

The Regulation of Glycolate Metabolism in Division Synchronized Cultures of *Euglena*¹

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ABSTRACT

Phosphoglycolate and phosphoglycerate phosphatases and glycolate dehydrogenase activities were determined in division synchronized *Euglena gracilis* strain Z cultures. Phosphoglycolate phosphatase activity remained nearly constant in the light but doubled in the dark, whereas phosphoglycerate phosphatase activity decreased by half in the light and increased 4-fold over the dark phase of the cycle. Glycolate dehydrogenase activity assayed by dye reduction increased over the light and remained constant during the dark phase, but when determined by the phenylhydrazine method, an assay dependent upon the presence of a natural hydrogen acceptor, activity decreased in the dark phase. The acceptor decayed in the dark in all cell-free extracts and 3-(3,4-dichlorophenyl)-1,1-dimethylurea inhibited light regeneration.

Enzyme activity regulated flow of carbon via the glycolate pathway; with cells at end of light phase and early dark phase, high levels of glycolate dehydrogenase activity were accompanied by an early and rapid labeling of glycerate. Degradation of early samples gave uniformly labeled glycolate and glycerate while phosphoglycerate was predominantly carboxyl labeled. At these stages in the division cycle, glycerate was formed from glycolate via the glycolate pathway, all the enzymes of the pathway being recorded in *Euglena*. In contrast to cells from end of dark phase and early light phase, times of maximum phosphoglycerate phosphatase activity, glycerate was predominantly carboxyl labeled as was phosphoglycerate, but glycolate was still uniformly labeled.

Glycolate excretion varied over the cycle, being maximal at end of dark phase, decreasing throughout the light phase until not detectable in early dark phase. When α -hydroxy-2-pyridinemethanesulfonate was used as a measure of glycolate biosynthesis, sufficient glycolate dehydrogenase was present to oxidize the glycolate produced at all stages over the cycle.

growth conditions, as is glycolate dehydrogenase activity in *Euglena* (4). The amount of ¹⁴C present in glycolate when division synchronized cultures of *Euglena* are allowed to fix ¹⁴CO₂ photosynthetically varies drastically over the cycle (3). This may result from variation in the capacity of the cells for the further metabolism of glycolate over the cycle. To investigate this we have determined whether activity of enzymes of glycolate metabolism regulates glycolate pool size in division synchronized cultures of *Euglena*.

MATERIALS AND METHODS

Axenic cultures of *E. gracilis* Klebs strain Z were grown, synchronized, and sampled as described before (3). Cell-free extracts were prepared by sonication (three 15 sec periods) of the cells suspended in 0.1 M potassium phosphate buffer (pH 8.2) and kept at 1 C. Cells were suspended in 0.1 M tris-acetate buffer, pH 7.0, containing a few milligrams of GSH for phosphatase assays. Cell debris was removed by centrifugation for 5 min at 5000g. Extracts were also prepared by passing a dense suspension in buffer at the required pH through an ice-cold French pressure cell at 20,000 psi. Protein was determined by the method of Lowry *et al.* (14).

Enzyme Assays. The following enzymes were measured as described in the references: glyoxylate-glutamate aminotransferase (11), serine hydroxymethyltransferase (17), D-glycerate dehydrogenase (13), glycerate kinase (8), phosphoglycolate phosphatase (1), 3-phosphoglycerate phosphatase (1), except that 3-phosphoglycerate was used as a substrate instead of phosphoglycolate. Conversion of serine into glycerate was demonstrated by incubating serine and pyruvate with enzyme in the presence of pyridoxal phosphate and NADH. The following were added in a volume of 3.0 ml: 200 μ moles potassium phosphate (pH 8.3); 20 μ moles pyruvate; 0.1 μ mole pyridoxal phosphate, 1 μ mole NADH and cell extract. Reaction was started by the addition of 20 μ moles serine (13). Preliminary assay showed the cell extract contained negligible lactate dehydrogenase activity.

Glycolate dehydrogenase was assayed by the DCPIP reduction method (20). Glycolate oxidation by cell extracts was also assayed by measuring an increase in absorbance at 324 nm consequent upon the formation of glyoxylate phenylhydrazine (12). This assay is dependent upon the presence in cell extracts of a natural hydrogen acceptor which decays in the dark. To study the effect of inhibitors on the regeneration of this acceptor, the cell extract was kept on ice in the dark until the ability to form glyoxylate phenylhydrazine had been lost (1 hr).

