ABSTRACT

GLYCOLATE METABOLISM IN GREEN ALGAE

by Edward Blake Nelson

Assimilation of exogenous glycolate by Scenedesmus obliquus was measured at stages in the life cycle of synchronized cultures. Glycolate uptake occurred only during the stage of cell division. This anion stimulated respiration and photosynthesis when the cells could assimilate it. Equal rates of glycolate metabolism occurred in the light and dark, and respiration of Scenedesmus was stimulated 25% to 100%. In the light and in the absence of CO_2, photosynthetic oxygen evolution increased 100% to 300% in the presence of glycolate. Glycolate and glyoxylate were equally effective in stimulating oxygen evolution. This stimulation was inhibited by CMU, an inhibitor of photosynthetic electron transport. It is proposed that the oxidation of glycolate to glyoxylate and the reduction of glyoxylate to glycolate is a terminal metabolic cycle, which accepts reducing equivalents from electron transport in photosynthesis and thereby stimulates oxygen evolution.

The ability of Chlamydomonas reinhardtii to excrete glycolate was found to be regulated by CO_2 availability during growth. While Chlamydomonas grown on 1% CO_2 in air

were capable of glycolate excretion, the ability to excrete glycolate decreased with increasing culture density. Cells grown on air (0.03% CO_2) did not excrete glycolate. Air grown cells would excrete glycolate in the presence of 10^{-2} M isonicotinyl hydrazide, an inhibitor of the glycolate pathway. When cultures grown on 1% CO_2 in air were transferred to air, they lost the ability to excrete glycolate in approximately 16 hours.

Levels of enzyme activities which might be involved in regulating glycolate excretion were examined in Chlamydomonas cultures grown on 1% CO_2 in air or on air. P-glycolate phosphatase (phosphoglycolate phosphohydrolase E.C. 3.1.3.18) activity was high and did not vary significantly on transfer from 1% CO_2 in air to air. Glycolate oxidase (glycolate: O_2 oxidoreductase E.C. 1.1.3.1) could not be detected in crude extracts of Chlamydomonas. A new enzyme, glycolate dehydrogenase (glycolate: acceptor oxidoreductase, no E.C. number), which catalysed the oxidation of glycolate to glyoxylate, was found in these Chlamydomonas. The activity of glycolate dehydrogenase increased two to four fold 16 hours after transfer of cells from 1% CO_2 in air to air. The increase in activity was inhibited by cycloheximide (5 µg/ml), an inhibitor of protein synthesis. These results suggest that CO_2 availability during growth of the algae, regulates glycolate excretion by controlling the levels of a glycolate dehydrogenase.

Edward Blake Nelson

Glycolate dehydrogenase was purified 10 fold from Chlamydomonas by Triton X-100 extraction of whole cells and $(NH_4)_2SO_4$ fractionation of the protein. The enzyme had no oxidase activity. No glyoxylate formation occurred in the absence of artificial electron acceptors. Only dichloroindophenol and phenazine methosulfate were found to serve as electron acceptors. The natural electron acceptors are unknown. The enzyme oxidized glycolate at pH 8.0 to 8.7 preferentially to all other α-hydroxy acids tested; and the K_m was 2.2×10^{-4} M. D-lactate was the second best substrate with a pH optimum at 8.7 and a K_m of 1.5×10^{-3} M. The enzyme was sensitive to sulfhydryl inhibitors. No requirement for co-factors was established, although by precedent a flavin is suggested.

Glycolate dehydrogenase activity was found in the following green algae; Chlamydomonas, Chlorella, Scenedesmus, Euglena, and Acetabularia. No glycolate oxidase was detected in these algae. The higher plants examined contained glycolate oxidase but no glycolate dehydrogenase. It is suggested that the enzyme oxidizing glycolate to glyoxylate in algae is a dehydrogenase and in plants an oxidase, which is located in peroxisomes. Procedures for differentiating between these two enzymes are outlined.

L-glutamate: glyoxylate aminotransferase (E.C. 2.6.1.4) was measured in crude extracts of Chlamydomonas. L-glutamate and L-alanine were the preferred amino donors.

Sufficient levels of activity were present to metabolize all glyoxylate formed by glycolate dehydrogenase.

The presence of glycolate dehydrogenase and glyoxylate aminotransferase in Chlamydomonas establishes enzymes for the glycolate pathway in green algae. This accounts for the labeling data in which glycolate-1-^{14}C was converted to glycine-1-^{14}C in green algae.

GLYCOLATE METABOLISM IN GREEN ALGAE

By

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bicine	N,N-bis(2-hydroxyethyl)glycine
p-CMB	p-chloromercuribenzoic acid
CMU	3-(p-chlorophenyl)-1,1-dimethylurea
DCIP	2,6-dichloroindophenol
EDTA	(Ethylenedinitrilo)tetraacetic acid
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
MES	2-(N-morpholino)ethanesulfonic acid
NAD^+	Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
PMS	phenazine methosulfate
tricine	N-tris(hydroxymethyl)glycine ethanesulfonic acid
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

The use of $^{14}CO_2$ for research in photosynthesis led to the discovery of the path of carbon in photosynthesis. It was originally observed that glycolate (α-hydroxyacetate) was rapidly formed during photosynthesis (10), and later work showed that it could be a major product of photosynthesis (97).

The determination of the direction of glycolate metabolism led to the formulation of the glycolate pathway (Figure 1). Applying the concept of "the unity of biochemistry," this pathway determined for higher plants was also attributed to green algae. Later work in a number of laboratories then suggested that the glycolate pathway did not exist or was not complete in green algae.

It has been the goal of the research reported in this thesis to ascertain the role of the glycolate pathway in green algae. It is felt that such information is important to our basic understanding of plant biochemistry and furthermore to our understanding of primary productivity in the biosphere. If glycolate accounts for approximately fifty percent of photosynthetic carbon metabolism as determined by Zelitch (104), then the problem of glyco-

late metabolism in algae is especially important.

LITERATURE REVIEW

The Glycolate Pathway in Higher Plants

Glycolate metabolism in higher plants has been demonstrated in three ways; 1) feeding experiments with glycolate-^{14}C, 2) $^{14}CO_2$ fixation experiments and, 3) inhibitor studies. The metabolic pathway derived from these studies, commonly called the glycolate pathway (92), is shown in Figure 1.

Specifically labeled glycolate-^{14}C fed to a number of green plants has given results consistent with the glycolate pathway (7, 60, 87). Glycolate-1-^{14}C gave glycine-1-^{14}C, serine-1-^{14}C and glycerate-1-^{14}C, while glycolate-2-^{14}C gave glycine-2-^{14}C, serine-2,3-^{14}C and glycerate-2,3-^{14}C (76). A precursor product relationship was found for these metabolites when glycolate-2-^{14}C was fed to wheat (76). The labeling pattern in glucose isolated after glycolate-^{14}C feeding was consistent with the operation of the glycolate pathway (46).

Photosynthetic $^{14}CO_2$ fixation with higher plants gave uniformly labeled glycolate at short times, during which 3-P-glycerate was still carboxyl labeled (10, 42). Labeling patterns of intermediates of the glycolate pathway, reflected synthesis from glycolate rather than 3-P-glycerate. Both glycine and serine have been found to be

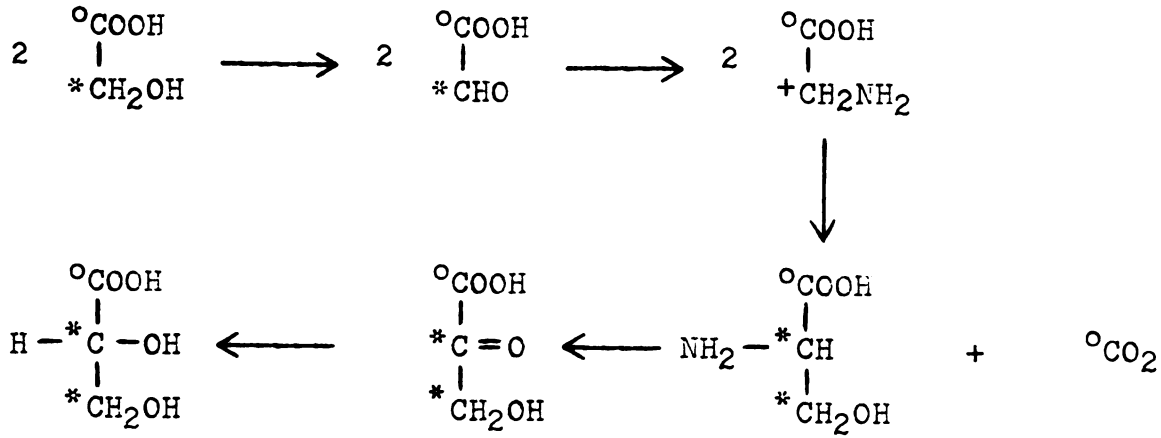


Figure 1. The Glycolate Pathway

uniformly labeled. If serine were derived from 3-P-glycerate, serine-1-^{14}C would be expected at short times.

Metabolism of glycolate via the glycolate pathway results in the loss of a one carbon unit. This one carbon piece is released as CO_2 in a process commonly called photorespiration. By use of an inhibitor of glycolate oxidation, an α-hydroxypyridinemethane sulfonate, Zelitch (104) has shown the release of CO_2 in the light by tobacco leaf disks was dependent on glycolate oxidation. Goldsworthy (36) obtained similar results with tobacco leaf segments using hydroxypyridinemethane sulfonate and another inhibitor of glycolate metabolism, isonicotinyl hydrazide.

The Glycolate Pathway in Algae

While it is well established in higher plants that glycolate is rapidly synthesized and metabolized, the metabolism of glycolate by algae has been an area of controversy. It was originally observed with Scenedesmus by Schou et. al. (83) that glycolate-^{14}C was assimilated both aerobically and anaerobically in both the light and dark. The products detected by chromatography were consistent with the glycolate pathway (Figure 1). Specific labeling data obtained from glycerate isolated after feeding glycolate-2-^{14}C was consistent with the pathway. Unfortunately no kinetic studies were done by Schou et. al. Other workers (85) have fed glycolate-^{14}C to Chlorella, however the data were not clear enough for

evaluation. The above mentioned work supported the presence of the glycolate pathway in algae.

Work in other laboratories suggested that algae lacked a glycolate pathway. Tolbert and Zill (89) observed that most of the glycolate synthesized was excreted by algae rather than metabolized. Numerous other laboratories have confirmed this (32, 61, 74). Labeling data from photosynthetic $^{14}CO_2$ fixation with algae showed serine to be carboxyl labeled rather than uniformly labeled as found in higher plants, indicating it came from 3-P-glycerate rather than glycolate (13, 43). Originally glycine was found to be labeled differently than glycolate during $^{14}CO_2$ fixation (43), however recent work shows them to be labeled identically (13). Also, it was observed that green algae lacked glycolate oxidase (42).

To obtain data to reconcile these differences, several laboratories attempted to do feeding experiments with glycolate-^{14}C. Unfortunately it has not been possible to obtain reproducible glycolate assimilation by green algae grown under normal laboratory conditions (16, 43, 57). Also cell free extracts of green algae would not metabolize glycolate-^{14}C or P-glycolate-^{14}C (42). The reasons for these observations are not clear. In order to obtain conditions which allow reproducible assimilation of exogenous glycolate, glycolate assimilation by synchronous cultures of Scenedesmus obliquus has been studied. Using this algae it has been

possible to obtain conditions which allow reproducible glycolate assimilation. Bruin (13) fed glycolate-1-^{14}C to these cells and degraded the glycine and serine formed. The labeling data was consistent with the operation of the glycolate pathway. This confirmed the original work of Schou et. al. (83). Thus it appears as though the glycolate pathway operates in green algae at least under certain conditions. Consistent with this is the recent detection of an enzyme from green algae which oxidizes glycolate to glyoxylate (55, 66, 106). It remains to be determined why the serine is carboxyl labeled.

Recent work by Merrett and his associates (37, 38, 58, 59) has suggested that an alternative pathway of glycolate metabolism might exist in Chlorella. Using acetate-^{14}C they observed the following pattern in short term feeding experiments:

acetate ⟶ glycolate ⟶ glycerate ⟶ serine

No glycine was detected. This pathway is identical to the way glycolate is metabolized by Pseudomonas (52). The overall significance of such a pathway in algae remains to be determined.

Several other items related to the glycolate pathway in algae deserve comment. If the pathway is operational then it is expected that green algae should be able to grow heterotropically on glycolate if they are able to assimilate it. It has been observed that glycolate will support slow growth of a phytoplankton strain of Chlorella (84) and

Chlorella ellipsoidea but not C. pyrenoidosa (82). Glycolate would not support dark growth of Euglena at pH 3.9 or 6.9 (23). At a physiological pH of 8, Dropp and McGill (28) found in testing 39 strains of algae that glycolate would not support growth of any of the strains. The lack of growth could be due to the high pH, which would keep glycolate in the anion form rather than as the free acid, which is the more readily absorbed form. Several of these strains would, however, grow on acetate at pH 8.0.

The effect of glycolate on cellular respiration has been studied by Myers (63) who found that glycolate stimulated respiration of Chlorella at pH 3.9 but not at pH 6.9. Sen (84) observed glycolate stimulated respiration at pH 6.1 with a phytoplankton strain of Chlorella which grew on glycolate. Anderson (2) observed stimulated respiration by glycolate using Prototheca zopfii, a colorless algae. No other studies to the author's knowledge have shown stimulated respiration by glycolate with algae. In this thesis and in publication (65) the stimulation of respiration by exogenous glycolate is reported. This occurs in cells of Scenedesmus which are capable of assimilating glycolate.

The Regulation of Glycolate Metabolism by Carbon Dioxide

The synthesis and excretion of glycolate by algae has been found to be very sensitive to CO_2 concentration and availability. Carbon dioxide effects glycolate metabolism

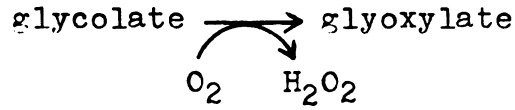
in two ways; one, by controlling the levels of metabolites and two, by regulating the activity of an enzyme which metabolizes glycolate. Numerous laboratories have observed that increasing the CO_2 concentration above 0.2% to 0.4% during photosynthesis lead to a decrease in glycolate synthesis, even though photosynthetic rates were not saturated (61, 74, 97). The effect has also been observed with higher plants (105) and isolated chloroplasts (90). It has been suggested that this phenomenon was observed because a proposed precursor of glycolate such as ribulose-1,5-diphosphate could be oxidized to glycolate when the carbon reduction cycle was not saturated (90, 97). In the experiments discussed above, the algae were grown under laboratory conditions with 1% CO_2 or higher in the atmosphere and then tested for glycolate synthesis or excretion at various CO_2 levels.

The second way in which CO_2 effects glycolate metabolism has not been extensively studied although it appears to be of equal physiological importance. Watt and Fogg (99) observed that Chlorella grown in 3% CO_2 excreted glycolate, while cells grown in 0.03% CO_2 (air) were capable of excreting glycolate only when the culture was very dilute. Two hypotheses could be proposed to explain Watt and Fogg's data: I) growth on air caused a drastic reduction in the cells' ability to synthesize glycolate or II) growth on 3% CO_2 caused a repression of enzymes metabolizing glycolate

and thus forced excretion rather than further metabolism. To differentiate between these two hypotheses, experiments have been carried out on the regulation of glycolate metabolism in Chlamydomonas by CO_2. The work supports the second hypothesis, that high CO_2 represses a glycolate metabolizing enzyme in Chlamydomonas.

Enzymes from Green Plants and Algae Oxidizing Glycolate

Glycolate oxidase was first described in green tissue by Clagett, Tolbert and Burris (19). This enzyme which appeared to be present in all green plant tissues oxidized glycolate to glyoxylate:



It also oxidized L-lactate to pyruvate; however the K_m for L-lactate was too high for the system to be of physiological importance. D-lactate was not oxidized by the enzyme (103). Low levels of glycolate oxidase were found in etiolated tissue, however in the light (31, 86, 87) or upon vacuum infiltration of glycolate into the tissue (53) the level of activity increased. Zelitch and Ochoa (103) showed that glycolate oxidase is a FMN requiring flavoprotein. The enzyme has been crystallized from spinach leaves and the active species has been shown to have a molecular weight of 270,000. No metal co-factors have been detected (34).

Recent work has established the sub-cellular localization of glycolate oxidase to be the peroxisome (92). This

microbody apparently contains catalase and most of the enzymes of the glycolate pathway. The close proximity of catalase and glycolate oxidase assures that H_2O_2 formed during the oxidation of glycolate will be rapidly decomposed.

The role of glycolate oxidase in algae metabolism is not clear. It was reported by Hess and Tolbert (43) that glycolate oxidase measured as an oxidase was absent in green algae that excreted glycolate. On the other hand Zelitch and Day (106) reported the presence of an enzyme in Chlorella and Chlamydomonas which oxidized glycolate to glyoxylate using DCIP as an electron acceptor. Lord and Merrett (55) also reported the presence of glycolate oxidase in Chlorella, but did not show that oxygen was consumed during the oxidation of glycolate to glyoxylate. Using Nitella, a Charophyte, Downton and Tregunna (27) reported a small amount of glycolate oxidase activity in crude extracts assaying with an oxygen electrode.

