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The involvement of stromal ATP in maintaining the pH gradient across the chloroplast envelope in the light

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The possible involvement of ATP in maintaining the pH gradient across the chloroplast envelope membrane was investigated by simultaneously measuring the stromal ATP concentration and the pH of the stroma and intrathylakoid spaces in intact isolated chloroplasts. Addition of exogenous ATP in the dark increased stromal pH by 0.3–0.4 pH units and increased the pH gradient across the thylakoid membrane by a similar amount. In the dark, dihydroxyacetone phosphate plus oxaloacetate increased stromal ATP to levels equal to those obtained in illuminated chloroplasts, but stromal pH was only increased by 0.1–0.3 pH units compared to an increase of 0.8–1.0 units in the light. The energy-transfer inhibitor, phlorizin, decreased stromal ATP in illuminated chloroplasts almost to dark levels, but did not decrease stromal pH. Inorganic pyrophosphate and an analog of ATP were used to exchange endogenous adenine nucleotides out of chloroplasts, and this also decreased the stromal ATP to dark levels without decreasing stromal pH in the light. Addition of 15–20 μM 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) reduced both the stromal pH and ATP content of illuminated chloroplasts to dark levels but lower concentrations of DCMU preferentially decreased stromal pH. It is concluded that the pH gradient across the chloroplast envelope is unlikely to be maintained by an electrogenic proton pump driven by ATP hydrolysis. Photosynthetic electron transport is required to maintain the pH gradients across both the chloroplast thylakoid and chloroplast envelope membranes.

Introduction

Photosynthetic electron transport is coupled to proton transport across the chloroplast thylakoid membrane resulting in an acidification of the intrathylakoid space in the light [1]. The chloroplast envelope is not freely permeable to protons, so the pH of the stroma increases in the light as a result of this proton uptake into the thylakoids [2,3]. The rise in stromal pH acts to regulate CO_2 fixation, since many of the carbon cycle enzymes have alkaline pH optima [4–6]. Furthermore, the light activation of certain carbon cycle enzymes is also pH dependent, hence stromal pH is an important factor in controlling the flow of carbon in photosynthesis [7–9]. Even a relatively small decrease in stromal pH can dramatically lower the rate of photosynthesis in isolated chloroplasts [5,9,10].

The mechanism by which an alkaline stromal pH is maintained in the light is not fully understood. In isolated choroplasts the stromal pH in the dark is below that of the medium by 0.5–1.0 units, and since this gradient is maintained in the presence of proton ionophores, it has been suggested that a Donnan distribution of protons is responsible [5]. In the light, the stromal pH is

Abbreviations: AMP-PCP, β,γ-methylene adenosine triphosphate; Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

0.1–0.4 units above the medium pH [3,5,9,10], and if the same Donnan potential exists across the envelope as in the dark the effective pH gradient across the envelope could be as high as 0.6–1.4 units. Initially, the uptake of protons into the intrathylakoid space would be sufficient to make the stroma alkaline, but the pH gradient across the chloroplast envelope would induce a passive inward flux of protons which would need to be balanced by an electrogenic transport of protons out of the chloroplast or else the pH gradient across the envelope would be dissipated. Such proton transport has been observed with intact chloroplasts as a light-dependent efflux of protons into the surrounding medium [3,9,11]. The efflux could be driven by an ATPase proton pump on the envelope membrane, and there is some evidence to support such a notion.

An Mg^{2+}-dependent ATPase activity is associated with envelope membranes [12–14], and is localised on the inner of the two membranes [15], which is most likely the site of the proton efflux. [5]. It has been reported that addition of exogenous ATP to darkened chloroplasts results in an increase in stromal pH [16], and the operation of an ATPase type proton pump has also been suggested from inhibitor studies with isolated chloroplasts [10]. In contrast, Gimmler et al. [17] reported that inhibition of phosphorylation by Dio-9 did not diminish the pH of the stroma. Light-dependent uptake of the glycerate anion, which is thought to follow the pH gradient across the envelope, was also not dependent on ATP [18]. In this paper, the possibility of an ATPase-type proton pump on the chloroplast envelope has been investigated by simultaneously measuring stromal ATP and the pH of the stroma and intrathylakoid spaces. The results suggest that ATP is not required to maintain the pH gradient across the chloroplast envelope in the light.

