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Photosynthetic Oxygen Exchange in Isolated Cells and Chloroplasts of C_3 Plants

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ABSTRACT

Photosynthetic O_2-production and photorespiratory O_2-uptake were measured, using stable isotope techniques, in isolated intact leaf cells of the C_3 plant *Xanthium strumarium* L., and isolated intact chloroplasts of *Spinacia oleracea* L (var. Yates 102). Considerable light dependent O_2-uptake was observed in both systems, a proportion of which could be suppressed by CO_2 (63% suppression in chloroplasts by 50 micromolar CO_2, 58% in cells by 100 micromolar CO_2 and 250 micromolar O_2). At low O_2, O_2-uptake was CO_2 insensitive. At high CO_2 up to 19% of total electron flow was to O_2 in cells and up to 14% in chloroplasts. O_2-uptake showed inhibition by KCN (61% in cells, 35% in chloroplasts by 0.2 millimolar KCN). O_2-uptake half saturated at 75 to 85 micromolar O_2 in cells and 50 to 65 micromolar O_2 in chloroplasts, at low CO_2. The results are discussed in terms of the RuP_2-oxygenase reaction and direct photoreduction of O_2 via a Mehler reaction.

Light-dependent O_2 uptake has been recognized as a feature of photosynthesis since the early observations of Hoch *et al.* (15) using isotopic O_2. The components of this O_2 uptake are believed to include RuP_2-oxygenase[1] (20), direct photoreduction of O_2 via a Mehler reaction (1, 12) or, alternatively, persistence of mitochondrial respiration during illumination (11).

Varying views have been expressed as to the relative magnitudes of the above uptake processes under a range of conditions for photosynthesis. Investigation into this area would provide data pertinent to the role of O_2 in two metabolic events. First, there has been considerable discussion concerning the relative roles of RuP_2-oxygenase *versus* the Mehler reaction and its photochemically generated H_2O_2 as the primary event of photorespiration (29). Second, there has been controversy over the relative magnitudes of cyclic and pseudocyclic (whole chain electron transport to O_2-Mehler reaction) photophosphorylation as mechanisms to generate the additional ATP required for CO_2 fixation and photorespiration (12).

The present studies address the above problems by examining the effects of CO_2 and O_2 concentration on the O_2 exchange (photosynthetic O_2-evolution and uptake) of isolated cells and chloroplasts in order to define the parameters of O_2 uptake in these systems.

MATERIALS AND METHODS

Spinach chloroplasts were prepared by the method of Lilley and Walker (18) from 5-week-old leaves of *Spinacia oleracea* L. (var. Yates 102) glasshouse grown in water culture. Leaf cells from *Xanthium strumarium* (glasshouse grown in soil) were isolated according to Sharkey and Raschke (26), and isolation was completed in less than 5 min after leaf detachment. O_2 exchange was measured using a Varian MAT GD 150/4 magnetic sector mass-spectrometer, continuously monitoring $^{18}O_2$ (mass 36), $^{16}O_2$ (mass 32), and argon (mass 40) as an internal reference gas (this machine has four collectors allowing each mass to be collected separately and simultaneously). Cells and chloroplasts were placed in a glass cuvette (similar in design to that of an O_2-electrode) in media depleted of O_2 by bubbling with argon. The cuvette was stoppered, and a bubble of $^{18}O_2$ was allowed to dissolve into the aqueous medium until the desired final total O_2 concentration ($^{18}O_2 + {}^{16}O_2$) had been reached. The bubble was then removed, and the experimental measurements of $^{16}O_2$ and $^{18}O_2$ changes were started. Gases were admitted to the analyzer by diffusion across a polythene membrane set in the base of the cuvette. Calibration of mass signals with regard to concentration of species in solution was made by bubbling liquid in the cuvette with known gas concentrations (*i.e.* 100% argon and 21% $^{16}O_2$ in air—as the signal response of the mass spectrometer is linear, then a single point calibration can be made).

Temperature was controlled through a water jacket maintained at 25°C. Calculations of O_2-uptake and evolution were carried out as given by Radmer and Kok (24) and Figure 1, with corrections based on the change in the argon signal for consumption of gas by the mass spectrometer. Leaks into and out of the cuvette to the atmosphere were negligible compared to the changes in signal levels caused by the photosynthetic reactions and mass spectrometer consumption. An example of a recorder tracing of the signal changes of masses 32, 36, and 40 following switching the light on is given in Figure 1.