Glycolate Excretion. Cells were harvested at times over the cycle (3) and resuspended in 0.01 M potassium phosphate buffer, pH 7.0. The cell suspension in thin walled glass tubes

Glycolate excretion by algae (18) was originally explained by Hess and Tolbert (9) as being the result of the absence of glycolate oxidase in algae. Since then the presence of glycolate dehydrogenase, allowing a further metabolism of glycolate, has been demonstrated in several green algae (4, 12, 15, 20). It was further shown by Nelson and Tolbert (15) that the activity of this enzyme in *Chlamydomonas* is markedly affected by the

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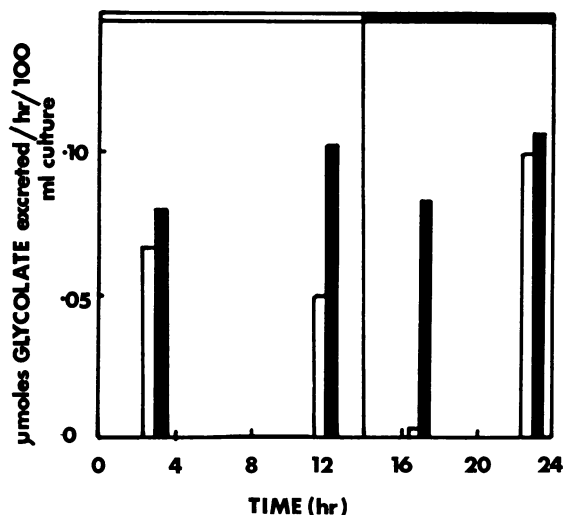


FIG. 1. Excretion of glycolate over the division cycle in synchronized *Euglena* cultures in the presence and absence of α -HPMS. □: No inhibitor; ■: 5×10^{-3} M α -HPMS.

at 25 C, illumination 10,000 lux, was gassed with air (7 liters/hr) and aliquots were removed at intervals into ice-cold centrifuge tubes. The cells were centrifuged; glycolate present in the supernatant fluid was estimated by the method of Calkins (2).

Degradation Procedures. 14 C-labeled glycerate, 3-phosphoglycerate,² and glycolate obtained in short term labeling experiments (3) were eluted from chromatograms, taken to dryness by vacuum distillation, and degraded by the procedure of Zelitch (19), except that ceric sulfate oxidations were allowed to proceed for 3 hr. 14 C-glycerate (Schwarz BioResearch Inc.) was purified by chromatography and used to check the efficiency of the degradation procedure. Eighty-five percent of 14 C was found in the carboxyl carbon, and 94% recovery was obtained. 14 C-glycolate (Radiochemical Centre, Amersham) was purified by chromatography and used to confirm the glycolate degradation procedure, in which 89% of the 14 C was present in the carboxyl carbon and 94% recovery was obtained. The recoveries from the degradation of samples were determined by the method of Zelitch (19) and confirmed by precipitation of the trapped 14 CO₂ using saturated barium chloride and gravimetric determination of the barium carbonate. The 14 CO₂ was released by acidification in a stoppered serum bottle, trapped in 0.2 ml of hydroxide of Hyamine (Packard Instrument Co.) contained in a detachable well, which was then transferred to a vial containing solvent-sciintillator and counted in a Packard Tri-Carb scintillation spectrometer.

RESULTS

Glycolate Excretion and Glycolate Dehydrogenase. Glycolate excretion was determined under conditions approximating those under which the cells were grown rather than conditions known to favor glycolate excretion. Excretion decreased towards the end of the light phase of 12-hr cells. This refers to time from beginning of light phase and not cell age. Early in the dark phase (17-hr cells) excretion was barely detectable. Throughout the rest of the dark phase the capacity for glycolate excretion increased until in 23-hr cells the rate was almost

² Abbreviations: α -HPMS: α -hydroxy-2-pyridinemethanesulfonate; P-glycolate: phosphoglycolate; P-glycerate: 3-phosphoglycerate; P-glycolate phosphatase: phosphoglycolate phosphatase; glycerate-3-phosphatase: 3-phosphoglycerate phosphatase.