Materials and Methods

Plant material

Spinach (*Spinacia oleracea* L. cv Hybrid 102) was grown in 12-cm pots filled with fine gravel. The pots were drip-irrigated once an hour with the nutrient solution described previously [19]. The plants were grown in a glasshouse, but the day-length was restricted to 14 h by a mechanical shutter. In the winter, supplementary lighting was provided by a mixture of fluorescent and incandescent lights to give a minimum light intensity of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. The plants were grown for 3–4 weeks before harvesting. Pea (*Pisum sativum* L. cv Massey Gem) seeds were germinated in vermiculite and grown in a glasshouse for 10–12 days.

Chloroplast isolation

Intact chloroplasts were isolated from spinach and purified on a two-step Percoll gradient [19]. All procedures were carried out at 0°C. Leaves (30–40 g) were ground for 3 s in a Polytron blender with 200 ml 330 mM sorbitol/5 mM $MgCl_2$/10 mM $Na_4P_2O_7$/2 mM isoascorbate/0.1% bovine serum albumin (pH 6.5). The brei was squeezed through two layers of Miracloth containing a layer of cotton wool and the filtrate was centrifuged at 1700 × *g* for 1 min. The pellets were resuspended in 6 ml of 330 mM sorbitol/2 mM EDTA/1 mM $MgCl_2$/1 mM $MnCl_2$/50 mM Hepes-KOH/0.2% bovine serum albumin (pH 7.6) and placed into two centrifuge tubes. Each was underlayered with 4 ml of the same medium plus 40% (v/v) Percoll, then the tubes were again centrifuged at 1700 × *g* for 1 min. Broken chloroplasts formed a band at the top of the Percoll layer, whereas intact chloroplasts were pelleted by this procedure. The supernatants were discarded and the pellets of intact chloroplasts were resuspended in the above medium. The same procedure was used for isolating intact chloroplasts from pea shoots, except that 60-g leaves were disrupted and the resuspension medium and Percoll pad contained in addition 10 mM KH_2PO_4.

The chloroplasts were greater than 95% intact based on penetration of ferricyanide [20]. The spinach chloroplasts exhibited rates of CO_2-dependent O_2 evolution of 120–200 μmol per mg Chl/h. The pea chloroplasts gave similar rates of CO_2-dependent O_2 evolution provided PPi and ATP were added to the assay medium [21].

Oxygen evolution

CO_2-dependent O_2 evolution was measured at 20°C with Hansatech O_2 electrodes. The assay medium contained 330 mM sorbitol, 2 mM EDTA,

1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes-KOH (pH 7.6), 4 mM $NaHCO_3$, 0.2 mM P_i, 1000 units per ml catalase, and chloroplasts equivalent to 40 μg chlorophyll in a total volume of 1 ml. The suspension was illuminated with white light (1500 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR measured with a Lambda Instruments quantum sensor). Chlorophyll was measured in 80% acetone by the method of Arnon [22].

pH measurements

The pH in the chloroplast stroma and intrathylakoid spaces were measured by silicone oil filtering centrifugation using 5,5-dimethyloxazolidine-2,4-dione and methylamine [3]. Chloroplasts were incubated at 20°C in the same medium as for oxygen evolution, except that the chlorophyll concentration was 50 $\mu g \cdot ml^{-1}$ and the medium also contained 0.5 mM 5,5-dimethyloxazolidine-2,4-dione and 0.03 mM methylamine. Four samples, each of 200 μl, were transferred to 0.4 ml microfuge tubes containing 20 μl 1N $HClO_4$ overlayered with 75 μl silicone oil (Wacker, AR180). The chloroplasts were incubated under a green safelight (dark) for a total of 1.5 min, then kept for a further 2 min in the dark or in white light (1500 $\mu E \cdot m^{-2} \cdot s^{-1}$) before being centrifuged for 15 s in a Beckman microfuge. For chloroplasts incubated in the light, illumination was continued during the centrifugation. Where inhibitors were added, they were present from the outset. Phlorizin and DCMU were dissolved in methanol and ethanol, respectively and an equal amount of alcohol was added to the controls. Three sets, each of four tubes, were processed for each treatment. The first contained ^3H_2O (1.5 μCi per ml) plus [^{14}C]sorbitol (0.5 μCi per ml) to determine the total volume pelleted and the volume external to the chloroplast (sorbitol space). From these measurements the stromal volume (sorbitol-impermeable space) was calculated. The second and third sets of tubes contained [^{14}C]5,5-dimethyloxazolidine-2,4-dione (0.6 μCi per ml) and [^{14}C]methylamine (0.2 μCi per ml) for determination of the stromal pH and intrathylakoid pH, respectively. Radioactivity in the pellet and supernatant fractions was determined by liquid scintillation counting, using external standard channels ratio for quench correction.

