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ATP Formation Onset Lag and Post-Illumination Phosphorylation Initiated with Single-Turnover Flashes. II. Two Modes of Post-Illumination Phosphorylation Driven by Either Delocalized or Localized Proton Gradient Coupling

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Abstract

Two modes of chloroplast membrane post-illumination phosphorylation were detected, using the luciferin-luciferase ATP assay, one of which was not influenced by added permeable buffer (pyridine). That finding provides a powerful new tool for studying proton-membrane interactions during energy coupling. When ADP and P_i were added to the thylakoid suspension after a train of flashes [similar to the traditional post-illumination phosphorylation protocol (termed PIP^- here)], the post-illumination ATP yield was influenced by pyridine as expected, in a manner consistent with the ATP formation, in part, being driven by protons present in the bulk inner aqueous phase, i.e., through a delocalized protonmotive force. However, when ADP and P_i were present during the flash train (referred to as PIP^+), and ATP formation occurred during the flash train, the post-illumination ATP yield was unaffected by the presence of pyridine, consistent with the hypothesis that localized proton gradients were driving ATP formation. To test this hypothesis further, the pH and flash number dependence of the PIP^- and PIP^+ ATP yields were measured, the results being consistent with the above hypothesis of dual compartment origins of protons driving post-illumination ATP formation.

Measuring proton accumulation during the attainment of the threshold energization level when no $\Delta\psi$ component was allowed to form (+ valinomycin, K^+), and testing for pyridine effects on the proton uptake, reveals that the onset of ATP formation requires the accumulation of about 60 nmol H^+ (mg Chl)$^{-1}$. Between that level and about 110–150 nmol H^+ (mg Chl)$^{-1}$, the accumulation appears to be absorbed by localized-domain membrane buffering groups, the protons of which do not equilibrate readily with the inner aqueous (lumen) phase. Post-illumination phosphorylation driven by the

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dissipation of the domain protons was not affected by pyridine (present in the lumen), even though the effective pH in the domains must have been well into the buffering range of the pyridine. That finding provides additional insight into the localized domains, namely that protons can be absorbed by endogenous low pK buffering groups, and released at a low enough pH ($\leqslant 5.7$ when the external pH was 8, $\leqslant 4.7$ at pH 7 external) to drive significant ATP formation when no further proton production occurs due to the redox turnovers. We propose that proton accumulation beyond the 110–150 nmol (mg Chl)$^{-1}$ level spills over into the lumen, interacting with additional, lumenal endogenous buffering groups and with pyridine, and subsequent efflux of those lumenal protons can also drive ATP formation. Such a dual-compartment thylakoid model for the accumulation of protons competent to drive ATP formation would require a gating mechanism to switch the proton flux from the localized pathway into the lumen, as discussed by R. A. Dilley, S. M. Theg, and W. A. Beard (1987) *Annu. Rev. Plant Physiol.* **38**, 348–389, and recently suggested by R. D. Horner and E. N. Moudrianakis (1986) *J. Biol. Chem.* **261**, 13408–13414. The model can explain conflicting data from past work showing either localized or delocalized gradient coupling patterns.

Key Words: ATP formation; localized and delocalized energy coupling; chloroplast membranes.

Introduction

The chemiosmotic hypothesis has provided an important model for understanding the coupling between the proton-generating events of electron transport and the proton-translocating ATP synthetases of oxidative and photosynthetic phosphorylation (Boyer *et al.*, 1977). Whether the proton gradient driving ATP formation is solely transmembrane and delocalized or if localized proton gradients can, in some circumstances, energize phosphorylation remains a controversial question (Ferguson, 1985; Haraux, 1985; McCarty, 1981; Melandri and Venturoli, 1986; Rottenberg, 1985; Westerhoff *et al.*, 1984; Dilley *et al.*, 1987). It is clear from the early work of Jagendorf and colleagues that protons originating in the inner aqueous phase of thylakoid membranes can drive ATP formation in a bulk-phase delocalized mode, as illustrated by the dark acid–base shift experiments (Jagendorf and Uribe, 1966) or from electron-transfer driven proton accumulation, as shown in the post-illumination ATP formation, (PIP),[3] experiments (Hind and Jagendorf, 1963). This concept was further developed by Nelson *et al.* (1971) and Avron (1972) inasmuch as low pK_a permeant buffers stimulated the PIP ATP yield.

[3]The abbreviations used are: CF_1, extrinsic membrane portion of the coupling factor complex; Chl, chlorophyll; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIP, post-illumination phosphorylation; PMS, phenazine methosulfate; Tricine, *N*-tris(hydroxylmethyl)methylglycine; ΔG_{ATP}, phosphoryl group transfer potential of ATP formation; $\Delta\psi$, transmembrane electrical potential difference.

The traditional PIP protocol involves illuminating the thylakoids under basal, noncoupled conditions (i.e., in the absence of ADP and P_i) and determining the ATP yield in the subsequent dark period after the addition of ADP and P_i.

Using the luciferin-luciferase assay system described in the foregoing paper (Beard and Dilley, 1988a), we observe, and will report here, the same type of PIP results; i.e., the low pK_a permeable buffer pyridine stimulated the PIP^- ATP yield when the external pH was high (i.e., 8) and the ADP and P_i were added in the dark after the proton accumulation in the light stage (in our case a train of single-turnover flashes). However, the PIP^+ ATP yield observed after a train of *phosphorylating* single-turnover flashes—the PIP^+ mode discussed in the preceding paper (Beard and Dilley, 1988a)—was insensitive toward pyridine. As pointed out in preliminary reports (Beard and Dilley, 1986a,b), those data suggest that the proton gradient driving PIP^+ was not in equilibrium with the bulk lumen phase, and that under those conditions a localized proton gradient can drive ATP formation.

In order to have more confidence in that interpretation of the PIP^+ results it is necessary to test for possible problems with, or artifacts in, the assay system. This report will do that by characterizing further the PIP^- and PIP^+ ATP yields, particularly in relation to: (1) external pH effects, (2) permeable buffer effects, and (3) the relationship between the onset and the extent of the PIP ATP yields and proton accumulation onto the endogenous, membrane-bound buffering groups as well as the pyridine-dependent buffering.

Materials and Methods

The procedures used in this study are primarily those used in the previous paper of this series (Beard and Dilley, 1988a). For proton pump measurements, thylakoids, 20 μg Chl/ml, were suspended at 10°C in 2.5 ml of reaction mixture consisting of 0.5 mM Tricine-KOH (pH 8.0), 50 mM sorbitol, 15 mM KCl, 3 mM $MgCl_2$, 0.1 mM methylviologen, and 400 nM valinomycin. For reactions carried out at pH 7.0, 0.5 mM MOPS was substituted for Tricine. Each reaction solution was adjusted to the appropriate pH before beginning the assay. Continuous recordings of pH were obtained using a Corning Model 12 pH meter. The sensitivity of such recordings was 0.045 pH units full scale. The lightly buffered thylakoid suspension was illuminated with saturating single-turnover flashes. Further details of the reaction conditions are given in the table and figure legends.