Cells were assayed in 0.1 M Hepes adjusted to pH 7.0 with KOH, and chloroplasts were assayed in 0.33 M sorbitol, 1 mM $MgCl_2$, 2 mM EDTA, 50 mM Hepes, and 0.5 mM KH_2PO_4, adjusted to pH 7.6 with KOH. Rates of O_2 exchange were measured after a linear rate was obtained unless otherwise specified. O_2 concentrations indicated and O_2 exchange rates were measured simultaneously; total O_2 varied less than 20% throughout an experiment. CO_2 concentrations indicated in the figures are initial values calculated from the added $NaHCO_3$ and the pH. Chl concentration ranged from 3 to 10 μg/ml in a 5 ml volume. Chl was estimated by the method of Arnon (3). Chloroplasts were 80 to 95% intact as measured by ferricyanide reduction (13). All chemicals were obtained from Sigma with the exception of $^{18}O_2$ (99% enrichment) which was obtained from Norsk-Hydro (Oslo).

RESULTS

O_2 uptake in cells and chloroplasts is considerably inhibited by increasing CO_2 concentrations. Figure 2 illustrates this effect of CO_2 concentration on O_2-uptake and O_2-evolution in spinach

[1] Abbreviation: RuP_2-oxygenase, ribulose-1,5-bisphosphate oxygenase (EC 4.1.1.39).

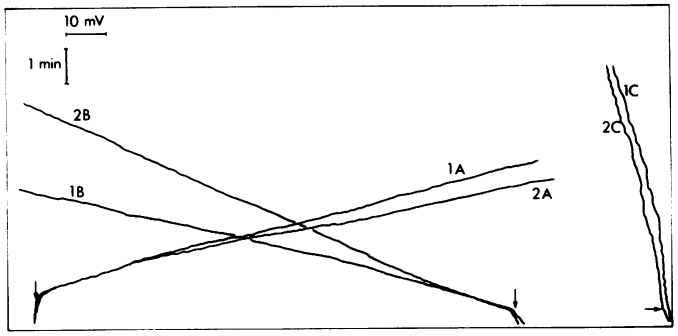

FIG. 1. Mass-spectrometer trace illustrating light on/off effect and effect of CO_2 concentration on O_2-exchange in *Xanthium* cells. Vertical axis is time, the horizontal axis is mass-spectrometer signal (mv). Trace 1 is of an experiment with no added CO_2 and 250 μM O_2, trace 2 with 50 μM CO_2 and 250 μM O_2. Traces 1A and 2A are changes in mass 32 signal, 1B and 2B are changes in mass 36 signal, and 1C and 2C are changes in mass 40 signal during an experiment. Traces 1C and 2C were made at double the sensitivity of traces A and B. Arrows indicate onset of illumination.

chloroplasts and *Xanthium* leaf cells. Experiments were performed at atmospheric (21% or 250 μM) O_2 concentration to provide conditions under which one would expect significant RuP-oxygenase-dependent O_2-uptake plus Mehler-type reaction. They were also done at low O_2 where the higher affinity Mehler reaction (4) might be expected to dominate the low affinity RuP_2-oxygenase (2, 5, 6). With 250 μM O_2, the rate of O_2-uptake decreased as CO_2 was increased. This uptake was inhibited some 63% in the chloroplasts (by 50 μM CO_2) and 58% in cells (by 100 μM CO_2).

O_2 uptake at low (35–60 μM) O_2 concentrations was not significantly affected by CO_2 in either cells or chloroplasts, suggesting that the O_2-uptake process, while accepting reducing power generated by the thylakoid reactions, is not necessarily competitively inhibited by CO_2. In the experiments presented here, different preparations of cells and chloroplasts were used, thus strict quantitative comparisons cannot be made between 250 μM O_2 and 35 to 50 μM O_2 data. However, the experiments under each condition were repeated several times with similar results and the data presented is representative of the responses obtained.

At saturating CO_2, it is possible to assume that all the O_2 uptake due to RuP_2-oxygenase has been inhibited, and that remaining is probably associated with direct photoreduction of O_2. If dark O_2 uptake is subtracted from the light-dependent rate, then an estimate of the amount of electron flow to O_2 occurring under these conditions can be calculated. These calculations also assume that all H_2O_2 produced is broken down to H_2O and O_2 and that one O_2 taken up represents the transport of four electrons and one O_2 evolved. In cells, at 250 μM O_2 and 100 to 180 μM CO_2, O_2-uptake represents approximately 19% of total electron flow. At 35 to 60 μM O_2, this value is 13%. For chloroplasts at 25 μM O_2 and 100 to 300 μM CO_2, O_2 uptake was supporting 12% of total electron flow, while at 35 to 60 μM O_2 this figure was 14%.