ATP

The stromal ATP content was determined using the luciferin-luciferase bioluminescence assay. Extracts for ATP determination were normally prepared by direct addition of $HClO_4$ to a rapidly stirred chloroplast suspension to mimimise any change in ATP concentration. Chloroplasts were incubated under the same conditions as for pH measurement (total volume, 0.4 ml) in a rapidly stirred glass chamber maintained at 20°C. The reaction was stopped by rapid injection of 50 μl of 12 M $HClO_4$ and stirred for a further 15 s before being transferred to an ice bath. Precipitated protein was removed by centrifugation and an aliquot of the supernatant was neutralised with K_2CO_3. The precipitate of $KClO_4$ was removed by centrifugation. ATP was determined using Boehringer CLS reagent in an LKB 1250 luminometer. For the experiment with pea chloroplasts (Table III), ATP would have been exported from the chloroplast into the assay medium, and it was important to know the level of ATP remaining in the chloroplast. In this case, the chloroplasts were separated from the medium by centrifuging through silicone oil under the same conditions as for pH measurements. The pellet fraction was treated as above and used for ATP determination.

Materials

Radioisotopes were obtained from Amersham, U.K. and Biochemicals from Sigma, U.S.A. The silicone oil was a kind gift from Wacker, Australia.

Results

For measurement of the pH in the stroma and intrathylakoid spaces in the dark it was found necessary to work under a green safelight. Even normal laboratory lighting (less than 10 $\mu E \cdot m^{-2} \cdot s^{-1}$, 400–700 nm) was sufficient to alter the pH significantly; for example, in one experiment, the pH of the stroma was 6.95 under green safelight, 7.46 in normal room lighting and 7.96 with saturating white light (1500 $\mu E \cdot m^{-2} \cdot s^{-1}$). The intrathylakoid pH values were 5.73 (green safelight), 5.30 (room light) and 5.00 (saturating light). This is consistent with the previous observation that alkalisation of the chloroplast stroma saturates at relatively low light intensities [3].

TABLE I

ATP-INDUCED INCREASE IN STROMAL pH IN THE DARK IN SPINACH CHLOROPLASTS

ATP and AMP-PCP were added to a final concentration of 2 mM, FCCP was 10 μM. The assay medium was pH 7.60 in all cases.

	Stromal pH	Intra-thylakoid pH	Transenvelope ΔpH	Trans-thylakoid ΔpH
Dark	7.03	5.88	−0.57	1.14
Dark + ATP	7.35	5.96	−0.25	1.39
Dark + AMP-PCP	7.04	6.10	−0.56	0.94
Dark + ATP + FCCP	6.75	6.18	−0.85	0.58
Light	7.91	5.18	0.31	2.73

Addition of ATP to darkened spinach chloroplats consistently increased the stromal pH by 0.3–0.4 units (Table I). Similar results were observed with pea chloroplasts, which have a higher rate of ATP transport than spinach chloroplasts [21,23]. In all experiments, the increase in stromal pH was accompanied by an approximately equal increase in the pH gradient across the thylakoid membrane (Table I). The pH changes were not observed with AMP-PCP, an analog of ATP which is transported by the chloroplast adenine nucleotide transporter but which cannot be hydrolysed [23]. The proton ionophore FCCP abolished the ATP-induced increase in stromal pH and the increase in transthylakoid ΔpH (Table I).

The ATP content of darkened chloroplasts can be increased by substrate phosphorylation using dihydroxyacetone phosphate provided the reducing equivalents are removed by providing oxaloacetate to reoxidise the NADPH formed by glyceraldehyde-3-phosphate dehydrogenase [4,24]. Table II shows the effect of DHAP plus oxaloacetate on the stromal ATP, stromal pH and intrathylakoid pH of spinach chloroplasts in the dark and light. In the dark, spinach chloroplasts contained 5–10 nmol ATP per mg Chl and this level was only decreased 10–15% by the addition of 2 mM glycerate suggesting that this reflected thermodynamically inactive ATP, possibly bound to the thylakoid coupling factor [24]. Illumination of the chloroplasts caused a 3–4 fold increase in stromal ATP concentration. As shown in Table II, addition of dihydroxyacetone phosphate plus oxaloacetate in the dark increased stromal ATP levels almost to the same extent as illumination of the chloroplasts. There was also a consistent increase in stromal pH by 0.1–0.4 pH units and this was accompanied by an approximately equal increase in transthylakoid ΔpH. In the light, dihydroxyacetone phosphate plus oxaloacetate also increased the stromal ATP content, stromal pH and the transthylakoid pH gradient. These effects were not observed when dihydroxyacetone phosphate or oxaloacetate were added singly. Similar results were

