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Temperature Dependence of Photosynthesis in *Agropyron smithii* Rydb.[1]

II. CONTRIBUTION FROM ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION

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ABSTRACT

As part of an analysis of the factors regulating photosynthesis in *Agropyron smithii* Rydb., a C_3 grass, the response of electron transport and photophosphorylation to temperature in isolated chloroplast thylakoids has been examined. The response of the light reactions to temperature was found to depend strongly on the preincubation time especially at temperatures above 35°C. Using methyl viologen as a noncyclic electron acceptor, coupled electron transport was found to be stable to 38°C; however, uncoupled electron transport was inhibited above 38°C. Photophosphorylation became unstable at lower temperatures, becoming progressively inhibited from 35 to 42°C. The coupling ratio, ATP/2e⁻, decreased continuously with temperature above 35°C. Likewise, photosystem I electron transport was stable up to 48°C, while cyclic photophosphorylation became inhibited above 35°C. Net proton uptake was found to decrease with temperatures above 35°C supporting the hypothesis that high temperature produces thermal uncoupling in these chloroplast thylakoids. Previously determined limitations of net photosynthesis in whole leaves in the temperature region from 35 to 40°C may be due to thermal uncoupling that limits ATP and/or changes the stromal environment required for photosynthetic carbon reduction. Previously determined limitations to photosynthesis in whole leaves above 40°C correlate with inhibition of photosynthetic electron transport at photosystem II along with the cessation of photophosphorylation.

In a previous study (12) on the temperature response of photosynthesis in *Agropyron smithii*, a C_3 prairie grass, Monson *et al.* showed that temperatures above 35°C inhibited net photosynthesis in whole leaves under light-limiting conditions. It was also found that kinetic properties of RuBP[3] carboxylase appear to be altered at high temperatures such that the Q_{10} of the V_{max} determined *in vivo* is lower than the Q_{10} of the V_{max} determined *in vitro*.

The photosynthetic electron transport chain is a component of energy conversion which may have multiple sites of high temperature inhibition which may affect the rate of net photosynthesis. High temperature inhibition of the photochemical production of ATP or NADPH or both could limit the turnover of the reductive pentose phosphate pathway, lowering the level of RuBP, and thus contribute to reduction of net photosynthesis at elevated temperatures. Likewise, high temperature limitations of the photochemical production of stromal activating factors, such as alkaline pH and elevated Mg^{2+} ion concentrations, could alter the kinetic properties of several stromal enzymes, including RuBP carboxylase. Alteration of the environment of the stromal enzymes could contribute to the reduction of net photosynthesis at elevated temperatures.

A number of investigations on the temperature sensitivity of the electron transport chain have been reported (1, 4, 6, 7, 9–11, 13–15, 19–23, 27), but few have attempted to correlate the temperature sensitivity of carbon fixation in intact leaves with the temperature sensitivity of the light reactions in isolated chloroplast membranes (1, 4). In this report, the temperature dependence of electron transport and photophosphorylation in isolated chloroplast thylakoids from *A. smithii* was investigated to test the hypothesis that temperature effect on electron transport and photophosphorylation contribute to the decline in whole leaf net photosynthesis at temperatures above 35°C.

MATERIALS AND METHODS

Plant Material. Sources of *Agropyron smithii* Rybd. and growth conditions are described elsewhere (12).

Preparation of Chloroplast Thylakoids. Thylakoids were prepared using 3- to 10-d-old leaves from native plants. Five g of leaves were cut into 3- to 5-cm segments. The segments were washed with deionized H_2O and placed in 100 ml of ice-cold grinding solution consisting of 0.33 M sorbitol, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.6) and 0.2% BSA. Leaf segments were blended for 3 to 5 s at the number five setting with a Polytron tissue homogenizer. The resulting homogenate was filtered through a 200-μm net, and the filtrate was placed in 50-ml tubes and centrifuged at 500*g* for 5 min in a bench top centrifuge at 4°C. The supernatant was poured from the pellets, and the pellets were resuspended in a minimal amount of grinding buffer. A typical thylakoid preparation contained 0.5 mg Chl/ml in 1 to 2 ml total volume.