Suppression of O_2-Uptake by KCN. It has been shown that KCN, in μM concentrations, inhibits both RuP_2-carboxylase and -oxygenase functions (19, 28), but does not significantly affect electron transport capacity (8, 23) or catalase activity (16). O_2 exchange at 250 μM O_2 and CO_2-free conditions was measured for both cells and chloroplasts, as a function of KCN concentration. In these experiments, extra catalase was added to the media and additions of H_2O_2 at 0.75 mM KCN showed that activity was sufficient to catalyze the rapid destruction of H_2O_2. Hence, it may be assumed that on a molar basis O_2-uptake via a Mehler reaction (forming superoxide and then peroxide) will release ½ O_2. O_2

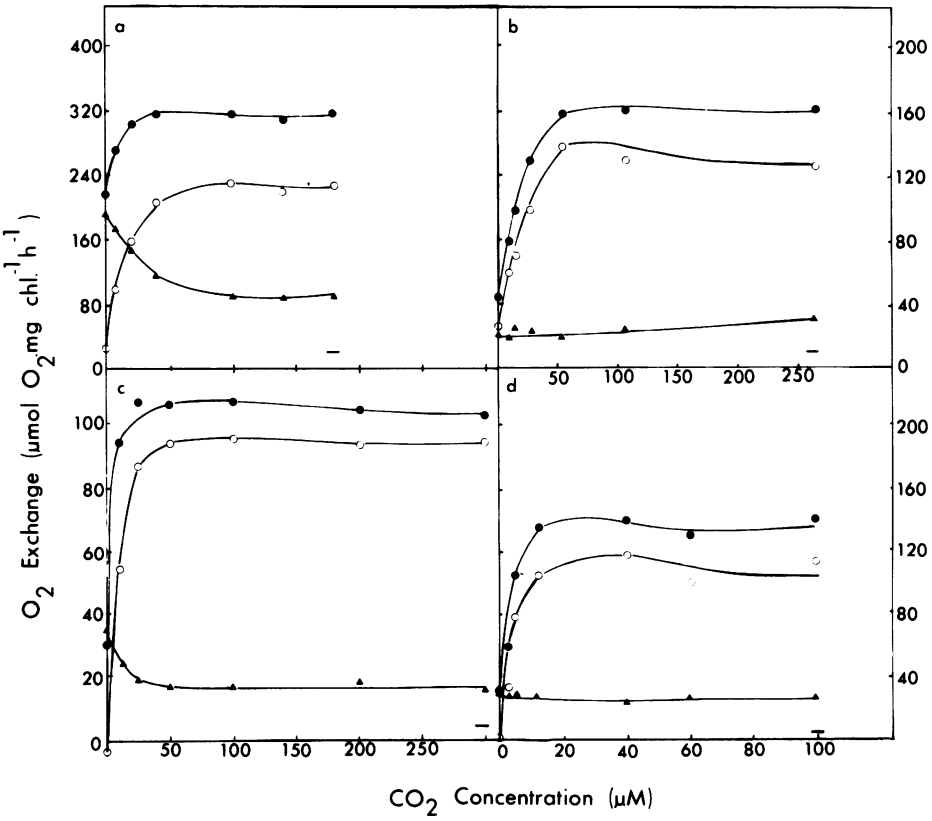

FIG. 2. Effect of CO_2 on O_2 exchange in isolated *Xanthium* cells (a and b) and intact spinach chloroplasts (c and d). a and c, experiments run at atmospheric O_2 (250 μM); b and d, run at 35 to 60 μM O_2; light intensity was 1,500 μE m^{-2} s^{-1}. Catalase was included (1,000 units/ml). ▲, O_2-uptake; ●, O_2-evolution; ○, net O_2-evolution. Horizontal line indicates dark O_2-uptake.

