Plant Physiol. (1976) 58, 315-319

Cytochemical Localization of Glycolate Dehydrogenase in Mitochondria of *Chlamydomonas*[1]

Received for publication March 29, 1976 and in revised form May 28, 1976

BELINDA B. BEEZLEY, PETER J. GRUBER, AND SUE ELLEN FREDERICK
Department of Biological Sciences, Mount Holyoke College, South Hadley, Massachusetts 01075

ABSTRACT

Mildly disrupted cells of *Chlamydomonas reinhardi* Dangeard were incubated in a reaction medium containing glycolate, ferricyanide, and cupric ions, and then processed for electron microscopy. As a result of the cytochemical treatment, an electron opaque product was deposited specifically in the outer compartment of mitochondria; other cellular components, including microbodies, did not accumulate stain. Incubation with D-lactate yielded similar results, while treatment with L-lactate produced only a weak reaction. Oxamate, which inhibits glycolate dehydrogenase activity in cell-free extracts, also inhibited the cytochemical reaction. These findings demonstrate *in situ* that glycolate dehydrogenase is localized in mitochondria, and thus corroborate similar conclusions reached on the basis of enzymic studies of isolated algal organelles.

One of the best resolved distinctions between the photorespiratory system of algae (10, 19) and that of higher plants (20) lies in the enzyme responsible for glycolate oxidation. Glycolate oxidase, the enzyme present in all land plants, and glycolate dehydrogenase, the enzyme characteristic of many green algae, differ with respect to immediate electron acceptors, utilization of alternate substrates, and inhibition by cyanide (2, 6, 11). Furthermore, the two appear to have different subcellular localizations. Glycolate oxidase is known to reside in the matrix of peroxisomes (1, 9, 18) while glycolate dehydrogenase is associated with membranes of organelles recently suggested to be mitochondria.

The latter conclusion is based upon studies of subcellular fractions isolated from green algae (7, 15, 16), diatoms (12), and *Euglena* (4). Some of these investigations have not ruled out contamination of mitochondrial fractions by other organelles; and none of them has excluded the presence of membranous components such as the plasmalemma, endoplasmic reticulum, and chloroplast envelope. In fact, the one report which presents electron micrographs of the isolated mitochondria underscores the possibility of contamination by extraneous vesicles (12).

The technique of ultrastructural cytochemistry is advantageous in that it can provide an *in situ* localization of enzyme activity. We have adapted a cytochemical method used previously to visualize a variety of other oxidoreductases (8, 13), applied the procedure to *Chlamydomonas*, and now report that in this green alga glycolate dehydrogenase does appear to be exclusively a mitochondrial enzyme.

MATERIALS AND METHODS

Cultures of *Chlamydomonas reinhardi* Dangeard (Indiana University Collection No. 90) were grown autotrophically in Sueoka high salt medium (17) at 25 C with constant bubbling of air. A 16 hr light-8 hr dark cycle was maintained, with a light intensity of 400 ft-c provided by fluorescent and incandescent sources. Cells were typically harvested during the log phase of growth.

Partial Disruption of Cells. In order to establish sonication conditions providing a degree of cell disruption optimal for the cytochemical work, cells were collected and treated according to Curtis *et al.* (5). Chilled algal suspensions were sonicated for various lengths of time at several power settings with a Sonifier Cell Disruptor, model W185 (Heat Systems-Ultrasonics Inc., Plainview, N. Y.). Rates of light-dependent O_2 evolution before and after addition of ferricyanide were measured for each sonicated sample with a YSI model 53 oxygen monitor. The percentages of intact cells remaining following sonication were determined by direct cell counts with a hemocytometer.

