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AN ANALYSIS OF PROTON FLUXES COUPLED TO ELECTRON TRANSPORT AND ATP SYNTHESIS IN CHLOROPLAST THYLAKOIDS

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Electron transport, phosphorylation and internal proton concentration were measured in illuminated spinach chloroplast thylakoid membranes under a number of conditions. Regardless of the procedure used to vary these parameters, the data fit a simple chemiosmotic model. Protons from Photosystem II did not appear to be utilized differently from those derived from Photosystem I. The maximal phosphorylation efficiency (P/e_2) for photophosphorylation in washed thylakoids under oxidizing conditions is likely to be 4/3. This value is consistent with a proton-to-electron-pair ratio of 4 for electron flow through both photosystems and a proton-to-ATP ratio of 3 for the chloroplast proton-ATPase.

Introduction

Illuminated chloroplast thylakoids couple ATP synthesis to electron transport from water to a number of electron acceptors. In the chemiosmotic hypothesis [1,2], a transmembrane electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) links ATP synthesis to electron flow. Electron flow is coupled to the accumulation of protons in the thylakoid interior and a $\Delta\tilde{\mu}_{H^+}$ is generated. The flow of protons down the gradient through a H^+-ATPase provides the driving force for ATP synthesis.

Among the basic parameters that describe the coupling between electron flow and ATP synthesis are the number of protons translocated per electron transferred (H^+/e^- ratio) and the number of protons translocated per ATP synthesized (H^+/ATP ratio). Measurements of proton appearance in the thylakoid lumen or disappearance from the medium following single turnover flashes suggest that the H^+/e^- ratio is 2 [3,4]. These observations are consistent with the notion that one H^+/e^- appears in the lumen as a consequence of water oxidation and another proton per electron from the oxidation of plastohydroquinone. However, under some circumstances, electron flow through the cytochrome b_6-f complex may be associated with the translocation of two protons per electron [5–7] and the purified cytochrome complex pumps protons after its incorporation into phospholipid vesicles [8].

Various approaches have been used to estimate the H^+/ATP ratio. A minimum H^+/ATP ratio of 3 was determined from estimates of the $\Delta G'$ for ATP synthesis and $\Delta\tilde{\mu}_{H^+}$ under conditions where the phosphorylation system was allowed to reach equilibrium in the light [9,10]. A wide range of values for the H^+/ATP ratio have been obtained when rates of proton accumulation coupled to ATP hydrolysis by thylakoids were estimated

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Abbreviations: ΔpH, pH of the medium minus pH of the thylakoid interior; R_e, rate of electron flow; R_p, rate of phosphorylation; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N′*-dicyclohexylcarbodiimide.

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through the use of pH electrodes (see for example, Ref. 11). In view of the slow response of pH electrodes, this approach is questionable. The maximum or intrinsic phosphorylation efficiency (P/e_2 ratio) is equal to $2 \times (H^+/e^-)/(H^+/ATP)$. Accurate measurement of the intrinsic P/e_2 ratio would place limits on the H^+/e^- and H^+/ATP ratios. Measured P/e_2 ratios are lower than the intrinsic ratio, since the coupling between electron flow and phosphorylation is not 100% efficient. Electron flow proceeds at an appreciable rate even in the absence of ADP and P_i, indicating that protons can leak out of the thylakoid lumen without driving ATP synthesis. Schröder et al. [12] and Portis et al. [13] measured the dependence of the rate of this leak on internal proton concentration under non-phosphorylating conditions and corrected observed P/e_2 ratios for its effect. Both groups obtained a constant P/e_2 after correction, indicating that passive proton efflux is the main dissipative process. For electron flow from water to Photosystem I acceptors, Schröder et al. [12] obtained a corrected P/e_2 of 1.0, whereas Portis and McCarty [14] and McCarty and Portis [15] obtained a value of 1.3. The latter value is consistent with an H^+/ATP ratio of 3 and a H^+/e^- ratio of 2.

In this paper, we present a further characterization of the relationships between nonphosphorylating electron flow, phosphorylation and $[H^+]_{in}$. Maximal P/e_2 ratios were also estimated under a wide variety of conditions.

Materials and Methods

Spinach chloroplast thylakoids were isolated as described [16] and were suspended in 0.3 M mannitol/0.02 M Tricine-NaOH (pH 8.0)/0.01 M NaCl. Intact (over 90%) chloroplasts were isolated from 11–16-day-old pea (*Pisum sativum*, var Progress No. 9) shoots as described [17]. Chlorophyll was determined in 80% acetone extracts using the extinction coefficients of Arnon [18].

ΔpH and $[H^+]_{in}$ were calculated from the steady-state extents of [^{14}C]hexylamine uptake, determined by a silicone-oil microcentrifugation method essentially as described previously [14,19]. The usual reaction mixtures contained (in 0.25 ml): 20 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.04 mM EDTA, 0.025 mM [^{14}C]hexylamine (1.3 mCi/mmol), thylakoids or chloroplasts equivalent to 0.1 mg chlorophyll/ml, [^{3}H]sorbitol (1 mCi/ml) and other additions as indicated. In many experiments, the sorbitol was omitted and the entire lower (glycerol/trichloroacetic acid) layer was taken for the determination of radioactivity. This simplified procedure gave quite similar results of the double-isotope method, since the correction for external [^{14}C]hexylamine carried through the silicone fluid with the thylakoids is less than 2% for the extents of uptake reported. 85–95% of the thylakoids penetrated the silicone fluid layer and a recovery of 90% was generally assumed in the calculation of ΔpH. Light intensities were varied with neutral density filters.

Electron flow from water to $Fe(CN)_6^{3-}$ (1 mM) was determined in a reaction mixture identical to that used in the estimation of ΔpH, except that neither [^{14}C]hexylamine nor [^{3}H]sorbitol was present. Aliquots were illuminated within the microcentrifuge and ferrocyanide production was assayed [20]. Electron flow from water to methyl viologen (0.2 mM) was estimated by determination of H_2O_2 production essentially according to Black and Brandt [21]. Photosystem I-dependent methyl viologen reduction was determined using tetramethylquinol as the electron donor [22] in the presence of superoxide dismutase and 2 mM DCMU. When phosphorylation was assayed, the reaction mixtures contained in addition 2 mM ADP/4 mM potassium phosphate buffer/0.2 μCi $^{32}P_i$. Incorporation of $^{32}P_i$ into organic phosphates was determined by the method of Lindberg and Ernster [23].

Nucleotides, bathophenanthroline sulfonate, carbonylcyanide *m*-chlorophenylhydrazone, phlorizin, Percoll, superoxide dismutase and catalase were purchased from the Sigma Chemical Company. Dimethyl- and tetramethylquinones were obtained from Aldrich. The silicone fluids were generous gifts of the Silicone Products Division, General Electric Company. [^{14}C]Hexylamine was obtained from Mallinckrodt Nuclear or from Amersham. Other isotopically labeled compounds were from New England Nuclear.

