6 units of a partially purified enzyme (DEAE pool). All substrates were 30 mM. Assays were for P_i release in 20 mM Bicine at pH 8.0.

Figure 15

The pH profiles of phosphate released from several substrates assayed with an ammonium sulfate fraction from Chlamydomonas reinhardtii.

a) o–o is activity with P-glycolate, and •–• is with the alkaline phosphatase substrate, p-nitrophenyl-P. b) o-o is activity with 3-P-glycerate, and •-• is with the acid phosphatase substrate, o-carboxyphenyl-P.



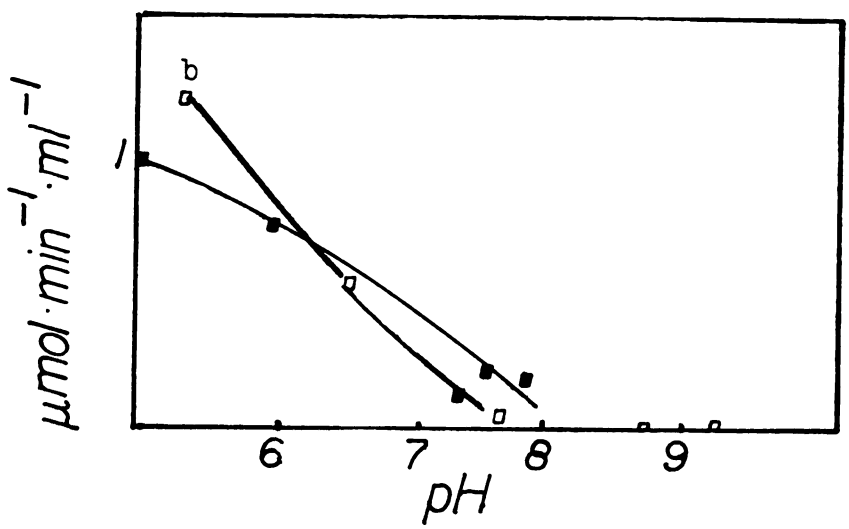


Figure 15

G. Attempts at Reactivation of P-Glycolate Phosphatase

Because P-glycolate phosphatase activity was lost after dialysis or DEAE-cellulose chromatography (Table 10), the possibility of the loss of an essential cofactor was explored. Various compounds were tested for their effect on enzyme activity in an ammonium sulfate fraction, a Sephadex G-50 preparation, and a dialyzed ammonium sulfate fraction of P-glycolate phosphatase from air grown Chlamydomonas. 1 mM DTT, 1 mM isocitrate, and 1 mM L and DL proline had no effect. Dialysate, with and without EDTA when added back to the enzyme, had no effect.

An ammonium sulfate precipitate was resuspended in 10 mM EDTA and dialyzed against 20 mM Bicine at pH 8.0 with 9 mM EDTA, and then dialyzed extensively against Bicine without EDTA. It was then assayed with various divalent cations which activate the higher plant enzyme. None of the cations tested worked as well as $MgCl_2$. The optimum Mg^{++} concentration was between 0.5-5.0 mM (Table 12).

It can be seen from Figure 16 that 100 mM KCl stimulated the P-glycolate phosphatase activity slightly, but was inhibitory at higher concentrations. Ammonium sulfate at a similar concentration also stimulated activity. P-glycolate phosphatase from blood is markedly stabilized by KCl (62).

H. Stability of P-Glycolate Phosphatase

The more purified enzyme, past the ammonium sulfate preparation step, was not stable to storage when frozen. Also the crude enzyme preparations could be stored only a week, but the loss of activity in the crude and purified preparations could have been due to different

TABLE 12

Effect of Some Salts on Dialyzed P-Glycolate Phosphatase from Chlamydomonas reinhardtii

Addition	pH 8 $\mu mol \cdot min^{-1} \cdot ml^{-1}$	pH 8.4
No addition	0.05	0
5 mM $MnCl_2$	0.12	0.07
5 mM $ZnCl_2$	0.09	0.02
5 mM $CoCl_2$	0.15	0.08
5 μM $MgCl_2$	0.14	0.01
50 μM $MgCl_2$	0.54	0.48
0.5 mM $MgCl_2$	0.73	0.72
5 mM $MgCl_2$	0.84	0.72
50 mM $MgCl_2$	0.67	0.47

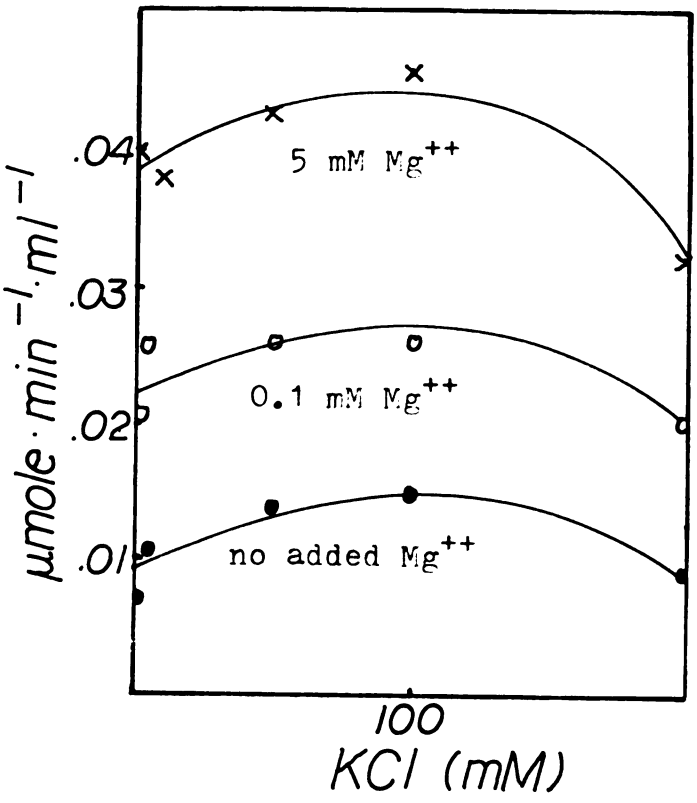


Figure 16

The effect of increasing ionic strength on P-glycolate phosphatase from Chlamydomonas reinhardtii.

causes. Since it has been reported that the tobacco enzyme was stabilized by citrate (56), citrate was tried as a stabilizer for the enzyme from Chlamydomonas. Neither 10 mM citrate or isocitrate slowed down the loss of activity at 50°C of an ammonium sulfate fraction at pH 8.0, with 2 mM $MgCl_2$. The ½ time of inactivation was 4 min. These acids also did not prevent loss of activity on purification when present in buffers.

Other compounds were tested for their effect on the storage of the enzyme and its stability to freeze-thawing (Table 13). Aliquots of a DEAE-cellulose pooled enzyme fraction frozen in the presence of various compounds was later assayed for activity. With no additions, 45-65% of the activity was lost after freeze-thawing. Including 5 mM DDT not only seemed to inhibit the reaction, it also caused a loss of most of the activity on storage. Both 2.5 mM $MgCl_2$ and 25% glycerol protected the activity with glycerol being slightly more effective. In the presence of 2% BSA, activity was slightly stimulated, and was not lost on freeze-thawing. The activity actually increased with ammonium sulfate over the course of the experiment.

TABLE 13

Stability of P-Glycolate Phosphatase from *Chlamydomonas reinhardtii* to Freeze-thawing

Treatment	before	Freeze-thaw 1	2
none	100% (100%)	34% (54%)	-- (53%)
25% Glycerol	186% (90%)	127% (83%)	115% (107%)
5 mM DTT	66% (47%)	5% (7%)	5% (4%)
60% Saturated $(NH_4)_2SO_4$	85% (78%)	144% (108%)	212% (118%)
2.5 mM $MgCl_2$	127% (81%)	97% (65%)	107% (82%)
10 mM citrate	129% (79%)	107% (51%)	90% (65%)
1 mg/ml BSA	202% (133%)	132% (113%)	132% (121%)

Activities are expressed in % relative to the control from the first day of the experiment. The treatments were done with an enzyme after a DEAE cellulose chromatography had 0.06 units/ml. In a second experiment (results in parentheses) the preparation had 0.07 units/ml.

CHAPTER IV

NADPH:GLYOXYLATE AND NADH:HYDROXYPYRUVATE REDUCTASES FROM *CHLAMYDOMONAS REINHARDTII*

LITERATURE REVIEW

Glyoxylate reductase activity was first described by Zelitch and Ochoa (63) in 1953 in extracts of spinach leaves. These extracts used glyoxylate to oxidize NADH and the enzyme was named by them "glyoxylate reductase" (EC 1.1.1.26). About the same time Vennesland's group (64) described an activity with NADH which converted hydroxypyruvate to D-glycerate, which they called D-glycerate dehydrogenase. This enzyme was purified to a crystalline form from both spinach and tobacco leaves and found to use only NADH at pH 6.5. In 1962 Zelitch and Gotto (65) showed that two activities, one for NADH and one for NADPH could be separable by ammonium sulfate fractionation. The NADH-linked activity had a high K_m for glyoxylate, but the NADPH-linked activity had a lower K_m for this substrate. Both activities from spinach or tobacco leaves had a pH optimum at around 6-6.5. In 1970 the properties of spinach leaf "glyoxylate (NADH) reductase" were extensively described in a series of papers from Kohn's lab (66,67, 68,69). The crystalline enzyme is available commercially.

In 1970 Tolbert *et al.* (70) showed that the two activities were located separately in the spinach leaf. The "glyoxylate (NADH) reductase" activity was located in the peroxisomes and was more specific

for hydroxypyruvate (K_m = 0.12 mM) than for glyoxylate (K_m = 15-35 mM) and was also specific for NADH. The NADPH activity was located in the chloroplasts and was not active with hydroxypyruvate. It had a K_m for glyoxylate of 0.13 mM. They suggested that the peroxisomal activity previously called "glyoxylate reductase" be renamed "hydroxypyruvate reductase" (or D-glycerate dehydrogenase) and that the name "glyoxylate reductase" be reserved for the chloroplastic NADPH specific activity. This is the nomenclature that will be used here.

In spinach leaves the activity of the hydroxypyruvate reductase was 10 $nmol \cdot min^{-1} \cdot g$ of $tissue^{-1}$ with hydroxypyruvate as substrate, and the activity of glyoxylate reductase (NADPH) with glyoxylate was 0.2 to 0.3 $nmol \cdot min^{-1} \cdot g$ of $tissue^{-1}$ (70).

It is highly debatable whether any substantial glyoxylate pool remains in the peroxisome. It is a highly reactive substance and should be immediately removed by the specific transaminases present to form glycine (1). It if were not, it would immediately be decarboxylated with any excess H_2O_2. It has been argued that 10% of the glyoxylate is decarboxylated in this way in leaf tissue (71), and that this is an important source of photorespiratory CO_2. Glyoxylate is also the source of oxalate in plants, and it is well known (72) that spinach plants grown on poor nitrogen containing soil have more oxalate-salts presumably since there is insufficient NH_3 to transaminate glyoxylate to glycine. Others have suggested the accumulation of oxalate may be because of necessity to chelate metal ions and detoxify the plant (72).

In any case, in green unicellular algae such as Chlamydomonas, which do not have glycolate oxidase in their peroxisomes, and which

apparently metabolize very little glycolate to glyoxylate, the necessity for glyoxylate reductase cannot be explained in this way. When the algae are grown on low nitrogen containing media (32), they can simply excrete the excess glycolate. The NADPH:glyoxylate reductase would provide a mechanism for utilization of excess photoreducing power as NADPH, if a source of glyoxylate were present. These algae grow best in lower light intensities and so perhaps they are very sensitive to the effects of high light, in which excess reducing power may be generated.

The peroxisomal hydroxypyruvate reductase is thought to act in the oxidative photosynthetic carbon cycle to generate glycerate which can be phosphorylated and enter the reductive photosynthetic carbon cycle in the chloroplasts. This would return 3 of every 4 carbon atoms which left the chloroplasts as glycolate due to the unavoidable side reaction of ribulose-P_2 carboxylase/oxygenase with O_2.

The low activity of NADPH:glyoxylate reductase does not seem to have a very vital role, and there is no known source of glyoxylate in the chloroplasts. A terminal oxidase system has been proposed with a cycle between glycolate oxidase in the peroxisomes and glyoxylate reductase in the chloroplasts (1). Such a cycle would link NADPH oxidation to H_2O_2 production in the peroxisomes. No physiological evidence for such a cycle has been found. Yokota and Kitoaka (72) presented evidence that in Euglena both activities (glycolate dehydrogenase and glyoxylate NADPH reductase) were present in the mitochondria. They disrupted mitochondria and added both rotenone and glycolate and got NADPH oxidation. They took this as evidence for the shuttle in Euglena mitochondria. The glycolate oxidizing system is

different in Euglena from both higher plants and from algae and cyanobacteria as is discussed elsewhere.

Another hypothesis for the presence of glyoxylate reductase is as a scavenger for the highly reactive glyoxylate should it be formed by any mechanism in the sensitive chloroplast.

MATERIALS AND METHODS

Chlamydomonas reinhardtii were grown with air or 5% CO_2 and harvested as described in Chapter 1. The 0.3% deoxycholate extracts were prepared as described in Chapter 1. The ammonium sulfate fractionation and cellulose column preparation were also done as described previously. Both NADPH:glyoxylate reductase and NADH:hydroxypyruvate reductase were assayed at 30° and at 340 nm in a Beckman spectrophotometer in 1 ml total volume. The assay contained: 0.7 ml of 0.2 M K phosphate at pH 6.2, 0.05 ml of 5 mg NADPH/ml, enzyme and water to 0.9 ml, and the reaction was started with 0.1 ml of 20 mM sodium glyoxylate. Hydroxypyruvate reductase was assayed the same way, except NADH and lithium hydroxypyruvate were used.

RESULTS

A. Presence of Glyoxylate Reductase and Hydroxypyruvate Reductase in Chlamydomonas reinhardtii

There was approximately 0.4 $\mu mol \cdot min^{-1} \cdot mg\ chlorophyll^{-1}$ of NADPH:glyoxylate reductase in the algal extracts. Hydroxypyruvate reductase was about 10 times more active in these algae than the glyoxylate reductase in both air and CO_2 grown cells (Table 14). This difference was pronounced in the experiment shown, but was not always this great.

TABLE 14

NADPH:Glyoxylate Reductase and NADH:Hydroxypyruvate Reductase in Air and 5% CO_2 Grown *Chlamydomonas reinhardtii*

	Air-grown $\mu mol \cdot min^{-1} \cdot$ wet weight^{-1}	5% CO_2-grown
NADPH:Glyoxylate reductase	0.19 (0.02)*	0.8 (0.06)
NADH:Hydroxypyruvate reductase	1.3 (0.14)	1.3 (0.10)

*Numbers in parentheses are $nmol \cdot min^{-1} \cdot mg$ protein^{-1}.

A reproducible difference in the level of glyoxylate reductase between air and 5% CO_2 grown cells was observed. The high-CO_2 grown cells had 2-4 fold more NADPH:glyoxylate reductase than did the air grown cells.

B. Direction of Glyoxylate Reductase Assay

Glyoxylate reductase was extractable by the same 0.3% deoxycholate extract which contained glycolate dehydrogenase and P-glycolate phosphatase. An attempt was made to cause glycolate reductase to proceed in the reverse direction as a glycolate dehydrogenase to see if it could account for glycolate oxidation in Chlamydomonas. The reaction was tried at pH 9.5 in 0.1 M Ches buffer with 1 mg $NADP^+$ and 12.5 mM glycolate in 1 ml. No reaction was seen, and addition of phenylhydrazine did not establish a reaction rate.