Cytochemical Assay for Glycolate Dehydrogenase. Once harvested, *Chlamydomonas* cells were rinsed twice with 50 mM K-phosphate (pH 7.2) and resuspended in this buffer, using approximately 1 ml packed cells/25 ml final volume. Aliquots of this suspension were then sonicated at power setting 3 for 5 sec with the sonifier described above. The partially disrupted cells were incubated in a cytochemical reaction medium in the dark at 25 C for 20 min, or in other cases for 2 or 5 min. The standard medium, mixed immediately before use in the order listed, consisted of the following ingredients (final concentrations in parentheses): 2.5 ml 0.2 M K-phosphate, pH 7.2 (50 mM); 2 ml 25 mM Na-K-tartrate (5 mM); 2 ml 25 mM $CuSO_4$ (5 mM); 0.4 ml 50 mM K-ferricyanide (2 mM); 0.4 ml 0.5 M Na-glycolate (20 mM); and 2.7 ml H_2O. In other experiments, glycolate was replaced by 0.4 ml of either H_2O, 0.5 M D(−)-lactate, or 0.5 M L(+)-lactate. For studies on oxamate inhibition, samples were preincubated for 30 min in a 50 mM K-phosphate buffer, pH 7.2, containing either 20 mM or 100 mM Na-oxamate. They were then incubated for 5 or 20 min in the standard cytochemical reaction mixture also containing 20 mM or 100 mM oxamate.

Although most cytochemical incubations were carried out using unfixed samples, several types of prefixation were tested: (*a*) 4% formaldehyde in 50 mM K-phosphate, pH 7.2, for 10, 20, and 30 min at room temperature; (*b*) 1% purified glutaraldehyde (Polysciences, Inc.) in 50 mM K-phosphate, pH 7.2, for 10 min at 4 C; and (*c*) 4% formaldehyde-1% glutaraldehyde in 50 mM K-phosphate, pH 7.2, for 20 min at 4 C. These prefixed samples were then rinsed twice in buffer prior to cytochemical treatment.

Preparation for Electron Microscopy. Following cytochemical incubation, the cells were rinsed once with 50 mM K-phosphate, pH 7.2, embedded in 2% agar, and rinsed two more times. The samples were fixed in 2% glutaraldehyde (buffered with 50 mM K-phosphate, pH 7.2) for 60 min at room temperature, then rinsed for at least 60 min with four or five changes of 50 mM K-phosphate, pH 7.2. Postfixation was in 2% OsO_4, buffered at pH 7.2 with 50 mM K-phosphate, for 60 min at room temperature. This was followed by dehydration in acetone and infiltra-

[1] This research was supported by a Cottrell College Science Grant from the Research Corporation and a Faculty Grant from Mount Holyoke College.

tion with Spurr's low viscosity resin (14). Silver gray sections were cut with a Sorvall MT-2 ultramicrotome, poststained with 2% aqueous uranyl acetate for 10 min followed by lead citrate for 5 min, and examined with a Philips EM-300 electron microscope.

Spectrophotometric Assays for Glycolate Dehydrogenase. Cell-free extracts of *Chlamydomonas* were prepared by passing cell suspensions through a prechilled French pressure cell at 12,000 to 15,000 p.s.i. The homogenate was centrifuged at 40,000*g* for 45 min, and the resulting supernatant was used for spectrophotometric assays. The reaction mixture used to determine glycolate-ferricyanide oxidoreductase activity contained 40 mM Na-PPi, pH 8.5, 1 mM K-ferricyanide, algal sample, and 20 mM substrate (either glycolate, D-lactate, or L-lactate). The reaction was initiated by addition of substrate, and reduction of ferricyanide was monitored at 410 nm. Assays for glycolate-DCPIP[2] oxidoreductase activity were performed as previously described (6).

RESULTS AND DISCUSSION

When previously sonicated *Chlamydomonas* cells are incubated, prior to fixation, in a complete ferricyanide reaction medium containing glycolate, an electron dense product is deposited in mitochondria. The greatly enhanced electron opacity of these mitochondria (Fig. 1) is readily apparent when they are compared to organelles of algal cells fixed routinely and not treated cytochemically (Fig. 2). The dense mitochondrial product is confined to the compartment between the inner and outer membranes, and normally fills it (Fig. 3). Here the stain typically forms a continuous amorphous layer which at higher magnification may appear as tightly packed globules. Occasionally, reaction product within an individual profile is "patchy," with some regions of the cristae stained heavily and others not at all. In a few cases there is also a marked diversity in the degree of reactivity among neighboring mitochondrial profiles within the same cell.

