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Light-Stimulated Changes in the Acidity of Suspensions of Oat Protoplasts

DEPENDENCE UPON PHOTOSYNTHESIS[1]

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ABSTRACT

Light induced an alkalinization and stimulated a subsequent acidification of the medium surrounding oat (*Avena sativa* L. cv Garry) leaf protoplasts. Blue light was less effective than would be predicted from photosynthetic action spectra. Nonetheless, 3-(3,4-dichlorophenyl)-1,1-dimethylurea prevented alkalinization and reduced acidification to the dark rate for protoplast suspensions exposed to all light regimes tested.

Alkalinization increased in parallel with initial rates of O_2 evolution as the quantum flux density of white light was raised to 75 microeinsteins per square meter per second. Alkalinization was accompanied by a decrease in the CO_2 content of the medium; therefore, it was attributed to photosynthetically induced CO_2 uptake. The effect of CO_2 depletion on the acidity of the medium appeared to be mainly restricted to the first 15 minutes of exposure to light. Consequently, subsequent pH changes primarily reflected a constant net proton efflux. Acidification occurred in the dark, but rates of acidification increased in response to increased light approximately in parallel with changes in a concomitant net O_2 efflux. The results indicated that protoplasts could acidify the medium in response to nonphotosynthetic activity, but that photosynthesis mediated light stimulation of acidification.

Plants acidify or alkalinize a surrounding medium in association with nutrient uptake (5, 10, 16), growth (23), photo- and geotropism (13), and maintenance of membrane potentials (18, 19). The phenomenon is considered to be important in the regulation of cytoplasmic pH (20). Acidification, in particular, is usually attributed to the activity of an electrogenic proton pump (19) and would be expected to require energy. Acidification is often dependent upon photosynthesis (7, 16, 18, 19); however, it may additionally occur in the dark (7, 16), it can be stimulated by phytochrome conversion (3), and acidification may be dependent upon mitochondrial respiration in photosynthesizing cells (2).

Consequently, acidification of the medium might reveal interactions between photosynthesis and respiration. This possibility has been tested by examining the photosynthetic and respiratory dependence of changes in the acidity of the medium surrounding oat leaf protoplasts.

Protoplasts were used for several reasons. First, they could be gradient-purified; therefore, they provided relatively pure, homogeneous suspensions of photosynthetically competent cells (4). Second, removal of cell walls reduced the buffering effect of the cell exterior (17). Third, respiratory and photosynthetic activity of mesophyll protoplasts has been reported to compare well with that of leaf tissue (12).

Photosynthesis and respiration must be limiting for the acidification response if acidification is to reflect changes in the rates of these activities. This paper, which is the first of two, reports on the photosynthetic dependence of an acidification response of oat protoplasts. It includes (a) a description of the system, (b) an evaluation of the extent to which photosynthetic activity was limiting for light-induced acidity changes, and (c) an assessment of the effect of an alkalinization response upon the apparent rate of acidification.

MATERIALS AND METHODS

Preparation of Protoplasts. Seedlings of *Avena sativa* L. cv Garry were grown at 21°C under a 16-h light and 8-h dark cycle. A mixture of cool white fluorescent and incandescent bulbs provided approximately 150 $\mu E/m^2 \cdot s$ white light. The seedlings were watered daily with half-strength Hoagland solution. Primary leaves were harvested 5 to 6 h after the beginning of the photoperiod, 7 d after planting.

Protoplasts were isolated and purified as described by Rubinstein (21) with the modification that the concentrations of T20 Dextran[2] and sorbitol in the gradient were 14% (w/w) and 0.4 M, respectively. One g of leaves yielded 12×10^6 protoplasts. The purified protoplasts were washed, centrifuged at 400*g* for 2.5 min, and resuspended in assay medium at a concentration of 2×10^6 protoplasts/ml. The assay and incubation medium was always 2 mM Hepes, 0.2 mM Pipes, 0.6 M sorbitol, 29 mM sucrose, 1 mM glucose, 1 mM $Ca(NO_3)_2$, 0.25 mM $MgCl_2$, 3 mM KCl, 1 mM KOH, and 1 mM NaOH (pH 7.2). Bicarbonate and phosphate, which are used in some protoplast media (12, 21), were omitted to avoid changes in the buffering capacity that could be caused by uptake of these compounds or equilibration of CO_2 with the air phase. Assays were performed on freshly prepared protoplasts.