In other ways glycolate dehydrogenase and NADPH:glyoxylate reductase appeared to be two different enzymes. Most of the glyoxylate reductase was found in the 45-60% saturated $(NH_4)_2SO_4$ precipitate compared with 30-45% for the glycolate dehydrogenase. These results do not rule out the possibility that glyoxylate reductase may act as a dehydrogenase in some unknown manner in vivo.

C. Assay Conditions for NADPH:Glyoxylate Reductase from Chlamydomonas reinhardtii

The conditions for assaying NADPH:glyoxylate reductase did not differ from those reported for the spinach enzyme (70). The pH optimum was at approximately pH 6.0 to 6.3 (Figure 17). Both substrates were inhibitory at high concentrations (Figures 18a and 19a), and the concentrations used for the standard assay were at the maximum of these curves (2 mM glyoxylate and 0.25 mM NADPH).

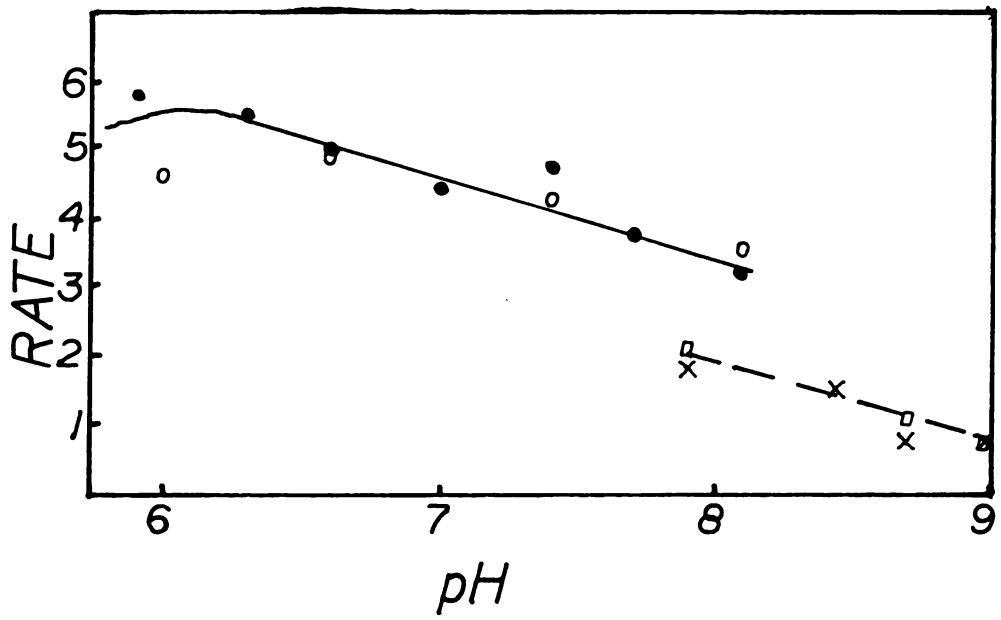


Figure 17

The pH profile of NADPH:glyoxylate reductase from Chlamydomonas reinhardtii.

The rates are relative. -o- 0.07 M KP_i; -●- 0.14 M KP_i; -□- 0.07 M KPP_i; -x- 0.14 mM KPP_i.

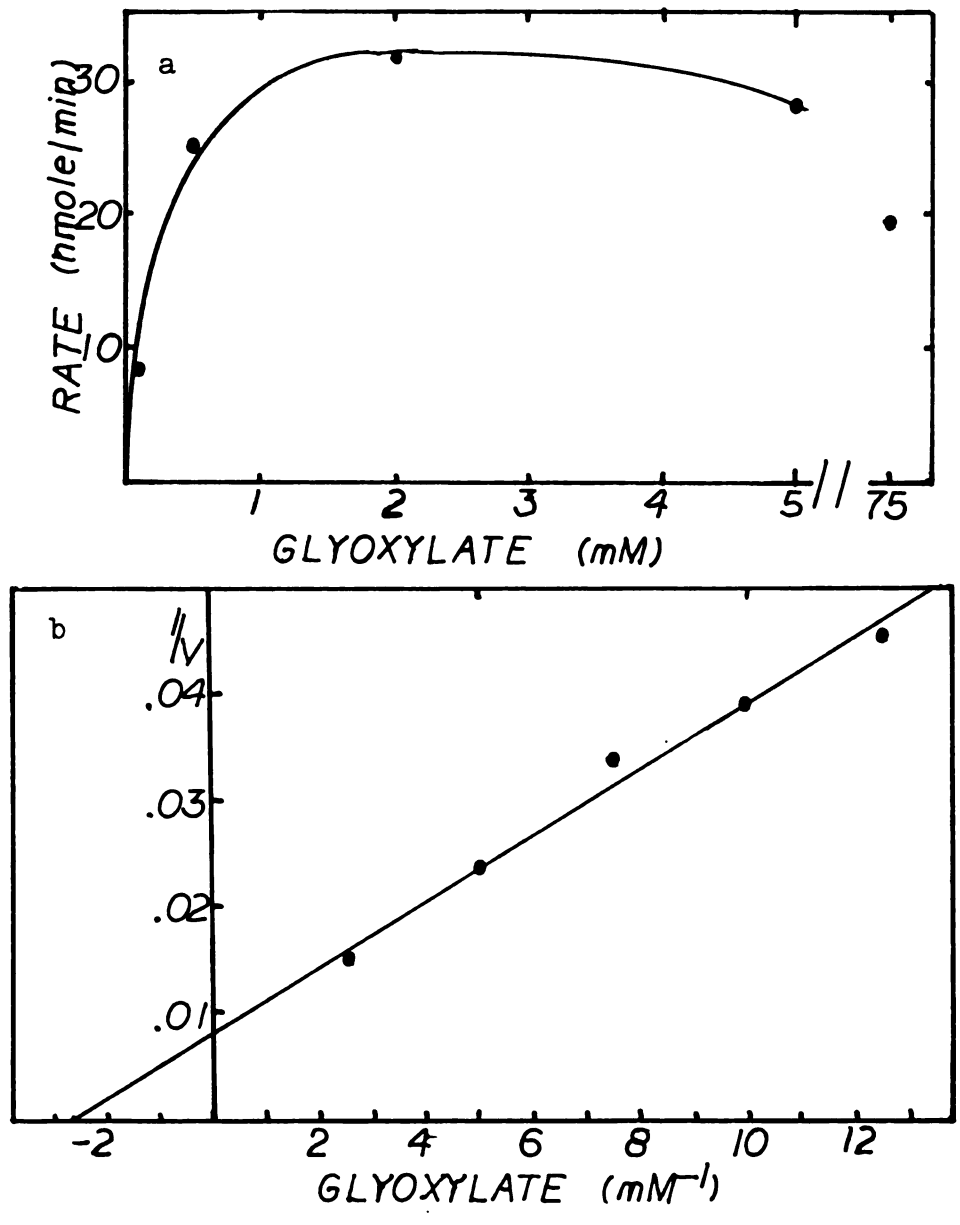


Figure 18

a. The rate of NADPH:glyoxylate reductase from Chlamydomonas reinhardtii with different glyoxylate concentrations.

b. Lineweaver-Burk plot for NADPH:glyoxylate reductase.

The concentration of NADPH was 0.245 mM.

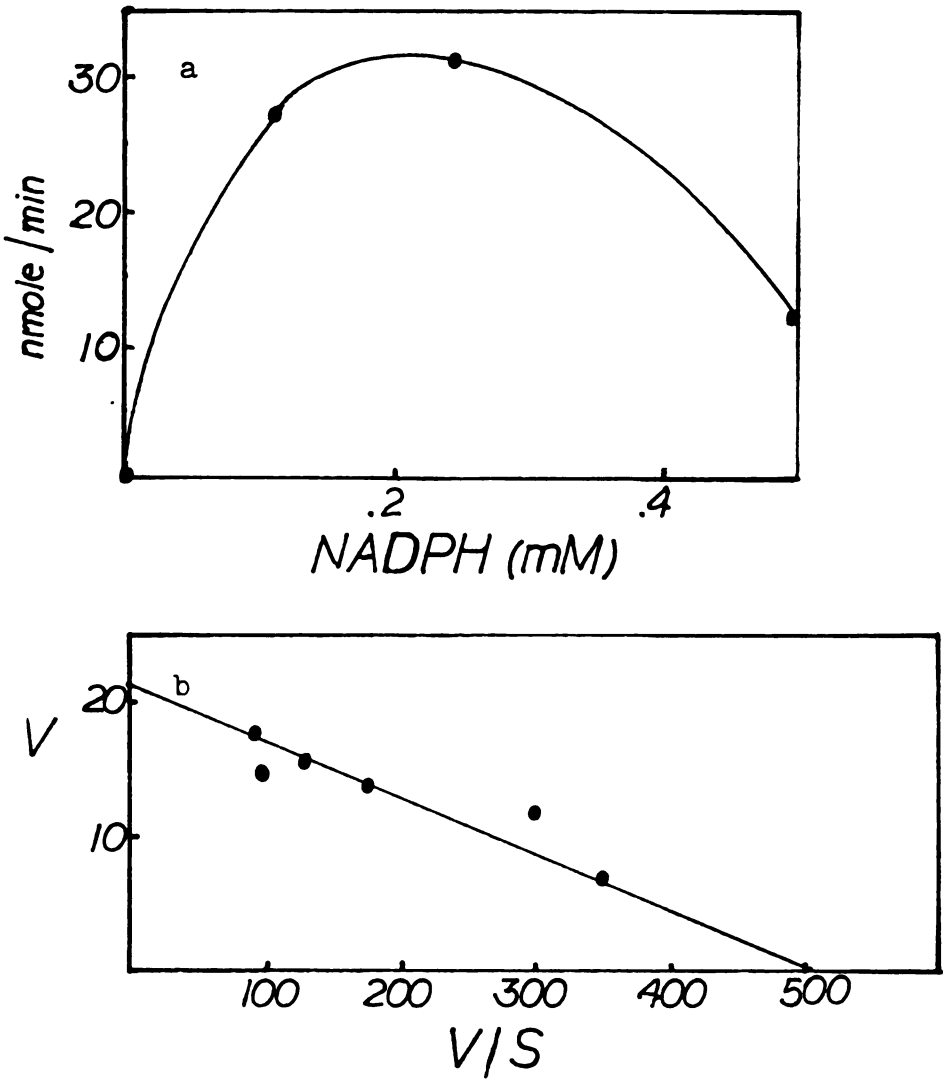


Figure 19

a. The rate of NADPH:glyoxylate reductase from Chlamydomonas reinhardtii with different NADPH concentrations.

The glyoxylate concentration was held at 2 mM.

b. Briggs-Haldane plot of NADPH:glyoxylate reductase.

The glyoxylate concentration was held at 1 mM.

The assay was proportional to the amount of enzyme added. In some cases the small rate of nonenzymatic oxidation of NADPH with glyoxylate present was significant relative to enzymatic rates and the endogenous rate without enzyme had to be subtracted. The non-enzymatic rate may amount to 1-3 $nmol \cdot min^{-1}$.

Concentrations of potassium phosphate up to 0.13 M did not cause any change in the activity (Figure 17).

D. Purification of NADPH:Glyoxylate Reductase from Chlamydomonas reinhardtii

The best method for solubilizing this enzyme from the cells was extraction with 0.3% deoxycholate, although 0.5% Triton X-100 was nearly as good (Table 15). Times between 20-60 min incubation with deoxycholate gave approximately equivalent results. These detergents were preferable to sonicating or using the French press to disrupt the whole cell because the chlorophyll and much of the protein in the cell was not solubilized.

The next step in the purification procedure after extraction from the algal cells was precipitation by 0.5% protamine sulfate of protein and nucleic acids which were discarded after centrifugation (Table 16). The supernatant was fractionated by ammonium sulfate precipitation, and the 45-60% pellet with the activity was resuspended in 5 mM glycylglycine buffer pH 8.7. The enzyme preparation was then dialyzed with several changes of a 50-fold dilution of the buffer. The hydroxypyruvate reductase activity was lost at this dialysis step. The glyoxylate reductase could not be absorbed onto DEAE-cellulose. A TEAE-cellulose column, equilibrated with the 5 mM glycylglycine

TABLE 15

Extraction Methods for NADPH:Glyoxylate Reductase from Chlamydomonas reinhardtii

Method	Activity $nmol \cdot min^{-1} \cdot g\ wet\ weight^{-1}$
Buffer only	3
0.5% Triton X-100	547
0.15% Deoxycholate	735
0.5 M KCl	153
1.0 M KCl	468
0.3% Deoxycholate	813

2-3 g of cells were resuspended in the extraction medium (6 mls) and at 0 min and subsequent times aliquots were removed and centrifuged in an Eppendorf Microfuge for 1/2 min. The supernatant was immediately removed and later assayed. Negligible activities were seen at 0 min. The time shown is the activity after 20 min.

TABLE 16

Purification of NADPH:Glyoxylate Reductase from Chlamydomonas reinhardtii

	Activity $\mu mol \cdot min^{-1} \cdot ml^{-1}$	Yield %	Specific Activity $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$	Ratio $\frac{280}{260}$	Fold Purification
Deoxycholate extract	0.15	100	0.11	0.7	1.0
Protamine Sulfate Supernatant	0.07	58	0.12	0.7	1.1
Ammonium Sulfate Precipitate	1.47	235	0.39	1.5	3.5
Dialysis	1.57	265	0.79	1.6	7.2
TEAE-cellulose Chromatography	0.50	142	7.14	1.3	64.9

buffer, was used to absorb the enzyme. It was eluted from the TEAE-cellulose column with a KCl gradient (Figure 20a).

Although glyoxylate reductase could be absorbed by and eluted from a hydroxylapatite column no purification was achieved due to a loss of activity. The activity also eluted at the void volume with most of the other proteins on a Sephacryl S-200 or on a Biogel A-0.5 M column. The glyoxylate reductase protein was retained on a Sepharose 4B column, but it still eluted with a major protein peak. Glyoxylate reductase acted as a protein of very large molecular weight (above 500,000 daltons), but this may be due to superficial agglomeration with other proteins or itself. A second smaller TEAE-cellulose column, with a shallower gradient, gave a better purification than that from the first column (Figure 20b). The activity eluted just before the main protein peak. A summary of the purification is in Table 16.

E. Kinetic Properties of NADPH:Glyoxylate Reductase from Chlamydomonas reinhardtii

The enzyme was inhibited at high substrate concentrations (Figures 18a and 19a). Lower concentrations were used to determine the approximate Michaelis constants for the enzyme (Figures 18b and 19b). These were 0.4 mM for glyoxylate and 0.043 mM for NADPH. These values are in the same range of activities as reported for the higher plant enzyme (70).

F. Inhibitors of NADPH:Glyoxylate Reductase from Chlamydomonas reinhardtii

Several possible substrate analogs and product analogs were tested for their action on the enzyme (Table 17). None of these

Figure 20

a. TEAE-cellulose fractionation of NADPH:glyoxylate reductase from Chlamydomonas reinhardtii.