The above mentioned work did not clarify whether glycolate oxidase was present in green algae or whether there was a different enzyme oxidizing glycolate which did not link to oxygen. Previous work by Schou et. al. (83) demonstrated that glycolate could be metabolized anaerobically in the dark by Scenedesmus. Nelson et. al. (65) also observed anaerobic glycolate metabolism by Scenedesmus and suggested that green algae at least had an enzyme oxidizing glycolate which did not link to oxygen. To clarify this work the

characteristics of an enzyme from Chlamydomonas which oxidizes glycolate have been studied. The results show that it is not an oxidase as found in higher plants and, furthermore, that it is different than the higher plant enzyme by other characteristics.

To avoid confusion and to follow the rules of the International Union of Biochemistry (73), the following enzyme terminology will be used. Glycolate oxidase (glycolate:O_2 oxidoreductase E.C. 1.1.3.1) will refer to enzymes which have been shown to link directly to oxygen, such as glycolate oxidase from spinach. Glycolate dehydrogenase (glycolate:DCIP oxidoreductase, no E.C. number) will refer to the enzyme isolated from green algae which does not link directly to oxygen.

Glycolate Excretion by Algae

One of the most interesting phenomena associated with glycolate synthesis and metabolism by algae is the observation that during photosynthesis under proper conditions, large quantities of glycolate are rapidly excreted into the medium. This observation has been made by a large number of research groups working under laboratory conditions (61, 74, 89, 97). Even of more significance, especially to the naturalist and ecologist is that this phenomena apparently occurs to some extent in nature. Work by Fogg and associates (32, 33) has shown that glycolate is excreted

by natural populations of phytoplankton and that detectable quantities of glycolate can be found in some natural water bodies. Watt (98) has shown that glycolate excretion in nature was highest under conditions optimum for photosynthesis.

The ability of algae to excrete glycolate is apparently not limited to green algae (Chlorophyceae). Hellebust (41) found excretion in all major classes of marine algae besides greens, including browns (Chrysophyceae), blue greens (Cyanophyceae), and diatoms (Bacillariophyceae). From Hellebust's work no relationship of pigment content to glycolate excretion can be made. Watt (100) has demonstrated glycolate excretion by the diatom, Stephanodiscus, under natural and laboratory conditions.

Studies on the regulation of glycolate metabolism by CO_2 are presented in this thesis and have been published (66). There are however other factors controlling excretion which are not understood. Chang (17) using synchronous cultures of Ankistrodesmus and Scenedesmus observed that glycolate excretion occurred during active growth, but not during cell division. This observation has since been confirmed (35, 95). Hess et. al. (45) observed glycolate excretion only during certain phases of the life cycle of Scenedesmus. It has been suggested that the changes in the level of a glycolate oxidizing enzyme through the cell's cycle could account for this observation (95).

Effects of Oxygen on Glycolate Metabolism

Oxygen affects glycolate synthesis and excretion and photosynthesis in an opposite manner. It was first observed by Benson and Calvin (10) that glycolate synthesis by green algae requires oxygen. Tolbert and Zill (89) demonstrated that glycolate excretion requires oxygen. Numerous other laboratories have confirmed these results. When oxygen was 90 to 100% of the atmosphere, glycolate accounted for 10% (8), 30% to 40% (20) or 92% (97) of the newly fixed CO_2 by Chlorella. While absolute amounts vary, all laboratories have observed this phenomena.

The relationship, if any, of enhanced glycolate synthesis to the observed inhibition of photosynthesis by high oxygen (the Warburg effect) (94) is not understood. Studies by Ellyard and Gibbs (30) with isolated spinach chloroplasts have shown that increasing oxygen concentration leads to increased glycolate synthesis. They were unable to determine the biochemical mechanism which controls this effect.

Effect of Light on Glycolate Metabolism

Increased light intensity increased glycolate synthesis in both algae (74, 98) and higher plants (104). The increase in glycolate synthesis occurred even when photosynthesis had been saturated with light (74). The quality of light also affected the glycolate system. Becker et. al. (9)

have observed that in blue light glycolate was not excreted by Chlorella, while in red light glycolate was excreted. Hess and Tolbert (44) have observed a complex effect of red and blue light on glycolate synthesis depending on growth conditions. At present no unifying theory can be proposed to account for the effect of different wavelengths of light on glycolate synthesis.

Glutamate: Glyoxylate Aminotransferase

The presence of an enzyme which converts glyoxylate to glycine is necessary for the completion of the glycolate pathway as shown in Figure 1. L-glutamate: glyoxylate aminotransferase activity has been shown in pea (21), tobacco (102), wheat (48), and spinach leaves (49) as well as endosperm tissue (22). Kisaki and Tolbert (49) have shown that the enzyme was localized in the peroxisome, an observation consistent with its role in the glycolate pathway. A requirement for the transaminase cofactor pyridoxal phosphate has been shown by Wilson et. al. (102) with tobacco leaf extracts.

To account for the conversion of glycolate-1-^{14}C to glycine-1-^{14}C in algae and an equal labeling in glycolate and glycine after photosynthetic $^{14}CO_2$ fixation (13), the participation of glutamate: glyoxylate aminotransferase is suggested. The presence of this aminotransferase to the author's knowledge has not been reported in green algae.

For this reason crude extracts of Chlamydomonas were examined to determine if this enzyme was present. The results presented in this thesis show the presence, level and amino donor specificity for glutamate: glyoxylate aminotransferase found in crude extracts of Chlamydomonas.

MATERIAL AND METHODS

Algae

Chlamydomonas reinhardtii Dangeard (-) (No. 90) was from the culture collection of algae at the University of Indiana. Cells were grown at 20°C with 1000 ft. candle light, in low-form Fernbach culture flasks in a growth chamber. The flasks containing 1500 ml culture media were shaken approximately 60 cycles/minute on a reciprocating Eberbach shaker while being aerated with gas from a tube inserted below the level of the culture media. Media were prepared as described previously (66, 71) and autoclaved before use. Flasks were gassed by bubbling into the culture medium either air or approximately 1% CO_2 in air.

Chlorella pyrenoidosa (strain 211/8p) was obtained from Prof. M. J. Merrett. This unusual strain is the same as that used by Merrett in his investigations (55, 59). The algae were routinely grown in low-form Fernback flasks on the same medium used in growing Chlamydomonas and at room temperature (23°C) and light (approximately 200 ft. candles). The cultures were continually aerated with air from a tube inserted below the level of the culture media.

Acetabularia mediterranea were obtained from Dr. P. Wolk of the A.E.C. Plant Research Laboratory. They had

been grown in sun light at room temperature on Erd-Schreiber media (75).

Euglena gracilis Klebs "Z" (No. 753) was obtained from the culture collection of algae at the University of Indiana. Cultures were grown at room temperature and light with continuous aeration. The media used was described by Cramer and Myers (23).

Scenedesmus obliquus (No. 393) was obtained from the culture collection of algae at the University of Indiana. This strain is the same as Gaffron's D-3. For work with synchronous cultures the algae were grown on a 16 hour day, 8 hour dark regime as previously described (17, 65). When cells were grown for enzymatic analysis, the mediawere the same as used for Chlamydomonas. The cells were then grown at room temperature and light with continuous aeration.

Nitella and Chara were obtained from local lakes in fall, 1969 by Dr. W. Wetzel. Nitella was also purchased from Carolina Biological Supply, Burlington, North Carolina.

Plants

Elodea was purchased from Carolina Biological supply house in Burlington, North Carolina and used within a day of arrival. Spinach was purchased from local markets. Marchantia polymorpha was obtained from Dr. Ole Björkman of the Carnegie Institute.

Measurement of Glycolate Excretion

Chlamydomonas were harvested by centrifugation at 1000 g for 10 minutes at 4°C, washed once in distilled water and then resuspended in buffer to approximately 2% (v/v). In most experiments 10 mM phosphate (pH 8.0) was used as the buffer. This pH was chosen because it has been observed that glycolate excretion is promoted by high pH (71). The cells resuspended in 15 ml were incubated at 20° for 10 min in the dark in a lollipop container after which the lights, 3,000 foot candles from flood lamps, were turned on with simultaneous addition of 0.5 ml of 0.2 M $NaHCO_3$ (final concentration 6.5 mM). Throughout the entire experiment, the cells were aerated with 100% O_2. At appropriate times, samples were removed and the cells spun out at 1000 g for 5 minutes. Aliquots of the supernatant were then assayed for glycolate (14). The assay could detect as little as 50 nmoles per ml. Pretreatment and resuspension of algae cells has been found not to affect their ability to excrete glycolate (99).

Measurement of Glycolate Assimilation

Scenedesmus obliquus was harvested at 1000 g, one time washed in distilled H_2O and then resuspended in 0.02 M phosphate pH 6.5, to give approximately a 2% v/v suspension. The cells were continually aerated and the temperature kept at 25°C. Light, when used, was at 3,000 foot candles

provided by GE flood lamps. Glycolate was added at zero time to give 5.5×10^{-4} M initial concentration. Glycolate was determined in the supernatant of aliquots after the cells had been spun out at 1000 g for three minutes (14).

Measurement of Oxygen Exchange

For oxygen exchange experiments Scenedesmus obliquus was harvested at selected stages in the life cycle, washed one time with distilled water and resuspended in phosphate, 0.02 M, pH 6.5 to give a 4% v/v suspension. Oxygen exchange was measured at 27°C on a Gilson Differential Respirometer, photosynthesis model, with flood lamps which delivered approximately 1000 foot candles light to the algae. Flask contents were 2 ml of the cell suspension and 0.5 ml H_2O or other additions in the main part of the flask and 0.5 ml of 0.02 M substrate in one side arm. In most experiments CO_2 was removed by 20% KOH with a filter paper wick in the center well.

Enzyme Assay Methods

All assays were carried out at 25°C. The spectrophotometric assays were done on a Gilford recording spectrophotometer. In all cases the enzymatic assays reported in this work were tested and found to be dependent on protein concentration over the range used. For assays done with the spectrophotometer or oxygen electrode initial rates were

determined. In the case of fixed time assays, they were shown to be linear with time.

P-glycolate Phosphatase
(Phosphoglycolate phosphohydrolase E.C. 3.1.3.18)

This enzyme was assayed by the method of Anderson and Tolbert (1). The assay mixture contained 0.5 ml cacodylate 0.20 M pH 7.0, 0.6 ml $MgSO_4$ 0.01 M, 1.0 ml 0.01 M P-glycolate (tricyclohexylammonium salt, General Biochemicals), water, and enzyme in 2.0 ml final volume. The reaction was terminated by addition of 1 ml of 10% trichloroacetic acid.

Glycolate Oxidase
(Glycolate: O_2 oxidoreductase E.C. 1.1.3.1)

Glycolate oxidase was assayed by following the reduction of DCIP anaerobically at 600 mμ (92). The assay mixture in a total volume of 2.5 ml contained 200 μmoles pyrophosphate pH 8.7, 0.3 μmole DCIP, enzyme and water or additional components. The reaction was initiated by addition of 20 μmoles of glycolate or other substrates from the side arm of the Thunberg cuvette. Changes in O.D. were converted to nmoles using an extinction coefficient of 21.9 $cm^{-1} \times mole^{-1} \times 10^{-3}$ for DCIP (4).

Two additional methods were used to assay glycolate oxidase and to characterize the enzyme as an oxidase. The

formation of glyoxylate phenylhydrazone at 324 mμ was measured similarly to that described by Hess and Tolbert (43). In 2.5 ml were 200 μmoles pyrophosphate, pH 8.7, 10 μmoles phenylhydrazine·HCl (previously neutralized), enzyme, water and other components. The reaction was initiated by addition of 20 μmoles glycolate.

Glycolate oxidase was also assayed by measuring the disappearance of oxygen with a Clark OPE oxygen electrode at 700 millivolts. The 3.2 ml volume reaction mixture in a continually stirred, temperature controlled chamber contained 250 μmoles pyrophosphate, enzyme, water, and other components. The reaction was initiated by addition of 20 μmoles glycolate. Oxygen concentration was calculated on the basis of solubility of air in water. All solutions for the assay were air saturated except the enzyme. At 25°C, the reaction mixture held 685 nmoles O_2 (40). Percent change in oxygen concentration recorded in 5 minutes was converted to nmoles O_2 x $minute^{-1}$.

Glycolate Dehydrogenase
(Glycolate: (DCIP) oxidoreductase)

Glycolate dehydrogenase was routinely assayed anaerobically by following DCIP reduction as described for glycolate oxidase. When measuring enzyme activity with the oxygen electrode the assay described for glycolate oxidase was used, with the addition of 8 μmoles of PMS (Sigma).

To determine electron acceptor specificity for glycolate dehydrogenase the following assay systems were tested.

NO_3^- reduction was assayed by looking for the appearance of NO_2^- (68). In an assay volume of 2.0 ml were 100 µmoles pyrophosphate pH 8.7, 6 µmoles KNO_3, 20 µmoles glycolate, water and enzyme. The reaction was terminated by boiling and a one ml aliquot was assayed for NO_2^-.

Cytochrome c reduction was assayed by looking for increased absorption at 550 nm. The assay in 2.5 ml final volume contained 200 µmoles pyrophosphate pH 8.7, 2 mg cytochrome c (Sigma type II), water, enzyme, and 20 µmoles glycolate. The assay was done anaerobically.

Potassium ferricyanide reduction by the enzyme was tested by looking for $Fe(CN)_6^{3-}$ reduction at 410 nm in an anaerobic Thunberg cuvette. The assay mixture contained in 2.5 ml final volume 200 µmoles pyrophosphate pH 8.7, 2.5 µmoles $K_3Fe(CN)_6$, freshly prepared, water, enzyme and 20 µmoles glycolate.

NAD^+ and $NADP^+$ reduction was tested by measuring increased absorbancy at 340 nm in an anaerobic Thunberg cuvette. The assay contained in 2.5 ml, 200 µmoles pyrophosphate pH 8.7, 0.8 µmoles NAD^+ (Sigma type III) or $NADP^+$ (Sigma type II), 20 µmoles glycolate, enzyme and water.

Methylene blue reduction was looked for at 668 nm in anaerobic Thunberg cuvettes. The assay in 2.5 ml

contained 200 μmoles pyrophosphate pH 8.7, 0.4 μmoles methylene blue, 20 μmoles glycolate, enzyme and water.

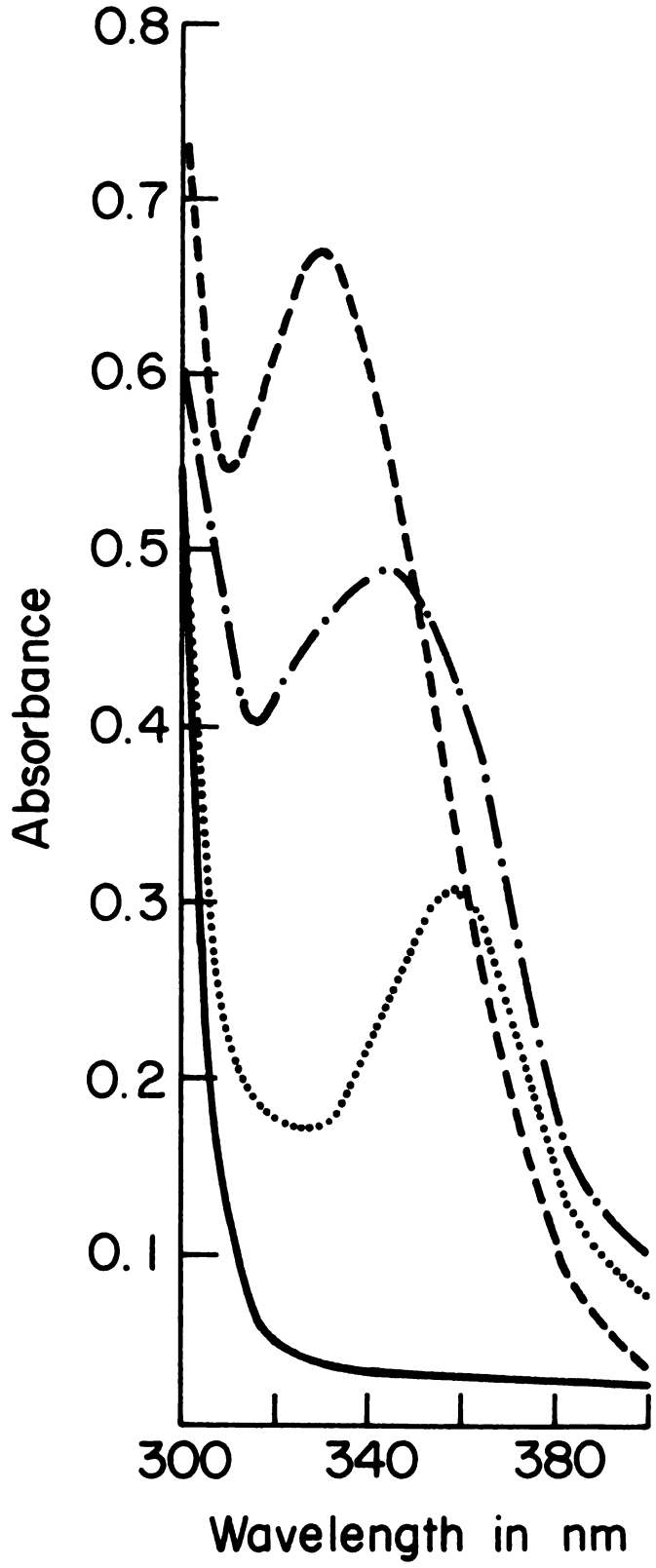
Glutathione reduction was measured by looking for the appearance of glyoxylate phenylhydrazone. The assay was carried out as described for glycolate oxidase with the addition of 2 μmoles glutathione (Sigma).

