ATP Formation Onset Lag and Post-Illumination Phosphorylation Initiated with Single-Turnover Flashes. III. Characterization of the ATP Formation Onset Lag and Post-Illumination Phosphorylation for Thylakoids Exhibiting Localized or Bulk-Phase Delocalized Energy Coupling

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Abstract

When 100 mM KCl replaced sucrose in a chloroplast thylakoid stock suspension buffer, the membranes were converted from a localized proton gradient to a delocalized proton gradient energy coupling mode. The KCl-suspended but not the sucrose-suspended thylakoids showed pyridine-dependent extensions of the ATP onset lag and pyridine effects on post-illumination phosphorylation. The ATP formation assays were performed in a medium of identical composition, using about a 200-fold dilution of the stock thylakoid suspension; hence the different responses were due to the pretreatment, and not the conditions present in the phosphorylation assay. Such permeable buffer effects on ATP formation provide a clear indicator of delocalized proton gradients as the driving force for phosphorylation. The pyridine-dependent increases in the onset lags (and effects on post-illumination phosphorylation) were not due to different ionic conductivities of the membranes (measured by the 515 nm electrochromic absorption change), H^+/e^- ratios, or electron transport capacities for the two thylakoid preparations. Thylakoid volumes and [^{14}C]pyridine equilibration were similar with both preparations. The KCl-induced shift toward a bulk-phase delocalized energy coupling mode was reversed when the thylakoids were placed back in a low-salt medium.

Proton uptake, at the ATP-formation energization threshold flash number, was much larger in the KCl-treated thylakoids and they also had a longer ATP formation onset lag, when no pyridine was present. These results are consistent with the salt treatment exposing additional endogenous buffering groups for interaction with the proton gradient. The concomitant appearance of the pyridine buffer effects implies that the additional endogenous buffering

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groups must be located on proteins directly exposed in the aqueous lumen phase.

   Kinetic analysis of the decay of the post-illumination phosphorylation in the two thylakoid preparations showed different apparent first-order rate constants, consistent with there being two different compartments contributing to the proton reservoirs that energize ATP formation. We suggest that the two compartments are a membrane-phase localized compartment operative in the sucrose-treated thylakoids and the bulk lumen phase into which protons readily equilibrate in the KCl-treated thylakoids.

Key Words: Phosphorylation; localized energy coupling; delocalized energy coupling; proton gradients.

Introduction

The sensitivity of the ATP formation onset lag to permeable buffers (a lag extension is caused by the extra buffering groups present in the thylakoid lumen), and even more so the effects of, say, pyridine on post-illumination phosphorylation (Nelson *et al.*, 1971; Avron, 1972; Vinkler *et al.*, 1980), are accepted as valid criteria for the presence of a transmembrane, delocalized proton gradient as the driving force for ATP formation. Thus, it is accepted that transmembrane delocalized gradients do drive ATP formation in the situations mentioned. The question is whether there *ever* are localized proton gradients as the energy coupling mode. If the ATP onset lag could be shown to not respond to added permeable buffers, then a localized proton would be indicated. Ten years ago conflicting reports were published concerning whether thylakoids showed localized coupling responses; Ort *et al.* (1976) showed no buffer effects on the lag, while Vinkler *et al.* (1980) and Davenport and McCarty (1980) reported lag extensions consistent with delocalized coupling. Naturally, questions arose as to whether the experimental protocols used to demonstrate the localized coupling (the more stringent and demanding hypothesis to test) were adequate to clearly distinguish the two cases. The accompanying two papers (Beard and Dilley, 1988; Beard *et al.*, 1988) present a broader experimental base for testing for localized or delocalized coupling responses. Single-turnover flash excitation with the luciferin-luciferase ATP detection system provides new tools to detect permeable buffer effects on phosphorylation energization.

   We have demonstrated earlier (Beard and Dilley, 1986a,b) that thylakoids with low salt concentrations in the resuspension buffer had properties

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[3]The abbreviations used are: Chl, chlorophyll; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; mOsm, milliosmolar; PIP^+, the post-illumination phosphorylation observed after a series of phosphorylating flashes; Tricine, N-tris(hydroxylmethyl)-methylglycine; $\Delta\psi$, transmembrane electrical potential difference.

indicative of localized proton gradient coupling; i.e., there were no pyridine-dependent extensions of the onset lag for ATP formation or pyridine effects on the post-illumination ATP yield. However, when those thylakoids were energized in the absence of ADP and P_i (i.e., basal, noncoupled conditions wherein the greatest proton accumulation is expected), addition of ADP and P_i subsequent to the energization period resulted in pyridine effects suggesting a component of bulk-phase delocalized protonic energy coupling (Beard *et al.*, 1988) as expected and as has been observed before (Ort *et al.*, 1976; Vinkler *et al.*, 1980). Thus, the assay used is fully competent to detect delocalized, bulk-phase proton gradient coupling.

Addition of high concentrations of KCl in the thylakoid resuspension buffer induced a sensitivity of the onset lag for ATP formation and post-illumination phosphorylation toward the permeable buffer pyridine (Beard and Dilley, 1986a,b) consistent with there being, in that case, a delocalized proton gradient. These results indicate that the conflicting reports in the literature concerning energy coupling may have been largely due to the different preparative techniques, assay conditions, and protocols employed.

This report provides further documentation that the KCl effect on the ATP onset lag and post-illumination phosphorylation were reasonably interpreted and that the data were not arising because of unforeseen artifacts possibly due to different membrane ionic conductivities, H^+/e^- ratios, or electron transport capacities introduced by the KCl treatment.

Materials and Methods

Sucrose- or KCl-Based Thylakoid Storage Media

Chloroplast thylakoids were isolated from growth chamber-grown spinach, and the chlorophyll content was determined as described previously (Ort and Izawa, 1973). The thylakoids were washed once in and resuspended in 200 mM sucrose, 5 mM Hepes (pH 7.5), 2 mM $MgCl_2$, and 0.5 mg bovine serum albumin/ml, or in a resuspension buffer where the sucrose was replaced with 100 mM KCl.

Flash Excitation, ATP Assay, Electron Transport Assay

Single-turnover flashes were used to initiate electron transfer, and luciferin-luciferase luminescence was used to follow ATP formation and post-illumination phosphorylation as described before (Beard and Dilley, 1988). Single-turnover flashes (10 μs width at half-peak height) were delivered at a rate of 5 Hz by a xenon lamp (EG&G FX-200).

Flash-induced electron transfer was determined by following the reduction of methyl purple (Graan and Ort, 1984) at 591 nm in the same reaction

cuvette as that used to measure luciferin luminescence. The measuring beam and actinic light were focused on a flexible, bifurcated light guide which was connected to the top of the 1-ml reaction chamber. The measuring beam exited through the glass bottom of the cuvette onto a light guide connected to an EMI 9558Q photomultiplier tube protected with a Corning 9782 filter. The signal was amplified with a Tektronix 5A22N differential amplifier and stored in a Nicolet 1072 signal averager. The signal was calibrated by the addition of $10–50\,\mu l$ of reaction buffer to change the concentration of methyl purple over a range of $0–0.5\,\mu M$. An equal fluid volume was then withdrawn so as to keep the path length constant. A plot of the change in signal vs. the change in concentration yielded a straight line (correlation > 0.99). The initial concentration of methyl purple was usually $15\,\mu M$ as determined by the absorption at 591 nm (pH 8) using a molar absorptivity of $74 \times 10^3\,M^{-1}cm^{-1}$ (Graan and Ort, 1984). Methyl purple was synthesized according to Graan et al. (1985).

Proton Transport in Flashing Light

Proton uptake under flashing light was determined by following the absorption change of cresol red at 575 nm. The measurement was performed in the same reaction chamber as used to follow methyl purple reduction. Thylakoids, $20–25\,\mu g$ Chl/ml, were suspended in a separate, stirred reaction vessel at 10°C in a reaction medium consisting of 0.25 mM (low buffer) or 5 mM Tricine (high buffer), 50 mM sorbitol, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 15 mM KCl, 10 mM methylviologen, 400 nM valinomycin, 0.1 mM ADP, 1500 units/ml hexokinase, 10 mM glucose, and $30\,\mu M$ cresol red. The pH was adjusted to 8.00 ± 0.05 with dilute KOH. An aliquot was transferred to the reaction chamber where it was energized with a train of flashes followed by addition of 5 nmol H^+ through a syringe needle port to calibrate the signal. Additionally, another aliquot, which was not flashed, was also calibrated with 5 nmol H^+. These calibrations yielded similar results. To improve the signal-to-noise ratio, four independent samples were flashed and summed. To get the signal resulting from pH changes only, the signal resulting from samples flashed in the presence of high Tricine was subtracted from the signal resulting from samples flashed in low Tricine.

515 nm Electrochromic Band Shift—$\Delta\psi$ Measurement

The electrochromic absorption change at 515 nm was followed in the same experimental setup as used to follow methyl purple reduction. The assay was performed under basal ($-$ ADP) conditions in the absence of valinomycin. To improve the signal, the signal from eight flashes fired at 0.1 Hz were summed.