Results

Comments on the Experimental Protocols

The sensitivity of the luciferin-luciferase ATP detection system allows direct observation of ATP formation (Lundin *et al.*, 1977) and post-illumination phosphorylation (Beard and Dilley, 1988a) in a thylakoid reaction mixture energized with single-turnover flashes. The traditional PIP experiment, where the electron (and H^+) transfers take place with no ADP present, amply demonstrated that bulk-phase delocalized gradients were formed in the light and were dissipated across the ATP synthetase complex in the dark ATP forming step (Hind and Jagendorf, 1963). The reader may consult Fig. 5 in the preceding paper (Beard and Dilley, 1988a) for a demonstration of such a PIP^- assay using the luciferin-luciferase technique. This type of PIP is referred to as PIP^-, the "$-$" indicating the absence of ADP and P_i (or presence in the case of PIP^+) during the light excitation period. In particular, it is the observed increase in the PIP ATP yield due to the presence of permeable buffers, such as pyridine (Nelson *et al.*, 1971) and phenylenediamine (Avron, 1972), that strongly supports the concept of bulk-phase delocalized energy coupling, just as does the succinate stimulation of the ATP yield in the dark acid–base experiments of Jagendorf and Uribe (1966). In the light stage of a PIP^- experiment some of the added pyridine, for example, which gets inside the lumen, become protonated, leading to increased H^+ uptake (Nelson *et al.*, 1971; Avron, 1972). The subsequent dark, phosphorylating stage therefore has more protons in the lumen than in the control case, due to the dissociation of the pyridine buffer in the dark stage, giving a greater ATP yield (Nelson *et al.*, 1971; Ort *et al.*, 1976; Vinkler *et al.*, 1980). Using arguments of this sort Vinkler *et al.* (1980) have correctly used permeable buffer effects on PIP^- ATP yields to probe events attributable to bulk-phase delocalized H^+ gradients. Horner and Moudrianakis (1983, 1986) have applied rapid-mixing and quenching techniques to examine permeable buffer effects on the light-stage ATP formation or the subsequent ATP formation occurring in the dark.

We will use the same logic and compare the effect of the permeable buffer pyridine on the PIP^- and PIP^+ ATP yields. Pyridine is an ideal buffer to use for this inasmuch as 5 mM concentration does not inhibit steady-stage photophosphorylation (Ort *et al.*, 1976), yet it enters the thylakoid lumen rapidly ($t_{1/2} \simeq 45$ sec, Beard and Dilley, 1988b). In preliminary reports (Beard and Dilley, 1986a,b), we have recently demonstrated that pyridine ($pK_a = 5.4$) did not extend the ATP onset lag and had no effect on the PIP^+ ATP yield—with thylakoids resuspended in the absence of KCl—concluding that those thylakoids utilized localized proton gradients in the PIP^+ energy

coupling event. To determine if a bulk-phase delocalized proton gradient operates in a PIP^- protocol, the effect of pyridine on the PIP^- ATP yield will be reported here. It is expected that the effect of a permeable buffer on the PIP yield will be dependent on (a) the external pH; (b) the pK_a of the permeant buffer in relation to the external pH; and (c) the time (number of flashes) of illumination, to give more or less of the protonated buffer species.

Additional background required to interpret the experiments is the concept of the energetic threshold protonmotive force necessary to initiate ATP formation. The protonmotive force required to overcome the energetic requirement necessary to activate the coupling factors (Hangarter *et al.*, 1986) or to phosphorylate ADP (ΔG_{ATP}) has been reported to be as low 140 mV (Gräber *et al.*, 1984) or as high as 165 mV (Hangarter and Good, 1982). This will depend on the ambient ΔG_{ATP} and the redox state of the CF_1 complex (Nalin and McCarty, 1984). When $\Delta\psi$ has been dissipated with valinomycin and K^+, this translates to a ΔpH requirement of 2.3 and 2.8 pH units respectively, if the H^+/ATP ratio is taken to be 3 (Hangarter and Good, 1982; Portis and McCarty, 1974). We will assume that, under our conditions of a low ΔG_{ATP} (< 30 kJ/mol) and a coupling factor in an oxidized state (i.e., dark adapted), the threshold ΔpH necessary to initiate ATP synthesis, in the presence of valinomycin, is 2.3 pH units.

Pyridine Effects on Post-Illumination Phosphorylation

Traditional PIP (PIP^-). The above discussion establishes the predictive effect of permeable buffers on PIP if bulk-phase delocalized gradients are involved. For pyridine, $pK_a = 5.4$ at 10°C (Perrin, 1964), when the external pH is 8, there will be significant buffering action in the pH range of 6, before the energetic threshold of 5.7 is reached (Fig. 1) (cf. Discussion for the calculation of the amount of protonated base in the lumen at pH 5.7). Pyridine is expected to retard the PIP^- yield at low flash numbers because it retards the pH dropping to the energetic threshold, and it should increase the PIP^- yield at longer flash regimes if bulk-phase delocalized proton gradients are involved and if the lumen pH drops below 5.7. A flash titration of PIP^- at an external pH of 8 (Fig. 2) indicates that pyridine affected the PIP^- ATP yield in the manner described above.

Starting at an external pH of 7, the prediction is for the pyridine to cause *more* retardation of PIP than when the external pH was 8. This is because the energetic threshold for ATP formation will be near pH 4.7 (internal) and about 85% of the pyridine present will absorb protons as the pH in the lumen drops (Fig. 1). This qualitative prediction is satisfied by the PIP^- flash titration shown in Fig. 3 for pH 7. Even with 150 flashes, the sample with pyridine was still giving a lower PIP^- ATP yield than the control. These

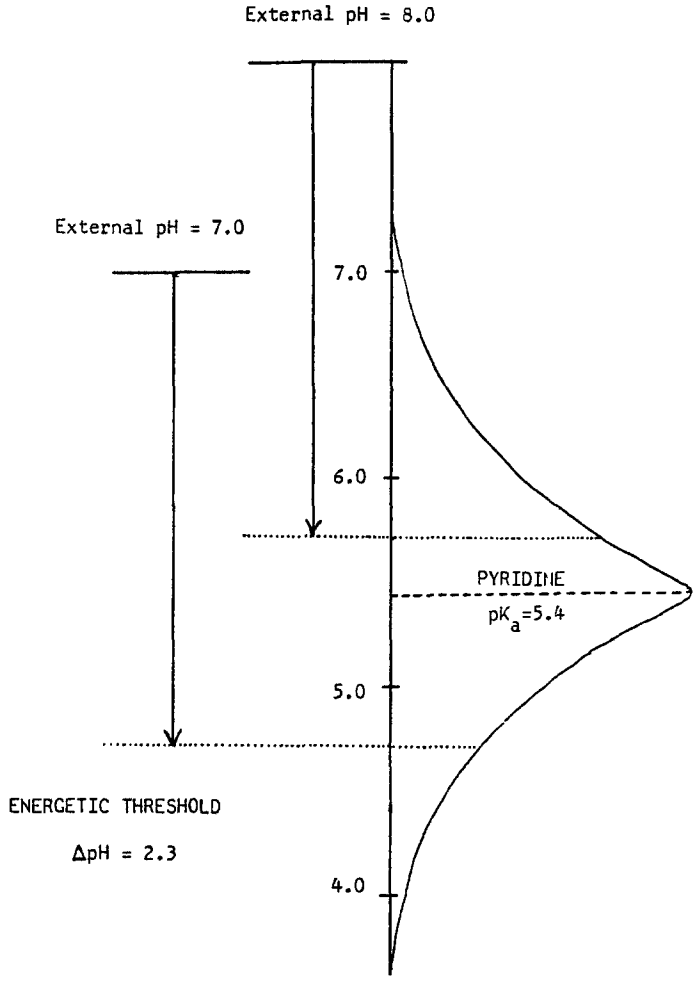

Fig. 1. The effect of pyridine on the buffering capacity of the thylakoid lumen. Pyridine, pK_a = 5.4, will influence the onset of ATP formation and post-illumination ATP formation depending on the pH of the external phase and the energetic threshold if a bulk-phase delocalized proton gradient drives ATP formation. Assuming that the energetic threshold is 2.3 pH units (Graber *et al.*, 1984) in the presence of valinomycin-K^+ to collapse $\Delta\psi$, then the lumen pH must drop to 5.7 and 4.7 for an external pH of 8 and 7, respectively. The pK_a of pyridine is alkaline enough so as to prolong the onset of ATP formation at either pH 7 or 8. At an external pH of 8, 35% of the pyridine would need to be protonated before the lumen pH could drop to 5.7, while 85% would need to be protonated before the energetic threshold (pH_{in} = 4.7) is reached at an external pH of 7. Whereas pyridine is expected to stimulate the post-illumination ATP yield at pH 8 because a large reservoir of protonated pyridine can drive ATP formation subsequent to a flash-induced phosphorylation, at pH 7 very little pyridine is available to allow for additional proton accumulation once the energetic threshold is reached.

