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Role of O_2 and Mitochondrial Respiration in a Photosynthetic Stimulation of Oat Protoplast Acidification of a Surrounding Medium[1]

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BARBARA M. KELLY
Department of Botany, University of Massachusetts, Amherst, Massachusetts 01003

ABSTRACT

Some photosynthetically stimulated acidification of the medium by oat (*Avena sativa* L. cv Garry) leaf protoplasts required respiration. The requisite respiration (a) had a low apparent affinity for O_2, (b) was blocked by cyanide plus salicylhydroxamic acid, (c) characterized protoplasts and mitochondria isolated from protoplasts, (d) could be induced in leaf segments, and (e) appeared to result from an inhibition of mitochondrial respiration that included the cytochrome pathway.

Carbon monoxide and cyanide prevented acidification of weakly photosynthesizing suspensions. Salicylhydroxamic acid had no effect on acidification, indicating a specific dependence upon cyanide-sensitive respiration. Photosynthesis stimulated acidification through stable products, and exogenously supplied O_2 stimulated acidification. The acidification response to O_2 was additive to the response to photosynthesis at subsaturating levels of light, indicating a common mode of action. Oligomycin prevented stimulation of acidification by low levels of photosynthetic activity; this stimulation appeared to be due to O_2-induced increases in mitochondrial energy production. Oligomycin only partially inhibited stimulation of acidification by higher levels of light; this stimulation appeared to be partially dependent upon photophosphorylation. Therefore, oligomycin-sensitive acidification of the medium appeared to reflect changes in mitochondrial energy production in photosynthesizing protoplasts.

The preceding paper provided evidence that photosynthetic activity mediated light stimulation of acidification of suspensions of oat leaf protoplasts (14). In addition, the system was described, protoplast viability was evaluated, and the validity of estimates of rates of acidification was assessed.

Some acidification occurred in the dark; it appeared to continue upon exposure to light and to continue to be independent of photosynthesis (14). This suggests a possible additional dependence upon mitochondrial respiration. Mitochondrial activity is difficult to assess in photosynthesizing cells (13, 23). This study was undertaken to determine if acidification of the medium could be used to detect changes in respiration as photosynthetic activity increased.

Acidification requires mitochondrial respiration in photosynthesizing *Asparagus* cells; however, rates of acidification appear to reflect a competition between acidification and photosynthesis for a limiting substrate rather than changes in rates of respiration (3). Acidification is commonly stimulated by photosynthesis (9, 19, 20).

Mitochondrial respiration in oat leaf protoplasts increased in response to high concentrations of O_2. This was examined through the use of inhibitors, isolation of mitochondria, and reduction of the apparent respiratory affinity for O_2 in leaf segments. The unusual (5) response to O_2 permitted manipulation of respiratory rates by O_2 as well as by inhibitors. This facilitated analysis of the acidification response to changes in mitochondrial respiration.

MATERIALS AND METHODS

Plant Material. Growth and harvesting of oat (*Avena sativa* L. cv Garry) seedlings and preparation of protoplasts were as described in the preceding paper (14). Protoplasts were used within a few hours of preparation.

Leaf segments were obtained by peeling the abaxial cuticle and epidermis from a section 4 cm long, 0.5 cm from the tip of the primary leaf; this section was sliced into 0.25-cm^2 segments. The segments (140 mg) were washed in 5 mM Hepes and transferred to 30 ml of the medium used for protoplasts with the modification that sorbitol was omitted (14). The segments were stirred until they no longer floated to the surface (about 2 h) and kept in the medium to complete a total equilibration time of 3.5 h. A portion of equilibrated segments, equivalent to about 0.2 mg Chl, was washed three times, suspended in 1.3 ml of sorbitol-free medium, and assayed for O_2 uptake.

The equilibration medium was maintained at about 250 nmol O_2/ml. For low O_2 treatments, the O_2 level was reduced by equilibrating the segments in sealed flasks in the dark. Protoplast lysates were prepared as follows: 1.5×10^6 protoplasts/ml were vortexed vigorously in sorbitol-free medium and centrifuged at 600*g* for 10 min; the supernatant was used for equilibration.

Isolation of Mitochondria. Mitochondria were obtained by homogenizing 6×10^7 protoplasts in 20 ml of Douce's isolation medium (7) with 10 strokes of a 0.5-inch Teflon homogenizer or by grinding 25 g of primary leaves with a mortar and pestle (1.5 min/3 g leaves) in Douce's isolation medium.