Results

Relation of nonphosphorylating electron flow to ΔpH

At a constant external pH, the internal proton concentration ($[H^+]_{in}$) was previously shown [12–14] to be proportional to the rate of electron flow under nonphosphorylating conditions. Since the rate of proton influx is given by the rate of electron transport multiplied by H^+/e, this result suggests that passive proton efflux is also proportional to $[H^+]_{in}$, since proton efflux and influx rates are equivalent at the steady state:

$$\text{Influx rate} = (H^+/e)\cdot R_e \qquad (1)$$

$$\text{Efflux rate} = k_H[H^+]_{in} \qquad (2)$$

$$(H^+/e)\cdot R_e = k_H[H^+]_{in} \qquad (3)$$

where R_e is the rate of electron flow and k_H is the apparent first-order rate constant for proton efflux. Since the external proton concentration ($[H^+]_{out}$) is always less than 1% of $[H^+]_{in}$ in our experiments, $[H^+]_{in}$ is an approximation of $[H^+]_{in} - [H^+]_{out}$. Thus, passive proton efflux can be thought of as proton diffusion through the membrane. However, we will show later that this process has some unusual properties. The transmembrane potential difference is near zero in the steady state [24–27] and has been ignored.

$[H^+]_{in}$ is proportional to R_e when the parameters are varied by decreasing light intensity [12–14]. This relationship also holds when light intensity is held constant and increasing amounts of dichlorophenyl 1,1-dimethylurea are used to inhibit R_e (Fig. 1). The protonophores gramicidin (not shown) and CCCP [14] decrease the $[H^+]_{in}$ supported by a given R_e, resulting in an increase in k_H. Thylakoids uncoupled by treatment with *o*-phenylenebismaleimide in the light [28] also have an increased k_H.

At pH 8, the linear relationship between $[H^+]_{in}$ and the rate of electron flow fails at high ΔpH values and further increases in the rate of electron flow result in little or no further increases in $[H^+]_{in}$ [13]. This second phase to the relationship between $[H^+]_{in}$ and R_e was ascribed to the induction at ΔpH values greater than about 2.9 of a second proton leak, occurring through the ATPase complex. This explanation was supported by the ability of ATP to prevent simultaneously the onset of this second phase and to prevent a conformational change in CF_1 [13]. Since ADP also prevents the leak at high ΔpH values, the effect of ATP cannot be ascribed to ATP-dependent H^+ uptake. Furthermore, neither the conformational change nor the second phase is seen in experiments at pH 7 [13]. Chloroplast thylakoids were reacted with DCCD, which prevents proton efflux through the ATPase complex by reacting with the CF_0 portion of the complex [16,29]. This treatment completely prevents onset of the second phase in the relationship between $[H^+]_{in}$ and the rate of electron flow, but leaves k_H unchanged (Fig. 2).

The energy transfer inhibitors, phlorizin and 4′-deoxyphlorizin, do not prevent the onset of the proton leak through the ATPase complex at high ΔpH values (not shown). Surprisingly, 2 mM phlorizin decreased the k_H taken from the linear portion of the curve relating $[H^+]_{in}$ to the rate of electron flow. Although 0.16 mM 4′-deoxyphlorizin inhibited energy transfer as well as 2 mM phlorizin, it did not alter the k_H, indicating that

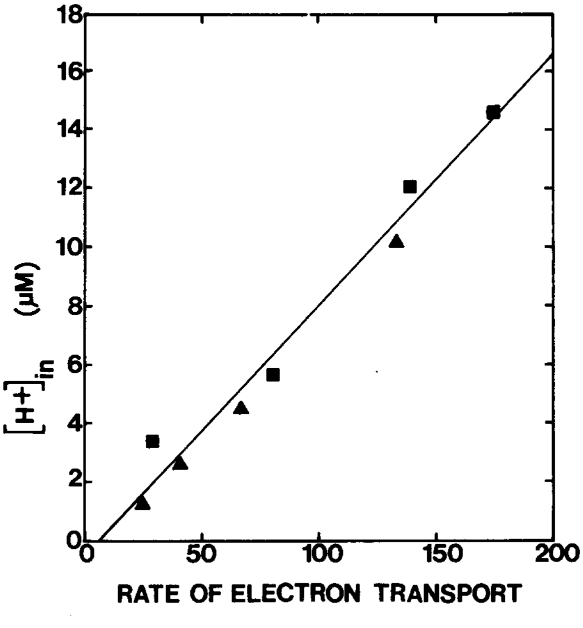

Fig. 1. Relationship between $[H^+]_{in}$ and rate of electron flow as varied by light intensity (■) and DCMU (▲). Electron flow from water to ferricyanide and ΔpH were determined either at a fixed light intensity ($1\cdot10^5 erg\cdot cm^{-2}\cdot s^{-1}$) in the presence of various concentrations (0 to 1 μM) of DCMU or in the absence of DCMU at light intensities ranging from 0.1 to $1.0\cdot10^5$ $erg\cdot cm^{-2}\cdot s^{-1}$. Methanol was present at 1% (v/v) in each case.

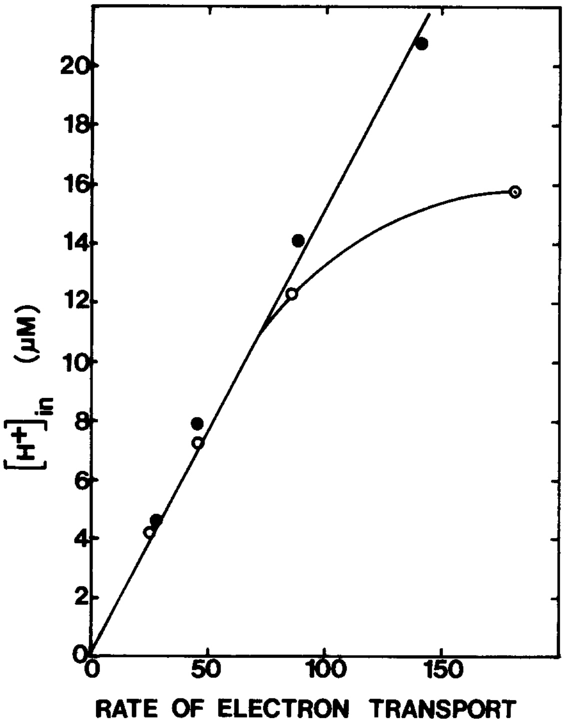

Fig. 2. Effect of DCCD on the relationship between $[H^+]_{in}$ and electron flow. Thylakoids (2.5 mg chlorophyll/ml) were incubated with 200 μM DCCD (●) or 1% methanol (○) for 15 min prior to assay of ΔpH and ferricyanide reduction. ATP (100 μM) was present in both cases during assay. The light intensity ranged from 0.1 to $1.5 \cdot 10^5$ erg·cm^{-2}·s^{-1}. Electron transport rates are expressed as μequiv/h per mg chlorophyll.

the effect is not due to energy transfer inhibition per se.

