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An Investigation into the Role of Photosynthesis in Regulating ATP Levels and Rates of H^+ Efflux in Isolated Mesophyll Cells[1]

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ABSTRACT

Aerated and stirred 10-ml suspensions of mechanically isolated *Asparagus sprengeri* Regel mesophyll cells were used for simultaneous measurements of net H^+ efflux and steady-state ATP levels.

Initial rates of medium acidification indicated values for H^+ efflux in the light and dark of 0.66 and 0.77 nanomoles $H^+/10^6$ cells per minute, respectively. When the medium pH was maintained at 6.5, with a pH-stat apparatus, rates of H^+ efflux remained constant. Darkness or DCMU, however, stimulated H^+ efflux by 100% or more. Darkness increased ATP levels by 33% and a switch from dark to light reduced ATP levels by 31%. In the absence of aeration, illumination prevented the accumulation of respiratory CO_2 and the buffering capacity of the medium was about 50% less than that found in the nonilluminated nonaerated medium. As a result, rates of pH decline were similar even though the dark rate of H^+ efflux was approximately 50% greater.

Proposals that photosynthesis stimulates H^+ efflux are based on changes in the rate of pH decline. The present data indicate that photosynthesis inhibits H^+ efflux and that changes in rates of pH decline should not be equated with changes in the rate of H^+ efflux.

Ion transport processes in leaf cells have not been as extensively investigated as those associated with root tissues. Nevertheless, leaf cells accumulate ions in their vacuoles. Concentrations as high as 200 mM have been reported and account for the osmotic water potential of leaf tissue (10). The source of metabolic energy for plasma membrane transport processes appears to be ATP. Published data suggest that an ATP requiring electrogenic H^+ efflux is involved in the generation of a major component of plant plasma membrane potentials (22, 24). This proton efflux produces a proton electrochemical gradient which drives the influx of major ions, sugars, and amino acids (21, 22, 24).

The relationships between photosynthesis, respiration, ATP levels, and rates of H^+ efflux are not understood. The view that light inhibits respiration by promoting a high cytosolic ATP/ADP ratio (9) is not consistent with recent reports that ATP/ADP ratios exert little influence on respiration in isolated pea leaf mitochondria (6), and that the cytosolic ATP/ADP ratio is actually higher when wheat leaf protoplasts are not illuminated (25). In addition, work with wheat leaves suggests that the rate of respiratory CO_2 efflux is similar with or without illumination (2), and work on green leaves of mung bean and *Scenedesmus* indicates that the rate of the Krebs cycle is as high in the light as the dark (4, 19). Thus, photosynthesis, respiration, or both may contribute ATP for energy-driven H^+ efflux at the plasma membrane of illuminated photosynthetic cells.

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Investigations into the influence of light on steady-state ATP levels in photosynthetic tissues have resulted in conflicting data. Reports indicate that light elevates ATP levels in *Lemna gibba* and maize mesophyll protoplasts (26, 29), reduces ATP in cotton cotyledons and tomato leaves (26, 27), and is without effect in barley and maize leaves (15, 16). In addition, the influence of light on the net rate of H^+ efflux from green cells is not clear. Light-stimulated acidification of the extracellular medium has been detected with bean and oat leaves. These results were interpreted as a stimulation of H^+ efflux by light although the buffering capacity of the nonaerated extracellular medium was not determined (8, 28). Light-stimulated acidification of the medium surrounding oat leaf protoplasts was interpreted as an artifact resulting from photosynthetic production of O_2 which was limiting for respiration (13, 14). Similarly, H^+ efflux from illuminated and aerated suspensions of *Asparagus* mesophyll cells appears to be dependent on respiration, not photosynthesis. H^+ efflux was eliminated by addition of the respiratory inhibitor oligomycin, whereas inhibition of photosynthesis with DCMU or darkness appeared to stimulate H^+ efflux. Interpretation of the data was complicated by the reduction in the rate of H^+ efflux as the external pH declined (3).

Limiting diffusion of CO_2 and O_2 within the extracellular medium in contact with the pH probe and the surface of cells or tissues may be responsible for much of the confusion. Diffusion rates of dissolved gases are considerably reduced. The diffusion coefficient for dissolved CO_2 is approximately 10,000 times lower than that in the gas phase. Consequently, gaseous diffusion may limit metabolic processes. For example, vacuum infiltration of leaf air spaces with liquid medium reduced the rate of photosynthesis by more than 90% (17). Apparent changes in the rate of H^+ efflux may occur if inadequate aeration results in the accumulation of respiratory CO_2 or photosynthetic depletion of CO_2. Changes in the CO_2 content of the medium will produce changes in pH which are independent of H^+ efflux (3, 13). In addition, apparent changes in the rate of H^+ efflux may occur if changing concentrations of CO_2 and bicarbonate result in a change in the buffering capacity of the medium. Under these circumstances, the rate of pH change can not be equated with the rate of H^+ efflux. Furthermore, light-stimulated H^+ efflux may arise indirectly if photosynthesis results in the production of O_2 limiting for respiration (13, 14). Limiting diffusion may occur when extracellular medium occupies leaf air spaces or because the fragile nature of mesophyll protoplasts precludes aeration.