double that for 12-hr cells (Fig. 1). Glycolate dehydrogenase activity increased 4-fold over the light phase of the division cycle and remained at this level throughout the dark phase (Fig. 2). Enzyme assay by phenylhydrazine formation gave lower levels of activity, the over-all rate probably being limited by the availability of hydrogen acceptor in these extracts (Fig. 2). Inhibition of glycolate oxidation by α -HPMS at stages over the division cycle did not reveal a doubling in the capacity of the culture for glycolate biosynthesis (Fig. 1); to determine accurately the peak of glycolate biosynthesis, the culture would have to be assayed at many more points over the division cycle. Similar levels of glycolate dehydrogenase activity were found in random air-grown cultures of *Euglena* as those in division synchronized cultures (Table I). Growth of cultures on 5% CO₂ decreased glycolate dehydrogenase activity.

Phosphoglycolate and Phosphoglycerate Phosphatases over the Cycle. Levels of P-glycolate phosphatase did not vary markedly in the light phase, after which activity doubled by the end of the following dark phase (Fig. 3). Assuming that glycolate is being formed by the hydrolysis of P-glycolate, changes in the level of this enzyme will not account for the changes in glycolate excretion (Table I), as activity is increasing when glycolate excretion is minimal (17 hr). Glycerate-3-

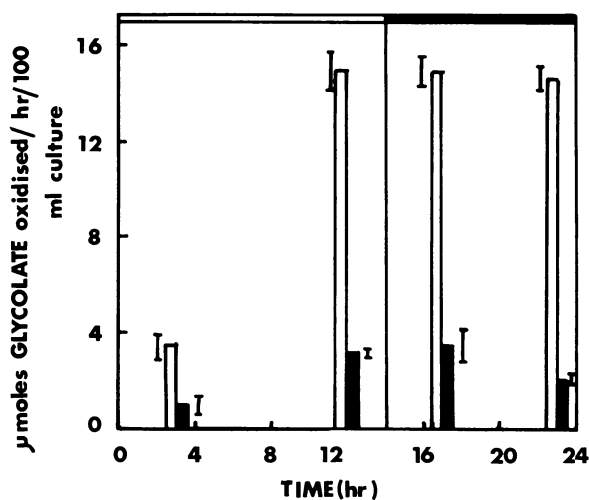


FIG. 2. Glycolate dehydrogenase activity over the division cycle in synchronized *Euglena* cultures. □: Assayed by dye reduction; ■: phenylhydrazine assay.

Table I. Glycolate Excretion and Glycolate Dehydrogenase Activity in Division Synchronized and Random *Euglena* Cultures

Culture Conditions	Excretion		Glycolate Dehydrogenase
	Control	5×10^{-3} M α -HPMS	
	$\mu\text{moles glycolate/hr} \cdot \text{mg protein}$		$\mu\text{moles glycolate oxidized/hr} \cdot \text{mg protein}$
Random 5% CO ₂ grown	0.50	0.48	0.16
Random air grown	0.16	0.40	2.50
Air-grown synchronous			
3-hr cells	0.54	0.64	3.20
12-hr cells	0.26	0.56	2.97
17-hr cells	0.01	0.42	2.68
23-hr cells	0.48	0.54	2.55

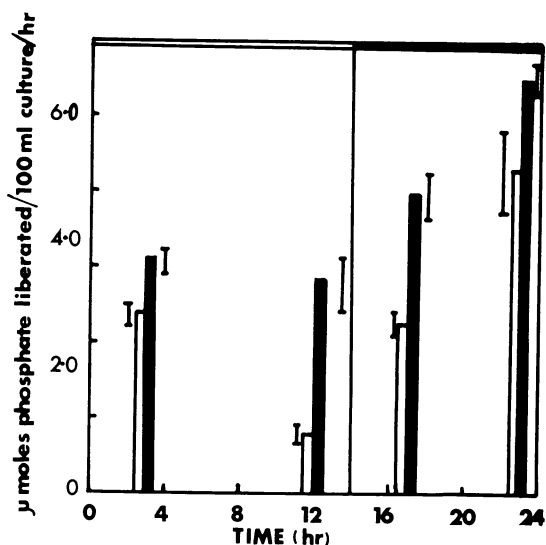


FIG. 3. Phosphoglycolate and phosphoglycerate phosphatase activities over the division cycle in synchronized *Euglena* cultures. □: Phosphoglycerate phosphatase; ■: phosphoglycolate phosphatase.