TABLE II

EFFECT OF DIHYDROXYACETONE PHOSPHATE PLUS OXALOACETATE ON THE ATP CONTENT AND STROMAL pH OF SPINACH CHLOROPLASTS

Dihydroxyacetone phosphate and oxaloacetate were each 1 mM. P_i was 0.2 mM, but was increased to 10 mM when dihydroxyacetone phosphate plus oxaloacetate were added.

	Stromal ATP nmol per mg Chl	Stromal pH	Intra-thylakoid pH	Trans-envelope ΔpH	Trans-thylakoid ΔpH
Dark	5.4	6.93	5.82	−0.67	1.11
Dark + DHAP + oxaloacetate	15.2	7.24	5.79	−0.36	1.45
Light	16.9	7.92	5.04	0.32	2.88
Light + DHAP + oxaloacetate	26.7	8.09	5.05	0.49	3.04

obtained with pea chloroplasts.

Energy-transfer inhibitors, such as phlorizin, directly inhibit the formation of ATP and should not effect electron transport nor the pH gradient across the thylakoid membrane [25,26]. As shown in Fig. 1, phlorizin inhibited CO_2-dependent oxygen evolution in spinach chloroplasts, 50% inhibition occurring at 0.3 mM and complete inhibition above 20 mM. Stromal ATP was less sensitive with 50% inhibition requiring 7 mM phlorizin. Nevertheless, 20 mM phlorizin decreased stromal ATP almost to the levels observed in the dark. In contrast, both stromal pH and the transthylakoid pH gradient were actually increased by 5–10 mM phlorizin. Stromal pH was not inhibited by the highest concentration of phlorizin used although there was a 20% decrease in the thylakoid pH gradient at 20 mM phlorizin.

An alternative method for decreasing stromal ATP content without affecting electron transport and the pH gradient across the thylakoid membrane would be to deplete the chloroplast of adenine nucleotides. Since the adenine nucleotide transporter is an obligatory counter-exchange type carrier, incubation of the chloroplasts with ATP analogs [23] or inorganic pyrophosphate [21] results in their uptake in exchange for chloroplast adenine nucleotides. Once outside the chloroplast the endogenous nucleotides are diluted 1000-fold in the assay medium and cannot compete with the analog for re-entry on the adenine nucleotide transporter. As expected, incubation of pea chloroplasts with PP_i or AMP-PCP depleted the chloroplast of ATP resulting in a stromal ATP content similar to that observed in the dark (Table III). In both cases, the stromal pH was actually increased despite the decreased ATP content. The pH gradient across the thylakoid membrane was not significantly altered by PP_i or AMP-PCP (Table III).

