Biochimica et Biophysica Acta, 1059 (1991) 16–27

ADONIS 000527289100175O

BBABIO 43425

Evidence that localized energy coupling in thylakoids can continue beyond the energetic threshold onset into steady illumination

M. Renganathan, R.S. Pan, R.G. Ewy, S.M. Theg *, F.C.T. Allnutt ** and R.A. Dilley

Department of Biological Sciences, Purdue University, West Lafayette, IN (U.S.A.)

(Received 22 February 1991)

Key words: Energy coupling; Proton gradient, localized, delocalized; (Thylakoid)

Energy transduction from proton gradients into ATP formation in chloroplast thylakoids has been hypothesized to be driven equally efficiently by localized domain $\Delta\tilde{\mu}_{H^+}$ or by a delocalized $\Delta\tilde{\mu}_{H^+}$ (Beard, W. A. and Dilley, R. A. (1988) J. Bioenerg. Biomembr. 20, 129–154). An important question is whether the apparent localized protonmotive force energy coupling mode can be observed only in the dark-to-light transient in the flash excitation protocol commonly used, or whether the localized energy coupling gradient can be maintained under conditions of continuous illumination ATP formation. The assay in the previous work was to use permeable amines, added to thylakoids in the dark, and observe the effect of the amine on the length of the energization lag (number of single-turnover flashes) required to initiate ATP formation in the dark-to-light transition. Amine buffers delayed the ATP onset in high-salt-stored membranes but did not delay the onset with low salt-stored membranes. This work tested whether permeable amines show the different effects in low- or high-salt-stored thylakoids which had attained a steady-state ATP formation rate (in continuous light) for 20–40 s prior to adding the amine. Hydroxyethylmorpholine was the preferred amine for such experiments, a suitable choice inasmuch as it behaves similarly to pyridine in the flash-induced ATP formation onset experiments, but it permeates more rapidly than pyridine and it has a higher pK_a, which enhances its buffering effects. With high-salt-stored thylakoids, 0.5 or 1.0 mM hydroxyethylmorpholine added after 40 s of continuous illumination caused a marked, but transient, slowing of the ATP formation rate, but little or no slowing of the rate was observed with low-salt-stored thylakoids (at similar phosphorylation rates for the two thylakoid samples). Those data indicate that in continuous illumination conditions the proton gradient driving ATP formation in thylakoids from the low-salt-stored treatment did not equilibrate with the lumen, but in thylakoids stored in high-salt the $\Delta\tilde{\mu}_{H^+}$ freely equilibrated with the lumen. That suggestion was supported by measurement of the luminal pH under coupling conditions by the [^{14}C]methylamine distribution method using low- or high-salt-stored thylakoids. Further supportive evidence was obtained from measuring the effect of permeable amine buffers on H^+ uptake under coupled and basal conditions with both types of thylakoid. Lumen volumes and uptake kinetics of [^{14}C]pyridine were shown to be essentially identical for low-salt- and high-salt-stored thylakoid preparations, indicating that the lack of permeable buffer effects observed with the low-salt-stored membranes under coupling conditions cannot be attributed to a lesser permeability of the amine across the low-salt-stored thylakoids, nor to a smaller lumen volume. Electron transport rates were essentially the same for both thylakoid types, and no differences were found in the decay of the 515 nm electrochromic shift. The data are consistent with the concept that thylakoids can maintain localized energy coupling responses in steady illumination

* Current address: Department of Botany, University of California, Davis, CA, U.S.A.
** Current address: Martek Corp., Columbia, MD, U.S.A.

Abbreviations: Chl, chlorophyll; HEM, hydroxyethylmorpholine; HS, LS, high-salt-stored and low-salt-stored chloroplasts, respectively; MA, methylamine; Pyr, pyridine; Val, valinomycin.

Correspondence: R.A. Dilley, Department of Biological Sciences, Lilly Hall of Life Sciences, Purdue University, West Lafayette, IN 47908, U.S.A.

conditions just as in the flash-induced ATP formation onset protocol. Thylakoids treated by storing in high-salt conditions show delocalized energy coupling in both the dark-to-light transient and in the steady illumination conditions.