Washed mitochondria were prepared as described by Ikuma and Bonner (12) with three modifications: (a) the mitochondria were not subjected to forces above 6000*g*, (b) 29 mM sucrose was added to the wash buffer, and (c) the chloroplasts were precipitated at 500*g*, washed, and reprecipitated. The mitochondria were usually obtained from the supernatant of the second 500*g* centrifugation. This produced a clean preparation when purified and did not alter the results. Mitochondria were purified through the use of Percoll gradients as described by Nishimura *et al.* (18), with the first and third modifications.

O_2 uptake was monitored for mitochondria suspended in 1.0 ml Douce's reaction medium (7). Chl contamination (mg Chl/mg

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protein) was 30% for washed mitochondria isolated directly from leaves, 3% for washed mitochondria isolated from protoplasts, and 0 for purified mitochondria.

Assays. Acidification and O_2 exchange were monitored as described in the preceding paper (14). Cyt oxidase was assayed by the polarographic method of Smith and Camerino (22), modified to include 0.5% Tween 20 in the reaction medium.

Proteins were determined by the method of Bradford (4) with the use of the prepared Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA). Chl was determined by the method of Wintermanns and de Mots (27); it was extracted from leaf segments by a 45-min immersion in 95% ethanol.

Treatment with O_2 or CO. The gases were added to suspensions during the acidification assay by flushing the air space with the pure gas and swirling the suspensions for 5 s. O_2 was added every 15 min; this maintained the medium at an O_2 concentration in excess of 250 nmol/ml. CO was added once, 45 min after the start of the assay. The gases were added to suspensions in the O_2 electrode chamber by bubbling the pure gas through the medium for 1 to 2 s.

Presentation of Data. Acidification is usually expressed as a percentage of the rate of acidification of the control, a suspension exposed to the optimum quantum flux density for this response, 50 $\mu E/m^2 \cdot s$ white light (14), and included in all experiments. The rate of acidification of controls varied from 5.8 to 15.1 neq proton/min·ml; the average rate was 10.6 ± 2.5 neq protons/min·ml (20 random experiments).

Unless otherwise stated, the data are expressed as the average of at least three experiments using separate protoplast preparations ±SD.

Chemicals. Oligomycin was dissolved in 20% ethanol. Antimycin was dissolved in 50% acetone. ADP, SHAM,[2] and KCN were dissolved in water and adjusted to pH 6.8 to 7.0. All were obtained from Sigma. The sources of other chemicals, as well as light sources and measurements, are given in the preceding paper (14).

RESULTS

O_2 Uptake by Protoplasts. The rate of O_2 uptake in the dark decreased as the O_2 concentration of the medium was reduced. The rate of uptake could be increased by permitting a few minutes of photosynthesis (Fig. 1) or by adding O_2 as described in "Materials and Methods." The O_2 uptake appeared to be due to mitochondrial respiration; at 200 nmol O_2/ml, uptake could be reduced 60% by treatment with 2 mM KCN and blocked by 2 mM SHAM plus 2 mM KCN (Fig. 2).

High concentrations of O_2 can stimulate alternative pathway activity (25). However, SHAM, an inhibitor of the alternative oxidase (1), had little effect on O_2 uptake when used alone (Fig. 2). Protoplast responses to CN and SHAM indicate (a) that respiration primarily occurred through the Cyt oxidase pathway in the absence of CN and (b) that CN-sensitive respiration required unusually high concentrations of O_2.

The coupling of protoplast respiration was investigated. The RC ratio, calculated as the ratio of the rate of respiration in the presence of CCCP to that in the presence of oligomycin (23), was 2.3 at 200 nmol O_2/ml (Fig. 2). In analogy with CN stimulation of alternative pathway activity (1, 24), treatment with oligomycin might stimulate other O_2 uptake mechanisms by reducing competition with Cyt oxidase. Therefore, the RC ratio was also calculated using SHAM plus oligomycin. The addition of SHAM reduced residual O_2 uptake and raised the RC ratio to 2.9. The RC values are consistent with moderate respiratory coupling to ATP production at the higher O_2 concentrations (23). Changes in