The glycolate dependent deposition of reaction product is restricted to mitochondria. Figure 1, representative of the many algal profiles studied, illustrates that no specific staining reaction is associated with other membranous components such as the plasma membrane, nuclear envelope, or microbody membrane; the endoplasmic reticulum and Golgi apparatus also react negatively. Moreover, no specific reaction product is deposited either in the matrix of other organelles, including microbodies, or in the general cytoplasm. Most cellular constituents may be observed at one time or another to contain random flecks of stain; and many of the cells examined do have some deposit of electron dense material associated with the chloroplast envelope and occasionally the thylakoids. This staining is not dependent upon or augmented by the presence of glycolate in the incubation medium, and is judged to be nonspecific.

Controls designed to test the validity of the cytochemical procedure as a marker of glycolate dehydrogenase activity included incubation with other potential substrates, omission of substrate from the reaction medium, and use of an inhibitor. In algae incubated with the alternate substrate D-lactate (6, 11), mitochondria contain a highly electron dense deposit (Fig. 4) which is virtually indistinguishable in amount and distribution from that in cells treated with glycolate (Fig. 3). This reaction product, as in the case of glycolate, is strictly limited to the mitochondria. Incubation with L-lactate, on the other hand, yields at most only a slight deposition of reaction product in mitochondria and no specific reaction with other cellular components. One of the most intense reactions observed with L-lactate is shown in Figure 5; even this reaction is much reduced from that occurring with either glycolate or D-lactate, and the product is disposed irregularly in the form of discrete globules rather than as a dense layer filling the intracristal space. When substrate is omitted from the cytochemical medium, algal samples lack the dense mitochondrial reaction product characteristic of samples treated with glycolate or D-lactate. On occasion, the mitochondria do stain slightly (Fig. 6), but with little more intensity or regularity than is displayed by nonspecific deposits elsewhere in the cell.

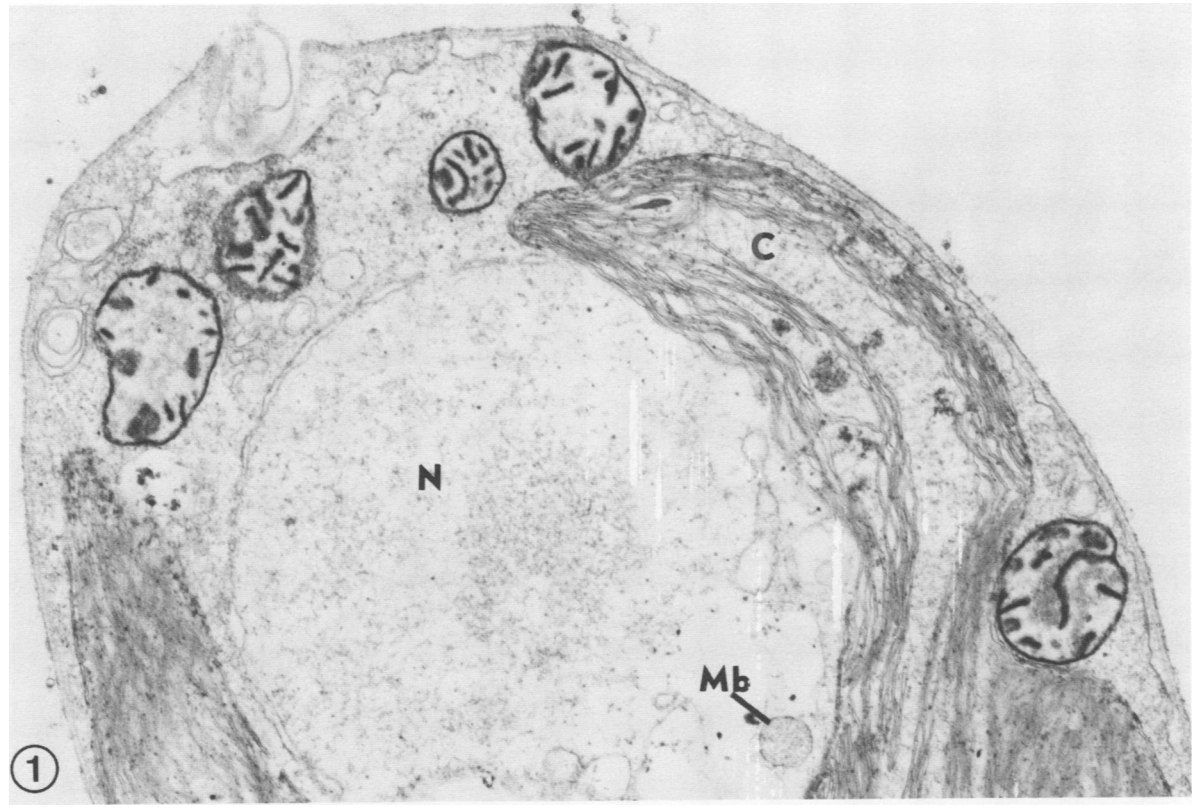
Oxamic acid, found in our laboratory to inhibit activity of glycolate dehydrogenase (unpublished results), has a definite inhibitory effect upon cytochemical staining of the mitochondria. When present in the standard incubation mixture at a final concentration of 20 mM, this compound lessens appreciably the amount of reaction product appearing in mitochondria; at a concentration of 100 mM, it almost completely inhibits the reaction (Fig. 7). Cyanide, an established inhibitor of glycolate dehydrogenase (6, 11), could not be used with the ferricyanide cytochemical procedure since it causes formation and precipitation of cupric ferrocyanide in the incubation medium.