In the present study, vigorously aerated and stirred suspensions of mechanically isolated mesophyll cells were used to investigate the role of photosynthesis in regulating steady-state levels of ATP and rates of net H^+ efflux.

MATERIALS AND METHODS

Asparagus sprengeri Regel was grown in vermiculite under greenhouse conditions. Mesophyll cells were isolated daily from

cladophylls using a technique of gentle mechanical disruption (5). Chl content of the resulting cell suspension was determined by the method of Arnon (1) and cell numbers measured using a hemocytometer and light microscope. A mean value of 40 μg Chl/10^6 cells was obtained. The fraction of cells with intact protoplasts was determined through their ability to exclude Evans Blue (11).

Photosynthetic rates and rates of H^+ efflux were measured using cell suspensions maintained at 30°C in water-jacketed glass vessels. Rates of net H^+ efflux from 10 to 12 × 10^6 aerated (500 ml/min) and stirred cells suspended in 10 ml 5 mM KCl, 5 mM NaCl, and 0.2 mM $CaCl_2$ were determined using a recording pH meter and determination of the buffering capacity of the medium (3). In addition, H^+ efflux was measured with an automated recording pH-stat method. The Radiometer (Copenhagen) apparatus consisted of a PHM 64 pH meter, a TT 80 titrator, an ABU 12 autoburette and an REC 61 recorder. The recorder pen moved at predetermined speed across the recording paper whereas automated incremental additions of alkali to maintain a constant pH were precisely coupled to movement of the paper. The rate of addition of $Ca(OH)_2$ in ml/min to maintain a constant preselected pH was used to calculate the net rate of H^+ efflux. The normality of the $Ca(OH)_2$ used varied between 0.7 and 1.1 mM and was determined daily by titrating it into 10 ml of standard 0.1 mM HCl to a pH 7 endpoint. The net rate of H^+ efflux was expressed as nmol $H^+/10^6$ cells·min or nmol H^+/mg Chl·min. Rates of photosynthetic O_2 production were determined with a calibrated Clark O_2 probe (YSI 4004) using 3 to 4 × 10^6 cells suspended in 7.5 ml 50 mM Hepes buffer (pH 7.2) in a closed system (3). Cell suspensions were illuminated with a 300-W reflector lamp (Sylvania) which gave an irradiance at the surface of the vessel of 1100 × 10^{-5} W cm^{-2}.

To determine steady-state levels of ATP, 0.5-ml aliquots were taken from the 10-ml cell suspension 5 min after addition of cells to the suspending medium and at subsequent 5-min intervals for an additional 25 min. Aliquots were transferred to 0.5 ml ice-cold 10% TCA containing 4 mM EDTA, agitated in a vortex for 30 s, and then stored on ice for 30 min. At this time, the cells were again agitated and the volume made up to 5 ml with 0.1 M Tris 2.0 mM EDTA (pH 7.75) prior to assay the same day. Assays were conducted using a firefly luciferase ATP monitoring reagent (LKB-Wallac 1243-200) which produced a light output of nearly constant intensity proportional to the concentration of ATP in the sample. Light intensity was measured using an LKB-Wallac 1250 Luminometer attached to an LKB-Wallac display unit with 0- to 10,000-mV output. To the sample holder was added with thorough mixing 0.8 ml of Tris-EDTA buffer, 0.1 ml of ATP monitoring reagent, and 0.1 ml of ATP sample or standard. The output was printed at 10-s intervals for 1 min and the maximum output recorded. During this period, decay of the signal was less than 10% of the maximal value if the ATP concentration used was no more than 10^{-6} M. The relationship between maximum output and concentration was found to be linear between 10^{-9} and 10^{-5} M ATP. The concentration of ATP in the 5-ml extracts was between 10^{-7} and 10^{-8} M. To account for any quenching due to the extract, the output from each sample was measured before addition of 0.1 ml 10^{-7} M ATP and recording of the increase in output. There was no significant loss of ATP when the extract was maintained on ice for 4 h. ATP concentrations were calculated as nmol ATP/10^6 cells or nmol ATP/mg Chl.

Stock solutions of oligomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, antimycin A, and DCMU were made up in 80% (v/v) ethanol, and 0.1-ml volumes were added to the 10-ml cell suspension as indicated. Addition of 0.1 ml of 80% (v/v) ethanol had no significant influence on the net rate of H^+ efflux. All concentrations quoted are final values after dilution with the cell suspension.