Table II. Percentage Distribution of ^{14}C in Products of Photosynthesis Formed by *Euglena* at Different Stages of the Division Cycle

Stage of Division Cycle	Time of Sampling	% Distribution of ^{14}C in Products					
		P-Glycerate		Glycerate		Glycolate	
		$\text{C}_1(-\text{COOH})$	$\text{C}_2 + \text{C}_3$	$\text{C}_1(-\text{COOH})$	$\text{C}_2 + \text{C}_3$	$\text{C}_1(-\text{COOH})$	C_2
3 hr	7 sec	96.6	3.4	64.5	35.5	47.7	52.3
	600	SD ¹	SD	34.8	65.2	SD	SD
	1200	50.8	49.2	SD	SD	SD	SD
12 hr	7	96.5	3.5	34.5	65.5	NDC ²	NDC
	120	SD	SD	SD	SD	44.1	55.9
	1200	41.4	59.6	35.9	64.1	SD	SD
17 hr	7	SD	SD	36.2	63.8	NDC	NDC
	15	93.5	6.5	SD	SD	NDC	NDC
	30	SD	SD	SD	SD	46.0	54.0
	600	SD	SD	35.2	64.8	SD	SD
	1200	40.4	59.6	SD	SD	48.6	51.4
23 hr	7	84.7	15.3	64.7	35.3	SD	SD
	1200	34.8	65.2	36.4	63.6	46.6	53.4

¹ SD: sample not degraded.

² NDC: not detected on chromatogram.

phosphatase activity varied over the cycle in a different manner than P-glycolate phosphatase activity, decaying in the light phase, followed by a 4-fold increase over the dark phase of the cycle (Fig. 3). Glycerate-3-phosphatase activity is minimal (12-hr stage) when maximum levels of glycolate dehydrogenase activity were observed. Variation in glycerate-3-phosphatase activity suggested that at some stages glycerate may be formed from P-glycerate while at other times it is formed by another pathway. When glycerate was degraded from 3-hr- and 23-hr cells (greatest PGA phosphatase activity), 64% of the ^{14}C was in the C-1 position, whereas with 12-hr and 17-hr cells the

glycerate formed was uniformly labeled in the early samples (Table II).

Glycolate Pathway in *Euglena*. The enzymes of the glycolate pathway and the operation of this pathway in higher plants are both well established (15). Recently Lord and Merrett (13) have shown the enzymes of this pathway to be present in *Chlorella*, and activities are great enough to account for the observed rate of incorporation of exogenous glycolate into cellular constituents. If photosynthetically formed glycolate is to be further metabolized in *Euglena*, besides glycolate dehydrogenase the other enzymes of the pathway must be present. The specific activities of enzymes catalyzing the metabolic sequence, glycolate \rightarrow glyoxylate \rightarrow glycine \rightarrow serine \rightarrow hydroxy-pyruvate \rightarrow glycerate \rightarrow P-glycerate are given in Table III. Glycerate kinase gave the lowest specific activity, probably as a result of phosphatase activity in the extracts. The specific activities of other enzymes, not previously recorded in *Euglena*, was greater than for *Chlorella* (13).

Effect of Light on Glycolate Dehydrogenase Activity in Cell-free Extracts. When glycolate dehydrogenase activity was assayed by the dye reduction method over the dark phase of the cycle, no reduction in activity was recorded compared with a reduction in activity when assayed by the phenylhydrazine method (Fig. 2). This suggested that the hydrogen acceptor present in cell-free extracts decreased in darkness. To test this, extracts were stored in the dark for 1 hr and then assayed. Throughout the assay the cuvette was kept in the dark except when placed in the spectrophotometer light beams for the necessary time to obtain a reading. Activity was very low but was restored upon illumination (Table IV). Assay of the enzyme by the dye reduction method was unaffected by dark incubation or illumination of the cell-free extract (Table IV). The restoration of activity upon illumination of dark stored extracts, when assayed by a method dependent upon the natural hydrogen acceptor, shows that light is involved in the generation of the acceptor. This was further investigated by determining the effect of inhibitors and uncouplers of photosynthetic electron transport upon the light-generated restoration of ac-

