Glycolate Stimulation of Oxygen Evolution During Photosynthesis[1]

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Received July 18, 1967.

Abstract. **Glycolate and glyoxylate stimulated 100 % to 300 % the rate of oxygen evolution by *Scenedesmus* in the light in the absence of added carbon dioxide. This stimulation occurred either aerobically or anaerobically, and was sensitive to CMU. Aerobic dark respiration was stimulated 25 % to 100 % by glycolate. This phenomenon was best demonstrated with synchronized *Scenedesmus* at the stage of cell division. For glycolate stimulation of oxygen evolution, a dark preincubation of 1 minute or less was necessary. In comparative test with other compounds of metabolism and photosynthesis, the stimulation of oxygen evolution was greatest by glycolate and glyoxylate. In a proposed scheme glyoxylate serves as a terminal hydrogen acceptor from NADPH produced by photosynthesis, and it thereby stimulates oxygen evolution when carbon dioxide is not available. Transformation of glycolate to glyoxylate in these cells would have to occur in the absence of oxygen.**

Similarities between photosynthetic electron transport and glycolate biosynthesis and metabolism have suggested that the 2 processes may somehow be directly related (18). Manganese deficiency preferentially inhibits oxygen evolution by algae (10) as well as glycolate formation by algae (8, 17). Glycolate formation occurs only in the light (9, 18). This is in contrast to some compounds of the photosynthetic carbon cycle which are formed in the dark for short periods of time after illumination ceases (14). Formation of large amounts of glycolate during photosynthesis in a high light intensity and high oxygen atmosphere, suggests that glycolate formation may be necessary to maintain in a reduced state some autooxidizable component of the chloroplast.

It has not been possible to evaluate directly the effect of added glycolate on photosynthesis or O_2 evolution with higher plants because of the immense activity of glycolate oxidase. More recently we have realized that respiration of random cultures of several algae was not stimulated by glycolate and that the form of glycolate oxidase previously described for higher plants was not detectable in the green unicellular algae which were examined (8). Nevertheless algae in the light biosynthesize and excrete large amounts of glycolate (9, 19), which makes it difficult to study the effect of added glycolate. Alterations upon addition of glycolate could best be tested when both the photosynthetic production of glycolate by the algae would be minimum, and when the algae could absorb the compound. These conditions appear optimum with synchronized cultures at the time of cell division or immediately afterwards (4, 9). In this report we describe the effect from addition of glycolate to these dividing cells.

Experimental Procedures

Algae. Stock cultures were obtained from the University of Indiana collection. *Ankistrodesmus braunii* (Naeg) Collins, number 245, and *Scenedesmus obliquus* (Gaffron D-3), number 393 were grown in 1 L batches of Medium V of Norris *et al.* (13) in 2.8 L low form Fernbach flasks. Cultures were aerated with 0.1 to 0.2 % CO_2 in air and shaken at 60 cycles per min. Illumination of 1100 ft-c was obtained from Sylvania Gro-lux lamps and the temperature was maintained at 30° ± 1°. The algal life cycle was maintained at 16 hr light and 8 hr dark. After each dark period the *Scenedesmus* were diluted 1 to 8 and the *Ankistrodesmus* 1 to 4. For experiments algae were harvested by centrifugation at 1000*g* for 5 to 10 min, washed once with a small amount of distilled water and recentrifuged in graduated test tubes to measure cell volume. Usually a 4 % v/v suspension of algae in 0.02 M phosphate buffer (pH 6.5) was prepared which gave a final suspension in the assay of 2.7 % cells. All cultures were examined microscopically before running an experiment.

Measurement of O_2 Exchange. Measurements of O_2 exchange at 27° were made on a Gilson Differential Respirometer, photosynthesis model with photoflood lamps which delivered approximately 1000 ft-c to the algae. Flask contents were 2 ml of the cell suspension and 0.5 ml of H_2O or other additions in the main part of the flask and 0.5 ml of 0.02 M substrate in the side arm. In most experiments CO_2

[1] Supported in part by NSF grant GB 4154 and published as journal article No. 4127 of the Michigan Agricultural Experiment Station.