In order to determine the stoichiometry between DCIP reduction and glyoxylate formation both aerobically and anaerobically, a method for the determination of glyoxylate by the phenylhydrazine assay in the presence of DCIP was developed. Normally phenylhydrazine and DCIP react at pH 8.7 leading to complete loss of DCIP color which is incompatable with the enzymatic assay. To avoid this problem the activity was measured in a standard DCIP reduction assay, but at fixed time intervals different assays were terminated by addition of 100 μl 12 N HCl. The mixtures were left standing for 20 minutes in which time the blue DCIP color disappeared. After this time 0.5 ml of 0.1 N phenylhydrazine·HCl was added to the assay mixture. After another 20 minutes the absorption due to glyoxylate phenylhydrazone was measured at 340 mμ.

Spectra in Figure 2 show the absorption of glyoxylate phenylhydrazone in the presence and absence of DCIP. The presence of DCIP and strong acid caused a shift in the absorption maximum of glyoxylate phenylhydrazone from 330 nm to 344 nm. The absorption spectrum of the product formed

Figure 2. Absorption Spectra of Glyoxylate Phenylhydrazone in Presence and Absence of DCIP

Spectra measured on a Beckman DB recording spectrophotometer. Samples were prepared as described in Methods and Materials. Each sample contained 2.5 ml final volume. (———), 200 µmoles pyrophosphate pH 8.7, and 10 µmole phenylhydrazine·HCl; (·····), 200 µmoles pyrophosphate pH 8.7, 0.30 µmoles DCIP, 100 µl 12 N HCl and 10 µmole phenolhydrazine·HCl; (-----), 200 µmoles pyrophosphate pH 8.7, 0.1 µmole glyoxylate, and 10 µmoles phenylhydrazine·HCl; (-·-·-), 200 µmoles pyrophosphate pH 8.7, 0.1 µmole glyoxylate, 0.3 µmoles DCIP, 100 µl 12 N HCl and 10 µmoles phenylhydrazine·HCl.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
300
340
380
Wavelength in nm

from the oxidation of glycolate by glycolate dehydrogenase in the presence of DCIP was identical with authentic glyoxylate phenylhydrazone. The absorption at 340 nm was linear with glyoxylate concentrations between 0 and 0.5 umoles when measured using the assay conditions described above.

Preparation of Cell Free Extracts

For experiments to determine the level of glycolate dehydrogenase and P-glycolate phosphatase in Chlamydomonas, the cells were harvested at 1000 g for 10 minutes at 2°C, washed once in distilled water and resuspended in 0.001 M phosphate pH 7.0, to give approximately 20-40% v/v cell suspension. The suspension was passed through a pre-cooled French pressure cell at 8000 to 12000 lb·inch^2. Cell debris was removed by centrifugation at 29,000 g for 10 minutes at 2°C. The supernatant was used for the assays. For determination of glycolate dehydrogenase in other algae, cells were treated as described above except that they were suspended in 0.1 M phosphate pH 7.5.

Acetabularia, Nitella, Chara, Elodea and Marchantia were washed with cold tap water, rinsed with cold buffer and cut into small pieces. The pieces were ground in a pre-cooled Potter-Elvehjem homogenizer with 0.1 M phosphate pH 7.5 at 2°C. The cell debris was removed by centrifugation at 10,000 g for 10 minutes at 2°C. The supernatant was used for the assays.

Glycolate Oxidase

Spinach glycolate oxidase was the gift of Dr. S. L. Vandor. This preparation was prepared by the method of Zelitch and Ochoa (103) through the acid precipitation step. The enzyme was kept frozen until use.

Purification of Glycolate Dehydrogenase

For the purification of glycolate dehydrogenase from air grown Chlamydomonas the enzyme was removed by a nonionic detergent treatment. Cells were harvested at 1,000 g, washed once in distilled water, washed once in 0.1 M phosphate pH 7.5 and resuspended approximately 10% v/v in 0.1 M phosphate pH 7.5 at 2°C containing 1% w/v Triton X-100 (Rohm and Haas). The mixture was stirred for 45 to 60 minutes in the cold room, after which the cell debris was removed by centrifugation at 29,000 g for 10 minutes at 2°C. The supernatant was used for further purification. All operation was carried out at 2° to 4°C.

To the Triton X-100 extract, solid $(NH_4)_2SO_4$ was added with constant stirring to give 35% saturation. The mixture was centrifuged at 39,000 for 10 minutes and yielded a green residue and a clear light yellow supernatant. The green residue was removed and discarded. The supernatant was 50% saturated with solid $(NH_4)_2SO_4$ and the precipitate, containing the enzyme was removed by centrifugation at 39,000 g for 15 minutes. The precipitate

was suspended in 1/10 the original volume of 0.1 M phosphate pH 7.5. This preparation was used for most studies concerning the characterization of glycolate dehydrogenase.

Glutamate: Glyoxylate Aminotransferase
(E.C. 2.6.1.4)

This enzyme was assayed at 25°C by following formation of glycine-^{14}C in the manner described by Kisaki and Tolbert (49). In a final assay volume of 1.25 ml were 20 µmoles glyoxylate-1,2-^{14}C, 12 µmoles amino donor, usually L-glutamate, 15 µmoles phosphate (pH 7.5), 0.1 µmole pyridoxal-5-phosphate (Sigma) and enzyme. The reaction was initiated by addition of glyoxylate, and terminated after 15 minutes by boiling. Glycine-^{14}C was separated from unreacted glyoxylate by passage of the boiled reaction mixture over a Dowex-1 acetate column (6 x 50 min) which was then washed with 2 ml of water. From the combined effluents 0.4 ml aliquots were counted for glycine-^{14}C in 15 ml Kinards solution (47) in a Packard Tri-Carb Scintillation counter. The counting efficiency was determined to be 71% with standard benzoic acid. Observed cpm were converted to µmoles glycine-^{14}C.

The glyoxylate-1,2-^{14}C (98% pure glyoxylate-1,2-^{14}C, Calbiochem) was prepared by dilution with a 1000 fold excess cold glyoxylate to give a 0.2 M solution containing 1 µc/ml. Control experiments demonstrated that glyoxylate-1,2-^{14}C was retained by the Dowex-1 acetate. Glycine-1,2-^{14}C was not

retained significantly by the column as control recovery experiments gave a minimum of 95% of the expected yield.

Enzyme was prepared for this assay by harvesting air grown Chlamydomonas, washing in distilled H_2O and resuspending 20-40% v/v in 0.02 M phosphate pH 7.5. The cells were put through French Press twice at 10,000 to 12,000 lb·inch^2. Cell debris was removed at 29,000 g for 10 minutes. The supernatant was used in the assay.

Protein and Chlorophyll Determination

Protein was determined by the method of Lowry et. al. (56) using bovine serum albumin as a standard. Chlorophyll was determined by the method of Arnon (5).

RESULTS AND DISCUSSION

Assimilation of Glycolate by Scenedesmus

The ability of Scenedesmus obliquus to assimilate exogenous glycolate was found to be related to the life cycle of the cells. As shown in Figure 3, glycolate assimilation was maximum at the stage of cell division and shortly afterwards. No assimilation was detected during the middle of the light phase of growth. Furthermore, glycolate assimilation could not be detected in random growth cultures of Scenedesmus, Ankistrodesmus, Chlorella (Warburg), or Chlamydomonas.

Glycolate assimilation by dividing cells was linear with time over the periods tested in both the light and dark (Figure 4). Assimilation was equally rapid in light or dark. Addition of 1×10^{-5} M CMU, an inhibitor of photosynthetic electron transport (101), had no effect on glycolate assimilation in the light (Figure 4). An inhibition by low pH on assimilation was examined. At pH 2.9 in 0.02 M phosphate no assimilation was observed while assimilation was observed at pH 6.5. This observation is inconsistent with Schou et. al. (83), who observed assimilation at pH 2.9. Phosphate was not required as equal assimilation was observed with 0.1 M MES pH 6.5 in both the light and dark.

Figure 3. Assimilation of Exogenous Glycolate by Synchronized Scenedesmus at Different Stages of Their Life Cycle

Cells were harvested at different stages of the life cycle, suspended 1% v/v in 0.02 M phosphate pH 6.5 and glycolate assimilation in the light measured as described in Methods and Materials. Initial glycolate concentration was 5.5×10^{-4} M. Stage of cell growth was determined by microscopic examination.

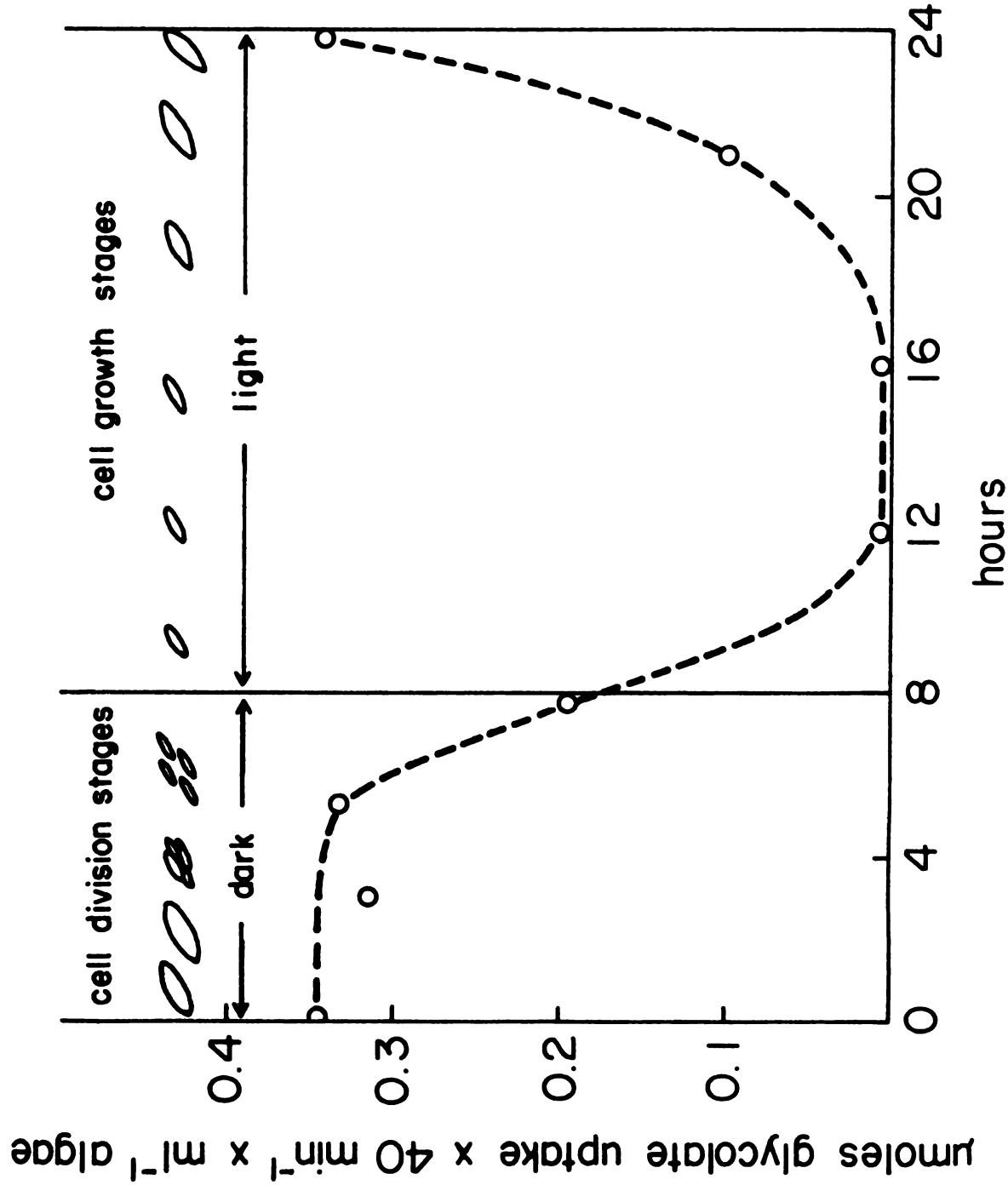
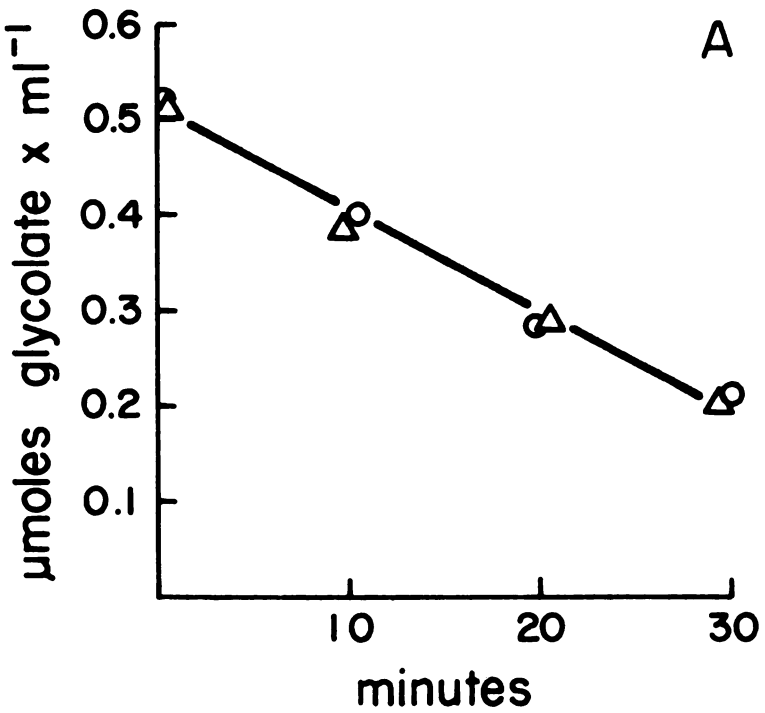

cell division stages
dark
cell growth stages
light
0.4
0.3
0.2
0.1
0
4
8
12
16
20
24
hours
μmoles glycolate uptake x 40 min⁻¹ x ml⁻¹ algae

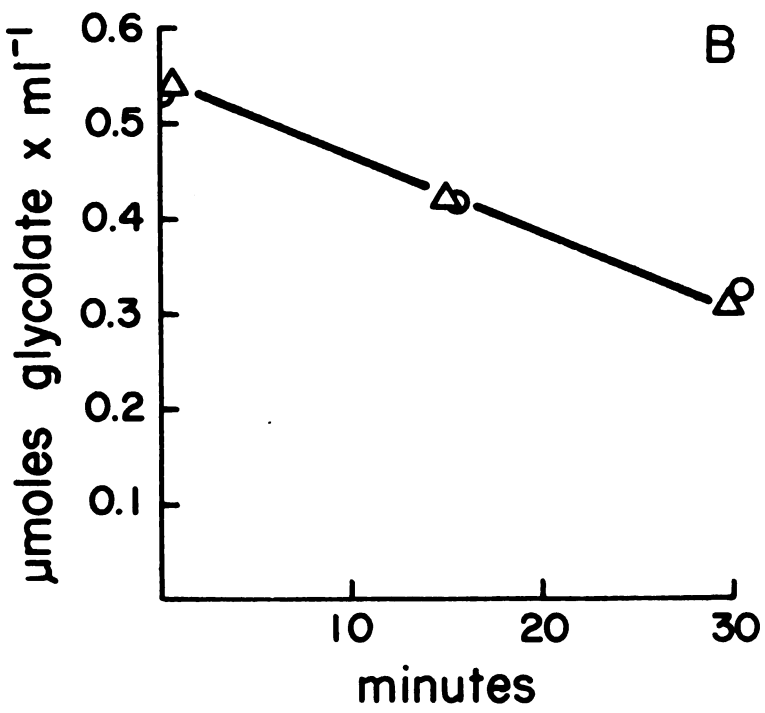
Figure 4. A. Glycolate Assimilation by Synchronized Scenedesmus in the Light and Dark

Scenedesmus was harvested at the stage of cell division, suspended to 2% v/v in 0.02 M phosphate pH 6.5 and glycolate assimilation in the light or dark measured as described in Methods and Materials. Initial glycolate concentration was 5.5×10^{-4} M: Δ - assimilation in dark; O - assimilation in the light.

B. Effect of CMU on Glycolate Assimilation by Synchronized Scenedesmus

Scenedesmus was harvested at the stage of cell division resuspended to 2% v/v in 0.02 M phosphate pH 6.5 and glycolate assimilation measured in light ± 5×10^{-5} M CMU as described in Methods and Materials. Initial glycolate concentration was 5.5×10^{-4} M. O - control; Δ - 1×10^{-5} M CMU.


A
0.6
0.5
0.4
0.3
0.2
0.1
µmoles glycolate x ml⁻¹
10
20
30
minutes


B
0.6
0.5
0.4
0.3
0.2
0.1
µmoles glycolate x ml⁻¹
10
20
30
minutes

This data confirms the ability of Scenedesmus to assimilate exogenous glycolate. The process does appear however to be limited to certain stages of the life cycle of the cells, at least for Scenedesmus. It is not yet clear why some workers have obtained glycolate assimilation with random cultures (61, 83), while others have not (16, 43, 57). The suggestion is made on the basis of my data that the cultures used previously were actually in a stage of unrecognized synchrony which permitted glycolate uptake.