Table III. Specific Activities of Enzymes Demonstrated in *Euglena* Extracts

Enzyme	Specific Activity
	$\frac{\mu\text{mole substrate transformed/hr}\cdot\text{mg protein}}{\text{protein}}$
Glycolate dehydrogenase	2.85
Glyoxylate-glutamate aminotransferase	8.82
Serine hydroxymethyltransferase	0.40
Conversion of serine into glycerate	36.10
Glycerate dehydrogenase	87.80
Glycerate kinase	0.14
Glycolate-P	2.71
Glycerate-3-P	1.39

Table IV. Effect of Dark Incubation and Subsequent Illumination upon Glycolate Dehydrogenase Activity

Assay	Specific Activity	
	Dark stored extract	Extract upon illumination
	$\frac{\mu\text{moles glyoxylate formed/mg protein}\cdot\text{hr}}{\text{hr}}$	
Dye reduction	2.71	2.75
Phenylhydrazine formation	0.09	0.35

tivity. The addition of DCMU gave the greatest inhibition of the restoration of activity (78%). The addition of quinacrine, which inhibits photosynthetic phosphorylation in isolated chloroplasts (5), caused a slight inhibition of activity (24%), while phlorizin, an energy transfer inhibitor (10), had no effect.

DISCUSSION

Levels of glycolate excretion in random cultures of *Euglena* (Table I) varied widely depending on the growth conditions, being inversely related to the activity of glycolate dehydrogenase. As with *Chlamydomonas reinhardtii* (15), lower glycolate dehydrogenase activity was recorded with cells grown on CO₂ compared with cells grown on air and this must be a factor in the greater glycolate excretion observed with cells grown on CO₂. Besides affecting the level of glycolate dehydrogenase in the cells, growth conditions may also affect the capacity of the cells for glycolate biosynthesis. If it is assumed that α -HPMS does not alter the rate of glycolate biosynthesis in the cells but only inhibits glycolate oxidation by glycolate dehydrogenase, then the rate of glycolate excretion in the presence of this inhibitor may be taken as a record of glycolate biosynthesis. The possibility that α -HPMS enhances the rate of glycolate biosynthesis seems unlikely, since with very low glycolate dehydrogenase levels, rates of glycolate excretion are similar in the presence and absence of α -HPMS (Table I). With random cultures of *Euglena* grown on air, glycolate dehydrogenase activity is much higher than the total rate of glycolate biosynthesis, yet some excretion of glycolate still takes place.

Glycolate excretion varies over the cycle with division synchronized *Euglena* and, as excretion was not forced, similar rates of excretion may operate in a natural environment. When glycolate dehydrogenase activity is determined over the cycle by the dye reduction method there is always sufficient enzyme to oxidize the glycolate being produced; even so, with 3-hr and 23-hr cells excretion still results (Fig. 1). When activity is determined by the phenylhydrazine method, it is minimal with 3-hr and 23-hr cells when excretion occurs, so at certain stages in the cycle glycolate oxidation may be limited by the availability of hydrogen acceptor. When rates of glycolate oxidation are high this corresponds to a peak period of biosynthesis in the cells, particularly DNA (6). With synchronous cultures of *Ankistrodesmus*, it has been found that glycolate excretion is inversely related to protein and nucleic acid synthesis (7).

In division synchronized *Euglena* cultures, activity of enzymes and labeling pattern of intermediates show that at some stages in the cycle the glycolate pathway is fully operative while at other times flow of carbon via this pathway is limited. Glycolate dehydrogenase, P-glycolate phosphatase, and glycerate-3-phosphatase show different patterns over the cycle which are related to their function in the cell. With 12-hr and 17-hr cells, the glycolate pathway is operative and label rapidly appears in glycerate; the glycerate is uniformly labeled (Table II), so it is probable that glycerate is derived from uniformly labeled glycolate via the glycolate pathway, all the enzymes of which are present (Table III). Kinetic experiments also support

the operation of this pathway; with cells at this stage, little ¹⁴C accumulates in glycolate but there is a rapid increase in label in glycine, serine, and glycerate (3). Most likely carboxyl-labeled glycerate results from phosphatase activity. Glycerate-3-phosphatase shows greatest activity in 23-hr and 3-hr cells when 64% of the ¹⁴C is in the C-1 position of glycerate, and P-glycerate is also predominantly carboxyl-labeled. Although these results were obtained with cultures synchronized by a light-dark regime, it is probable that the same sequence of biochemical events occurs during growth and division of algae in light; in continuous light, cell division and development are random, so variation in the amount of carbon being metabolized by the glycolate pathway would not be detected.