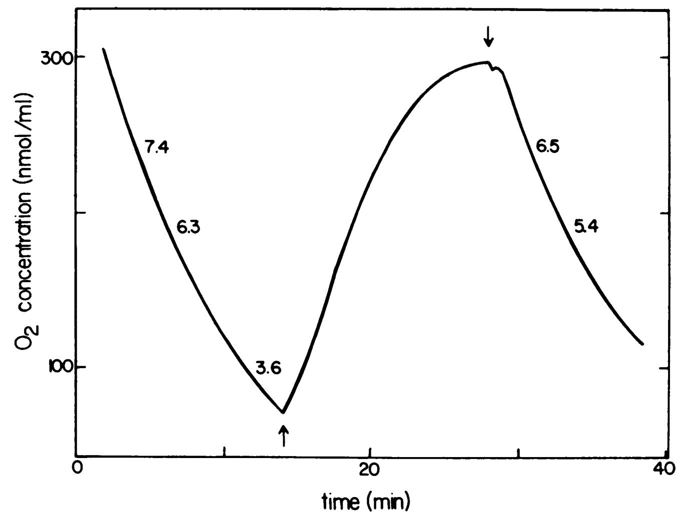

FIG. 1. Effect of O_2 concentration on rates of O_2 uptake by protoplasts. O_2 uptake was monitored polarographically after bubbling O_2 for 1 to 2 s through suspensions that had been incubated at room temperature for 1 h in the dark. Values are rates of uptake in μmol O_2/mg Chl·h. 1 ml = 2 × 10^6 protoplasts = 200 μg Chl. ↑, light on; ↓, light off.

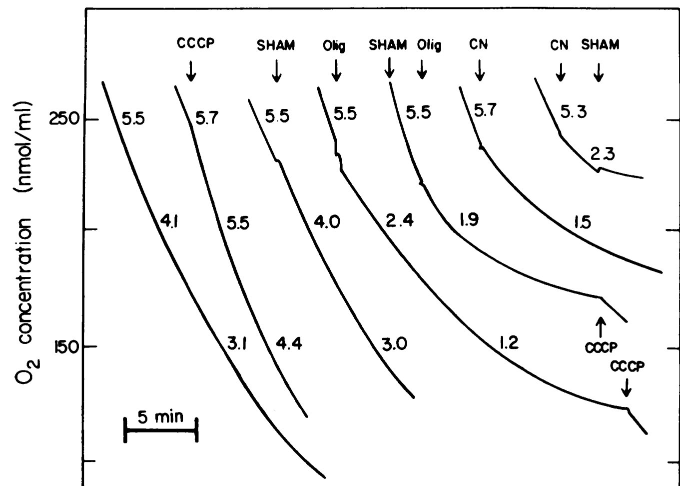

FIG. 2. Effect of inhibitors and uncoupling agents on O_2 uptake by protoplasts. Suspensions were prepared, uptake was measured, and protoplast concentrations were as described in Fig. 1. KCN and SHAM = 2 mM; oligomycin (Olig) = 20 ng/ml; CCCP = 2 μM. Values are rates of uptake in μmol O_2/mg Chl·h.

rates of O_2 uptake (Fig. 2) indicate that both coupled and uncoupled respiration increased as O_2 concentrations were raised.

Protoplasts took up O_2 at 5.9 ± 0.7 μmol O_2/mg Chl·h at an O_2 concentration of 250 nmol/ml. This is approximately the rate of respiration of *Nicotiana* leaves and protoplasts (17) and segments of oat leaves (6.0 ± 1.3 μmol O_2/mg Chl·h). Thus, the oat protoplasts appeared to require 250 nmol O_2/ml for normal rates of coupled, CN-sensitive respiration.

Cytochrome Oxidase Activity and Mitochondrial O_2 Uptake. The affinity of Cyt oxidase for O_2 is too high to permit stimulation by 250 nmol O_2/ml. Therefore, the response to O_2 was further examined.

Cyt oxidase, assayed in detergent-treated protoplasts and mitochondria, took up O_2 at a constant rate from 250 to nearly 0 nmol O_2/ml (data not shown). The uptake could be blocked by 2 mM KCN. This indicates that the solubilized enzyme had an apparently normal (5) affinity for O_2.

The apparent O_2 affinity of isolated mitochondria varied with

[2] Abbreviations: SHAM, salicylhydroxamic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; P:O, ADP addition: amount of O_2 uptake.

