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ON THE RELATIONSHIP OF H^+ TRANSPORT TO PHOTOPHOSPHORYLATION IN SPINACH CHLOROPLASTS*

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SUMMARY

In *Spinacea oleracea* chloroplasts, rapid ATP formation decreased the rate and extent of the dark decay of the H^+ gradient, relative to that measured when photophosphorylation was limited or inhibited. Photophosphorylation rates were controlled by using arsenate in place of phosphate, inhibition with poly-L-lysine (a compound which inhibits ATP formation at concentrations not inhibitory to the H^+ pump), and by mild heat treatment. These techniques clearly showed that significant differences occur in the rate and extent of the dark H^+ efflux (decay) with very little difference in the initial rate of H^+ uptake.

These data were interpreted as evidence that ATP formation occurs within a compartment in contact with that into which H^+ is deposited by the light-driven H^+ uptake (pump). The reaction $H^+ + ADP^{3-} + HPO_4^{2-} \rightarrow ATP^{4-}$ taking place within this compartment with subsequent release of ATP into the outer phase would reduce the steady-state H^+ gradient and thereby reduce the H^+ efflux observable in the dark. This interpretation implicates the H^+ pump as an obligatory part of the ATP-forming mechanism in spinach chloroplasts, and would rule out H^+ transport as an energy utilization alternative to (or competitive with) photophosphorylation.

INTRODUCTION

A great deal of attention has recently been given to energy-linked ion movements in chloroplasts[1–6] and mitochondria (see ref. 7 for review). The MITCHELL chemiosmotic hypothesis[8] considers that ion transport (particularly H^+) is a precursor to ATP formation, in that the energy developed in an electrochemical potential gradient is utilized as the main driving force for the esterification reaction. An opposing viewpoint holds that ion transport is an expression of an alternative way of using the energy derived from electron transport. From chloroplast research there is considerable evidence showing that the H^+ transport has the general characteristics predicted by chemiosmotic theory[3,6], although evidence is available which disagrees with the postulated mechanism of H^+ transport[9,10]. Mitochondrial researchers, led by BRIERLEY *et al.*[11], have evidence that ion transport is competitive with phosphorylation.

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KARLISH AND AVRON[12] have performed experiments with chloroplasts, designed to test the MITCHELL hypothesis, in which they studied the effect of various conditions related to stimulation and inhibition of ATP formation on the decay of the proton gradient in the dark. Their rationale is that if MITCHELL's hypothesis is correct, there should be a decrease in the steady-state extent of the proton gradient corresponding to the utilization of H^+ to drive the synthesis of ATP. Using conditions of with and without ADP and with and without $MgCl_2$ at low salt concentrations, they found significant stimulation in the extent of the proton gradient (measured as the amount of the dark decay) when ADP or $MgCl_2$ was added to an otherwise complete phosphorylating mixture. On the basis of these data, they suggested that the proton movements are not consistent with either the chemiosmotic hypothesis of MITCHELL[8] or the chemical intermediate theory (see ref. 13 for a synopsis of this theory). We will present evidence which casts strong doubt on their interpretation as it might apply to spinach chloroplasts (some of the differences might be a reflection of the difference between lettuce and spinach). Our evidence consists of three parts: (a) We find that an active phosphorylating condition does reduce the observable proton gradient relative to a less active phosphorylating condition. (b) Under the low salt conditions used by KARLISH AND AVRON[12], the addition of ADP or $MgCl_2$ leads to a significant stimulation in electron transport, which in itself could account for the stimulations of the H^+ transport they observed. (c) Under low salt conditions, addition of $MgCl_2$ induces the chloroplast grana membrane to be less permeable to protons, thus altering the steady-state proton gradient in the direction of allowing a greater retention of H^+.