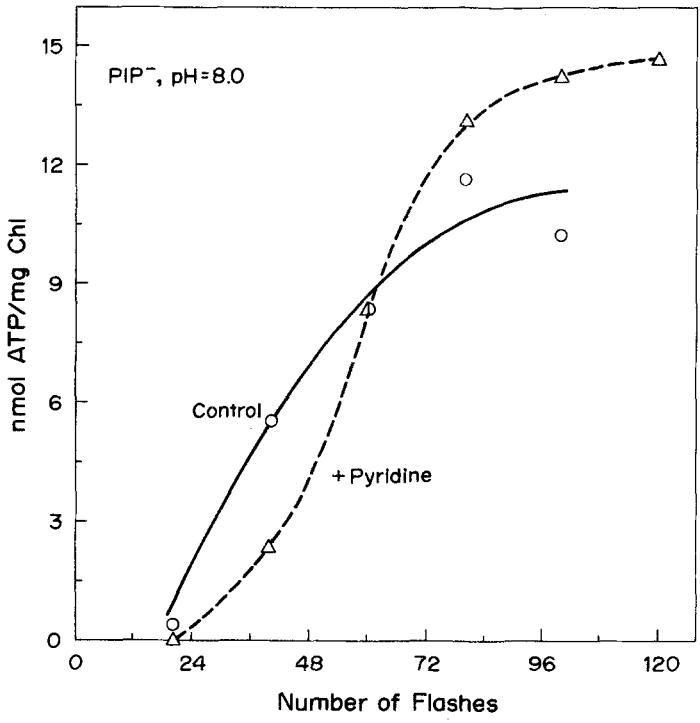

Fig. 2. Flash titration of the PIP^- ATP yield at pH 8 in the absence or presence of pyridine. Conditions are as in Table I. The pH of the reaction mixture was adjusted at 10°C with and without 5 mM pyridine. (○) control; (△) + pyridine. Pyridine is shown to stimulate PIP^- at high flash number sequences and inhibit PIP^- at low flash number sequences; see also Table I.

Table I. Effect of Pyridine on the PIP^- ATP Yield[a]

pH	Number of flashes	Pyridine	PIP^- ATP yield (nmol ATP/mg Chl)
7	125	−	21 ± 3
		+	14 ± 0.7
8	100	−	11.8 ± 0.4
		+	16.7 ± 0.5

[a]The reaction medium consisted of 50 mM Tricine-KOH (pH 8.0), 10 mM sorbitol, 3 mM $MgCl_2$, 400 nM valinomycin, 0.1 mM methylviologen, 5 mM DTT, with or without 5 mM pyridine, and 10 μM diadenosine pentaphosphate. After the last flash, ADP and KH_2PO_4 were added to a final concentration of 0.1 mM and 2 mM, respectively. For the assays at pH 7, 50 mM MOPS-KOH replaced Tricine. The pH of the reaction mixture was adjusted at 10°C with and without 5 mM pyridine. The flash rate was 5 Hz. The values reported are the means ± S.E. of three determinations.

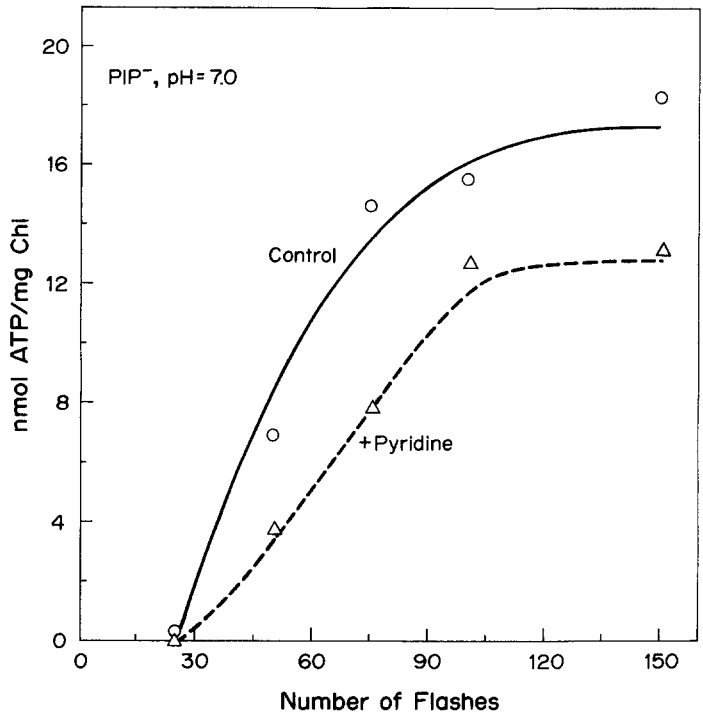

Fig. 3. Flash titration of the PIP^- ATP yield at pH 7 in the absence and presence of pyridine. Conditions are as in Table I. Due to residual ATP present in the ADP, 2.6 nmol ATP/mg Chl were subtracted from the initial PIP^- ATP yield. The reaction mixture was pH adjusted at 10°C with or without 5 mM pyridine. (○) control; (△) + pyridine. Pyridine is seen to inhibit the PIP^- ATP yield at all flash sequences performed; see also Table I.

effects are documented in Table I for pH 7 and 8 conditions, with several observations made with a specific number of flashes. Hence, we agree with the earlier conclusions (Vinkler *et al.*, 1980) that PIP^- ATP formation can be driven by proton gradients arising in the lumen bulk phase.

Post-Illumination Phosphorylation After a Train of Phosphorylating Flashes (PIP^+). Pyridine *did not* affect the PIP^+ ATP yield at either pH 7 or 8, as shown in Figs. 4 and 5, where the PIP^+ ATP yield is plotted as a function of flash number. Due to the data scatter in the flash titrations, many identical flash sequences were measured (Table II) to get sufficient data for a given flash number, with the same thylakoid preparation, to determine if significant differences existed which might have been obscured with the single observations necessitated by time constraints in producing data such as in Fig. 4 or 5. Table II demonstrates that 5 mM pyridine had no significant effect on PIP^+ at either pH 7 or 8, in contrast to the marked pyridine effect on PIP^- (Table I).