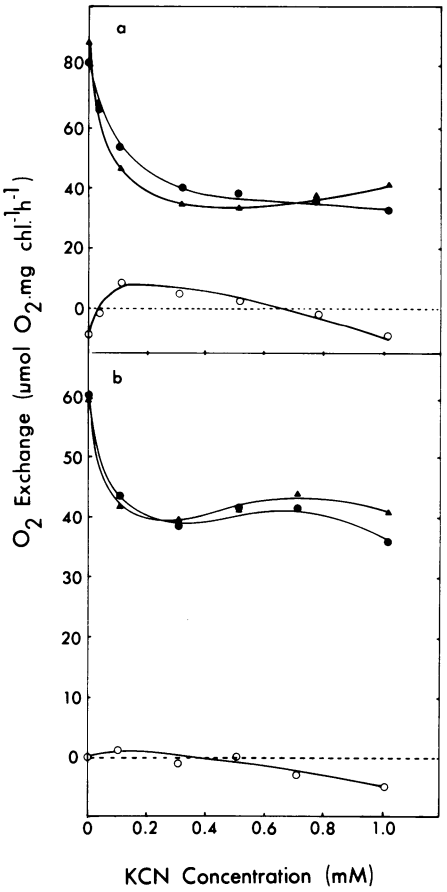

FIG. 3. Effect of KCN concentration on O_2 exchange in isolated *Xanthium* cells (a) and spinach chloroplasts (b) at 250 μM O_2 and CO_2-free conditions. Catalase was included (2,000 units/ml). Light intensity was 1,000 μE m^{-2} s^{-1}. ▲, O_2-uptake; ●, O_2-evolution; ○, net O_2-evolution. Horizontal line indicates dark O_2-uptake.

uptake and evolution were substantially inhibited over the range from 0 to 0.5 mM KCN (Fig. 3), and above 0.5 mM both uptake and evolution remain constant, maintaining an exchange ratio of approximately 1 or a net uptake of 0. The inhibition was greater in cells (61%) than it was in chloroplasts (35%). At 0.5 to 1 mM KCN, the O_2-uptake can be stimulated some 2- to 3-fold by 5 mM NH_4Cl (data not shown), indicating that the potential rate of O_2-uptake under these conditions is coupled to photophosphorylation reactions.

The data in Figure 3 while suggesting that considerable O_2-uptake is associated with RuP_2-oxygenase activity, also indicates that there is direct O_2-uptake through a Mehler type reaction at low CO_2 concentrations.

The Effect of O_2 Concentration on O_2-Uptake. O_2 uptake in *Xanthium* cells and spinach chloroplasts showed similar responses to O_2 concentration at high and low CO_2 (Fig. 4). At high CO_2 (200 μM), O_2-uptake increased up to 250 μM O_2 and was half saturated by less than 100 μM O_2. Chloroplasts saturated at about 150 μM O_2 and were half saturated below 75 μM O_2. At low CO_2, O_2-uptake was greater in both the cells and chloroplasts, and was half saturated by 75 to 85 μM O_2 in the cells and 50 to 65 μM O_2 in the chloroplasts.

At low CO_2, increasing O_2 generally stimulated gross O_2-uptake and gross O_2-evolution, and inhibited net O_2-evolution. This is consistent with the stimulation of RuP_2-oxygenase activity. At high CO_2 (Fig. 4, a and c), however, increasing O_2-stimulated gross O_2-evolution, O_2-uptake, and net O_2-evolution. This was more pronounced in the cells where an increase from 65 to 275 μM O_2 stimulated net O_2-evolution by about 50%.

DISCUSSION

A major portion of the O_2-uptake of both *Xanthium* cells and spinach chloroplasts can be suppressed by both CO_2 (Fig. 2) and KCN (Fig. 3). It can be argued that such responses are consistent with the predicted effect on RuP_2-oxygenase activity. It is difficult, however, to assume unequivocally from such data that this suppressible portion represents only oxygenase activity, as O_2 uptake via a Mehler type reaction may show a similar response. It can be envisaged how increasing CO_2 would increase NADP levels, thus decreasing photoreduction of O_2, while KCN inhibition would slow ADP regeneration, thus limiting the rate of coupled electron transport, again decreasing O_2-uptake. That O_2-uptake is suppressed by CO_2 in the range of 0 to 100 μM, with a $K_i(CO_2)$ of around 20 to 30 μM for cells and 10 to 15 μM for chloroplasts is also consistent with O_2-uptake due to RuP_3-oxygenase activity, but again it is not conclusive.