RESULTS

The exclusion of Evans Blue by isolated cells indicated that over 85% maintained an intact plasma membrane. Approximately 11 × 10^6 cells suspended in 3 ml 5 mM KCl, 5 mM NaCl, and 0.2 mM $CaCl_2$ were added to 7 ml of similar medium. Over the next 30 min, the ATP level of the stirred and aerated cell suspension remained relatively constant. Levels declined by 8% in the dark and by 25% in the light. The ATP levels were particularly stable after the initial 10-min period. Although ATP levels within any one cell preparation were relatively constant, there was considerable variation between measurements on different days when different cell preparations were used. ATP levels after 5-min aeration varied between 0.56 and 1.70 nmol ATP/10^6 cells or 16.8 to 51.0 nmol ATP/mg Chl. The mean level of ATP was found to be approximately 35% higher in the absence of illumination (Table I). Changes in illumination within the 30-min experimental period also indicated that ATP levels were higher in nonilluminated cells (Fig. 3).

On addition of cells to the unbuffered salt solution, the pH rose rapidly and peaked at a pH between 6.0 and 6.6 after 1 to 2 min. The pH then started to decline (Figs. 1 and 4) and

Table I. *ATP Levels in Cells Maintained in Continuous Darkness or Continuous Illumination*

Aliquots of cell suspension for ATP analysis were removed 5 min after addition of cells to the suspending medium and at subsequent 5-min intervals. The decline in pH of the bathing medium was measured simultaneously (*e.g.* Fig. 4).

	Period of Aeration (min)					
	5	10	15	20	25	30
	nmol ATP/10^6 cells					
Dark						
Expt. 1	0.81	0.87	0.82	0.71	0.65	0.60
Expt. 2	0.70	0.66	0.67	0.63	0.68	0.71
Expt. 3	1.38	1.23	1.30	1.2	1.35	1.35
Mean	0.96	0.92	0.93	0.84	0.89	0.88
Light						
Expt. 1	0.56	0.46	0.48	0.46	0.45	0.45
Expt. 2	0.99	0.85	0.88	0.85	0.80	0.78
Expt. 3	0.59	0.53	0.46	0.47	0.40	0.38
Mean	0.71	0.61	0.61	0.59	0.55	0.53

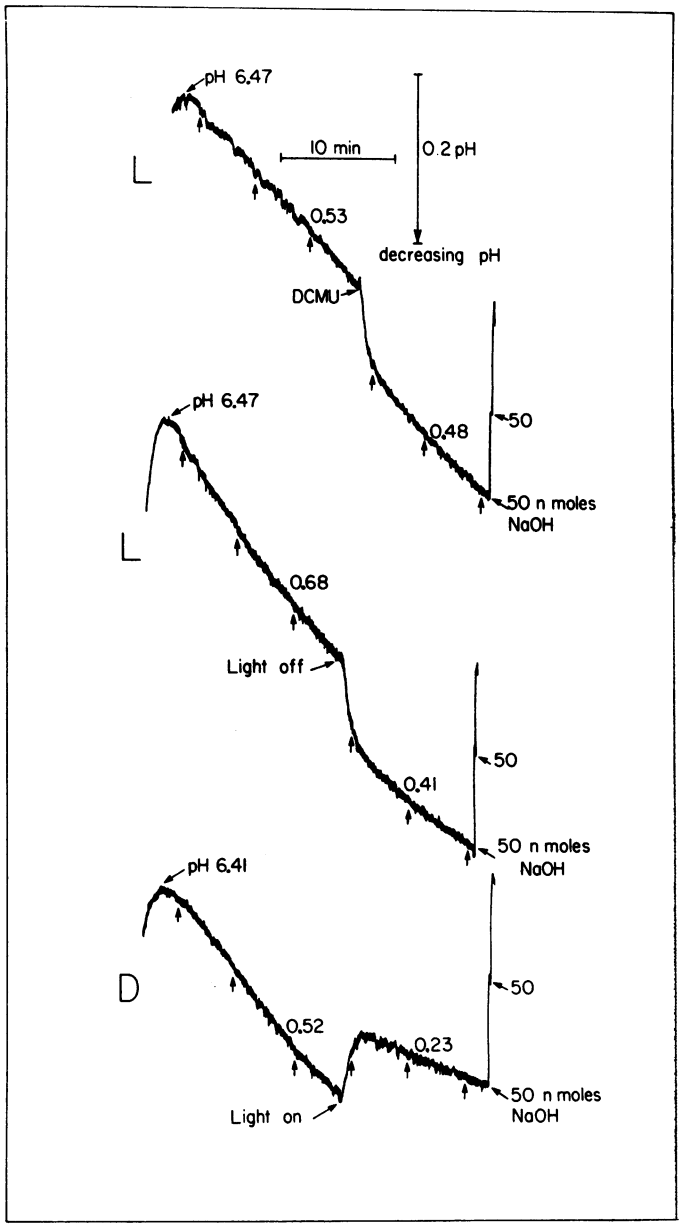

FIG. 1. Influence of photosynthesis on net rates of H^+ efflux. L (light) or D (dark) indicates the initial condition for the experiment. Conditions were changed by addition of DCMU (1 μM) or a change in illumination as indicated. Rates of acidification are expressed in nmole $H^+/10^6$ cells·min. Unlabeled arrows indicate the times samples of cell suspension were removed for ATP analysis.