Calcofluor white ST (0.1%, w/v), dissolved in the assay medium was used to check for the presence of cell walls (14). The stain was applied for 5 min. The preparations were washed twice and observed with a Reichert Zetopan epifluorescent microscope, using a UG1 excitation filter (maximum transmission = 350 nm) and a KV-418 barrier filter (passes wavelengths above 400 nm). Walled cells in a macerated tissue suspension showed a green fluorescence that was absent from the protoplast preparation.

Chl was determined by the method of Wintermanns and de Mots (24).

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[2] Abbreviations: T20 dextran, mol wt 20,000 dextran; *c*, speed of light; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; *h*, Planck's constant; λ, wavelength of light; Pipes, 1,4-piperazinediethanesulfonic acid.

Acidification Assay. Prior to the assay, 0.5 ml of the protoplast suspension was incubated in the dark for 1 h at room temperature, in a 75 × 12 mm glass tube. During the assay, the suspensions were exposed to the appropriate light regime and swirled every 7.5 min, and the pH was taken every 15 min with a Radiometer pH meter, equipped with a type 1-D4 glass electrode.

The experiments were done at room temperature (20 to 34°C; average = 25°C). Water baths were used to maintain a uniform temperature for samples exposed to various light regimes during a single experiment. To compare results obtained at different times, the data usually are expressed in terms of the control, a suspension that was exposed to 50 $\mu E/m^2 \cdot s$ white light and included in all experiments. The rate of acidification and the amount of alkalinization of the control varied from 5.8 to 15.1 neq protons/min·ml and 106 to 293 neq protons/ml, respectively (20 random experiments). The average values for these experiments are given in Table I.

CO_2 Concentrations. The CO_2 concentration was determined from the increase in pH that could be induced by bubbling N_2 through a supernatant obtained by centrifuging the protoplast suspension at 600*g* for 5 min.

O_2 Exchange. O_2 evolution or consumption was monitored with a Yellow Springs Instrument O_2 electrode, model 5331. The protoplasts were incubated in the dark for 1 h at room temperature before being transferred to a 1-ml Clark O_2 electrode chamber. To test O_2 evolution in the presence of additional CO_2, CO_2-enriched air was bubbled through the suspensions for 30 s prior to measuring the O_2 concentration.

Calculations. The rate of acidification is defined by the slope between the 45- and 90-min points on the curve obtained when the change in acidity of the medium is plotted against time (Fig. 1).

The amount of alkalinization is determined by an initial deviation from a constant rate of acidification: amount of alkalinization = rate of acidification × 15 min − Δ neq H^+/ml at 15 min. (The rate of acidification from 30 to 45 min is used for suspensions exposed to quantum flux densities above 50 $\mu E/m^2 \cdot s$.) The rate of alkalinization of CCCP-treated suspensions is defined by the slope of the curve representing the change in acidity of the medium.

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

Light. Quantum flux density was measured with a LiCor photometer LI 190S quantum sensor. When necessary, the equivalent value in w/m^2 was determined by the formula $E = hc/\lambda$. The readings for a given light source and distance were highly consistent; however, variability was introduced into quantum flux densities reported for O_2 evolution due to the difficulty of estimating the amount of light within the O_2 electrode chamber.

White light was provided by two 40-w cool white fluorescent bulbs. Broad bands of different wavelengths were provided by projectors equipped with the following Kodak Wratten filters: No. 98 (406 to 470 and 700^+ nm; maximum = 432 nm), No. 74 (496 to 586 and 700^+ nm; maximum = 520 nm), and No. 29 (610 to 700^+ nm; maximum = 665 to 700^+ nm). These filters were used with far-red blocking filter No. 35-5461-3 (Ealing Corp., S. Natick, MA) to prevent transmission of wavelengths above 690 nm.

