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Photosynthetic Oxygen Reduction in Isolated Intact Chloroplasts and Cells from Spinach[1]

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THOMAS V. MARSHO AND PAUL W. BEHRENS
Department of Biological Sciences, University of Maryland Baltimore County (UMBC), Catonsville, Maryland 21228

RICHARD J. RADMER
Martin Marietta Laboratories, 1450 South Rolling Road, Baltimore, Maryland 21227

ABSTRACT

The time course of light-induced O_2 exchange by isolated intact chloroplasts and cells from spinach was determined under various conditions using isotopically labeled O_2 and a mass spectrometer. In dark-adapted chloroplasts and cells supplemented with saturating amounts of bicarbonate, O_2 evolution began immediately upon illumination. However, this initial rate of O_2 evolution was counterbalanced by a simultaneous increase in the rate of O_2 uptake, so that little net O_2 was evolved or consumed during the first ~ 1 minute of illumination. After this induction (lag) phase, the rate of O_2 evolution increased 3- to 4-fold while the rate of O_2 uptake diminished to a very low level. Inhibition of the Calvin cycle, *e.g.* with DL-glyceraldehyde or iodoacetamide, had negligible effects on the initial rate of O_2 evolution or O_2 uptake; both rates were sutained for several minutes, and about balanced so that no net O_2 was produced. Uncouplers had an effect similar to that observed with Calvin cycle inhibitors, except that rates of O_2 evolution and photoreduction were stimulated 40 to 50%.

These results suggest that higher plant phostosynthetic preparations which retain the ability to reduce CO_2 also have a significant capacity to photoreduce O_2. With near-saturating light and sufficient CO_2, O_2 reduction appears to take place primarily via a direct interaction between O_2 and reduced electron transport carriers, and occurs principally when CO_2-fixation reactions are suboptimal, *e.g.* during induction or in the presence of Calvin cycle inhibitors. The inherent maximum endogenous rate of O_2 reduction is approximately 25 to 50% of the maximum rate of noncyclic electron transport coupled to CO_2 fixation. Although the photoreduction of O_2 is coupled to ion transport and/or phosphorylation, this process does not appear to supply significant amounts of ATP directly during steady-state CO_2 fixation in strong light.

Efficient photosynthetic CO_2 fixation is dependent upon the light-driven production of NADPH and ATP in appropriate stoichiometric amounts. One means for assuring that correct stoichiometries are maintained would be to vary the rate of photosynthetic electron flow through pathways differing in their ATP:NADPH production ratios (10). The possible occurrence of coupled electron flow pathways other than the noncyclic transport from water to NADPH, *e.g.* pseudocyclic or cyclic electron transport, has been recognized for some time (2, 16). The extent to which these pathways operate *in vivo*, however, is still unresolved.

Studies with washed broken chloroplast preparations have shown that rates of electron transport to O_2 are generally quite low unless certain exogenous electron acceptors are added. In contrast, algae or intact isolated chloroplasts display a potentially significant endogenous capacity for pseudocyclic (and cyclic) transport (see, *e.g.* 1, 8, 19, 20, 24). Under certain conditions, algae have been shown to carry out pseudocyclic electron transport (O_2 reduction) at rates approaching saturated rates of noncyclic electron transport (24). A number of direct and indirect measurements with isolated intact chloroplasts have suggested that higher plant photosynthetic tissues also possess a substantial endogenous capacity for O_2 reduction (5, 6, 12, 14, 15, 17). Here, we determined the magnitude and kinetics of O_2 evolution and reduction in isolated intact chloroplasts and whole cells obtained from spinach using a mass spectrometer with a fast mass stepper system. Significant rates of O_2 reduction are shown to occur principally when CO_2 fixation reactions are suboptimal.

MATERIALS AND METHODS

Intact chloroplasts were isolated from greenhouse-grown spinach as described previously (26). Preparations contained more than 70% intact chloroplasts as determined by the ferricyanide reduction method (13) and fixed CO_2 at rates in excess of 100 μmol/mg Chl · h in saturating light. Chloroplasts were routinely assayed in a 0.33 M sorbitol, 50 mM Hepes-KOH (pH 8.0) medium containing 10 mM $NaHCO_3$, 5 mM $Na_4P_2O_7$, 2 mM EDTA, 0.25 mM K_2HPO_4, and catalase (195 units/ml). Other additions are indicated in the figure legends.