Thylakoid Volume and Pyridine Content

Entry of [^{14}C]pyridine into the lumen space was determined by centrifugal filtration through silicon oil as described by Flores et al. (1983). Uptake was initiated by thylakoid addition (75 μg Chl/ml) to the radioactive reaction mixture. The reaction mixtures consisted of 10 mM sorbitol, 50 mM Tricine (pH 8.0), 3 mM MgCl$_2$, 1 mM KH$_2$PO$_4$, 5 mM pyridine, 400 nM valinomycin, 10 μM DCMU, and 0.5 μCi/ml of [^{14}C]pyridine or 0.5 μCi/ml [^{14}C]inulin or 2 μCi/ml [^{3}H]H$_2$O. Aliquots (0.1 ml) were removed from the suspensions at intervals and placed in 0.4-ml microfuge tubes above a 0.1-ml layer of silicone oil [Versilube F-50 and SF-96(50); 2/1 (vol./vol.], which in turn was layered over 20 μl of 10% perchloric acid in 0.4 M NaCl. Uptake was stopped by centrifuging the thylakoids in a Beckman microfuge for 35 s which pelleted more than 90% of the thylakoids. This was performed at 10°C. Chlorophyll recovery was determined by centrifugation of duplicate samples into 0.02 ml of 10% sucrose, 3% Triton X-100, and resuspension of the bottom phase in 80% acetone. The internal concentration of the [^{14}C]pyridine was calculated from the percentage of added label appearing in the bottom phase, the total volume of water entering the bottom phase, which was measured with [^{3}H]H$_2$O, and the calculated lumen volume. The latter was calculated as the difference between the [^{14}C]inulin permeable space and the [^{3}H]H$_2$O permeable space.

[^{14}C]pyridine was purchased from Amersham. Thin-layer chromatography with an acetone solvent system (Damani et al., 1978) indicated a radiochemical purity > 80%. [Carboxyl-^{14}C]inulin and [^{3}H]H$_2$O were purchased from ICN.

Results

KCl Effect on Flash Titration of PIP ATP Yield

Flash titrations of the sensitivity of PIP$^+$ toward the permeable buffer pyridine (pK_a = 5.4 at 10°C) for thylakoids stored in KCl-containing buffer are presented in Figs. 1 and 2 for pH 8 and 7, respectively. The pyridine effect on PIP$^+$ ATP yield for these thylakoids was very different than for thylakoids stored in sucrose-containing buffer [Figs. 4 and 5 of the previous paper, reproduced as Fig. 1B and 2B for ease of comparison (Beard et al., 1988)]. In fact, Figs. 1A and 2A are more similar to the PIP$^-$ flash titration shown in Figs. 2 and 3 of the previous paper, and reproduced as Fig. 1C and Fig. 2C. In the previous paper, we pointed out that the proton gradient energizing the PIP$^-$ ATP yield follows the predicted pattern for a *delocalized* gradient, which interacts with added pyridine in an understandable way. Obviously, the KCl-treated thylakoids show PIP$^+$ flash titrations most reasonably understood as indicative of a bulk-phase delocalized proton gradient
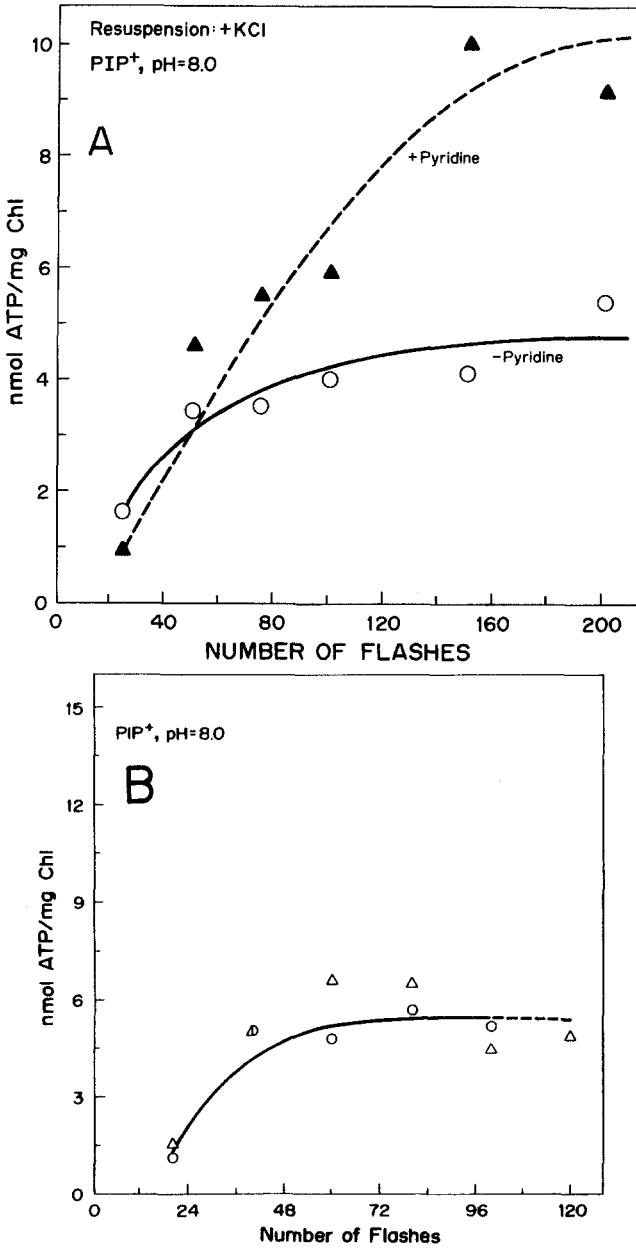
Fig. 1. A. Flash titration of the PIP$^+$ ATP yield at pH 8 for KCl-isolated thylakoids in the absence or presence of pyridine. Conditions are as in Table I. The reaction mixture was pH adjusted at 10°C with or without 5 mM pyridine. (O) Control; (▲) +pyridine. (B and C) Figures are from the preceding paper in this series (Beard *et al.*, 1988). (B) Fig. 5; (C) Fig. 2; both (B) and (C) were from experiments with sucrose-isolated thylakoids.
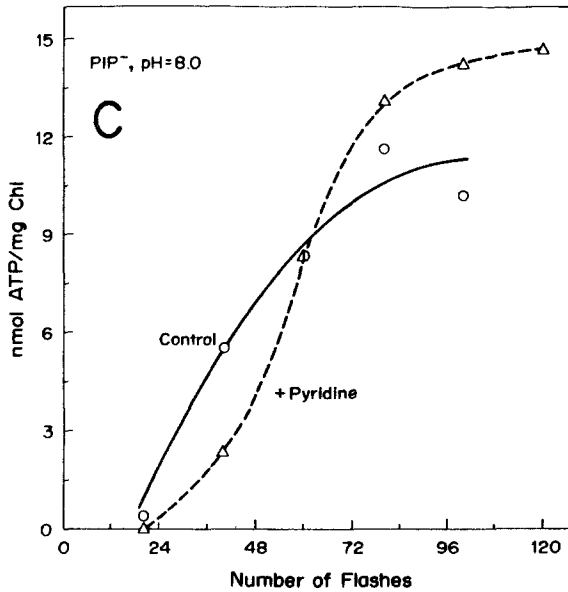
Fig. 1. Continued.

serving as the intermediate between electron transfer-mediated proton accumulation and the ATP synthetases. This is in contrast to the data for PIP$^+$ ATP yield in sucrose-treated thylakoids which suggested a localized proton coupling pattern described in the foregoing paper (Beard et al., 1988).

NaCl Can Replace KCl

Thylakoids isolated in the presence of 100 mM NaCl in place of the KCl also showed the delocalized energy coupling pattern (Table I), although to a somewhat lesser extent.