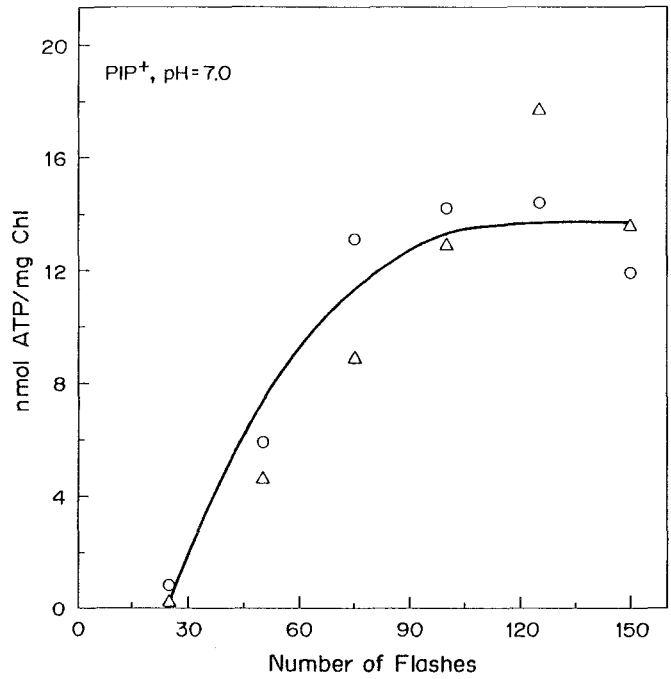

Fig. 4. Flash titration of the PIP^+ ATP yield at pH 7 in the absence and presence of pyridine. Conditions are as in Table II. The reaction mixture was pH adjusted at 10°C with or without 5 mM pyridine. (○) control; (△) + pyridine. PIP^+ appears not to be sensitive to pyridine; see also Table II.

Table II. Effect of Pyridine on the PIP^+ ATP Yield[a]

pH	Number of flashes	Pyridine	PIP^+ ATP yield (nmol ATP/mg Chl)
7	100	−	14 ± 1.4
		+	14 ± 2.4
8	80	−	6.3 ± 0.5
		+	6.4 ± 0.7

[a]The conditions are as in Table I except that the ADP and KH_2PO_4 were present in the reaction mixtures during the flash sequences. The values reported are the means ± S.E. of four determinations (pH 7) or six determinations (pH 8).

Proton Accumulation During the Pre-Energetic Threshold and the Post-Threshold ATP Forming Periods. The simplest interpretation of the pyridine effects on PIP^- and lack of pyridine effect on PIP^+ is that the initial proton accumulation occurs within membrane phase localized domains. If ATP formation can be driven by a protonmotive force constrained by the

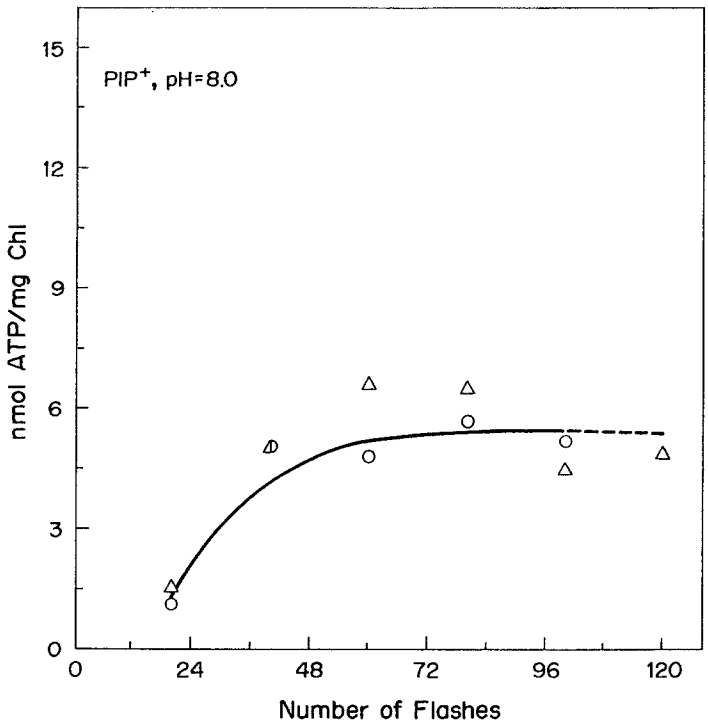

Fig. 5. Flash titration of the PIP^+ ATP yield at pH 8 in the absence and presence of pyridine. Conditions are as in Table II. The reaction mixture was pH adjusted at 10°C with or without 5 mM pyridine. (○) control; (△) + pyridine. PIP^+ appears not to be sensitive to pyridine; see also Table II.

domains, then that could account for the lack of pyridine effect on the PIP^+. If ATP formation is not possible (e.g., no ADP added), the domains may fill to capacity and further proton accumulation could spill over into the lumen. A way of testing this concept and obtaining a measure of the capacity of the localized domains is to measure the proton uptake and the pyridine effect on it, under basal and coupled conditions. Table III shows such measurements, and for greater clarification we will introduce here the use of KCl-treated thylakoids (Beard and Dilley, 1986a), which do not show localized domain effects, as a comparison to the low-salt, high sucrose-treated thylakoids which do show localized coupling, used for this study. The next paper in this series gives details on the KCl versus sucrose-treated thylakoids (Beard and Dilley, 1988b). The sucrose-treated thylakoids will be dealt with first in presenting this analysis.

We determined proton accumulation under basal or coupled conditions with a pH electrode or with cresol red. An ADP regenerating system,

Table III. Effect of Pyridine on Proton Uptake[a]

		pH	Frequency/Number of flashes given	Proton uptake [nmol H^+ (mg Chl)$^{-1}$]		
				Control	+ Pyridine	Difference
A. Basal	1.	7.0	5/125	368 ± 20	461 ± 20	93
	2.	8.0	5/100	220 ± 12	327 ± 16	107
	3.	8.0	1/100	107 ± 13	109 ± 7	2
	4.[b]	8.0	1/100	150 ± 8	150 ± 9	0
	5.	8.0 KCl-treated	1/100	147 ± 2	206 ± 13	59
B. Coupled	6.	7.0	5/125	285 ± 19	348 ± 23	63
	7.	8.0	5/100	126 ± 15	150 ± 8	24
	8.[b]	8.0	1/100	109 ± 3	107 ± 6	0
	9.[b]	8.0 KCl-treated	1/100	108	167	59

[a]The reaction conditions for basal proton uptake (A) are given under Materials and Methods. For coupled proton uptake (B) 2 mM KH_2PO_4, 0.1 mM ADP, 10 mM glucose, and 15 units/ml of Sigma F-300 hexokinase were included in the basal reaction mixture, while at pH 7.0, MOPS was excluded. The pH of the reaction mixture was adjusted to the desired pH prior to flash illumination with dilute HCl. Pyridine was present at a concentration of 5 mM. Results are the means ± S.E. of four determinations.

[b]Data for lines 4, 5, 8, and 9 were taken at a different time than the other data, using the cresol red pH determination technique (Beard and Dilley, 1988b), which may account for the larger H^+ accumulation for the basal case. The data for lines 5 and 9 were obtained using KCl-treated thylakoids as described in the accompanying paper (Beard and Dilley, 1988b). The data are presented here to show that the pyridine effect of enhancing H^+ uptake *can* occur at 1-Hz flash frequency, provided that the thylakoids are of the type that show delocalized proton gradient coupling.

hexokinase and glucose, was used to cancel pH changes that would have occurred owing to net formation of ATP, thus allowing observation of pH changes solely due to the proton uptake into the membrane. Table III indicates that at pH 8 and 5-Hz flash frequency, there was a significant decrease (close to 50%) in the proton accumulation under phosphorylating conditions in the presence or absence of pyridine, as compared to basal (nonphosphorylating) conditions (compare line 2 with line 7). This is consistent with an increased proton flux through the ATP synthetases during phosphorylation, decreasing the proton electrochemical gradient (Davenport and McCarty, 1984; Pick *et al.*, 1973; Portis and McCarty, 1974).