Introduction

Energy coupling in cellular organelles and bacteria utilizes the redox-generated protonmotive force to drive ATP formation [1]. One of the important, unanswered questions concerning the mechanisms involved in $\Delta\tilde{\mu}_{H^+}$-dependent coupling has to do with localized versus delocalized proton gradients, with various reports giving support for one or the other coupling mode [1,2]. Although arguments against localized $\Delta\tilde{\mu}_{H^+}$ coupling continue to be raised [3,4], independent work in several laboratories strongly supports the notion that the initial light-induced energization of thylakoids can occur via localized H^+ gradients [5–9], based on the failure of added permeable buffers to delay the onset of ATP formation. Horner and Moudrianakis [7,8] suggested that, while a localized $\Delta\tilde{\mu}_{H^+}$ clearly seemed to be responsible for the initial energization, after several hundred milliseconds a delocalized $\Delta\tilde{\mu}_{H^+}$ may come into play. This raises the important question of whether the localized $\Delta\tilde{\mu}_{H^+}$ energy coupling can ever be maintained in longer time frames, or whether the intrinsic mechanisms for proton processing obligatorily lead to proton flux into the lumen. This question is drawn into sharper focus by our recent results showing that by treating thylakoids with certain high K^+ or Na^+ salt regimes [9–11] or by disturbing the Ca^{2+} ions normally associated with thylakoids [11,12] the permeable amine approach can also clearly detect – in the onset of energization – delocalized gradient energization [9,13,14]. Thus, we can ask whether the localized coupling response is a special case limited to only the early periods of energization.

On the one side of the issue are studies with uncouplers, employing steady-state conditions (usually less than 2 min of illumination) which gave evidence for localized coupling [15–17]. However, still other approaches have yielded a large body of evidence clearly supporting delocalized energy coupling (Refs. 3, 13, 14, 18, and see review, Ref. 1, for other references). Clearly there is a need for further work testing for the occurrence of localized $\Delta\tilde{\mu}_{H^+}$ coupling in continuous illumination. To this end, we have here adapted the permeable buffer approach, previously used with considerable success in the flash energization studies, to steady-state phosphorylation experiments so as to compare the results with the transient (flash) energization method and the other steady-state methods. We thought this would be a particularly incisive approach when utilized with thylakoids stored under the low-salt and high-salt regimes which, in the flash excitation experiments, so clearly showed localized or delocalized energy coupling modes, respectively.

Materials and Methods

Thylakoid isolation

Thylakoids were isolated from 14–21-day-old peas and stored in either low- or high-salt conditions as described previously [9]. The osmotic strength of the suspension media was maintained by 200 mM sucrose (low-salt storage) or 100 mM KCl (high-salt storage) in the low- and high-salt-stored samples, respectively, in addition to 5 mM Hepes-KOH (pH 7.5), 3 mM $MgCl_2$ and 0.1% defatted bovine serum albumin. This difference persisted only in the storage buffer, both types of sample were subsequently transferred to and assayed in identical media for each experiment with a chlorophyll concentration of 20 μg ml^{-1} or less. Chlorophyll concentration was always greater than 2 mg ml^{-1} in the storage, thus the maximum KCl carried over into the assay buffer was 1 mM or less.

ATP formation

Luciferin-luciferase method. Phosphorylation under steady illumination was assayed with the luciferin-luciferase method as in Refs. 9, 10 in a 0.8 ml volume stirred, water-jacketed thermostated (10 °C) cuvette. The medium contained: 50 mM Tricine-KOH (or NaOH when nonactin was used to collapse the $\Delta\Psi$) pH 8.0 or 50 mM Mops-KOH (pH 7.0 or 7.5, see figure legends), 3 mM $MgCl_2$, 1 mM KH_2PO_4, 0.1 mM methylviologen, 0.1 mM ADP, 5 μM diadenosine pentaphosphate (to inhibit adenylate kinase activity), 10 μl luciferin-luciferase (LKB ATP monitoring kit, see Ref. 9), 200 nM valinomycin and HEM or pyridine as indicated in table or figure legends. Chlorophyll, as isolated thylakoids, was kept very low (0.8 to 2 μg, as indicated in the figure legends) to avoid excessive ATP formation during the 1 min light period, which would desensitize the luciferase. The light source was a 500 W projection bulb delivered to the cuvette through one or two Corning red CS-2-64 filters and focused on a light guide giving 240 mW cm^{-2} for the maximum intensity at the cuvette using two red filters.