Intact cells were prepared from freshly harvested spinach leaves sliced into small (0.5 × 2.0 cm) strips. Approximately 2 g of leaf strips were vacuum-infiltrated in 20 ml of media containing 0.8 M sorbitol, 20 mM Mes (pH 5.8) buffer, 12.5 mM K_2SO_4, and 0.75% Macerase (obtained from Calbiochem). Leaf strips were digested in 75 ml of the infiltration medium (maintained at 15 C) in an apparatus similar to that described by Servaites and Ogren (23). Released cells were pelleted (100*g*), washed twice with 50 mM Hepes-KOH (pH 7.8) buffer containing 0.8 M sorbitol and 1 mM $MgCl_2$, and subsequently resuspended in a small volume of the washing medium containing 5 mM DTT. Preparations contained ≥ 85% intact (plasmolysed) cells as judged microscopically after staining with 2.5% Evans Blue. Cells were normally assayed in a medium consisting of 0.7 M sorbitol, 50 mM Hepes-KOH (pH 8.0) buffer, 10 mM $NaHCO_3$, and 1 mM $MgCl_2$. Saturated rates of net O_2 evolution were routinely 50 to 70 μmol O_2/mg Chl · h.

O_2 exchange was measured polarographically or with a mass spectrometer using isotopically labeled O_2 (99 atom% $^{18}O_2$, obtained from Bio-Rad Laboratories). The instrumentation and

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expressions used for calculating rates of O_2 evolution and O_2 uptake have been described earlier (20, 21). All experiments were done using broad-band saturating orange-red light (Schott OG530 and appropriate heat filters) at approximately 20 C. Experiments with the mass spectrometer were generally run at elevated O_2 tensions (see figure legends).

RESULTS

O_2 Reduction In Isolated Chloroplasts. Figure 1A shows the calculated rates of O_2 evolution and O_2 uptake characteristically observed when dark-adapted chloroplasts were illuminated with near-saturating light. O_2 evolution began immediately upon illumination (within the $\leq$ 3-s response time of the instrument). However, this initial rate (V_o) of O_2 evolution was counterbalanced by a comparable light-induced rate of O_2 uptake, so that little net O_2 was evolved. After 1 to 2 min in continuous light, the rate of O_2 evolution increased 3- to 4-fold to a maximum steady-state rate (V_m) while the rate of O_2 uptake diminished to a relatively insignificant level. Figure 1B shows the integrated time course of net O_2 evolution computed from the data of Figure 1A. These results are consistent with the typical induction lag measured with dark-adapted intact chloroplasts (or with whole cells) using a standard O_2 concentration electrode.

One could postulate that the observed light-driven O_2 uptake occurred via O_2 uptake reactions associated with the Calvin cycle, *e.g.* ribulose bis-P carboxylase/oxygenase. The results shown in Figure 2A indicate that this is not the case. Inhibition of the Calvin cycle with DL-glyceraldehyde (3, 25) had minimal effects on the light-induced rate of O_2 uptake or evolution (compared to V_o rates observed in Fig. 1A). Both rates were sustained in continuous light for several minutes so that, observed polarographically, little net O_2 would be evolved. Similar results were obtained when net CO_2 fixation (and net O_2 evolution) was inhibited with high phosphate concentrations (data not shown). As illustrated in Figure 2B, light-induced rates of O_2 evolution and concomitant O_2 uptake were increased approximately 50% by the uncoupler methylamine.

Results equivalent to those of Figure 2B were also obtained with methylamine in the absence of DL-glyceraldehyde (data not shown); the uncoupler eliminated CO_2 fixation (and net O_2 evolution), and O_2 evolution and an equivalent O_2 uptake were sustained during illumination at a rate ~ 50% higher than that observed during the induction lag or in the presence of Calvin cycle inhibitors. These results are in agreement with numerous suggestions that pseudocyclic electron transport is coupled to ion transport and/or ATP synthesis (5–7, 10).