In a number of experiments, $[H^+]_{in}$ extrapolated to zero at a finite rate of electron transport. This may be due to underestimation of ΔpH caused by efflux of hexylamine during centrifugation as the chloroplasts accumulate at the silicone/reaction mixture interface. Evidence for this explanation comes from the general correlation between the extent of uncoupling and the size of the x intercept (unpublished observations, and Ref 14). If the rates of proton and hexylamine efflux are appreciable relative to the rate at which thylakoid membranes pass through the silicone-oil layer, a situation that is more likely to occur in the presence of uncouplers, $[H^+]_{in}$ would be underestimated. To account for the observed data, the underestimation would have to have a constant term, independent of the actual $[H^+]_{in}$.

TABLE I

DEPENDENCE OF k_H UPON EXTERNAL pH

k_H was estimated from slopes of plots of $[H^+]_{in}$ vs. rate of electron flow (water-to-ferricyanide). Light intensity was used to vary these parameters. The units for k_H are μequiv/h per mg chlorophyll per μM $[H^+]_{in}$.

External pH	k_H	$k_H/[H^+]_{out}$ ($\times 10^7$)
7.0	1.1	1.10
7.5	5.4	1.71
8.0	26.3	2.63
8.5	55.3	1.74
9.0	147.7	1.48

In 35 experiments at pH 8.0, in the absence of uncouplers, k_H was 21 ± 8 μequiv/h per mg chlorophyll per μM $[H^+]_{in}$ and ranged from 11 to 41. The k_H values determined at a variety of external pH values are given in Table I. The k_H increased by a factor of 100 as the pH increases from 7 to 9. Over this pH range, $[H^+]_{in}$ at a given low light intensity also decreases by a factor of 100. Therefore, the proton efflux rate is nearly independent of external pH. The observations that both ΔpH and the rate of electron flow are independent of pH over this range suggest that passive proton efflux is a function of ΔpH, and not of $[H^+]_{in}$ alone. These observations may be reconciled with Eqn. 2 if the leak rate is expressed as k_H^o ($[H^+]_{in}/[H^+]_{out}$), where k_H^o is a pH-independent constant. In Table I, k_H^o was calculated for each external pH by multiplying the observed k_H by $[H^+]_{out}$. k_H^o is, as expected, relatively invariant. Low light intensities were used in these experiments to avoid the proton efflux through the ATPase that is induced at high ΔpH values. This leak has a considerable pH dependence and can account for the pH dependence of nonphosphorylating electron transport at saturating light intensities [13].

Photophosphorylation in relation to ΔpH

If ADP and P_i are present in the reaction mixture, the proton flux through the ATPase coupled to ATPase synthesis must be the difference between the two terms of Eqn. 3:

$$(H^+/e) \cdot R_e - k_H[H^+]_{in} = (H^+/ATP) \cdot R_p \qquad (4)$$

where R_p is the rate of ATP synthesis and (H^+/ATP), the proton to ATP ratio. The k_H can be measured by the experiments of the type presented in Figs. 1–3. Thus, if $[H^+]_{in}$ is measured under phosphorylating conditions, electron flow can be corrected for the leak (i.e., nonphosphorylating electron flow) and the intrinsic P/e_2 ratio can be calculated. As any two points should unambiguously fix the k_H and intrinsic P/e_2 ratio, Eqn 4 can be rearranged and multiplied by 2 to solve for the P/e_2 ratio

$$\frac{P}{e_2} = \frac{2R_p}{R_e} = \frac{2(H^+/e)}{(H^+/ATP)} - \frac{2k_H[H^+]_{in}}{(H^+/ATP)R_e} \qquad (5)$$

If the measured P/e_2 is plotted against $[H^+]_{in} \div R_e$, the points so generated should form a line of slope $-2 \times k_H \div (H^+/ATP)$ and of y intercept $2 \times (H^+/e^-) \div (H^+/ATP)$, the intrinsic P/e_2 ratio. The k_H can be calculated from such a plot if the value of either H^+/e^- or H^+/ATP is known. Thus, there is no need to perform a separate experiment of the type shown in Figs. 1 and 2 to determine k_H. This considerably simplifies the experiments designed to measure the intrinsic P/e_2. Portis and McCarty [14] found that the P/e_2 corrected for leakage was constant at 1.3 when the observed P/e_2 was varied with either light intensity or 4′-deoxyphlorizin. The same corrected P/e_2 was found with either Eqn. 4 or 5, and the k_H calculated from Eqn. 5 agreed quite well with that calculated from Eqn. 3 if both experiments were performed [15]. It must be stressed that these experiments were carried out under conditions where $[H^+]_{in}$ was always a linear function of nonphosphorylating electron flow. The maximum $[H^+]_{in}$ reached at an external pH of 8.0 was less that 10 μM and $[H^+]_{in}$ is still linear with respect to R_e at and below this $[H^+]_{in}$.

Each of the three proton fluxes was varied as a test of the adequacy of Eqn. 5. The rate of proton pumping into the lumen can be varied by changing the rate of electron flow. This has been done by varying the light intensity and and by adding DCMU at constant light intensity. In these experiments, the P/e_2 extrapolates to 1.3. The k_H calculated from the experiment in which light intensity was varied is the same as that in which DCMU concentration was altered (Fig. 3). Electron flow exclusively through Photosystem I can be assayed in DCMU-poisoned chloroplasts if reduced tetramethylquinone is used as an electron donor and methyl viologen is the electron acceptor [22]. Presumably, only one proton is translocated per electron transported through Photosystem I [22,30]. The P/e_2 for this Photosystem I reaction was varied with light intensity. The plot of P/e_2 vs. $[H^+]_{in}/R_e$ showed an extrapolated P/e_2 half that of the water-to-ferricyanide reaction (Fig. 4). The k_H usually was about 50% larger than that of the water-to-ferricyanide controls. In a series of four experiments, the Photosystem I reaction gave an extrapolated P/e_2 of 0.60 vs. 1.22 for the water-to-ferricyanide controls. An effort was made to isolate a pure Photosystem II reaction by using