METHODS AND MATERIALS

Spinacea oleracea chloroplasts were prepared from plants grown in an environment-controlled chamber as described before[14]. Assays for chlorophyll, H^+ uptake and photophosphorylation were similar to those previously used[3,14]. Electron transport was assayed by measuring O_2 uptake with a Clark-type O_2 electrode using an amplifier from Chemtronics, Inc. NaN_3 was used to inhibit the enzymic breakdown of H_2O_2 (by endogenous catalase or peroxidase) formed by the oxidation of methyl viologen used as an electron acceptor[15].

The technique of measuring the H^+ uptake and efflux (decay) rates involved drawing a tangent to the trace at the point where the light was turned on or off. This value (ΔpH) may be converted to ΔH^+ and multiplied by the buffer capacity to yield the actual rate of H^+ flux.

RESULTS

Effect of $MgCl_2$ and ADP on electron transport

As will be seen below, our data and interpretation are contrary to those of KARLISH AND AVRON[12]. We feel that the experimental protocol the latter authors used, introduced a significant difference in electron transport rate between the control and the experimental treatments. Such a difference in electron transport could explain their apparent stimulation in the extent of the H^+ decay under phosphorylating conditions. Fig. 1 shows that both the proton pump and electron transport are stimulated by the addition of 1 mM $MgCl_2$ to a low KCl concentration medium con-

taining 0.2 mM ADP and 0.2 mM AsO_4^{3-}. The conditions here are similar to those used by KARLISH AND AVRON, except that we use methyl viologen in place of pyocyanine in order to measure electron transport. However, we also found the same effect on the proton pump when we used pyocyanine under these conditions.

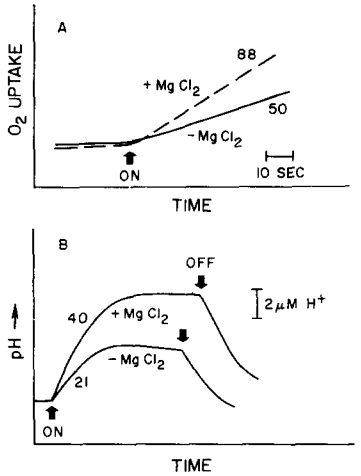

Fig. 1. Effect of $MgCl_2$ on electron transport and H^+ pump under low salt conditions. A. Reaction mixtures for measuring O_2 uptake contained: 40 mM KCl, 0.2 mM ADP, 0.2 mM AsO_4^{3-}, 20 μg/ml chlorophyll as chloroplasts, 0.5 mM NaN_3, 5 mM Tris–HCl (pH 7.9) and 50 μM methylviologen in a total volume of 5 ml. 1 mM $MgCl_2$ was present in the assay as indicated in the figure. The numbers by the traces represent the specific activity in μmoles e^- transported/h per mg chlorophyll. B. Reaction mixtures for measuring H^+-pump activity were similar to those of (A) except no Tris–HCl was added. Red light used as in Fig. 2.

Addition of ADP to chloroplasts suspended in low salt with the other phosphorylation cofactors present also results in a similar stimulation of electron transport and H^+ uptake as seen in Table I. In both cases above, the stimulation of electron transport affected by the addition of $MgCl_2$ or ADP can account for the observed stimulation in the extent and rate of H^+ gradient decay reported by KARLISH AND AVRON[12]. It is therefore quite doubtful that their interpretation is correct, *i.e.*, they concluded that adding the missing component for a complete phosphorylation reaction resulted in an increased efficiency of H^+ pumping. Recent experiments have shown that electron transport is very sensitive to ionic strength in the concentration range of monovalent and divalent salts used here[16], a fact consistent with our results.

Somewhat smaller stimulations of the H^+ pump and electron transport are obtained by adding ADP or $MgCl_2$ to chloroplasts suspended in high salt concentration (0.1 M KCl). Table II shows the effect of either ADP or $MgCl_2$ addition to otherwise complete phosphorylation mixtures. That the stimulations are less than those found under low salt conditions is consistent with the fact that part of the stimulation obtained under low salt conditions is due to a non-specific salt effect. This salt effect would be saturated by 0.1 M KCl, hence the ADP and $MgCl_2$ effects under these conditions would be the classical stimulatory effect of the cofactors of photophosphorylation on electron transport (and therefore on the H^+ pump).