KCl Concentration in the ATP Assay Medium

An important question to address is why the sensitivity of the PIP$^+$ ATP yield toward pyridine was not observed if thylakoids were isolated in the absence of KCl (Beard and Dilley, 1986a,b; Beard et al., 1988). The flash titrations for the two cases were presented earlier (Beard et al., 1988), and the data were interpreted as consistent with a localized mode of energy coupling in thylakoids stored in a sucrose-based media. However, could there be subtle effects of the KCl isolation responsible for the data interpreted as indicating a delocalized energy coupling? One such possibility is that a different electric field component develops in the flash train of the sucrose-treated membranes compared to the KCl-treated membranes.
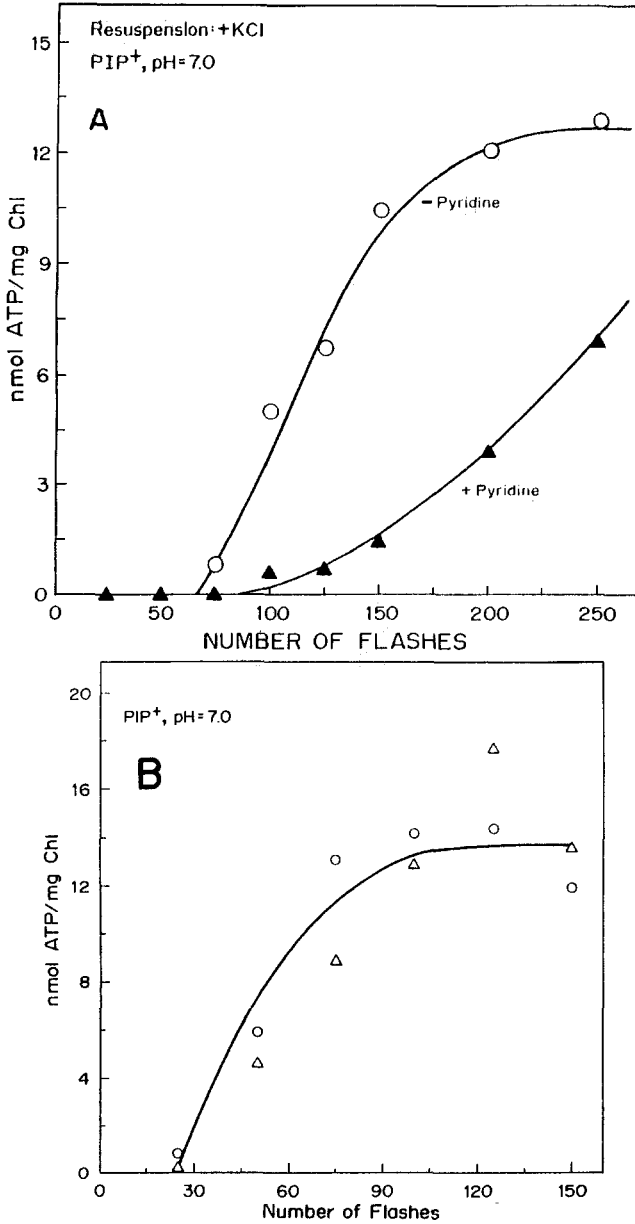
Fig. 2. A. Flash titration of the PIP$^+$ ATP yield at pH 7 for KCl-isolated thylakoids in the absence or presence of pyridine. Conditions are as in Table I except that 50 mM MOPS-KOH replaced Tricine. The reaction mixture was pH adjusted at 10°C with or without 5 mM pyridine. (O) Control; (▲) +pyridine. (B and C) Figures are from the preceeding paper in this series (Beard *et al.*, 1988). (B) Fig. 7; (C) Fig. 3; both (B) and (C) were from experiments with sucrose-isolated thylakoids.
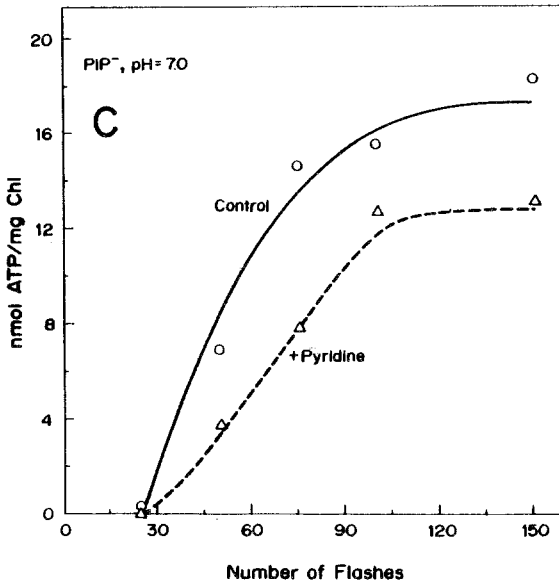
Fig. 2. Continued

Table I. Effect of NaCl Resuspension and Pyridine on the Onset of ATP Formation and Post-Illumination ATP Formation[a]

Pyridine	Onset$_A$/Onset$_E$	Yield/flash	Flash yield	PIP$^+$
−	15/28	0.70	51	6.4
	($\pm 1/\pm 1$)	(± 0.02)	(± 1)	(± 0.6)
+	20/40	0.63	41	8.2
	($\pm 1/\pm 2$)	(± 0.06)	(± 2)	(± 0.4)

[a]Thylakoids were isolated as described under Materials and Methods, except that 100 mM NaCl replaced the sucrose in the resuspension buffer. Thylakoids were then assayed for pyridine-dependent increases of the onset of ATP formation and post-illumination phosphorylation. Thylakoids were excited with 100 flashes delivered at a rate of 5 Hz, and the ATP level was monitored by luciferin-luciferase luminescence. The reaction mixture included 50 mM Tricine-KOH (pH 8.0), 10 mM sorbitol, 3 mM MgCl$_2$, 1 mM KH$_2$PO$_4$, 0.1 mM methylviologen, 0.1 mM ADP, 5 mM DTT, 400 nm valinomycin, and 10 μM diadenosine pentaphosphate. The flash lag was determined by the first detectable rise in luciferin luminescence (actual onset = O$_A$) or by the back-extrapolation of the steady rise in the flash-induced luminescence increase to a horizontal line drawn at the base of the nonphosphorylating flashes (extrapoled lag = O$_E$). Values given are the means ± S.E. (parentheses) of at least five observations. The ATP yield/flash was determined from the linear rise in luciferin luminescence. The flash yield and PIP$^+$ are expressed as nmol ATP/mg Chl, while the yield/flash is expressed as nmol ATP/(mg Chl flash).

Monitoring the electrochromic absorption change (515–540 nm) demon-strated that a significant electrical potential *did not* develop during the early number of flashes, or after a group of flashes where a significant PIP was observed in the presence of valinomycin (see Fig. 4 of the accompanying paper, Beard and Dilley, 1988). Table II shows that this was verified by the lack of an effect that doubling the K^+ concentration in the *assay buffer* had on the ATP formation onset lag or the PIP ATP yield. It should be noted that thylakoids isolated in the presence or absence of KCl were always assayed under identical osmotic and ionic conditions. The carryover of KCl with KCl-isolated thylakoids accounts for less than a 0.5 mM increase in the K^+ concentration in the reaction buffer, and the thylakoids ($\pm$ KCl-isolated) were incubated with valinomycin 3 min in the reaction buffer before the flash train was initiated. Therefore, it is unlikely that different reservoirs of internal K^+ can account for the contrasting results observed with the two thylakoid preparations.

Decay Kinetics of the PIP+ Signals

It is known from earlier studies that the energized state responsible for driving PIP decays with the apparent first-order kinetics of the H^+ gradient relaxation (Hind and Jagendorf, 1963; Izawa, 1970; Hangarter and Good, 1984). Recent advances in understanding the effects that buffers trapped in the lumen may have on the apparent rate constant of proton efflux predict that increasing the amount of a trapped buffer (endogenous or exogenous) with a pK near the lumen pH developed in the energized state should slow down the proton efflux kinetics (Schonfeld and Kopeliovitch, 1985;

Table II. Effect of an Additional 15 mM K^+ in the Assay Buffer and Pyridine on the Onset of ATP Formation and Post-Illumination Phosphorylation[a]

K^+ concentration	Pyridine	Onset$_A$/Onset$_E$	Yield/flash	Flash yield	PIP+
15 mM	—	23/30	0.54	39	4.1
30 mM	—	21/29	0.48	34	4.2
15 mM	+	24/36	0.50	32	4.4
30 mM	+	23/33	0.47	33	4.4

[a]Thylakoids were isolated in a resuspension buffer with 200 mM sucrose and assayed for pyridine-dependent increases of the onset of ATP formation and post-illumination phosphorylation in an assay buffer which included 15 mM K^+ or in an assay buffer with an additional 15 mM $KHCO_3$. Luciferin-luciferase was used to follow the ATP formed from 100 flashes delivered at 5 Hz. The specific reaction conditions and the determination of the onset parameters (Onset$_A$/Onset$_E$) are described in Table I. Values given are the means of two determinations. The flash yield and PIP+ are expressed as nmol ATP/mg Chl, while the yield/flash is expressed as nmol ATP/(mg Chl flash).

Whitmarsh, 1987). We will use this analysis to test for the hypothesized delocalizing effect of the KCl treatment.