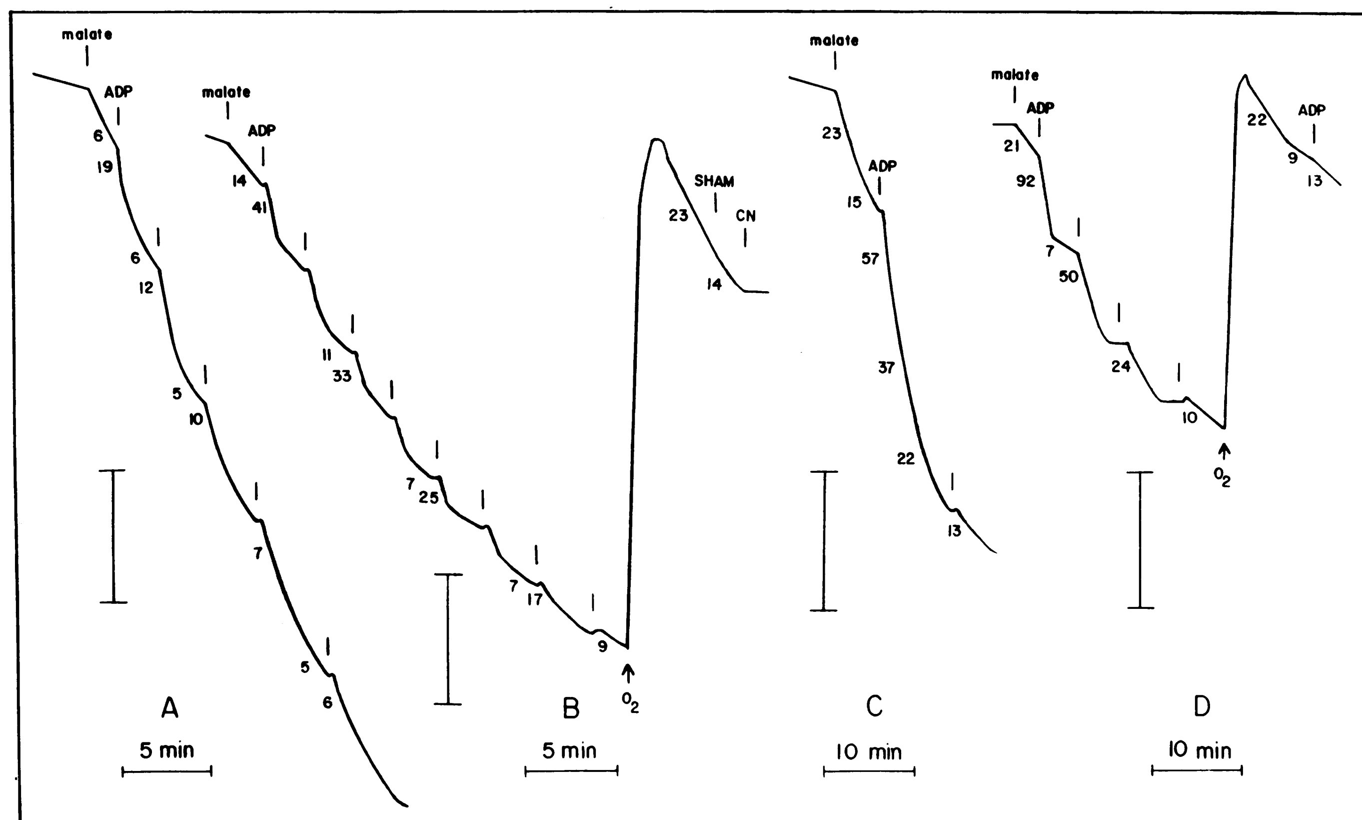

FIG. 3. Malate oxidation by mitochondria. Mitochondria were prepared and suspended in reaction medium as described in "Materials and Methods." Pure O_2 was bubbled through the medium for 1 to 2 s where indicated. Malate = 30 mM; KCN and SHAM = 2 mM. Values are rates of uptake in nmol O_2/mg protein·min. Vertical bars = 50 nmol O_2/ml. Initial nmol O_2/ml: A and C ≃ 260; B and D ≃ 240. A, Washed mitochondria isolated directly from leaves, 100 μM ADP; B and C, washed mitochondria isolated from protoplasts; B, 100 μM ADP; C, 300 μM ADP; D, purified mitochondria isolated from protoplasts, 150 μM ADP; |, Repeated addition.

the substrate being oxidized and the source of the mitochondria. When oxidizing malate, mitochondria took up O_2 at a reduced rate at lower O_2 concentrations (Fig. 3). For mitochondria isolated from protoplasts, (a) the rate of O_2 uptake could be stimulated slightly by raising the O_2 concentration, (b) the ability to use ADP appeared to decrease at lower O_2 concentrations, as indicated by increased P:O ratios, and (c) the apparent affinity for O_2 was lower than that of mitochondria isolated directly from leaves. Purification of these mitochondria increased the O_2 requirement (Fig. 3).