A measure of the proton accumulation which can initiate and sustain phosphorylation, but not show a pyridine effect on ATP formation parameters (or on the proton uptake), is a measure of the capacity of the putative localized proton-buffering domain. Varying the flash frequency (1 or 5 Hz) under basal and coupled conditions showed that a proton accumulation up to 150 nmol H^+/mg chl occurred before pyridine buffering effects were noticed, even under basal conditions where the maximum H^+ accumulation should occur (line 4, Table III). In those measurements the H^+ uptake reached a steady state by 80–90 flashes with either 1 or 5 Hz flash rate. Different chloroplast preparations gave somewhat different effects; note that the data for lines 1–3, 6, and 7 were from a different experiment than that for lines 4, 5, 8, and 9. In the former set, the H^+ accumulation where the pyridine effects began to be noticed may have been closer to 120 nmol H^+/mg chl. When the proton accumulation in the absence of pyridine was over 200 nmol H^+/mg chl at pH 8, pyridine addition significantly increased the H^+ uptake (line 2, for 5 Hz, basal). With coupling conditions at pH 8, 5-Hz flashes (line 7) gave only 126 nmol H^+/mg chl, with perhaps some increase due to pyridine.

In the other series of measurements, the basal situation gave 150 nmol H^+/mg chl (line 4) with 1-Hz flashes, and pyridine did not give an increased H^+ uptake. The 1-Hz flash train under coupling conditions decreased the H^+ uptake (line 8) to about 110 nmol/mg chl, still with no pyridine effect. The decreased H^+ uptake is expected inasmuch as 1-Hz flashes drive ATP formation effectively (Fig. 3 of the previous paper, Beard and Dilley, 1987a) with a lag of about 20 flashes (+ valinomycin, K^+). Hence, a ΔpH of $\geqslant 2.3$ units must have occurred between the proton gradient source and the outer medium for the last 80 flashes of the 1-Hz flash train.

It could be suggested that the lumen equilibrated with the acid side pH of $\leqslant 5.7$, but the 1-sec dark time between flashes was long enough to allow the lumen protons to efflux to an extent that no pyridine effects were detected. This can be ruled out in theory by the argument that the $t_{1/2}$ for proton efflux in the dark is > 10 sec, so a net pyridine buffering action should be detectable in a 1-Hz flash train. The point can be ruled our more clearly by an experiment,

using the KCl-treated thylakoids, which allow a rapid lumen equilibration of the proton gradient that drives ATP formation (cf. the next paper in this series for the details of this, Beard and Dilley, 1988b). Line 5, Table III shows that under basal conditions, pH 8, and 1-Hz excitation, pyridine increased the H^+ uptake from 147 to 206 nmol H^+/mg chl. Compare these data to line 4, from the same series of measurements using sucrose-treated thylakoids, where no pyridine effect was observed with a 1-Hz flash sequence. Even under coupling conditions, where the H^+ accumulation for the control was lowered to 108 nmol H^+/mg Chl (line 9), the KCl-treated thylakoids showed a large pyridine-induced increase in proton accumulation. Those results clearly show that pyridine can effectively increase the observed H^+ uptake in a 1-Hz flash train. Thus, the failure of the sucrose-treated thylakoids to show a pyridine-dependent increase in proton accumulation in the 1-Hz flash excitation can be explained by the protons not being able to equilibrate readily with the lumen phase in that case.

The pH 7 data in Table III (lines 1 and 6) show the expected greater H^+ uptake compared to pH 8 conditions. Moreover, even though pyridine did not significantly affect the PIP^+ ATP yield nor the ATP formation energization flash lag (Fig. 4), there was a significant spillover of protons into the lumen under coupling conditions to allow the 22% increased H^+ uptake (line 6).

Discussion

Delocalized Proton Gradient Coupling Involvement in PIP^-

Nelson *et al.* (1971) have demonstrated that pyridine can increase the extent of proton uptake in combination with an increase in the yield of ATP in a PIP^- protocol involving an alkaline pH jump. Ort *et al.* (1976) and Vinkler *et al.* (1980) have demonstrated that with short preillumination periods, pyridine initially inhibited the post-illumination yield of ATP but ultimately increased the yield if the preillumination period was extended sufficiently. Those results are most reasonably interpreted as indicative of pyridine affecting bulk-phase delocalized proton gradients. Similar pyridine effects on the PIP^- ATP yields were observed in the experiments reported here when thylakoids were illuminated with a train of single-turnover flashes in the absence of ADP and P_i (PIP^-). Figure 2 illustrates that at pH 8 about 60 flashes were required to overcome the buffering capacity of pyridine and drop the lumen pH below a ΔpH of 2.3 (the energetic threshold) and initiate PIP^- ATP formation. After 60 flashes, further protonation of pyridine increased the reservoir of protons at ΔpH values exceeding 2.3; thus a greater

PIP^- ATP yield occurred in the presence of pyridine. With less than 60 flashes, pyridine buffered the protons accumulated in the inner bulk phase at pH values above about 5.7, so in that case protons released from pyridine in the dark upon relaxation of ΔpH were energetically incompetent for ATP formation, hence the lower PIP^- ATP yield. At pH 7, the PIP^- ATP yield was decreased by pyridine, as compared to the yield in its absence, out to 150 flashes (Fig. 3). This is reasonably explained as due to pyridine buffering protons shuttled to the lumen at pH values above 4.7, the energetic ΔpH threshold, resulting in a decreased PIP^- ATP yield. We will return to the interesting point concerning why pyridine did not inhibit the PIP^- ATP yields more fully; the issue will involve the suggestion that some of the membrane phase buffering groups are spatially separated from the bulk inner space.

Localized Proton Gradient Coupling Involvement in PIP^+

It is significant that 5 mM pyridine did not affect the PIP^+ ATP yields at either pH 7 or 8 (Figs. 4 and 5, Table II), and for both pH conditions this was true considerably beyond the flash numbers where saturation of the PIP^+ yield occurred. This is important, for it establishes that the system had reached a type of steady state for the given flash frequency, so the absence of a pyridine effect on PIP^+ cannot be due to a possible transient state response. When ATP formation occurred during the flash train, the *pH of the inner bulk phase apparently did not drop low enough to significantly protonate pyridine* so as to allow pyridine to serve as a source of energetically competent protons to drive PIP^+ phosphorylation. If the energetic threshold for ATP formation was 2.3 pH units, then under these conditions the lumen pH must have remained above 5.7 when the external pH was 8, or above 4.7 when the external pH was 7, during the ATP-producing flashes.

At 10°C pyridine has a pK_a of 5.4 (Perrin, 1964), so under these conditions significant amounts of unprotonated pyridine would have been present to buffer protons prior to attaining the energetic threshold internal pH (see below). Therefore, the lack of an effect of pyridine on the PIP^+ ATP yield indicates that the protons which drive this phosphorylation are not derived from the lumen. The only other source of a proton gradient would be sequestered proton domains which are not accessible to pyridine.