The observation by KARLISH AND AVRON that the H^+-pump activity is stimulated by $MgCl_2$ addition in the presence of DIO-9 (an inhibitor of coupled electron

TABLE I

STIMULATION OF ELECTRON TRANSPORT AND H^+ PUMP BY ADDITION OF ADP UNDER LOW SALT CONDITIONS

The reaction mixtures were as follows: H^+-pump assay; 30 mM NaCl, 0.4 mM AsO_4^{3-}, with or without 0.2 mM ADP, 0.5 mM $MgCl_2$, 50 μM methylviologen and 30 μg chlorophyll as chloroplasts per ml in a total volume of 7.0 ml at pH 7.9. O_2 assay, the same as for the H^+-pump assay *plus* 0.4 mM NaN_3, and 5 mM Tris–HCl (pH 7.9).

	Effect of ADP addition		
	*− ADP**	*+ ADP**	*Δ***
H^+ uptake rate	60%	100%	
	25	42	17 μmoles/h per mg chlorophyll
O_2 uptake	56%	100%	
	36	64	28 μmoles e^-/h per mg chlorophyll
H^+ uptake extent	64%	100%	
	0.053	0.084	0.031 μmole/mg chlorophyll
H^+ decay rate	98%	100%	
	28	29	1 μmole/h per mg chlorophyll
H^+ decay extent	Equal to uptake		

* Values are given as percentages or as absolute values.
** Values given as the absolute increase on addition of ADP.

TABLE II

STIMULATION OF ELECTRON TRANSPORT AND H^+ PUMP BY ADDITION OF (a) ADP OR (b) $MgCl_2$ UNDER HIGH SALT CONDITIONS

(a) The reaction mixtures were similar to those of Table I with 0.100 M KCl present. NaN_3 was present in both assay systems. (b) The reaction mixtures were similar to those of Fig. 1 except 0.10 M KCl was used, and NaN_3 was present in both assay systems.

(*a*)	*Effect of ADP addition*		
	*− ADP**	*+ ADP**	*Δ***
H^+ uptake rate	83%	100%	
	70	84	14 μmoles/h per mg chlorophyll
O_2 uptake	66%	100%	
	125	190	65 μmoles e^-/h per mg chlorophyll
H^+ uptake extent	69%	100%	
	0.16	0.23	0.07 μmole/mg chlorophyll
H^+ decay rate	83%	100%	
	38	46	8 μmoles/h per mg chlorophyll
H^+ decay extent	Equal to uptake		
(*b*)	*Effect of $MgCl_2$ addition*		
	*− $MgCl_2$**	*+ $MgCl_2$**	*Δ***
H^+ uptake	69%	100%	
	67	98	31 μmoles/h per mg chlorophyll
O_2 uptake	73%	100%	
	70	95	25 μmoles e^-/h per mg chlorophyll
H^+ uptake extent	83%	100%	
	0.19	0.23	0.04 μmole/mg chlorophyll
H^+ decay rate	83%	100%	
	44	53	9 μmoles/h per mg chlorophyll
H^+ decay extent	Equal to uptake		

* Values are given as percentages or as absolute values.
** Values given as the absolute increase on addition of ADP or $MgCl_2$.

transport[17]) could also be explained as being due to the stimulation of the basal electron transport (which is not inhibited by DIO-9) as well as the coupled electron transport[18]. SHAVIT AND AVRON[16] have shown the stimulation of the basal or non-phosphorylating electron transport by added salts.