Flash-induced ATP formation was assayed as described previously [9,19] using a medium similar to that mentioned above except that the chlorophyll content was 20 μg ml^{-1}.

[^{32}P]P_i method. Phosphorylation rates for some experiments were determined by the [^{32}P]P_i incorporation method [20]. The assay conditions were as above,

with 50 mM Tricine-KOH (pH 8.0) as the pH control buffer, but without the luciferin-luciferase materials or the diadenosine pentaphosphate.

H^+ uptake

The H^+ electrode method was used with an Orion combination pH electrode and a Corning Model 12 pH meter. The medium consisted of 100 mM sorbitol, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 0.1 mM ADP (or no ADP for basal conditions), 0.1 mM methylviologen, 200 nM valinomycin, hexokinase at 15 units ml^{-1}, 10 mM glucose (see below), and thylakoids equivalent to 40 μg Chl ml^{-1}. Illumination was with saturating intensity of $CuSO_4$ filtered white light (Fig. 6) or for Fig. 3, red (Corning 2-64) light at saturation or at reduced-intensity (240 mW cm^{-2}, close to that given to the thylakoids in the phosphorylation assay using luciferin-luciferase). Temperature was 10 °C. Hexokinase and glucose were included as an ADP-regenerating system, permitting the recording of pH changes caused by the redox-driven H^+ accumulation without interference from pH increases that accompany net ATP formation.

Electrochromic absorbance changes and electron transport

Electric field-indicating absorption changes at 515 nm were measured as before [19]. Rates of electron transport were determined spectrophotometrically using methyl purple as the electron acceptor [21]. All experiments were performed at 10 °C in the same instrument [9], thereby maintaining the same cuvette geometry for the different types of measurement, i.e., luminescence and absorption.

[^{14}C]Pyridine uptake

Pyridine uptake into low- or high-salt-stored thylakoids was determined using the silicone oil centrifugation technique employed previously [10,22] and as described in the legend of Fig. 2. [^{3}H]H_2O was used to estimate the total water (internal or lumen water and externally trapped fluid) and [^{14}C]inulin was used to measure the externally trapped fluid.

Transmembrane ΔpH measurement

ΔpH was determined by the distribution of methylamine (MA) using the silicone oil centrifugation method [22] (at 10 °C) using 30 μM [^{14}C]MA and 1 min exposure to heat-filtered white light in a medium with 50 mM Tricine-KOH (pH 8.0) 10 mM sorbitol, 20 μg Chl ml^{-1}, 5 mM P_i, 0.5 mM ADP, 3 mM $MgCl_2$, 200 nM valinomycin and 0.1 mM methylviologen.

Results and Discussion

Rationale for the experimental approach

We used a variation of the permeable buffer protocol to examine whether lumen protons always drive ATP synthesis in continuous light, after 20–40 s of illumination have established a steady-state condition. We reasoned that if a permeable buffer, added during steady-state illumination, could cross the thylakoid membrane quickly enough, it would transiently lower the steady-state ΔpH existing between the lumen and the external medium. If photophosphorylation was being driven by this delocalized transmembrane ΔpH, then ATP synthesis should slow down upon amine entry into the lumen in response to the drop in transmembrane ΔpH. If, on the other hand, the protonmotive force was derived from a localized domain proton gradient which did not equilibrate with the lumen, then the rate of ATP formation should not be affected by the addition of the buffer. It is particularly relevant to compare the response to buffer addition in the low and high salt-stored thylakoids which show, by the ATP formation onset criterion we have used [9], either the localized or delocalized coupling mode, respectively. It is important to note that, as mentioned in the Materials and Methods section, all experiments with low- and high-salt-stored chloroplasts were performed in the identical phosphorylation media, and that the stated salt conditions applied only to the media used to store the samples after isolation.