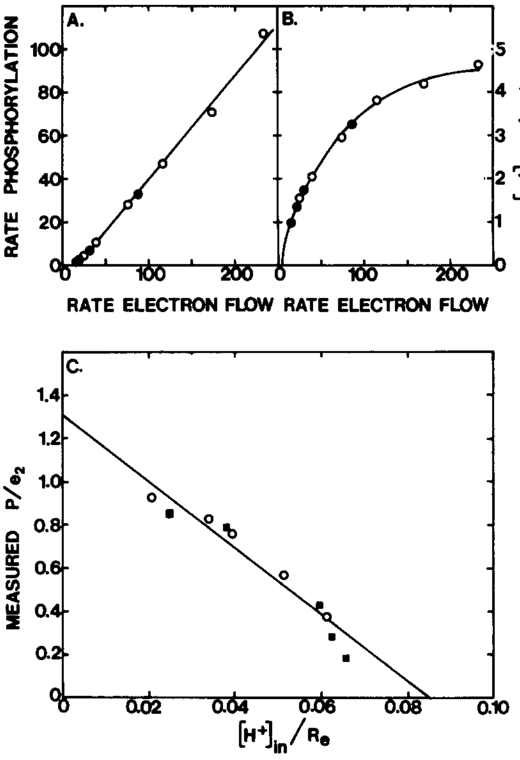

Fig. 3. Relationship between $[H^+]/R_e$ and P/e_2 as varied by light intensity and DCMU. Phosphorylation, electron flow (water to ferricyanide) and ΔpH were assayed at light intensities ranging from 0.06 to $1.0 \cdot 10^5$ erg·cm^{-2}·s^{-1} in the absence of DCMU (○) or at $0.5 \cdot 10^5$ erg·cm^{-2}·s^{-1} in the presence of DCMU (0–0.7 μM) (●,■). Electron flow and phosphorylation rates are expressed as μmol/h per mg chlorophyll In (C), P/e_2 extrapolates to 1.31; correlation coefficient is 0.97.

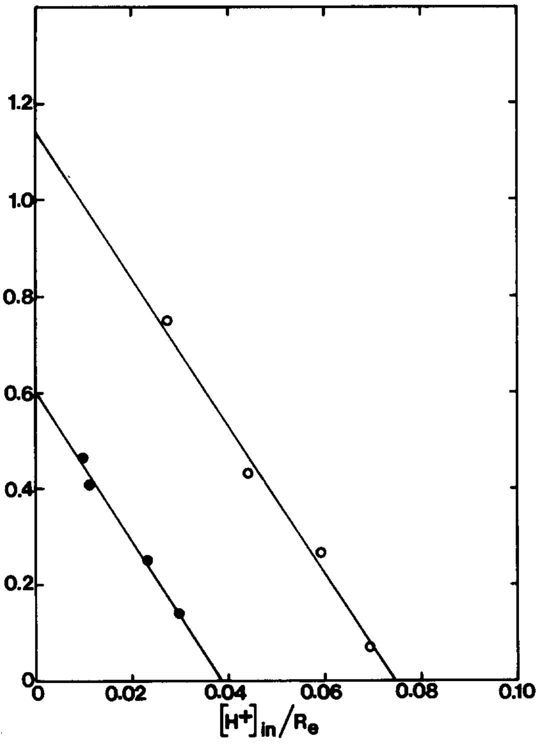

Fig. 4. Comparison of the relationship between $[H^+]_{in}/R_e$ and P/e_2 during electron flow through Photosystem I alone or through both Photosystems. Electron flow from either water to ferricyanide (P/e_2 extrapolates to 1.15) or tetramethylquinol to methyl viologen (extrapolation to 0.60), phosphorylation and ΔpH were assayed at various light intensities ((0.1–1.0)·10^5 erg·cm^{-2}·s^{-1}). All incubations contained 4% (v/v) of ethylene glycol/ethanol (1:1, v/v), since this mixture was used to dissolve the quinol and DCMU. Methyl viologen was present at 0.5 mM, tetramethylquinol at 0.9 mM, DCMU at 2 μM and superoxide dismutase at 0.1 mg/ml. This concentration of superoxide dismutase produced the maximum effect on the P/e_2 ratio. Rates of electron flow are expressed as μequiv/h per mg chlorophyll and $[H^+]_{in}$ as μM.

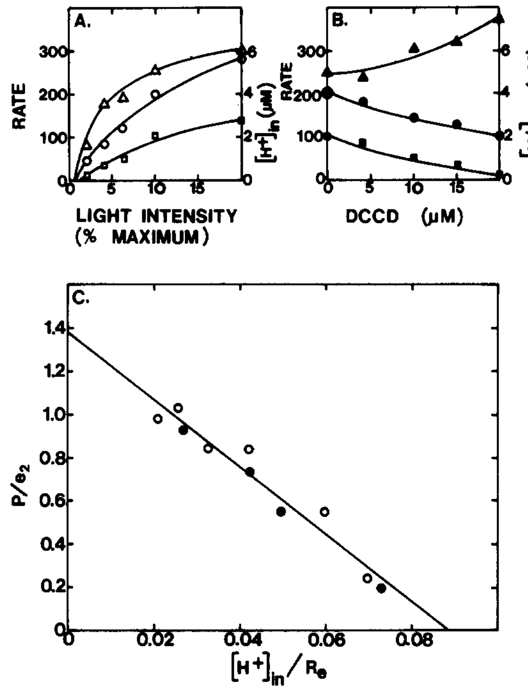

Fig. 5. Effect of DCCD on the relationship between $[H^+]_{in}/R_e$ and P/e_2. Methanolic solutions of DCCD were diluted 1:100 with thylakoid suspensions (2.5 mg chlorophyll/ml) to give final DCCD concentrations of 0.1–0.5 mM (● in (C)). Methanol was also added to the controls. (○ in (C)). The suspensions were incubated for 30 min at 0°C prior to assay. The light intensity varied from 0.1·10^5 to 1.0·10^5 erg·cm^{-2}·s^{-1} for controls and was fixed at 0.5·10^5 erg·cm^{-2}·s^{-1} for DCCD-treated samples. Phosphorylation (□,■), ΔpH (△,▲) and electron flow (water to ferricyanide) (○,●) were assayed. Rates are expressed as μmol/h per mg chlorophyll. P/e_2 extrapolates to 1.38; corr. coeff. 0.97.

dimethylquinone as an acceptor. Electron flow to Photosystem I was blocked with trifluralin or nitroflurophen, since the standard inhibitor, dibromomethylisopropyl benzoquinone reacts with hexylamine [31]. The extrapolated P/e_2 varied from 0.3 to 0.8. Since the k_H was uniformly higher than in water-to-ferricyanide controls and since it is difficult to assess the magnitude of the leak through the inhibited site to Photosystem I, these values are not reliable.

The efflux of protons through the ATPase complex can be limited by energy-transfer inhibitors, as already shown with 4′-deoxyphlorizin [14]. The same effect may be realized with increasing concentrations of DCCD to vary the P/e_2 ratios (Fig. 5). Both the P/e_2 and k_H are similar to those of controls in which light intensity is varied. The rate of phosphorylation can also be inhibited by lowering the phosphate concentration. This was done at two different light intensities. The P/e_2 and k_H calculated from these data do not differ from those of controls in which light intensity was varied at saturating phosphate (not shown).