Measurement of Electron Transport and Photophosphorylation. Electron transport was measured polarographically using a Rank Brothers O_2 electrode. Photophosphorylation was determined by following incorporation of ^{32}Pi into ATP according to the method of Avron (3). The cuvette housing the O_2 electrode was water jacketed for temperature control and contained a magnetic stirrer. Solution temperature was monitored using a thermocouple and a Wescor TH 65 digital thermometer. Illumination of the cuvette was provided by a single 500-w flood lamp. The light was filtered through 8 cm of water to minimize heat irradiation. The light intensity was 100 nE cm^{-2} s^{-1} (PAR) at the surface of the cuvette, as measured with a quantum sensor (model 1776; LiCor Instru-

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[3] Abbreviation: RuBP, ribulose 1-5-bisphosphate.

ments).

The reaction medium consisted of 0.1 M sorbitol, 50 mM KCl, 5 mM $MgCl_2$, 50 mM Hepes/KOH (pH 7.8), 2 mM KH_2PO_4, 2 mM ADP, 100 μM NaN_3, and 50 μM methyl viologen and 10^5 dpm ^{32}Pi. The reaction volume was 1 ml. In all experiments, chloroplasts were added to give a final concentration of 10 to 20 μg Chl/ml. Chl was determined by the method of Arnon (2). After 1 min illumination, 0.8 ml of the reaction mixture was removed and added to a test tube containing 0.2 ml 20% TCA.

PSI electron transport was measured in a medium containing 0.1 M sorbitol, 50 mM KCl, 5 mM $MgCl_2$, 50 mM Hepes (pH 7.8), 10 mM sodium ascorbate, 10 μM DCMU, 100 μM NaN_3, and 30 μM 2,6-dichlorophenolindophenol. Superoxide dismutase (150 units) was included in the assay medium to eliminate O_2 uptake contributions due to superoxide radical reactions (16).

Steady-state proton uptake was monitored using a glass pH electrode and Corning digital 112 pH meter attached to a chart recorder. The magnitude of the light-induced pH rise was calibrated by injections of known volumes of standardized 10 mM HCl.

RESULTS

Thermal Stability of Noncyclic Photophosphorylation and Electron Transport. Photophosphorylation coupled to noncyclic electron transport was measured at the preincubation temperature for 1-min reaction times following periods of 15 s, 1 min, and 3-min preincubations (Fig. 1). At temperatures of 34°C or lower, the photophosphorylation rates were relatively insensitive to preincubation time; however, at 38 and 42°C, large decreases in the relative rates of ATP formation occurred as the preincubation time was increased. It was important, therefore, to assess the temperature effects in the context of the time dependence of the onset of inhibition.

The temperature dependencies of photophosphorylation and electron transport were determined at three different preincubation times (Fig. 2). The temperature response of photophosphorylation rate showed similar patterns at all three preincubation times; the rates increased with temperature up to an optimum, then decreased at higher temperatures. The curves for the 1- and 3-min preincubation conditions were virtually parallel throughout the temperature range, though the magnitude of photophosphorylation rates in the 3-min preincubation assays was 20 to 50 μmol mg Chl^{-1} h^{-1} (25–50%) lower than the rates in the 1-min preincubation assays. The optima for 1- and 3-min preincubation times was about 34°C. The temperature response curve obtained under 15-s preincubation conditions showed continually increasing rates of photophosphorylation up to 38°C. Photophosphorylation decreased markedly at 42°C at all three preincubation times.

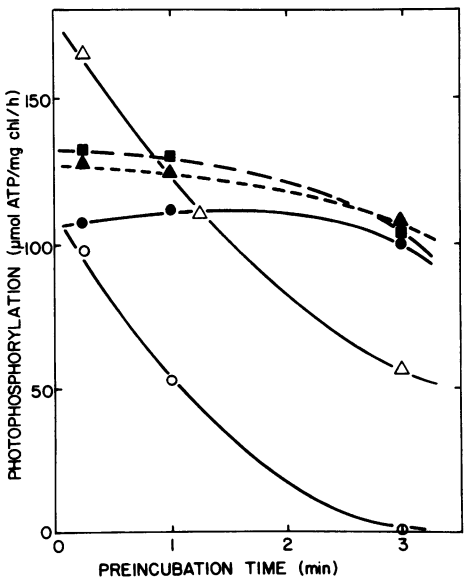

FIG. 1. The dependence of noncyclic photophosphorylation on preincubation time. Chloroplasts thylakoids were prepared as described in "Materials and Methods" and added to the temperature equilibrated reaction mixture at zero time. One min illumination at the same temperature followed the indicated preincubation times. (●), 26°C; (▲), 30°C; (■), 34°C; (△), 38°C; (○), 42°C.