The PIP$^+$ signal measured with our technique decayed exponentially with an apparent $t_{1/2}$ which was dependent on external pH, composition of the thylakoid resuspension buffer, and the presence or absence of pyridine. Figure 3A demonstrates that pyridine slows the phosphorylating proton flux for the KCl-treated thylakoids when the external pH was 8, consistent with the pyridine increasing the buffering capacity of the internal bulk aqueous phase (Schonfeld and Kopeliovitch, 1985; Whitmarsh, 1987). However, at an external pH of 7, pyridine had no effect on the decay kinetics of PIP$^+$ (Fig. 3B). The severe ($\approx 75\%$) inhibition of the PIP$^+$ yield after 175 flashes at pH 7 (Fig. 2A) indicates that pyridine must have exerted so much buffering that the pH in the lumen was reaching the pH 4.7 energetic threshold in only a small proportion of the thylakoids. Both of those observations can be understood if most of the protons which were buffered by pyridine in the pH 7 cases were not energetically competent to drive ATP formation, apparently because most of the buffering occurred at pH values above pH 4.7, the energetic threshold. That is, at pH 4.7, more than 80% of the pyridine buffering capacity has already been used, so that a relatively small amount of protonated pyridine is available at pH values less than 4.7. In the absence of pyridine, the apparent $t_{1/2}$ values for the decay of PIP$^+$ are 0.8 and 3.9 s at pH 8 and 7, respectively (Fig. 3). The apparent $t_{1/2}$ values for thylakoids isolated in the absence of KCl only increased by 400 ms (0.8 to 1.2 s) as the external pH was lowered 1 pH unit (8 to 7) (Fig. 4), indicating that the increase in the apparent $t_{1/2}$ with KCl-isolated thylakoids cannot be accounted for solely by a possible change in the $t_{1/2}$ of the luciferase reaction upon lowering the external pH. Figure 4 also indicates that pyridine had no effect on the PIP kinetics when thylakoids were isolated in the absence of KCl. Therefore, the much slower kinetics of PIP at pH 7 and the pyridine effects on PIP kinetics at pH 8, with thylakoids isolated in the presence of KCl, compared to the sucrose-treated case, are most reasonably understood as due to the different origins (bulk phase compared to membrane localized) of the protons contributing to the proton flux driving phosphorylation.

Pyridine Equilibration

[^{14}C]Pyridine uptake was measured in both types of thylakoid preparations, to assess the possibility that the low salt-treated thylakoids may have in some way excluded pyridine from the lumen relative to the KCl-isolated membranes, and thus would show less effect of pyridine on the onset lag length and PIP$^+$ yield. Recall that the assay media were always identical for both types of thylakoids except for the slight amount of KCl (0.5 mM

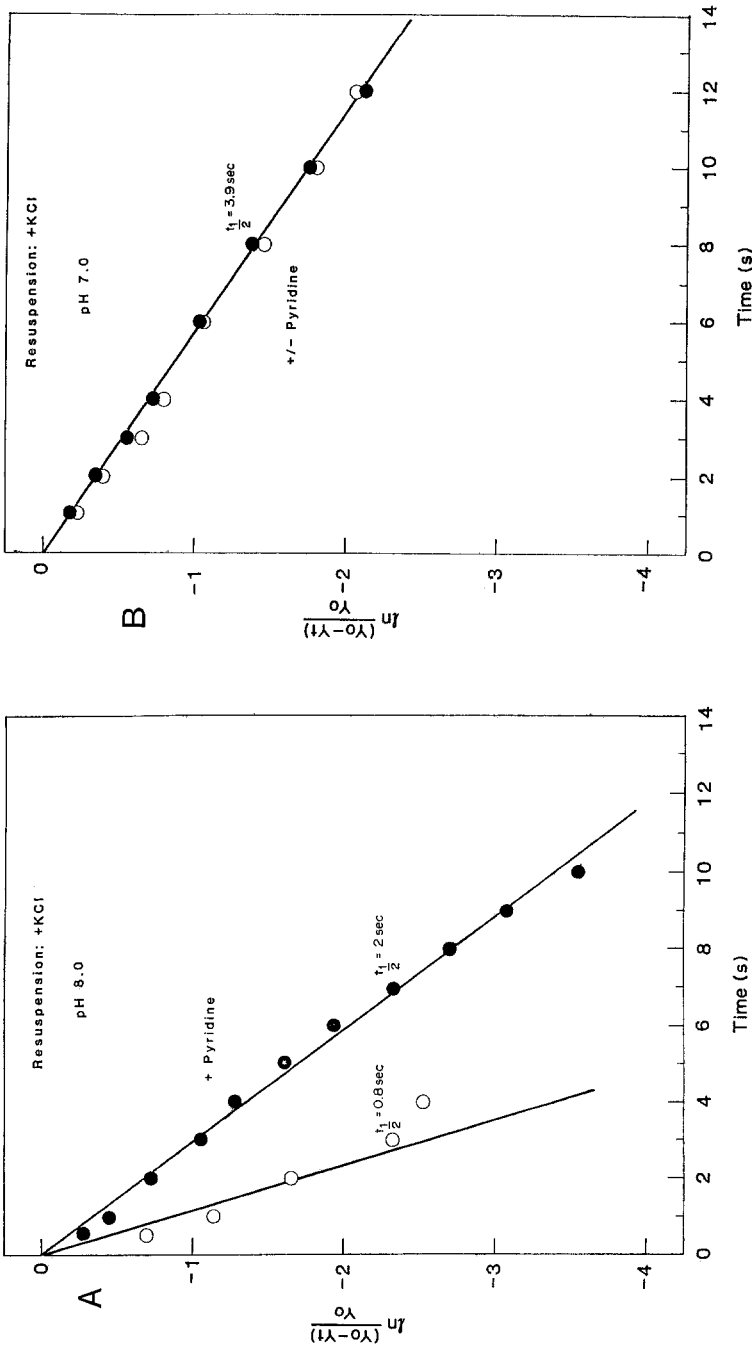**Fig. 3.** The effect of pyridine on the apparent decay of PIP$^+$ with KCl-isolated thylakoids. Y_o refers to the maximum ATP yield and Y_t refers to the ATP yield at time t after the last flash. The time course of PIP$^+$ resulting from 125 flashes at pH 8, ±pyridine (A) and 175 flashes at pH 7, ±pyridine (B) is presented. The apparent rate constants and ($t_{1/2}$, s) are: (A) 0.87/s (0.8 s), − pyridine; 0.35/s (2.0 s), + pyridine; (B) 0.18/s (3.9 s), − pyridine; 0.18/s (3.9 s), + pyridine. (O) Control; (●) − pyridine; (◐) + pyridine.
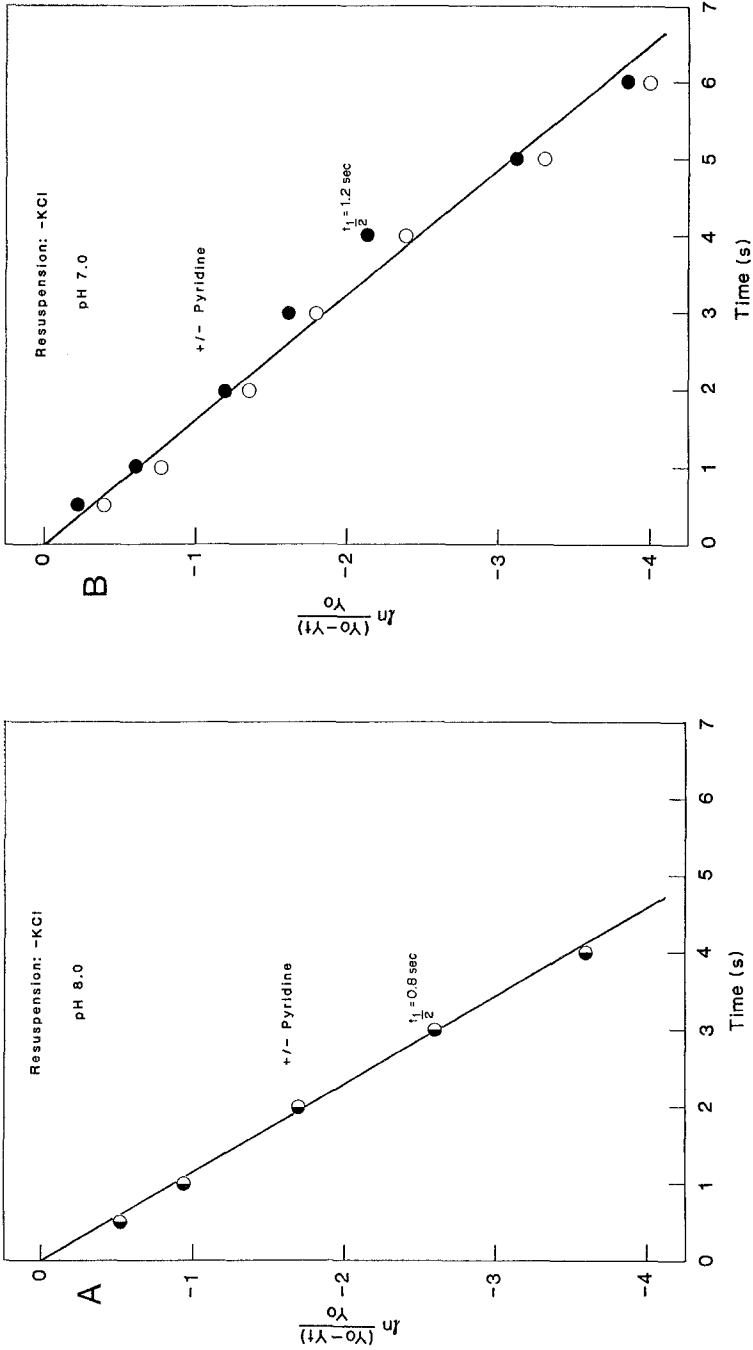
Fig. 4. The effect of pyridine on the apparent decay of PIP^+ with sucrose-isolated thylakoids. Y_o refers to the maximum ATP yield and Y_t refers to the ATP yield at time t after the last flash. The time course of PIP^+ resulting from 100 flashes at pH 8, $\pm$ pyridine (A) and 125 flashes at pH 7, $\pm$ pyridine (B) are presented. The apparent rate constants and ($t_{1/2}$, s) are: (A) 0.87/s (0.8 s) and (B) 0.58/s (1.2 s) $\pm$ pyridine.
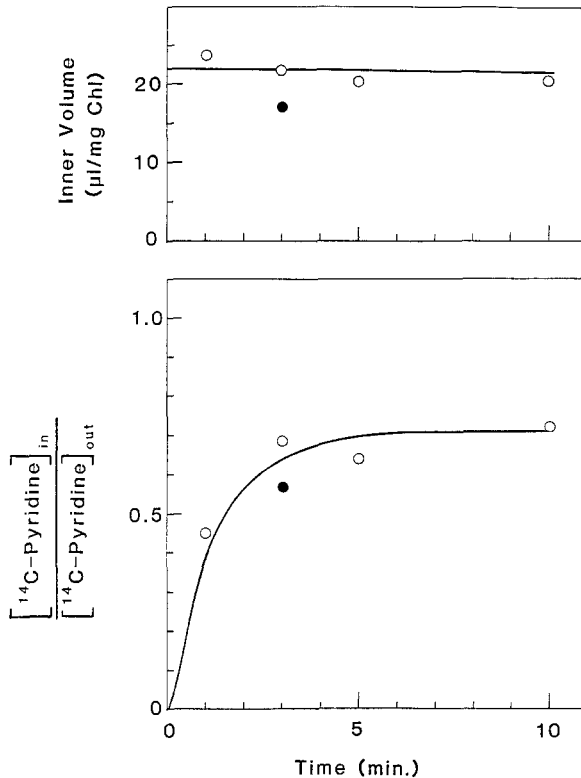
Fig. 5. The intrathylakoid volume and uptake of [^{14}C]pyridine for thylakoids isolated in the absence and presence of KCl. The apparent $t_{1/2}$ for the radioactive label was about 45 s. Conditions are described under Materials and Methods. (O) Sucrose-isolated thylakoids; (●) KCl-isolated thylakoids.