The reduction of ferricyanide that is stimulated by glycolate and D-lactate and detectable in intact cells by cytochemical methods can be observed spectrophotometrically in cell-free preparations of *Chlamydomonas*. This activity is relatively difficult to measure, especially with this algal species, but low rates have been detected consistently. Glycolate and D-lactate serve equally well as substrates, while L-lactate gives rates of ferricyanide reduction approximately equal to controls which lack substrate. These results agree with the substrate specificities established for glycolate dehydrogenase using the DCPIP assay; in *Chlamydomonas* glycolate and D-lactate are oxidized readily, but L-lactate at only 10 to 25% of that rate (11). Both ferricyanide and DCPIP reduction with either 20 mM glycolate or 20 mM D-lactate as substrate are inhibited completely in the presence of 20 mM oxamate. Thus, spectrophotometric and cytochemical results with respect to substrates and inhibitor correlate well, and it appears reasonable to attribute the cytochemical reaction to the activity of glycolate dehydrogenase.

Several experimental variables were considered in designing the standard cytochemical procedure. First, it was necessary to devise a method that would facilitate the entry of cytochemical reagents into cells, since initial experiments yielded positive results in only a low percentage of the cells. The technique of mild sonication, used to advantage by Curtis *et al.* (5) to study photosynthetic activities in *Chlamydomonas,* was chosen. We varied the duration and intensity of sonication, and monitored the accompanying change in membrane permeability and degree of cell disruption. This was accomplished by (*a*) measuring the Hill activity, *i.e.* the ferricyanide induced evolution of O_2, of the sonicated samples, and (*b*) counting with a light microscope the percentage of intact cells that remained following sonication. The sonication conditions finally adopted for the cytochemical studies were those which caused visible breakage of approximately half the cells and resulted in the appearance of substantial Hill activity. When these sonicated cells were incubated in the cytochemical medium and later viewed with the electron microscope, all degrees of breakage could be observed. We found that generally only those cells showing a certain amount of disruption exhibited a positive reaction. The possibility was considered that sonication might alter the localization of the enzyme. However, when unsonicated samples were incubated in the cytochemical medium, the very low percentage of cells that did react exhibited a staining pattern identical with that of the sonicated ones.

Second, the fixation protocol was varied in attempts to improve the precision of the results. In all cases fixation of sonicated samples prior to the cytochemical incubation failed to yield a more precise enzyme localization, and it did not enhance the

[2] Abbreviation: DCPIP: 2,6-dichlorophenolindophenol.