continued at a diminishing rate for several h until a constant pH between 4.0 and 5.0 was attained (data not shown). The initial mean value for the net rate of H^+ efflux in the dark was 0.77 nmol $H^+/10^6$ cells·min or 27.9 nmol H^+/mg Chl·min. In the light, corresponding values were 0.66 nmol $H^+/10^6$ cells·min and 21.7 nmol H^+/mg Chl·min.

The influence of photosynthesis on H^+ efflux and ATP levels was determined by inhibiting photosynthesis with a switch from light to dark or with addition of 1 μM DCMU. This concentration of DCMU completely inhibited photosynthetic O_2 production as measured with an O_2 electrode (data not reported). In complementary experiments, photosynthesis was initiated by switching the cell suspension from dark to light. Inhibition of photosynthesis by darkness or DCMU was accompanied by an immediate pH decline of approximately 0.1 pH units over a 2-min period. Initiation of photosynthesis was accompanied by an equally rapid alkalinization of about 0.1 pH unit (Fig. 1). These changes indicate that photosynthesis reduced the steady-state CO_2 concentration in the stirred and aerated cell suspension resulting in an increase of pH on illumination, and a decrease when photosynthesis was inhibited. Apart from these small transient effects on pH, switching from dark to light was followed by a reduction in the mean rate of acidification of 58% and switching from light to dark by a mean value of 29.9%. Thus, after the switch in illumination, rates of H^+ efflux in the dark were approximately 80% greater than those in the light (Fig. 1). This observation was confirmed when the rate of H^+ efflux was measured at pH 6.5 with the pH-stat apparatus. Darkness or 1 μM DCMU resulted in a transient rapid rate of acidification followed by a linear rate of acidification which was 70 to 200% greater than the initial rate. A switch from darkness to light resulted in alkalinization of the medium and H^+ efflux was not recorded for 20 min until the pH returned to 6.5. At this point H^+ efflux resumed at a rate which was approximately 50% less than the previous dark rate (Fig. 2). A switch from dark to light reduced ATP levels by 31%,

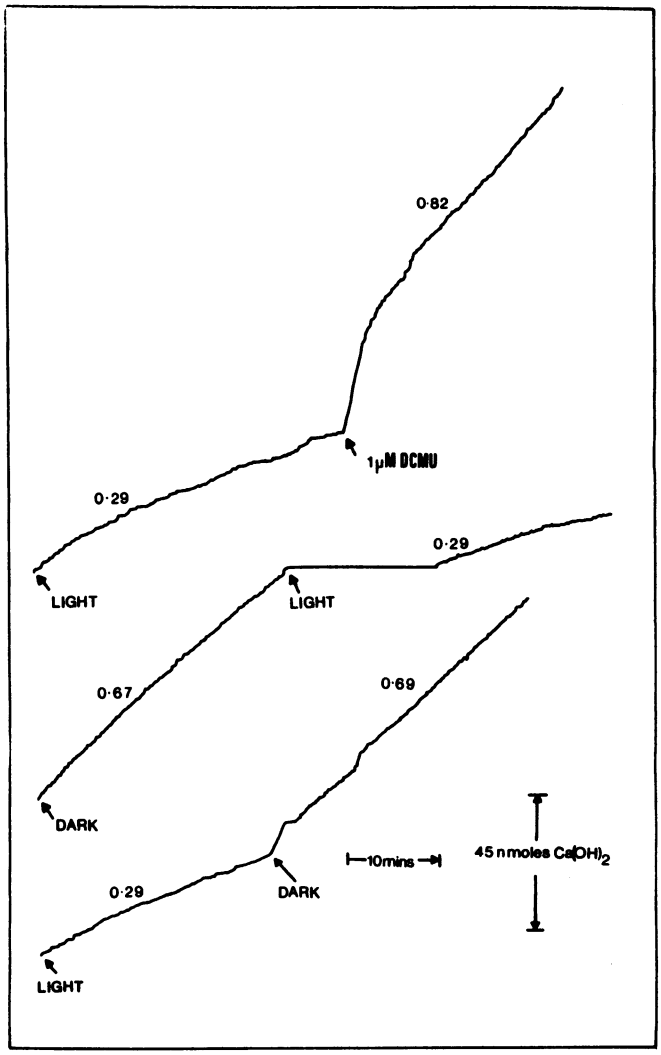

FIG. 2. Influence of photosynthesis on net rates of H^+ efflux at pH 6.5. The initial conditions of illumination are indicated. Conditions were changed by addition of 1 μM DCMU (upper trace), a switch to illumination (middle trace), or a switch to darkness (lower trace). The pH of 6.5 was maintained by an automated recorded addition of $Ca(OH)_2$. Rates of acidification are expressed in nmol $H^+/10^6$ cells·min.

whereas a switch from light to dark resulted in a 33% increase in the level of ATP within 10 min (Fig. 3). This increase is particularly significant when contrasted with the slow decline in ATP levels with time (Table I).