NADH (1.5 mM) was oxidized at a constant rate to nearly 0 nmol O_2/ml by mitochondria isolated directly from leaves (Fig. 4). Utilization of NADH by mitochondria isolated from protoplasts was somewhat variable; however, rates of O_2 uptake were usually constant to about 150 nmol O_2/ml and decreased below that level (Fig. 4).

Succinate was not readily oxidized by mitochondria isolated from protoplasts (Fig. 4). However, when isolated directly from leaves, mitochondria oxidized succinate at a constant rate from 250 to nearly 0 nmol O_2/ml (O_2 uptake = 22 nmol O_2/mg protein·min; RC = 0).

Mitochondria isolated from protoplasts had good initial RC values (2.2 to 4.4) and P:O ratios (2 to 3). The data suggest that oat protoplast mitochondria had a reduced ability to use substrate.

O_2 Uptake by Leaf Segments. When leaf segments were equilibrated aerobically with sorbitol-free medium, O_2 uptake curves were obtained that were linear to nearly 0 nmol O_2/ml, irrespective of the number of segments (Fig. 5).

The rate of O_2 uptake became dependent upon the O_2 concentration of the medium if leaf segments were equilibrated (a) with medium containing 0.6 M sorbitol, (b) at low O_2 concentrations, or (c) with a protoplast lysate. Combining sorbitol and low O_2 treatments further reduced the apparent respiratory affinity for O_2 (Fig. 5). The O_2 uptake increased upon addition of fresh medium at a higher O_2 concentration and was sensitive to CN plus SHAM (data not shown). These data indicate that the apparent respiratory affinity for O_2 could be reduced by the osmoticum (14) and O_2 concentration of protoplast suspensions and by endogenous substances.

O_2 Stimulation of Acidification. The medium of suspensions kept in the dark averaged 115 ± 17 nmol O_2/ml 60 min after the start of the acidification assay. Maintaining the O_2 concentration at a minimum of 250 nmol/ml nearly doubled the rate of respiration (Fig. 1) and doubled the rate of acidification (Table I).

A further increase to a minimum of 300 nmol O_2/ml did not alter the rate of acidification (data not shown) or the percentage of protoplasts remaining intact through the assay (82 ± 24 and 81 ± 30% for a minimum of 250 and 300 nmol O_2/ml, respectively). Therefore, the acidification response to 250 nmol O_2/ml appeared to be the maximum response to exogenously supplied O_2. The response to O_2 was two-thirds of the maximum response to photosynthesis.

O_2 stimulation of acidification was additive to stimulation by suboptimal levels of photosynthesis, but O_2 did not enhance the acidification response to optimal photosynthetic activity (Table I). (The acidification response to different quantum flux densities of light is given in the preceding paper [14].) This suggests that O_2 stimulated acidification through a common pathway with photosynthesis.

Therefore, photosynthetic stimulation of acidification was tested

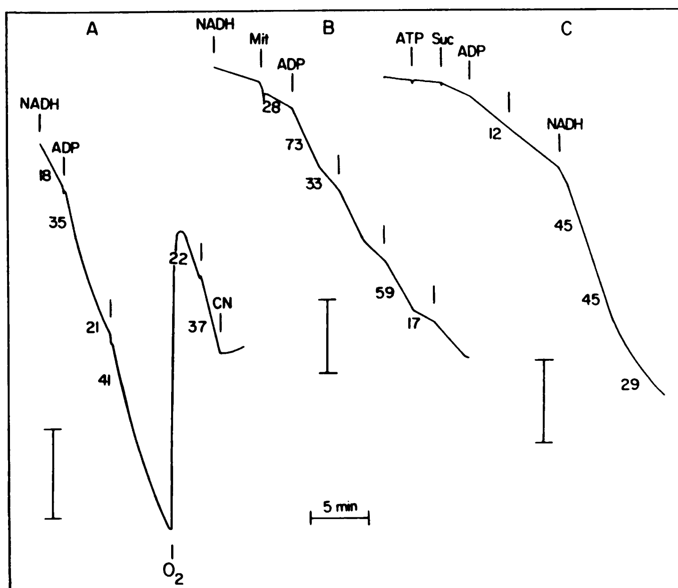

FIG. 4. NADH and succinate (Suc) oxidation by isolated mitochondria (Mit). Preparation and O_2 addition were as in Figure 3. NADH = 1.5 mM; succinate = 10 mM; ADP and ATP = 150 μM; KCN = 2 mM. Values are rates of uptake in nmol O_2/mg protein·min. Vertical bars = 50 nmol O_2/ml. Initial nmol O_2/ml: A ≃ 220, B and C ≃ 250. A, Washed mitochondria isolated directly from leaves; B and C, washed mitochondria isolated from protoplasts. |, repeated addition.