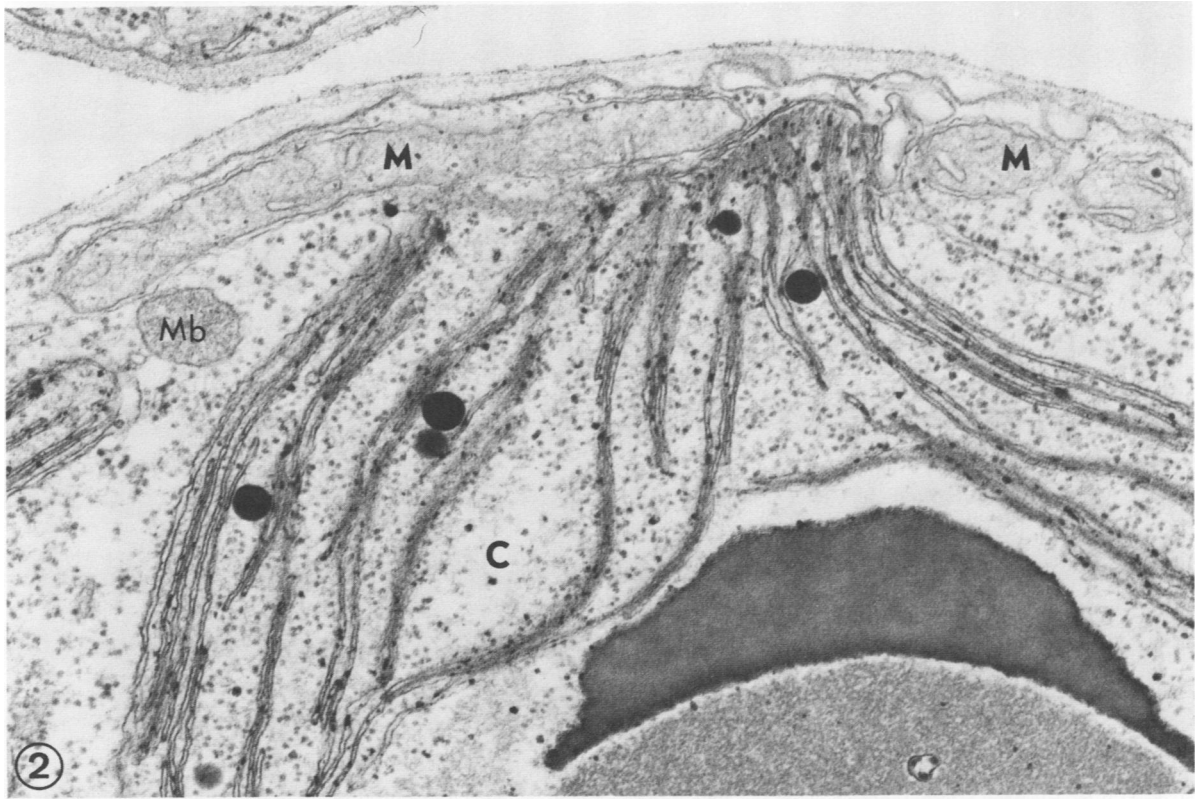


FIG. 1. Portion of a *Chlamydomonas* cell that was partially disrupted by mild sonication, then incubated for 20 min in a complete cytochemical medium containing glycolate. A dense reaction product has accumulated in the compartment between the inner and outer membranes of the mitochondria, which are somewhat swollen due to the sonication. Note that reaction product is absent from the mitochondrial matrix, as well as from the nucleus (N), chloroplast (C), and microbody (Mb). 30,000 X.

FIG. 2. Appearance of an intact (unsonicated) *Chlamydomonas* cell that was fixed routinely and not treated cytochemically. Mitochondria (M), one microbody (Mb), and part of a chloroplast (C) are visible in this electron micrograph. 52,000 X.