Effect of $MgCl_2$ on chloroplast permeability

The addition of 0.5 mM $MgCl_2$ to a low salt (10 mM KCl) suspension of chloroplasts apparently can render the membranes less permeable to H^+. In Fig. 2 the rate of decay of the light-induced H^+ gradient is plotted *vs.* the extent of the gradient. In this experiment the extent was varied by using different light intensities. Similar results were found when the extent was varied by giving different lengths of illumination. The rationale behind this experiment is that, since the dark decay of the H^+ gradient is a passive process, the slope of the line for this plot should be greater under conditions leading to greater permeability. Fig. 2 shows that the addition of $MgCl_2$ results in a plot of lesser slope, and hence the chloroplast thylakoid membrane is probably less permeable under these conditions. This effect could also contribute to the greater H^+ accumulation found by KARLISH AND AVRON[12] due to addition of $MgCl_2$.

Other evidence that low ionic strength leads to greater chloroplast membrane permeability is indirectly seen in experiments concerning the interaction of KCl and gramicidin on the inhibition of photophosphorylation and H^+ uptake. It has been shown that gramicidin causes mitochondrial membranes to become quite permeable to monovalent cations[19,20]. Fig. 3 shows that gramicidin greatly increases the dark decay rate of H^+ accumulated in the light. This effect is probably due to an increase in the membrane permeability to H^+ induced by gramicidin. As seen in Fig. 4, gramicidin is much more inhibitory toward H^+ uptake (Fig. 4a), and photophosphorylation (Fig. 4b) at low KCl than at high KCl. The greater inhibition at low salt could be due to the combined 'permeability stress' of gramicidin *plus* the low salt, *i.e.*,

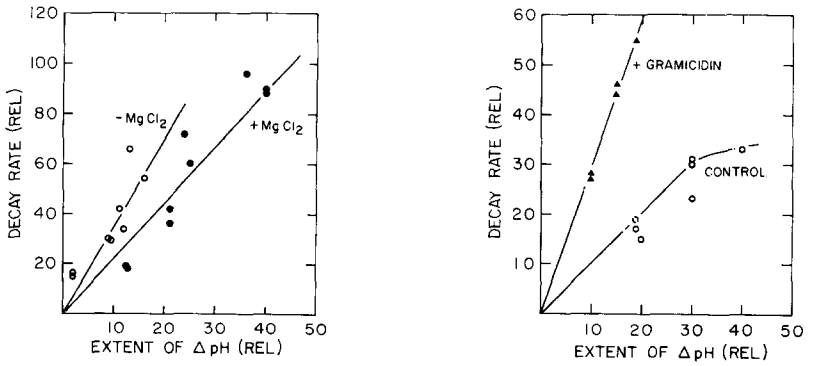

Fig. 2. Effect of $MgCl_2$ on chloroplast permeability to H^+. The slope of a plot of extent of ΔpH *vs.* the decay rate is a measure of the rate of passive efflux of H^+ through the membrane, *i.e.*, the membrane permeability to H^+. Reaction mixtures contained: 10 mM KCl, ± 1 mM $MgCl_2$, 15 μM pyocyanine, and 25 μg/ml chlorophyll as chloroplasts, in a total volume of 5 ml. The pH was 7.0, temperature 18°. Red light through Corning 2304 and CS-I-69 infrared filters was used at a maximum intensity of $1.5 \cdot 10^5$ erg·cm^{-2}·sec^{-1}. The ΔpH values are extents given in chart paper lines.

Fig. 3. Effect of gramicidin on chloroplast permeability to H^+ ions. The reaction conditions were similar to those of Fig. 2 with 100 mM KCl, 0.5 mM $MgCl_2$, ± 0.15 μM gramicidin. The initial pH was 6.2.

the limited extent of the H^+ gradient due to low salt concentration may be reduced further by the H^+ leakage induced by gramicidin. Gramicidin may also interact more strongly with the chloroplast membrane under low salt conditions, and thereby cause a greater increase in permeability. Other workers have shown that chloroplasts suspended in low salt have more extended lamellae with few or no stacked grana discs[21,22]; perhaps this phenomena is related to the greater sensitivity toward gramicidin observed with these chloroplasts under low salt conditions.