Uncouplers increase the rate of the proton leak, as reflected by increases in k_H calculated under basal conditions. Light intensity was caried in the presence of a constant concentration of CCCP and

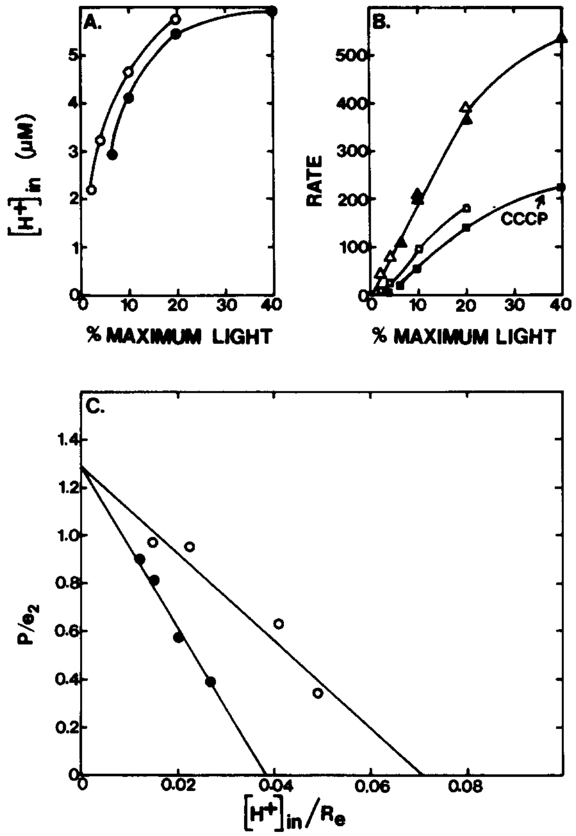

Fig. 6. Effect of CCCP on the relationship between $[H^+]_{in}/R_e$ and P/e_2. Electron flow (water to ferricyanide) (△,▲), phosphorylation (□,■) and ΔpH were assayed in the presence (filled symbols) and absence (open symbols) of 0.25 µM CCCP at light intensities ranging from $0.1 \cdot 10^5$ to $2.0 \cdot 10^5$ erg·cm^{-2}·s^{-1}. Rates are expressed as µmol/h per mg chlorophyll. P/e_2 extrapolates to 1.29.

the P/e_2 plotted against $[H^+]_{in}/R_e$. The extrapolated P/e_2 is the same as that of controls which lacked CCCP, but as expected, the calculated k_H is higher than that of controls (Fig. 6). Similar results are obtained in thylakoids treated with *o*-phenylenebismaleimide in the light, a treatment that uncouples photophosphorylation [28]. The k_H increases with uncoupler concentration. The concentration of uncoupler must be kept low to allow an appreciable range of P/e_2 ratios to be represented, and to keep $[H^+]_{in}$ in a range in which it can be accurately measured.

On plots of P/e_2 vs. $[H^+]_{in}/R_e$ datum points obtained at low light intensity sometimes fall below and to the left of a line extrapolated from higher light intensities. This is especially true in the presence of uncouplers and is likely to result from underestimation of $[H^+]_{in}$. If increasing concentrations of DCCD are used to vary P/e_2 instead of light intensity, $[H^+]_{in}$ is kept high and the underestimation of $[H^+]_{in}$ is of minor importance.

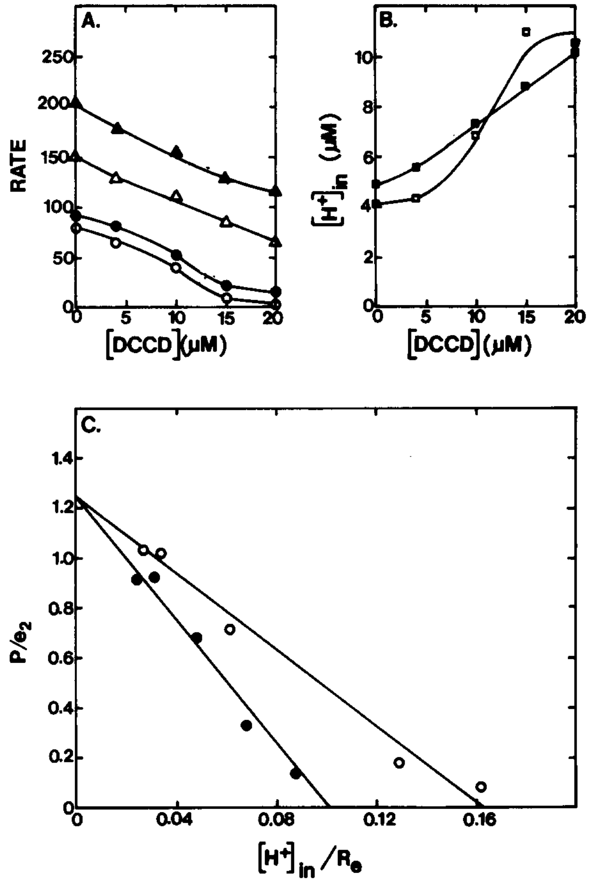

Fig. 7. Effect of gramicidin on the relationship between $[H^+]_{in}/R_e$ and P/e_2. Electron flow (water to methyl viologen) (△, ▲), phosphorylation (○,●) and ΔpH were determined in aliquots of the thylakoids that had been treated with DCCD in the presence (filled symbols) and absence (open symbols) of 0.7 nM gramicidin as described in the legend to Fig. 6. The concentrations of DCCD and gramicidin as described in the legend to Fig. 6. The concentrations of DCCD and gramicidin shown are the final concentrations in the reaction mixtures. Methanol was present at 1% (v/v) during the preincubation. Light intensities were $5 \cdot 10^4$ and $9 \cdot 10^4$ erg·cm^{-2}·s^{-1} for control and gramicidin-treated thylakoids, respectively. The rate of phosphorylation is higher in the presence of gramicidin because of the higher light intensity. Phosphorylation was less in the gramicidin-treated thylakoids when the assays were carried out at the same light intensity. Rates are expressed as µmol/h per mg chlorophyll. P/e_2 extrapolates to 1.24.

The linear relationship between observed P/e_2 ratios and $[H^+]/R_e$ is maintained over a wide range in the presence of gramicidin when DCCD is used (Fig. 7).

The P/e_2 was varied by decreasing the light intensity at different external pH values and then plotted against $[H^+]_{in}/R_e$ (Fig. 8). The extrapolated P/e_2 shows no systematic variation with external pH. As in the investigation of the relationship between $[H^+]_{in}$ and the rate of basal electron flow, the calculated k_H increased by roughly 10-fold for each unit increase in external pH. This reflects the lack of a strong dependence, at low light intensities, of ΔpH and the rates of electron transport and phosphorylation upon external pH. At pH values below 7, the plot of P/e_2 vs. $[H^+]_{in} \div R_e$ is not linear, but rather curves over as light intensity increases, reaching a maximal P/e_2 value near 0.5 at pH 6.7. Substitution of methyl viologen for ferricyanide did not change this behavior, so it is not due to Photosystem II-mediated reduction of ferricyanide. It may reflect the inability of hexylamine to behave as an ideal indicator of ΔpH at pH values at which the concentration of free base is very low [31].