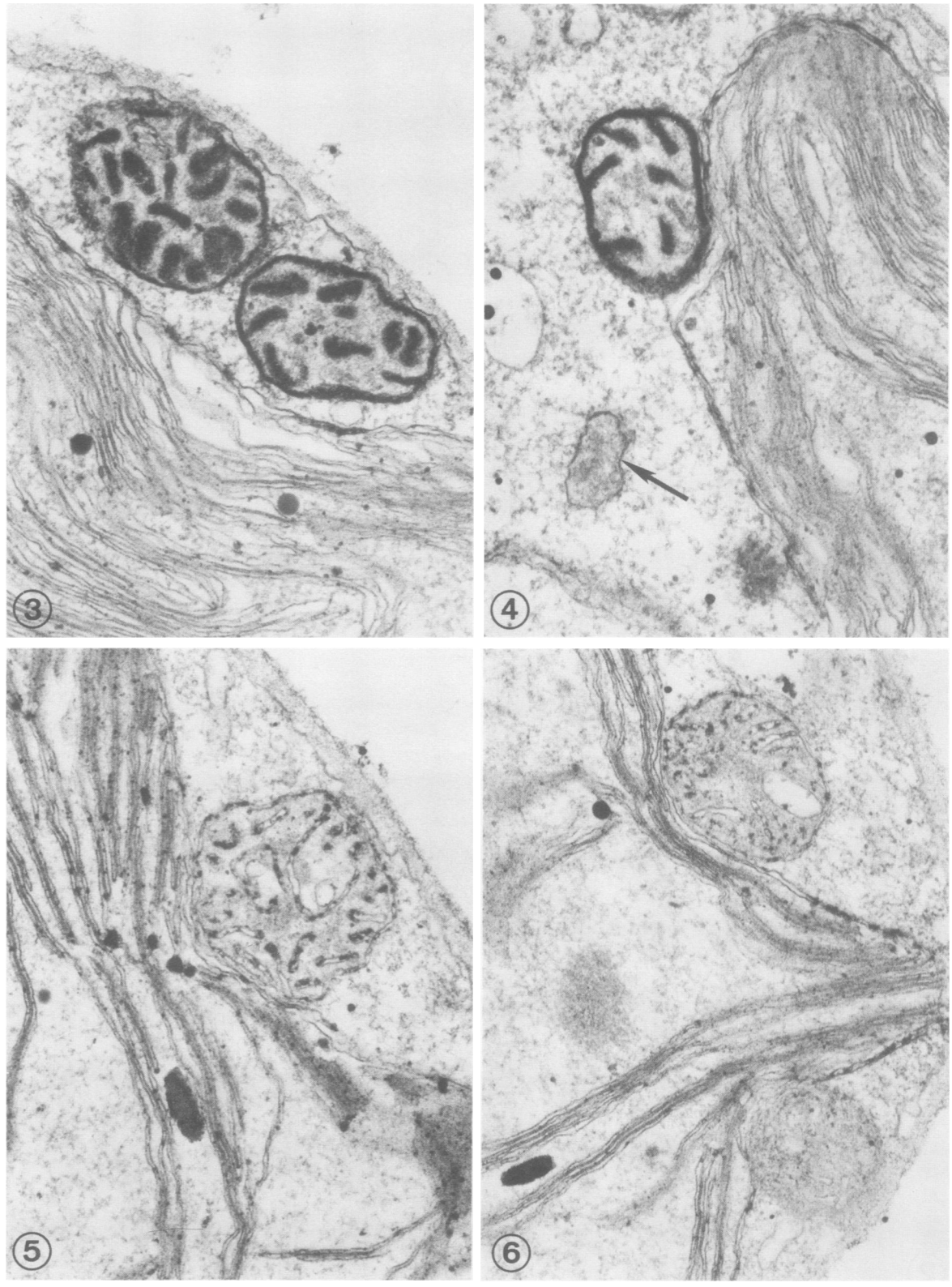
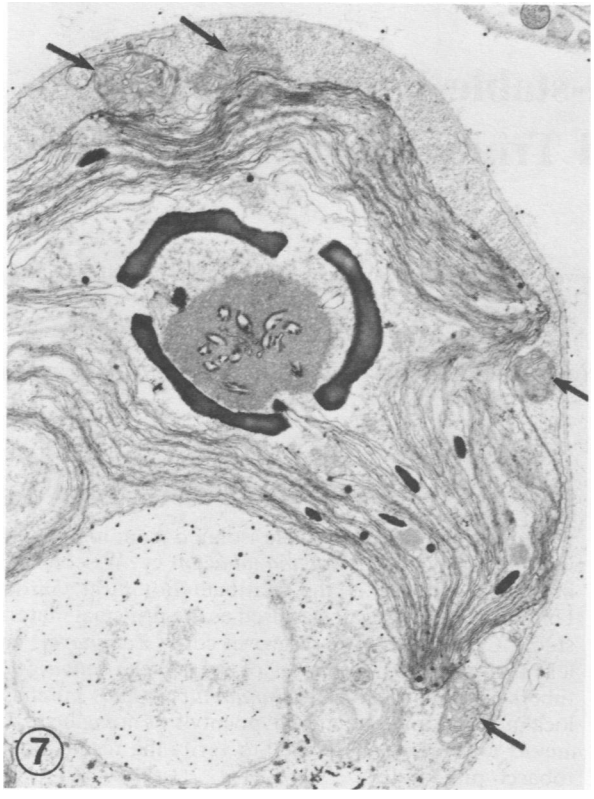