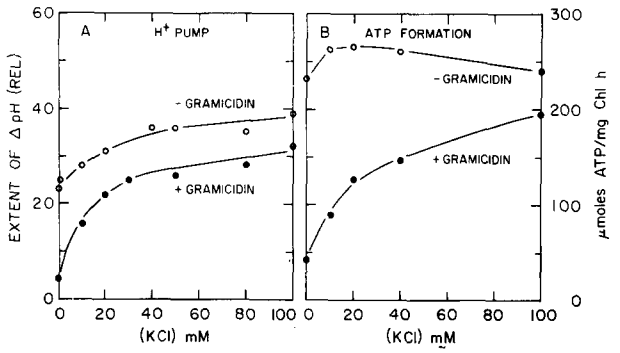

Fig. 4. Effect of KCl and gramicidin on photophosphorylation and H^+-pump rates. A. Reaction conditions for the H^+-pump assay were similar to those of Fig. 2 with 0.5 mM $MgCl_2$, varying KCl concentration and with or without $2 \cdot 10^{-9}$ M gramicidin. The specific activity of the −gramicidin control at 100 mM KCl was 95 μmoles H^+ per h per mg chlorophyll. B. Reaction conditions for the photophosphorylation assays consisted of: 2 mM $MgCl_2$, 0.33 mM ADP, 0.67 mM K_2HPO_4, 20 μg per ml chlorophyll as chloroplasts, 5 mM Tris–HCl (pH 7.8), varying concentrations of KCl and $3.3 \cdot 10^{-7}$ M gramicidin where indicated. Photophosphorylation was assayed by the ^{32}P method[5].

H^+ decay vs. photophosphorylation

Two techniques have recently been developed which permit one to measure the H^+ pump under conditions similar to those required for photophosphorylation. AsO_4^{3-} may be used in place of phosphate in a complete phosphorylation system, and the pH changes due only to the H^+ pump may be observed[3]. A new uncoupler of photophosphorylation, poly-L-lysine[14], will inhibit ATP formation at concentrations which do not inhibit the H^+ pump, again allowing measurement of the H^+ pump in the presence of ADP and HPO_4^{2-} at pH 7.8.

Fig. 5 shows the effect of varying the efficiency of ATP formation by using different ratios of PO_4^{3-} to AsO_4^{3-}. With no AsO_4^{3-} added, the ATP formation rate (here measured by the ΔpH after 10 sec illumination) is optimal and the decay rate is not very fast (Trace A). As the AsO_4^{3-} concentration increases (and PO_4^{3-} decreases), ATP formation is depressed and the H^+ decay in the dark is accelerated both in rate and extent (Traces B, C and D). While not shown clearly, the extent of decay of Curves A and B was essentially complete at the points where the traces terminate, the downward deflection after that point being due to pH drift caused by CO_2 equilibration. This indicates that the extent of H^+ efflux under the rapid phosphorylating condition is only about one-third the extent realized in the AsO_4^{3-} case (Trace D).

Similar results were found when poly-L-lysine was used to inhibit ATP formation. Fig. 6 shows that progressive inhibition of ATP formation with increasing polylysine, results in stimulating the rate and extent of decay of the proton gradient.

In these experiments polylysine is functioning as an uncoupler and not as an

energy-transfer inhibitor. Evidence for this is that O_2 uptake in a typical experiment was stimulated 164%, and the H^+ uptake rate was stimulated 140%.