The concurrent temperature response curve for electron transport showed electron transport rates increasing with temperatures up to 38°C under all three preincubation conditions (Fig. 2B). A decline in the electron transport rate occurred at 42°C at all three preincubation times, with the decline in rate being most drastic with the 3-min preincubation.

The ratio of ATP formed per electron pair transported, a measure of the coupling efficiency of the thylakoids, is shown in Figure 3 as a plot of the data of Figure 2. ATP/$2e^-$ shows a relatively constant ratio below 35°C at all three preincubation times, while at temperatures above 35°C, the ratio declines. For the 3-min preincubation, the ratio declined from 1.2 to 0.6 to 0 at 34, 38, and 42°C, respectively. Preincubation times of 1 min and of 15 s resulted in similar but less drastic declines at temperatures above 35°C.

An assessment of the relative stability of coupled and gramicidin-uncoupled noncyclic electron transport at high temperatures was carried out with 1-min preincubations. The rate of coupled electron transport (-gramicidin) was similar to that in Figure 2, while gramicidin stimulated electron transport 4- to 5-fold at temperatures below 38°C (Fig. 4). Above 38°C, the magnitude of the uncoupler stimulation decreased, and at 42°C, electron transport was totally inhibited in the presence of gramicidin. A similar effect was observed using 10 mM NH_4Cl as the uncoupler (data not shown).

Thermal Stability of PSI and Cyclic Photophosphorylation. PSI electron transport was found to increase linearly with temperatures above 35°C and remained stable and increasing to 48°C (Fig. 5). Cyclic photophosphorylation, coupled to PSI electron transport was found to have roughly the same temperature response as noncyclic photophosphorylation (Fig. 6).

Thermal Stability of Proton Uptake. The higher temperature stability of both noncyclic and cyclic electron transport relative to photophosphorylation suggested that temperature-dependent membrane permeability changes might explain the high temperature inhibition of photophosphorylation. Net proton uptake is seen to be relatively constant with temperatures up to 35°C (Fig. 7). Above 35°C, proton uptake decreased in a temperature dependence similar to that of photophosphorylation (Figs. 2 and 6). The temperature dependence of proton uptake was most similar to the temperature dependence of the coupling ratio, ATP/$2e^-$ in the noncyclic electron transport system (Fig. 3). Other experiments (not shown) indicated that the high-temperature-induced reduction in proton uptake is not due to dissociation of coupling factor.

DISCUSSION

We have demonstrated that photophosphorylation exhibits a temperature- and time-dependent lability. Below 35°C cyclic and noncyclic phosophosphorylation are stable for at least 3 min (Figs. 1 and 6) exhibiting an increasing activity with increasing temperature. Above 35°C time of pretreatment is a more critical element with a 15-s treatment increasing the rate at 38°C (Fig. 2) and