FIG. 3, 4, 5, AND 6. Electron micrographs showing the substrate specificity of the cytochemical reaction mediated by glycolate dehydrogenase. All incubations were carried out at 25 C for 20 min. Fig. 3: 20 mM glycolate. 63,000 X. Fig. 4: 20 mM D-lactate. Note that the microbody (arrow) lacks reaction product, as does the chloroplast. 64,000 X. Fig. 5: 20 mM L-lactate. 51,000 X. Fig. 6: no substrate. 48,000 X.

general fine structure of the cells. Algae fixed in 4% formaldehyde for 10 to 30 min prior to cytochemical incubation showed a consistent and specific deposit of stain within the mitochondria (Fig. 8), but contained an increased amount of nonspecific deposits in the chloroplasts compared to unfixed samples. Prefixation with glutaraldehyde, either alone or combined with formaldehyde, completely eliminated the appearance of reaction product in mitochondria. That glutaraldehyde destroyed enzyme



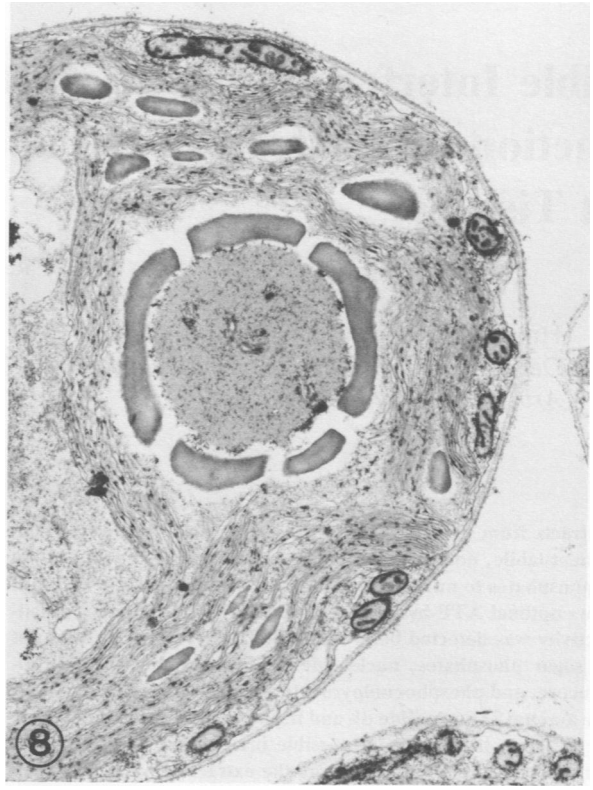


FIG. 7. Appearance of a partially disrupted cell incubated in the presence of 20 mM glycolate and 100 mM oxamate. The four mitochondria (arrows) visible in this section contain little or no reaction product. 15,000 X.

FIG. 8. Portion of a sonicated cell which was prefixed in 4% formaldehyde for 30 min, then incubated for 20 min in a cytochemical medium containing glycolate. The mitochondria are as reactive as those in unfixed cells, but the chloroplast shows an increased accumulation of nonspecific deposits. 21,000 X.

activity could be confirmed by spectrophotometric assays of homogenates of samples briefly exposed to this fixative; these preparations failed to show glycolate-dependent reduction of ferricyanide.

Finally, shorter incubation times and lower incubation temperatures were employed in the hope of confining the reaction product to one of the two mitochondrial membranes. However, in all cases the granules of cupric ferrocyanide still occupied the compartment between the inner and outer membranes. Thus, our cytochemical results by themselves leave open the possibility of an inner membrane, outer membrane, or even intermembrane localization of glycolate dehydrogenase. However, an inner membrane localization seems most likely since (*a*) cell fractionation data indicate that the enzyme is associated with a membrane (7), and (*b*) the presence of reaction product throughout the cristae, even following very brief incubations, speaks against an outer membrane localization.