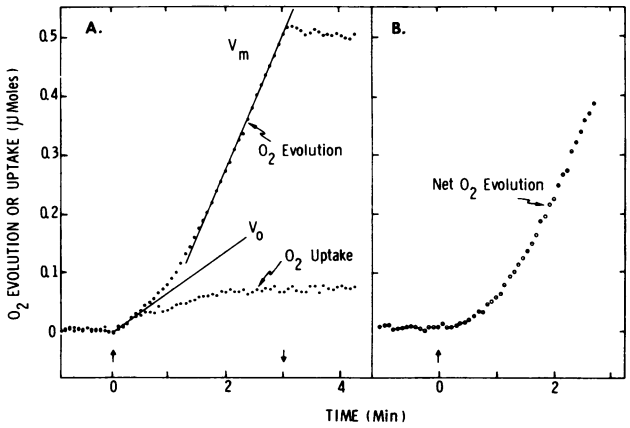

FIG. 1. A: light-induced O_2 evolution and O_2 uptake in intact chloroplasts. B: time course of net O_2 evolution computed from the data of Figure 1A. Standard chloroplast assay medium. Saturated net rates of O_2 evolution for this chloroplast preparation were 110 μmol O_2/mg Chl · h. Actinic light on (↑) and off (↓). Total Chl concentration, 100 μg/ml. Initial total O_2 concentration 0.610 mM. Each point in this and the succeeding figures represents the value of O_2 evolution and O_2 uptake measured and computed as described in reference 21.

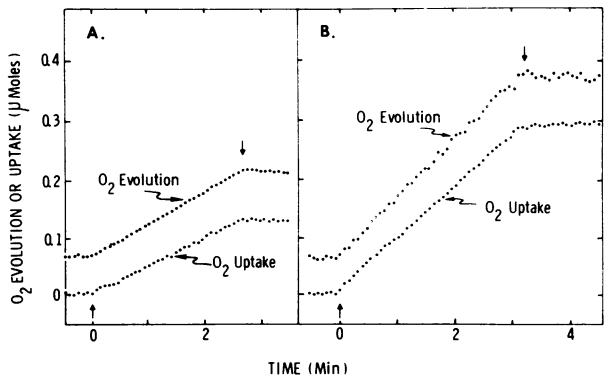

FIG. 2. Light-induced O_2 evolution and O_2 uptake in intact chloroplasts: (A) in the presence of 10 mM DL-glyceraldehyde, and (B) in the presence of 10 mM DL-glyceraldehyde plus 30 mM methyl amine. Other conditions as in Figure 1. Actinic light on (↑) and off (↓). Initial O_2 concentration of 0.814 mM and 0.516 mM in A and B, respectively. Note that the plot of O_2 evolution was offset for clarity.

Our results indicate that the broken chloroplasts ($\leq$ 30%) present in our intact chloroplast preparations do not contribute significantly to the observed rates of O_2 uptake. The light-induced rate of O_2 uptake decreased to a low steady-state following induction (Fig. 1A). This decrease correlates with the increase in the rate of O_2 evolution, suggesting that electron transport to O_2 is diminished by noncyclic electron transport leading to CO_2 reduction. This conclusion is further corroborated by the results shown in Figure 3. Intact chloroplasts which were osmotically shocked (in the reaction vessel) had an O_2 uptake rate that was 32% of that observed with intact chloroplasts in the presence of DL-glyceraldyhyde; washed broken chloroplasts preparations (type C[9]) had an even lower rate (14% of the DL-glyceraldehyde control). As expected, neither of these broken chloroplast preparations showed net O_2 evolution. These observations are consistent with the suggestions (1, 20) that the catalyst(s) involved in electron transport to O_2 is loosely membrane-bound.

O_2 Reduction in Intact Cells. We also measured the magnitude and kinetics of O_2 reduction in isolated intact spinach cells to determine whether the results obtained with isolated chloroplasts accurately reflected O_2 reduction *in vivo*. The results of a set of experiments, performed under conditions similar to those with isolated chloroplasts, are shown in Figures 4 and 5. It was necessary in these whole cell experiments to substitute the Calvin cycle inhibitor iodoacetamide for DL-glyceraldehyde and the uncoupler CCCP[2] for methylamine. With minor exceptions the whole cell data are comparable to those obtained with intact chloroplasts. Under optimum conditions for CO_2 fixation (Fig. 4A), O_2 evolution and O_2 uptake again began immediately upon illumination; O_2 uptake subsequently declined in the light to a very low steady-state rate; the initial rates of O_2 evolution and uptake did not quite balance. This is also apparent in the integrated time course for net O_2 evolution plotted in Figure 4B. Similar net O_2 kinetics (albeit with a slightly longer induction) were observed for this cell preparation when measured polarographically under approximately the same conditions (dashed curve in Fig. 4B). Presumably in both cases, CO_2 fixation was not completely inactivated at the onset of illumination.

A sustained light-induced rate of O_2 uptake, similar to that observed initially with bicarbonate, was obtained when the Calvin cycle was inhibited with iodoacetamide (Fig. 5A). Likewise, the rate of O_2 uptake was stimulated 40 to 50% by the uncoupler

[2] Abbreviation: CCCP: carbonylcyanide *m*-chlorophenylhydrazone.