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was removed by 20 % KOH with a filter paper wick in the center well. When $NaHCO_3$ or CO_2 was used, the KOH in the center well was omitted. O_2 exchange was also measured polarographically using a Clark oxygen electrode. A 1 % suspension of cells in phosphate buffer was stirred, and illuminated with approximately 1000 ft-c from 2 photoflood lights. Two cells with electrodes were used simultaneously; 1 recorded the blank and the other contained algae with glycolate. Results obtained with the polarograph were quantitatively the same as with the respirometer. In anaerobic experiments the flasks were flushed with prepurified N_2 for 20 min in the dark and then the manometers were closed and glycolate was added from the side arm. Dark incubation was continued for 5 additional min while the manometer pressure stabilized, after which time the lights were turned on and O_2 evolution measured. Light intensity was varied by neutral density filters of sheets of exposed film whose absorption spectra was constant between 400 and 700 nm.

Results

Stimulation of O_2 Evolution. Addition of glycolate (3.3 μM final concentration) to a 2.7 % suspension of *Scenedesmus* (harvested immediately after cell division) in 20 mM phosphate at pH 6.5 stimulated the rate of dark respiration 25 to 100 % as measured by oxygen uptake (fig 1). Upon the addition of 1000 ft-c of white light, a 100 to 300 % stimulation of oxygen evolution occurred as compared to controls without substrate. The length of time that this stimulation continued varied among the algal preparations. It usually lasted 10 to 15 min after which the stimulated rate decreased to about 25 % above the controls. With excess glycolate (as provided by 3.3 μM), the phenomenon was reproducible upon repeating the dark period followed again by light (fig 1). In fact, during the second and third dark-light cycle the stimulation of O_2 evolution in the light with glycolate became more pronounced with those preparations in which a weak initial stimulation had been observed.

A period of dark respiration before photosynthesis was necessary in order to observe the light dependent stimulation of oxygen evolution. When the length of the dark period was varied between 1 min and 30 min the same amount of stimulation was observed. Thus only a short dark period of 1 min or less was necessary to obtain maximum stimulation. These data indicate a rapid saturation of the intermediate(s) involved in this phenomena.

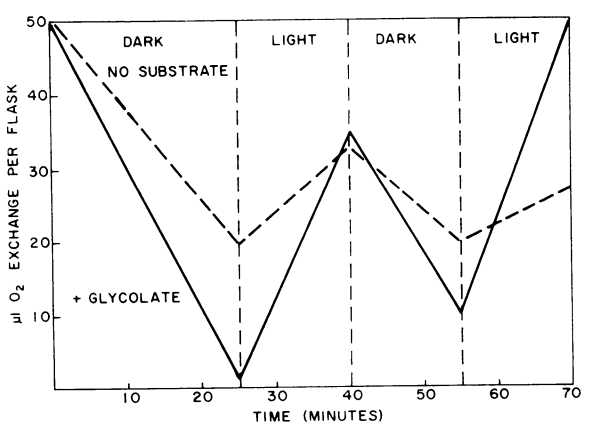

FIG. 1. Effect of glycolate on O_2 evolution in light and O_2 uptake in the dark by *Scenedesmus*. Each flask contained 3 ml of a 2.7 % suspension of synchronized cells at the stage of cell division in phosphate buffer at pH 6.5 and ± 3.3 × 10^{-3} M glycolate.

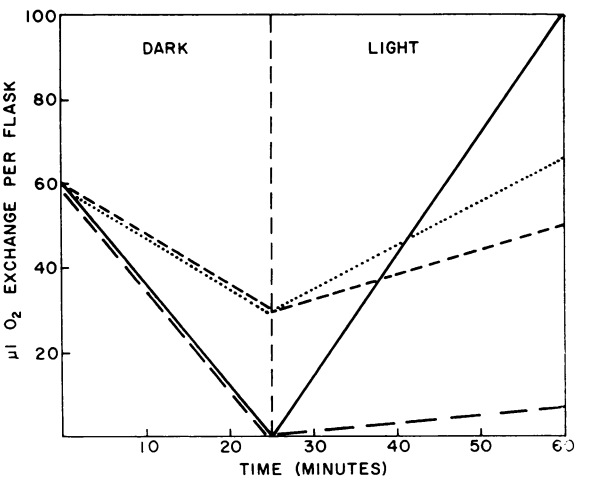

FIG. 2. CMU inhibition of glycolate stimulation of oxygen evolution in the light. Experimental conditions were similar to those in figure 1 except that 6 × 10^{-6} M CMU was added. ——— glycolate; — — — glycolate + CMU, ... control; - - - - - control + CMU.