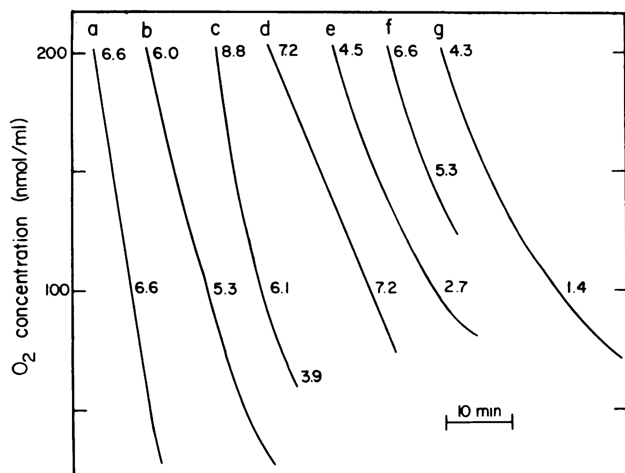

FIG. 5. O_2 uptake by leaf segments. After 3.5 h of equilibration with medium as described in "Materials and Methods," peeled leaf segments were washed and suspended in fresh, sorbitol-free medium, and O_2 uptake was monitored polarographically. Values are rates of O_2 uptake in μmol/mg Chl·h. Equilibration: with sorbitol-free medium, a, d, and e; medium containing 0.6 M sorbitol, b and g; protoplast lysate, c and f. Equilibration at low O_2 levels, a and g. μg Chl/ml: a = 286; b = 264; c = 179; d = 66; e = 140; f = 104; g = 130.

for a dependence upon stable products. Acidification continued at nearly maximal rates in suspensions that were transferred to the dark after a 45-min exposure to 50 $\mu E/m^2 \cdot s$ white light (Table II). When extra O_2 was supplied to prevent rapid depletion of photosynthetically generated O_2 (Fig. 1), the transferred protoplasts acidified the medium at the maximum rate (Table II). These data indicate that all photosynthetic stimulation of acidification was dependent upon stable products, one of which was O_2.

Acidification Response to Respiratory Inhibitors. Inhibitors were used to test for a dependence upon mitochondrial respiration. Ten μM antimycin A (data not shown) or 2 mM KCN (Table III) prevented acidification. SHAM (2 mM) had no effect when used alone (rate of acidification = 98 ± 27% that of untreated controls) or when used with CN (Table III). These data indicate (a) that acidification was not dependent upon alternative pathway activity, and (b) that acidification required mitochondrial respiration when suspensions were not exposed to light.

CO was used to test the respiratory dependence of photosynthetically stimulated acidification since CN (8, 13) and antimycin (11) inhibit photosynthesis. CO inhibited neither initial rates of O_2 evolution nor subsequent maintenance of O_2 levels (data not shown). CO prevented acidification of suspensions that were

Table I. *O_2 Stimulation of Acidification*

Changes in acidity were monitored by measuring the pH of the medium every 15 min; the change in pH from 45 to 90 min after the start of the assay determined the rate of acidification. O_2 was added every 15 min by flushing the air space above the suspensions with the pure gas and mixing the sample. Protoplast concentration was 2×10^6/ml (200 μg Chl/ml). Values are the average of at least three experiments using separate protoplast preparations ±SD.

White Light Regime	Rate of Acidification
$\mu E/m^2 \cdot s$	*% stimulation by 250 nmol O_2/ml*
0	100 ± 56
5	63 ± 13
10	39 ± 15
50[a]	−6 ± 12
150	6 ± 20
50 +2 μM DCMU	93 ± 54

[a] Optimal quantum flux density for acidification.

Table II. *Stimulation of Acidification by Stable Products of Photosynthesis*

Rates of acidification were determined, O_2 was added, and protoplast concentration was as described in Table I. Values are the average of four experiments using separate protoplast preparations ±SD.

Treatment		Acidification Rates
0 to 45 min	45 to 90 min	(45–60 min)
		% control[a]
Light[b]	Dark	87 ± 26
Light +2 μM DCMU	Dark	28 ± 9
Light	Dark + O_2	114 ± 13
Dark	Dark + O_2	65 ± 34

[a] Suspensions exposed to light for 90 min.
[b] 50 $\mu E/m^2 \cdot s$.