Concentrations of oligomycin and antimycin A which do not inhibit photosynthetic O_2 evolution in *Asparagus* cells were determined. Antimycin A (0.04 μM) eliminated net H^+ efflux in the light within 10 min, whereas the oligomycin concentration employed (1.0 μg/ml) inhibited H^+ efflux slowly and was not complete until 12.5 to 15 min had elapsed (Fig. 4). These observations were confirmed when antimycin A and oligomycin were used to inhibit linear rates of H^+ efflux obtained with illuminated cells maintained at pH 6.5 with the pH-stat apparatus (Fig. 5). The same concentrations of inhibitor reduced ATP levels in the light by 25 to 30%. However, in the presence of oligomycin, the ATP level appeared to drop slowly, whereas the effect of antimycin A appeared to be complete within 5 min (Fig. 6). Higher concentrations of oligomycin (10 μg/ml) and antimycin A (1.0 μM) resulted in similar 25 to 30% reductions in the ATP levels of illuminated cells (data not shown).

With stirring but without aeration, similar rates of pH decline were obtained in the light and dark. However, the mean value for the buffering capacity of the nonilluminated cell suspension was 590 nmol OH^-/pH unit, whereas the corresponding value for the illuminated cell suspension was 323 nmol OH^-/pH unit. Consequently, calculated rates of H^+ efflux were 60% higher when illumination was absent (Fig. 7). The initial rapid acidification rate when cells were first added to the nonaerated nonilluminated cell suspension (Fig. 7) and the rapid 0.1 to 0.2 pH rise on resumption of aeration (data not shown) indicate the accumulation of CO_2 and bicarbonate under these conditions. This accumulation would account for the observed increase in buffering capacity when the cell suspension is neither illuminated nor aerated (Fig. 7). Thus, light can reduce the buffering capacity of the medium surrounding photosynthetic cells demonstrating that its influence on the rate of H^+ efflux cannot be determined by measurement of the rate of pH change in the medium.

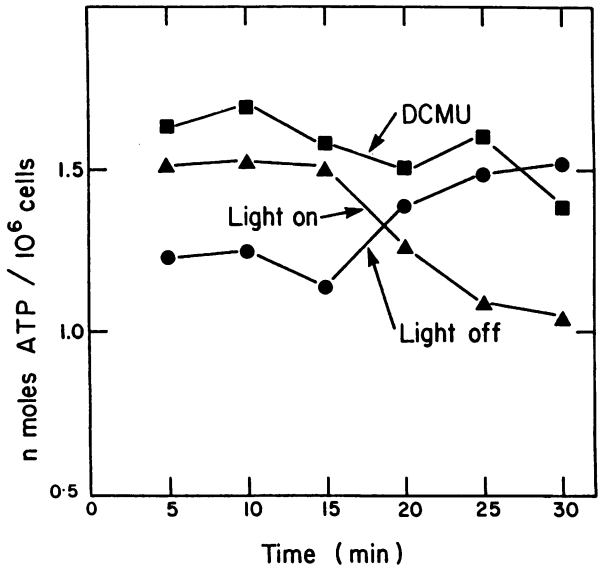

FIG. 3. Influence of photosynthesis on ATP levels. Aliquots of cell suspension for ATP analysis were removed 5 min after addition of cells to the suspending medium and at subsequent 5-min intervals. The decline in pH of the bathing medium was measured simultaneously (Fig. 1). DCMU (1 μM) was added to a suspension of illuminated cells at the time indicated (■). Illumination of cell suspensions ceased at the time indicated (●). Illumination of cells maintained in the dark began at the time indicated (▲). The data represent mean values from three separate experiments.