Effect of Light Intensity and CMU. In order to ascertain whether the increased oxygen evolution was due to enhanced photosynthetic electron flow the effect of the inhibitor of photosynthesis, CMU (3-*p*-chlorophenyl-1,1,dimethylurea), at 6 × 10^{-6} M final concentration was tested. As shown in figure 2, the stimulation of oxygen evolution by glycolate was completely inhibited by CMU. No inhibition of the glycolate stimulated dark respiration by CMU at this concentration was observed. These data indicate that the observed stimulation in the light was due to enhanced photosynthetic electron flow.

With increasing light intensity there was an increase in the rate of oxygen evolution by the algae with or without glycolate (table I). The manometric values as given are not corrected for respiration. Values in parenthesis are the sum of the measured dark respiration and light dependent oxygen evolution and indicate the maximum possible rate of O_2 evolution in the light. By either set of values the rate of oxygen evolution increased with increasing light intensity, but the stimulation of oxygen evolu-

Table I. *Effect of Light Intensity on Stimulation of O_2 Evolution*

Experimental conditions are outlined in Experimental Procedure and figure 1. Values are manometric readings and are the average of 2 flasks. This experiment represents the maximum rates observed in 4 trials with different batches of algae. Values in parenthesis are calculated as the sum of the dark respiration plus the rate in the light.

Light	No glycolate	With glycolate	Glycolate stimulation
ft-c	*μl O_2 exchange/10 min*		
0	—24	—35	—11
380	+14 (38)	+35 (70)	+21 (32)
750	+29 (53)	+54 (89)	+25 (36)

tion attributable to glycolate was nearly constant. This observation that the stimulation of oxygen evolution by glycolate was not related to light intensity between 380 and 750 ft-c indicates that either the phenomena was saturated at a low light intensity or that the glycolate effect was a dark reaction.

Anaerobic Conditions. When the cells were made anaerobic by dark incubation under nitrogen, the stimulation of oxygen evolution in the light by glycolate still occurred (fig 3). Because the cells were anaerobic for 20 min before adding the glycolate and turning on the lights, the stimulation was probably not linked to aerobic respiration, such as oxidation of glycolate to glyoxylate by a form of glycolate oxidase requiring oxygen.

Bicarbonate Effect. Addition of 1 mM $NaHCO_3$ to the *Scenedesmus* cells stimulated O_2 evolution in the light to a maximum value. Similar results were obtained at pH 6.5 in phosphate buffer with an atmosphere of approximately 0.6 % CO_2 as generated by a $NaHCO_3$-$NaCO_3$-arsenite mixture in side arms of the Warburg vessels (7, 21). Stimulation of O_2 evolution by glycolate as described in the previous

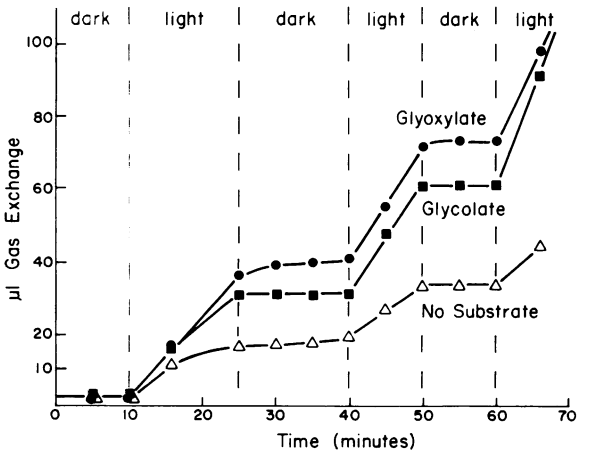

FIG. 3. Stimulation of O_2 evolution under anaerobic conditions. Experimental conditions were similar to those in figure 1, except that the flasks were gassed with N_2 for 20 min before adding glycolate.

sections was generally about 50 to 66 % of the maximum rate obtained with $NaHCO_3$. The stimulation of O_2 evolution by $NaHCO_3$ and by glycolate were not additive. As will be discussed later, the glycolate stimulation was probably not due to its prior conversion to CO_2. In the presence of sufficient CO_2, adequate glycolate production by the cells from photosynthesis may occur, even though excess glycolate is not excreted. Thus no further stimulation from added glycolate would occur.