The details of the electron transport system associated with glycolate dehydrogenase in green algae remain to be elucidated. In *Euglena* and three species of diatoms, the enzyme appears to work in conjunction with a mitochondrial electron transport chain that terminates in O_2 (4, 12), and in *Euglena* ATP is apparently a by-product (3). A more complete characterization of glycolate oxidation and other steps in the glycolate pathway of green algae is desirable. Conceivably it could help clarify, or at least place in a new perspective, some of the puzzling features of the photorespiratory process in higher plants.

LITERATURE CITED

1. BURKE, J. L. AND R. N. TRELEASE. 1975. Cytochemical demonstration of malate synthase and glycolate oxidase in microbodies of cucumber cotyledons. Plant Physiol. 56: 710-717.
2. CODD, G. A., J. M. LORD, AND M. J. MERRETT. 1969. The glycollate-oxidizing enzyme of algae. FEBS Lett. 5: 341-342.
3. COLLINS, N., R. H. BROWN, AND M. J. MERRETT. 1975. Oxidative phosphorylation during glycollate metabolism in mitochondria from phototrophic *Euglena gracilis*. Biochem. J. 150: 373-377.
4. COLLINS, N. AND M. J. MERRETT. 1975. The localization of glycollate-pathway enzymes in *Euglena*. Biochem. J. 148: 321-328.
5. CURTIS, V. A., J. J. BRAND, AND R. K. TOGASAKI. 1975. Partial reactions of photosynthesis in briefly sonicated *Chlamydomonas*. I. Cell breakage and electron transport activities. Plant Physiol. 55: 183-186.
6. FREDERICK, S. E., P. J. GRUBER, AND N. E. TOLBERT. 1973. The occurrence of glycolate dehydrogenase and glycolate oxidase in green plants. An evolutionary survey. Plant Physiol. 52: 318-323.
7. GRUBER, P. J., S. E. FREDERICK, AND N. E. TOLBERT. 1974. Enzymes related to lactate metabolism in green algae and lower land plants. Plant Physiol. 53: 167-170.
8. HAND, A. R. 1975. Ultrastructural localization of L-α-hydroxy acid oxidase in rat liver peroxisomes. Histochemistry 41: 195-206.
9. HUANG, A. H. C. AND H. BEEVERS. 1973. Localization of enzymes within microbodies. J. Cell Biol. 58: 379-389.
10. MERRETT, M. J. AND J. M. LORD. 1973. Glycollate formation and metabolism by algae. New Phytol. 72: 751-767.
11. NELSON, E. B. AND N. E. TOLBERT. 1970. Glycolate dehydrogenase in green algae. Arch. Biochem. Biophys. 141: 102-110.
12. PAUL, J. S., C. W. SULLIVAN, AND B. E. VOLCANI. 1975. Photorespiration in diatoms. Mitochondrial glycolate dehydrogenase in *Cylindrotheca fusiformis* and *Nitzschia alba*. Arch. Biochem. Biophys. 169: 152-159.
13. SHNITKA, T. K. AND A. M. SELIGMAN. 1971. Ultrastructural localization of enzymes. Annu. Rev. Biochem. 40: 375-396.
14. SPURR, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31-43.
15. STABENAU, H. 1974. Verteilung von Microbody-Enzymen aus *Chlamydomonas* in Dichtegradienten. Planta 118: 35-42.
16. STABENAU, H. 1974. Localization of enzymes of glycolate metabolism in the alga *Chlorogonium elongatum*. Plant Physiol. 54: 921-924.
17. STARR, R. C. 1971. The culture collection of algae at Indiana University—additions to the collection July 1966-July 1971. J. Phycol. 7: 350-362.
18. TOLBERT, N. E. 1971. Microbodies—peroxisomes and glyoxysomes. Annu. Rev. Plant Physiol. 22: 45-74.
19. TOLBERT, N. E. 1974. Photorespiration. *In:* W. D. P. Stewart, ed., Algal Physiology and Biochemistry. U. of California Press, Berkeley. pp. 474-504.
20. ZELITCH, I. 1971. Photosynthesis, Photorespiration, and Plant Productivity. Academic Press, New York.