contribution) accompanying the dilution of the KCl in the stock suspension. We have already demonstrated that pyridine can increase the number of protons taken up under a train of flashes with thylakoids isolated in the *absence* of KCl, indicating that pyridine was present in the lumen phase (Beard *et al.*, 1988). Figure 5 indicates that pyridine equilibrates across the thylakoid membrane of the low salt-treated sample with a half time of about 45 sec. Thus, at 3 min, the standard incubation time for the phosphorylation experiments, pyridine equilibration would be reached for the low-salt case, the one of most concern vis-a-vis pyridine being adequately equilibrated. Experiments with high KCl-treated thylakoids showed a similar time course (data not shown). The single 3-min point for the high-KCl case (solid circle in Fig. 5) indicates the similarity to the response at that time. The composition of the assay medium results in an osmotic strength of about

70 mOsm and the volumes reported here agree with those reported earlier under similar osmotic conditions (Ort *et al.*, 1976). The lack of total equilibration of the pyridine label may have been due to an impermeable impurity resulting from radiation decay (see Discussion). In any case, 100 mM KCl-treated thylakoids exhibited a slightly lower equilibration of label at 3 min than did the sucrose-treated thylakoids. Therefore, the greater ATP onset lag sensitivity of KCl-isolated thylakoids toward pyridine cannot be accounted for by a greater concentration of pyridine present at the onset of the flash train.

Possible Uncoupling Effects, Electron Transport Measurements

Hangarter and Ort (1985) have shown that reducing the number of electrons transferred per flash, by attenuating the intensity of the flashes or with the electron transport inhibitor DCMU, resulted in an increase in the number of flashes required to accumulate enough protons to reach the energetic threshold. Similarly, uncouplers at concentrations which did not effect steady-state photophosphorylation significantly extended the lag for the onset of ATP formation (Ort, 1978). To determine if the longer onset lags observed for ATP formation with KCl-isolated thylakoids were due to a decrease in electron transfer, the number of electrons transferred per 30 flashes was determined by following the reduction of methyl purple, a photosystem I acceptor. Figure 6 illustrates that the same number of electrons were transferred in a sequence of 30 flashes independent of the composition of the stock resuspension buffer ($\pm$ KCl).

The possibility of different ionic conductivities of the thylakoids prepared in the two resuspension buffers was tested by measuring the decay of the electrochromic absorption change at 515 nm under basal conditions ($-$ADP) in the absence of valinomycin. Figure 7 indicates that thylakoids isolated in the absence or presence of KCl have similar absorption change decay kinetics. Therefore, an inherent uncoupling or a reduction in the number of electrons transferred per flash cannot account for the greater number of flashes required to reach the energetic threshold for KCl-isolated thylakoids.

H^+/e^- Ratios

The longer lag for the onset of ATP formation with KCl-isolated thylakoids could be due to a lower H^+/e^- ratio as compared to thylakoids isolated in the absence of KCl. The number of protons taken up per flash in the external medium is a test for this possibility and was examined by following the absorption change of cresol red at 575 nm. Figure 8 illustrates typical cresol red signals resulting either from 29 flashes fired at 5 Hz for
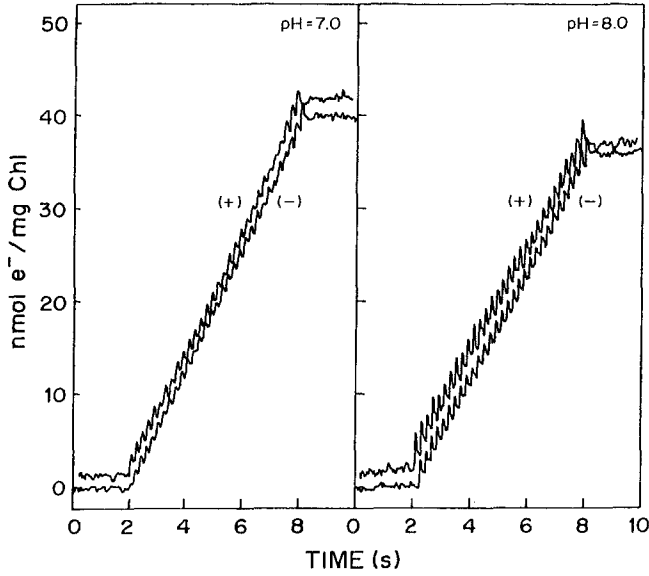
**Fig. 6.** The number of electrons transferred per flash as monitored by the $\Delta A_{591\,nm}$ of methyl purple for thylakoids isolated in the absence (−) and presence (+) of KCl. Conditions are as outlined in Materials and Methods.
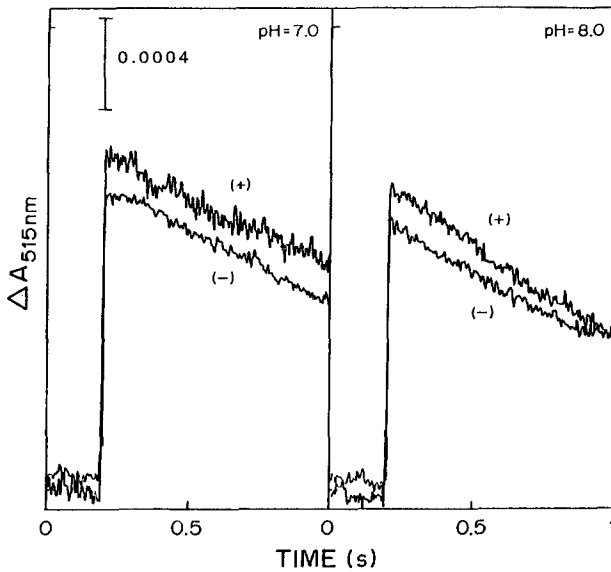
Fig. 7. The electrochromic absorption change at 515 nm for thylakoids isolated in the absence (−) and presence (+) of KCl. Conditions are described under Materials and Methods.
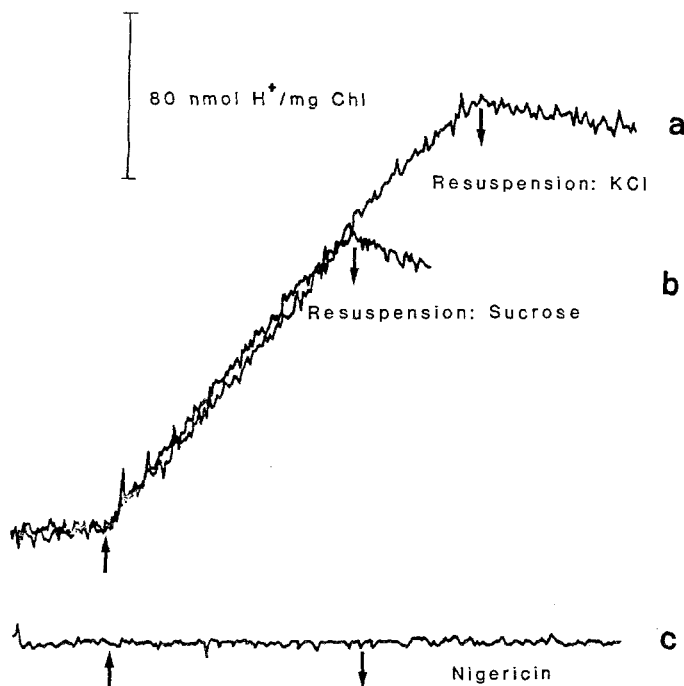
Fig. 8. Cresol red absorption changes at 575 nm as a monitor of proton uptake for thylakoids isolated in the absence and presence of KCl. Conditions are described under Materials and Methods. Thylakoid samples were given a sequence of flashes which was sufficient to overcome the energetic threshold to initiate ATP formation as determined by examining the onset of ATP formation with the aid of luciferin luminescence. KCl-isolated thylakoids required 49 flashes to initiate ATP formation, trace a; sucrose-isolated thylakoids required 29 flashes to overcome the energetic threshold, trace b; $10\,\mu M$ nigericin completely eliminates any net H^+ uptake as monitored by cresol red (note that the time scale is expanded 2 times with trace c).