Hydroxyethylmorpholine as a permeable buffer

An appropriate buffer to use for the steady-state experiments reported here should have two properties, one being that it duplicate the thoroughly studied effects of pyridine on the ATP formation onset lag [5] in flash excitation in low and high salt-stored thylakoids [9,10] (i.e., giving localized or delocalized responses, respectively), and the other being that it cross the membrane rapidly enough to provide a significant pulse of neutral amine buffer to the lumen. Pyridine has been the principal permeable amine buffer used in the studies of the ATP formation onset lags [5,7,9,14]. Its advantages are that at 5 mM it does not cause detectable uncoupling [5], whereas imidazole [5,7] and Tris [5] have significant uncoupling activity. However, pyridine is not ideal for the steady-state experiment because, added at 5 mM, it causes a downward shift in the luciferin-luciferase signal, even when added in dark conditions (data not shown). Tricine cannot be used for this experiment owing to its very low permeability ($t_{1/2}$ = 1 h [6]).

Hydroxyethylmorpholine (HEM) is a more suitable amine buffer for the present purposes because (a) it satisfies the two criteria mentioned above – and it does not shift the luminescence signal the way pyridine does – (b) it has a higher pK_a (6.3) making its buffering action come into play more quickly (relative to the pH 8 starting conditions) for a given amount of buffer, and (c) it seems to permeate membranes faster than pyridine (although we do not have a direct measure of its

TABLE I

Effects of hydroxyethylmorpholine compared with those of pyridine effects on ATP formation onset lags using 5 Hz flash trains with low- and high-salt-stored thylakoids.

Thylakoids were assayed for the ATP formation onset lag as specified in Materials and Methods using 5 Hz flashes and 20 μg Chl ml^{-1}. Either HEM or pyridine or both were added to the reaction mixture 1 min prior to turning on the flash train. The error terms (S.D.) for the onset lags were generally calculated to be ± 1 or 2 or occasionally ± 3. The ATP formation onset lag was measured by two parameters [9], the first number being the flash on which the first detectable ATP formation occurred (16 of 16/24, first line) and the second number the flash number at the intersection of the back-extrapolated line of the rising signal to the baseline (24 of 16/24, first line).

Thylakoid storage	HEM (mM)	Pyr (mM)	ATP formation onset lag (No. of flashes)	ATP yield per flash nmol (mg Chl flash)$^{-1}$
Low-salt	–	–	16/24	0.85
Low-salt	+0.5	–	18/29	0.86
Low-salt	–	+5	16/27	0.85
Low-salt	+0.5	+5	17/30	0.85
High-salt	–	–	26/39	0.76
High-salt	+0.5	–	40/59	0.60
High-salt	–	+5	33/48	0.53
High-salt	+0.5	+5	52/66	0.33

permeability as no radioisotope is available), a useful property for the present experimental approach. Table I shows that 0.5 mM HEM is similar to 5 mM pyridine in having little or no effect on the ATP onset lag parameter in low-salt-stored thylakoids. However, with high-salt-stored thylakoids, 0.5 mM HEM caused about a 14/20 flash lag increase, somewhat larger than the 7/8 flash increase owing to 5 mM pyridine. These results are similar to the previously documented pyridine effects using low- or high-salt-stored thylakoids, interpreted as showing localized or delocalized energy coupling, respectively [9,10]. In the experiment shown in Table I, 1 min incubation with either HEM or pyridine was given, whereas in the past work with pyridine a 3 min incubation was given prior to beginning the flash train. That probably accounts for the slightly smaller effect of pyridine (usually $\approx$ 10/20 flash increases were observed) with the high-salt-stored membranes. The $t_{1/2}$ for pyridine entry is about 1 min (Ref. 10; cf. below). The longer flash lag induced by HEM (in the high-salt-stored thylakoids) could be caused by its faster rate of penetration, as well as owing to its pK_a being 0.8 units higher than that of pyridine. The latter point means that HEM will exert a larger buffering effect than pyridine in the pH range from 6.7 to 5.7, in delaying the drop of the internal pH down to the threshold energization values of near 5.7 [9,19].

Comments on the differential effect of the amines on the ATP yield per flash in the two thylakoid types, reported in Table I, will be made at the end of the next section.