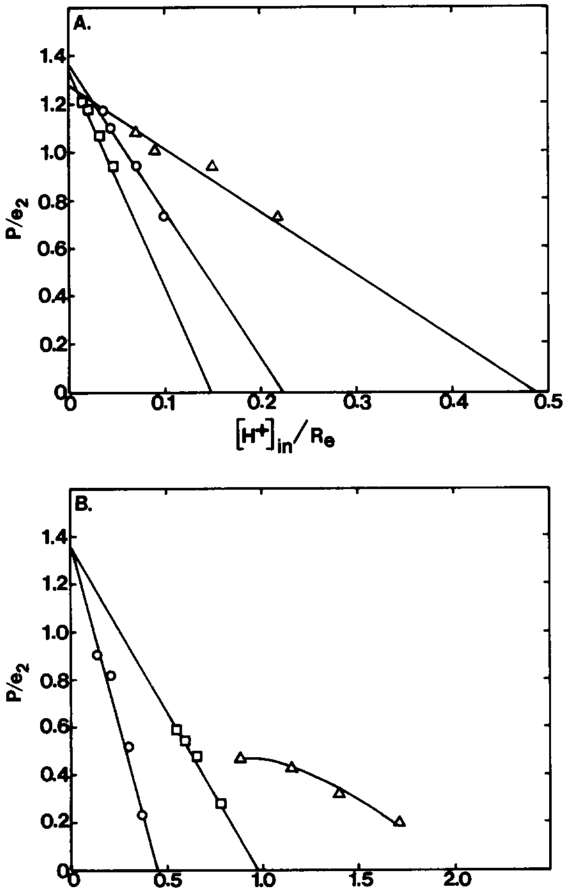

Fig. 8. Influence of external pH on the relationship between $[H^+]_{in}/R_e$ and P/e_2. Electron flow from water to ferricyanide, phosphorylation and ΔpH were determined at the pH values shown at light intensities ranging from $0.1 \cdot 10^4$ to $1.0 \cdot 10^5$ erg·cm^{-2}·s^{-1}. The buffers used were (A) Tricine-NaOH for pH 8.4 (□), 8.0 (○) and 7.7 (△); (B) Hepes-NaOH, for pH 7.4 (○) and 7.0 (□), Mes-NaOH for pH 6.7 (△). Rates are expressed as μequiv/h per mg chlorophyll.

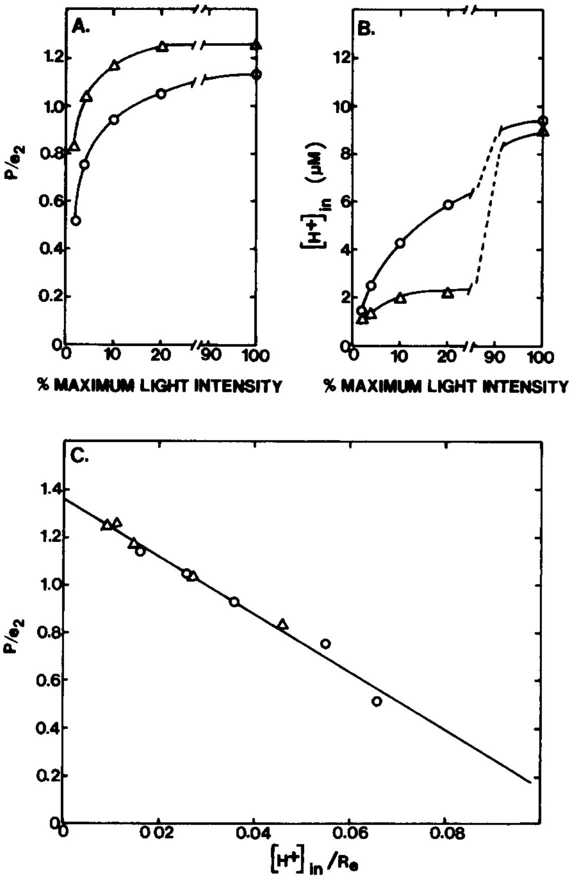

Fig. 9. Comparison of the relationship between $[H^+]_{in}/R_e$ and P/e_2 for freshly lysed chloroplasts (△) and washed thylakoids (○). Electron flow from water to ferricyanide, phosphorylation and ΔpH were assayed at several light intensities of 0.1–$5.0 \cdot 10^5$ erg·cm^{-2}·s^{-1} in either washed peak thylakoids or in intact pea chloroplast lysed in the reaction mixture. Rates are expressed as μequiv/h per mg chlorophyll. P/e_2 extrapolates to 1.36; corr. coeff., 0.99.

In most experiments, the highest measured P/e_2 was between 0.9 and 1.1, so the extrapolation to obtain the intrinsic P/e_2 is large. Intact chloroplasts were isolated from pea leaves [17] and lysed by addition to the reaction medium. The resulting suspensions of especially fresh thylakoid membranes gave higher measured P/e_2 ratios, but the extrapolated P/e_2 was the same as that found for chloroplasts isolated without an outer membrane by diluting the Percoll supernatant with a buffered mannitol solution and pelleting the chloroplasts (Fig. 9). the k_H values for the two preparations were also the same.

In 80 experiments, k_H calculated from the P/e_2 vs. $[H^+]_{in} \div R_e$ plots at pH 8 in the absence of uncouplers was 19 ± 9, with a range from 8 to 41 μequiv/h per mg chlorophyll per μM $[H^+]_{in}$. As with the k_H calculated from plots of $[H^+]_{in}$ vs. the rate of basal electron transport, the k_H determined by this method was reproducible from day to day with thylakoid membranes isolated from a given batch of spinach leaves, as long as the leaves remained fresh. In 80 experiments, the extrapolated P/e_2 was 1.25 ± 0.11, and ranged from 0.95 to 1.42.

Discussion

The results presented in this paper provide further support for the concept that the coupling between ATP synthesis and electron flow at the steady state may be described quantitatively in terms of proton fluxes. Proton influx is linked to electron flow and two pathways of proton efflux from the thylakoid lumen occur. One pathway is dissipative and appears to be passive, while the other is coupled to ATP synthesis.

Since the passive leak is unaffected by DCCD, it probably does not occur through the H$^+$-ATPase. Phlorizin and quinones when present at high concentrations decrease k_H, probably by partitioning into the membrane and altering its properties. No specific inhibitor of the leak has been found. The dependence of the passive leak on pH suggests that the lack rate may be described as:

$$\text{rate of passive H}^+\text{ movement} = k_H^0 \frac{[H^+]_{in}}{[H^+]_{out}} \qquad (6)$$

where k_H^0 is nearly independent of pH. Using a quite different experimental approach, Gräber and Witt [32] deduced a similar rate equation for H$^+$ efflux. Since our experiments were performed over a limited range of pH and ΔpH, Eqn. 6 may not be a complete description of the process. The dependence of proton efflux rate on ΔpH is unusual and is not predicted by the simplest models for proton transport. If, however, H$^+$ transport is assumed to be asymmetric, models can be generated for carrier or channel-mediated H$^+$ efflux in which the transport rate depends on ΔpH [33].