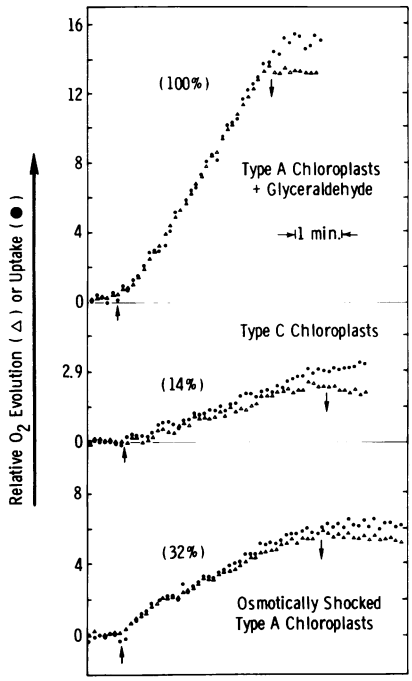

FIG. 3. Light-induced O_2 evolution (open markers) and O_2 uptake (solid markers) in three types (9) of chloroplast preparations. Broken (type C) chloroplasts were prepared according to reference 22. Osmotically shocked chloroplasts were prepared by placing intact (type A) chloroplasts in distilled H_2O for 5 min and then diluting with an equal volume of twice-concentrated assay medium. Standard assay medium containing 10 mM DL-glyceraldehyde and 50 μg/ml total Chl. Actinic light on (↑) and off (↓). Note that the middle trace was plotted on a different relative scale and that the numbers in parentheses refer to relative absolute rates. Initial O_2 concentration of 0.431 mM, 0.202 mM, and 0.306 mM in upper, middle, and lower experiments, respectively.

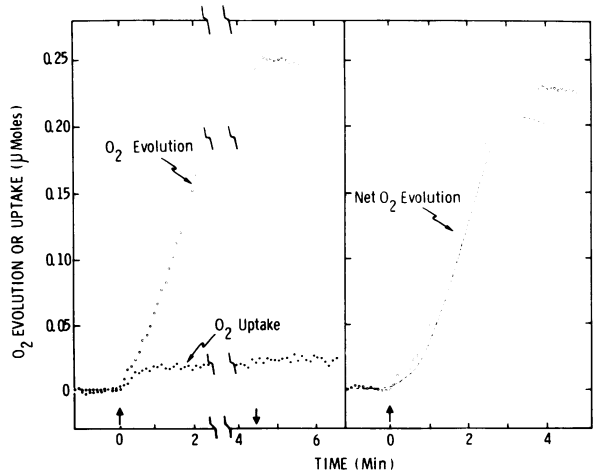

FIG. 4. A: light-induced O_2 evolution and O_2 uptake in isolated spinach cells. B: time course of net O_2 evolution computed from the data of Figure 4A (open markers) or recorded polarographically (dashed curve). Standard cell assay medium. Net rates of O_2 evolution for this cell preparation were 55 μmol O_2/mg Chl · h. Actinic light on (↑) and off (↓). Total Chl concentration, 75 μg/ml. Initial O_2 concentration of 0.323 mM in A and 0.237 mM in polarographic measurement.

CCCP (Fig. 5B). In both cases, rates of O_2 uptake were accompanied by comparable (slightly greater) rates of O_2 evolution such that, as observed polarographically, little net O_2 was evolved.

Preliminary studies with isolated soybean cells have shown O_2 exchange kinetics similar to those described above for spinach. We should note, however, that our soybean preparations had

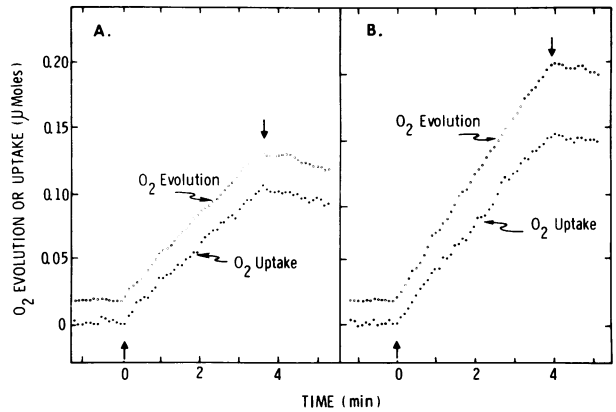

FIG. 5. Light-induced O_2 evolution and O_2 uptake in isolated spinach cells: (A) in the presence of 5 mM iodoacetamide and (B) in the presence of 1 μM CCCP. Other conditions as in Figure 4. Actinic light on (↑) and off (↓). Initial O_2 concentration of 0.408 mM and 0.468 mM in A and B, respectively. Note that the plot of O_2 evolution was offset for clarity.

higher maximum net rates of photosynthesis (70–100 μmol O_2/mg Chl · h) and required turgid cells, *i.e.* lower sorbitol concentrations, for maximum rates of CO_2 fixation, compared to spinach (see also 23).