Effects of adding hydroxyethylmorpholine to a steady-state ATP formation system

Following the rationale given above, HEM was added to a thylakoid suspension containing the phosphorylation components and the luciferase-luciferin ATP assay system while the thylakoids were illuminated with continuous light (and attained steady state ATP formation). Fig. 1 shows such an experiment at high and low light intensity (ATP formation rates) and with either low- or high-salt-stored thylakoids. The HEM addition does not, by itself, cause any perturbation in the luminescence signal (whereas pyridine, for reasons not understood, causes a downward shift in the luminescence signal level, data not shown). With low salt-stored thylakoids, adding 1 mM HEM to a steady-state phosphorylating system caused little or no change in the signal (Fig. 1A) or a slight transient decrease in the signal (Fig. 1C). In contrast, adding 1 mM HEM to an assay utilizing high-salt-stored thylakoids caused an easily detected transient drop in the rate of ATP formation at high (Fig. 1B) and even more so at low (Fig. 1D) light intensities. The rate of ATP formation recovered to nearly the pre-HEM addition rate after 6 and 12 s, respectively, for the high- and low-light intensity cases. The rates of steady-state ATP formation prior to adding HEM were determined on at least three separate assays and were observed for the respective conditions to be: 1A, 230 ± 18 μmol ATP (mg Chl hr)$^{-1}$; 1B, 249 ± 15; 1C, 48 ± 6; and 1D, 61 ± 2.

The observed transient slowing of the ATP formation rate after adding HEM to high-salt-stored thylakoids is consistent with the notion that the protonmotive force driving ATP formation was formed between the thylakoid lumen and the external phase, and that the added (neutral) HEM penetrated to the lumen and became protonated, thus decreasing the driving force (ΔpH) on ATP formation. The transient slowing of the ATP formation rate by HEM is more pronounced and requires a longer time for recovery to the new steady-state ATP formation rate at low compared to high light intensity. This effect may be related to the balance between the rate of entry of HEM and the rate of H^+ production by the redox reactions. A parameter to measure this HEM-dependent loss of ATP formation is the displacement (d in the trace D of Fig. 1) between the two steady-state rates, before and after HEM addition, where d is taken close to the time when the rate recovers to the new steady-state value. For the experiment shown in Fig. 1, that parameter, determined from triplicate measurements, (in units of nmol ATP (mg Chl)$^{-1}$) was as follows: A, 78 ± 16; B,