Dark Respiration. Glycolate stimulated the rate of dark respiration with *Scenedesmus* if the cells were harvested during the stage of cell division. Little or no stimulation was observed when cells were collected during the other stages. As shown in figure 1, respiration was stimulated 25 to 100 %. To our knowledge, this is the first report of glycolate stimulating dark respiration by unicellular green algae.

Table II. *Comparison of Different Substrates for Stimulation of O_2 Evolution and Respiration*

Data are expressed a 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	O_2 evolution in light	O_2 uptake in dark
	Activity ratio as substrate/glycolate	
Organic Acids		
Glyoxylate	1.0	1.0
Acetate	0.5	2.5
P-Glycolate	<0.1	<0.1
Lactate	0.2	1.0–1.2
Formate	<0.1	0.5–1.0
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

Substrate Specificity and Concentration. Glycolate and glyoxylate were equally effective in stimulating oxygen evolution and respiration (table II). In this paper we have referred to the phenomena as being caused by glycolate. While the actual effector might be glycolate or glyoxylate the relatively large amounts of glycolate formed in photosynthesis (18) leads us to consider glycolate of primary importance. Further glyoxylate could have been reduced to glycolate, since the algae contain both NADH and NADPH glyoxylate reductase (2). No other organic substrate has to this date been tested which stimulated oxygen evolution in the light to the same extent as did glycolate and glyoxylate. It was presumed that other compounds entered the cells since they stimulated respiration. Acetate and glucose

stimulated dark respiration better than glycolate or glyoxylate, but were less effective than glycolate in stimulating oxygen evolution. These data indicate that the glycolate effect was not one of general respiration where increased carbon dioxide production caused enhanced oxygen evolution rates.

Organic substrates biochemically related to glycolate such as P-glycolate, glycine, serine, glycerate and lactate were also less active (table II). Though not exhaustive this list suggests that the effect of glycolate or glyoxylate is rather specific for stimulating oxygen evolution. It is of interest that 3-P-glycerate was the second most active effector of stimulated oxygen evolution. Walker and Hill (20) have reported that 3-P-glycerate reduces the lag period for carbon dioxide fixation and oxygen evolution by isolated chloroplasts. In their case 3-P-glycerate probably resulted in the build up of photosynthetic carbon cycle intermediates which had been lost in the isolation of the plastids.

Stimulation of O_2 evolution by a suspension of 2.7 % *Scenedesmus* cells was maximum between 10^{-2} and 10^{-3} M final concentration of glycolate. At 10^{-4} and 10^{-1} M stimulation was reduced 10 to 25 %, and at 1 M glycolate, both dark respiration and O_2 evolution in the light were severely inhibited even in the presence of $NaHCO_3$.

Condition of the Algae. In order to observe good stimulation of oxygen evolution and respiration by glycolate it was necessary to use cells at the stage of division or immediately afterwards, from synchronized cultures of *Scenedesmus* or *Ankistrodesmus*. Generally cell division occurred 2 to 6 hr after the beginning of the dark period. While some stimulation of oxygen evolution has been observed in a few cases at other stages of cell growth, only the stage of division or immediately following gave consistent and reproducible results. Successful experiments have not been achieved with *Chlorella*, which were not fully synchronized.

It appears that at the stage of cell division and immediately afterwards there is something unique about the algae that results in the measurement of glycolate stimulation of oxygen evolution and respiration. Other observations indicate that metabolic changes relating to glycolate metabolism and photosynthesis occur during the stage of cell division. Chang (4) found glycolate synthesis at this stage to be minimum when compared to the entire life cycle of both *Scenedesmus* and *Ankistrodesmus*. Exogenously added glycolate is assimilated primarily at this stage of the life cycle (Nelson and Tolbert unpublished). Senger and Bishop (16) have observed that, in synchronous cultures of *Scenedesmus*, photosystem II drops in efficiency during the stage of cell division.