An important point to discuss is whether the transmembrane ΔpH could have been large enough to energize ATP formation for the PIP^+ case at pH 8, but perhaps no pyridine effects were observed because the pH inside "hovered" near the energization threshold of 5.7 and the pyridine buffering may not have made a detectable effect at that internal pH. An argument against this interpretation derives from the data of Fig. 5, where it is

illustrated that the PIP^+ ATP yield was measurable at about 20 flashes (5 Hz), the same number of flashes required to initiate ATP formation; i.e., to reach the energetic threshold when valinomycin and K^+ are present. If we assume that bulk-phase delocalized protons drive ATP formation, the inner aqueous phase must be assumed, in that case, to have reached a pH of 5.7 at the onset of ATP formation and PIP^+. But Fig. 5 also shows that the PIP^+ ATP yield increased about 3- to 4-fold as the number of flashes increased from 20 to near 50. Given that the PIP^+ ATP formation is rather slow (apparent $t_{1/2} \simeq 1$ s, Beard and Dilley, 1988a), the increase in PIP^+ yield with flash number implies that, if the inner aqueous space were the source of the protons driving the PIP^+, then: (a) the pH of the lumen must have dropped more acidic than 5.7 with flashes 20 to 50; (b) there was ample time for pyridine inside to be protonated [in fact, more neutral pyridine should have entered as some pyridine inside was converted to the cationic form (Nelson *et al.*, 1971)]; and (c) at 5 mM pyridine and 20 μl/mg Chl of lumen water (Beard and Dilley, 1988b) there is a total of 100 nmol pyridine/mg Chl in the lumen. At pH_{in} of 5.7, 36 nmol/mg Chl would be protonated, leaving 64 nmol/mg Chl as available buffer as the pH drops below 5.7. Pyridine protonated at or below 5.7 would be a source of energetically competent protons upon deprotonation, so an additional PIP^+ yield due to the pyridine would be expected if the lumen were the location of the energetic gradient in the PIP^+ experiment. Additionally, (d) if a bulk-phase proton gradient was required to energize the system, then a delay in the onset of ATP formation should have occurred until the 36 nmol of pyridine base/mg Chl (about an eight-flash extension) were protonated. Such an extension was not observed (Beard and Dilley, 1986a). Lastly, (e) the same lack of a pyridine effect on the PIP^+ ATP yield when the external pH was 7 strongly argues against the above possibility, because most of the pyridine (85%, amounting to at least 85 nmol/mg Chl if no further pyridine uptake occurred) would have to be protonated before the energetic threshold ($pH_{in} = 4.7$) was reached (Fig. 1). This should have significantly delayed the onset of the PIP^+, but it did not (Fig. 4). At pH 7, a small inhibition of the PIP^+ ATP yield at low flash numbers may be perceived in Fig. 4, but because no inhibition was exhibited between 100 and 125 flashes, and because Table II shows no difference in PIP^+ yield, we conclude that there was no effect.

The logic of points (a)–(e) is compelling, but in the absence of any other data, one could hold out for some other explanation: some inexplicable effect that precludes pyridine showing a delay in PIP^+ yield, even while the essential proton gradient was established between the lumen and the external phase. What would be convincing, of course, would be to switch the system into a condition wherein, using the same PIP^+ assay, one *could* demonstrate pyridine effects on PIP^+. This would establish that the flash-energized

system, and the assay used, have the intrinsic capacity to detect delocalized protonmotive force-driven PIP^+. The following paper in this series (Beard and Dilley, 1988b) presents just that situation, thus giving further credence to the conclusion supported by points (a)–(e) above.

Complete protonation of pyridine cannot account for the lack of sensitivity of the PIP^+ ATP yield to pyridine at high flash numbers. In a PIP^- experiment at pH 7, which shows a greater capacity for proton accumulation than in the PIP^+ case, even at 150 flashes (Fig. 3), the ATP yield was still lowered in the presence of pyridine, i.e., pyridine was still influencing the buffering capacity of the lumen. The same was true even for PIP^- yields with 20-s continuous high light exposure in the presence of pyridine at pH 7. This is consistent with the notion that additional pyridine can accumulate in the lumen during proton accumulation, further inhibiting lumenal acidification (Nelson *et al.*, 1971).

Table IIIA confirms that pyridine stimulated the total number of protons taken up by the sucrose-treated thylakoids when illuminated, in the absence of ADP and P_i, by single-turnover flashes delivered at 5 Hz. Interestingly, "extra" proton accumulation was also demonstrated at 5 Hz (employing a hexokinase and glucose trap) under phosphorylating conditions (Table IIIB). A question relevant to this point is whether the observed pyridine-dependent extra H^+ accumulation occurred in all of the thylakoid vesicles to a slight extent or whether a subpopulation of phosphorylation-inactive vesicles experienced a much greater H^+ accumulation and hence more of a pyridine effect. If the latter case were true, those inactive vesicles would not show ATP yield in any event, hence no pyridine effect on PIP^- would be expected, but there was a pyridine effect on PIP^-. If the former case holds, then this indicates that some protons may be shuttled to the lumen even under phosphorylating conditions, but are absorbed by the buffering capacity of the lumen at pH values above the energetic threshold pH, so as not to effect the yield of ATP in a PIP^+ protocol.

Proton Uptake During the Flash Sequence

Thylakoids required the accumulation of about 60 nmol H^+/mg Chl before ATP formation was initiated, as reported by Hangarter and Ort (1985) and reaffirmed by this work. However, with low-salt, high sucrose-treated thylakoids prepared as described under Materials and Methods, significant pyridine effects on proton uptake were not observed until the total proton accumulation was 2–2.5 times that required for the onset of ATP formation. Even when proton accumulation was examined under basal conditions, where the dark time between flashes was increased to 1 s, no significant stimulation of proton uptake was observed in the presence of pyridine (Table IIIA)

although the total proton accumulation was near 110 in one case and 150 nmol H^+/mg Chl in the other case. This indicates that the lumen pH was more alkaline than the putative localized domain experiencing the energetic threshold pH of 5.7, which must have been reached after the accumulation of only 60 nmol H^+/mg Chl. This was not the case for the KCl-treated thylakoids (described in detail in the accompanying paper, Beard and Dilley, 1988b), which showed a large pyridine-dependent H^+ uptake at 1-Hz flash frequency, and when the control H^+ uptake was only 108 nmol H^+/mg Chl in the coupled state (line 9, Table III), or 147 nmol/mg Chl in the basal case (line 5, Table III). This is an important control because it rules out the possibility that the failure to see the pyridine effect on H^+ uptake at 1 Hz with the low-salt, high sucrose-treated thylakoids was due to a transient lumenal pyridine buffering effect that relaxed by the end of the 1-s dark period between flashes. The implication for the thylakoids prepared in low-salt, high-sucrose media is that the *primary energetic proton gradient and the endogenous buffering groups which interact with the protons in that gradient, up to near 150 nmol H^+/mg Chl, must occur in sequestered domains within the membrane, rather than be freely associated with the lumen.*

Carboxyl groups of membrane proteins could constitute a sequestered buffering domain in the pK_a range < 6. The thylakoid membrane exhibits its greatest buffering capacity in the range of pH 5.3 down to around 4.8, with much less buffering power above pH 5.7 (Walz *et al.*, 1974). If such groups occurred in squestered domains of small volumes, unavailable to buffers such as pyridine, then the PIP^+ and the PIP^- data and the effects of pyridine on them can be understood. This is supported by the analysis below.