The column was 1.5x0.5 cm, and was developed with 800 ml of a 0-0.3 M KCl gradient in 5 mM glycylglycine at pH 8.5. Fractions 100-107 were pooled.

b. A second TEAE-cellulose fractionation of NADPH:glyoxylate reductase from Chlamydomonas reinhardtii.

This column was 1.5x8.0 cm. The pooled fraction from the first column was diluted 3-fold with buffer and developed with 200 ml of a 0-0.2 M KCl gradient in buffer.

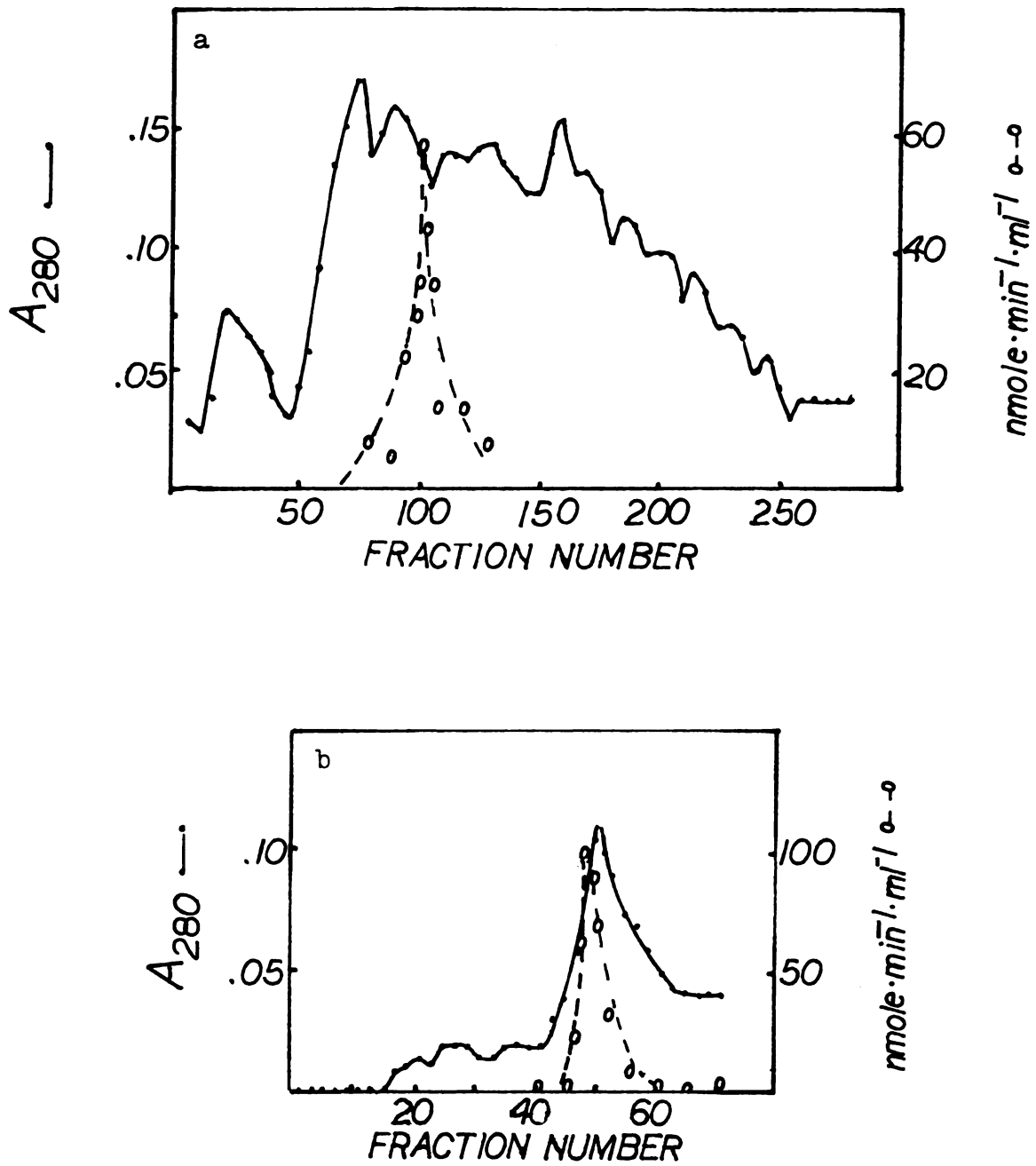

a
.15
.10
.05
A_{280}
60
40
20
nmole·min⁻¹·ml⁻¹
50
100
150
200
250
FRACTION NUMBER
b
.10
.05
A_{280}
100
50
nmole·min⁻¹·ml⁻¹
20
40
60
FRACTION NUMBER

Figure 20

TABLE 17

Effect of Some Inhibitors on NADPH:Glyoxylate Reductase at pH 6.2 and 8.1

Additions to Standard Assay	pH 6.2	pH 8.1
Control	(100%)	(100%)
1 mM AOA	42%	39%
1 mM Oxamate	53%	79%
1 mM Oxalate	92%	123%
12.5 mM Glycolate	50%	78%
10 mM D-lactate	102%	89%
50 mM $NaHCO_3$	41%	33%
0.7 mM $NADP^+$	89%	72%
1.4 mM NAD^+	94%	97%

The additions were made before the glyoxylate was added, and then the endogenous rate was measured. Glyoxylate was added to start each reaction.

substances was a substrate or activator of the enzyme. High CO_2, AOA, and oxamate were inhibitory. The products, glycolate and $NADP^+$ were slight inhibitors. AOA was studied further (Figure 21), and an approximate K_i of 1 mM was obtained. It acted as a competitive inhibitor, as would be expected from a non-reactive substrate analog.

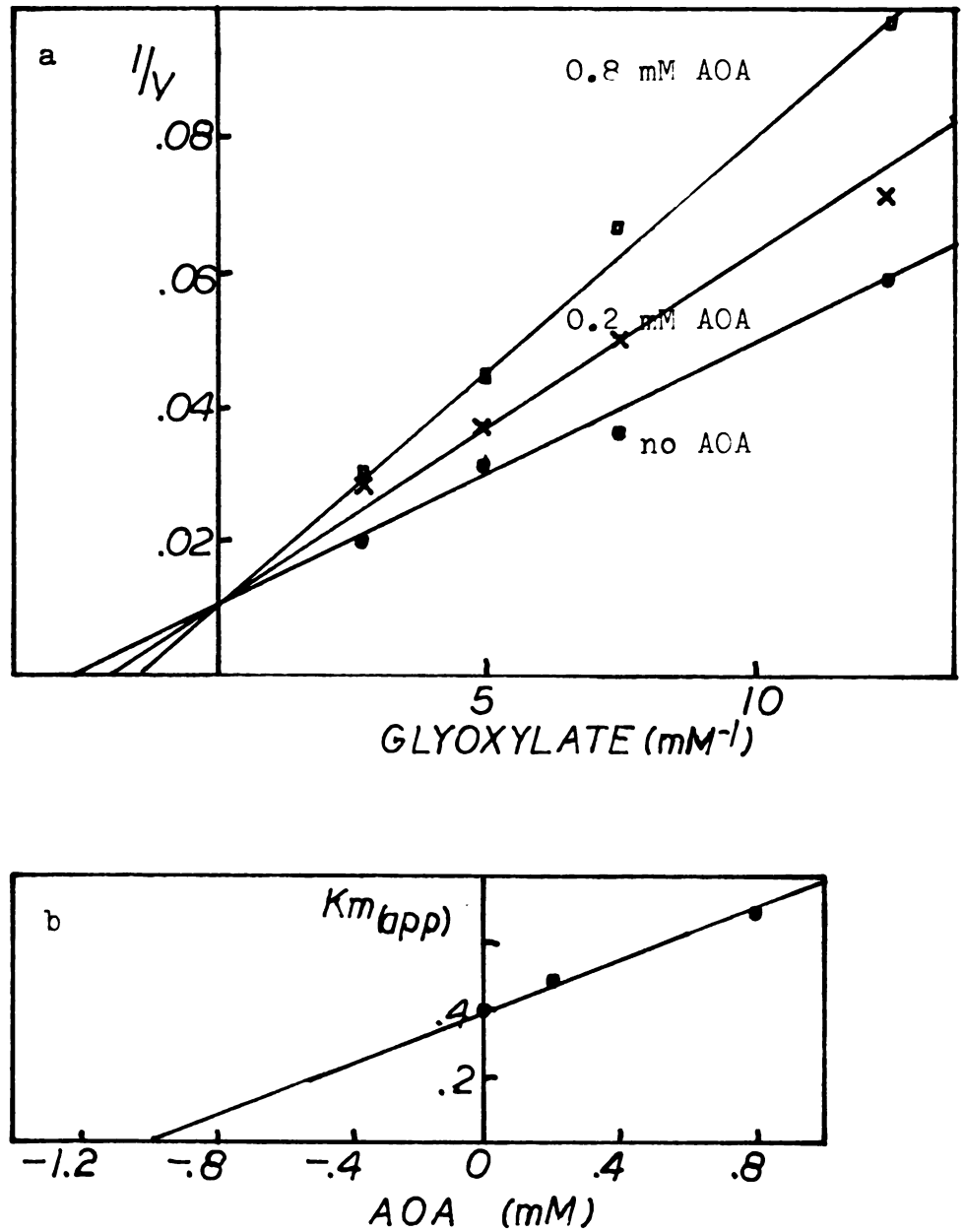


Figure 21

AOA inhibition of NADPH:glyoxylate reductase from Chlamydomonas reinhardtii.

a. The AOA exhibits competitive inhibition. The K_m (apparent) were plotted in b, below.

b. A Dixon plot of AOA inhibition. The K_i was 1 mM.

CHAPTER V

STIMULATION OF GLYCOLATE EXCRETION BY AMINOOXYACETATE

It has been known for some time that Chlamydomonas and other algae excrete glycolate (2,8). When the algae photosynthetically fix $^{14}C\text{-}HCO_3^-$, labelled glycolate is present in the medium within a few minutes. The reason for this excretion is not known. Why do some algae (and all higher plants) metabolize the large amount of glycolate produced as the inevitable product of ribulose-P_2 carboxylase/oxygenase in the oxidative photosynthetic carbon cycle (Figure 1), and other unicellular algae excrete much of the glycolate? Is there a reason for this glycolate excretion, or is it a simple way of disposing of this product that is unavailable to higher plants?

Inhibitors that block certain steps of the oxidative photosynthetic carbon cycle have been used to study this pathway. Glycolate oxidase has been inhibited by sulfonates, particularly by α-hydroxypyridinemethanesulfonate (73), and by α-hydroxy-3-butynoate (45). Algae have also been treated with HPMS, which results in glycolate accumulation (76) and increased excretion (74). Glycine to serine conversion can be blocked with isonicotinyl hydrazide and glycine hydroxamate (12,2). Aminotriazole has been used to inhibit catalase (1). Glyoxylate in large enough concentration can cause a decrease in photorespiration (75). The level of NH_4^+ available can cause

differences in products. Recently Krampitz (unpublished) has used aminooxyacetate (AOA), an aminotransferase inhibitor, to inhibit the glycolate pathway in Chlorella. AOA caused large increases in the amount of excreted glycolate. Presumably AOA acted by blocking the transaminase reactions in the glycolate pathway. The large amounts of glycolate formed would indicate that there is a large flux of carbon normally going through the glycolate pathway. This conclusion is contradicted by the low amount of glycolate dehydrogenase which can be detected.

This part of my research dealing with AOA has been written up with others for publication and is enclosed as an Appendix.

The Chlamydomonas cells were freshly harvested and exposed to the experimental treatments and $NaH^{14}CO_3$ while in the light. Aliquots of the cells were removed at various time points, rapidly centrifuged, and killed in hot methanol. The aliquots were later analyzed by two-dimensional paper chromatography.

The cells grown both on air and 5% CO_2 exhibited glycolate excretion. Nearly all the glycolate observed was in the supernatant fraction, and not in the cells. The excretion of ^{14}C-glycolate was greatly stimulated by 1 mM AOA. Most of the labelled product in the supernatant was glycolate, although under some circumstances a significant amount (up to 20% in air grown cells) was observed in the C-4 acids, principally malate. The amount of labelled glycolate in the supernatant was as much as 40% of the total labelled soluble products after 10 min of photosynthesis by the high-CO_2 cells treated with AOA. The presence of 0.2 M ammonium nitrate prevented this AOA stimulation of glycolate excretion.

These data are consistent with the idea that AOA is inhibiting the transaminases of the glycolate pathway, particularly those that convert glyoxylate to glycine. This can be overcome by the presence of enough NH_4^+ in the cells to overcome any competitive inhibition. Thus, AOA stimulates glycolate excretion in much the same way as does HPMS, by blocking its further metabolism. If this were the case, glycolate metabolism in these algae should normally be large, but the activity of glycolate dehydrogenase usually is not nearly sufficient to account for this much carbon flow through the glycolate pathway.

CONCLUSIONS

Several enzymes of glycolate metabolism were studied in the green unicellular alga Chlamydomonas reinhardtii. Glycolate dehydrogenase, P-glycolate phosphatase, and NADPH:glyoxylate reductase were partially purified and some properties studied. A D-lactate dependent NBT staining band from Chlamydomonas and spinach leaf extracts is described. The effect of growing Chlamydomonas with air or high CO_2 was studied in respect to the levels of glycolate dehydrogenase, P-glycolate phosphatase, NADPH:glyoxylate reductase and NADH:hydroxypyruvate reductase present. The levels of glycolate excretion and lactate production were also examined.

Glycolate dehydrogenase, unlike glycolate oxidase, does not couple directly to O_2 in vitro. The oxidation of glycolate by Chlorella vulgaris was found to be similar to the Chlamydomonas activity, and not coupled directly to oxygen, despite a report that Chlorella vulgaris contained glycolate oxidase (35). The Chlamydomonas glycolate dehydrogenase was purified up to a maximum specific activity of 25 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ by deoxycholate extraction, ammonium sulfate fractionation, and dialysis. The enzyme lost activity upon further purification. The glycolate dehydrogenase did not reduce cytochrome c, although this compound did inhibit the assay of the enzyme with DCPIP. The enzyme also coupled to NBT on polyacrylamide

gels, and to MTT, a soluble analog, in a spectrophotometric assay. It also can be assayed by ferricyanide reduction. Glycolate dehydrogenase had a pH optimum at 8.7, and its K_m for glycolate was 45 μM. It was inhibited by α-hydroxybutynoic acid, a flavoprotein inhibitor, indicating that a flavin may be part of the enzyme complex. Glycolate oxidase is known to contain FMN (1).

The instability and low activity of the glycolate dehydrogenase, and the various results obtained by different investigators of this enzyme, has made it a difficult enzyme with which to work. If it is part of the mitochondrial electron transport chain, as is indicated by cytochemical studies (18) and work on the Euglena system (28), it may well be membrane bound and in association with other components of the chain. Indeed, the success of the isolation with detergents and the fact that only detergent or French press gave reliable activity, would tend to substantiate this conclusion. Membrane association might account for the different results obtained with this enzyme, as different methods or conditions of isolation have produced particles with more or less of the mitochondrial electron transport chain components included. The fact that one or more of the natural electron acceptors are missing could be the reason for the low activity of this enzyme observed in vitro. It may be far more active in situ. The activity lost during purification may be due to loss of an essential cofactor, conformational change, or degradation.