A second proton leak becomes apparent at medium pH values in the range of 7.8–8.3 when the rate of electron flow is sufficient to generate a ΔpH of 2.8 or above. This induced leak is partially prevented by low concentrations of ADP or ATP [13] and is totally prevented by DCCD. These results suggest that the induced proton leak is through the H$^+$-ATPase. The induction of the leak at alkaline pH values can account for the pH dependence of nonphosphorylating electron flow at saturating light intensities. Measurements of proton fluxes across thylakoids with pH electrodes confirm the presence of a proton leak through CF$_1$ [34]. A considerable increase in proton permeability with increasing light intensity, which was not mediated by CF$_1$, was also detected. This apparent effect, however, can be attributed to the analysis of the data [35]. The proton leak through the H$^+$-ATPase might be expected to complicate the analysis of ATP synthesis in terms of proton fluxes. However, phosphorylation decreases ΔpH sufficiently at the light intensities used in these experiments to prevent development of the leak. As the light intensity and, thus, $[H^+]_{in}$ are increased beyond the highest levels used in these experiments, the induced leak sets in and further increases in R_e are not accompanied by increases in R_p. This is apparent in P/e_2 vs. $[H^+]_{in}/R_e$ as points at low $[H^+]_{in}/R_e$ with low P/e_2 ratios.

The observation that internal proton concentration is a linear function of the rate of nonphosphorylating electron transport suggests that, under the conditions of our experiments, H^+/e does not vary with light intensity. It has been demonstrated [5–7] that electron flow through the cytochrome b_6-f complex can translocate two protons per electron. However, the translocation of an extra proton by the complex appears to occur at low values of ΔpH and at relatively low redox potentials. No indication of an extra proton was obtained when methyl viologen replaced ferricyanide as the electron acceptor, when DCMU was used instead of light intensity to vary R_e and when tetramethylquinol was used as the electron donor. Thus, ΔpH may be the major factor that inhibits proton pumping by the cytochrome complex. Even at the lowest light intensities used, the ΔpH was at least 2 units. We have no explanation for the difference between the results of Rathenow and Rumberg [36] and ours.

Phosphorylation, electron flow and $[H^+]_{in}$ were measured under a wide variety of conditions that affect electron flow, the H^+-ATPase, or the permeability of the membrane to protons. No matter how these parameters were varied, the data fit well with Eqn. 5 which describes the dependence of observed P/e_2 ratios on the internal proton concentrations. The fact that the dependence of observed P/e_2 ratios on $[H^+]_{in}/R_e$ is similar whether DCCD and DCMU (or light intensity) is used is of interest. As the DCCD concentration is raised, $[H^+]_{in}$ increases because of the inhibition of H^+ efflux coupled to ATP synthesis. In contrast, $[H^+]_{in}$ decreased with decreasing light intensity or increasing DCMU concentration. Thus, the coupling between electron transport and ATP synthesis may be described satisfactorily in terms of transmembrane proton fluxes. Our system is, however, not readily applicable to the study of the effects of uncouplers. Uncouplers cause a marked increase in the proton permeability of thylakoids and, therefore, also increase the rate at which accumulated hexylamine cation would leak out of the thylakoid lumen. Uncouplers would augment loss of hexylamine during centrifugation and, therefore, cause an underestimation of ΔpH.

In contrast to the observations from the Kouchkovsky's laboratory [37,38], this laboratory has consistently found that phosphorylation responds in the same manner to ΔpH whether light intensity, DCMU or uncoupler is used to vary these parameters or whether a Photosystem I or Photosystem II electron transfer reaction supports photophosphorylation. For example, Portis and McCarty [19] showed that pyocyanine-supported and methyl-viologen-supported phosphorylation varied in the same way with ΔpH. In this paper, we show that DCMU and light intensity have identical effects on phosphorylation and ΔpH. Moreover, phosphorylation coupled to electron flow through both Photosystems (H_2O ferricyanide) responded to ΔpH in a similar manner as that linked to electron flow through Photosystem I alone (tetramethyl quinol to methyl viologen). Our results do not support the microchemiosmotic notion in which lateral resistance to proton movement from appressed membranes has been postulated [37]. The discrepancy between our results and those of Haraux et al. [38] may be traced to the use by Haraux et al. of 9-aminoacridine to measure ΔpH. Although it is clear that 9-aminoacridine fluorescence responds to ΔpH, its use as a quantitative indicator for ΔpH has been questioned. Moreover, dibromomethylisopropylbenzoquinone has been used in experiments in which discrepancies between the response of phosphorylation and ΔpH to light intensity and to inhibition of electron flow. The quinone appears to interact to some extent with 9-aminoacridine. The absorption and fluorescence spectra of 9-aminoacridine are altered by the presence of the quinone.

When the passive leak is zero, all of the protons exit thylakoids through the H^+-ATPase and the P/e_2 ratio is then at its maximal value. In Eqn. 5, the P/e_2 ratio would approach $2(H^+/e)/(H^+ATP)$ as $[H^+]_{in}/R_e$ approaches zero. Thus, the intercept on the ordinate of plots of observed P/e_2 ratios vs. $[H^+]/R_e$ gives the maximal P/e_2 ratio. The P/e_2 is a measure of the fraction of protons that exit via the ATPase as opposed to via the passive leak. The rate of phosphorylation at a given external pH is given roughly by $C \cdot [H^+]_{in}^3$ [14], where C is a constant of proportionality. Since phosphorylation increases with the third power of $[H^+]_{in}$, at high ΔpH values most protons will exit via the ATPase and the P/e_2 will be high. Minor changes in k_H, such as those induced by the low concentrations of uncouplers used in this study, from batch to batch of spinach, have little impact upon the P/e_2 because of the overwhelming importance of the third-order term. As light intensity and thus $[H^+]_{in}$ decrease the importance of the leak and the magnitude of k_H increase, and increases in k_H have a major effect upon the P/e_2 (Fig. 8). Thus, both the size of k_H and of $[H^+]_{in}$

influence the P/e_2. Another factor is made clear in Fig. 9. The P/e_2 ratio is considerably larger in the freshly lysed chloroplasts than in the control thylakoids, despite the fact that their k_H values are the same. This reflects the fact that the freshly lysed chloroplasts have a much large proportionality constant C relating the rate of phosphorylation to $[H^+]_{in}^3$ than do controls. This favors phosphorylation just as an increase in k_H favors the leak process. Among other things, this change means that a lower ΔpH is required to drive the same overall rate of proton efflux, so ΔpH is lower in the freshly lysed chloroplasts, much as it would be if uncoupler has been added to increase k_H. This result dramatically illustrates the idea that it is not the k_H per se that determines the P/e_2, but rather the overall balance between the two efflux pathways, which is a function of k_H, C and $[H^+]_{in}$.