PIP^- Corrected for PIP^+

Since the PIP^+ ATP yield is postulated to be originating from localized proton buffering domains associated with the thylakoid membrane, this ATP yield should also contribute to the ATP yield in a PIP^- protocol. That is, protons which fill the localized domain buffering groups before additional protons spill over into the lumen, should exert their capacity to contribute to PIP ATP yield in a PIP^- experiment. To obtain a qualitative picture of lumenal proton processing, the PIP^+ ATP yields from the curves drawn in Figs. 4 and 5 were subtracted from the total PIP^- ATP yields of the curves drawn in Figs. 2 and 3 for pH 7 and 8, respectively. The corrected PIP^- ATP yields are presented in Figs. 6 and 7.

At an external pH of 8 (Fig. 7), correcting for the PIP^+ yield, pyridine makes no contribution to the PIP^- ATP yield below about 60 flashes; i.e., the lumen pH did not drop below 5.7 during the first 60 flashes in the presence of pyridine. At about 60–70 flashes, when the bulk-phase transmembrane

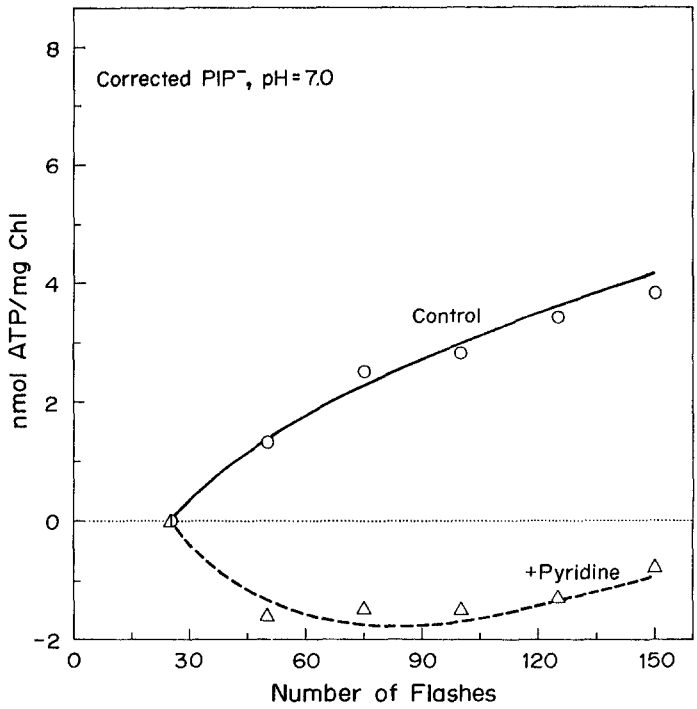

Fig. 6. Flash titration of the PIP^- ATP yield corrected for PIP^+ at pH 7. Data were extrapolated from the curve in Fig. 4 (PIP^+) and subtracted from the corresponding points extrapolated from the curves in Fig. 3 (PIP^-). (○) control; (△) + pyridine.

ΔpH had reached the energetic threshold, "extra" proton accumulation due to pyridine apparently served as a reservoir of energetically competent protons, and the PIP^- yield was increased. Figure 6 illustrates that at pH 7 pyridine never allowed the internal pH to drop below the energetic threshold pH of 4.7, and all the PIP^- ATP yield was accounted for as that which occurred in the PIP^+ mode; i.e., all the protons driving ATP formation orginated from endogenous buffering groups in the sequestered domains. This is consistent with the ability of pyridine to accumulate in the lumen during the illumination, making it difficult to lower the internal pH. The corrected PIP^- curves of Figs. 6 and 7 are similar to the PIP^+ titrations of thylakoids isolated in the presence of high KCl and which exhibit a bulk-phase delocalized protonic energy coupling [cf. Figs. 1 and 2 of the following paper (Beard and Dilley, 1988b)]. During the early flash numbers at pH 8 (Fig. 7) and for the entire flash titration in Fig. 6, the corrected PIP^- ATP yields are observed to fall below zero. This is probably due to a combination of factors: the scatter in the data (the PIP^+ experiments were performed on

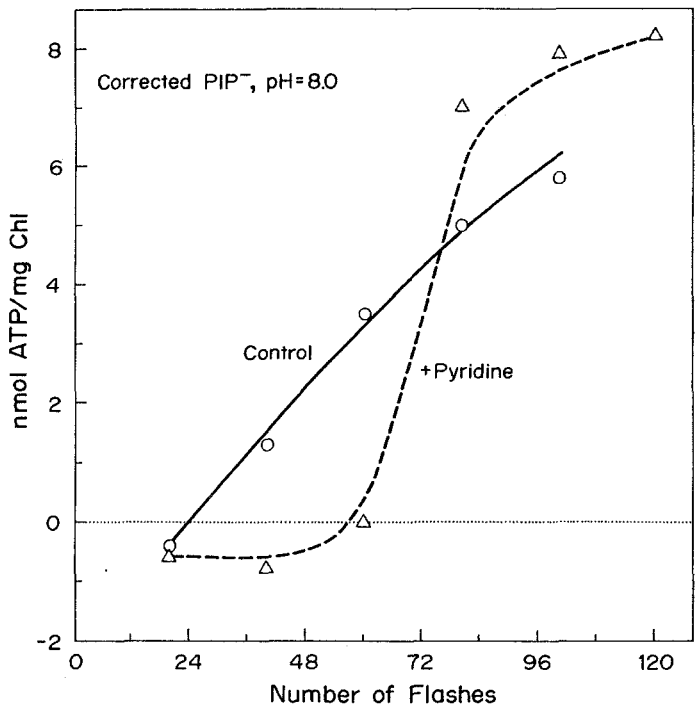

Fig. 7. Flash titration of the PIP^- ATP yield corrected for PIP^+ at pH 8. Data were extrapolated from the curve in Fig. 5 (PIP^+) and subtracted from the corresponding points extrapolated from the curve in Fig. 2 (PIP^-). (○) control; (△) +pyridine.

different days) and/or minor contributions of lumenal protons giving rise to PIP^+ ATP yield which are within the experimental errors of our measurements. These figures confirm and emphasize the effects that pyridine has on lumenal protons.

Under the conditions used here, PIP^+ was not sensitive to pyridine at either pH 7 or 8 (Beard and Dilley, 1986a,b). This is in contrast to Horner and Moudrianakis (1983, 1986), who observed no change in the photophosphorylation onset lag in the presence of valinomycin-K^+ and imidazole, but found PIP^+ to be severely inhibited. This apparent discrepancy may be due to a different method of illumination; Horner and Moudrianakis (1983, 1986) selected a continuous flash of tens of milliseconds with PMS/ascorbate as a cyclic electron flow mediator as compared to single-turnover flashes with methylviologen as a noncyclic electron acceptor used in the present study. They concluded that protons can be utilized through intramembrane routes to the ATP synthetase, but also indicated that protons may leak into the lumen before finding a path to an ATP synthetase. The continuous

illumination and PMS/ascorbate probably allowed for a greater proton accumulation in the inner bulk phase, making the PIP^+ ATP yield sensitive to the added permeable buffer. More recently, de Kouchkovsky *et al.* (1986) have demonstrated that imidazole can have a "delocalizing" effect on protons which normally show a localized behavior in a steady-state measurement. We have also noted that 1 mM imidazole severely inhibits PIP^+ at low flash numbers using single-turnover flashes and may at times stimulate PIP^+ with long flash sequences (unpublished observation), in agreement with the "delocalizing" effect exhibited by imidazole (De Kouchkovsky *et al.*, 1986).