Because glycolate dehydrogenase is as active with D-lactate as with glycolate, it is possible that the physiological activity could be with D-lactate. It may be analogous to the D-lactate dehydrogenases in bacteria, which are involved in transport (77). This could be

true whether its natural substrate was glycolate or D-lactate. The Chlamydomonas cells were found to produce large amounts of lactate.

The algal extracts contained material which electrophoresed differently from glycolate dehydrogenase in native polyacrylamide gels, but gave a strong NBT activity stain dependent on D-lactate, and not glycolate, or L-lactate. This D-lactate activity was always far more prominent on the gels than glycolate dehydrogenase. D-lactate activity was present in only one of two fractions of glycolate dehydrogenase separated by DEAE-cellulose chromatography. The "D-lactate band" on polyacrylamide gels existed as two closely spaced bands of equal intensity. The material at this location also was active with glycerate or other straight chain α-hydroxy acids as substrates. The D-lactate activity band on gels was present in spinach leaf homogenates. In spinach, it was localized in the mitochondrial fraction. All attempts to assay this enzyme activity spectrophotometrically failed.

Another enzyme of glycolate metabolism that was studied was P-glycolate phosphatase. It was 100 times more active than glycolate dehydrogenase. It required Mg^{++} for activity, and was specific for P-glycolate. The pH optimum of the algal P-glycolate phosphatase was different from that of the higher plant enzyme. Instead of an acidic pH optimum (6.3), it had an optimum at pH 7.5 to 8 in crude extracts and 8.7 in ammonium sulfate fractions. The enzyme lost two-thirds of its activity on dialysis, which was not restored by Mg^{++}. The pH profile upon dialysis or chromatography on DEAE-cellulose also extended over a broad range with a slight maximum at pH 7-8. This

change may be due to residual nonspecific phosphatases, or to loss of a cofactor. The higher plant P-glycolate phosphatase has different pH optima according to which divalent cation was present (57). Bound citrate also shifts the pH curve. Neither of these factors could be found to be responsible for changes in the enzyme from Chlamydomonas. The Chlamydomonas enzyme also lost activity, analogous to that lost upon dialysis, or upon any purification step beyond ammonium sulfate. Consequentially, although the enzyme was purified 200-fold, only 8% yield was obtained, and the protein was not at all pure by the criterion of gel electrophoresis.

Pea leaves have been reported to contain two isozymes of P-glycolate phosphatase, but only one form was seen in Chlamydomonas.

Another enzyme of glycolate metabolism present in Chlamydomonas was NADPH:glyoxylate reductase. It too was present at 100 times the activity of glycolate dehydrogenase. It is also much more active in algae than in spinach. This enzyme could theoretically produce large amounts of glycolate, if there were a source of glyoxylate in the cells. No such source is known. If the reaction could run in the reverse direction, this enzyme could act as a glycolate dehydrogenase. This was not observed. The NADPH:glyoxylate reductase from Chlamydomonas was purified over 60-fold. It had a pH optimum of pH 6-6.3. The K_m for glyoxylate was 0.4 mM and the K_m for NADPH was 43 μM. Both substrates were inhibitory at high concentrations. These properties are similar to those of the spinach leaf enzyme (70). The NADPH:glyoxylate reductase from Chlamydomonas was inhibited by AOA at K_i of 1 mM. It was also inhibited by oxamate, high glycolate, and high $NaHCO_3$. It was not inhibited by oxalate, D-lactate or NAD^+.

Several experiments were done with Chlamydomonas reinhardtii grown both with air and high-CO_2. On a gram wet weight basis high-CO_2 cells had more glyoxylate reductase, and less glycolate dehydrogenase and P-glycolate phosphatase than did the air-grown cells. High CO_2 cells also contained more lactate, and excreted more glycolate. AOA caused both air and CO_2 grown cells to excrete up to 40% of their recently fixed carbon as glycolate.

In high CO_2-grown cells the balance of the competition between CO_2 and O_2 for the ribulose-P_2 carboxylase/oxygenase would tilt towards the carboxylating activity. Therefore, less P-glycolate would be produced, and less P-glycolate phosphatase and glycolate dehydrogenase would be needed. The reason for the presence of high amounts of glyoxylate reductase in these cells is not known. It would be consistent with glyoxylate production as a by-product of reductive photosynthetic carbon cycling. However, all indications are that this is not the case. The only known source of glyoxylate in these cells is the glycolate dehydrogenase, with a 100-fold lower activity. There could be an unknown source for this substrate, or perhaps the enzyme is only present as insurance to keep glyoxylate pools down to a minimum.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. N.E. Tolbert (1971) Annu. Rev. Plant Physiol. 22, 45-74.

2. N.E. Tolbert (1981) Annu. Rev. Biochem. 50, 133-157.

3. N.E. Tolbert (1974) in Algal Physiology and Biochemistry (Stewart, W.D.P., ed.), pp. 474-504, Blackwell Scientific Publications, Oxford.

4. N.D.H. Lloyd, D.T. Canvin and D.A. Culver (1977) Plant Physiol. 59, 936-40.

5. R.G.S. Bidwell (1977) Can. J. Bot. 55, 809-818.

6. K. Roberts, M. Gurney-Smith and G.J. Hills (1972) J. Ultrastructure Res. 40, 599-613.

7. A.A. Benson and M.J. Calvin (1950) J. Exper. Bot. 1, 63-68.

8. N.E. Tolbert and L.P. Zill (1956) J. Biol. Chem. 222, 895-906.

9. J.L. Hess and N.E. Tolbert (1967) Plant Physiol. 42, 371-379.

10. M.J. Lord and M.J. Merrett (1968) Biochim. Biophys. Acta 159, 534-544.

11. I. Zelitch and P.R. Day (1968) Plant Physiol. 43, 289-291.

12. E.B. Nelson and N.E. Tolbert (1969) Biochim. Biophys. Acta 184, 263-270.

13. G.A. Codd, J.M. Lord and M.J. Merrett (1969) FEBS Lett. 5, 341-342.

14. E.B. Nelson and N.E. Tolbert (1970) Arch. Biochem. Biophys. 141, 102-110.

15. S.E. Frederick, P.J. Gruber and N.E. Tolbert (1973) Plant Physiol. 52, 318-323.

16. G.L. Floyd and J.L. Salisbury (1977) Amer. J. Bot. 64, 1294-1296.

17. H. Stabenau (1974) Planta 118, 35-42.

18. B.B. Beezley, P.J. Gruber and S.E. Frederick (1976) Plant Physiol. 58, 315-319.

19. J.S. Paul and B.E. Volcani (1976) Planta 129, 59-61.

20. J.S. Paul and B.E. Volcani (1975) Plant Sci. Lett. 5, 281-285.

21. J.S. Paul, C.W. Sullivan and B.E. Volcani (1975) Arch. Biochem. Biophys. 169, 152-159.

22. H. Stabenau (1974) Plant Physiol. 54, 921-924.

23. A-K.J. Sallal and G.A. Codd (1975) FEBS Lett. 56, 230-234.

24. B. Grodzinski and B. Colman (1976) Plant Physiol. 58, 199-202.

25. G.A. Codd and A-K.J. Sallal (1978) Planta 139, 177-181.

26. N. Collins, R.H. Brown, and M.J. Merrett (1975) Biochem. J. 150, 373-377.

27. A. Yokota, Y. Nakano and S. Kitoaka (1978) Agric. Biol. Chem. 42, 115-120.

28. A. Yokota, Y. Nakano and S. Kitoaka (1978) Agric. Biol. Chem. 42, 121-129.

29. J.S. Paul and B.E. Volcani (1974) Arch. Micro. 101, 115-120.

30. J.M. Lord (1972) Biochim. Biophys. Acta 267, 227-237.

31. D.W. Fry and K.E. Richardson (1979) Biochim. Biophys. Acta 567, 482-491.

32. K.E. Cooksey (1971) Plant Physiol. 48, 267-269.

33. B. Davis and M.J. Merrett (1975) Plant Physiol. 55, 30-34.

34. N. Collins and M.J. Merrett (1975) Biochem. J. 148, 321-328.

35. W. Kowallik and G.H. Schmid (1971) Planta 96, 224-237.

36. G.M. Orth, N.E. Tolbert and E. Jiminez (1966) Plant Physiol. 41, 143-147.

37. J. McD. Armstrong (1964) Biochim. Biophys. Acta 86, 194-197.

38. A. Bensadoon and D. Weinstein (1976) Anal. Biochem. 70, 241-250.

39. C. Schrarrenberger, A. Oeser and N.E. Tolbert (1973) Arch. Biochem. Biophys. 154, 438-448.

40. R.G. Martin and B.N. Ames (1961) J. Biol. Chem. 236, 1372-1379.

41. P. Tuengler, T. Stain and G.L. Long (1980) Proc. Nat. Acad. Sci. USA 77, 5832-5836.

42. C.T. Walsh, A. Schonbrunn, O. Lockridge, V. Massey, and R.H. Abeles (1972) J. Biol. Chem. 247, 6004-6006.

43. P.J. Jewess, M.W. Kerr and D.P. Whitaker (1975) FEBS Lett. 53, 292-296.

44. G. Fendrich and S. Ghisla (1982) Biochim. Biophys. Acta 702, 242-248.

45. O. Warburg, H-S. Gewitz and W. Volker (1957) Zeit. fur Natur. 12, 722-724.

46. P.J. Gruber, S.E. Frederick and N.E. Tolbert (1974) Plant Physiol. 53, 167-170.

47. T. Betsche (1981) Biochem. J. 195, 615-622.

48. T. Kobayashi, Y. Inamori, I. Tanabe and A. Obayashi (1974) Nippon Nogei Kagaku Kaishi 48, 261-267.

49. T. Kobayashi, I. Tanabe and A. Obayashi (1974) Nippon Nogei Kagaku Kaishi 48, 269-273.

50. L. Ornstein (1964) Ann. N.Y. Acad. Sci. 121, 332-349.

51. B.J. Davis (1964) Ann. N.Y. Acad. Sci. 121, 404-429.

52. B. Grodzinski and B. Colman (1972) Phytochem. 11, 1281-1285.

53. N.E. Tolbert (1974) Methods in Enzymology 31, 734-746.

54. K.E. Richardson and N.E. Tolbert (1961) J. Biol. Chem. 236, 1285-1290.

55. D.E. Anderson (1968) Ph.D. Thesis, Michigan State University.

56. J.T. Christeller and N.E. Tolbert (1978) J. Biol. Chem. 253, 1780-1785.

57. J.T. Christeller and N.E. Tolbert (1978) J. Biol. Chem. 253, 1786-1790.

58. J.T. Christeller and N.E. Tolbert (1978) J. Biol. Chem. 253, 1791-1798.

59. C. Verin-Vergeau, P. Baldy and G. Calvalié (1979) Phytochem. 18, 1279-1282.

60. C. Verin-Vergeau, P. Baldy, J. Peuch and G. Calvalié (1980) Phytochem. 19, 763-767.

61. L.F. Leloir and C.E. Cardini (1957) Methods in Enzymology, 3, 844.

62. Z.P. Rose and J. Salon (1979) Biochem. Biophys. Res. Comm. 87, 869-875.

63. I. Zelitch and S. Ochoa (1953) J. Biol. Chem. 201, 707-718.

64. H.A. Stafford, A. Magaldi and B. Vennesland (1954) J. Biol. Chem. 207, 621-629.

65. I. Zelitch and A.M. Gotto (1962) Biochem. J. 84, 541-546.

66. L.D. Kohn, W.A. Warren, and W.R. Carroll (1970) J. Biol. Chem. 245, 3821-3830.

67. L.D. Kohn and W.A. Warren (1970) J. Biol. Chem. 245, 3831-3839.

68. W.A. Warren and L.D. Kohn (1970) J. Biol. Chem. 245, 3840-3849.

69. L.D. Kohn (1970) J. Biol. Chem. 245, 3850-3858.

70. N.E. Tolbert, R.K. Yamazaki and A. Oeser (1970) J. Biol. Chem. 245, 5129-5136.

71. B. Halliwell (1976) FEBS Lett. 64, 266-270.

72. A. Yokota and S. Kitoaka (1979) Biochem. J. 184, 189-192.

73. I. Zelitch (1957) J. Biol. Chem. 224, 251-260.

74. N.E. Tolbert and J.L. Hess (1966) J. Biol. Chem. 241, 5712-5715.

75. D.J. Oliver and I. Zelitch (1977) Science 196, 1450-1451.

76. J.L. Hess and N.E. Tolbert (1966) J. Biol. Chem. 241, 5705-5711.

77. H.R. Kaback (1974) Science 186, 882-892.

APPENDIX

AMINOOXYACETATE STIMULATION OF GLYCOLATE FORMATION AND EXCRETION BY CHLAMYDOMONAS

Aminooxyacetate Stimulation of Glycolate Formation and Excretion By *Chlamydomonas*[1]

N.E. Tolbert, M. Harrison, N. Selph
Department of Biochemistry
Michigan State University
East Lansing, MI 48824

1. Research supported by a National Science Foundation grant, PCM 7815891. Published as journal article xxxx of the Michigan Agricultural Experiment Station.

Abbreviation: AOA for aminooxyacetate

Acknowledgement: We thank L.O. Krampitz for first telling us about the stimulatory effect of aminoooxyacetate on glycolate formation and for exchange of information during the course of this work.

Abstract

Aminooxyacetate (1 mM) did not inhibit photosynthetic $^{14}CO_2$ fixation by Chlamydomonas reinhardtii but greatly stimulated the biosynthesis and excretion of glycolate. Similar results were obtained from cells grown with 5% CO_2 or low CO_2 (air). After 2 minutes with air-grown cells ^{14}C-glycolate increased from 0.3% of the total ^{14}C fixed by the control to 11.7% in the presence of aminooxyacetate and after 10 minutes from 3.8% to 41.1%. Ammonium nitrate (0.2 mM) in the media blocked the aminooxyacetate stimulation of glycolate excretion. Chromatographic analyses of the labeled products in the cells and supernatant media indicated that aminooxyacetate also completely inhibited the labeling of alanine while some pyruvate accumulated and was excreted. A high percentage (35%) of initial $^{14}CO_2$ fixation was into C_4 acids. Initial products of $^{14}CO_2$ fixation included phosphate esters as well as malate, aspartate and glutamate in treated or untreated cells. Lactate was also a major early product of photosynthesis, and its labeling was reduced by aminooxyacetate. Since lactate was not excreted, glycolate excretion seemed to be specific. When photosynthesis was inhibited by DCMU labeled organic and amino acids but not phosphate esters were lost from the cells. Aminooxyacetate did not inhibit the enzymes associated with glycolate synthesis from ribulose bisphosphate.

In 1956 Tolbert and Zill (25) reported that Chlorella excreted only glycolate among the products of photosynthetic $^{14}CO_2$ fixation. Glycolate biosynthesis and excretion is increased by high light intensity, high O_2 and low CO_2 concentration and high pH (20). Excretion normally amounts to 2 to 5% of the total ^{14}C fixation at pH 7 to 8 over a 10 min period. From many subsequent investigations, excretion of glycolate has been recognized as a general but unexplained phenomenon of unicellular algae (27,28). In higher plants glycolate is also produced in chloroplasts and excreted into the cytoplasm to be metabolized in the peroxisomes (15). There is no evidence that glycolate excretion by algae or chloroplast is an active process but only the unionized acid (pK_a 3.8) difuses these membranes across (11,24). Cells grown with saturating levels of CO_2 (1 to 5%) excrete more glycolate than air-grown cells, when either are tested with low levels of $NaH^{14}CO_3$ (6). At first it was thought that air grown Chlorella did not excrete glycolate, but they will do so at a high O_2/CO_2 ratio (3) or after a short time lag of about 30 min ( ), or if glycolate metabolism is blocked by inhibitors (see Discussion).