The relationship between glycolate assimilation and glycolate excretion is of interest. Chang (17) using the same cultures of Scenedesmus as used in this thesis and also using synchronous Ankistrodesmus observed that glycolate excretion was maximal during the light or active growth stage, and that little or no excretion could be detected when the cells were in the dark or division stage. These observations have been confirmed (35, 95). The active stage of growth is the stage where no glycolate assimilation occurs (Figure 3). Thus there appears to be an inverse relationship between glycolate excretion and assimilation. It is not known whether glycolate assimilation is regulated by factors involved in uptake or whether ability or inability to be metabolized by the cell is the regulating factor in assimilation.

Effects of Glycolate on Photosynthesis and Respiration

The effects of exogenously added glycolate on the rates of photosynthesis and respiration by Scenedesmus measured at a time when cells were capable of assimilating glycolate have been published (65). These results will only be summarized here. As shown in Figure 5, glycolate stimulated respiration approximately 100% over controls. When compared to other substrates (Table 1), acetate and glucose were two to three times more effective in stimulating respiration. Similar results have been obtained by Sen (84) with Chlorella. The level of stimulated respiration by glycolate was much higher with Scenedesmus than with Chlorella however.

Glycolate stimulated oxygen evolution during photosynthesis by Scenedesmus taken at a time when they were capable of glycolate assimilation (Figure 5). Compared to other substrates tested, Table 1, glycolate was the best substrate. The observation that glucose and fructose can stimulate oxygen evolution has been confirmed (72). Glycolate stimulated oxygen evolution was inhibited by 6×10^{-6} M CMU, an inhibitor of photosynthetic electron transport (101). A scheme to account for these observations is shown in Figure 6. The necessary enzymes to operate the postulated cycle have been found in green algae (12, 66). It should be noted that the proposed cycle will only account for stimulated oxygen evolution if the enzyme oxidizing

Figure 5. Effect of Glycolate on Oxygen Evolution in the Light and Respiration in the Dark by Scenedesmus

Each flask contained 3 ml of a 2.7% suspension of synchronized Scenedesmus at the stage of cell division in 0.02 M phosphate, pH 6.5 and ± glycolate 3.3 x 10-3 M.

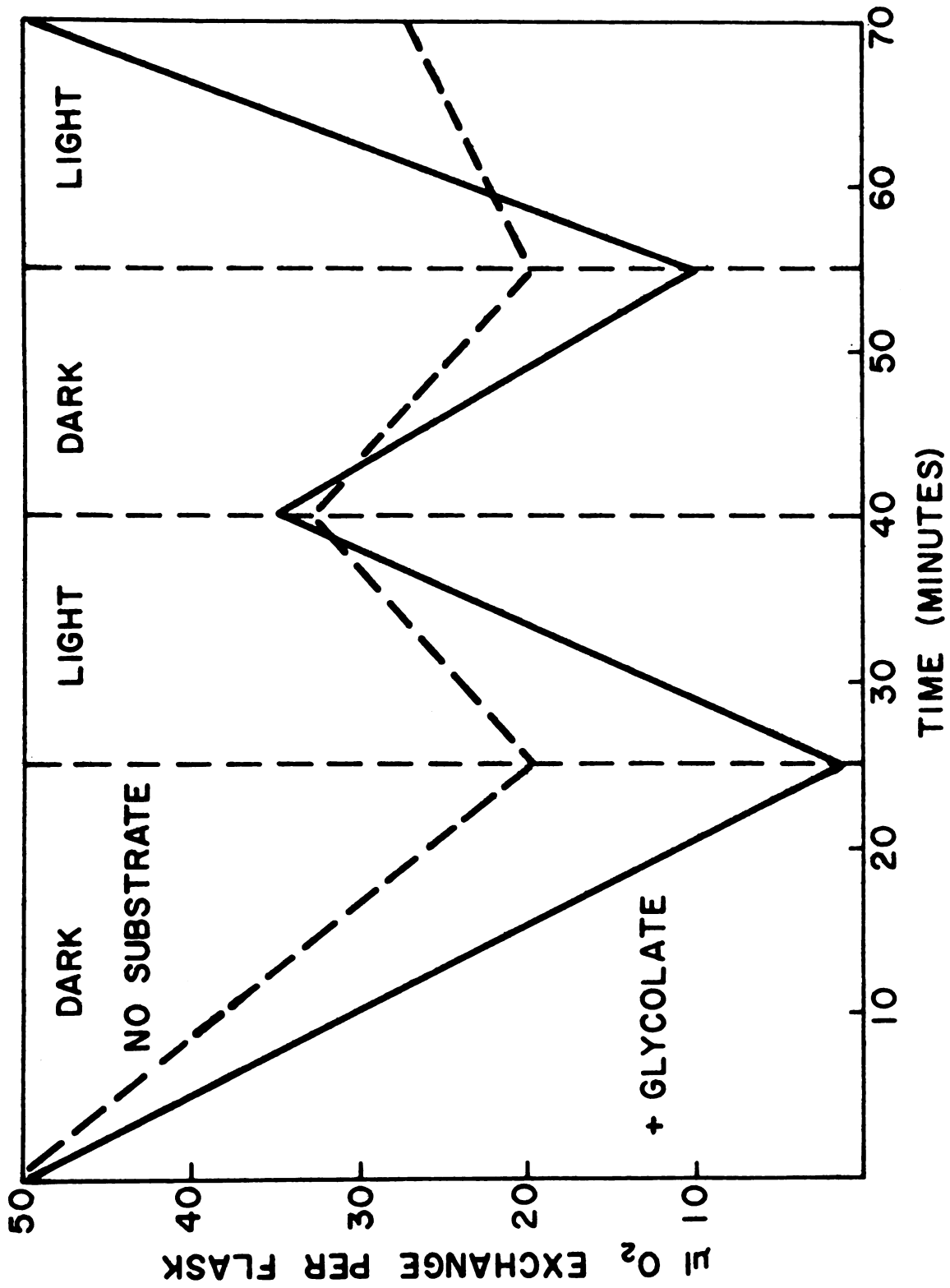
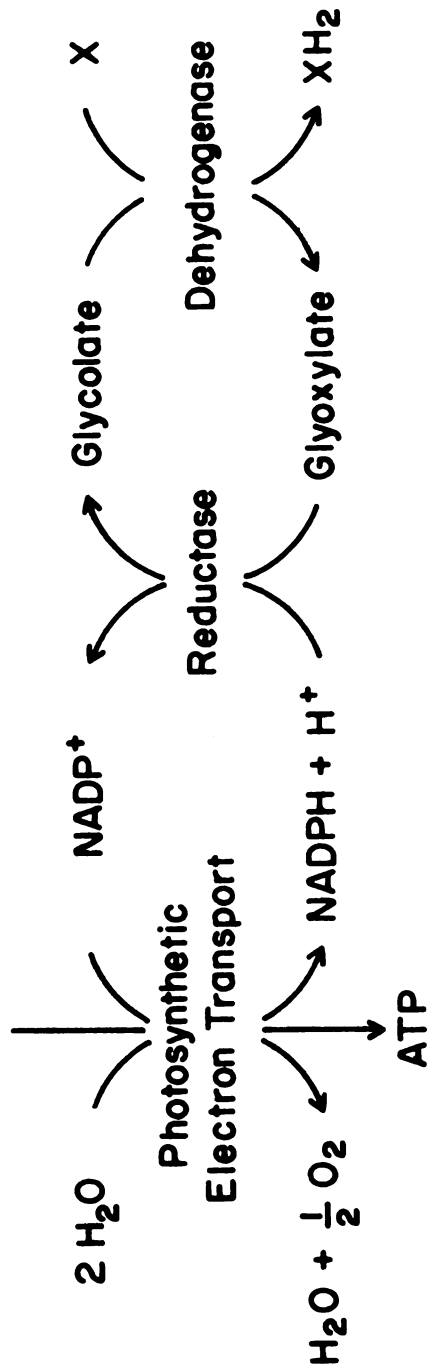

DARK
NO SUBSTRATE
LIGHT
DARK
LIGHT
+ GLYCOLATE
50
40
30
20
10
μl O2 EXCHANGE PER FLASK
10
20
30
40
50
60
70
TIME (MINUTES)

Table 1. Comparison of Different Substrates for Stimulation of Oxygen Evolution and Respiration

Data are expressed as ratio of activity with each substrate as compared with glycolate. Final substrate concentrations were 3.3 mM and rates were corrected for controls without added substrates.

Substrate	Oxygen Evolution in Light	Oxygen Uptake in Dark
	Activity ratio as substrate/glycolate	
Organic Acids		
Glycolate	1.0	1.0
Glyoxylate	1.0	1.0
Acetate	0.5	2.5
P-Glycolate	<0.1	<0.1
D,L-Lactate	0.2	1.0-1.2
Formate	<0.1	0.5-1.0
D,L-Glycerate	0.5-0.7	0.5-1.0
3-P-Glycerate	0.5-0.7	0.5-1.0
Amino Acids		
L-Serine	0.5	1.0
Glycine	0.5	1.0
Sugars		
D-Glucose	0.4	2.0-3.0
D-Ribose	0.2	0.3
D-Fructose	0.2	0.8

Figure 6. A Proposed Scheme to Account for Glycolate Stimulation of Oxygen Evolution


2 H2O
Photosynthetic
Electron Transport
H2O + 1/2 O2
NADP+
NADPH + H+
ATP
Reductase
Glycolate
Glyoxylate
Dehydrogenase
X
XH2

glycolate is not an oxidase. Otherwise stimulated oxygen evolution and oxygen uptake due to glycolate oxidation would be equal and no net stimulation would be observable. Since the proposal of this cycle in algae (65), the enzyme in green algae oxidizing glycolate has been found to be a dehydrogenase (this thesis) and not an oxidase. A scheme similar to this has been proposed by Asada et. al. (6) to account for glyoxylate stimulated photophosphorylation in isolated chloroplasts.

Glycolate Excretion by Chlamydomonas

Chlamydomonas reinhardtii, grown on air supplemented with 1% CO_2 was capable of excreting glycolate when tested under the optimum conditions described in Methods and Materials. Glycolate excretion at detectable levels was not found when cells were grown on air. The ability of cultures grown on air supplemented with 1% CO_2 to excrete glycolate dropped off with increasing culture density (Figure 7). This also occurred in older cultures, an observation which confirmed a previous report (89). This phenomenom may be partially related to availability of CO_2 per cell as the culture density increases. It was found that transferring cultures from CO_2 supplemented air to only air resulted in the disappearance within about 15 hours of glycolate excretion by the cells during the test assay (Figure 8). Likewise when cultures were transferred

Figure 7. Glycolate Excretion by Chlamydomonas Grown on 1% CO_2 in Air Relative to the Age and Density of the Culture

Cells were harvested and tested for glycolate excretion in the light at pH 8.0 (0.02 M phosphate) as described in Methods and Materials. Culture density is expressed on the basis of µg chlorophyll per ml of culture.

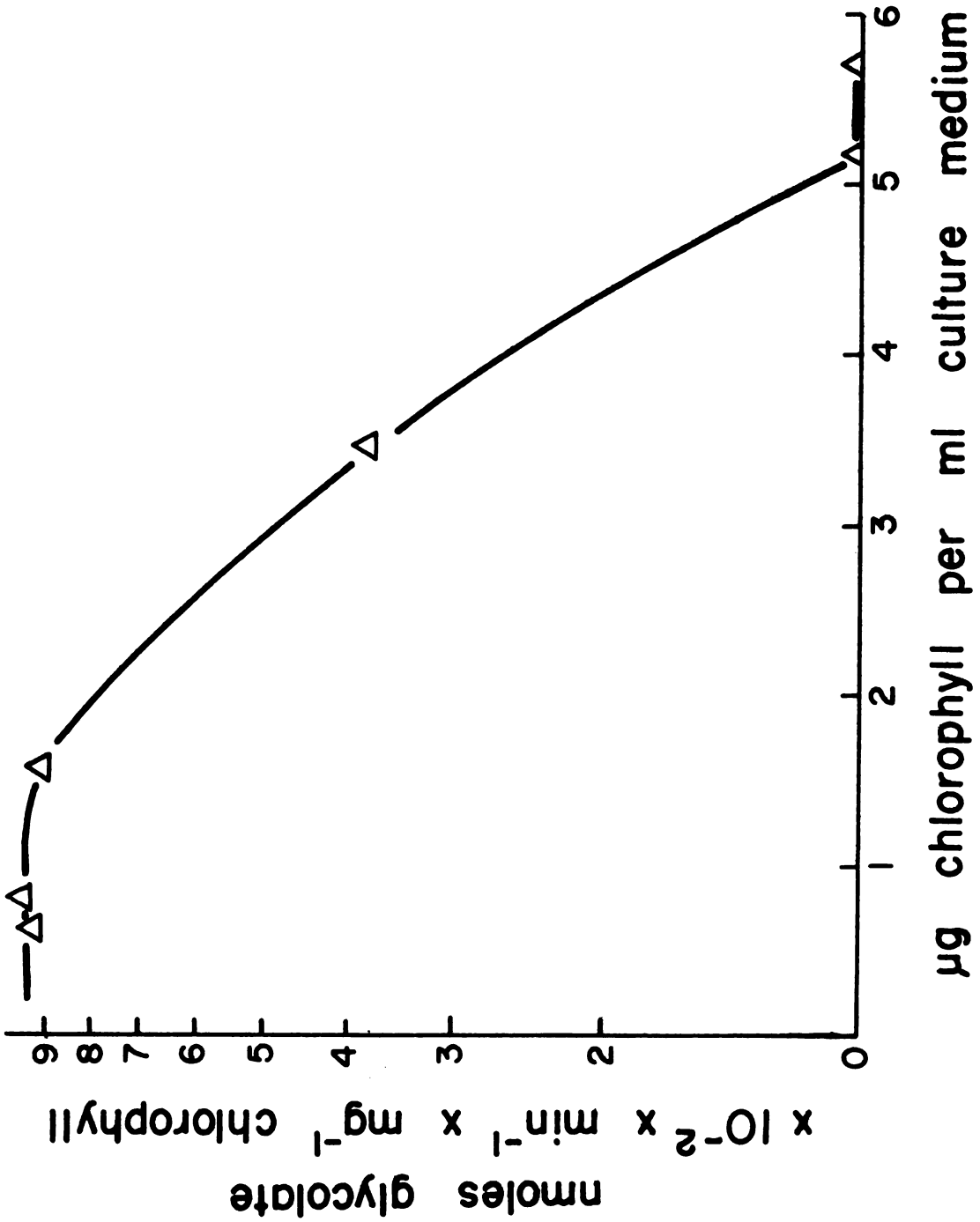
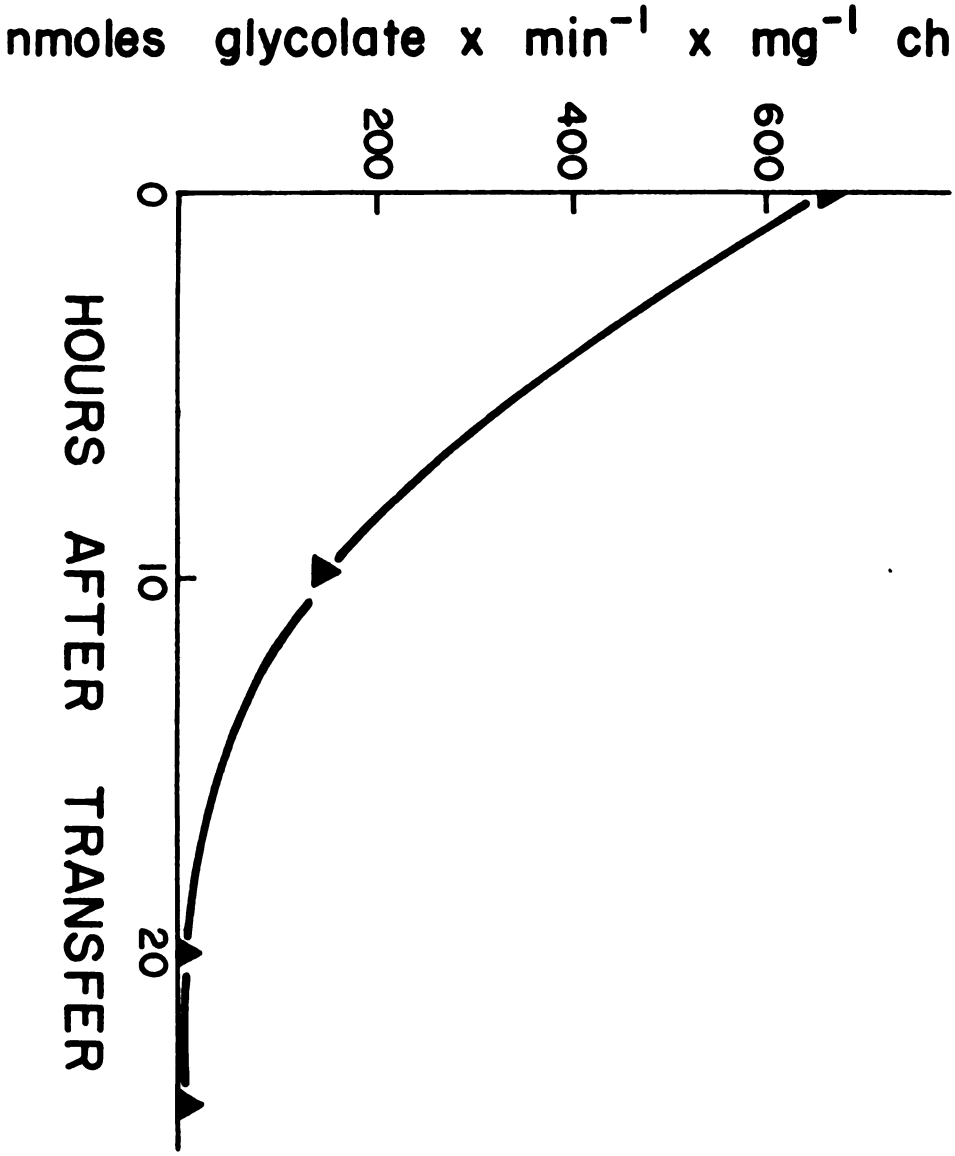

µg chlorophyll per ml culture medium
0
1
2
3
4
5
6
nmoles glycolate
x 10⁻² x min⁻¹ x mg⁻¹ chlorophyll
2
3
4
5
6
7
8
9

Figure 8. Decrease in Ability of Chlamydomonas to Excrete Glycolate After Being Transferred from 1% CO_2 in Air to Air

Cultures growing on 1% CO_2 in air were transferred to air (0.03% CO_2). At appropriate times, a portion of the culture was removed, harvested and resuspended to 2% v/v in 0.02 M phosphate pH 8.0. Glycolate excretion in the light was then measured as described in Methods and Materials.


nmoles glycolate x min^{-1} x mg^{-1} chlorophyll
200
400
600
0
10
20
HOURS AFTER TRANSFER

from air to CO_2 supplemented air, they regained the ability to excrete glycolate within about 15 hours.