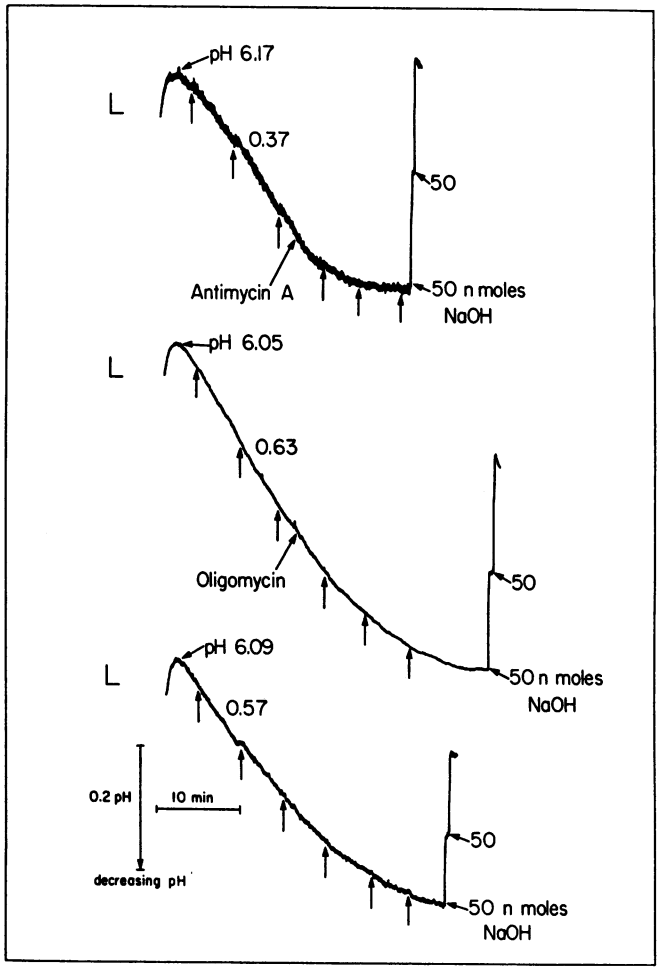

FIG. 4. Influence of inhibitors of oxidative phosphorylation on net rates of H^+ efflux in the light. Oligomycin (1.0 μg/ml) and antimycin A (0.04 μM) were added as indicated. L indicates illumination throughout the experiment. Rates of acidification are expressed in nmol $H^+/10^6$ cells · min. Unlabeled arrows indicate the times samples of cell suspension were removed for ATP analysis.

DISCUSSION

Previous work with aerated nonilluminated *Asparagus* mesophyll cells has shown that the inhibitors of oxidative phosphorylation oligomycin and antimycin A rapidly eliminated H^+ efflux and reduced ATP levels by 60% or more (3, 23). In addition, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which renders membranes permeable to protons, resulted in alkalinization of the suspending medium indicating that acidification results from H^+ efflux against a proton electrochemical gradient (3). These results support suggestions that H^+ efflux from plant cells is driven by a plasma membrane located outwardly directed ATP dependent proton pump (22, 24).

The large day-to-day variation in the ATP content of the cells was obtained even though each measurement was calibrated by adding standard ATP to the assay system. The extraction process did not result in a detectable loss of ATP from standard solutions, and re-extraction of the pellet after the initial extraction did not result in significant levels of ATP. There was no loss of sample ATP during storage on ice prior to the assay. Consequently, the variation observed probably represents real differences in the

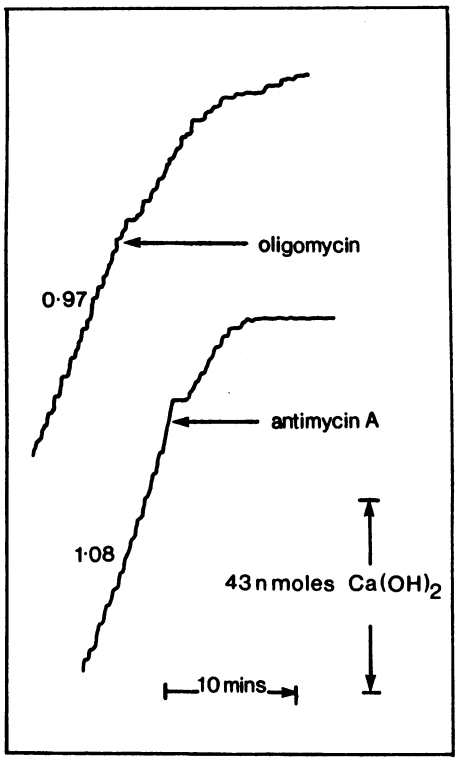

FIG. 5. Influence of inhibitors of oxidative phosphorylation on net rates of H^+ efflux from illuminated cells maintained at pH 6.5. Illumination was continuous. Oligomycin (1.0 μg/ml) and antimycin (0.04 μM) were added as indicated. The pH of 6.5 was maintained by an automated recorded addition of $Ca(OH)_2$. Rates of acidification are expressed in nmol $H^+/10^6$ cells·min.