Table III. *Effect of CN and SHAM on Acidification*

Rates of acidification were determined, O_2 was added, and protoplast concentration was as described in Table I. KCN (2 mM) and SHAM (2 mM) additions were made 45 min after the start of the assay. Values are the average of at least three experiments using separate protoplast preparations ±SD.

Treatment	Rate of Acidification		
	No addition	CN	CN + SHAM
		% control[a]	
Dark + O_2	75 ± 14	2 ± 4	6 ± 7
50 $\mu E/m^2 \cdot s$ white light	100	23 ± 22	22 ± 15

[a] Untreated suspensions exposed to 50 $\mu E/m^2 \cdot s$ white light.

Table IV. *Inhibition of Acidification by CO*

Rates of acidification were determined, O_2 was added, and protoplast concentration was as described in Table I. CO was added once, alone or with O_2, 45 min after the start of the assay. Values are the average of at least three experiments using separate protoplast preparations ±SD.

Treatment	No Addition	CO	CO + O_2
		% control[a]	
Dark	33 ± 12	8 ± 8	64 ± 1
17 $\mu E/m^2 \cdot s$ green light	70 ± 22	9 ± 9	103 ± 4
5 $\mu E/m^2 \cdot s$ white light	55 ± 14	39 ± 20	

[a] Untreated suspensions exposed to 50 $\mu E/m^2 \cdot s$ white light.

Table V. *Effect of Oligomycin on Increases in Acidification Induced by O_2 and Photosynthesis*

Rates of acidification were determined, O_2 was added, and protoplast concentration was as described in Table I. Rates are defined in terms of the rate of acidification of suspensions exposed to 50 $\mu E/m^2 \cdot s$ white light. Values are the average of at least three experiments using separate protoplast preparations.

White Light Regime	Rate of Acidification	
	−Oligomycin[a]	+Oligomycin[a]
$\mu E/m^2 \cdot s$	*% stimulation over dark rate*	
0	0	0
0 + O_2	100 ± 18	0
10	91 ± 17	0
10 + O_2	133 ± 9	0
35	173 ± 8	0
50	203	36 ± 17
100	158 ± 7	58 ± 2
150	145 ± 11	73 ± 9
50 +2 μM DCMU	15 ± 11	0

[a] 20 ng/ml.

exposed to low levels of green light (Table IV).

Inhibition of acidification by CO could be reversed by increasing the O_2 concentration or by exposure to white light (Table IV). The amount of light was restricted to maintain a low O_2 concentration (O_2 evolution in μmol/mg Chl·h; 5 $\mu E/m^2 \cdot s$ white light = 0.6 ± 0.2; 17 $\mu E/m^2 \cdot s$ green light = 1.2 ± 0.6). Thus, the results of two separate tests are consistent with an inhibition due to CO-Cyt oxidase binding (21).

Low levels of white light weakly reversed CO inhibition of O_2 uptake by DCMU-treated protoplasts (% reversal: 5 $\mu E/m^2 \cdot s$ = 14 ± 9, 7 $\mu E/m^2 \cdot s$ = 16 ± 8; 10 $\mu E/m^2 \cdot s$ = 28 ± 16). To quantitate reversal, O_2 uptake was tested at about 180 nmol O_2/ml (uptake in μmol O_2/mg Chl·h: ≃ 4; +CO ≃ 2). Thus, alternative pathway activity may have obscured some changes in uptake. Nonetheless, this response to light provides some support for the evidence that CO inhibition of acidification was due to inhibition of mitochondrial respiration. Therefore, the data indicate that low levels of photosynthetic activity required concomitant mitochondrial respiration to stimulate acidification.

Oligomycin (20 ng/ml) weakly inhibited photosynthesis (13 ± 16%); therefore, it was used to evaluate the dependence of acidification upon coupled respiration. Oligomycin inhibited O_2 uptake by less than 50% at the O_2 concentration of some samples (Fig. 2); however, it prevented stimulation of acidification by O_2 or low levels of light (Table V). These data suggest a specific dependence upon mitochondrial energy production.

Oligomycin was less effective at higher quantum flux densities of light (Table V). The oligomycin-resistant acidification was sensitive to DCMU. Thus, photosynthetic stimulation of acidification appeared to be less dependent upon mitochondrial energy production in these suspensions.