The theory has developed that glycolate excretion represents an unnecessary photosynthetic end product that is elminated by excretion (26). This theory is based upon the fact that those unicellular algae that excret glycolate do not contain an active peroxisomal glycolate oxidase, as in higher plants, for its conversion to glycine and serine by the glycolate pathway portion of the oxidative photosynthetic carbon cycle (19,27). Instead these algae contain a mere trace of glycolate dehydrogenase (8,19) in membranes of their mitochondria or chloroplasts. These algae produce a limited amount of glycine and serine during photosynthesis, and because the serine is carboxyl labeled, the serine it has been proposed that serine can be formed by both the glycolate and glycerate pathways (27). Excess glycolate seems to be excreted,

and since algae grown on high CO_2 have half as much glycolate dehydrogenase as when grown on air (19), they should excrete more of the glycolate.

Several inhibitors have been used to block the glycolate pathway and to force glycolate excretion by algae. Krampitz (16) introduced the pyridoxal phosphate inhibitor, aminooxyacetate or AOA ($COOH-CH_2ONH_2$), to inhibit the glyoxylate aminotransferase of the glycolate pathway with resultant accumulation and excretion of glycolate. In its zwitter ion form AOA apparently rapidly enters cells. Our present investigation was initiated because the amount of glycolate produced in the presence of AOA seemed unusually large, and the mechanism of its inhibition of glycolate metabolism is unknown.

Materials and Methods

Chlamydomonas reinhardtii, Dangeard, (-) strain (N.90) was obtained from the R.C. Starr algal collection at the University of Texas and 1 L cultures in 3 L Fernback flasks were grown in a phosphate and NH_4NO_3 medium at pH 7.4 as described previously (20). Fresh cultures were started from the original slant every few months. During growth the algae were continuously mixed on an Eberbach shaker and aerated with about 3 to 5% CO_2 in air or with air alone, corresponding with the experimental designation of algae grown on high CO_2 or on low CO_2. The algae were grown continuously in 150 μeinstein/M^2/s of light from fluorescent and incandescent lamps. The temperature of the growth medium was regulated between 21 to 23°C by fans which moved air over the flasks. Growth curves as cells/mm^3 of Chlamydomonas were monitored (data not shown) and the algae were harvested during the middle or late part of the log phase of growth, which was in about 48 hours for the cultures on high CO_2. An aliquot of cells were counted with a Neubauer cell counter. In general cultures with approximately 5000 cells/mm^3 were used from the log phase of

growth, while cultures with 7000 or more cells/mm^3 were considered to be in the stationary phase. A fraction of the air grown cells always appeared motile, while the cells grown on high CO_2 were generally non-motile.

Cells were harvested as rapidly as possible (about 10 to 30 min) and tested immediately. The first centrifugation was in 300 ml bottles at 1000 x g for 5 min, and then the algae were washed by resuspension in 1/3 volume of water and recentrifuged. The pellet was then resuspended in about 20 ml water with a disposable pasteur pipette and put into a pre-weighed centrifuge tube and centrifuged at about 10,000 x g for 10 min at 4°C. After decanting the supernatant and drying the tube with a paper towel, the tube was weighed to establish cell wet weight. The final algal suspension was prepared in 3 mM potassium phosphate buffer at pH 7.5 unless otherwise designated. A range from 1 to 10 g wet weight of cells per 100 ml was tested, but in general 1 or 2% suspensions were used. Cell suspensions were used for photosynthesis experiments immediately and never later than 2 h after harvest.

Aliquots of 1 to 3 ml of the algal suspension were placed in a photosynthetic vessel consisting of either flattened test tubes or 2 cm diameter glass vials with a flat bottom so that the light path through the solution was about 2 cm. Most experiments were run with a plastic block which held 6 vials in a circulating water bath at 25°C, and the contents of each vial were mixed by a magnetic stirrer. For experiments in test tubes the samples were mixed by a stream of CO_2 free air. Illumination of 1500 μEinstein/m^2/s from a projector with a built in infra red heat filter was used in most experiments. Lower light intensities were obtained by moving the projector or by use of neutral density filters.

After 2 min for light adaptation, 10 to 20 ul aliquots of $NaH^{14}CO_3$ were added to each algal suspension to provide the final bicarbonate

concentrations. When AOA or NH_4NO_3 was added, it was present during this 2 min adaptation period. At time intervals, 100 to 500 µl aliquots of the algae suspension were removed and the cells were separated from the supernatant fluid by centrifugation for 10 sec in a microfuge or by sedimentation through silicon oil (9). Similar results for the distribution of fixed ^{14}C were obtained by both methods and because centrifugation was simpler, the silicon oil procedure was not used in latter work. Both the supernatant and cell fractions were immediately mixed with 60% methanol and boiled. Aliquots were quantitatively assayed for glycolate by the Calkins procedure (25). Aliquots of the supernatant were added to counting vials containing 200 µl of 2 N HCl. The rest of the supernatant was stored separately in acid for chromatographic analysis. Aliquots of the cell fraction were also put into counting vials with acid. All samples were allowed to stand for at least 2 h in the strong acid for removal of CO_2 and then the fixed ^{14}C was measured with a scintillation counter and a Triton X-100 based scintillant.

Aliquots of the supernatant and of the cells were chromatographed for product identification on Whatman 1 paper as described earlier (2). Water saturated phenol was the first solvent and butanol:proprionic acid:water the second solvent. Before the paper was completely dry after the second solvent development, it was sprayed with a 1 M $NaHCO_3$ solution over the general area of the acids, to prevent volatilization of glycolic acid. The radioactive compounds were located by exposure to X-ray film for about 2 weeks, and the spots were cut out of the chromatograms and into 5 mm^2 pieces and placed into scintillation vials for counting of each compound. Compound identification was based upon the standard chromatograph map of R_F values (2) and co-chromatography with carriers, which were identified on the chromatograms by appropriate spray tests. Several experimental parameters affecting ^{14}C excretion and the time and rate of $^{14}CO_2$ fixation, as discussed in the results, were explored to establish the experimental conditions.

Results

All experiments were replicated with Chlamydomonas reinhartii grown on air as well as on 5% CO_2. The rates of CO_2 fixation were linear for 10 or 20 minutes with 1 to 3 mM $NaHCO_3$ in a 2% algae suspension (Figure 1 and 2). In general labeling of glycolate from $^{14}CO_2$ had an initial lag period of several minutes relative to ^{14}C incorporation into the phosphate esters and C_4 acids as previously observed (22). This lag in glycolate labeling was more pronounced with cells grown on 5% CO_2. The lag is attributed to the time to label ribulose-P_2, the glycolate precursor, during which time unlabeled glycolate was being formed.

In the accompanying paper (16), Krampitz has noted that a 10% Chlorella pyrenoidosa culture aerated with 0.2% CO_2 in air produces enough glycolate in an hour with AOA to form about a 10 mM solution. Using the Calkins colorimetric assay for glycolate we observe in our procedure the production of about 75 ug glycolate per ml per hour by 3 to 5 Chlamydomonas with 1 or 2 mM AOA and the addition of 1 mM $NaHCO_3$ every 15 min. This amount of glycolate is equal to 1 mM solution and is comparable with Krampitz's results. The rest of this report deals only with the excretion of photosynthetic $^{14}CO_2$ fixation products.

The distribution of fixed ^{14}C, after a time period of $^{14}CO_2$ photosynthesis, between the cellular fraction and the supernatant media was measured after rapid separation of the two fractions by centrifugation (Figure 1 and 2). This simple technique was more rapid than filtration of the algae on a celite bed as originally used to study glycolate excretion by algae (25) or chloroplasts (15), but the results were essentially the same. Data in Figures 1 and 2 and Tables 1 and 2 are expressed first on the basis of percentage of the total $^{14}CO_2$ fixed photosynthetically that was excreted. Then %

^{14}C distribution among the different products of both the cell and supernatant fractions are presented. The total % of fixed ^{14}C in a compound is the sum of that in the cells plus that in the supernatant. Glycolate represented over 90% of the ^{14}C-labeled content in the media after $^{14}CO_2$ fixation periods of longer than 2 min by algae grown on either high or low CO_2. In exposures of 2 min or shorter to $^{14}CO_2$ the glycolate pool was not highly labeled and little ^{14}C was excreted although unlabeled glycolate was probably being excreted. Malate, aspartate, glutamate, and phosphate esters contained most of the ^{14}C label in the first 2 min, and the presence of about 1% of these labeled acids and amino acid products in the media was a significant percentage of the small amount of ^{14}C excreted. However, with time, only the amount of ^{14}C-glycolate in the media increased, as if the small pool of the other labeled products represented some background cell leakage or breakage. In subsequent sections the phrases glycolate excretion or excreted ^{14}C are interchanged, but detailed chromatographic analysis of every sample was not always run. The % of the of the ^{14}C in glycolate increased with time for several reasons. (a) The glycolate excreted was an end product that accumulated and with time contained an ever larger percentage of the ^{14}C. In this respect labeling of glycolate was similar to the label into sucrose, while label into other organic acids and phosphate esters decrease in percentage of total ^{14}C fixed with time. (b) In the first 2 min of $^{14}CO_2$ fixation there was a lag in the appearance of ^{14}C in glycolate, since it is assumed to be formed from ribulose-P_2 which must first be labeled by the reductive photosynthetic carbon cycle. (c) Toward the end of the experiments (i.e. after 20 min) when the CO_2 concentration had dropped due to utilization of the $NaHCO_3$ and when the pH had increased, a higher percentage of the ^{14}C appeared in glycolate

presumably because there was less CO_2 for the competition between the ribulose-P_2 carboxylase versus oxygenase reaction (see next section).

As reviewed in the introduction, glycolate excretion has been reported to occur in much larger amounts with algae grown on high CO_2. Later reports concluded that excretion occurred only from such CO_2-adapted algae. In our current experiments considerable glycolate excretion occurred by air grown cultures of Chlamydomonas, although more glycolate production in general seemed to occur with CO_2 grown cultures (compare Figure 1 with Figure 2).

Bicarbonate Concentration and pH

The pH and $NaH^{14}CO_3$ concentration were two related factors greatly affecting glycolate excretion. Addition of $NaHCO_3$ to an unbuffered 2% algal suspension in the light resulted in a rapid pH increase (Figure 3) which rose to between 9 and 10 within a few min. This has been attributed to HCO_3^- uptake in exchange for OH^- (7). The rate of alkalinity increase in the supernatant was dependent on both $NaHCO_3$ concentration and cell density. Upon increasing the cell density from 0.5% to 2% the rate of increase of pH increased. The rate of pH rise was much greater at 10 mM $NaHCO_3$ than at 1 mM $NaHCO_3$ for a given cell density, and this faster rate of pH increase was against the increased buffer capacity of 10 mM $NaHCO_3$. Because of the HCO_3^-/OH^- exchange, the pH of the algal suspension quickly rose above 8.3 (pH of $NaHCO_3$) into a range which was inhibitory to photosynthesis. This shift with 1 or 2% algae to an unfavorably high pH occurred within about 1 min with 10 mM $NaHCO_3$ and within 5 to 10 min with 1 mM $NaHCO_3$. An initial rate of $^{14}CO_2$ fixation in the first minute after adding $NaH^{14}CO_2$ was found to be faster than in the buffers we examined. However in the unbuffered solution the rate of photosynthesis slowed down very quickly, making comparison of rates difficult to interpret.

When the rate of CO_2 fixation decreased due to either a pH increase or to ultilization of the added $NaHCO_3$, the percentage of the fixed $^{14}CO_2$ that was excreted as glycolate increased (data not shown)(20). As the $NaH^{14}CO_3$ was used up in a buffered solution after 20 min the rate of ^{14}C fixation decreased but the % ^{14}C excreted increased. The rate of photosynthesis will be linear until CO_2 becomes limiting, but the rate of glycolate excretion is exponential. These results are attributed to preferential ribulose-P_2 oxygenase activity for glycolate synthesis over limited ribulose-P_2 carboxylase activity in the absence of sufficient CO_2. Consequently it was not possible to study variables effecting glycolate excretion without pH control by either titration with acid, use of $^{14}CO_2$ rather than $NaH^{14}CO_3$, or the use of buffer. In this report we have used buffers for convience.

Effect of Buffers on Glycolate Excretion

To investigate glycolate excretion the pH must be controlled as explained in the previous section. A limited investigation of buffers indicated that they all seemed to inhibit the initial rate of ^{14}C fixation, but that Hepes and Tris at pH 7.4 seemed more inhibitory than phosphate. Chlamydomonas in 50 mM Hepes initially fixed ^{14}C at less than 10% of the rate without buffer, but the use of 1 mM Hepes resulted in only a 10 to 20% inhibition. Increased ^{14}C excretion by algae in Hepes buffer was sometimes observed but the products were not analyzed chromatographically. After this limited investigation, we elected to use 3 mM phosphate at pH 7.5 for the photosynthetic media. Higher concentration of around 10 mM would have provided better buffering, but would have limited subsequent chromatographic analyses of the excreted products. During a normal photosynthetic experiment with 1 mM $NaH^{14}CO_3$ added to the phosphate buffer at pH 7.5, about 25% of the total

^{14}C was lost over the 20 min test period, presumably due to CO_2 escaping from the stirred or aerated algal medium. For this reason experiments were performed in a ventilated hood. Generally after 20 minutes most of the ^{14}C had been fixed or lost. Higher concentrations of 3 and 10 mM $NaHCO_3$ did not increase the initial rate and, due to CO_2 loss as a gas from the open flasks, did not greatly prolong the linear period for photosynthesis.

Effect of Cell Age and Storage Condition After Harvest

If the algal cells were harvested and washed as described in the methods section, and then allowed to let stand in water in the dark for 1 to 2 h, the medium became acidic, decreasing to pH 4.5. Cells two or more h after harvest had slower rates of CO_2 fixation and were less able to increase the media pH upon addition of $NaHCO_3$. Aerating the cells, storage in light, or use of buffers did not prevent deterioration of their photosynthetic capacity. Rather than investigate this aspect further, only cells freshly harvested within 1 h were used in all experiments.

Production of Acids and Amino Acids During $^{14}CO_2$ Fixation

Benson and Calvin (1) using Chlorella first observed a large percentage of the early products of $^{14}CO_2$ fixation to be in organic acids. After 1950 when the phosphate esters of the reductive photosynthetic carbon cycle were considered to be the early product of $^{14}CO_2$ fixation, the rates and the significance of labeling the acids by these algae was largely ignored. These compounds include malate, aspartate, glutamate, glutamine, citrate, succinate and fumarate (Table 1 and 2). The early ^{14}C labeling of these acids during photosynthesis seems similar to their labeling by C_4 plants. Recently re-investigation of $^{14}CO_2$ fixation products by algae has emphasized the large amount of ^{14}C incorporation into the acids as well as into phosphate esters. Our data in Table 1 and 2 for Chlamydomonas grown on either low or

high CO_2 is supportive of this labeling pattern which needs further investigation. The large percentage of 25 to 50% of the total ^{14}C in these acids during initial $^{14}CO_2$ photosynthesis indicates that the acid labeling is not simply due to slow dark respiration superimposed on photosynthesis.