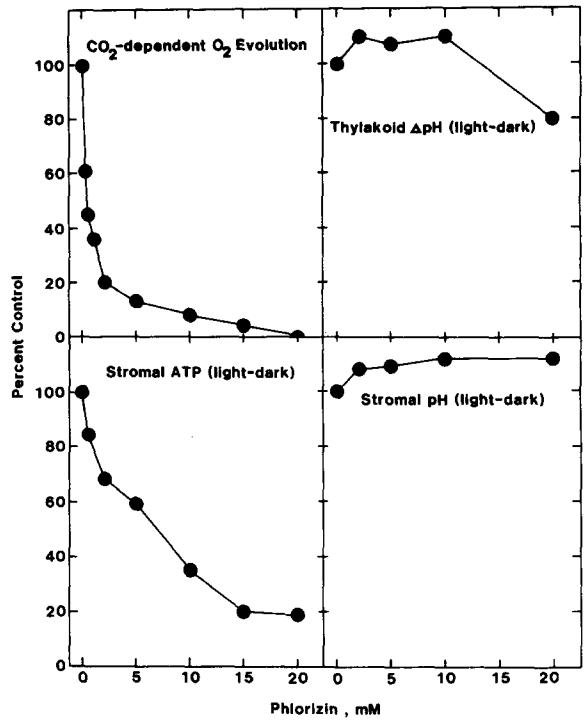

Fig. 1. Effect of phlorizin on CO_2-dependent O_2 evolution, stromal ATP, pH gradient across the thylakoid membrane and pH of the stroma in spinach chloroplasts. Dark values were subtracted and the results plotted as a percentage of the control, thus 100% represents the control value in the light minus that in the dark and 0% represents the control value in the dark. The respective control values were 140 (light) and 0 (dark) μmol O_2 per mg Chl/h, 21.8 (light) and 6.0 (dark) nmol ATP per mgChl, 3.01 (light) and 0.92 (dark) pH units, and pH 7.96 (light) and 6.74 (dark).

TABLE III

EFFECT OF PP_i (5 mM) AND AMP-PCP (1 mM) ON THE ATP CONTENT AND STROMAL pH OF PEA CHLOROPLASTS

The chloroplasts were separated from the assay medium by centrifugation through silicon oil for determination of stromal ATP.

	Stromal ATP (nmol per mg Chl)	Stromal pH	Intra-thylakoid pH	Trans-envelope ΔpH	Trans-thylakoid ΔpH
Dark	3.2	6.52	6.02	−1.08	0.50
Light	15.0	7.70	5.14	0.10	2.55
Light + PP_i	4.8	7.81	5.29	0.21	2.52
Light + AMP-PCP	3.8	7.79	5.22	0.19	2.57

The effect of DCMU, an inhibitor of non-cyclic electron transport, on CO_2-dependent oxygen evolution, stromal ATP, stromal pH and the pH gradient across the thylakoid membrane is shown in Fig. 2. Oxygen evolution was inhibited more than 90% by 1 μM DCMU and completely abolished at 5–10 μM DCMU. In contrast, stromal ATP was only decreased 5–10% by low concentrations of DCMU (1–5 μM) and reduction of stromal ATP to dark levels required 20 μM DCMU. Addition of 5 μM DCMU decreased both stromal pH and the pH gradient across the thylakoid by approx. 50% but reduction to dark levels only occurred with 20 μM DCMU. In some experiments, the differential effect of lower concentrations of DCMU (5–10 μM) on stromal ATP and stromal pH were more marked with little or no decrease in stromal ATP, but a 40–80% reduction in stromal pH.

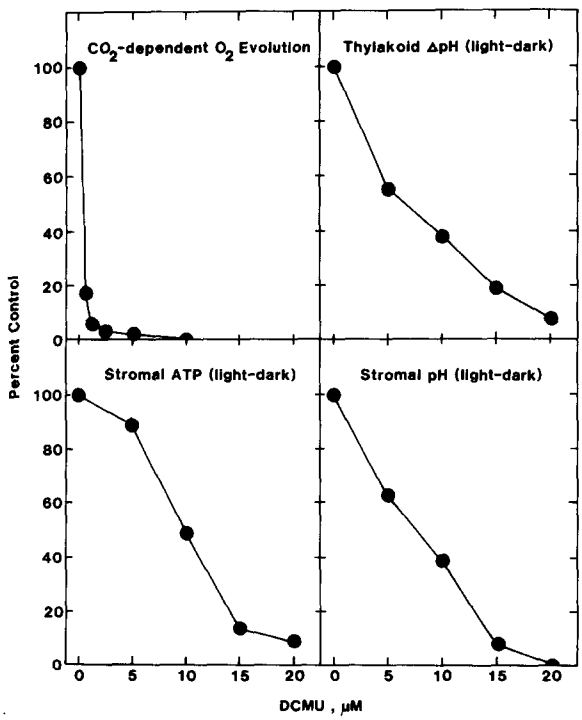

Fig. 2. Effect of DCMU on CO_2-dependent O_2 evolution, stromal ATP, pH gradient across the thylakoid membrane and pH of the stroma in spinach chloroplasts. Results are expressed as described in Fig. 1. The respective control values were 149 (light) and 0 (dark) μmol O_2 per mg Chl/h, 32.1 (light) and 9.4 dark) nmoles ATP per mg Chl, 2.80 (light) and 1.10 (dark) pH units, and pH 7.94 (light) and 6.98 (dark).