In order to determine whether the absence of glycolate excretion in air grown cultures was due to the absence of glycolate synthesis or the inability of the cells to excrete glycolate, experiments with isonicotinyl hydrazide were performed. Pritchard et. al. (74) have shown that isonicotinyl hydrazide stimulated glycolate excretion by Chlorella grown on air supplemented with 4% CO_2. They proposed that isonicotinyl hydrazide acted as an inhibitor of the glycolate pathway, thus forcing excretion of glycolate. Air grown Chlamydomonas, when treated with 0.02 M isonicotinyl hydrazide in 1 mM phosphate (pH 7.0), excreted glycolate (Figure 9), but not in the absence of this inhibitor. Therefore the absence of glycolate excretion by air grown cultures was not due to their inability to synthesize glycolate, but rather their failure to excrete it.

Glycolate Dehydrogenase: Detection and Assay

Cells of Chlamydomonas were harvested and the crude extracts tested for glycolate oxidase activity using the three assays described in Methods and Materials. No activity in crude extracts could be detected with the oxygen electrode or phenylhydrazone assays (Table 2). Glycolate dehydrogenase (glycolate: DCIP oxidoreductase) activity was found. All glycolate dehydrogenase activity was soluble after centrifuging crude extracts 10 minutes at 29,000 g.

Figure 9. Effect of Isonicotinyl Hydrazide on Glycolate Excretion by Air Grown Chlamydomonas

Cells grown on air were harvested and suspended to 2% v/v in 0.02 M isonicotinyl hydrazide, 0.001 M phosphate (pH 7.0) and tested for ability to excrete glycolate in the light as described in Methods and Materials. The control was from the same culture and was treated identically except that isonicotinyl hydrazide was omitted.

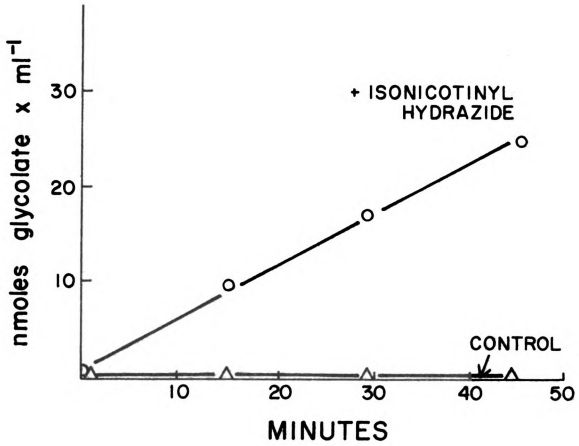

nmoles glycolate x ml⁻¹
30
20
10
+ ISONICOTINYL
HYDRAZIDE
CONTROL
10
20
30
40
50
MINUTES

Table 2. Absence of Endogenous Inhibitors of Glycolate Oxidase and Dehydrogenase in Crude Extracts of Chlamydomonas

Activity determined in 29,000 g supernatant of French Press extracts buffered with 1 mM phosphate pH 7.0. Assays described in Methods and Materials.

	nmoles x min^{-1}
Phenylhydrazone Assay	
crude extract Chlamydomonas	0
spinach glycolate oxidase	15.1
combined	15.3
Oxygen Electrode Assay	
crude extract Chlamydomonas	0
spinach glycolate oxidase	20.4
combined	20.4
DCIP Reduction Assay	
air grown Chlamydomonas crude extract	57.5
1% CO_2 grown Chlamydomonas crude extract	8.5
combined extracts	64.8

Tests were run to determine if endogenous inhibitors of glycolate dehydrogenase or glycolate oxidase were present in the crude extracts of Chlamydomonas cells (Table 2). Glycolate oxidase from spinach leaves was tested with an equivalent amount of algal enzyme on the basis of DCIP reduction, and no inhibition of oxygen uptake or glyoxylate formation by the spinach enzyme was observed. When extracts of Chlamydomonas grown on 1% CO_2 in air, which had DCIP reductase activity were mixed with extracts of air grown cells, no inhibition of DCIP reduction with glycolate was observed (Table 2). Therefore it appears that natural inhibitors of glycolate dehydrogenase or glycolate oxidase are not present in Chlamydomonas extracts.

During the course of this investigation glycolate dehydrogenase was recognized as a DCIP reductase (106), and although assayed for by DCIP reduction it had been called glycolate oxidase in spite of the fact that no oxidase activity was observed. On the basis of the data presented in Table 2 and in the Electron Acceptor Specificity section of this thesis the glycolate dehydrogenase of Chlamydomonas is not an oxidase as is found in higher plants. Thus while Chlamydomonas lacks glycolate oxidase, it has a glycolate dehydrogenase which carries out the oxidation of glycolate to glyoxylate.

Levels of Glycolate Dehydrogenase and P-Glycolate Phosphatase in Chlamydomonas

In view of the above data on glycolate excretion, it seemed reasonable to examine the levels of enzymes involved with glycolate metabolism to see if alterations in their activity could account for the difference in excretion ability. P-glycolate phosphatase has been suggested to be involved in glycolate synthesis (91). The level of this enzyme did not change significantly when cells were transferred from 1% CO_2 supplemented air to air (Figure 10), therefore it does not appear as though the phosphatase is involved in the regulation of excretion ability.

The effect on glycolate dehydrogenase of transferring Chlamydomonas from 1% CO_2 supplemented air to air was determined (Figure 10). The enzyme level increased three fold on the basis of either protein or chlorophyll, simultaneous with the loss of the ability of the cells to excrete glycolate.

The levels of glycolate dehydrogenase determined on the basis of chlorophyll and protein in five separate experiments are shown in more detail in Table 3. Glycolate dehydrogenase activity was at least two to four times higher in air grown cells than in cells that were capable of glycolate excretion during growth on air with CO_2.

To determine if the increased activity of glycolate dehydrogenase was due to enzyme activation or new protein

Figure 10. Changes in Specific Activity of P-Glycolate Phosphatase and Glycolate Dehydrogenase (Glycolate: DCIP Oxidoreductase) in Chlamydomonas after Transfer from 1% CO_2 in Air to Air

Specific activities of the enzymes on the basis of chlorophyll and protein were determined in 29,000 g supernatant from French Press crude extracts. The extracts were prepared in 0.001 M phosphate pH 7.0. Enzymes were assayed as described in Methods and Materials:
● - P-glycolate phosphatase, μmoles x min^{-1} x mg^{-1} chlorophyll;
□ - phosphatase, μmoles x min^{-1} x mg^{-1} protein;
△ - glycolate: DCIP oxidoreductase, nmoles x min^{-1} x mg^{-1} chlorophyll;
▼ - reductase, nmoles x min^{-1} x mg^{-1} protein.

P-GLYCOLATE PHOSPHATASE
μmoles x min⁻¹ x mg⁻¹ chlorophyll
10² x μmoles x min⁻¹ x mg⁻¹ protein
HOURS AFTER TRANSFER
REDUCTASE
PHOSPHATASE
REDUCTASE
GLYCOLATE: DCPIP REDUCTASE
0.5 x nmoles x min⁻¹ x mg⁻¹ protein
10⁻² x nmoles x min⁻¹ x mg⁻¹ chlorophyll

Table 3. Glycolate Dehydrogenase in Chlamydomonas Grown on Air or 1% CO_2 in Air

Specific activity of glycolate dehydrogenase in 29,000 g supernatant of French Press extracts determined by DCIP reduction on basis of chlorophyll or protein as described in Methods and Materials. Data from five cultures of air grown and five grown on 1% CO_2 in air capable of excreting glycolate.

	Air Grown		1% CO_2 in Air	
	Chlorophyll	Protein	Chlorophyll	Protein
	nmoles x min^{-1} x mg^{-1}		nmoles x min^{-1} x mg^{-1}	
range	260-520	2.8-4.9	80-150	0.7-1.7
average	388	3.8	122	1.1

synthesis, an inhibitor of protein synthesis, cycloheximide (29), was used at a concentration of 5 µg/ml. This inhibitor should prevent the increase in levels of glycolate dehydrogenase if new protein synthesis is involved, when cultures are transferred to air from air supplemented with CO_2. It has been shown that cycloheximide does not effect the CO_2 fixation ability of Chlorella (62). In the presence of cycloheximide levels of glycolate dehydrogenase did not increase (Figure 11). This result suggested that the increase in glycolate dehydrogenase was due to de novo synthesis of the enzyme.

This data suggests that the level of glycolate dehydrogenase present in Chlamydomonas controls whether the algae will excrete glycolate or metabolize it. Cells grown on high CO_2 excrete glycolate and have low dehydrogenase levels, while cells that do not excrete glycolate have elevated dehydrogenase levels. Both types of cells synthesize glycolate as shown by the isonicotinyl hydrazide data. This is further supported by the observation that levels of P-glycolate phosphatase did not vary significantly between high and low CO_2 grown cells (Figure 10). Therefore the availability of CO_2 during growth regulates glycolate excretion by enhancing further glycolate metabolism rather than effecting glycolate synthesis.

Figure 11. Effect of Cycloheximide on Specific Activity of Glycolate Dehydrogenase After Transfer of Chlamydomonas from 1% CO_2 in Air to Air

Cultures were transferred from 1% CO_2 in air to air and cycloheximide (5 ug/ml culture medium) was added after 4 hours. Specific activity of glycolate dehydrogenase was determined by the DCIP reduction assay in the 29,000 g supernatants from French Press extracts as described in Methods and Materials. Extracts were prepared in 0.001 M phosphate pH 7.0:

● - control, nmoles x min^{-1} x mg^{-1} protein;
△ - control, nmoles x min^{-1} x mg^{-1} chlorophyll;
□ - cycloheximide, nmoles x min^{-1} x mg^{-1} protein;
+ - cycloheximide, nmoles x min^{-1} x mg^{-1} chlorophyll.

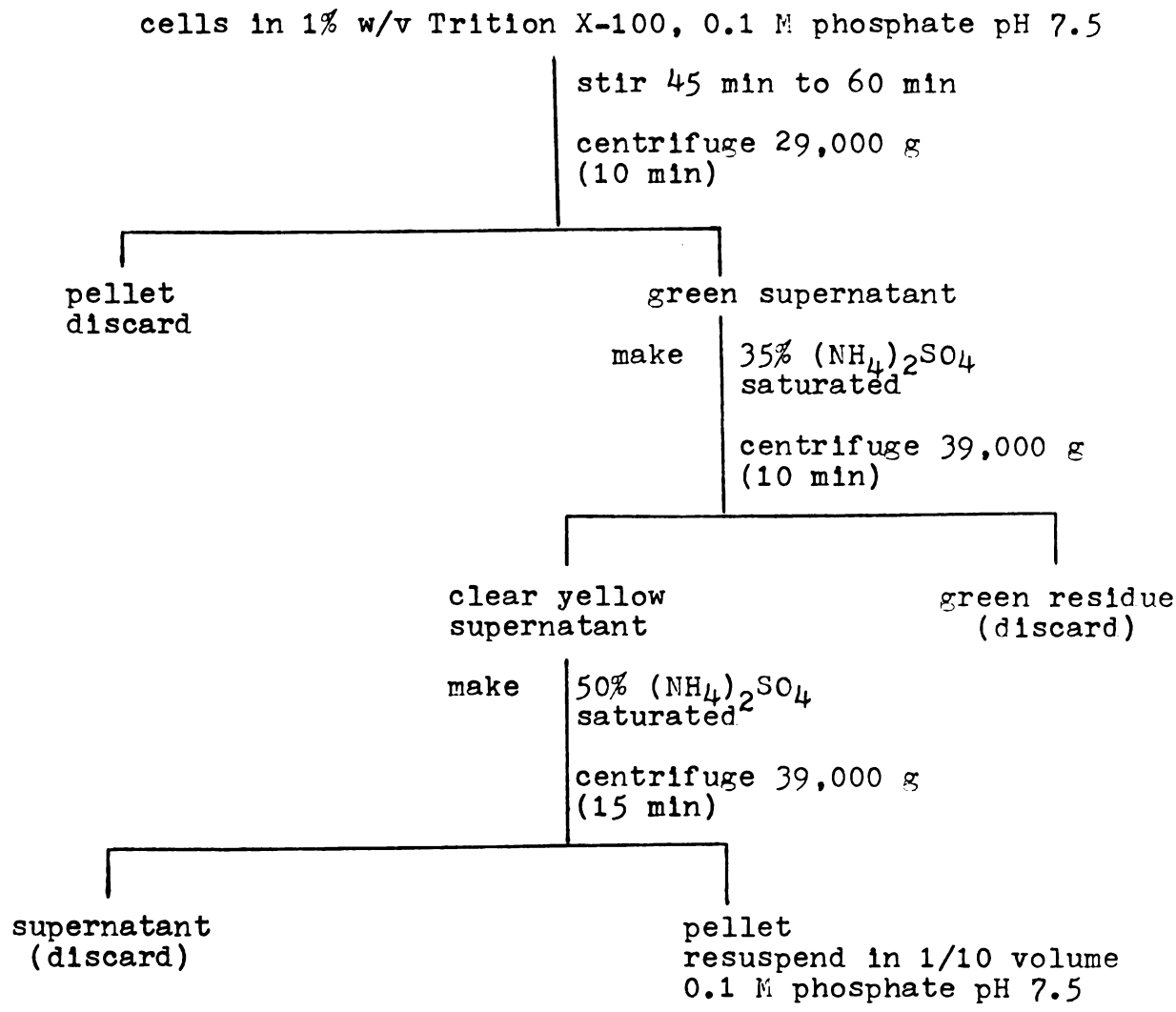
nmoles x min⁻¹ x mg⁻¹ protein
nmoles x min⁻¹ x mg⁻¹ chlorophyll
HOURS AFTER TRANSFER
ADDITION OF CYCLOHEXIMIDE
0
5
10
15
20
25
1
2
3
100
200
300

Purification of Glycolate Dehydrogenase

Glycolate dehydrogenase was purified from air grown Chlamydomonas according to the scheme shown in Figure 12. The enzyme was recovered in relatively good yield in a preparation free of chlorophyll and other major interferring pigments. The results of a typical purification are shown in Table 4. While this table shows only a 3.3 fold purification, it must be remembered that the preparation begins with the Triton X-100 extract. The Triton X-100 extract preparation was a partial purification step in itself. When compared with the activity found in crude extracts (Table 3), it can be seen that Triton X-100 extraction gave approximately a three fold purification. Using this method of calculation, purifications as high as 30 fold have been obtained.

The use of Triton X-100 to release glycolate dehydrogenase from cells was studied. As shown in Figure 13, there was an initial release of approximately 50% of the enzyme. The rest of the activity was more slowly released until a maximum of 82% of the total in French Press extracts was obtained. Whether examined on the basis of protein or chlorophyll a similar release rate was obtained. As can be seen from Figure 13, shorter time periods of Triton X-100 extraction could lead to increased purification. This occurs, however, with a decrease in enzyme recovery, and generally the longer extraction time for more recovery was used.