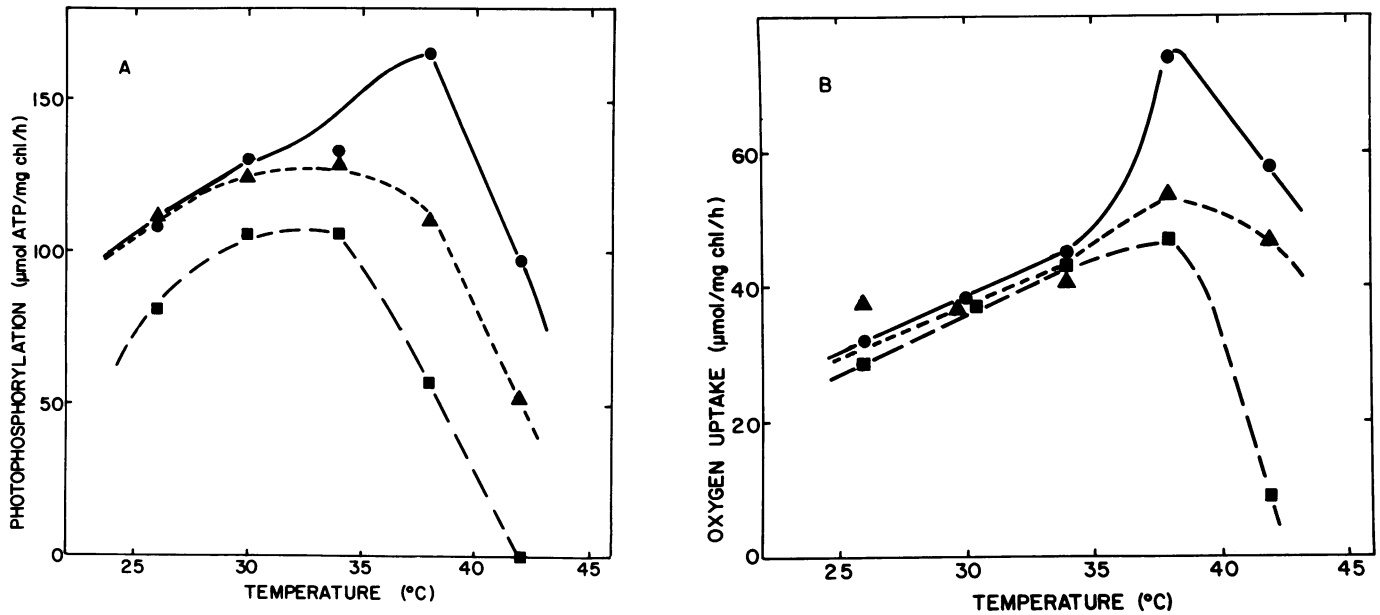

FIG. 2. The temperature dependence of noncyclic photophosphorylation (A) and accompanying electron transport (B) at three different preincubation times. Conditions are as in Figure 1. Photophosphorylation and electron transport were determined in one experiment. 15-s preincubation (●); 1-min preincubation, (▲); 3-min preincubation (■).

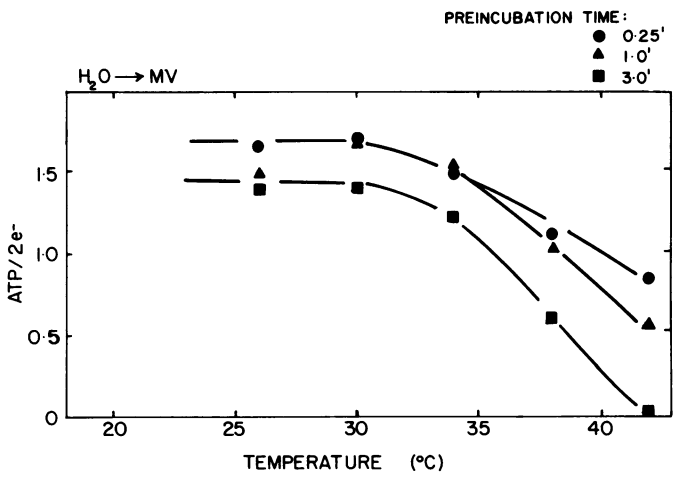

FIG. 3. The temperature dependence of the coupling ratio, ATP/2e⁻. Electron transport was calculated as four equivalents per O_2. The data and symbols are as in Figure 2.

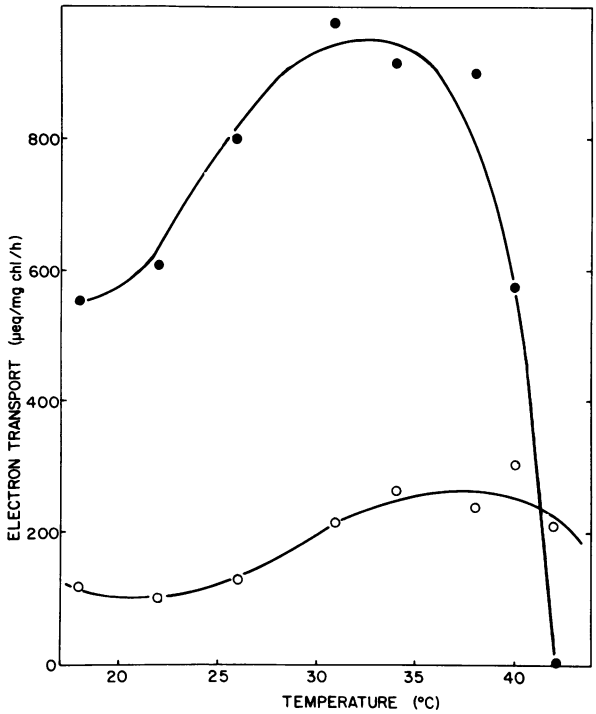

FIG. 4. The temperature dependence of noncyclic electron transport in the presence (●) and absence (○) of 10 μM gramicidin. Conditions are as in Figure 1. Ten μl of 1 mM gramicidin (in ethanol) were added to the reaction mixture before the addition of chloroplasts in the experiments which included gramicidin. The preincubation time was 1 min.