In 80 experiments in which phosphorylation, electron flow (through both Photosystems) and $[H^+]_{in}$ were varied by a number of means, including light intensity, DCMU, external pH, 4′-deoxyphlorizin and phosphate concentration, a maximal P/e_2 value of 1.25 ± 0.12 was obtained. This value is consistent with an H^+/e ratio of 2 and an H^+/ATP ratio of 3. The fact that the maximal P/e_2 ratio for phosphorylation coupled to electron flow through Photosystem I alone was nearly precisely half of that for phosphorylation through both Photosystems is most consistent with an overall H^+/e^- ratio of 2. Under conditions of our assays, neither cyclic electron flow nor extra proton translocation by the cytochrome b_6-f complex appears to operate. These pathways appear to work at low ambient redox potentials [6,7] and ΔpH values. An H^+/e value of 2 is in accord with previous measurements [3,4]. There is independent evidence for an H^+/ATP ratio for thylakoids of 3 [9,10,39–41]. An H^+/ATP ratio of 3 for submitochondrial particles was also obtained recently [42]. Thus, the maximal P/e_2 ratio for non-cyclic electron flow through both Photosystems is likely to be 4/3. Since 3 ATP and 2 NADPH are required for CO_2 fixation by the reductive pentose-phosphate cycle, it is apparent that either cyclic electron flow or increased proton translocation by the cytochrome b_6-f complex contributes to ATP synthesis in vivo.

In summary, our results are consistent with the notion that photophosphorylation and electron flow are coupled by the proton gradient. The response of the system to a wide variety of inhibitors or treatments may be quantitated in terms of proton fluxes.

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References

1 Mitchell, P. (1961) Nature 191, 144–149

2 Mitchell, P. (1966) Biol. Rev. Cambridge Phil. Soc. 41, 445–502

3 Junge, W. and Ausländer, W. (1974) Biochim. Biophys. Acta 333, 59–70

4 Fowler, C.F. and Kok, B. (1974) Biochim. Biophys. Acta 357, 199–307

5 Velthuys, B.R. (1979) Proc. Natl. Acad. Sci. USA 76, 2765–2769

6 Hind, G., Crowther, D., Shahak, Y. and Slovacek, R.E. (1981) Proceedings of the Fifth International Congress on Photosynthesis, 1984, Vol. 2, pp. 87–97

7 Bouges-Bocquet, B. (1981) Biochim. Biophys. Acta 635, 327–340

8 Hurt, E.C., Jauska, G. and Shahak, Y. (1982) FEBS Lett. 149, 211–216

9 Avron, M. (1978) FEBS Lett. 96, 225–232

10 McCarty, R.E. (1978) in The Proton and Calcium Pumps (Azzone, G.F., Avron, M., Metcalfe, J.C., Quagliarello, E. and Silliprandi, N., eds.), pp. 65–70, Elsevier, Amsterdam

11 Carmeli, C. (1970) FEBS Lett. 7, 297–300

12 Schröder, H., Siggel, V. and Rumberg, B. (1973) Proceedings of the Third International Congress on Photosynthesis, pp. 919–930

13 Portis, A.R., Jr., Magnusson, R.P. and McCarty, R.E. (1975) Biochem. Biophys. Res. Commun. 67, 877–884

14 Portis, A.R., Jr. and McCarty, R.E. (1976) J. Biol. Chem. 251, 1610–1617

15 McCarty, R.E. and Portis, A.R., Jr. (1976) Biochemistry 15, 5110–5114

16 McCarty, R.E. and Racker, E. (1967) J. Biol. Chem. 242, 3436–3439

17 Howitz, K.T. and McCarty, R.E. (1982) Plant Physiol. 70, 949–952

18 Arnon, D.I. (1949) Plant Physiol. 24, 1–15

19 Portis, A.R., Jr. and McCarty, R.E. (1974) J. Biol. Chem. 249, 6250–6254

20 Punnett, T., Iyer, R.V. and Ellinwood, B.W. (1964) Anal. Biochem. 7, 328–334

21 Black, M.J. and Brandt, R.B. (1974) Anal. Biochem. 58, 246–254

22 Izawa, S. and Pan, R.L. (1978) Biochem. Biophys. Res. Commun. 83, 1171–1177

23 Lindberg, O. and Ernster, L. (1956) Methods Biochem. Anal. 3, 1–24

24 Schröder, H., Muhle, H. and Rumberg, B. (1971) Proceedings of the Second International Congress on Photosynthesis, pp. 919–930

25 Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–63

26 Vredenberg, W.J. and Tonk, W.J.M. (1975) Biochim. Biophys. Acta 387, 580–587

27 Davenport, J.W. (1983) Ph.D. Thesis, Cornell University

28 Weiss, M.A. and McCarty, R.E. (1977) J. Biol. Chem. 252, 8007–8012

29 Nelson, N., Eytan, E., Notsani, B.-E., Sigrist, H., Sigrist-Nelson, H. and Gittler, C. (1977) Proc. Natl. Acad. Sci. USA 74, 2375–278

30 Trebst, A. (1974) Annu. Rev. Plant Physiol. 25, 423–458

31 Portis, A.R., Jr. and McCarty, R.E. (1973) Arch. Biochem. Biophys. 156, 621–625

32 Gräber, P. and Witt, H.T. (1976) Biochim. Biophys. Acta 423, 141–163

33 Hansen, U.-P., Gradmann, D., Sanders, D. and Slayman, C.L. (1981) J. Membrane Biol. 63, 165–190

34 Ho, Y.K. and Wang, J.H. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 241–248, Elsevier-North Holland, New York

35 Abbot, M.S. and Dilley, R.A. (1983) Arch. Biochem. Biophys. 222, 95–104

36 Rathenow, M. and Rumberg, B. (1980) Ber. Bunsenges. Phys. Chem. 84, 1059–1062

37 Haraux, F. and De Kouchkovsky, Y. (1982) Biochim. Biophys. Acta 679, 235–247

38 Haraux, F., Sigalat, C., Moreau, A. and De Kouchkovsky, Y. (1983) FEBS Lett. 155, 248–251

39 Handgarter, R.P. and Good, N.E. (1982) Biochim. Biophys. Acta 681, 397–404

40 Dewey, T.G. and Hammes, G.G. (1981) J. Biol. Chem. 256, 8941–8946

41 Gräber, P. (1980) Biochem. Biophys. Res. Commun. 96, 1232–1239

42 Berry, E.A. and Hinkle, P.C. (1983) J. Biol. Chem. 258, 1474–1486