Discussion

The alkalisation of the stroma in darkened chloroplasts by exogenous ATP (Table I) is similar to that reported by Champigny and Joyard [16]. The transport of ATP into the chloroplasts would actually be expected to acidify the stroma assuming that ATP^{4-} enters together with a proton in exchange for efflux of ADP^{3-}. Such an acidification was not observed with ATP nor with the analog, AMP-PCP. The fact that the ATP analog did not increase stromal pH suggests that the ATP-induced alkalisation of the stroma was not related to adenine nucleotide transport, since AMP-PCP is also exchanged by the chloroplast adenine nucleotide transporter [23]. The proton ionophore FCCP reversed both the alkalisation of the stroma and the increase in thylakoid pH gradient induced by exogenous ATP, suggesting that the changes in proton gradient across both the envelope and thylakoid membranes were not an artifact of the measurement technique. Phosphorylation of endogenous adenine nucleotides by addition of DHAP plus oxaloacetate resulted in a similar increase in stromal pH to that induced by exogenous ATP (Tables I and II). These results indicate that increased stromal ATP can cause an alkalization of the stroma in the dark which is suggestive of the operation of an ATPase proton pump on the envelope membrane. It should be stressed, however, that the rise in stromal pH was less than 40% of that brought about by illumination despite the fact that stromal ATP was raised to the same level as that attained in the light (Table II). The increase in stromal pH was in all instances accompanied by an approximately equal increase in the pH gradient across the thylakoid membrane and this may offer an alternative explanation for the ATP-induced alkalisation of the chloroplast stroma. If the ATP were hydrolysed by the chloroplast coupling factor, rather than by the envelope ATPase, this would increase the pH gradient across the thylakoid membrane and this pH gradient could be transmitted to the envelope membrane via connections of the stroma lamellae to the inner envelope membrane [5].

It is possible that the mechanism for transporting protons across the envelope membrane is only fully operative in the light and this could explain

the failure of elevated dark levels of stromal ATP to cause an equivalent alkalisation of the stroma to that observed in the light. The experiments of Fig. 1 and Table III were designed to test this possibility by decreasing the stromal ATP concentration in illuminated chloroplasts without altering other parameters such as the proton gradient across the thylakoid membrane. This was achieved with the energy-transfer inhibitor phlorizin (Fig. 1) and by exchanging the endogenous adenine nucleotides out of the chloroplast with PP_i or the ATP analog, AMP-PCP (Table III). In both cases, stromal ATP was decreased virtually to dark levels without decreasing the stromal pH. From the stromal ATP content and chloroplast volume, the ATP concentration in the stroma would have been 0.6–1.3 mM in the light and 0.1–0.3 mM in the dark in these experiments. It could be argued that the chloroplast envelope ATPase might have such a high affinity for ATP that this decrease in stromal ATP would not alter its activity. This seems unlikely, since the reported affinity of the enzyme for ATP is not high (K_m (ATP) 0.4–0.8 mM) [13,14]. Furthermore, the level of ATP remaining in both treatments was similar to that in darkened chloroplasts which supports previous evidence that this ATP is bound within the chloroplast and not free to participate in biochemical reactions in the stroma [24]. The most logical conclusion from these experiments is that ATP is not required for the maintenance of an elevated stromal pH in the light, and hence that the envelope ATPase does not function as a proton pump. This is supported by the preferential decrease in stromal pH at low DCMU concentrations (Fig. 2). Under these circumstances the decreased demand for ATP would result from a lack of NADPH to drive CO_2 fixation and the ATP level may also be elevated by the contribution of cyclic photophosphorylation. Higher concentrations of DCMU completely reversed the light-induced alkalisation of the stroma indicating that electron transport is required to maintain the pH gradient across the chloroplast envelope.

If an envelope membrane ATPase is not responsible for the proton efflux from chloroplasts, alternative mechanisms need to be explored. The possibility that the proton gradient across the thylakoid membrane is transmitted to the envelope membrane via connections between the two membranes [5] is an intriguing one and could explain the ATP-induced rise in stromal pH in the dark (Tables I and II). This hypothesis is difficult to test, however, and the proposed connections are not obvious from normal electron micrographs. An alternative possibility would be some other kind of proton pump on the chloroplast envelope, possibly driven by an electron-transport chain. The plasmalemma of corn root protoplasts has recently been shown to contain a redox reaction driven by an NADH oxidase which results in proton efflux and potassium influx [27] and the possibility of a similar system on the chloroplast envelope is currently being investigated.

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