having little effect at 40°C (Fig. 6). High temperature inactivation is more readily noted in 1- and 3-min treatments with noncyclic phosphorylation inhibited beyond 35°C (Fig. 2). Cyclic phosphorylation is more resistant requiring a 3-min preincubation at 40°C to achieve a significant reduction in the rate (Fig. 6). Electron transport has greater thermal stability. Noncyclic electron flow involving both photosystems is maximally stable to 38°C (Fig. 2B) while PSI electron transport remains stable at least to 48°C (Fig. 5).

The data indicate that gramicidin-uncoupled noncyclic electron transport is thermal-labile at lower temperatures than coupled electron transport (Fig. 4). This result is understood in terms of the known sensitivity of the water oxidation apparatus to high internal pH. Reimer and Trebst (18) demonstrated that alkaline sensitivity of the water oxidation apparatus is observed only when the proton gradient is abolished by the presence of an uncoupler. In our experiment, the presence of gramicidin should bring the pH of the internal space of the thylakoids to 7.8. Since the water oxidation step is also heat labile (27), the combination of high internal pH and high temperatures would rapidly inactivate the water oxidation system. Dependence of heat-inactivation of ferricyanide reduction on pH has been reported for spinach thylakoids (14).

The differential relative temperature sensitivity of electron transport and photophosphorylation is best illustrated in Figure 3

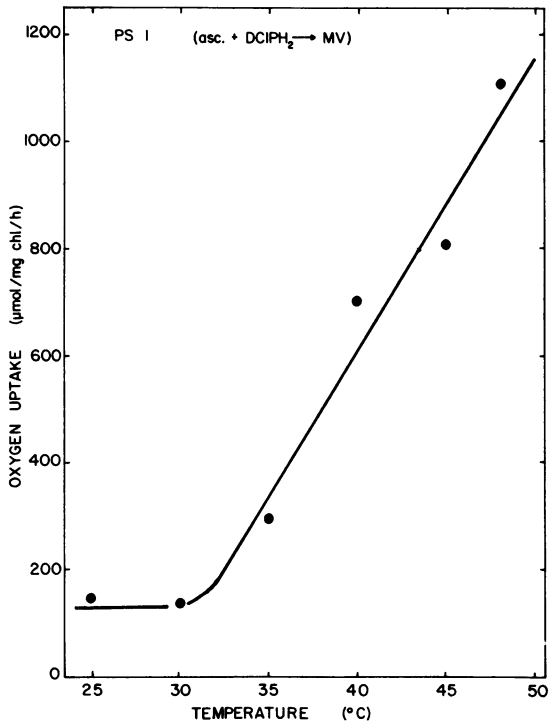

FIG. 5. The temperature dependence of PSI-mediated electron transport. Chloroplasts were prepared as in "Materials and Methods" and preincubated at the indicated temperature for 1 min. Illumination time was 1 min. The reaction medium composition is given in "Materials and Methods."