DISCUSSION

Mitochondrial energy production, as reflected by an acidification of the medium by oat leaf protoplasts, increased in response to photosynthetically produced O_2 and appeared to decrease after photosynthetic activity reached moderate levels. The evidence is as follows: (a) the apparent O_2 affinity of coupled, CN-sensitive mitochondrial respiration was low enough for high concentrations of O_2 to stimulate respiration; (b) mitochondrial respiration was required for most photosynthetic stimulation of acidification; (c) photosynthesis partially stimulated acidification by generating O_2; and (d) acidification was less sensitive to oligomycin in suspensions with moderate photosynthetic activity.

Protoplasts required 250 nmol O_2/ml to sustain apparently normal rates of coupled, CN-sensitive respiration. SHAM-sensitive and uncoupled respiration also increased at high O_2 concentrations, particularly after coupled or CN-sensitive respiration was blocked. Alternative pathway activity may increase in response to high concentrations of O_2 (25); however, CN-sensitive respiration should not be affected (5). And indeed, solubilized Cyt oxidase had an apparently normal (5) affinity for O_2.

Mitochondria isolated from protoplasts had RC values and P:O ratios that were consistent with intact, well coupled organelles. Nonetheless, they required higher concentrations of O_2, and they appeared to use all substrates less efficiently than mitochondria isolated from leaves.

Leaf segments had a high apparent respiratory affinity for O_2. However, this could be reduced by exposure to the osmoticum and O_2 concentrations of protoplast suspensions or a sorbitol-free protoplast lysate. Thus, the high osmoticum and low O_2 concentrations to which protoplasts were subjected may have decreased mitochondrial ability to use substrate. It is also possible that these conditions increased the effective concentration of endogenous substances able to alter the respiratory affinity for O_2.

Morisset (16) found exposure to high osmoticum or low O_2 concentrations to inhibit subsequent mitochondrial respiration in tomato roots. There, too, the apparent affinity of Cyt oxidase for O_2 was high; however, mitochondrial morphology was altered. Morisset concluded that ultrastructural changes had been induced that affected the *in situ* activity of the entire electron chain. This may also have occurred in the oat protoplasts. If so, the apparent affinity for O_2 could be reduced without affecting the activity of solubilized enzymes.

Evidence that protoplast acidification of the medium was dependent upon mitochondrial respiration was obtained by examining the acidification response to respiratory inhibitors and O_2. Acidification was stimulated by O_2. CO and CN prevented acidification, including that stimulated by low levels of photosynthesis; SHAM had no effect. Therefore, most acidification was concluded to be dependent upon CN-sensitive mitochondrial respiration.

Oligomycin inhibited acidification more effectively than it inhibited respiration. This could result from nonmitochondrial mediation of acidification since oligomycin inhibits a plasmalemma proton pump in some tissues (6). However, the sensitivity of acidification to oligomycin decreased with increasing light at quantum flux densities that were supraoptimal for the acidification response to photosynthesis. This observation is more consistent with expected changes in mitochondrial energy production (13) than in the activity of a plasmalemma pump. Consequently, acidification was concluded specifically to require coupled respiration.

Photosynthesis stimulated acidification through its stable products, one of which was O_2. Therefore, inasmuch as protoplast respiration could increase in response to the appropriate concentrations of O_2, photosynthetic stimulation of acidification ap-

peared to result, in part, from increased mitochondrial energy production.

The maximum acidification response to photosynthesis was greater than to O_2 alone, indicating some dependence upon another product. Photophosphorylation can be supported by the Mehler reaction (8) and, thus, should occur (2) even in CO_2-limited (14) protoplasts. The concentration of oligomycin used does not inhibit photophosphorylation (26). Therefore, the increased resistance to oligomycin at higher levels of light is consistent with some dependence of acidification upon photophosphorylation after photosynthesis became moderately active.

Contrary to expectations (13), tricarboxylic acid cycle activity and mitochondrial O_2 uptake continue at the dark rate in some photosynthesizing tissues (10, 15). Mitochondrial energy production usually cannot be estimated in photosynthesizing cells, but it is expected to be minimal (13, 23). The respiratory dependence of protoplast acidification would be consistent with some reduction in mitochondrial energy production in the more actively photosynthesizing suspensions.

The abnormal protoplast respiratory requirement for high concentrations of O_2 facilitated demonstration of a relationship between respiration and acidification; however, it hinders extrapolation beyond the protoplast system. Oat leaves also have an acidification response that occurs in the dark but can be stimulated by photosynthesis; the stimulation by photosynthesis is enhanced by high osmoticum (9). Therefore, this acidification response appears to be similar to that of the protoplasts; thus, it might provide information more applicable to respiration in the intact photosynthesizing plant.

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