Under low CO_2 conditions and 250 μM O_2, cells exhibited quantitatively greater O_2-uptake and evolution rates than did chloroplasts while electron transport in chloroplasts was much more dependent on the presence of CO_2, and O_2 did not appear to be as efficient as electron acceptor as in the cells. This difference may be due to the existence of an intact photorespiratory carbon-oxidation cycle in cells, while in chloroplasts, phosphoglycolate and glycolate produced under low CO_2 will be excreted and act as a carbon drain from the system (17). In cells, the O_2-uptake observed will represent more than that due to oxygenase activity alone. Assuming one representation of the events occurring in the photosynthetic-photorespiratory pathway interchange (20), it can be calculated that oxygenase activity is in fact equal to gross uptake observed divided by 1.75. Thus, the difference in relative inhibition by KCN or CO_2 of O_2-uptake between cells and chloroplasts will be largely eliminated if this is taken into account.

Another potential contributing factor to uptake in cells is the persistence of mitochondrial 'dark' respiration in the light, recently supported by the isotope O_2 exchange measurements of Gerbaud and André (11) with whole wheat plants. However, in these experiments, a large proportion of tissue included in the gas exchange measurements was nonphotosynthetic and undoubtedly contributed significantly to the O_2-uptake observed. In the cells and chloroplasts examined here, dark uptake rates were considerably lower than light dependent O_2-uptake rates as were the DCMU inhibited O_2-uptake rates. It would seem that in these systems, dark respiration is not a significant contributor to the observed O_2-uptake in the light.

The K_m O_2 for O_2-uptake has previously been measured in a number of different photosynthetic systems ranging from algae (25) to intact leaves (9). In all these systems, the values measured fall within the range of 75 to 140 μM O_2, with saturation occurring between 250 and 375 μM O_2. These are very similar to the results presented in Figure 4. These values are less than that for the *in vitro* response of RuP_2-oxygenase, 320 to 625 μM (2, 5, 6), and considerably more than that of the *in vitro* Mehler reaction, 2.5 to 12.5 μM (4). Thus, it is difficult to interpret precisely such an uptake response.

Recent reports by a number of workers (21, 22, 27) have strongly emphasized the notion that pseudocyclic photophosphorylation is a minor pathway contribution to the production of additional ATP within the chloroplast. Instead, cyclic photophosphorylation mediated by electron flow from ferredoxin to Cyt b_6 and plastoquinone has been proposed to act as the major energy balancing reaction (27). Pseudocyclic electron flow to O_2 has been proposed to play a role in the operation of cyclic photosphosphorylation by ensuring that intermediates such as plastoquinone and Cyt b_6 do not become over-reduced and are 'poised' so that cyclic flow can occur. In this role, pseudocyclic flow to O_2, although never quantitated in these systems, does not represent more than a few percent of whole chain electron transport (O_2-evolution), and is