DISCUSSION

Our results with intact chloroplasts and cells from spinach support the earlier suggestion (20) that the direct photoreduction of O_2 appears to be a reaction common to all oxygenic photosynthetic organisms. Under the conditions of our experiments, *i.e.* near-saturated light, ample CO_2 and relatively high O_2 tensions, significant endogenous rates of O_2 reduction appear to occur principally when rates of CO_2 fixation are limited, *e.g.* during the initial lag phase following the onset of illumination or in the presence of components which interfere with normal CO_2 assimilation (DL-glyceraldehyde, methylamine, etc.). Thus, as in algae, O_2 appears to compete with CO_2 for photochemically generated reducing equivalents. The coupling of O_2 reduction to ion transport and/or phosphorylation and the normally low steady-state rate of O_2 uptake (in the presence of saturating amounts of CO_2) suggest that the electron flow via this pathway is tightly controlled and may principally function to prime the CO_2 reduction system. In weak light the direct photoreduction of O_2 has been reported to account for approximately 40% of the net steady-state rate of O_2 evolution during CO_2 reduction in intact spinach chloroplasts (5), although a more recent report from the same laboratory (11) indicates a much lower relative steady-state rate of O_2 uptake ($\leq$ 16%) with increased light intensities. Consistent with our findings, it was suggested that the relative direct contribution of pseudocyclic electron transport to the ATP demands of the Calvin cycle at steady-state in high light is minimal.

The most striking difference we observed between algae and higher plant systems is the considerably lower rates of O_2 photoreduction in spinach. We typically find endogenous O_2 reduction rates of approximately 25 to 50% of the maximum rate of noncyclic electron transport compared to O_2 uptake rates of $\geq$ 80% of V_{max} in algae. Our calculated rates of O_2 reduction in both cases represent minimum estimates. The sampling system used in these experiments does not necessitate equilibration across a gas-liquid interface, but incomplete equilibration of the O_2 isotopes between the inside and outside of the chloroplasts (or cells) will result in an underestimation of the rate of O_2 reduction. However, the relatively simpler isolated chloroplast system and the reported (27) rapid equilibration of O_2 across chloroplast membranes particularly at elevated O_2 tensions as used in the present experiments would suggest that the observed differences in maximum rates of O_2 reduction are not an artifact.

The lower rates of O_2 reduction in spinach are not a result of substrate (O_2) limitations; preliminary experiments (unpublished) suggest an apparent K_m for O_2 similar to that found for *Scenedesmus* (21). It is interesting to note that previous studies with whole leaves (17, 18) have shown that rates of O_2 evolution are roughly equivalent to rates of O_2 uptake when measured in strong light at CO_2 compensation, and that these rates are approximately 30% of the rates of O_2 evolution observed at high CO_2 concentrations. These relative rates of O_2 exchange are strikingly similar to those we found in isolated chloroplasts and cells (comparing steady-state O_2 exchange rates with sufficient CO_2 in the presence and absence of Calvin cycle inhibitors), although one cannot exclude a possible involvement of carbon metabolism, *e.g.* glycolate metabolism in the whole leaf experiments. In this regard, recent studies with leaves and intact chloroplasts from spinach have suggested that a considerable fraction of O_2 uptake (at CO_2 compensation) can be associated with glycolate formation (4).

Under the conditions of our experiments, uncouplers weakly stimulated (~50%) electron transport to O_2. This increase is considerably less than can typically be demonstrated for noncyclic electron transport to other acceptors in intact chloroplasts, *e.g.* NO_2^- and oxaloacetate. We assume that this simply reflects a lower turnover rate for O_2 reduction in higher plants.