thylakoids isolated in a resuspension buffer lacking KCl (Fig. 8b) or 49 flashes for thylakoids isolated in the presence of KCl (Fig. 8a). The pH-indicating signal was derived by subtracting the signal observed in the presence of a high concentration of Tricine from that obtained with a low concentration. Addition of $10\,\mu M$ nigericin completely quenched any absorption change (Fig. 8c). Figure 8 clearly demonstrates that thylakoids isolated with and without KCl take up the same number of protons per flash. Since the number of electrons transferred to methyl purple per flash was also identical for the two thylakoid preparations (Fig. 6), the H^+/e^- ratios were the same.

Energization Threshold Proton Buffering Pools

Given the results of the above control experiments, we are left with the best explanation for the KCl treatment increasing the ATP onset lag as due

to an increase in the proton buffering during the energization threshold. Hangarter and Ort (1985, 1986a) have demonstrated that this threshold buffering pool was about 60–80 nmol H^+/mg Chl for thylakoids which were isolated in a resuspension buffer lacking KCl and was the same whether PSI or PSII alone, or both photosystems, were used to drive proton accumulation. When both types of thylakoids ($\pm$ KCl) were isolated in parallel, we always observed a greater lag for the onset of ATP formation with KCl-isolated thylakoids than for sucrose-isolated thylakoids. However, the magnitude of the lag was dependent on the illumination history of the spinach leaves from which the thylakoids were isolated (Table III). Spinach leaves that were harvested when the growth chamber lights were on yielded thylakoids having lags significantly shorter than when thylakoids were isolated from spinach leaves that were dark-adapted at least 3 hours before thylakoid isolation (Table III). After a thylakoid preparation, the lag for the onset of ATP formation was determined so as to determine the number of flashes required to overcome the energetic threshold. The size of the threshold proton accumulation was then determined in a separate reaction mixture by following proton uptake (with cresol red) given by that number of flashes. The number of electrons transferred was also determined for each thylakoid preparation, allowing the calculation of the H^+/e^- ratio. In all cases, KCl-isolated thylakoids exhibited a longer lag for the onset of ATP formation as

Table III. Effect of Leaf Dark Adaptation and KCl Thylakoid Isolation on the Onset of ATP Formation, H^+/e^- Ratio, and the Size of the Threshold Buffering Pool at pH 8.0[a]

Leaf treatment	Thylakoid isolation	Onset for ATP formation (number of flashes)	H^+/e^-	Size of threshold buffering pool (nmol H^+/mg Chl)
Illuminated	− KCl	13 ± 2	3.0 ± 0.5	48 ± 7
	+ KCl	28 ± 1	3.3 ± 0.5	108 ± 3
Dark adapted	− KCl	29 ± 1	3.0 ± 0.5	128 ± 28
	+ KCl	47 ± 3	3.3 (*)	199 (*)

[a]The reaction conditions are described in Table I and under Materials and Methods. Spinach leaves, under illumination, were harvested from a growth chamber, and the thylakoids were isolated immediately or dark-adapted at least 3 hours before isolation. Thylakoids were resuspended in a resuspension buffer with 100 mM KCl or where the KCl was replaced with 200 mM sucrose. The proton uptake was determined with cresol red as a monitor for external pH changes, and the number of electrons transferred per flash was determined by following $\Delta A_{591\,nm}$ with methyl purple as a photosystem I acceptor. The number of flashes to initiate ATP formation was determined by following the increase in luciferin-luciferase luminescence as before, except that the flash intensity was diminished by a factor of 2 with a bifurcated light guide which was used in the cresol red and methyl purple measurements. The flash rate was 5 Hz. The values are the average of at least three independent observations, except in one case, which was based on one determination (*). The standard errors are shown. The size of the threshold buffering pool was determined by monitoring the number of protons taken up by the threshold number of flashes determined in the ATP formation assay.

compared to sucrose-isolated thylakoids, independent of the illumination history of the spinach leaves. The H^+/e^- ratios were always measured to be around 3, consistent with an additional H^+ being accumulated by the cytochrome b_6/f complex, possibly through the operation of a proposed Q-cycle or redox-linked proton pump (Cramer *et al.*, 1987). Ort and colleagues (Graan and Ort, 1981; Hangarter and Ort, 1986) have also observed an H^+/e^- ratio near 3, with flash excitation.

Reversal of the KCl Effect

The proton gradient delocalizing effect of the KCl treatment was reversed by simply washing away the high-salt resuspension buffer (Table IV). Parallel thylakoid preparations ($\pm$ KCl in the resuspension buffer) were assayed as before (Beard and Dilley, 1986a) for the pyridine-dependent extension of the

Table IV. Reversal of the KCl-Induced Bulk Phase Delocalization of the Protonmotive Force[a]

	Thylakoid isolation	Pyridine	$Onset_A/Onset_E$	Yield/flash	Flash yield	PIP^+
A.	$-$ KCl	$-$	18/26	0.56	43	4.0
			($\pm 1/\pm 1$)	(± 0.02)	(± 3)	(± 0.4)
		$+$	20/31	0.54	38	4.0
			($\pm 1/\pm 1$)	(± 0.03)	(± 1)	(± 0.3)
B.	$+$ KCl	$-$	28/41	0.46	42	3.5
			($\pm 1/\pm 2$)	(± 0.01)	(± 5)	(± 0.4)
		$+$	41/69	0.39	23	8.2
			($\pm 2/\pm 3$)	(± 0.01)	(± 1)	(± 0.2)
Thyalkoids from A and B Washed and resuspended in a sucrose resuspension buffer.						
A′.	$-$ KCl	$-$	19/26	0.43	33	2.5
			($\pm 1/\pm 1$)	(± 0.1)	(± 1)	(± 0.2)
		$+$	21/33	0.39	27	2.7
			($\pm 2/\pm 4$)	(± 0.04)	(± 2)	(± 0.2)
B′.	$+$ KCl	$-$	20/29	0.45	44	2.7
			($\pm 1/\pm 1$)	(± 0.02)	(± 1)	(± 0.3)
		$+$	22/35	0.42	39	3.1
			($\pm 2/\pm 3$)	(± 0.01)	(± 1)	(± 0.2)

[a]Thylakoids were isolated in a resuspension buffer with 200 mM sucrose (A) or where the sucrose was replaced 100 mM KCl (B). Thylakoids were then assayed for pyridine-dependent increases of the onset of ATP formation and post-illumination phosphorylation. Luciferin-luciferase was used to follow the ATP formed from 100 flashes delivered at 5 Hz. The A and B thylakoid suspensions were diluted with the sucrose-buffer media, centrifuged, and resuspended in the sucrose-buffer media. The thylakoids were then assayed for pyridine-dependent effects as above. The specific reaction conditions and the determination of the onset parameters ($Onset_A/Onset_E$) are described in Table I. Values given are the means $\pm$ S.E. of at least three observations. The flash yield and PIP^+ are expressed as nmol ATP/mg Chl, while the yield/flash is expressed as nmol ATP/(mg Chl flash).

ATP formation onset lag and the pyridine stimulation of PIP$^+$. As before, thylakoids stored in sucrose-based media were insensitive to the presence of pyridine while thylakoids stored in the presence of 100 mM KCl exhibited pyridine-dependent effects on both the ATP formation onset lag and PIP$^+$ (Table IV). Both thylakoid preparations were then diluted in a resuspension buffer with sucrose as the osmoticum to about 100 μg Chl/ml, and centrifuged 4 min at 2500 × g and resuspended in 1 ml of the *sucrose*-containing buffer. Assaying the thylakoids for the effect of pyridine on the ATP formation onset lag and PIP$^+$ ATP yield indicated that *both* thylakoid preparations were returned to the pyridine-*insensitive* mode. The high KCl-induced shift toward delocalized energy coupling had been returned to a behavior consistent with localized energy coupling.