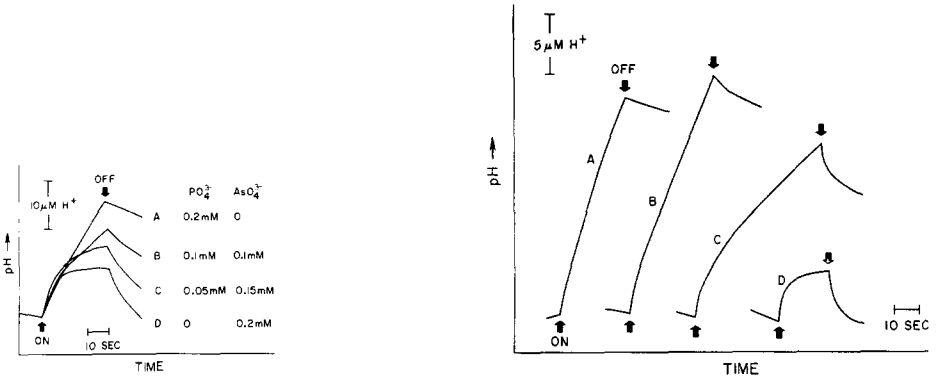

Fig. 5. Effect of AsO_4^{3-} on photophosphorylation and the H^+ gradient. Reaction conditions were: 0.1 M KCl, 0.5 mM $MgCl_2$, 15 μM pyocyanine, 0.2 mM ADP, 20 μg/ml chlorophyll as chloroplasts, and various concentrations of K_2HPO_4 and K_2HAsO_4 as shown. Total volume was 10 ml, the pH was 7.9 and a red light was used.

Fig. 6. Effect of polylysine on photophosphorylation and the H^+ gradient. Reaction conditions were similar to those of Fig. 5 but with 0.3 mM K_2HPO_4 (A), control; (B), $+1 \cdot 10^{-8}$ M 195000 mol. wt. poly-L-lysine; (C), $+4 \cdot 10^{-8}$ M polylysine; (D), $+8 \cdot 10^{-8}$ M polylysine.

Fig. 7 shows that heating chloroplasts to 55° for 10, 15 and 20 sec (Traces B, C and D, respectively) prior to assaying also resulted in a greater rate and extent of H^+ pump decay as the phosphorylation rate progressively decreases.

It should be emphasized that the extent of the H^+ gradient decay in the controls (Trace A in Figs. 5, 6 and 7) is much less than the extent of the experimental reactions. This suggests that the difference in decay rates observed is not due to differences in permeability of the membrane to H^+; if such were the case the final decay extents would be the same. Some of the downward deflection in the decay phases is accounted for by the pH shift which accompanies atmospheric CO_2 equilibration into the reaction mixtures. This is also seen in some of the traces prior to turning the light on (Figs. 5 and 6B, C and D).

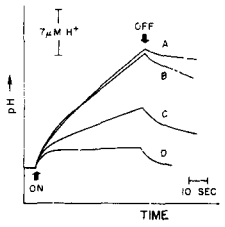

Fig. 7. Effect of heating chloroplasts on photophosphorylation and the H^+ gradient. Reaction conditions were similar to those of Fig. 6. Aliquots of the chloroplasts suspension were heated for the following times prior to assay: (A), control, no heating; (B), 10 sec at 55°; (C), 15 sec at 55°; (D), 20 sec at 55°. The heat treatment was accomplished by adding 0.2 ml of chloroplasts to a preheated test tube which was gently agitated in the water bath for the indicated time, followed by rapid cooling to ice-bath temperatures, and immediately assayed.