Figure 12. Purification Scheme for Glycolate Dehydrogenase*



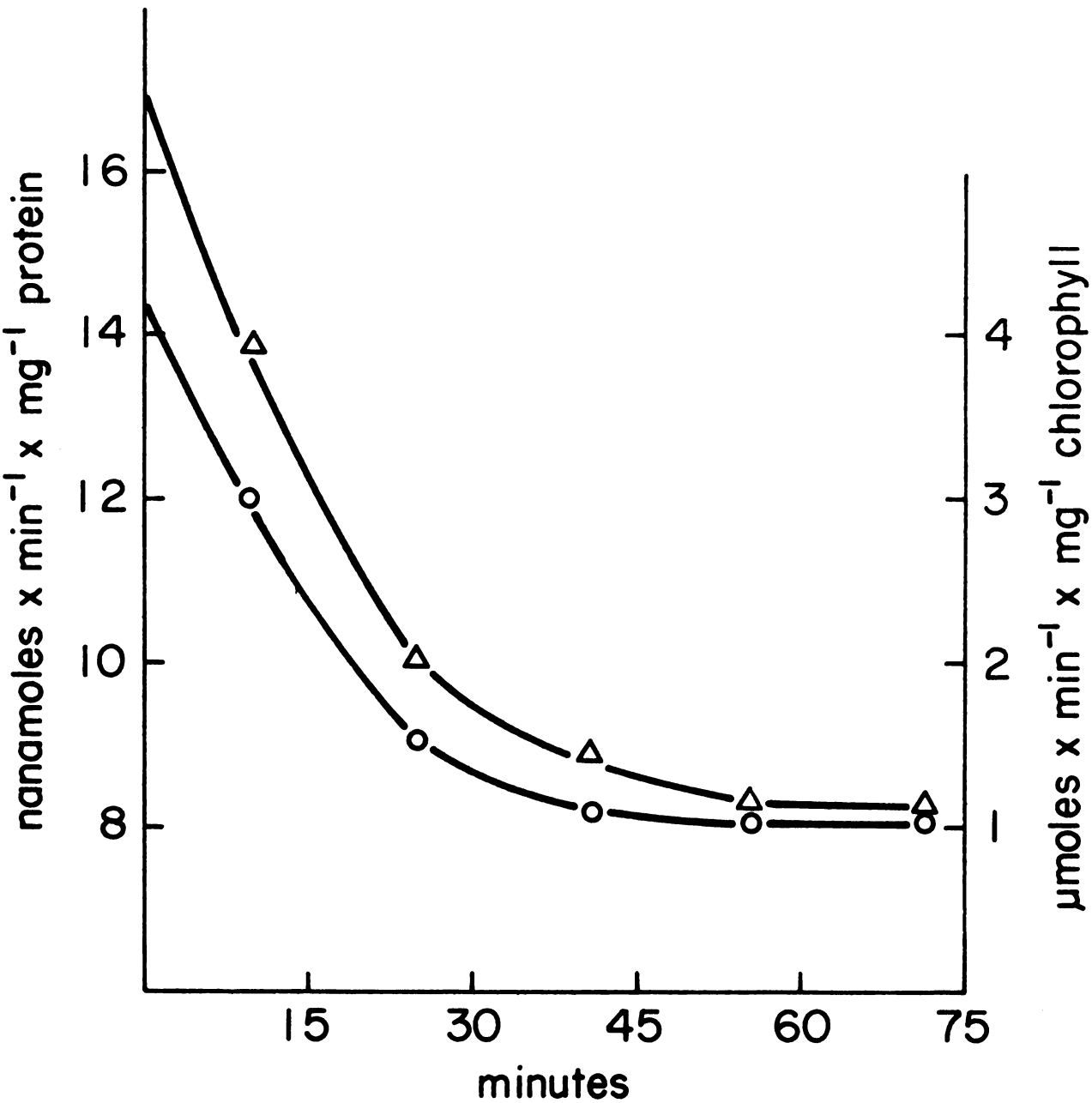
*All work done at 2°-4°C.

Table 4. Purification of Glycolate Dehydrogenase

Fraction	Volume	Total Units	Specific Activity (DCIP Reduced)	% Recovery	Purification
	ml		(nmoles x min^{-1} x mg^{-1} protein)		
Triton X-100 extract	100	4020	11.5	100	---
1st $(NH_4)_2SO_4$ supernatant	110	3660	15.5	91	1.3
2nd $(NH_4)_2SO_4$ supernatant	110	<100			
2nd $(NH_4)_2SO_4$ pellet	12	3500	38.1	87	3.3

Figure 13. Specific Activity of Glycolate Dehydrogenase Released from Chlamydomonas by 1% Triton X-100

Glycolate dehydrogenase activity was determined by DCIP reduction in the 29,000 g supernatant of Triton X-100 extracts. Extracts were prepared by stirring cells (10% v/v) in 1% Triton X-100 w/v, 0.10 M phosphate pH 7.5. O - μmoles x min^{-1} x mg^{-1} chlorophyll; Δ - nmoles x min^{-1} x mg^{-1} protein.


nanamoles x min⁻¹ x mg⁻¹ protein
16
14
12
10
8
μmoles x min⁻¹ x mg⁻¹ chlorophyll
4
3
2
1
15
30
45
60
75
minutes

Further purification of glycolate dehydrogenase was not obtained in many attempts. This was due to the instability of the preparations and the lack of large amounts of starting material. Consequently studies with the enzyme were run on the $(NH_4)_2SO_4$ fractionated preparation and referred to as the partially purified dehydrogenase.

Substrate Characterization of Glycolate Dehydrogenase

The affinity of partially purified glycolate dehydrogenase for glycolate and other substrates was tested. The results showed glycolate to be the preferred substrate, while D-lactate, L-lactate, glyoxylate and D,L-α-hydroxybutyrate also served as substrates (Table 5). The affinity of the enzyme for D-lactate is of special interest since other workers have shown that glycolate oxidase from higher plants does not oxidize D-lactate, while it does oxidize L-lactate at a rate of about 60% that of glycolate (79, 103). This rate of oxidation with spinach glycolate oxidase was confirmed. The affinity of glycolate dehydrogenase for glyoxylate and D,L-α-hydroxybutyrate was similar to that found for higher plants for glycolate oxidase (79).

Mixed substrate experiments were carried out to determine if activities observed with glyoxylate, D and L-lactate could be attributed to one or more enzymes. The rates of oxidation of mixtures of substrates were not additive (Table 6). The results indicated that all substrates

Table 5. Substrate Specificity of Glycolate Dehydrogenase

Relative rates of DCIP reduction with glycolate taken as 100. Substrates at 8 mM final concentration. 16 μmoles of D,L mixtures were added to give 8 mM final concentration of each.

Substrate	Relative Activity
Glycolate	100
D-Lactate	50-70
L-Lactate	10-25
Glyoxylate	30-50
D,L-α-Hydroxybutyrate	50-75
Glycine	0
meso-Tartrate	0
D,L-Malate	0
D,L-α-Phenyl Lactate	0
D,L-β-Phenyl Lactate	0
P-Glycolate	0
D,L-Glycerate	0

Table 6. Mixed Substrate Assays with Glycolate Dehydrogenase

Relative rates of DCIP reduction with glycolate taken as 100. All substrates at 8 mM final concentration.

Substrate	Relative Rates	
	Found	Predicted
Glycolate	100	
Glyoxylate	34	
Glyoxylate + Glycolate	82	67
L-Lactate	22	
L-Lactate + Glycolate	63	61
D-Lactate	66	
D-Lactate + Glycolate	83	83

were oxidized by the same enzyme. The slightly high value obtained with glyoxylate can be explained by contamination of the glyoxylate with glycolate.

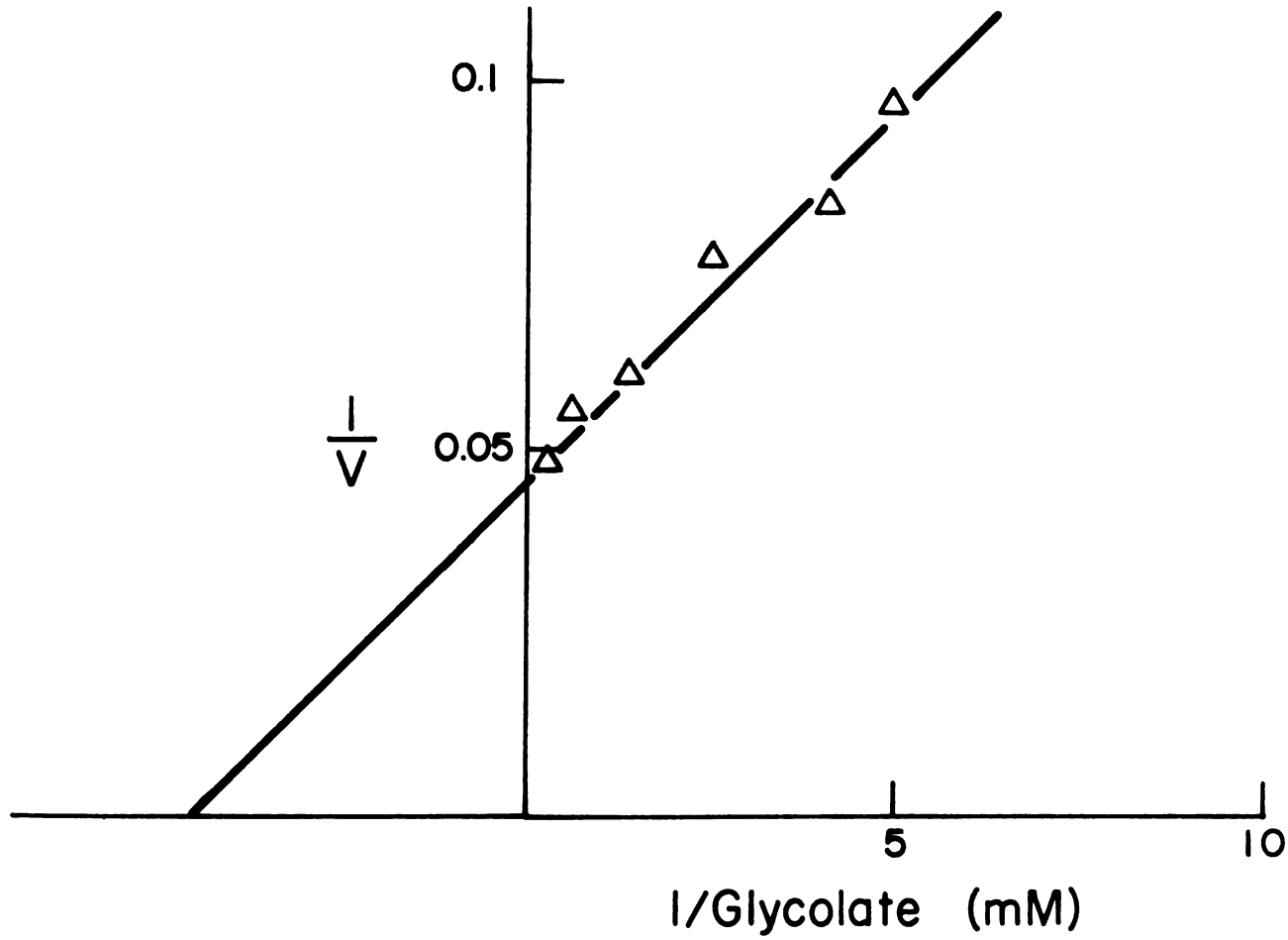
The K_m's for glycolate and D-lactate were determined using the method of Lineweaver and Burke (54). The K_m for glycolate oxidation by glycolate dehydrogenase from Chlamydomonas was 2.2×10^{-4} M (Figure 14). A high K_m of 1.5×10^{-3} M for D-lactate oxidation was obtained (Figure 14). Thus the oxidation of D-lactate may be of no physiological importance to the glycolate dehydrogenase system.

Electron Acceptor Specificity of Glycolate Dehydrogenase

The ability of the partially purified glycolate dehydrogenase to reduce various electron acceptors was investigated. The results indicated that only DCIP and PMS, of all the acceptors tested, would link to glycolate dehydrogenase (Table 7). No oxidase activity could be detected with either the oxygen electrode or the phenylhydrazone assay. Glyoxylate formation was measured in relationship to DCIP reduction anaerobically and also measured aerobically in the presence and absence of DCIP (Figure 15). Aerobic glyoxylate formation was dependent on the presence of DCIP, no glyoxylate formation was detected in its absence. This experiment confirmed the results of Zelitch and Day (106), who reported a 1:1 stiochiometry between DCIP reduction and glyoxylate formation.

Figure 14. Determination of the K_m's for Glycolate and D-Lactate

Glycolate dehydrogenase activity measured by following DCIP reduction as described in Methods and Materials.


0.1
0.05
1/V
5
10
1/Glycolate (mM)

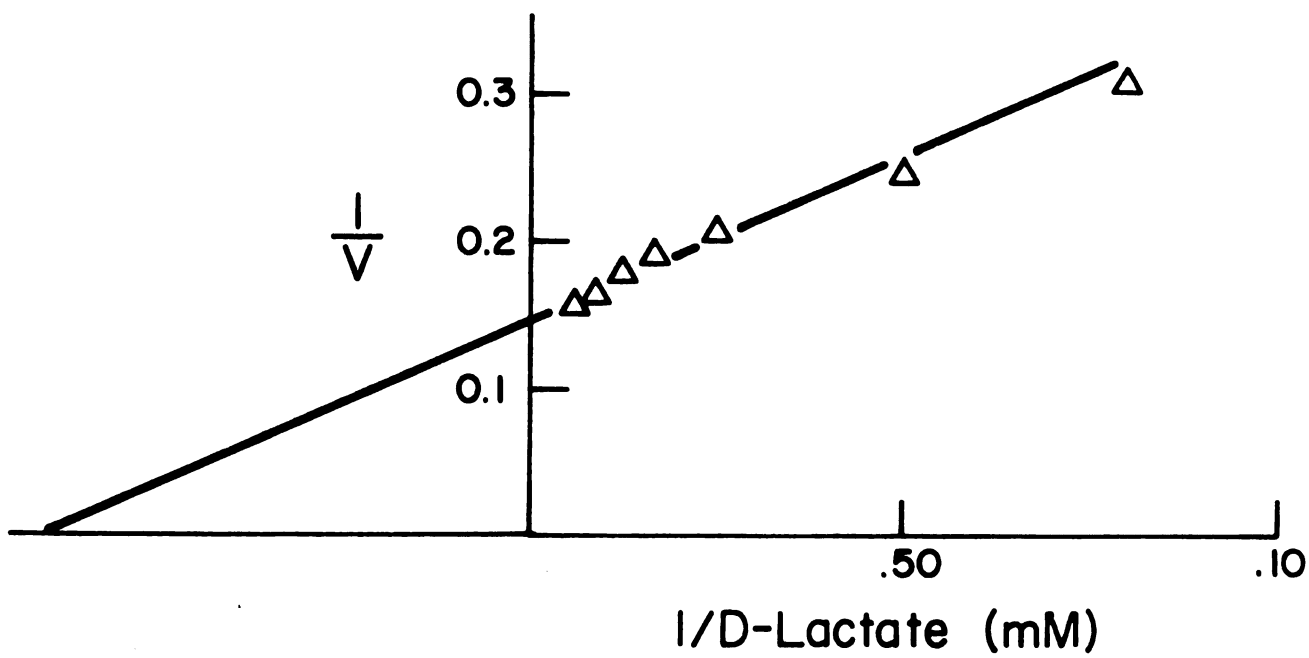

0.3
0.2
0.1
1/V
.50
.10
1/D-Lactate (mM)

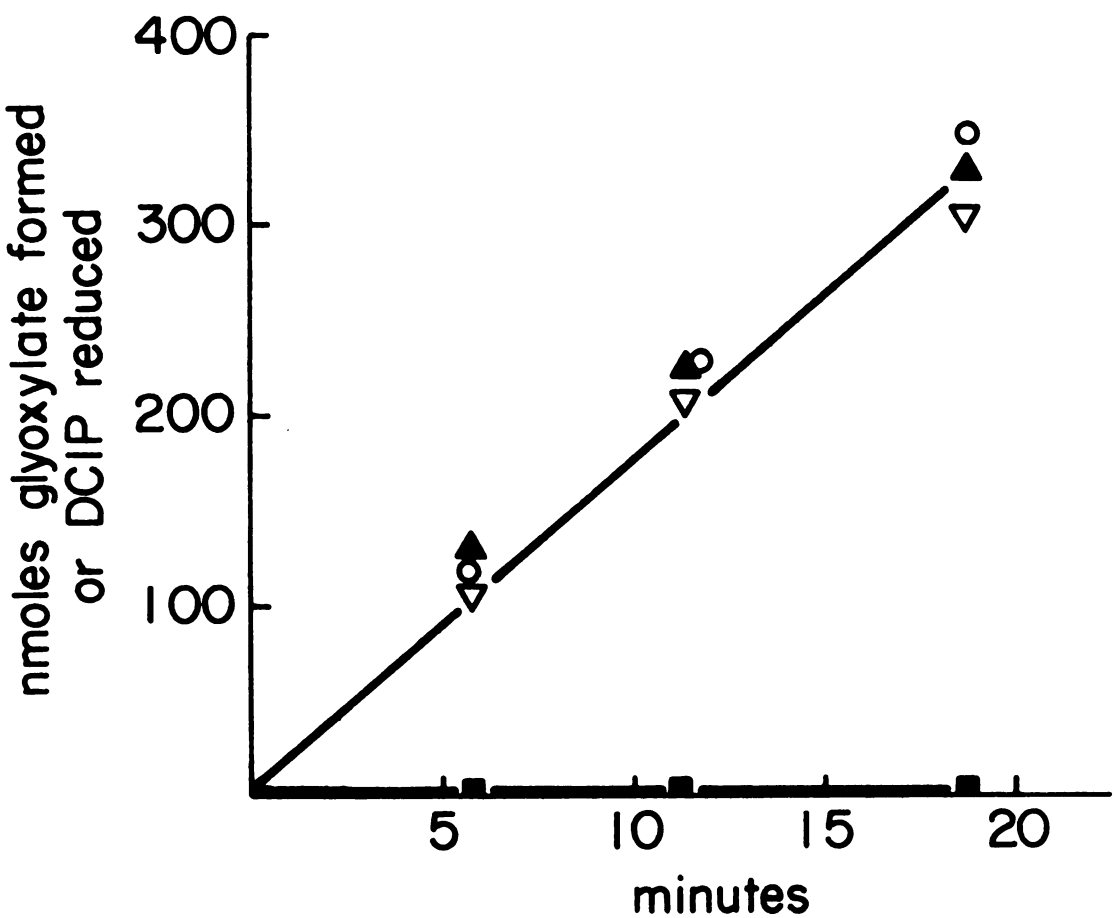
Table 7. Electron Acceptor Specificity of Glycolate Dehydrogenase

Assays carried out as described in Methods and Materials. Activity expressed relative to DCIP reduction.