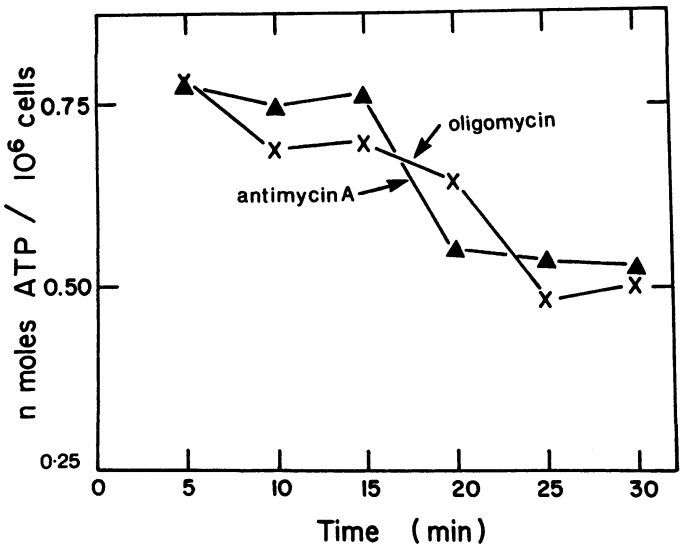

FIG. 6. Influence of inhibitors of oxidative phosphorylation on ATP levels in illuminated cells. Aliquots of cell suspension for ATP analysis were removed 5 min after addition of cells to the suspending medium and at subsequent 5-min intervals. The decline in pH of the bathing medium was measured simultaneously (Fig. 4). Oligomycin (1.0 μg/ml, ×) and antimycin A (0.04 μM, ▲) were added as indicated. The data represent mean values from three separate experiments.

ATP content of cells from different preparations. Previous workers also concluded that large variation in measured ATP levels in *Chara corallina* (12) and red beet tissue (20) represented inherent variation in the ATP content of these cells.

Work with wheat leaf protoplasts indicated 63 nmol ATP/mg Chl in the dark and 70.4 nmol ATP/mg Chl in the light (25). In maize leaf protoplasts, values in the dark were 3.90 nmol ATP/10^6 protoplasts and in the light were 5.04 nmol ATP/10^6 protoplasts (29). In the present study, aerated and stirred cells were used and steady-state ATP levels were somewhat lower (see "Results"). In addition, the ATP level was reduced in the light (Table I, Fig. 3). Lower ATP levels in the light have been reported for 7 and 14-d-old cotton cotyledons and tomato leaf tissue (26, 27). The steady-state level of ATP will depend on the rate of ATP synthesis and also the rate of ATP utilization in photosynthetic CO_2 reduction. For example, ATP levels were higher in illuminated bean leaves which were deprived of CO_2 (18). The rate of ATP utilization will decline, and ATP levels may rise if CO_2 levels in a nonaerated medium become limiting for photosynthesis. The fragile nature of protoplasts precludes aeration (25, 29). The availability of CO_2 may explain the contrasting reports on the influence of photosynthesis on ATP levels (15, 16, 26, 27, 29). In the present study, cells were stirred and vigorously aerated to maintain the supply of CO_2 for photosynthesis.

DCMU or darkness resulted in a reproducible and large stimulation of the net rate of H^+ efflux (Fig. 2). Thus, photosynthesis in addition to reducing ATP levels also resulted in inhibition of H^+ efflux. This result is consistent with a previous suggestion that light inhibits H^+ efflux from isolated *Asparagus* mesophyll cells (3). It contrasts, however, with reports that light stimulates efflux of H^+ from sections of bean leaves (28) and oat leaves (8). Light stimulation of H^+ efflux from oat leaf protoplasts was attributed to the stimulation of respiration when photosynthesis resulted in the production of O_2 (13, 14). In all cases, the onset of illumination resulted in an initial alkalinization (3, 8, 13, 14, 28; Fig. 1) which has been shown to correspond with photosynthetic removal of CO_2 (3, 13). The contentious issue is the effect of illumination on the subsequent rate of H^+ efflux. Clearly, species and physiological differences between leaf sections (8, 28), mesophyll protoplasts (13, 14), and mesophyll cells (3) may be important. However, failure to aerate the extracellular medium in contact with the cell surface and the pH probe (8, 13, 14, 28) may result in misleading data when the influence of light on H^+ efflux is investigated. O_2 levels may become limiting for respiration when nonaerated isolated protoplasts and cells are maintained in a nonilluminated cell suspension medium (3, 13, 14). Thus, photosynthetic production of oxygen may stimulate energy-dependent processes such as H^+ efflux. The present data demonstrate that photosynthetic removal of CO_2 reduces the buffering capacity of the extracellular medium (Fig. 7). The pK of the CO_2 bicarbonate buffer system is 6.35 so that the range of buffering corresponds closely with the 5 to 7 pH range over which measurements of acidification are normally made (3, 8, 13, 14, 28). Consequently, if the buffering capacity of the medium is not determined, rates of pH decline should not be equated with rates of H^+ efflux (8, 28). Light-stimulated removal of CO_2 may lower the buffering capacity and result in an apparent stimulation of H^+ efflux. In the present study, vigorous aeration resulted in a similar buffering capacity for the illuminated and nonilluminated cell suspensions (Fig. 1). The accumulation of respiratory CO_2 and buffering capacity in a nonilluminated extracellular medium will depend on many factors including aeration and the amount of tissue in the medium. In the present study, the weight of tissue was less than 0.06 g fresh weight per ml of medium. The corresponding value for work on oat leaves was 0.66 g/ml (8).