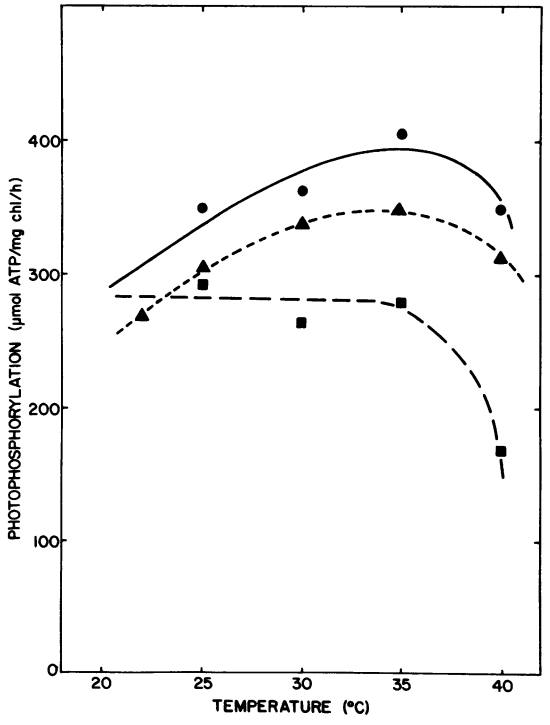

FIG. 6. The temperature dependence of cyclic photophosphorylation. Chloroplasts were prepared as in "Materials and Methods" and preincubated for 15 s (●), 1 min (▲), and 3 min (■). The reaction medium was as in "Materials and Methods" with 50 μM pyocyanine replacing methyl viologen and sodium azide.

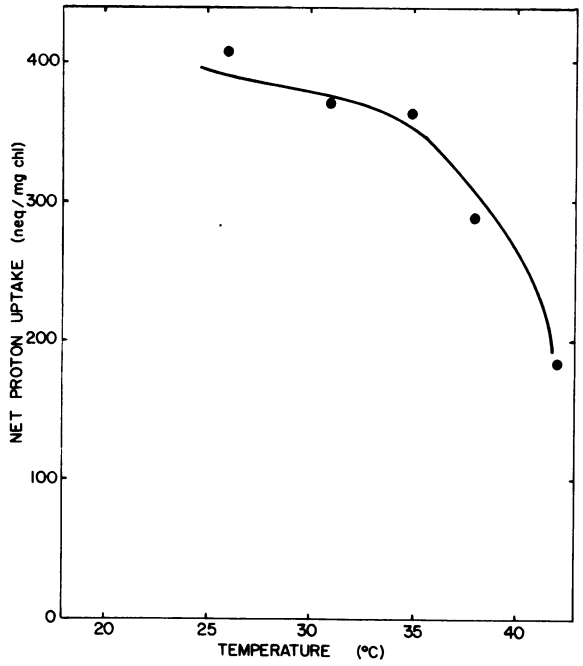

FIG. 7. The temperature dependence of net proton uptake. Conditions were as in Figure 6, with the omission of ADP and phosphate and the substitution of 0.1 mM Hepes/KOH (pH 7.8) for 50 mM Hepes/KOH. The chloroplast concentration was 29 μg Chl/ml.

which shows the coupling ratio decreasing continuously from 35 to 42°C. Since net proton uptake shows a similar temperature sensitivity (Fig. 7), it is reasonable to conclude that temperature-dependent changes in the permeability of the thylakoid membranes causes a thermal uncoupling at higher temperatures. Thermal uncoupling of electron transport in chloroplasts has been reported previously in studies using isolated thylakoids (6, 7, 13, 14, 19–23) and intact chloroplasts (11). Thermal uncoupling has been observed both in experiments where chloroplasts received a heat pretreatment before analyzing for light-dependent activities at room temperature (6, 7, 14, 19, 20, 22, 23) and in experiments where analysis of activities was conducted at high temperature (13, 21). Some of these studies have demonstrated that high temperature inhibition of photophosphorylation occurs along with inhibition of proton uptake (13), acid-base phosphorylation (14), and ΔpH (20). A few experiments have been reported showing high temperature inhibition of photophosphorylation that is not accompanied by a decrease in ΔpH (7, 11). In these experiments we concluded that high temperature increased counter ion permeability of the thylakoid membrane, thus accelerating counter ion flux to proton transport and inhibiting phosphorylation. It should be mentioned that Weis (24, 25) has recently concluded that high temperatures increase thylakoid counter ion permeability without inhibiting ATP formation. His conclusions are based on (a) experiments with intact spinach chloroplasts in which high temperature pretreatment increases ΔpH and light scattering while increasing the ratio of ATP to ADP (24), and (b) experiments with intact spinach leaves showing high temperature pretreatment effects on the kinetics of light scattering changes and electrochromic pigment absorption shift (25).