Chemicals. DCMU was obtained from E. I. DuPont and dissolved in 20% methanol. Mes, Hepes, Pipes, and CCCP were obtained from Sigma. CCCP was dissolved in 20% ethanol. Calcofluor was a gift from Peter K. Hepler (Botany Department, University of Massachusetts).

RESULTS

Description of the Response to Light. Protoplasts that were kept in the dark acidified the medium. Upon exposure to light, the rate of acidification increased following a brief alkalinization (Fig. 1).

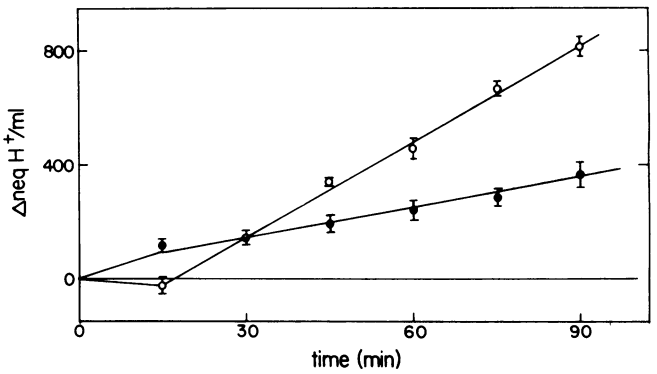

FIG. 1. Changes in the acidity of protoplast suspensions. The change in acidity from the start of the assay was determined from pH measurements of the medium made every 15 min. Suspensions were mixed every 7.5 min. Vertical bars, variation in duplicate samples. 1 ml = 2×10^6 protoplasts = 200 μg Chl. (○), 50 $\mu E/m^2 \cdot s$ white light; (●), dark.

The increase in pH was replaced by a temporary reduction in the rate of acidification in weak light.

The rate of acidification and amount of alkalinization were determined as described in "Materials and Methods." The average values for samples kept in the dark or exposed to 50 $\mu E/m^2 \cdot s$ white light are given in Table I. Mixing the suspensions continuously did not alter the results. Cell counts and the sustained ability to take up O_2 indicated good protoplast viability throughout the assay (Table I). Evans blue dye (1.25%) induced some rupture of the protoplasts (16 ± 8% before and 24 ± 2% after the assay). At the completion of the assay, the dye was excluded by 98 ± 2% of the intact protoplasts. Therefore, alkalinization and acidification of the medium appeared to be a response of relatively homogeneous suspensions of viable protoplasts to light.

Response to Different Wavelengths and DCMU. Red, green, and blue light were compared with respect to their ability to stimulate acidification of the protoplast suspensions (Table II). The relative efficiency of red and green light at stimulating acidification was nearly equal to the relative efficiency with which these wavelengths stimulate photosynthesis in oats (11); in comparison, blue light was much less effective. The irradiances required to stimulate 60% of the maximum acidification response also induced an alkalinization equal to approximately 40% of the control, a suspension exposed to 50 $\mu E/m^2 \cdot s$ white light (data not shown).

DCMU (2 μM) prevented alkalinization and reduced the rate of acidification to that of suspensions kept in the dark in samples exposed to all light regimes tested (Tables I and II). DCMU had no effect on the acidification of suspensions kept in the dark. These data indicate that photosynthetic activity was required for the stimulation of acidification and induction of alkalinization by red, green, blue, and white light. Inasmuch as both alkalinization and acidification responded poorly to blue light, the data are also consistent with a relatively inefficient photosynthetic response to blue light.

Quantum Flux Density Requirements for Acidity Changes and Photosynthesis. The quantum flux density requirements for acidity changes were compared with those of O_2 evolution to determine whether photosynthesis was limiting for acidification and alkalinization. Initial rates of O_2 evolution increased linearly with increasing light to the highest quantum flux density tested, 150 $\mu E/m^2 \cdot s$ white light. The initial rates of O_2 evolution were consistent with good rates of photosynthesis for the amount of light. The rate of O_2 evolution of suspensions exposed to 100 $\mu E/m^2 \cdot s$ white light was one-third and one-ninth, respectively, of that reported for *Nicotiana* leaf discs exposed to 9 times as much light (12) and spinach chloroplasts that were exposed to 15 times as much light

Table I. *Effect of Light and Continuous Mixing on Acidification, Alkalinization, and Protoplast Viability*

Acidity changes were measured as described in Figure 1, except that mixing was continuous where indicated. Rates of acidification were determined from the change in acidity of the medium from 45 to 90 min after the start of the assay. The amount of alkalinization is the deviation from the rate of acidification 15 min after the start of the assay. O_2 uptake was measured polarographically. Intact protoplasts were determined from cell counts. Values are the average of at least three experiments using separate protoplast preparations ±SD. 1 ml = 2×10^6 protoplasts = 200 μg Chl.