Electron Acceptor	Relative Affinity
DCIP	100
PMS	78
$K_3Fe(CN)_6$	0
NAD^+	0
$NADP^+$	0
Methylene Blue	0
Oxygen	0
Glutathione	0
Cytochrome c	0
NO_3^-	0
FMN	0
FAD	0

Figure 15. Glyoxylate Formation in Relation to DCIP Reduction, the Requirement of DCIP for Glyoxylate Formation

Glyoxylate formation measured by the glyoxylate phenylhydrazone assay in presence and absence of DCIP as described in Methods and Materials:
▲ - anaerobic glyoxylate formation + DCIP;
▽ - anaerobic DCIP reduction;
○ - aerobic glyoxylate formation + DCIP
■ - aerobic glyoxylate formation - DCIP.


400
300
200
100
nmoles glyoxylate formed
or DCIP reduced
5
10
15
20
minutes

The K_m for DCIP reduction with glycolate as the substrate was 1.7×10^{-5} M (Figure 16) determined by the method of Lineweaver and Burke (54). For PMS the K_m was 3×10^{-5} M (Figure 17) determined by a V versus S plot. It is noteworthy that the K_m for DCIP is approximately 20 times lower for glycolate dehydrogenase as compared to the K_m of DCIP for glycolate oxidase whose DCIP K_m is 3.8×10^{-4} M.

Flavin or Pyridine Nucleotide Cofactors

Glycolate oxidase is a flavoprotein (103) as are various other α-hydroxyacid dehydrogenases (3, 15, 24, 70, 80). Attempts were made to demonstrate a flavin co-factor for glycolate dehydrogenase from Chlamydomonas. Addition of 2×10^{-4} M FMN or FAD to the assay had no effect on the rate of DCIP reduction using partially purified enzyme. Treatment of the $(NH_4)_2SO_4$ purified enzyme with acid $(NH_4)_2SO_4$ at pH 4.5 or 5.0 following the method of Zelitch and Ochoa (103) resulted in the complete loss of activity. Neither FMN nor FAD was able to restore activity. Addition of NAD^+ or $NADP^+$ did not stimulate the rate of DCIP reduction. Treatment of the enzyme with acid washed charcoal did not cause a loss of activity.

These results do not demonstrate that either a flavin or a pyridine nucleotide is a cofactor for glycolate dehydrogenase. By precedent the involvement of a flavin co-factor is indicated, however, this remains to be established in the case of glycolate dehydrogenase from Chlamydomonas.

Figure 16. Determination of the K_m for DCIP

Glycolate dehydrogenase assayed following DCIP reduction as described in Methods and Materials. DCIP concentration determined spectrophotometrically before assaying activity.

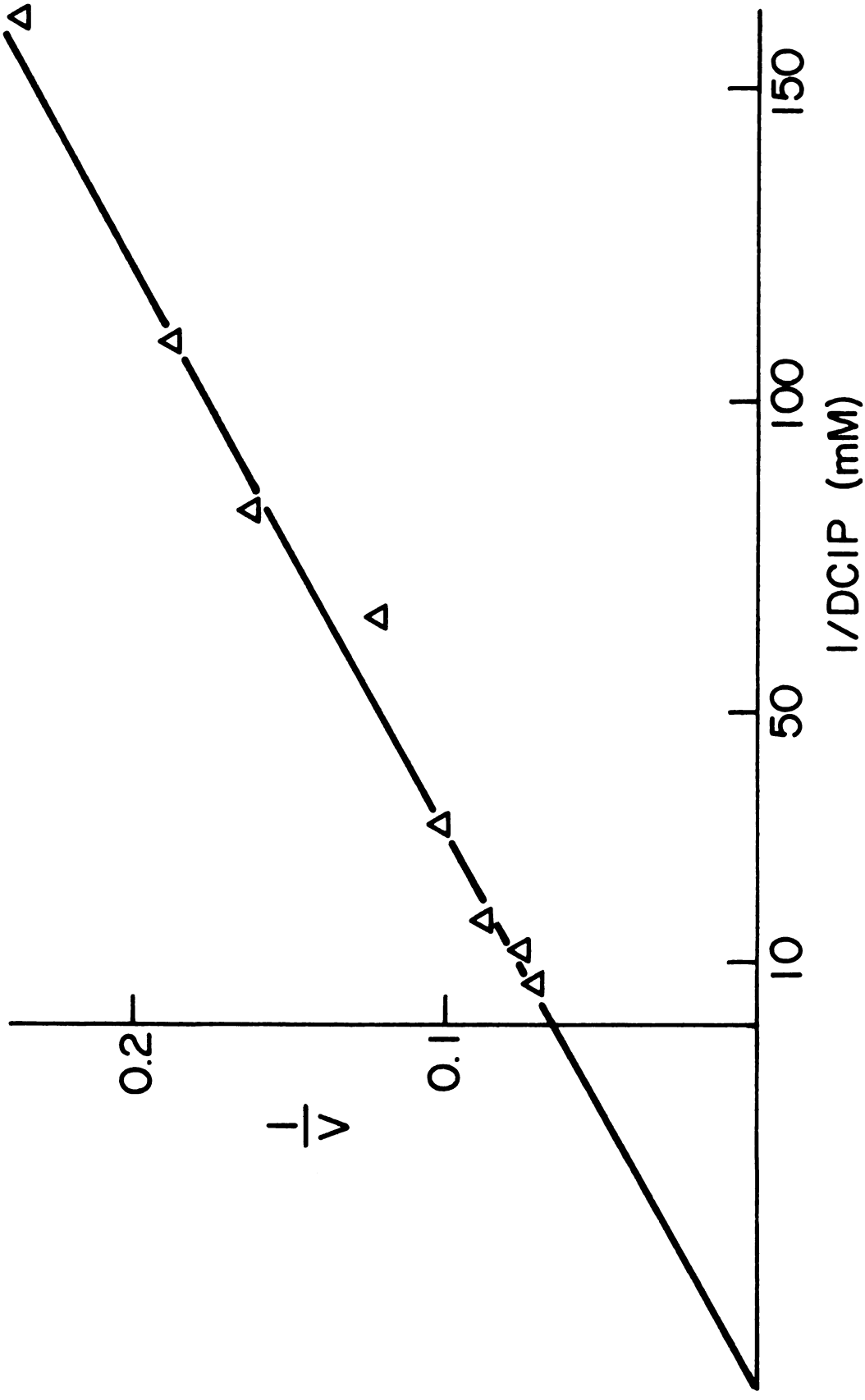
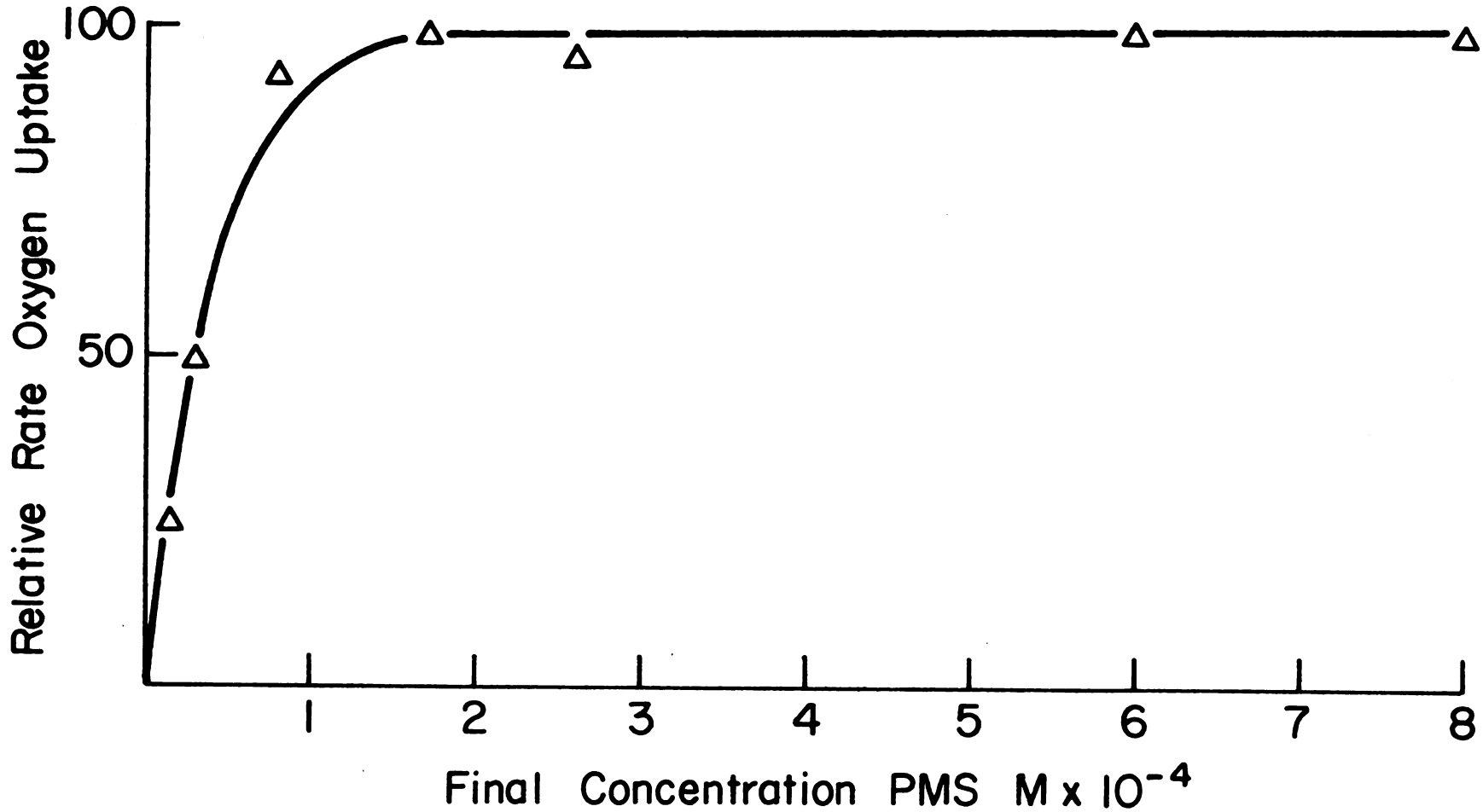

0.2
0.1
1/V
10
50
100
150
1/DCIP (mM)

Figure 17. Substrate Saturation Curve for PMS

Glycolate dehydrogenase assayed following oxygen reduction with an oxygen electrode and described in Methods and Materials. PMS was prepared fresh and kept dark before added to the assay. Glycolate was at a saturating level (6.2×10^{-3} M).


Relative Rate Oxygen Uptake
100
50
1
2
3
4
5
6
7
8
Final Concentration PMS M x 10^{-4}

pH Optimum and the Effect of Ionic Strength on Glycolate Dehydrogenase

The pH curves for the oxidation of glycolate and D-lactate are shown in Figure 18. The pH optimum for glycolate extended over a broad range between 8.0 and 9.0 and was similar to that observed for partially purified glycolate oxidase (19, 103). When determining these optima the change in extinction coefficient of DCIP with decreasing pH was taken into account (4). The optimum for D-lactate oxidation was at pH 8.7.

As glycolate oxidase purified from spinach was very sensitive to ionic strength when DCIP reduction was assayed (34), the effect of increasing ionic strength on glycolate dehydrogenase was tested. Enzyme was dialysed against 0.1 M phosphate pH 7.5 to remove $(NH_4)_2SO_4$. The dialysed enzyme was then assayed with NaCl or $(NH_4)_2SO_4$ added to the assay mixtures. No effect on the reduction of DCIP was observed with these salts even when the ionic strength of the assay mixture was doubled.

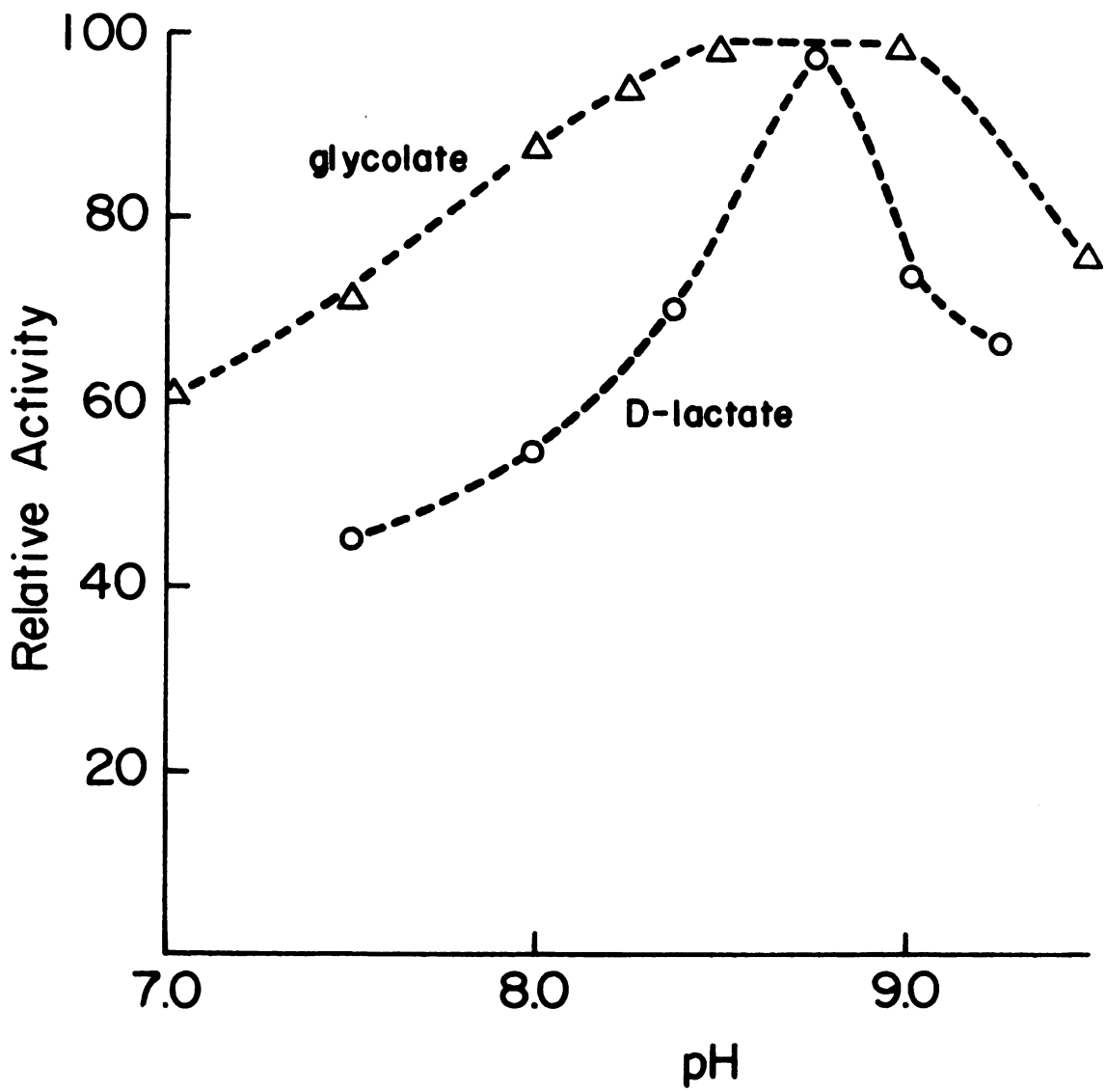
No significant difference in the rate of DCIP reduction by glycolate dehydrogenase was observed when the following buffers were tested at pH 8.7, 0.1 M pyrophosphate, bicine, tricine, glycylglycine and Tris.

Effect of Inhibitors on Glycolate Dehydrogenase

Glycolate dehydrogenase was sensitive to inhibitors

Figure 18. pH Curve for the Oxidation of Glycolate and D-Lactate by Glycolate Dehydrogenase

Rates were determined by measuring DCIP reduction. Buffer used was 0.1 M pyrophosphate/phosphate adjusted to desired pH. Final pH was determined after completion of the assay.


100
80
60
40
20
Relative Activity
glycolate
D-lactate
7.0
8.0
9.0
pH

which effect sulfhydryl groups such as $CuSO_4$, N-ethylmaleimide and, p-CMB (Table 8). Glycolate oxidase from higher plants has been shown to be relatively insensitive to p-CMB (34, 69), however, glycolate oxidase from rat liver (64) and renal cortex (80), have been found to be inhibited by these sulfhydryl inhibitors.