Concluding Remarks—Hypothesis

The hypothesis most simply explaining our results is that in thylakoids prepared and stored in low-KCl media, the *initial proton gradient energization occurs in membrane-localized domains which are connected directly to the CF_0–CF_1 complexes*. Protons filling those domains can efflux through the CF_0–CF_1 and drive ATP formation as in the PIP^+ mode presented above, without equilibrating with the lumen, provided that conditions are favorable for CF_1 function. If CF_1 is not functional (−ADP, for example) or is operating at its capacity, the localized domain fills to its buffer capacity, about 110–150 nmol H^+/mg Chl, and additional protons spill over into the lumen, perhaps by the operation of a regulated gating device, acidifying it and interacting with buffering groups present. Horner and Moudrianakis (1986) have suggested such a gating mechanism, and it had been suggested in a general sense even earlier (Dilley and Schreiber, 1984). Protons in the lumen, at sufficient concentration to achieve the $\Delta pH \simeq 2.3$ units, can efflux through the CF_0–CF_1, driving ATP formation, as in the PIP^- mode presented above. Some proton spillover into the lumen may occur while the localized domains are filling, perhaps due to leaks or nonfunctional CF_0–CF_1 units.

The results clearly show that post-illumination ATP formation can be driven by either localized (the PIP^+ mode) or delocalized proton gradients (as in the PIP^- mode).

When valinomycin and K^+ keep the $\Delta\psi$ suppressed, proton accumulation of about 60 nmol (mg Chl)$^{-1}$ is sufficient to attain the energetic threshold protonmotive force $\geqslant 2.3$ ΔpH units in the localized domain. The buffering groups involved in binding the 60 nmol H^+ (mg Chl)$^{-1}$ do not include the $pK \simeq 7.8$ localized amine groups identified from previous work (Dilley *et al.*, 1987; Laszlo *et al.*, 1984), but are likely to be lower pK groups, such as carboxyls, which do not retain protons in metastable pools when the external pH is near 8. Between 60 and near 110–150 nmol H^+ (mg Chl)$^{-1}$ the accumulated protons can drive increasing PIP^+ ATP yields, but under the

conditions used here the lumen is not a contributing source of protons for driving that ATP formation. Proton accumulation after about 55–60 flashes in the presence of pyridine (Fig. 7), predicted to be between 150–200 nmol H^+ (mg Chl)$^{-1}$, may be required before the lumen reaches a pH below 5.7 (the ΔG_{ATP} energetic threshold), sufficient to drive PIP^- (sensitive to pyridine). In the next paper of this series, it will be seen that a 100 mM KCl washing treatment abolishes the localized gradient-driven PIP, making both PIP^+ and PIP^- ATP yields appear to be driven by proton gradients which rapidly equilibrate with the lumen bulk phase. This result makes even more compelling the notion of a type of switch, which may be regulated by conditions such as ion concentration, whereby protons are less or more easily shunted from the membrane domains to the lumen.

Acknowledgments

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References

Avron, M. (1972). In *Proc. IInd Intl. Congress on Photosynthesis Research* (Forti, G., Avron, M., and Melandri, A., eds.), Dr. W. Junk, The Hague, pp. 861–871.

Beard, W. A., and Dilley, R. A. (1986a). *FEBS Lett.* **201**, 57–62.

Beard, W. A., and Dilley, R. A. (1986b). In *Proc. VIIth Intl. Congress on Photosynthesis* (Biggins, J., ed.), Martinus Nijhoff/Dr. Junk Publishers, The Hague, Vol. 3, pp. 165–168.

Beard, W. A., and Dilley, R. A. (1988a). *J. Bioenerg. Biomembr.* **20**, 85–106.

Beard, W. A., and Dilley, R. A. (1988b). *J. Bioenerg. Biomembr.* **20**, 129–154.

Boyer, P. D., Chance, B., Ernster, L., Mitchell, P., Racker, E., and Slater, E. (1977). *Annu. Rev. Biochem.* **46**, 955–1026.

Davenport, J. W., and McCarty, R. E. (1984). *Biochim. Biophys. Acta* **766**, 363–374.

De Kouchkovsky, Y., Sigalat, C., and Haraux, F. (1986). In *Proc. VIIth Intl. Congress on Photosynthesis* (Biggins, J., ed.), Martinus Nijhoff/Dr. Junk Publishers, The Hague, Vol. 3, pp. 169–172.

Dilley, R. A., and Schreiber, U. (1984). *J. Bioenerg. Biomembr.* **16**, 173–193.

Dilley, R. A., Theg, S. M., and Beard, W. A. (1987). *Ann. Rev. Plant Physiol.*, **38**, 348–389.

Ferguson, S. J. (1985). *Biochim. Biophys. Acta* **811**, 47–95.

Gräber, P., Junesch, U., and Schatz, G. H. (1984). *Ber. Bunsenges. Phys. Chem.* **88**, 599–608.

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

Hangarter, R., and Ort, D. R. (1985). *Eur. J. Biochem.* **149**, 503–510.

Hangarter, R., Grandoni, P., and Ort, D. R. (1986). In *Proc. VIIth Intl. Congress on Photosynthesis* (Biggins, J., ed.), Martinus Nijhoff/Dr. Junk Publishers, The Hague, Vol. 3, pp. 205–212.

Haraux, F. (1985). *Physiol. Veg.* **23**, 397–410.

Hind, G., and Jagendorf, A. T. (1963). *Proc. Natl. Acad. Sci. USA* **49**, 715–722.

Horner, R. D., and Moudrianakis, E. N. (1983). *J. Biol. Chem.* **258**, 11643–11647.

Horner, R. D., and Moudrianakis, E. N. (1986). *J. Biol. Chem.* **261**, 13408–13414.

Jagendorf, A. T., and Uribe, E. (1966). *Proc. Natl. Acad. Sci. USA* **55**, 170–177.

Laszlo, J. A., Baker, G. M., and Dilley, R. A. (1984). *J. Bioenerg. Biomembr.* **16**, 37–51.

Lundin, A., Thore, A., and Baltscheffsky, M. (1977). *FEBS Lett.* **79**, 73–76.
McCarty, R. E. (1981). In *Chemiosmotic Proton Currents in Biological Membranes* (Skulachev, V. P., and Hinkle, P., eds.), Addison-Wesley, Reading, Massachusetts, pp. 271–281.
Melandri, B. A., and Venturoli, G. (1986). In *Encyclopedia of Plant Physiology, New Series* (Staehelin, L. A., and Arntzen, C. J., eds.), Springer-Verlag, Berlin, Vol. 19, pp. 560–569.
Nalin, C. M., and McCarty, R. E. (1984). *J. Biol. Chem.* **258**, 7275–7280.
Nelson, N., Nelson, H., Naim, Y., and Neumann, J. (1971). *Arch. Biochem. Biophys.* **145**, 263–267.
Ort, D. R., Dilley, R. A., and Good, N. E. (1976). *Biochim. Biophys. Acta* **449**, 108–124.
Perrin, D. D. (1964). *Austl. J. Chem.* **17**, 484–488.
Pick, U., Rottenberg, H., and Avron, M. (1973). *FEBS Lett.* **32**, 91–94.
Portis, A. R., Jr., and McCarty, R. E. (1974). *J. Biol. Chem.* **249**, 6250–6254.
Rottenberg, H. (1985). In *Modern Cell Biology* (Satir, B., ed.), Alan R. Liss, New York, Vol. 4, pp. 47–83.
Vinkler, C., Avron, M., and Boyer, P. D. (1980). *J. Biol. Chem.* **255**, 2263–2266.
Walz, D., Goldstein, L., and Avron, M. (1974). *Eur. J. Biochem.* **47**, 403–407.
Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F., and Kell, D. B. (1984). *Biochim. Biophys. Acta* **768**, 257–292.