Treatment	Rate of Acidification	Amount of Alkalinization	Intact Protoplasts	Rate of O_2 Uptake
	neq/min·ml	*neq/ml*	%[a]	%[a]
50 $\mu E/m^2 \cdot s$ white light	10.6 ± 2.5	187 ± 56	77 ± 18	82 ± 17
Continuous mixing	9.6 ± 2.2	163 ± 71	80 ± 17	
Dark	3.3 ± 1.3	0 ± 0	84 ± 19	78 ± 11

[a] Value after the assay as a percentage of the initial value.

Table II. *Effect of DCMU on Acidification and Alkalinization Responses to Different Wavelengths*

Protoplasts were exposed to red (610 to 690 nm; maximum = 665 to 690 nm), green (496 to 586 nm; maximum = 520 nm), and blue (406 to 470 nm; maximum = 432 nm) light. These irradiances stimulated 60% of the rate of acidification and 40% of the amount of alkalinization of the control, an untreated suspension exposed to 50 $\mu E/m^2 \cdot s$ white light. The variation in irradiance within the experimental area is shown. Acidity changes were determined and protoplast concentration was as described in Figure 1 and Table I. Values are the average of at least three experiments using separate protoplast preparations ±SD.

Color	Irradiance	+2 μM DCMU	
		Rate of acidification	Amount of alkalinization
	w/m^2	*% control*	
Red	1.5–2.0	37 ± 7	0 ± 0
Green	2.0–2.5	35 ± 12	8 ± 19
Blue	5.5–7.0	23 ± 8	6 ± 11
White	10[a]	38 ± 11	5 ± 12
Dark	0	33 ± 8	0 ± 0

[a] Equivalent to 50 $\mu E/m^2 \cdot s$.

(6).

Increases in alkalinization paralleled increases in initial rates of O_2 evolution as the quantum flux density was raised; however, the alkalinization response was saturated by 75 $\mu E/m^2 \cdot s$ white light (Fig. 2). This strongly suggests that photosynthetic activity was limiting only for the alkalinization of suspensions exposed to low levels of light.

Both the rate of acidification and the concomitant O_2 level of the suspensions increased as the amount of light was raised to 50 $\mu E/m^2 \cdot s$ (Figs. 3 and 4). These data suggest that photosynthetic activity was limiting for the acidification response to low levels of light.

After 20 min of exposure to quantum flux densities above 50 $\mu E/m^2 \cdot s$, a net O_2 influx reduced the O_2 concentration of the medium (Fig. 4). This was confirmed by measuring the O_2 concentration of suspensions that had been exposed to light for 60 to 75 min under the conditions of the acidification assay (data not shown). The O_2 influx could be alleviated by adding CO_2 to the suspensions (Fig. 4). Therefore, the data are consistent with increased photorespiration and Mehler reaction in these suspensions (1).

The rate of acidification decreased following the change in photosynthetic activity in suspensions exposed to quantum flux densities above 50 $\mu E/m^2 \cdot s$ (Figs. 4 and 5). This suggests that photosynthesis was also limiting for the acidification response to higher levels of light.