It is not clear why light inhibits H^+ efflux from aerated suspensions of *Asparagus* mesophyll cells. Evidence indicates that H^+ efflux from plant cells is inhibited when cytoplasmic H^+ concentrations are reduced (22). In *Asparagus* mesophyll cells, the light-mediated reduction of cytoplasmic H^+ levels has been demonstrated (7). Thus, inhibition may reflect a reduced cytoplasmic

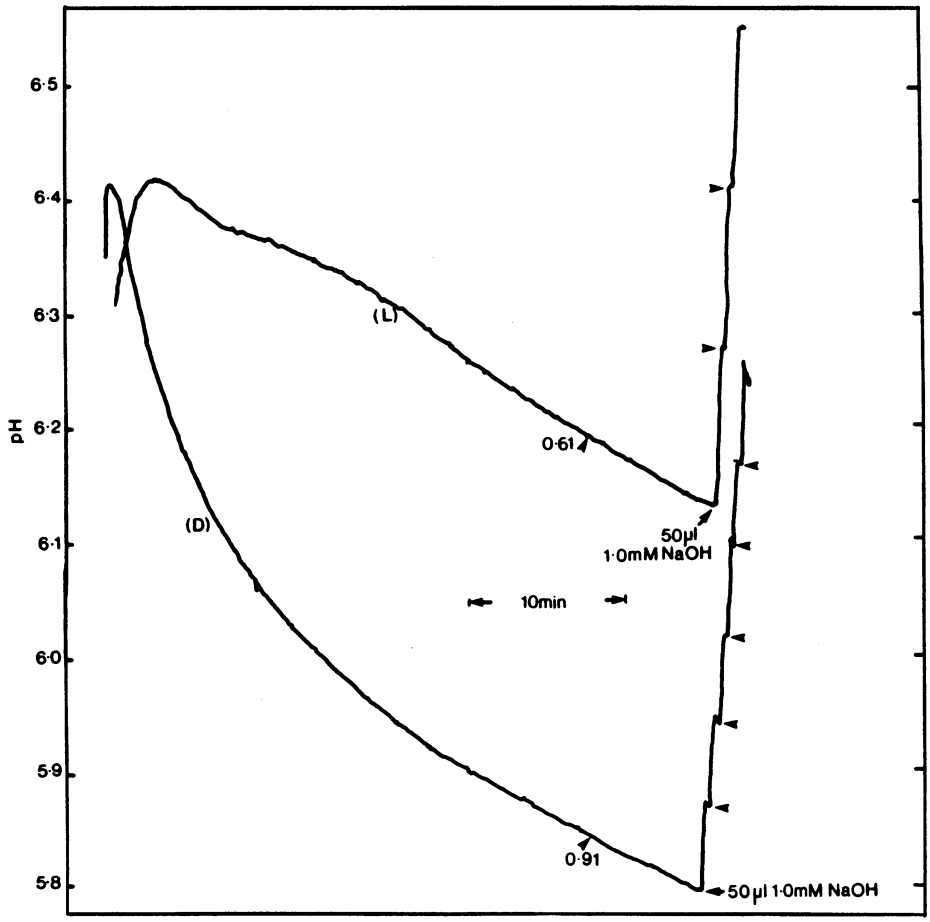

FIG. 7. Influence of photosynthesis on H^+ efflux and the buffering capacity of a nonaerated cell suspension medium. Illuminated (L) or nonilluminated (D) cell suspensions were stirred but not aerated. Rates of acidification are expressed in nmol $H^+/10^6$ cells·min.

level of H^+. Alternatively, the reduction of ATP levels in illuminated cells (Fig. 3) may indicate reduction of cytosolic ATP concentrations to levels which inhibit the plasma membrane ATPase driving H^+ efflux. Illumination reduces cytosolic ATP levels in wheat leaf protoplasts (25).

The inhibitors of mitochondrial oxidative phosphorylation antimycin A and oligomycin were applied to illuminated cell suspensions at concentrations which did not inhibit photosynthetic O_2 evolution. The efflux of H^+ was rapidly eliminated (Figs. 4 and 5) and ATP levels were reduced by 25 to 30% (Fig. 6). The results suggest that oxidative phosphorylation drives H^+ efflux even when aerated mesophyll cells are illuminated (3). Similarly, light-stimulated H^+ efflux from oat leaf protoplasts was attributed to O_2 production and an increase in mitochondrial activity (13, 14). The results are consistent with reports that respiration rates are similar in illuminated and nonilluminated photosynthetic tissue (2, 4, 19).

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