LITERATURE CITED

- ANDERSON, D. E. AND N. E. TOLBERT. 1966. Phosphoglycolate phosphatase. In: W. A. Wood, ed., *Methods in Enzymology*, Vol. 9. Academic Press, New York, pp. 646-650.
- CALKINS, V. P. 1943. Microdetermination of glycolic and oxalic acid. *Ind. Eng. Chem. Anal. Ed.* 15: 762-763.
- CODD, G. A. AND M. J. MERRETT. 1971. Photosynthetic products of division synchronized cultures of *Euglena*. *Plant Physiol.* 47: 635-639.
- CODD, G. A., J. M. LORD, AND M. J. MERRETT. 1969. The glycolate oxidizing enzyme of algae. *FEBS. Lett.* 5: 341-342.
- DILLEY, R. A. AND L. P. VERNON. 1964. Changes in light-absorption and light scattering properties of spinach chloroplasts upon illumination: relationship to photophosphorylation. *Biochemistry* 3: 817-824.
- EDMUNDS, L. N. 1965. Studies on synchronously dividing cultures of *Euglena gracilis* Klebs (strain Z). II. Patterns of biosynthesis during the cell cycle. *J. Cell. Comp. Physiol.* 66: 159-182.
- GIMMLER, H., W. ULLRICH, J. DOMANSKI-KADEN, AND W. URBACH. 1969. Excretion of glycolate during synchronous culture of *Ankistrodesmus braunii* in the presence of disalicylidene-propanediamine or hydroxypyridinemethanesulfonate. *Plant Cell Physiol.* 10: 103-112.
- HATCH, M. D. AND C. R. SLACK. 1969. NADP-specific malate dehydrogenase and glycerate kinase in leaves and evidence for their location in chloroplasts. *Biochem. Biophys. Res. Comm.* 34: 589-593.
- HESS, J. L. AND N. E. TOLBERT. 1967. Glycolate pathway in algae. *Plant Physiol.* 42: 371-379.
- IZAWA, S., G. D. WINGET, AND N. E. GOOD. 1966. Phlorizin, a specific inhibitor of photophosphorylation and phosphorylation-coupled electron transport of chloroplasts. *Biochem. Biophys. Res. Comm.* 22: 223-226.
- KISAKI, T. AND N. E. TOLBERT. 1969. Glycolate and glyoxylate metabolism by isolated peroxisomes or chloroplasts. *Plant Physiol.* 44: 242-250.
- LORD, J. M. AND M. J. MERRETT. 1968. Glycolate oxidase in *Chlorella pyrenoidosa*. *Biochim. Biophys. Acta* 159: 543-544.
- LORD, J. M. AND M. J. MERRETT. 1970. The pathway of glycolate utilization in *Chlorella pyrenoidosa*. *Biochem. J.* 117: 929-937.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurements by the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- NELSON, E. B. AND N. E. TOLBERT. 1969. The regulation of glycolate metabolism in *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta* 184: 263-270.
- RABSON, R., N. E. TOLBERT, AND P. C. KEARNEY. 1962. Formation of serine and glyceric acid by the glycolate pathway. *Arch. Biochem. Biophys.* 98: 154-163.
- TAYLOR, R. T. AND H. WEISSBACH. 1965. Radioactive assay for serine transhydroxymethylase. *Anal. Biochem.* 13: 80-84.
- TOLBERT, N. E. AND L. P. ZILL. 1956. Excretion of glycolic acid by algae during photosynthesis. *J. Biol. Chem.* 222: 895-906.
- ZELITCH, I. 1965. The relation of glycolic acid synthesis to the primary photosynthetic carboxylation reaction in leaves. *J. Biol. Chem.* 240: 1869-1876.
- ZELITCH, I. AND P. R. DAY. 1968. Glycolate oxidase activity in algae. *Plant Physiol.* 43: 289-291.