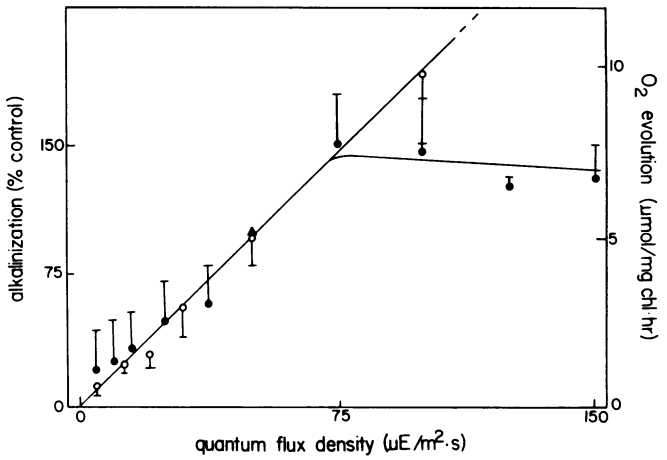

FIG. 2. Quantum flux density requirements of alkalinization and initial rates of O_2 evolution. Alkalinization was determined as described in Table I. O_2 evolution was measured polarographically. Each point is the average of at least three experiments using separate protoplast preparations; vertical bars, 0.5 SD. (○), initial rates of O_2 evolution; (●) alkalinization; (▲), control.

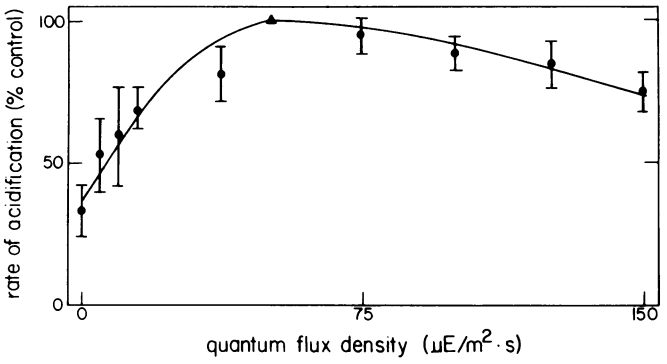

FIG. 3. Acidification response to different quantum flux densities of light. Acidification was determined as described in Table I. Each point is the average of at least three experiments using separate protoplast preparations; vertical bars, SD. (▲), control.

Proton and CO_2 Flux. The role of CO_2 flux in acidification and alkalinization was assessed by comparing changes in acidity and CO_2 levels of the medium. The CO_2 concentration was estimated from the increase in pH produced by purging the solution with N_2 as described in "Materials and Methods."

Both alkalinization and decreases in the CO_2 content of the medium were restricted to suspensions that were exposed to light. The amount of alkalinization was approximately equal to the

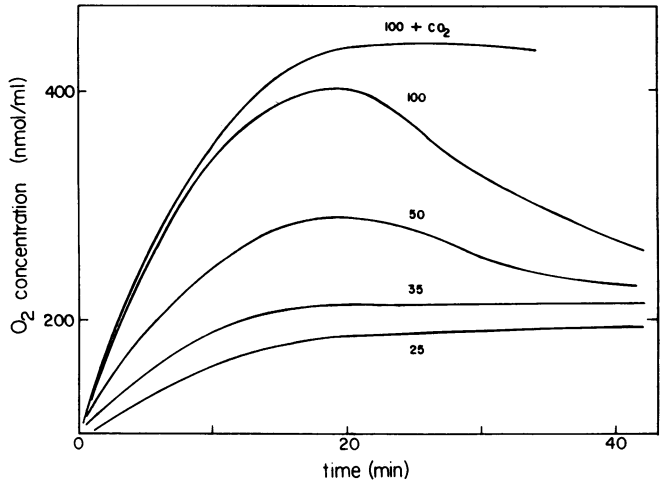

FIG. 4. Changes in the O_2 concentration of protoplast suspensions at different quantum flux densities of light. O_2 levels were monitored polarographically. CO_2 was added by bubbling CO_2-rich air through the suspension for 30 s. Values = $\mu E/m^2 \cdot s$ white light. 1 ml = 2×10^6 protoplasts = 200 μg Chl.