The effect of metal complexing agents on glycolate dehydrogenase gave equivocal results. o-Phenanthroline was found to inhibit 70% of the activity at 4 mM (Table 8) when added directly to the DCIP assay mixture (pH 8.7). Incubation of the enzyme at 4°C with 10 mM o-phenanthroline at pH 7.5 (0.1 M phosphate) gave only 50% inhibition after 24 hours. o-Phenanthroline was not separated from the enzyme before assaying, making the o-phenanthroline concentration in the assay 2×10^{-4} M. Dialysis against 10 mM o-phenanthroline for two hours at pH 8.1 (0.1 M phosphate) caused a complete loss of activity, compared to controls lacking o-phenanthroline. After removal of the o-phenanthroline by continued dialysis, no reactivation occurred by incubation of the inhibited enzyme at 4°C up to two hours with 5×10^{-4} M $ZnCl_2$ or 5×10^{-4} M $FeCl_3$. Also addition of 5×10^{-5} M $ZnCl_2$, $FeCl_3$ or $MgCl_2$ to the assay directly with inhibited dialysed enzyme did not lead to reactivation. In both types of reactivation experiments, 0.1 M phosphate pH 8.1 was used as the assay buffer rather than 0.1 M pyrophosphate pH 8.7 in order to

Table 8. Effect of Enzyme Inhibitors on Glycolate Dehydrogenase

Inhibition measured after addition of inhibitor directly to the DCIP reduction assay mixture. Enzyme was in the presence of inhibitor for 10 minutes before addition of glycolate.

Inhibitor	Concentration	% Inhibition
N-Ethylmaleimide	5×10^{-5} M	35%
	1×10^{-4} M	60%
	5×10^{-4} M	100%
$CuSO_4$	1×10^{-4} M	20%
	1×10^{-3} M	60%
p-CMB	1×10^{-5} M	35%
	2×10^{-5} M	73%
	5×10^{-5} M	100%
o-Phenanthroline	1×10^{-3} M	25%
	4×10^{-3} M	71%
	8×10^{-3} M	90%
m-Phenanthroline	8×10^{-3} M	0%
EDTA	1.5×10^{-2} M	0%
KCN	5×10^{-4} M	47%
	1×10^{-3} M	85%
8-Hydroxyquinoline Sulfonate	1.5×10^{-2} M	30%

prevent binding of the cation by pyrophosphate. Inhibition by o-phenanthroline could be avoided by addition of 10 mM mercaptoethanol or cysteine to the dialysing mixture. m-Phenanthroline had no inhibitory effect (Table 8), indicating o-phenanthroline inhibition was not due to non-specific interactions. Work in Horecker's laboratory with rabbit muscle fructose-1,6-diphosphate aldolase has shown similar inhibitory results with o-phenanthroline at pH's above 8.0 (50). This aldolase is not a metalloenzyme. It was inhibited, however, by o-phenanthroline and the o-phenanthroline inhibition was relieved by addition of sulfhydryl reagents. On the basis of the aldolase work it is suggested that o-phenanthroline inhibition of glycolate dehydrogenase could be due to enhanced sulfhydryl oxidation rather than metal complexing.

No inhibition of glycolate dehydrogenase was observed with EDTA after incubation in 10 mM EDTA for up to 24 hours at pH 7.5. Addition of EDTA directly to the assay mixture up to 16 mM had no effect on the rates. 8-Hydroxyquinoline sulfonate gave 30% inhibition at 15 mM when added directly to the assay (Table 8). The high concentrations necessary for inhibition indicated non-specific interactions rather than metal complexing.

Cyanide caused inhibition of activity (Table 8). Cyanide has been shown to inhibit the Zn-flavoprotein-α-hydroxy acid dehydrogenase of yeast (24). Cyanide did not

inhibit glycolate oxidase isolated from spinach, however it inhibited glycolate oxidase from liver, an enzyme which has no metal co-factor (64). This type of inhibition by cyanide is felt to be due to sulfhydryl interactions rather than metal complexing (26). Thus the use of cyanide does not distinguish between metalloenzymes and sulfhydryl sensitive enzymes.

On the basis of the data it is not possible to state definitely that glycolate dehydrogenase is or is not a metalloprotein.

Distribution of Glycolate Dehydrogenase

The work reported so far in this thesis has been done exclusively with glycolate dehydrogenase isolated from Chlamydomonas. To determine if glycolate dehydrogenase is present in other green algae and perhaps in lower forms of plants, tests were run on several other green algae and plants. Both glycolate oxidase and glycolate dehydrogenase were assayed from crude extracts by measuring DCIP reduction, D and L lactate specificity and the effect of cyanide on DCIP reduction. These assays should distinguish between the two enzymes as glycolate oxidase oxidizes L-lactate but not D-lactate, and is not effected by cyanide, while glycolate dehydrogenase oxidizes D-lactate and is inhibited by cyanide. The results of the survey are presented in Table 9.

Table 9. Survey of Algae and Plants for Glycolate Dehydrogenase*

	Substrate Affinity			Cyanide Inhibition (2 mM)		Specific Activity
Algae	Glycolate	D-Lactate	L-Lactate	Glycolate	D-Lactate	Glycolate Dehydrogenase
	Activity Relative to Glycolate			% Inhibition Compared to Controls		nmoles x min^{-1} x mg^{-1} Protein
Chlamydomonas	100	50-70	10-25	100%	100%	3-5
Chlorella	100	70	5	100%	100%	3-5
Scenedesmus	100	88	0	100%	100%	11
Acetabularia	100	123	5	100%	100%	13
Euglena	100	200	25	100%	25%	12
Plants						
Elodea	100	0	43	0	---	---
Marchantia	100	0	62	0	---	---
Spinach	100	0	40-60	0	---	---

*Activity measured in crude extracts by DCIP reduction as described in Methods and Materials. Cyanide added directly to assay mixture.

There appeared to be no detectable glycolate dehydrogenase in any of three higher plants tested including Elodea, an aquatic plant. Of the Chlorophyta tested all contained glycolate dehydrogenase. Euglena gave a more complex result. On the basis of cyanide inhibition data there appeared to be in Euglena another enzyme beside glycolate dehydrogenase for oxidizing D-lactate which was less cyanide sensitive. With Euglena extracts additive rates were obtained when D-lactate and glycolate were used in mixed substrate assays, which confirmed the presence of two enzymes. The presence of a D-lactate dehydrogenase in Euglena has been reported by Price (73). It is suggested that this enzyme could account for the different results obtained with Euglena. No glycolate oxidase or dehydrogenase could be detected in crude extracts of Nitella or Chara. Thus I was unable to confirm the report of Downton and Tregunna (27) on the presence of glycolate oxidase in Nitella.

On the basis of this limited survey it appears as though glycolate dehydrogenase may represent the form of the enzyme in algae, and glycolate oxidase the form in higher plants.

Glutamate: Glyoxylate Aminotransferase

The presence of glutamate: glyoxylate aminotransferase in Chlamydomonas was established. The formation of

glycine by crude extracts was linear with time and protein concentration under the assay conditions. The conversion was dependent on the presence of an amino donor (Table 10), pyridoxal-5-phosphate was stimulatory in some experiments, but not in all. Table 11 shows the reactivity of various amino donors as substrates for the transamination of glyoxylate. L-Glutamate and L-alanine were the preferred donors. Similar results by this assay have been obtained for higher plants (49) and castor bean endosperm extracts (22).

The level of enzymatic activity on a protein basis was determined for several preparations to be 60 to 80 nanomoles x min^{-1} x mg^{-1} protein with L-glutamate as the amino donor. This activity is higher than that reported for spinach peroxisomes which ranged between 5 to 15 nanomoles x min^{-1} x mg^{-1} protein (13). It is suggested that non-specific activity of other aminotransferases could account for some of this high activity.

Table 10. Glutamate: Glyoxylate Aminotransferase Activity in Crude Extracts of Chlamydomonas

Complete assay contained in 1.25 ml 20 μmoles glyoxylate-1,2-^{14}C, 12 μmoles L-glutamate, 15 μmoles phosphate pH 7.5 and 0.1 μmoles pyridoxal-5-phosphate.

Treatment	nmoles Glycine Synthesized
Complete assay	199
- Pyridoxal-5-phosphate	153
- L-Glutamate	<10

Table 11. Specificity of Amino Group Donor for Glycine Formation by Crude Extracts of Chlamydomonas

Relative rates of glycine formation with L-glutamate taken as 1000. Each assay contained 12 μmoles L amino donor or 24 μmoles donor when D,L substrate mixtures were used.

Amino Donor	Glycine Formed
L-glutamate	1000
L-alanine	1000-1200
D,L-serine	490
D,L-asparagine	400
L-ornithine	350
L-arginine	220
D,L-glutamine	199
D-alanine	112
D,L-aspartate	104
D,L-γ-aminobutyrate	94
D,L-tryptophan	70
D,L-valine	44
L-leucine	42
β-alanine	35

GENERAL DISCUSSION

The Role of Glycolate Excretion and Assimilation in Nature

From the data on glycolate metabolism by Chlamydomonas and Scenedesmus it appears that glycolate is both excreted and assimilated by green algae. It is mainly excreted if the algae are grown on high CO_2 and mainly metabolized if the cells are grown on air or low CO_2. Glycolate excretion, which occurs to a limited extent in nature, is observed even though the cells grow at low CO_2 levels (41, 98, 99). The importance of this phenomena in nature has not been fully evaluated. Fogg (33) has suggested that glycolate excretion is important in maintaining trophic relationships in aquatic habitats. In addition glycolate metabolism in the cell appears to be important in regulation of net photosynthesis as illustrated in publication (65) and in Figure 6. Using the biochemical mechanisms proposed in this scheme, the algae assimilating glycolate can carry on non-cyclic photophosphorylation when CO_2 is unavailable or very limiting. Thus the presence of exogenous glycolate which was previously excreted can serve as a reserve pool for generating ATP when other acceptors are unavailable. The reabsorption of glycolate under high light intensity by natural

populations of algae has been demonstrated (33). It remains to be determined whether this reabsorption leads to increased non-cyclic photophosphorylation.

The Regulation of Enzyme Levels by CO_2 Availability

While CO_2 is the natural substrate for photosynthesis little attention has been given to its role in controlling metabolic events by effecting enzyme levels. The levels of glycolate dehydrogenase have been found to be regulated by the availability of CO_2 (Table 2). The activity of this enzyme would determine the ability of the cell to excrete glycolate. In nature where CO_2 levels are normally low, this phenomenon can be of significant consequence in maintaining efficient carbon assimilation ability. Low CO_2 in the culture would lead to maximum glycolate dehydrogenase activity and therefore little or no excretion of the substrate. Thus newly fixed CO_2 would all be used for cell growth rather than be excreted into the media.

Work by Graham and Whittingham (39) has suggested that CO_2 also regulated the amount of another enzyme which was related to efficient photosynthesis. When all photosynthetic tests were carried out at low CO_2 concentrations, they observed that Chlorella grown on high levels of CO_2, 3%, had different initial products of photosynthetic CO_2 fixation and slower rates of fixation than Chlorella grown on low CO_2. The enzyme suspected of regulating this differ-

ence was thought to be carbonic anhydrase (77). In this laboratory we have observed that growth of Chlamydomonas on 1% CO_2 leads to a 20 fold decrease in carbonic anhydrase levels compared to controls grown on air (67). The role of carboic anhydrase in photosynthesis remains to be evaluated. Thus it appears as though at least the activity or amount of two enzymes, glycolate dehydrogenase and carbonic anhydrase, are controlled by CO_2 availability during growth.

Enzymes of the Glycolate Pathway in Algae

The presence of enzymes carrying out the first three steps of the glycolate pathway (Figure 1) has been demonstrated in green algae. Work described in this thesis and by other workers (55, 106) demonstrated the presence of glycolate dehydrogenase. The presence of glutamate: glyoxylate aminotransferase in Chlamydomonas was also confirmed (Table 10). Serine hydroxymethyltransferase has been reported to be present in Chlorella (Warburg) and Chlamydomonas by Bruin (13). The activity of the various enzymes of the glycolate pathway in green algae extracts are listed in Table 12. The levels of glycolate dehydrogenase and serine hydroxymethyltransferase are about equal and appear to be the limiting enzymes of the pathway. Relative to these two enzymes glutamate: glyoxylate aminotransferase appears to be available in excess.

Table 12. Activity of Enzymes of the Glycolate Pathway in Green Algae

Enzyme	Cell Source	Specific Activity in Crude Extracts
Glycolate Dehydrogenase	*Chlorella* *Chlamydomonas*	3-5 nanomoles x min^{-1} x mg^{-1} protein
Glutamate: glyoxylate aminotransferase	*Chlamydomonas*	60-80 nanomoles x min^{-1} x mg^{-1} protein
Serine hydroxymethyltransferase[1]	*Chlorella* *Chlamydomonas*	2-6 nanomoles x min^{-1} x mg^{-1} protein

[1]Data from reference 13.

The presence of serine hydroxymethyltransferase would promote the synthesis of serine from glycine, however, in $^{14}CO_2$ fixation experiments with algae, serine appeared to be derived from 3-P-glycerate (13, 43). This is not due to low activity of the transferase since the levels found in algae are equal to that found in crude extracts of spinach (13). Apparently the reaction between glycine and serine proceeds very slowly in algae, while there is rapid synthesis of serine from 3-P-glycerate. More research is needed to determine the actual mechanism involved in synthesis of serine by green algae.

The maximum activity of glycolate dehydrogenase observed was not enough to metabolize the maximum amount of glycolate synthesized (compare Figures 7 and 8 with Table 2). The reason for this imbalance is not understood, but two explanations may be proposed. The amount or rate of glycolate production in air grown cells may be lower. Also there may be an alternative pathway for glycolate metabolism in air grown cells not involving glycolate dehydrogenase. As some glycolate excretion occurs in nature (33), it appears probable that at some stage of the life cycle of the algae there is excess glycolate production in relation to its ability to be metabolized. This stage of the life cycle is when glycolate excretion could occur.

Comments on Glycolate Dehydrogenase From Green Algae

The presence of glycolate dehydrogenase in several green algae has been demonstrated (Table 6). Whether or not glycolate dehydrogenase will be found in other algae such as blue greens remains to be determined. Evidence suggests that glycolate oxidase rather than glycolate dehydrogenase is present in Nitella (27). Glycolate dehydrogenase activity has been reported in Neurospora, however, the ability of the enzyme to link to oxygen was not tested (18).

One of the major problems related to the presence of glycolate dehydrogenase is the question of its natural electron acceptor. The data reported herein shows that it does not link to oxygen. While Lord and Merrett (55) first reported the presence of this enzyme in Chlorella by assaying for glyoxylate aerobically in the absence of DCIP, they have now confirmed that there was no glycolate oxidase activity present in their preparations (M. J. Merrett, personal communication). Therefore their preparations must have contained sufficient quantities of the natural electron acceptor to allow the reaction to proceed. In our laboratory, no formation of glyoxylate could be detected using Merrett's strain of Chlorella under identical conditions described by Lord and Merrett (55). The reason for this difference has not been resolved. Kolesnikov et. al. (50) have suggested that quinones could be the natural electron

acceptors for glycolate oxidase from green plants. While it remains to be established, it is a reasonable hypothesis that glycolate dehydrogenase from green algae might also link to quinones *in vivo*.

The role of D-lactate in algal metabolism is of interest. Glycolate dehydrogenase will oxidize D-lactate perferentially over L-lactate (Table 5). D-lactate dehydrogenase activity is present in *Euglena* (Table 9). Two other groups have reported enzymes in algae capable of metabolising D-lactate. Price (73) has reported an NAD^+ linked D-lactate dehydrogenase in heterotrophic grown *Euglena*. Warburg et. al. (96) have described an NAD^+ linked D-lactate dehydrogenase in *Chlorella*. Furthermore, they isolated D-lactate from these cells. Yeast possess two enzymes for metabolizing D-lactate depending on whether growth is aerobic or anaerobic. Both enzymes are Zn requiring FAD flavoproteins, they differ in their electron acceptor specificity (24, 70). The yeast enzymes, like glycolate dehydrogenase, have no oxidase activity. The ability of these unicellular organisms to metabolize D-lactate is significant. It remains to be determined if the glycolate dehydrogenase system is also involved in D-lactate metabolism. The high K_m for D-lactate (1.5×10^{-3} M) suggests it is not involved.

Preliminary evidence suggests that D-lactate oxidation carried out by flavoprotein α-hydroxy acid dehydrogen-

ases is not localized in the peroxisome while L-lactate is oxidized by α-hydroxy acid oxidases localized in the peroxisome. Acetabularia contains glycolate dehydrogenase (Table 4), and Acetabularia has been reported on the basis of electron microscopic evidence to lack peroxisomes (11). There are no reports of peroxisomes in any green algae to the author's knowledge. Tetrahymena, a unicellular organism has L-lactate oxidase localized in the peroxisome (25). Glycolate oxidase from green plants is a peroxisomal enzyme (92). Liver and kidney peroxisomes contain L-α-hydroxy acid oxidases (25, 64, 81) and although liver and kidney also have D-lactate dehydrogenase activity (15, 93), this activity is not localized in the peroxisome (25). The reason peroxisomal metabolism is limited to L-acids and glycolate appears to be that flavoproteins oxidizing these substrates form H_2O_2, which must be destroyed by catalase also localized in the peroxisome. Enzymes preferentially oxidizing D-acids and glycolate do not form H_2O_2 (70, 93); therefore close proximity to catalase is not necessary. The reason why flavoproteins oxidizing D-acids and glycolate do not link to oxygen while those oxidizing L-acids and glycolate do remains to be determined.

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