Discussion

Hypothesis: The KCl Effect Induces an Obligatorily Delocalized Proton Gradient

Because the permeable buffer (pyridine, hydroxyethylmorpholine, and 4-picoline all gave similar effects with the low and high salt-treated thylakoids) stimulation of post-illumination phosphorylation in the PIP$^-$ or "traditional" protocol so convincingly shows that delocalized proton gradients in the lumen can drive ATP formation, the data of Figs. 1 and 2 of this work support the hypothesis that the KCl-treated thylakoids drive ATP formation also via a delocalized protonmotive force, even in the PIP$^+$ protocol. It is particularly important to point out that measuring the effects of pyridine on the PIP$^+$ ATP yield and the ATP onset lag at both pH 7 and 8 (Beard and Dilley, 1986b, 1986a, respectively) gives a measure of confidence that the buffering effects in the high KCl-treated thylakoids are sensibly attributed to the buffer in respect to its pK (5.44) and the need for a 2.3 ΔpH to develop to reach the energetic threshold for ATP formation. That is, starting at pH 8, pyridine stimulates the PIP$^+$ ATP yield (Fig. 1), but at pH 7 (with the limited number of flashes used here), pyridine inhibits the PIP$^+$ yield (Fig. 2). Pyridine exerts most of its buffering action between pH 6 and 4.8; and in these experiments, depending only on the ΔpH part of the protonmotive force, for pH 7 outside, the pH on the acid side of the CF$_0$–CF$_1$ must be ≤ 4.7 to attain the energetic threshold. Therefore, it is expected that in the pH 7 case pyridine will have a strong retarding effect on the pH dropping below 4.7, and hence most of the protons will be buffered by pyridine (≈ 84% of the pyridine buffering occurs above pH 4.7) above pH 4.7. This explains why the + pyridine curve for PIP$^+$ yield in Fig. 2A is

so much depressed below the control curve. Most of the protons dissociating from the pyridine in the post-illumination phase will do so *above* pH 4.7, and they would not be at a sufficiently energetic level to drive ATP formation. Even at 250 flashes at 5 Hz the $+$ pyridine curve has not reached the control PIP^+ yield value, although it is rising steeply, indicating that with more flashes (or certainly in steady illumination) the PIP^+ yield in that case would exceed the control, as it does in the pH 8 PIP^+ experiments (Fig. 1A). These are consistent, predictable effects of permeable buffers, also discussed in the previous paper in this series.

The pyridine effects on PIP^+ for KCl-treated thylakoids closely resemble the PIP^- data for sucrose-treated thylakoids, and the point is clear (cf. the previous paper in this series, Beard *et al.*, 1988) that delocalized proton gradients drive at least part of the PIP^- ATP formation. By the same criteria, however, the PIP^+ protocol used with sucrose-containing rather than KCl-containing thylakoid storage buffer clearly *did not* utilize a delocalized protonmotive force for driving ATP formation. The results suggest the existence of two compartments, the lumen and a membrane-localized domain, both of which can be the source of the proton gradient that drives ATP formation. For reasons that are not yet understood, thylakoids kept in sucrose rather than KCl-containing buffer maintain the energy coupling proton fluxes in domains not accessible to added pyridine buffer. It is unclear how the KCl storage treatment causes an apparent switching of the H^+ fluxes *obligatorily* into the lumen. (See Note Added in Proof.)

The effects of the permeable buffer reported here are not unique to pyridine. Hydroxyethylmorpholine (pK 6.3) showed a pattern very similar to that reported for pyridine when used with sucrose- or KCl-treated thylakoids (Theg and Dilley, 1986). 4-Picoline (pK 6.1) at 1 mM was compared to 5 mM pyridine in an experiment with low salt- and high salt-treated thylakoids, measuring the ATP onset lag, PIP^+ ATP yields, and the ATP yield per flash. By all three parameters the 4-picoline gave responses essentially identical to the pyridine (data not shown). Therefore, the effects are due to general permeable buffer properties, not attributable to unique interactions of pyridine with thylakoid membranes. In selecting an appropriate permeable buffer for such experiments, it is critical to use only those which exert little or no uncoupling action in the concentration range used. Imidazole and tris, for instance, are not good choices because of their uncoupling activity, even at 3 and 20 mM, respectively (Ort *et al.*, 1976).

Checks for Possible Trivial Explanations for the KCl vs. Sucrose Treatment Differences

Because of the uncertainties in explaining the events, it is important to consider what other types of evidence may support or disprove the

hypothesis; i.e., could there be trivial explanations for the different responses to pyridine buffering?

Kinetics. First, it should be pointed out that energy-linked proton efflux from two compartments is also suggested by the kinetics of the PIP^+ signal (Figs. 3A and 4A). At pH 8, pyridine decreased the apparent first-order rate constant of the PIP^+ yield in the KCl-treated thylakoids, but had no effect on the rate constants in the sucrose-treated case. This is most simply explained as due to lumenal pyridine, in the former case, interacting with the protons which energized the PIP^+, and slowing the proton efflux and consequently the PIP kinetics. This argument rests on the analysis of Schonfeld and Kopeliovitch (1985) and Whitmarsh (1987), who have shown that a change in the lumenal buffer content will change the rate constant of proton efflux. The sucrose-treated thylakoids, having the same lumen volume and pyridine content as the KCl-treated sample (Fig. 5), did not show a pyridine effect on the PIP^+ kinetics (Fig. 4A). This is consistent with the notion that the pyridine in that case did not reach the compartment containing the proton gradient which drove the PIP^+. The similar kinetics ($\pm$ pyridine) of PIP^+ for pH 7.0 in both types of thylakoids, and for pH 8 with the sucrose-treated thylakoids, argues against pyridine deleteriously affecting the membranes, acting as an uncoupler, or interacting with the CF_0-CF_1 in a non-specific manner. This is also indicated by the fact that pyridine ultimately stimulates PIP^+ or PIP^-. Some permeable buffers—3 mM imidazole for example—are moderately active as uncouplers (Ort *et al.*, 1976) and they are not well suited for the present purposes. Pyridine, however, was shown to have no uncoupling action at 5 mM (Table III of Ort *et al.*, 1976).

The endogenous buffering groups that influence the PIP^+ yield (probably carboxyl groups with pKs in the range of 4.7–5.2) are likewise affected by KCl treatment in a way consistent with the treatment exposing more buffering power to the accumulated protons. The nearly threefold decrease in the rate constant of PIP^+ decay for pH 7 conditions compared to pH 8 (Figs. 3B and 4B), caused by the KCl treatment, is the expected effect of increasing the concentration of available buffering groups (Whitmarsh, 1987). The simplest interpretation of that effect, and consistent with the data on the pyridine effects, is that protons have access to carboxyl groups in the lumen phase after the KCl treatment.

Relative Pyridine Contents. A trivial argument for the lack of a pyridine effect on ATP onset lags or PIP^+ with sucrose-treated thylakoids, based on there being less lumenal pyridine in that case, cannot be valid, as it was shown (Fig. 5) that the lumen volume and the pyridine content were essentially identical for the two types of thylakoids. The lack of complete equilibration was probably due to the insoluble impurities in the pyridine resulting from radioactive decay. TLC indicated that more than 80% of the

cpm migrated with an R_f expected of pyridine (Damani *et al.*, 1978). At pH 7 or 8, nearly all of the pyridine should be unprotonated and therefore permeable to the thylakoid membrane (Nelson *et al.*, 1971). Additional support indicating that pyridine was permeable to thylakoids stored in the sucrose-based media was the observation that pyridine increased the accumulation of protons into the lumen, and increased the PIP$^-$ ATP yield, as expected (Beard *et al.*, 1988).

Possible Differences in Electrical Potential. The question can be raised whether the longer ATP onset lags in KCl-treated thylakoids could be caused by a more complete suppression of a $\Delta\psi$ component—and hence a requirement for more flashes to give a greater ΔpH component—due to more K$^+$ ions available to the valinomycin and more rapid suppression of the $\Delta\psi$. The data indicate that this cannot be the explanation, for the following reasons:

1. The assays were done in media with 50 mM Tricine KOH (giving about 15 mM K$^+$). The amount of K$^+$ contributed by the KCl-treated thylakoids to the reaction media was about 0.5 mM. Moreover, *doubling* the [K$^+$] in the reaction media by adding 15 mM KHCO$_3$ had no effect on the onset lag or the PIP$^+$ yield (Table II).

2. The 515 nm electrochromic absorption change was completely suppressed in the sucrose-treated thylakoids (Fig. 4 of the first paper of this series, Beard and Dilley, 1988), using the standard conditions of the phosphorylation assays.