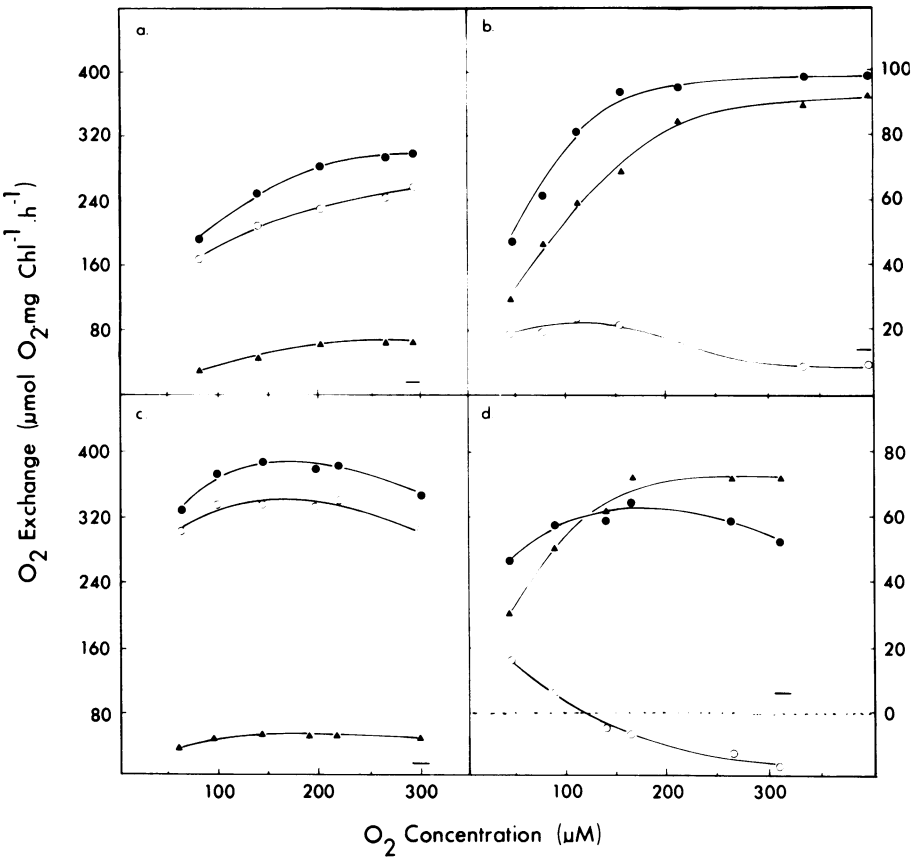

FIG. 4. Effect of O_2 concentrations on O_2 exchange in isolated *Xanthium* cells (a and b) and spinach chloroplasts (c and d). a and c, experiments done at 200 and 300 μM CO_2, respectively; b and d, done at 4 μM and 1 μM CO_2. Catalase was included (1,000 units/ml). Light intensity was 1,500 μE m^{-2} s^{-1}. ▲, O_2-uptake; ●, O_2-evolution; ○, net O_2-evolution.

not quantitatively important as an ATP producing mechanism.

The best quantitative estimate that one can make of Mehler reaction *in vivo* is at high CO_2 where RuP_2-oxygenase activity is suppressed. Presumably, this is where it will be lowest, due to the higher NADP levels; however, it is possible at this point to ask the question whether O_2-uptake here is sufficient to support pseudocyclic electron flow and thus pseudocyclic photophosphorylation at such a rate to provide the ATP necessary for the PCR cycle. It is possible to calculate how much flow to O_2 in theory would be necessary under these conditions if we assume that (a) the ATP to $2e^-$ ratio is 1.33, (b) the stoichiometry of O_2 uptake to electron flow is 4 (due to catalase and superoxide dismutase activity), and (c) to fix CO_2 via the carbon reduction cycle requires 1.5 ATP per NADPH (assuming no other energy consuming reactions such as photorespiration or nitrate reduction).

Using these assumptions, O_2-uptake via pseudocyclic electron transport should represent 11.3% of gross O_2-evolution for an ATP-NADPH ratio of 3:2. In Figure 1, O_2-uptake in chloroplasts was 11% total O_2 evolution at high CO_2 and 250 μM O_2, and 18% in cells. At 35 to 60 μM O_2, these values were 16 and 12.5%, respectively. Thus, in all cases O_2-uptake was sufficient to support pseudocyclic photophosphorylation at a rate which would balance the ATP requirements of the chloroplast during CO_2 fixation.

Aside from the data presented here, only the results of Heber *et al.* (14) and Marsho *et al.* (21) represent an attempt to quantitate the '*in vivo*' Mehler reaction. Heber *et al.* (14) found that in spinach chloroplasts O_2-uptake was between 9.5 and 27% of total O_2 evolution at saturating CO_2 and light intensities ranging from 9 to 120 wm^{-2}. However, Marsho *et al.* (21), using cells and chloroplasts of spinach, found that at saturating CO_2, O_2-uptake was only between 2 and 3% of total O_2-evolution. Results presented here are similar to those of Heber *et al.* (14) and support the concept that pseudocyclic electron flow to O_2 is potentially a major energy balancing mechanism within the chloroplast.

One of the arguments for a major involvement of cyclic photophosphorylation in energy balance is the accepted notion that Mehler reaction O_2-uptake is too low to be an important energy balancing reaction. Clearly, evidence here and that presented by Heber *et al.* (14) is opposed to this. O_2 uptake measurements on whole leaves (7, 9) of C_3 plants at high CO_2 also indicate that there is sufficient O_2-uptake via a Mehler-type reaction to be quantitatively important in energy balancing. With regard to this point, it is important to note stimulation of both gross O_2-evolution and net O_2-evolution in *Xanthium* cells (Fig. 4a) at high CO_2, by increasing O_2 concentrations. O_2 uptake increased over the same range and may certainly be invoked quantitatively in the production of extra ATP. Chloroplasts (Fig. 4c), while not stimulated to the same extent as cells, increased again over the same range of O_2 concentrations that increased O_2-uptake. Such stimulation of net O_2-evolution on CO_2 fixation at high CO_2 has been noted by Cornic and Louason (10) in intact leaves. Thus, reasonably high levels of O_2 appear to be required for maximum rates of photosynthesis. We do not argue that cyclic photophosphorylation does not occur at all *in vivo*; however, we feel that future studies in this area should consider the fact that there is sufficient Mehler reaction-based pseudocyclic electron flow to balance the energy requirements of the chloroplast.

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