Effect of Aminooxyacetate Upon Glycolate Biosynthesis and Excretion

The rate of photosynthetic $^{14}CO_2$ fixation by a 2% air grown Chlamydomonas cell suspension with 1 mM $NaHCO_3$ and in high light intensity was not greatly effected by 1 mM AOA (Figure 1). In these experiments the amount of the total ^{14}C fixed that was excreted increased from 4% by the air grown cells after 30 min to 30% after 30 min with 1 mM AOA. Excretion by the cells grown on 5% CO_2 was also increased AOA (Figure 2). Although the untreated cells grown on high CO_2 excreted more of the total ^{14}C as glycolate than the air grown and untreated cells, both cultures excreted the same % of the total ^{14}C as glycolate when treated with AOA. This result would be consistent with the hypothesis that the ability to metabolize glycolate by the cells regulates glycolate excretion; when this metabolism was blocked by AOA, both cells grown on low or high CO_2 made and excreted the same amount of glycolate.

The products of CO_2 fixation by AOA were altered in two major ways. Most dramatic was that the total ^{14}C in glycolate increased from 0.3% after 2 min to 11.7% with AOA and from 3.8% after 10 min to 41.1% with AOA (Table 1). The maximum % of the total ^{14}C in glycolate generally varied from 20 to 75% dependent upon unknown factors. In longer term experiments when $NaHCO_3$ became limiting, which increased glycolate production normally, the % of fixed ^{14}C in glycolate could reach values of 50 to 75% of the total ^{14}C if AOA were present. Most of this massive production of glycolate was

excreted outside the cells into an end product pool. However glycolate in the cells treated with AOA could amount to 10% of the ^{14}C retained in the cell. Somewhat similar results were obtained with the algae grown on 5% CO_2 (Table 2).

Previous literature has indicated that algae grown on high CO_2 produce more ^{14}C-glycolate when tested with low levels of $NaH^{14}CO_3$. A comparison of results in Tables 1 and 2 hardly support this concept. Both the air grown and CO_2 grown cells produced and excreted about the same percentage of the newly fixed $^{14}CO_2$ as glycolate with or without AOA. Since the two experiments for Tables 1 and 2 were done with different ^{14}C specific activity, no comparison between total fixation can be done, but in general both types of algae initially fixed about the same amount of CO_2 per cell per unit of time.

Effect of Aminooxyacetate Upon Other Products of Photosynthesis

Lactate was a major product of $^{14}CO_2$ fixation by Chlamydomonas (Table 1 and 2). This observation has not been pursued in the past, although Warburg et al. (29) reported in 1957 that D-lactate was a major product from CO_2 fixation by Chlorella, and lactate dehydrogenase is present in algae (8). AOA reduced the % of the total ^{14}C in lactate while increasing the % ^{14}C in glycolate. Lactate was not excreted, where as glycolate, its structural analogue was mostly excreted. These observations suggests a specific translocator mechanism for glycolate.

A definite metabolic change from AOA treatment of air-grown Chlamydomonas was the complete blockage of ^{14}C-alanine formation and a nearly corresponding increase of ^{14}C-pyruvate (Table 1). Part of the pyruvate was excreted. In control cells the pool size of pyruvate was too small to be detected by the chromatographic procedure. AOA also blocked alanine formation

by Chlamydomonas grown on high CO_2, although pyruvate accumulation was not accumulation was not noted. This action of AOA would seem to be a direct inhibition of the aminotransferase reaction from pyruvate to alanine.

With increased glycolate biosynthesis from AOA, ^{14}C accumulation decreased particularly significantly in the phosphate esters comprising the reductive photosynthetic carbon cycle. This would be consistent with the biosynthesis of P-glycolate from ribulose-P_2. How the $^{14}CO_2$ fixation rate was maintained under these circumstances is not clear, but it is assumed that the smaller pools of phosphate esters were sufficient to maintain the rate of CO_2 fixation.

The early CO_2 fixation products by Chlamydomonas reinhardtii included malate, aspartate, glutamate and other acids of the tricarboxylic acid cycle (succinate and fumarate). With AOA an initial larger percentage of ^{14}C incorporation went into these C_4 acids before labeling of glycolate, although AOA did not alter the % distribution of ^{14}C into these acids as much as into glycolate and alanine. The % ^{14}C incorporated into aspartate was significantly increased by AOA from 6.5% to 22.3% after 2 min of photosynthesis and from 4.7 to 25.3% of the ^{14}C in the cell after 10 min. Aminotransferase reactions are involved in aspartate and glutamate formation and metabolism, so the reason for aspartate accumulation in the presence of AOA is not known. The % ^{14}C in glutamate within the cells was probably not altered significantly by AOA.

The presence of a little malate, aspartate and glutamate in the supernatant from the cells is to be noted. Since the control cells initially excreted only 1 or 2% of the total ^{14}C fixed, the small amount of these excreted acids represent 20 to 24% of the total ^{14}C excreted. In the AOA treated cells, which excreted large amounts of glycolate, the presence of this

small amounts of malate represented only a few % of the total excretion. In order to simplify the results, the limited amount of these other organic acids and amino acids in the supernatant has been disregarded. However it is to be noted that none of the phosphate esters appeared outside of the cells.

Effect of light, DCMU or Darkness on Glycolate Biosynthesis and Excretion

Glycolate biosynthesis is light dependent (26), just as is $^{14}CO_2$ fixation. Assuming that glycolate is formed by ribulose-P_2 oxidation, the dependency on light could be explained by regeneration of ribulose-P_2 and the activation of the carboxylase/oxygenase. Indeed DCMU or darkness blocks both CO_2 fixation and glycolate biosynthesis (26). In the older DCMU experiments, the algae or plants were poisoned prior or simultaneous with addition of $^{14}CO_2$, and as a consequence no ^{14}C fixation occurred. If glycolate were being formed from endogenous products it would not have been labeled with ^{14}C. In the present experiments the Chlamydomonas cells with AOA were pre-labeled with $^{14}CO_2$ for 10 minutes and then treated with DCMU or put in darkness. DCMU or darkness stopped $^{14}CO_2$ fixation and in fact the total fixed ^{14}C in the cells suspension decreased, presumably from dark respiration (Figure 4). DCMU or darkness did not stop ^{14}C excretion into the medium. As a result the percentage of the ^{14}C in the medium increased with time (Figure 4). Paper chromatographic analysis of the excreted products indicated that, whereas glycolate was the primary excreted product in the absence of DCMU, in the presence of DCMU the cells lost all of their products labeled with ^{14}C (Table 3). In the presence of DCMU other products, particularly malate, glutamate and succinate were lost from the cell and account for the continued excretion. Thus the loss of total ^{14}C and the excretion of products of respiration are consistent with continued respiratory

metabolism, but no photosynthesis or glycolate formation or excretion occurred in the presence of DCMU.

Effect of Aminooxyacetate Concentration, Ammonium Nitrate and O_2 On Glycolate Excretion

AOA concentrations between 1 and 10 mM had no great effect on the rate of CO_2 fixation by Chlamydomonas (Table 4). Since 1 mM AOA was as effective as 10 mM for stimulating glycolate formation and excretion, in all other experiments 1 mM AOA was used.

As reported by Krampitz (16), addition of 1 to 2 mM NH_4Cl to the algal medium inhibited glycolate excretion. As shown in Table IV, addition 0.2 mM NH_4NO_3 to the Chlamydomonas blocked AOA stimulation of ^{14}C excretion of new fixed $^{14}CO_2$ higher concentrations of AOA (10 mM) however did stimulate ^{14}C excretion in the presence of 0.2 mM NH_4NO_3 (Table 4) . Similar results were found with Chlamydomonas in the log phase of growth or resting phase of development. Paper chromatographic analysis of the ^{14}C fixation products at the end (20 min) of the experiment with or without AOA indicated that the major ^{14}C products of the cells treated with NH_4NO_3 were aspartate and glutamate in both the cells and in the supernatant. Similar accumulation of amino acids by algae and leaves during photosynthesis in the presence of excess NH_4NO_3 has been detailed (17). The results indicate that any increased excretion with higher levels of AOA and NH_4NO_3 is not the result of more glycolate formation and excretion but rather increase cell leakage. AOA stimulation of glycolate production was blocked by NH_4NO_3.

Oxygen stimulated ^{14}C excretion by Chlamydomonas, (Table V), as expected from the increased rate of glycolate biosynthesis by ribulose-P_2 oxygenase in competition with the carboxylase reaction. The old cells grown on

5% CO_2 excreted much more, than cells grown on air. The combination of increased O_2 and AOA resulted in more ^{14}C excretion than from either alone. With AOA and O_2 the air-grown algal excreted as much ^{14}C as the CO_2-grown cells, as was also observed in the experiments on the effect of AOA upon glycolate excretion. It appears as if both O_2 and AOA stimulated glycolate biosynthesis and excretion, but that the amount did not exceed similar percentages of fixed $^{14}CO_2$ for both algal cultures.

Carbonic Anhydrase and Glycolate Excretion

Ethoxyzolamide, a carbonic anhydrase inhibitor, causes a 7 fold increase in glycolate excretion while inhibiting photosynthesis 73% by Chlorella (13). Diamox, another carbonic anhydrase inhibitor, also stimulated glycolate excretion by the blue green algae, Coccochloris peniocystis (12). These results seem similar to the increase in glycolate production at pH over 8 or when the $NaHCO_3$ is near depletion during photosynthesis (previous section), and can be attributed to decrease CO_2 availability relative to O_2. Increased glycolate excretion has been attributed to the absence of carbonic anhydrase in 5% CO_2-grown algae or the inhibition of the anhydrase, which lowers the effective CO_2 level in the cell to favor the ribulose-P_2 oxygenase reaction over the carboxylase. That AOA might be functioning in a similar way was explored by testing it as an inhibitor of carbonic anhydrase. 1 mM AOA did not significantly inhibit bovine carbonic anhydrase. From a sonicated homogenate of Chlamydomonas cells an ammonium sulfate fraction for carbonic anhydrase was prepared (21). AOA also did not inhibit this algal carbonic anhydrase. Jahnke (13) has pointed out that the carbonic anhydrase inhibitors have little effect on CO_2-grown Chlorella because they contain little carbonic anhydrase. Since AOA caused both air grown and CO_2 grown Chlamydomonas to excreted glycolate, it would appear that the primary cite of action of AOA is not upon carbonic anhydrase.

Discussion

Mode of Action of Aminooxyacetate

The known mode of AOA inhibition is to combine with pyridoxal phosphate to inhibit aminotransferase reactions. Thus it should inhibit glyoxylate transamination to glycine, a reaction coupled to glutamate, or a serine:glyoxylate aminotransferase in the oxidative photosynthetic carbon cycle (28). The reversal of the AOA stimulation of glycolate excretion by NH_4NO_3 is consistent with a stimulation of the glyoxylate aminotransferase reactions by additional amino groups available from the excess NH_4NO_3. AOA likewise inhibited the formation of alanine, which led to the accumulation and excretion of pyruvate. It is puzzling why AOA did not block the biosynthesis of aspartate or glutamate during photosynthesis by Chlamydomonas, which would have involved aminotransferase reactions, and if anything AOA stimulate the formation of these amino acids. AOA did not inhibit CO_2 photosynthetic fixation by Chlamydomonas.

Glycolate biosynthesis, excretion and conversion to glycine and serine combined normally amounted to about 2 to 10% of the ^{14}C fixed in 10 or 20 min from $NaH^{14}CO_3$ by Chlamydomonas cells in 3 mM phosphate buffer at pH 7.5. Most of the glycolate would be excreted and in our current experiments so little (< 1%) ^{14}C was present in glycine and serine that these compounds were not included in Tables 2 and 3 which listed the main labeled products of CO_2 fixation. Glycolate excretion has been attributed to an insufficient system for its metabolism; glycolate oxidase is absent and glycolate dehydrogenase activity is very small. Chlamydomonas contained adequate ribulose-P_2 carboxylase/oxygenase and P-glycolate phosphatase for glycolate biosynthesis (23). They also contain 1 to 2 mmoles$\cdot hr^{-1}\cdot mg^{-1}$ chlorophyll of NADPH glyoxylate reductase (23) and an active glutamate or

alanine glyoxylate aminotransferase for glycine formation. Thus the aminotransferase inhibitor, aminooxyacetate (AOA) should have inhibited the conversion of glyoxylate to glycine and the glyoxylate could be reduced to glycolate by the NADPH:glyoxylate reductase. We never observed the accumulation of significant quantities of glyoxylate on the chromatograms of Chlamydomonas products, even with AOA treatment.

Comparison of Aminooxyacetate With Other Inhibitors of Glycolate Metabolism

Previous experiments with air-grown algae had pointed out that glycolate excretion could be forced by inhibitors of the glycolate pathway, such as isonicotinyl hydrazide or hydroxypyridine methane sulfonate (6,14,19). With air grown cells 0.2 to 1 mM isonicotinyl hydrazide increased glycolate excretion at pH 7.0 from nil to about 30 $nmol \cdot hr^{-1} \cdot ml^{-1}$ of 2% (v/v) cells (19). Isonicotinyl hydrazide increased glycolate excretion by Chlorella to 17 μg glycolate$\cdot mg^{-1}$ dry weight$\cdot hr^{-1}$ (7,21), but the hydrazide had no effect on glycolate excretion by the blue green algae, Coccochloris peniocystis (12). α-Hydroxypyridinemethanesulfonate, an inhibitor of glycolate oxidase as well as glycolate dehydrogenase, stimulates glycolate excretion by green algae (13), blue green algae (12) and Rhodospirillum rubrum (5). Thus the increase in glycolate excretion with AOA would seem to be consistent with the use of other inhibitors of the glycolate pathway. However the magnitude of glycolate excretion with AOA seems to be much greater than with the other inhibitors.

Although AOA ($COOH\text{-}CH_2ONH_2$) is an analog of glycolate, its possible hydrolysis to glycolate cannot account for the large amount of ^{14}C-glycolate produced from $^{14}CO_2$ for two reasons. The AOA was not labeled with ^{14}C, and the amount of excreted glycolate exceeded the amount of AOA added.

Speculation about stimulated glycolate excretion by aminooxyacetate

Even with the above consistencies for AOA inhibition of the glycolate pathway, the immense increase in glycolate excretion by Chlamydomonas cells during photosynthesis in the presence of 1 mM AOA can not be readily explained. In the experiment with cells grown on air (Table 2), total production of glycolate after 2 min increased from 0.3% of the total ^{14}C in controls to 11.7% by cells treated with AOA or from 3.8% after 10 min to 41% with AOA treatment. At longer times of 20 to 40 min, 50 to 75% of the total photosynthate was converted into glycolate and excreted. This phenomenon occurs with Chlamydomonas cells grown on either air or high CO_2. There is no evidence with these algae for such a large flow of carbon through the glycolate pathway to glycine. Glycine and serine were hardly labeled. In addition there is no enzyme of significant activity for converting glycolate to glyoxylate. Upon addition of ^{14}C-glycolate to homogenate of Chlamydomonas only trivial amounts were converted to glycine, glyoxylate or CO_2. ^{14}C-glycolate is not taken up and metabolized by the whole algae. Therefore it would appear that there is little glycolate metabolism in these algae and that greatly increased glycolate excretion could hardly be due to AOA inhibition of the aminotransferase for glyoxylate conversion to glycine. Chlamydomonas also contain 1 to 2 $\mu mol \cdot hr^{-1} \cdot mg^{-1}$ chlorophyll of NADPH:glyoxylate reductase when assayed at its optimum of pH 6.2 but AOA inhibited it with a K_i of 1 mM (23). This activity is about 1 to 2% of the rate of photosynthetic CO_2 fixation and would appear to be inadequate to account for the fast rate (40% of photosynthesis) of glycolate excretion in the presence of AOA. This arguement also tends to exclude biosynthesis of glyoxylate by some unknown pathway, as a precursor for the excreted glycolate. All these facts suggest that AOA might be effecting glycolate biosynthesis rather than its

metabolism. However, 1 mM AOA did not inhibit purified ribulose-P_2 carboxylase or oxygenase activities from spinach leaves, P-glycolate phosphatase, glycolate dehydrogenase, or carbonic anhydrase. Its point(s) of action is unknown.