Uncoupling or Electron Transport Inhibition. An alternative explanation for the increased lag for the onset of ATP formation with KCl-stored compared with sucrose-stored thylakoids is a decreased H$^+$ accumulation per flash with KCl-isolated thylakoids. This could occur due to an inhibition of electron transport (Hangarter and Ort, 1985), an uncoupling (Ort, 1978), or a change in the H$^+$/e$^-$ ratio. However, Fig. 6 indicates that KCl treatment had no effect on the number of electrons transferred to methyl purple per flash. Uncoupling was not a problem, because Fig. 7 shows that both thylakoid preparations had the same rate of decay of the electrochromic absorption change (a measure of the ionic conductivity of the thylakoid membrane). The lack of a KCl-induced uncoupling was also suggested by the observation that the same number of protons was taken up per flash for the two thylakoid preparations, as monitored by the alkalization of the external suspending phase measured with cresol red (Fig. 8 and Table III). Therefore the H$^+$/e$^-$ ratios were the same for both thylakoid preparations.

Proton Buffering Domains at the Energetic Threshold

The longer lags for the onset of ATP formation and the pyridine sensitivity for the KCl-treated thylakoids are most reasonably explained as due to

additional buffering groups becoming available for proton interaction, lead-
ing to a larger proton accumulation prior to reaching the threshold ener-
gization. The size of the threshold buffering domain was dependent on the
illumination history of the spinach from which the thylakoids were isolated
(Table III). This is consistent with the activation of the CF_1 owing to the
thioredoxin system reducing a critical disulfide bridge in the γ subunit of CF_1
(Mills and Mitchell, 1982; Nalin and McCarty, 1984). The diminished size of
the threshold buffering domain may therefore result from an ATPase-
induced creation of a Δp or a diminished energetic requirement to activate the
CF_1 complex (Hangarter et al., 1986; Mills and Mitchell, 1982). In either
case, parallel thylakoid preparations ($\pm$ KCl) indicated that although the size
of the threshold buffering domain was dependent on the illumination history
of the leaves from which the thylakoids were isolated, KCl-stored thylakoids
always had a larger threshold buffering domain, by 60–70 nmol H^+/mg Chl,
than thylakoids isolated in the absence of KCl (Table III). Hangarter and Ort
(1985, 1986a), using thylakoids similar to our sucrose-treated case, have
estimated the size of the threshold buffering pool to be about 60–80 nmol
H^+/mg Chl, independent of the portion of the electron transport chain used
to drive H^+ accumulation (external pH about 8.4 at 4°C). Although not
measured directly, Ort and colleagues (Hangarter et al., 1986; Hangarter and
Ort, 1986) have indicated that the size of the threshold buffering pool was
diminished, when the CF_1 complex was reduced with a DTT preactivation,
because the number of flashes required to reach the energetic threshold was
diminished.

Although variable, a slow dark ATP hydrolysis (< 100 nmol ATP/mg
Chl h) was sometimes observed with thylakoids isolated from leaves which
were harvested under illumination. Other groups have shown that ATPase-
dependent proton pumping can decrease the ATP onset lag (Galmiche and
Girault, 1982; Schreiber and Del Valle-Tascon, 1983). Therefore, if some
ATP hydrolysis occurred in the thylakoids prepared from illuminated leaves,
we cannot conclude definitively that the diminished onset lag observed with
CF_1-activated thylakoids was solely because the energetic requirement for
CF_1 activation was lowered with thylakoids isolated from illuminated leaves.

Table IV illustrates that the KCl-induced shift from a localized to a
bulk-phase delocalized energy coupling was reversible. By simply washing the
KCl-treated thylakoids in a sucrose-resuspension buffer, the KCl-induced
delocalization of energy coupling was reversed (i.e., the KCl-treated, sucrose-
washed thylakoids did not exhibit pyridine-dependent increases in the lag for
the onset of ATP formation or the PIP^+ ATP yield). This may indicate that
protein conformations which were perturbed during storage of thylakoids in
the presence of high concentrations of KCl and allowed H^+ equilibration
with the lumen, reverted back to their native state upon washing the KCl

away. The reversibility of the phenomena indicates that the availability of the lumen phase as a part of the pathway for proton processing from the redox centers to the coupling factor complexes may be regulated. A switch or gating device to regulate proton distribution between a localized phase and the lumen, possibly sensitive to K^+ concentration, was already suggested by Sigalat *et al.* (1985) and Horner and Moudrianakis (1986)

Concluding Remarks

The physiological significance of regulating the participation of delocalized or localized proton processing pathways could be to avoid overacidification of the lumen during extended periods of light exposure. As discussed in more detail by Dilley *et al.* (1987), thylakoids can respond to energization by uptake of salts and water, leading to extensive swelling (Packer *et al.*, 1965; Nishida *et al.*, 1966). The swelling response does not always occur, for reasons that are not clear (Dilley and Deamer, 1971), but a regulated switch for partitioning of protons between a localized path and the lumen may be involved. This leads directly to the question of H^+ : cation exchange proteins possibly being involved in these phenomena. The easily-used assay system reported here for detecting localized or delocalized proton gradient coupling, and the experimental protocols for switching the thylakoids from one mode to the other, provide convenient tools for further study of these and related questions.

Note Added in Proof

Recent results (G. Chiang and R. A. Dilley, 1987, *Biochemistry*, **26**, 4911–4916) suggest that Ca^{++} ions on the lumen side of the thylakoid membrane are involved in gating the H^+ fluxes between localized (Ca^{++} in place, perhaps binding to special carboxyl groups) and delocalized gradients (Ca^{++} displaced, for example by K^+ or H^+ interaction with the putative gate carboxyl groups).

Acknowledgments

References

Avron, M. (1971). *In Proc. IInd Intl. Cong. on Photosynthesis* (Forti, G., Avron, M., and Melandri, A., eds.), Dr 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. (1988). *J. Bioenerg. Biomembr.* **20**, 85–106.

Beard, W. A., Chiang, G., and Dilley, R. A. (1988). *J. Bioenerg. Biomembr.* **20**, 107–128.

Cramer, W. A., Widger, W. R., Black, M. T., and Girvin, M. E. (1987). In *Electron Transfer Mechanisms and Oxygen Evolution* (Barber, J., ed.), Elsevier, Amsterdam, in press.

Daemani, L. A., Patterson, L. H., and Garrod, J. W. (1978). *J. Chromatogr.* **155**, 337–348.

Davenport, J. W., and McCarty, R. E. (1980). *Biochim. Biophys. Acta* **589**, 353–357.

Dilley, R. A., and Deamer, D. (1971). *Bioenergetics* **2**, 33–38.

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

Flores, S., Graan, T., and Ort, D. R. (1983). *Photobiochem. Photobiophys.* **6**, 293–304.

Galmiche, J. M., and Girault, G. (1982). *FEBS Lett.* **146**, 123–128.

Graan, T., and Ort, D. R. (1981). *Biochim. Biophys. Acta* **637**, 447–456.

Graan, T., and Ort, D. R. (1984). *J. Biol. Chem.* **259**, 14003–14010.

Graan, T., Flores, S., and Ort, D. R. (1981). In *Energy Coupling in Photosynthesis* (Selman, B., and Selman-Reimer, S., eds.), Elsevier North-Holland, Amsterdam, pp. 25–34.

Graan, T., Ort, D. R., and Prince, R. C. (1985). *Anal. Biochem.* **144**, 193–198.

Hangarter, R., and Good, N. E. (1984). *Biochemistry* **23**, 122–130.

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

Hangarter, R., and Ort, D. R. (1986). *Eur. J. Biochem.* **158**, 7–12.

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, 205–212.

Hind, G., and Jagendorf, A. T. (1963). *Proc. Nat'l. Acad. Sci. (USA)* **49**, 715–722.

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

Izawa, S. (1970). *Biochim. Biophys. Acta* **223**, 165–173.

Izawa, S., and Hind, G. (1967). *Biochim. Biophys. Acta* **143**, 377–390.

Mills, J. D., and Mitchell, P. (1982). *FEBS Lett.* **144**, 63–67.

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.

Nishida, K., Tamai, N., and Ryogama, K. (1966). *Plant Cell Physiol.* **7**, 415–428.

Ort, D. R. (1978). *Eur. J. Biochem.* **85**, 479–485.

Ort, D. R., and Izawa, S. (1973). *Plant Physiol.* **52**, 595–600.

Ort, D. R., Dilley, R. A., and Good, N. E. (1976). *Biochim. Biophys. Acta* **449**, 108–124.

Packer, L., Siegenthaler, P. A., and Nobel, P. S. (1965). *Biochem. Biophys. Res. Commun.* **18**, 474–477.

Schonfeld, M., and Kopeliovitch, B. S. (1985). *FEBS Lett.* **193**, 79–82.

Schreiber, U., and Del Valle-Tascon, S. (1983). *FEBS Lett.* **150**, 32–37.

Sigalat, C., Haraux, F., de Kouchkovsky, F., Hung, S. P. N., and de Kouchkovsky, Y. (1985). *Biochim. Biophys. Acta* **809**, 403–413.

Theg, S. M., and Dilley, R. A. (1986). In *Proc. VIIth Intl. Congress on Photosynthesis* (Biggins, J., ed.), Martinus Nijhoff/Dr. Junk Publishers, The Hague, Vol. 3, pp. 161–164.

Vinkler, C., Avron, M., and Boyer, P. D. (1980). *J. Biol. Chem.* **255**, 2263–2266.

Whitmarsh, C. J. (1987). *Photosynthesis Res.*, in press.