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LITERATURE CITED

1. ALLEN JF 1977 Oxygen—a physiological electron acceptor in photosynthesis? Curr Adv Plant Sci 9: 459–469
2. ARNON D 1961 Cell-free photosynthesis and the energy conversion process. *In* WD McElroy, B Glass, eds, Light and Life. Johns Hopkins Univ Press, Baltimore, pp 489–565
3. BAMBERGER ES, M AVRON 1975 Site of action of inhibitors of carbon dioxide assimilation by whole lettuce chloroplasts. Plant Physiol 56: 481–485
4. BERRY JA, CB OSMOND, GH LORIMER 1978 Fixation of $^{18}O_2$ during photorespiration. Plant Physiol 62: 954–967
5. EGNEUS H, U HEBER, U MATTHIESEN, M KIRK 1975 Reduction of oxygen by the electron transport chain of chloroplasts during assimilation of carbon dioxide. Biochim Biophys Acta 408: 252–268
6. FORTI G, P GEROLA 1977 Inhibition of photosynthesis by azide and cyanide and the role of oxygen in photosynthesis. Plant Physiol 59: 859–862
7. FORTI G, AT JAGENDORF 1961 Photosynthetic phosphorylation in the absence of redox dyes: oxygen and ascorbate effects. Biochim Biophys Acta 54: 322–330
8. GLIDEWELL SM, JA RAVEN 1975 Measurement of simultaneous oxygen evolution and uptake in *Hydrodictyon africanum*. J Exp Bot 26: 479–488
9. HALL DO 1972 Nomenclature for isolated chloroplasts. Nature New Biol 235: 125–126
10. HEBER U 1976 Energy coupling in chloroplasts. J Bioenerget Biomembr 8: 157–172
11. HEBER UH, EGNEUS U HANCK, M JENSEN, S KOSTER 1978 Regulation of photosynthetic electron transport in intact chloroplasts and leaves of *Spinacia oleraceae* L. Planta 143: 41–49
12. HEBER U, CS FRENCH 1968 Effects of oxygen on the electron transport chain of photosynthesis. Planta 79: 99–112
13. HEBER U, KA SANTARIUS 1970 Direct and indirect transfer of ATP and ADP across the chloroplast envelope. Z Naturforsch 256: 718–728
14. HUBER S, G EDWARDS 1975 The effect of oxygen on CO_2-fixation by mesophyll protoplast extracts of C_3 and C_4 plants. Biochem Biophys Res Commun 67: 28–34
15. KAISER W 1976 The effect of hydrogen peroxide on CO_2 fixation of isolated intact chloroplasts. Biochim Biophys Acta 440: 476–482
16. MEHLER AH 1951 Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. Arch Biochem Biophys 33: 65–77
17. MULCHI CL, RJ VOLK, WA JACKSON 1971 Oxygen exchange of illuminated leaves at carbon dioxide compensation. *In* MD Hatch, CB Osmond, RO Slatyer, eds, Photosynthesis and Photorespiration. Wiley-Interscience, New York, pp 458–471
18. OZBUN JL, RJ VOLK, WA JACKSON 1964 Effects of light and darkness on gaseous exchange of bean leaves. Plant Physiol 39: 523–527
19. PATTERSON COP, J MYERS 1973 Photosynthetic production of hydrogen peroxide by *Anacystis nidulans*. Plant Physiol 51: 104–109
20. RADMER RJ, B KOK 1976 Photoreduction of O_2 primes and replaces CO_2 assimilation. Plant Physiol 58: 336–340
21. RADMER RJ, B KOK, O OLLINGER 1978 Kinetics and apparent K_m of oxygen cycle under conditions of limiting carbon dioxide fixation. Plant Physiol 61: 915–917
22. SCHWARTZ M 1966 *N*-Tetramethyl-*p*-phenylenediamine as a catalyst of photophosphorylation. Biochim Biophys Acta 112: 204–212
23. SERVAITES JC, WL OGREN 1977 Rapid isolation of mesophyll cells from leaves of soybean for photosynthetic studies. Plant Physiol 59: 587–590
24. SIMONIS W, W URBACH 1973 Photophosphorylation *in vivo*. *In* L Machlis, ed, Annu Rev Plant Physiol, Vol 24. Annu Reviews Inc, Palo Alto, pp 89–114
25. SLABAS AR, DA WALKER 1976 Inhibition of spinach phosphoribulokinase by DL-glyceraldehyde. Biochem J 153: 613–619
26. SOKOLOVE PM, TV MARSHO 1977 Slow fluorescence quenching of type A chloroplasts: relationship to electron-flow with CO_2 as acceptor. FEBS Lett 75: 28–32
27. STEIGER HM, E BECK, R BECK 1977 Oxygen concentration in isolated chloroplasts during photosynthesis. Plant Physiol 60: 903–906