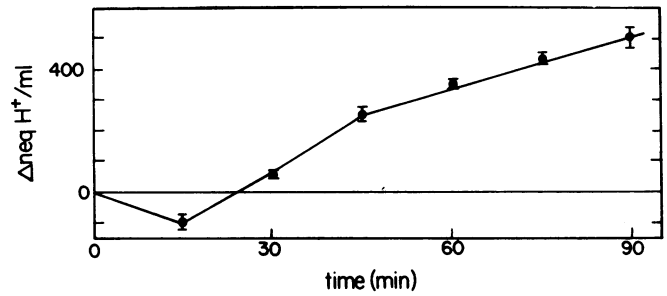

FIG. 5. Acidification response to supraoptimal levels of light. Acidity changes were determined and protoplast concentration was as described in Figure 1. Quantum flux density = 100 $\mu E/m^2 \cdot s$ white light. Vertical bars, variation between duplicate samples.

Table III. *CO_2 and Acidity Levels of the Medium*

CO_2 was purged with N_2 from medium decanted from suspensions of 2×10^6 protoplasts/ml. The decrease in acidity after removal of CO_2 defined the neq CO_2/ml. The acidity level is defined as the increase in acidity over the cell-free medium. Values are the average of four experiments using separate protoplast preparations ±SD.

	CO_2 Concentration	Acidity Level
	neq/ml	
Before assay[a]	258 ± 87	601 ± 93
After assay, dark	307 ± 187	933 ± 129
After assay, light[b]	77 ± 64	1349 ± 205
Cell-free medium	120 ± 70	0
+1300 neq CO_2/ml[c]	1440 ± 28	1300

[a] Suspensions were kept in the dark for 1 h at room temperature before all assays.

[b] 50 $\mu E/m^2 \cdot s$ white light.

[c] The amount of CO_2 was determined by the acidity.

decrease in CO_2 (Tables I and III). In addition, the maximum alkalinization (289 ± 64 neq H^+/ml; 12 experiments) approximated the CO_2 concentration of the medium at the start of the assay (Table III). These data suggest that protoplasts induced alkalinization by taking up CO_2 from the medium.

Suspensions were back-titrated to detect changes in the buffering capacity due to organic acid efflux. Following the assay, the acidity of the medium could be returned to the original value by an OH^- concentration equal to 92 ± 6% of the increase in acidity. Suspensions were also spun at 600*g* for 10 min and the pH of the supernatant was lowered to 3.0 with HCl. The OH^- concentration required to return the pH to 6.0 for suspensions that had been assayed was 118 ± 16% of that required to effect the same change in pH in supernatant obtained before the assay (3 experiments, single protoplast preparation). These data indicate that acidification was primarily due to OH^- or H^+ flux. In accordance with convention (19), the flux is referred to as proton efflux.

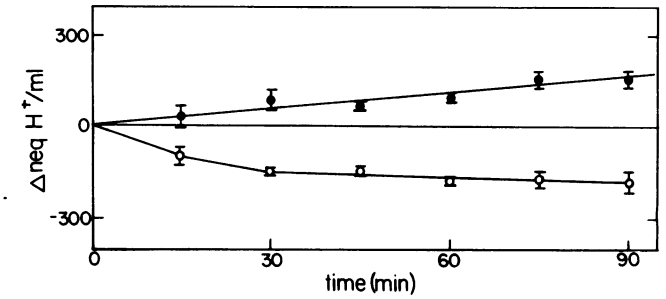

FIG. 6. Changes in acidity of CCCP-treated suspensions. CCCP (10 μM) was added 15 min before the start of the assay. Acidity changes were determined and protoplast concentration was as described in Figure 1. Vertical bars, variation between duplicate samples. (○), 100 $\mu E/m^2 \cdot s$ white light; (●), dark.

Alkalinization of CCCP-Treated Suspensions. The pH of the medium reflects changes in the CO_2 concentration caused by photosynthetic or respiratory activity in carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-treated *Chlamydomonas* suspensions (15). A related uncoupling agent, CCCP (22), was used to minimize active proton extrusion by the protoplasts and, thus, facilitate analysis of the effect of CO_2 uptake on the pH of the medium.

Protoplasts that were treated with 10 μM CCCP weakly acidified the medium when kept in the dark and alkalinized it when exposed to light (Fig. 6). Rates of O_2 evolution and uptake after the assay (60%, single experiment, and 58 ± 4%, respectively, of the initial rate) indicated over 60% viability. Nonetheless, the alkalinization was reduced by 90% after the first 30 min (Fig. 6).