Discussion

Carbon dioxide acts as the natural electron acceptor for the NADPH produced by photosystem I. As mentioned its presence in the medium greatly enhanced oxygen evolution and completely eliminated the glycolate stimulation. Nitrate has also been shown to accept electrons from photosynthesis and to stimulate oxygen evolution in the absence of carbon dioxide (5). Glyoxylate has been shown to enhance electron flow in isolated spinach chloroplasts by serving as an electron acceptor for NADPH (1), as catalyzed by glyoxylate reductase. With glycolate and glycolate oxidase a cyclic process could occur that would stimulate electron flow in the absence of CO_2. This Mehler type of reaction (11), as shown in figure 4 with O_2 as terminal electron acceptor, would not enhance net oxygen evolution, however, since glycolate oxidase would utilize an equivalent amount of oxygen for each glyoxalate produced. A cyclic process such as this has been suggested for *Chorella* by Butt and Peel (3) and for higher plants by Asada *et al.* (1) and Zelitch and Walker (22).

While the explanation given in the previous paragraph could account for the data observed for glyoxylate with the algae which have no glycolate oxidase, it will not account for the data observed with glycolate. The observation, that the stimulation of oxygen evolution by glycolate occurs under anaerobic conditions, indicates that oxygen is not necessary for the formation of the active species, if it were glyoxylate. The presence of an enzyme linking the oxidation of glycolate to glyoxylate with oxygen has not been observed in algae (8). Previous work indicates that glycolate metabolism occurs anaerobically in *Scenedesmus* (15). Products of the

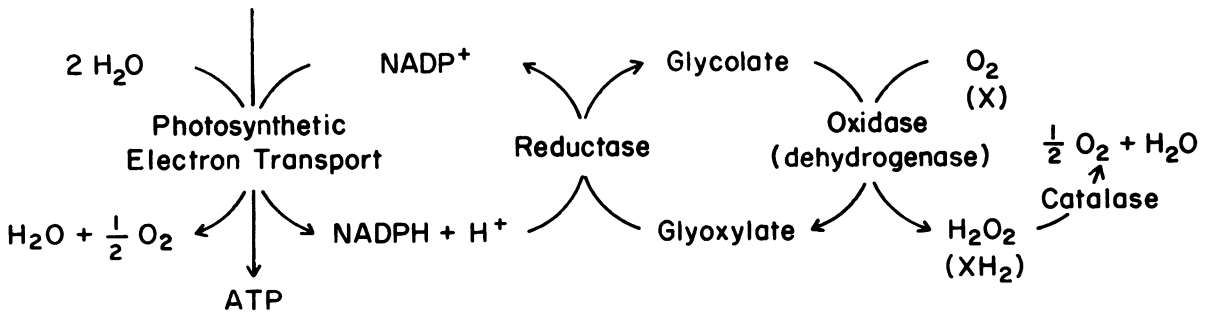

FIG. 4. A proposed scheme to account for glycolate stimulation of oxygen evolution.

glycolate pathway, glycine, serine, and P-glycerate, were produced during dark anaerobic glycolate-^{14}C assimilation (15). Thus it appears that glycolate oxidation may occur in *Scenedesmus*, but that it is not necessarily linked to oxygen. For this reason, it is probable that glycolate is oxidized anaerobically in these cells to glyoxylate, which in turn is reduced by NADPH produced photosynthetically, thereby stimulating oxygen evolution. In the oxidation of glycolate, the ultimate, hydrogen acceptor in lieu of oxygen is not known. Some time ago Kolesnikov *et al.* (12) indicated that quinones could act as acceptors for glycolate oxidation to glyoxylate. Such an oxidation might effect the oxidation reduction level of quinones in the photosynthetic electron transport chain, and therefore indirectly rates of oxygen evolution.

Labeling data of Schou *et al.* (15) indicates that glycolate passes through the glycolate pathway and into the Krebs cycle when experiments were done aerobically. Therefore glycolate stimulation of dark respiration is most likely due to enhanced mitochondrial respiration. Respiration in the dark was stimulated equally by both glycolate or glyoxylate (table II). If glycolate oxidation to glyoxylate involved oxygen uptake an enhanced rate of oxygen uptake over glyoxylate would have been expected, but this was not observed.

The importance of these phenomena in nature has not been evaluated. Glycolate concentrations in natural waters have been determined to be in the range of 0.1 mg to 1.0 mg/liter (6). While this concentration is below the amount used in this investigation, an extremely high algae concentration of 2.7 % was used. Reabsorption of excreted glycolate can occur in natural populations of algae in the light (6). Thus the possibility exists that glycolate under conditions of low CO_2 occurring in nature might stimulate oxygen evolution. This process would give the cells a method of making ATP by a non-cyclic process when no other natural electron acceptor was available.