DISCUSSION

Using the above three techniques of limiting or inhibiting phosphorylation, it is seen that there is an inverse relation between phosphorylation activity and the rate and extent of decay of the H^+ gradient. It seems clear from these data that when ATP formation is most rapid, there is a significant decrease in the rate and extent of H^+ efflux. This indicates that there is a lower steady-state concentration of H^+ within the grana in the rapid phosphorylating condition. These data are the opposite of those obtained by KARLISH AND AVRON[12]. We believe, however, that their results can be explained on the basis of the stimulating effects on electron transport due to adding $MgCl_2$ or ADP to an otherwise complete phosphorylation system in a low salt medium (Fig. 1, Table I). The stimulation of electron transport due to Mg^{2+} or ADP additions are shown to be sufficient to account for the stimulation in H^+ uptake, seen as a greater extent and a faster rate of decay. An additional effect, which is rather difficult to evaluate, is that of permeability changes brought about by addition of $MgCl_2$ to chloroplasts suspended in low salt media. We have shown (Fig. 2) that $MgCl_2$ added to such a suspension results in a lower permeability of the chloroplast membranes to H^+, which would increase the steady-state gradient of H^+ capable of being retained. The effects of $MgCl_2$ on H^+ permeability are corroborated by experiments in which gramicidin was used to increase membrane permeability (Fig. 3). Gramicidin increases mitochondrial membrane permeability to monovalent cations such as H^+, K^+, Na^+, and Cs^+ (refs. 19 and 20) and similar effects have been shown recently with chloroplasts[5]. The similarity in effect of gramicidin and low salt concentration (without added $MgCl_2$) on the decay rate of the H^+ gradient suggests that a permeability barrier is being affected in both cases. It is not surprising therefore, that low salt concentration and gramicidin act in an additive manner in the inhibition of the H^+ pump and photophosphorylation (Fig. 4).

The observation that increasing the phosphorylation activity decreases the steady-state extent of the H^+ gradient is consistent with either the chemical intermediate theory[13] or the chemiosmotic theory[8,25]. The simplest interpretation of Figs. 5, 6 and 7 is that the H^+ pump is an alternative transport activity which utilizes the energy which otherwise would be used for ATP formation. This would follow from the chemical intermediate theory, which as developed for mitochondria, visualizes ion transport utilizing a high-energy intermediate in a competitive manner with ATP formation. However, other data reviewed below lend support to the concept that the H^+ uptake is a required part of the ATP-forming mechanism and not an alternative use of energy. Previous experiments have shown that under phosphorylating conditions the initial rate of H^+ uptake is as fast as under non-phosphorylating conditions[3]. This would not be expected if the H^+ pump and ATP formation were competitive. This fact together with the observation that at light intensities comparable to those used here, a lag of about 2–3 sec occurs before ATP formation begins, with no lag in H^+ uptake[23,24], was interpretated as evidence that the H^+ pump is an obligatory prerequisite for ATP formation in spinach chloroplasts[3]. Since a 3-sec lag is approximately the time required for the H^+ pump to reach one-half of the steady-state H^+-uptake value[2,3], it is logically consistent that the lag in ATP formation may be due to the time required to accumulate a certain threshold amount of H^+. Other data have shown that in spinach chloroplasts, any treatment which inhibits the H^+ pump, correspondingly

affects photophosphorylation[1,3,26], a necessary but not sufficient condition to prove the H^+ pump an obligatory part of the ATP-forming process. Additional evidence supporting this interpretation was obtained from experiments using ADP and AsO_4^{3-} in place of PO_4^{3-} (ref. 3). In chloroplasts, AsO_4^{3-} + ADP substitute for PO_4^{3-} + ADP, so as to give a similar increment of coupled electron transport which is also inhibited back to the basal rate by phlorizin[18]. Taking this as evidence that AsO_4^{3-} + ADP utilizes the same steps of the energy-conservation mechanism as does PO_4^{3-} + ADP, the H^+ pump activity measured in the presence of AsO_4^{3-} + ADP (where no net H^+ uptake due to ATP formation interferes) should reflect competition between ATP synthesis and H^+ pump activity if it occurs. Earlier work indicated that no such competition exists[3]. This is corroborated by the data of Table II where addition of ADP or $MgCl_2$ to an otherwise complete phosphorylation mixture at 0.1 M KCl (AsO_4^{3-} replaced PO_4^{3-}), stimulated the initial rate of H^+ uptake and electron transport. If competition existed between the H^+ pump and ATP formation, such an addition should have decreased the initial rate of the H^+ pump as coupling was accomplished.