DCMU (2 μM) inhibited alkalinization by 80 ± 9%, indicating a dependence upon photosynthesis. A greater percentage of the total alkalinization occurred during the first 15 min as quantum flux densities were raised (data not shown); this is consistent with faster depletion of CO_2 by greater photosynthetic activity. CCCP-treated suspensions had one-fourth the rate of O_2 evolution of normal suspensions. Thus, a greater percentage of the total alkalinization could be expected to occur initially in untreated suspensions exposed to the same level of light. Consequently, the data are consistent with minimal, or no, change in acidity due to CO_2 uptake during the latter part of the assay in normal suspensions.

DISCUSSION

The results indicated that photosynthetically dependent CO_2 uptake and proton efflux produced the alkalinization and subsequent acidification of suspensions of oat leaf protoplasts that were exposed to light.

Light-induced alkalinization can result from proton influx (8, 18) as well as CO_2 uptake (15). Alkalinization of the protoplast suspensions was attributed to photosynthetically induced CO_2 uptake because (a) it could be prevented by DCMU, and (b) it appeared to reflect changes in, and to be limited by, the CO_2 concentration of the medium.

Inhibition by DCMU showed the acidification response to a variety of light regimes to be dependent upon photosynthesis. In

addition, the amount of acidification was similar to other photosynthetically stimulated acidification responses (7, 16) and greater than acidification reported to be dependent upon phytochrome conversion (3) or mitochondrial respiration (2).

Acidification and alkalinization responded weakly to blue, as compared with red or green, light. The results were consistent with a weak photosynthetic response to blue light that would not have been predicted from photosynthetic action spectra for oats (11). Nonetheless, the apparent photosynthetic response to white light compared well with other studies (12). The discrepancy was not resolved.

The effect of increasing light on alkalinization and acidification indicated that photosynthesis was limiting for both responses at low levels of light. The initial alkalinization became limited by the CO_2 content of the medium at quantum flux densities above 75 $\mu E/m^2 \cdot s$ white light. However, a reduction in apparent photosynthetic activity at higher quantum flux densities of light was followed by a reduction in the rate of acidification. Consequently, photosynthesis appeared to continue to be limiting for the acidification of suspensions exposed to higher levels of light.

Acidification appeared to be due to proton efflux. Changes in the pH of the medium should have reflected a combination of proton efflux and CO_2 uptake. Nonetheless, two lines of evidence indicated that concomitant CO_2 uptake did not introduce a significant error in estimates of the rate of acidification. First, the initial CO_2 content of the medium was estimated to be nearly depleted after 15 min of exposure to optimal light conditions. Thus, CO_2 would be subsequently supplied by equilibration with the air phase; this would prevent CO_2 uptake from altering the pH of the medium during the 45- to 90-min period that determined the rate of acidification. Second, the rate of alkalinization of CCCP-treated suspensions 45 to 90 min after exposure to light was low enough to suggest that uptake of CO_2 had little direct effect on the pH during this period.

Net proton efflux was assumed to be relatively constant from the start of the assay. This assumption was used to calculate the amount of alkalinization; it is consistent with two observations. First, there was a constant increase in the rate of acidification as white light increased to 25 $\mu E/m^2 \cdot s$. This suggests that the response to light represented a stimulation of an acidification response that was continuous upon transition from dark to light. Second, when the change in acidity is plotted as a function of time, the portion of the curve indicating the rate of acidification extrapolates to a value on the y axis that corresponds to the estimated reduction in the CO_2 content of the medium (Fig. 1 and Table III). The simplest interpretation is that a constant rate of proton efflux was initially obscured by the effect of CO_2 uptake on the pH.

In summary, the results support the following conclusions: (a) light stimulated a net proton efflux that was partially dependent upon photosynthesis but also occurred in the dark, (b) photosynthetic activity was limiting for the acidification response to light, and (c) CO_2 uptake had little effect on the pH of the medium during the part of the assay used to estimate the rate of acidification. The second paper reporting on this study provides evidence that some photosynthetic stimulation of acidification required mitochondrial respiration (9). Therefore, acidification of the medium appears to be a possible means for detecting mitochondrial respiration in photosynthesizing protoplasts.

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