Literature Cited

1. ASADA, K., S. KITOH, R. DEURA, AND A. KASAI. 1965. Effect of α-hydroxysulfonates on photochemical reactions of spinach chloroplasts and participation of glyoxylate in photophosphorylation. Plant Cell Physiol. 6: 615–29.
2. BRUIN, W. J., J. L. HESS, C. SWANSON, AND N. E. TOLBERT. 1966. Phosphoglycolate phosphatase and D-glycerate dehydrogenase from algae. Plant Physiol. 41: xxxviii.
3. BUTT, V. S. AND M. PEEL. 1963. The participation of glycolate oxidase in glucose uptake by illuminated *Chlorella* suspensions. Biochem. J. 88: 31 p.
4. CHANG, W. 1967. Excretion of organic acids during photosynthesis by synchronized algae. Ph.D. thesis, Michigan State University.
5. DAVIS, E. A. 1953. Nitrate reduction by *Chlorella*. Plant Physiol. 28: 539–44.
6. FOGG, G. E. 1963. The role of algae in organic production in aquatic environments. British Phycological Bulletin 2: 195–210.
7. GOOD, N. E. 1963. Carbon dioxide and the Hill reaction. Plant Physiol. 38: 298–304.
8. HESS, J. L. AND N. E. TOLBERT. 1967. Glycolate pathway in algae. Plant Physiol. 42: 371–79.
9. HESS, J. L., N. E. TOLBERT, AND L. PIKE. 1967. Glycolate biosynthesis by *Scenedesmus* and *Chlorella* in the presence or absence of $NaHCO_3$. Planta 74: 278–85
10. KESSLER, E. 1955. On the role of manganese in the oxygen-evolving system of photosynthesis. Arch. Biochem. Biophys. 59: 527–29.
11. KOK, B. 1965. Photosynthesis: The path of energy. In: Plant Biochemistry, J. Bonner and J. E. Varner, eds. Academic Press, New York. p 903–60.
12. KOLESNIKOV, P. A., E. I. PETROCHENKO, AND S. V. ZORE. 1958. Enzymatic reduction of quinone by glycolic acid. Dokl. Akad. Nauk U.S.S.R. 123: 729–32.
13. NORRIS, L., R. E. NORRIS, AND M. CALVIN. 1955. A survey of the rates and products of short-term photosynthesis in plants of nine phyla. J. Exptl. Botany 6: 67–74.
14. PEDERSON, T. A., M. KIRK, AND J. A. BASSHAM. 1966. Light-dark transients in levels of intermediate compounds during photosynthesis in air-adapted *Chlorella*. Physiol. Plantarum 19: 219–31.
15. SCHOU, L., A. A. BENSON, J. A. BASSHAM, AND M. CALVIN. 1950. The path of carbon in photosynthesis XI. The role of glycolic acid. Physiol. Plantarum 3: 487–95.
16. SENGER, H. AND N. I. BISHOP. 1967. Quantum yield of photosynthesis in synchronous *Scenedesmus* culture. Nature 214: 140–42.
17. TANNER, H. A., T. E. BROWN, H. C. EYSTER, AND R. W. TREHARNE. 1960. A manganese dependent photosynthesis process. Biochem. Biophys. Res. Commun. 3: 205–10.
18. TOLBERT, N. E. 1963. Glycolate pathway. In: Photosynthetic Mechanisms in Green Plants. NSF-NRC Publication 1145. p 648–62.
19. TOLBERT, N. E. AND L. P. ZILL. 1956. Excretion of glycolic acid by algae during photosynthesis. J. Biol. Chem. 222: 895–906.
20. WALKER, D. A. AND R. HILL. 1967. The relation of oxygen evolution to carbon assimilation with isolated chloroplasts. Biochim. Biophys. Acta 131: 330–38.
21. WARBURG, O. H., A. GEISSLER, AND S. LORENZ. 1962. Neue Method zur Bestimmung der Kohlensäuredrucke Über Bicarbonate-Carbonatgemischen. In: Weiterentwicklung der Zellphysiologischen Methoden, Georg Thieme Verlag, Stuttgart. p 578–81.
22. ZELITCH, I. AND D. A. WALKER. 1964. The role of glycolic acid metabolism in opening of leaf stomata. Plant Physiol. 39: 856–62.