The large amount of the total ^{14}C incorporation into glycolate during photosynthesis in the presence of AOA and the lack of evidence for much carbon flow normally from glycolate to glycine in unicellular algae, leads to speculation that the glycolate might be arising from some other source than ribulose-P_2. However ^{14}C (10) and ^{18}O (18) distribution in glycolate formed by algae has been consistent with its formation by ribulose-P_2 oxygenase from carbohydrates of the photosynthetic carbon cycle (28).

References

1 Benson AA, M Calvin 1950 Carbon dioxide fixation by green plants. Ann Rev Plant Physiol 1:25-42

2 Benson AA, JA Bassham, M Calvin, M Goodale, TC Haas, VA Haas, W Stepka 1950 The path of carbon in photosynthesis V. Paper chromatography and radioautography of the products. J Am Chem Soc 72:1710-1718

3 Berry J, J Boynton, A Kaplan, M Badger 1976 Growth and photosynthesis of Chlamydomonas reinhardtii as a function of CO_2 concentration. Carnegie Institution Year Book 75:423-432

4 Chang W-H, NE Tolbert 1970 Excretion of glycolate, mesotartrate and isocitrate lactone by synchronized cultures of Ankistrodesmus braunii. Plant Physiol 46:377-385

5 Codd GA, BM Smith 1974 Glycolate formation and excretion by the purple photosynthetic bacterium Rhodospirillum rubrum. FEBS Let 48:105-108

6 Colman B, AG Miller, B Grodzinski 1974 A study of the control of glycolate excretion in Chlorella. Plant Physiol 53:395-397

7 Findenegg GR 1977 Interaction of glycolate$^-$, HCO_3^-, Cl^- and H^+ balance of Scenedesmus obliquus. Planta 135:33-38

8 Gruber PJ, SE Frederick, NE Tolbert 1974 Enzymes related to lactate metabolism in green algae and lower land plants. Plant Physiol 53:167-170

9 Heldt, HW 1980 Measurement of metabolite movement across the envelope and of the pH in the stroma and the thylakoid space in intact chloroplasts. Methods of Enzymol 69:604-613

10 Hess, JL, NE Tolbert 1967 Glycolate biosynthesis by Scenedesmus and Chlorella in the presence or absence of $NaHCO_3$. Plant 74:278-285

11 Howitz KT, RE McCarty 1982 pH dependence and kinetics of glycolate uptake by intact pea chloroplasts. Plant Physiol 70:949-952

12 Ingle RK, B Coleman 1976 The relationship between carbonic anhydrase activity and glycolate excretion in the blue green algae Coccochloris peniocystis. Plant 128:217-223

13 Jahnke LS 1981 Indirect effects of α-hydroxypyridinemethanesulfonate and cyanide on photosynthesis and glycolate excretion in Chlorella. Z Pflanzenphysiol 104:373-383

14 Kaplan A, JA Berry 1981 Glycolate excretion and the oxygen to carbon dioxide net exchange ratio during photosynthesis in Chlamydomonas reinhardtii. Plant Physiol. 67:229-232

15 Kearney PC, NE Tolbert 1962 Appearances of glycolate and related products of photosynthesis outside of chloroplasts. Arch Biochem Biophys 98:164-171

16 Krampitz, LO 1983 The accompanying paper.

17 Lawyer AL, KL Cornwell, PO Larsen, JA Bassham 1981 Effects of carbon dioxide and oxygen on the regulation of photosynthetic carbon metabolism by ammonium in spinach mesophyll cells. Plant Physiol 68:1231-1236

18 Lorimer GH, CB Osmond, T Aakazawa, S Asami 1978 Mechanism of glycolate synthesis by Chromatium and Chlorella. Arch Biochem Biophys 185:49-56

19 Nelson EB, NE Tolbert 1969 The regulation of glycolate metabolism in Chlamydomonas reinhardtii. Biochem Biophysica Acta 184:263-270

20 Orth GM, NE Tolbert, E Jiminez 1966 Rate of glycolate formation during photosynthesis at high pH. Plant Physiol 41:143-147

21 Rickli EE, SAS Ghazanfar, BH Gibbons, JT Edsall 1964 Carbonic Anhydrase from human erythrocytes. J Biol Chem 239:1065-1078

22 Schou L, AA Benson, JA Bassham, M Calvin 1950 The path of carbon in photosynthesis XI. The role of glycolic acid. Physiol Plantarum 3:487-495

23 Selph, NL 1982 Enzymes of glycolate metabolism in Chlamydomonas reinhardtii. Ph.D. Thesis, Michigan State University

24 Takabe T, T Akazawa 1981 Mechanism of glycolate transport in spinach leaf chloroplasts. Plant Physiol 68:1093-1097

25 Tolbert NE, LP Zill 1956 Excretion of glycolic acid by algae during photosynthesis. J Biol Chem 222:895-906

26 Tolbert NE 1963 Glycolate pathway in Photosynthesis mechanisms in green plants, publication 1145, Nat Acad Sci - Nat Res Council, p 648-662

27 Tolbert NE 1974 Photorespiration by algae. in: Algal physiology and biochemistry, ed SDD Stewart, Blackwell Scientific Publications Ltd, Oxford, England. p. 474-504

28 Tolbert NE 1980 Photorespiration. in: The Biochemistry of Plants, eds P Stumpf, E Conn, II 488-525, Academic Press

Table 1. ^{14}C Distribution Among Products from Air Grown Chlamydomonas with Aminooxyacetate Treatment. See figure 1 for total fixation and excretion at other time points

	No Treatment				1 mM AOA			
	2 min		10 min		2 min		10 min	
Total ^{14}C fixed ml	7.88×10^5 cpm		3.72×10^6 cpm		7.67×10^5		3.39×10^6	
Supernatant	1.2%		2.2%		4.5%		20.7%	

Products	Cells	Supernatant	Total	Cells	Supernatant	Total	Cells	Supernatant	Total	Cells	Supernatant	Total
	%	%	%	%	%	%	%	%	%	%	%	%
glycolate	0	72.6	0.3	0.9	77.7	3.8	9.2	46.5	11.7	10.6	86.7	41.1
phosphate esters	25.3	0	25.2	37.9	0	29.2	10.7	0	10.0	17.7	0	10.5
UDPG	0	0	0	2.8	0	2.1	0.4	0	0.4	1.4	0	0.8
malate	14.9	24.4	14.9	13.0	20.0	30.0	20.5	5.9	19.5	11.5	3.0	8.0
glutamate	14.8	0	14.7	15.9	2.3	12.3	9.4	0	8.7	19.7	1.1	12.2
aspartate	6.5	0	6.4	4.7	0	3.6	22.3	0.5	20.8	25.3	2.1	15.9
lactate	26.3	0	26.2	14.6	0	11.2	16.6	0	15.5	9.2	1.8	6.2
succinate	5.8	0	5.8	3.8	0	3.0	6.6	0	6.1	1.6	0	0.9
fumarate	1.1	0	1.1	1.0	0	0.8	1.1	0	1.0	0.4	0	0.2
glutamine	0	0	0	1.7		1.3	0.5	0	0.5	1.2	0.9	1.1
alanine	5.4	0	5.4	3.6	0	2.8	0.5	0	0.5	0	0	0
pyruvate	0	0	0	0	0	0	2.3	47.1	5.2	1.4	4.5	2.7

Table II. ^{14}C Distribution Among Products from High CO_2 Grown Chlamydomonas with Aminooxyacetate Treatment. See figure 2 for total fixation and excretion at other time points

	No Treatment						1 mM AOA					
	2 min			10 min			2 min			10 min		
Total ^{14}C fixed ml	1.04×10^5			4.68×10^5			9.58×10^4			4.97×10^5		
Supernatant	0%			2.8%			1.0%			16.6%		
Products	Cells	Supernatant	Total	Cells	Supernatant	Total	Cells	Supernatant	Total	Cells	Supernatant	Total
	%	%	%	%	%	%	%	%	%	%	%	%
glycolate	0	0	0	3.0	97.8	12.7	0	100	0.9	5.3	99.6	37.3
phosphate Esters	12.0	0	12.0	25.9	0	23.3	12.9	0	12.8	16.0	0	10.5
UDPG	0	0	0	1.8	0	1.7	0	0	0	0	0	0
malate	16.9	0	16.9	13.9	2.2	12.7	33.5	0	33.2	14.1	0.4	9.4
glutamate	10.0	0	10.0	5.4	0	4.8	19.2	0	19.0	23.2	0	15.3
aspartate	4.7	0	4.7	1.2	0	1.1	34.5	0	34.2	21.1	0	13.9
lactate	51.3	0	51.3	34.9	0	31.3	0	0	0	15.4	0	10.2
succinate	0	0	0	3.1	0	2.8	0	0	0	3.9	0	2.6
fumarate	0	0	0	0.4	0	0.3	0	0	0	1.0	0	0.7
glutamine	0	0	0	0	0	0	0	0	0	0	0	0
alanine	5.1	0	5.1	10.4	0	9.3	0	0	0	0	0	0
pyruvate	0	0	0	0	0	0	0	0	0	0	0	0

Table III. Distribution of ^{14}C Among Excreted Products From Chlamydomonas Treated with DCMU

compound	after 20 min		after 40 min		after 20 min control plus 20 min with DCMU	
	cpm	%	cpm	%	cpm	%
glycolate	29,093	89.60	62,081	94.5	30,271	59.2
phosphate esters	0		0		3,835	7.5
malate	2,576	7.9	2,674	4.1	9,205	18.0
glutamate	376	1.2	478	0.7	3,013	5.9
aspartate	0		0		350	0.7
lactate	208	0.6	0		633	1.2
succinate	0		465	0.7	3,241	6.3
glutamine	0		0		544	1.1
other	210	0.6	0		51	0.1
total in aliquot	32,463	99.9	65,698	100.0	51,143	100

Table IV. Effect of Aminooxyaceate and Ammonium Nitrate on Glycolate Excretion By Chlamydomonas Grown With Low CO_2

Algal Media and time	Treatment							
	0		1 mM AOA		3 mM AOA		10 mM AOA	
Control	Total cpm	% excreted	Total cpm	% excreted	Total cpm	% excreted	Total cpm	% excreted
10 min	6.5×10^4	0.2	8.0×10^4	5.4	7.5×10^4	6.7	6.7×10^4	5.8
20 min	1.3×10^5	0.4	1.4×10^5	13.4	1.4×10^5	13.2	1.2×10^5	12.2
Plus 0.2 mM NH_4NO_3								
10 min	5.5×10^4	0.4	6.4×10^4	0.2	6.6×10^4	0.2	6.3×10^4	2.5
20 min	1.1×10^5	0.5	1.6×10^5	0.3	1.3×10^5	1.2	1.2×10^5	8.6

Table V. Effect of O_2 And Aminooxyacette On ^{14}C Excretion by Chlamydomonas.

	Air-Grown Cells		5% CO_2-Grown Cells	
O_2 Concentration	0	1 mM AOA	0	1 mM AOA
	% ^{14}C excreted			
0 (N_2)	1.3	5.1	2.0	4.8
21% (air)	1.0	8.4	8.0	13.7
100%	3.0	24.9	16.4	22.7

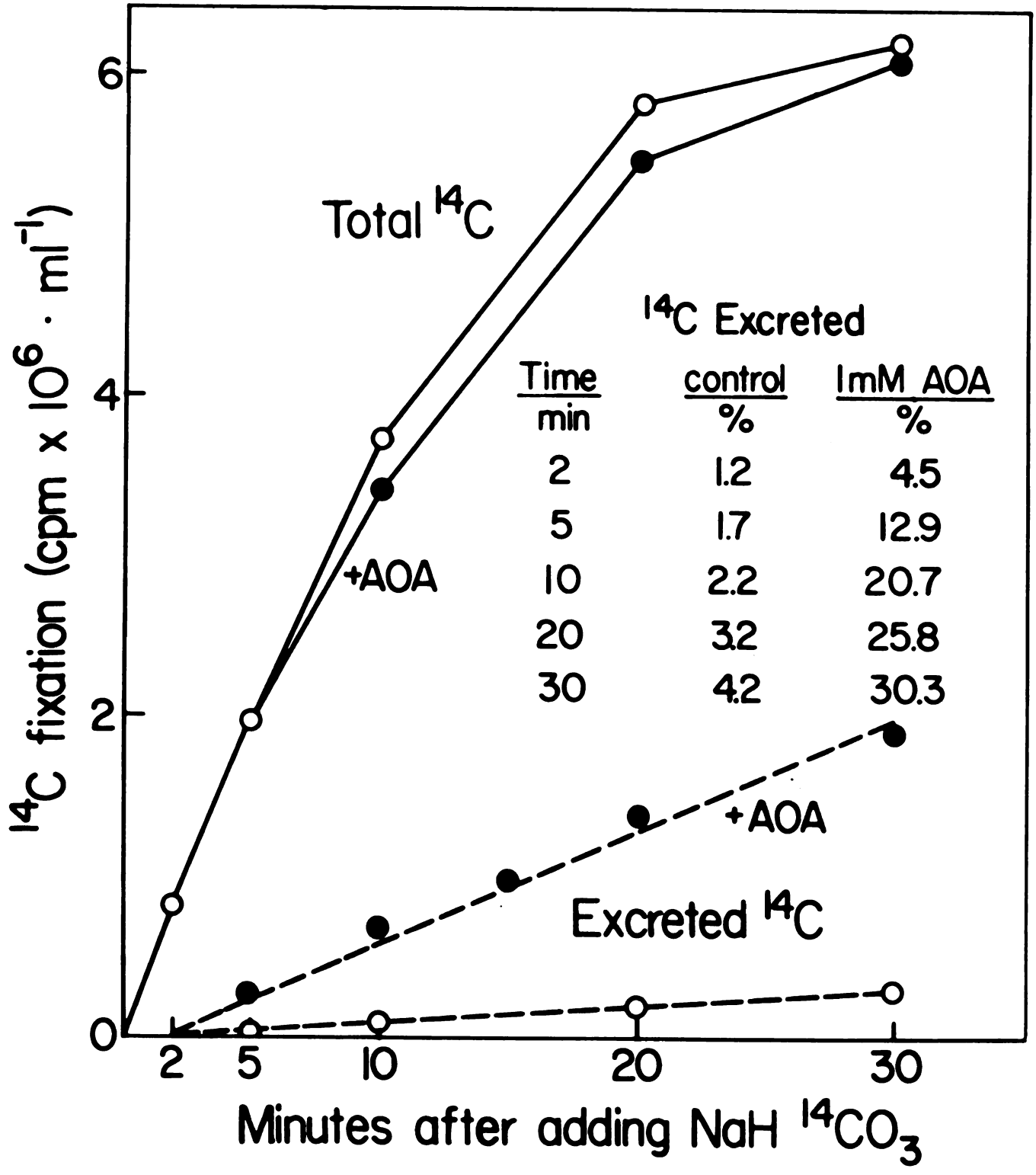
Air-grown cells were 3 days old and entering the stationary phase. CO_2-grown cells were 5 days old and in the stationary phase. Values are % of total ^{14}C fixed that was excreted after a period of 20 min of photosynthesis with 3 mM $NaH^{14}CO_3$. Since the experiments were run in the light and in tubes open to the air strictly anaerobic conditions were not obtained when gassing with N_2.