Assuming the above interpretation to be correct, the diminished decay rate and extent of H^+ efflux in the dark, observed here under phosphorylating conditions, suggests that there may be a difference in the fate of H^+ taken up in the coupled compared to non-coupled conditions. One explanation is that the H^+ are being utilized as MITCHELL has suggested[8,25], *i.e.*, the high internal H^+ concentration provides a 'sink' for OH^-, postulated to be released inwards from the membrane upon esterification of ADP by P_i (this is most clearly proposed in ref. 25). Any other formulation of the MITCHELL hypothesis would likewise require the efflux of the H^+ as ATP was formed, out of consideration for conservation of energy (*i.e.*, the first law of thermodynamics).

Another explanation, which we favor, is that the esterification reaction which utilizes a proton, *i.e.*:

$$H^+ + ADP^{3-} + HPO_4^{2-} \rightarrow ATP^{4-}$$

takes place in a compartment in contact with that into which the H^+ transport mechanism deposits H^+. As the ATP is released into the suspension phase, H^+ would effectively be taken from the inner compartment to the outer phase, thus lowering the internal concentration, the consequence of which would be a decreased gradient,

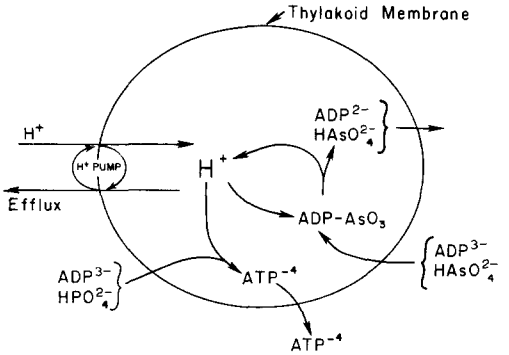

Fig. 8. A diagramatic model depicting two modes of H^+ efflux from the chloroplast. See text for details.

observable as a decrease in the extent and rate of the dark H^+ efflux, relative to a non-phosphorylating control. In the case of AsO_4^{3-} and ADP it is assumed that the transient species, ADP–AsO_3, hydrolyzes before it can be transported out of the grana space, thus returning the H^+ to the internal pool where it can contribute to the H^+ gradient. The model in Fig. 8 shows schematically the proposed relationship between the H^+ pump and the phosphorylating system of spinach chloroplasts. This model would explain our present data. It remains to be demonstrated whether ATP formation occurs within the membrane as suggested here. If that is the case, then the H^+ pump could facilitate the entry of ADP–Mg^{1-} and HPO_4^{2-} by contributing to a net positive charge within the membrane and/or provide counter-ions and thereby aid the entry of the anions ADP–Mg^{1-} and HPO_4^{2-}. This type of involvement of the H^+ transport in the energy-conservation mechanism was discussed by DILLEY[3], and recently in more detail by KARLISH AND AVRON[12]. The latter authors postulated that the main function of the H^+ pump is to allow co-transport of HPO_4^{2-} or $HAsO_4^{2-}$ and ADP^{3-}, and that this could lead to stimulation of net H^+ uptake, in the presence of these cofactors. However, as shown above, the addition of $MgCl_2$ or ADP used for their experimental treatments gave enough stimulation in electron transport to account for the increased uptake of H^+.

Since we have shown that the steady-state gradient of H^+ decreases under phosphorylating conditions, it seems unlikely that co-transport of ADP and PO_4^{3-} would be the main function of the H^+ pump. Our evidence suggests that one function of the H^+ pump is rather to supply H^+ being utilized by the formation of ATP. This would keep the pH of the internal space from being driven to high alkalinity, a condition which would tend to slow down the esterification. Other possible functions of the H^+ pump will be discussed elsewhere.

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