In the present study, high temperature inhibition of photophosphorylation can be termed 'thermal uncoupling' only in the sense that the ATP/2e$^-$ decreased (Fig. 3) and proton transport decreased (Fig. 7). Electron transport is increased (Figs. 2B and 4) but not stimulated to gramicidin-uncoupled rates at temperatures that inhibit photophosphorylation. In Figure 4, the rate of gram-

icidin-uncoupled electron transport is much higher than the rate of coupled electron transport at all temperatures above 35°C (except for 42°C). Thus, 'uncoupling' in the classical sense (*i.e.* stimulation of electron transport and simultaneous inhibition of phosphorylation) is not observed to occur as a consequence of high temperature.

The temperature range that produces this apparent uncoupling in *A. smithii* thylakoids (35–40°C) is identical to the temperature range that has been reported to induce uncoupling in spinach thylakoids (13, 21). This temperature range is also the range in which CO_2-saturated photosynthesis in whole leaves of *S. smithii* becomes limited by temperature (12). Inasmuch as the enzymes of the reductive pentose phosphate pathway have been found to be stable at these temperatures (4), high temperature effects on photophosphorylation may limit photosynthesis *in vivo* by limiting the regeneration of the substrate for RuBP carboxylase.

The conclusions of this study are correlated with the conclusions in the accompanying study (12), of the temperature response of whole leaf photosynthesis in *A. smithii*. In the accompanying study, reversible decreases in the quantum yield of photosynthesis were found to occur between 35 and 42°C. Since the quantum yield measurements are indicative of light-dependent reactions, the reversible decreases may be correlated with the decline in $ATP/2e^-$ found in this study (Fig. 3). Above 42°C, the quantum yield was found to be irreversibly inhibited, correlating with the lability of photophosphorylation (Figs. 2A and 6).

Another physiological consequence of thermal uncoupling can be predicted from a consideration of the light activation of stromal enzymes required for CO_2 reduction. Proton movement into the thylakoids during light-driven electron transport causes an alkalinization of the stroma (8). Also, counter ion flux of Mg^{2+} into the stroma from the thylakoids in response to proton transport raises the stromal concentration of this ion (17). Several enzymes of the reductive pentose phosphate pathway, including RuBP carboxylase, fructose 1,6-bisphosphatase, and sedoheptulose 1,7-bisphosphatase are activated by these stromal changes induced by proton transport into the thylakoids (5). A report by Weis (24) supports the hypothesis that a decrease in light activation of RuBP carboxylase occurs at elevated temperatures, and that this decrease in light-activation is accompanied by changes in thylakoid membrane properties. It is likely that thermally induced proton leakage in the thylakoid membrane could cause an inhibition of light-dependent stromal alkalinization and/or an alteration in Mg^{2+} fluxes such that activation of the carboxylase would be inhibited.

Effects on RuBP carboxylase would be most important in normal air (*i.e.* below CO_2 saturation), when the carboxylation reaction is rate limiting to net photosynthesis. In the previous report, temperature inhibition of whole leaf photosynthesis in normal air occurred from 25 to 47°C. Much of this inhibition was due to O_2 inhibition of photosynthesis. Even under conditions that eliminated O_2 inhibition, the Q_{10} of whole leaf photosynthesis was only 1.0 to 1.1 between 25 and 40°C, even though the Q_{10} of RuBP carboxylase from the *in vitro* assay was about 2.0 in this temperature range. Werdan *et al.* (26) have shown that photosynthetic carbon reduction is highly sensitive to stromal pH, thus suggesting that small decreases in proton uptake by the thylakoids contributing to small changes in stromal pH and Mg^{2+} could result in larger effects on carbon reduction. In Figure 7, the magnitude of proton uptake declined only slightly between 25 and 35°C (~1.5%/degree). This makes it unlikely that a decrease in alkalinization of the stromal pH is the cause of decreased activation of RuBP carboxylase or another enzymes in the reductive pentose phosphate pathway in this temperature region. The large drop in proton uptake between 35 and 40°C (~10%/degree) could, however, prevent alkalinization of the stroma and decrease stromal Mg^{2+} levels which could inhibit the carbon reduction cycle at either of the bisphosphatases or RuBP carboxylase steps, causing the decreased photosynthetic capacity observed in the previous study (12).

In summary, high temperature causes an apparent uncoupling of electron transport that may result from the increased thylakoid permeability to protons. This causes a decrease in ATP production required for the regeneration of the CO_2 acceptor. Studies on isolated protoplasts and intact chloroplasts support the contention that the high temperature limitation of photosynthesis observed in whole leaves is a consequence of a limitation in ATP production.

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