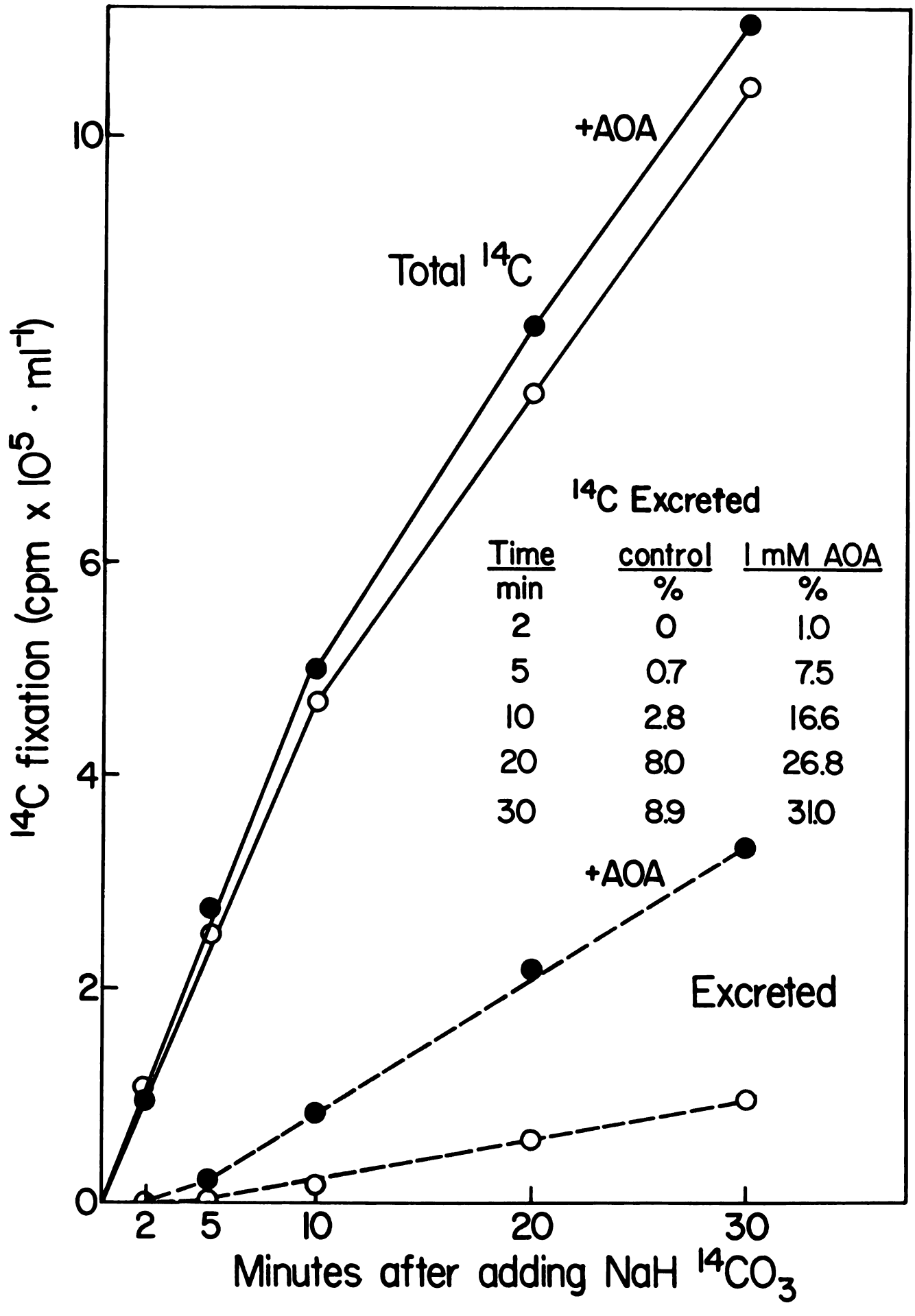
Figure 1. Rate of $^{14}CO_2$ Fixation And Glycolate Excretion By Chlamydomonas Grown On Air When Treated With 1 mM Aminooxyacetate. A 2% Suspension containing 4 x 10^4 cells/mm^3 from the log phase of growth were given 1 mM $NaH^{14}CO_3$ at zero time - total ^{14}C fixed; --- ^{14}C excreted; open circles no treatment; closed circles 1 mM AOA was added 2 min prior to addition of $NaH^{14}CO_3$.

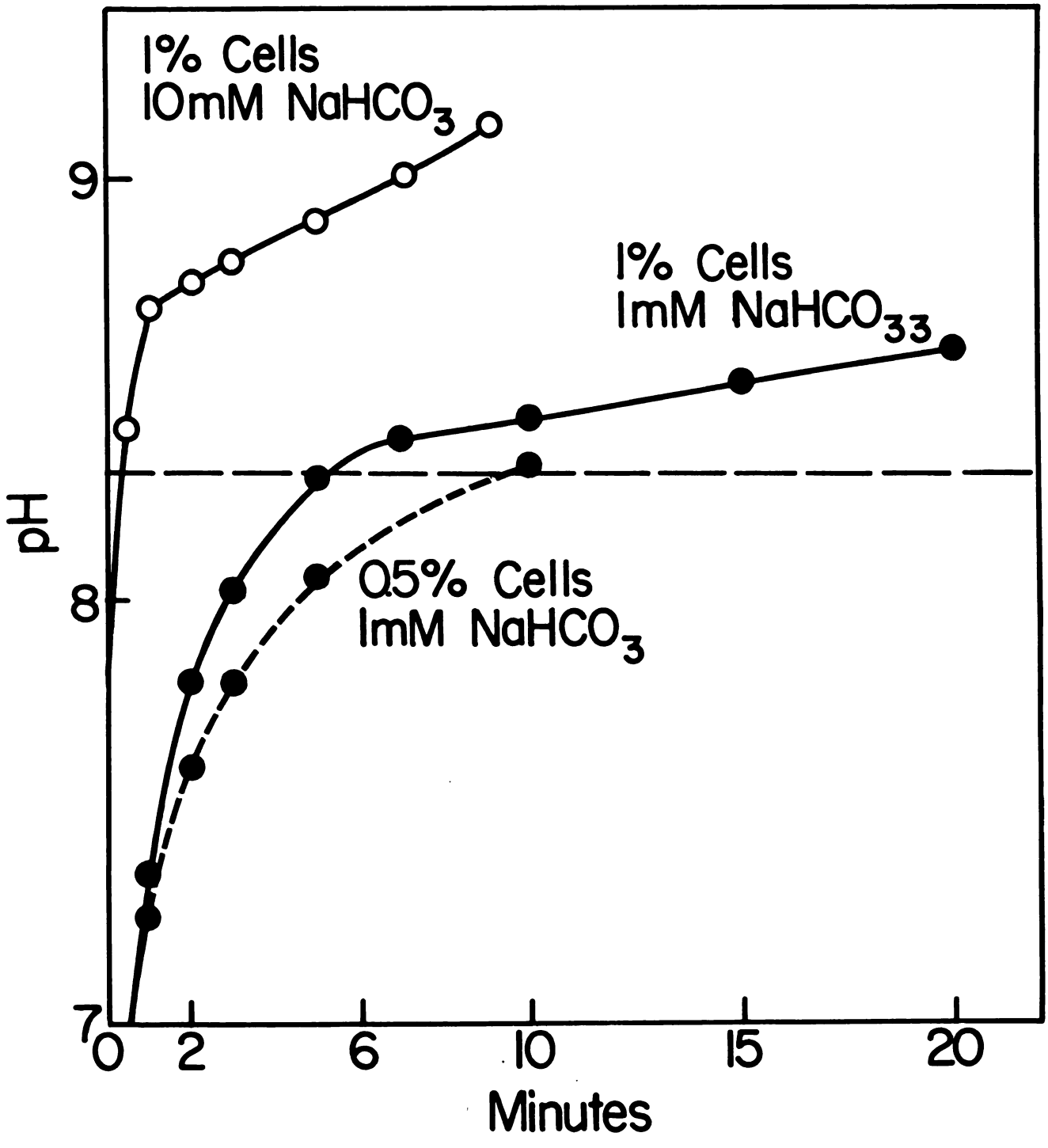
Figure 2. Rate of $^{14}CO_2$ Fixation And Glycolate Excretion By Chlamydomonas Grown With 5% CO_2 when treated With 1 mM Aminooxyacetate. Experimental conditions similar to those in figure 1 except that the experiment were run at a different periods with $NaH^{14}CO_3$ of lower specific activity.

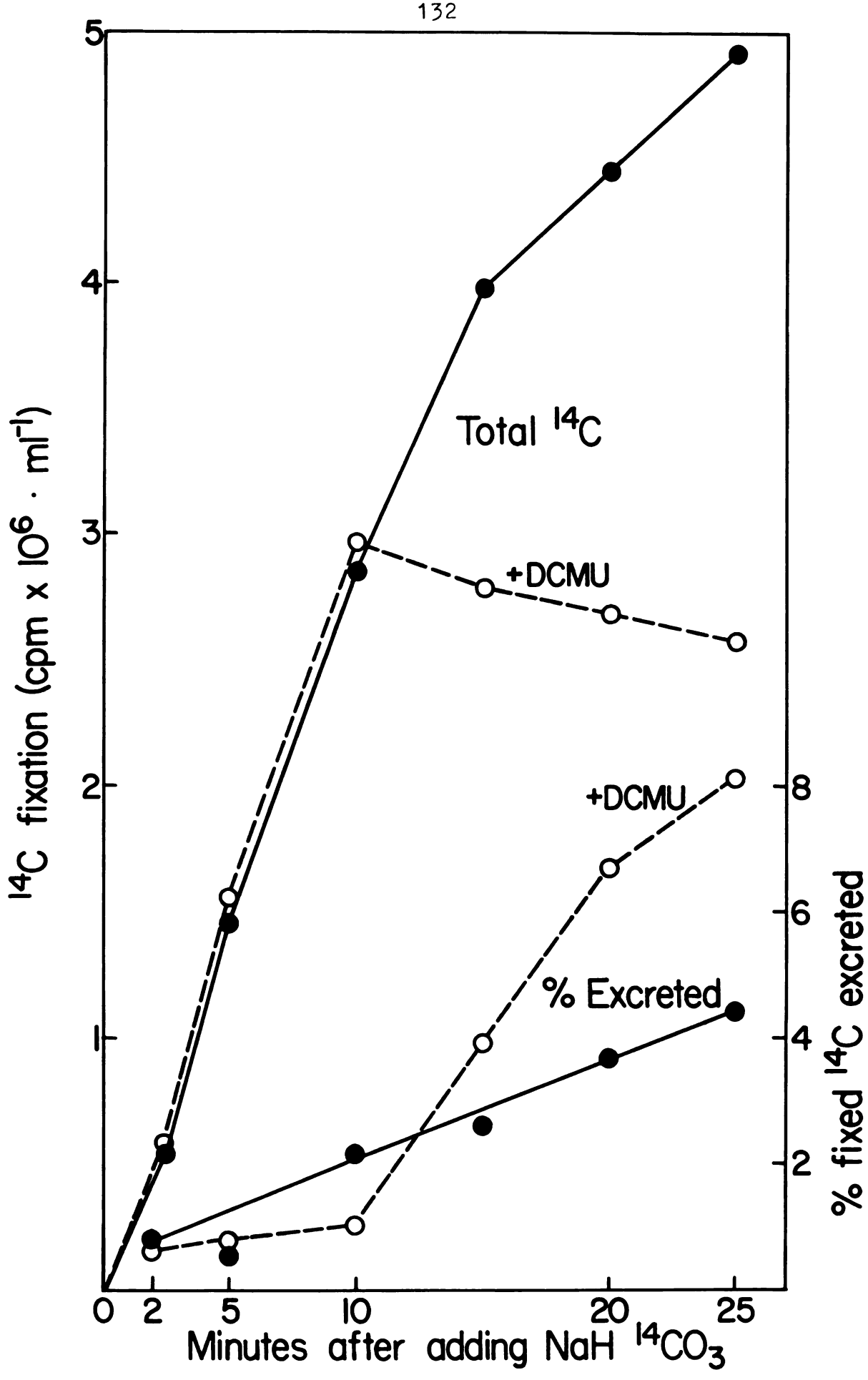
Figure 3. Increase In pH By Air-Grown Chlamydomonas Cells In Water In The Light Upon Addition of $NaHCO_3$. The horizontal dashed line indicates the pH of a $NaHCO_3$ solution. The first Pka of a bicarbonate solution is 6.3 and the second is 9.4. The pH before adding $NaHCO_3$ was about 5.5 from the natural acidity of the algal suspension. pH was measured by electrodes in the algal suspension. --- 0.5% cells in 1 mM $NaHCO_3$; —— 1% cells in 1 mM $NaHCO_3$; —— 1% cells in 10 mM $NaHCO_3$. The pH increase with a 2% cell suspension and 10 mM $NaHCO_3$ was similar to that with 1% cells and 10 mM $NaHCO_3$.

Figure 4. Effect of DCMU on Photosynthetic $^{14}CO_2$ Fixation and Excretion of ^{14}C By Air-Grown Chlamydomonas.


Total ^{14}C
+AOA
^{14}C Excreted
Time min
control %
1mM AOA %
2 1.2 4.5
5 1.7 12.9
10 2.2 20.7
20 3.2 25.8
30 4.2 30.3
+AOA
Excreted ^{14}C
^{14}C fixation (cpm x 10^6 · ml^{-1})
0
2
4
6
2 5 10 20 30
Minutes after adding NaH $^{14}CO_3$


+AOA
Total ^{14}C
^{14}C Excreted
Time min | control % | 1 mM AOA %
2 | 0 | 1.0
5 | 0.7 | 7.5
10 | 2.8 | 16.6
20 | 8.0 | 26.8
30 | 8.9 | 31.0
+AOA
Excreted
^{14}C fixation (cpm x 10^5 · ml^{-1})
0
2
4
6
10
2
5
10
20
30
Minutes after adding NaH $^{14}CO_3$


1% Cells
10mM NaHCO3
1% Cells
1mM NaHCO33
0.5% Cells
1mM NaHCO3
pH
9
8
7
0
2
6
10
15
20
Minutes


Total ^{14}C
+DCMU
+DCMU
% Excreted
^{14}C fixation (cpm x 10^6 · ml^{-1})
% fixed ^{14}C excreted
Minutes after adding $NaH\,^{14}CO_3$
0
2
5
10
20
25
1
2
3
4
5
2
4
6
8