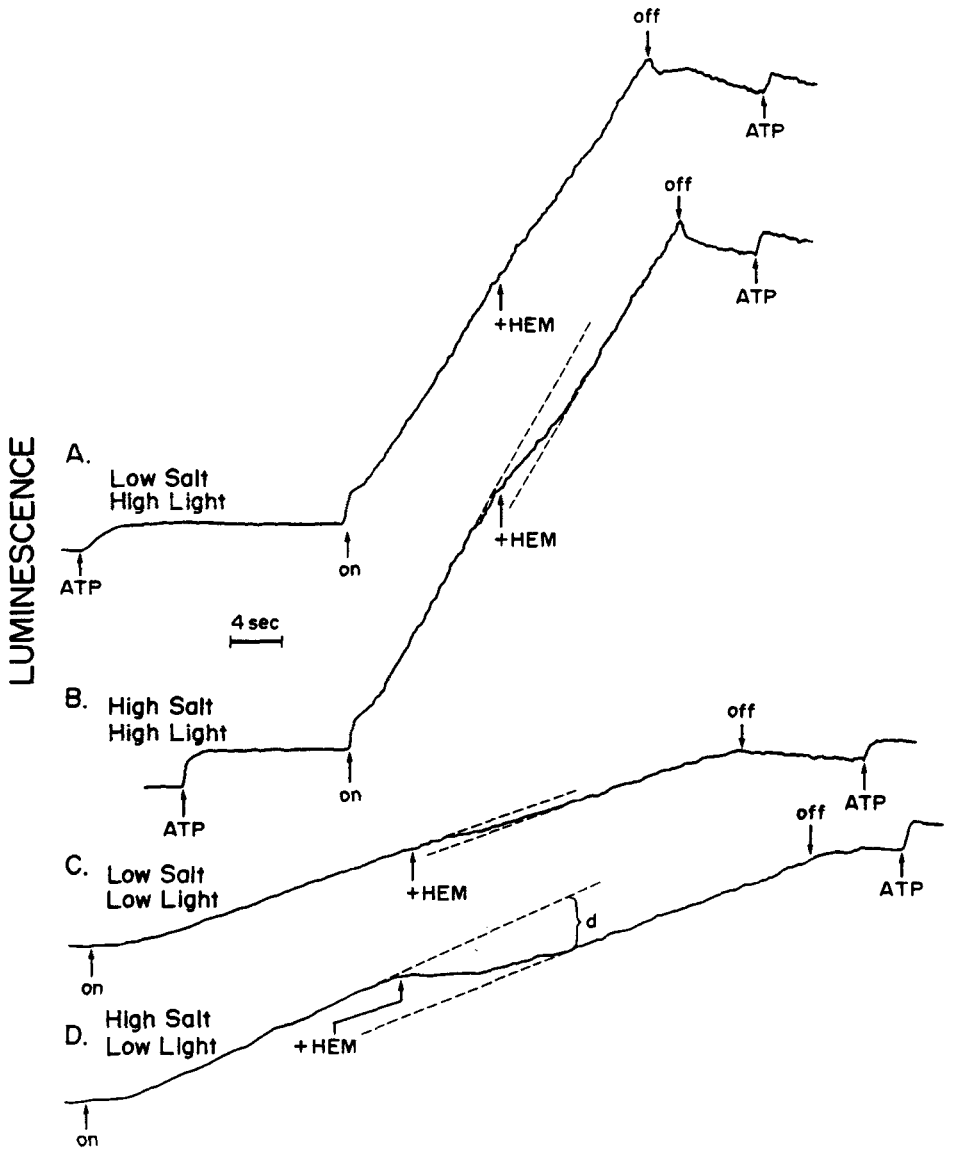

Fig. 1. Effect of adding the permeable amine HEM on ATP formation rates with low- or high-salt-stored thylakoids. Steady-state ATP formation was assayed by detecting luciferin-luciferase luminescence at 10 °C using 0.8 ml reaction mixtures which contained 1 μg Chl/ml from either low-salt- (LS) or high-salt- (HS) stored thylakoids, 10 mM sorbitol, 50 mM Tricine-NaOH (pH 8.0], 3 mM $MgCl_2$, 1 mM KH_2PO_4, 5 mM DTT, 0.1 mM MV, 5 μM A_2P_5, 0.1 mM ADP (Dowex purified), 20 nM nonactin, 10 μl of luciferin/luciferase complex (from a stock of 1.5 ml LKB vial) and 1 mM HEM (when indicated). The assay was initiated by either saturating red light (defined by two Corning CS 2-64 filters) with intensity of 240 mW cm^{-2} or at a lower intensity of 30 mW cm^{-2}. The upward arrow indicates light on and the downward arrow indicates the light off. In traces A and B, the assay was calibrated by standard ATP before the onset of phosphorylation, and for all traces standard ATP (0.23 μM added) at the end of the run. The chart speed was decreased by 6-fold after switching off the actinic light.

220 ± 40; C, 44 ± 12; and D, 250 ± 70. Clearly, the high-salt-stored samples (B and D) showed a much larger effect of the HEM than the low-salt-stored samples (A and C). One explanation for this difference is that low-salt-stored thylakoids can utilize a localized protonmotive force rather than a delocalized gradient. However, another possible source for the different response of the two thylakoid samples to the amine is that the neutral amines do not penetrate the membranes as readily in the low-salt- as in the high-salt-storage case. As shown above (Table I), pyridine and HEM share similar significant effects on the phosphorylation onset lags, and the ATP yield per flash, in high-salt-stored membranes, but had little or no effect on either parameter with the low-salt-stored membranes. If pyridine (or HEM) had a slower permeation rate into the lumen of thylakoids stored in the low-salt condition, then one could question whether the differences found in the experiments of Table I and Fig. 1 are to be explained by that mechanism, as Borchard and Junge [4] have suggested, rather than by the localized or delocalized hypothesis we have proposed [9,10].

We were aware of that possibility and for that reason pyridine uptake experiments were reported earlier [10] and no difference was detected for [^{14}C]pyridine entry into low- or high-salt-stored thylakoids, but only a single time of incubation was reported for high-salt-stored thylakoids. It was considered important to repeat those experiments and report them in more detail because the recent suggestion by Borchard and Junge [4] called into question whether the low-salt-stored thylakoids might have a lower permeability to pyridine than high-salt-stored thylakoids.

Kinetics of amine buffer penetration in low- and high-salt-stored thylakoids

[^{14}C]Pyridine uptake was measured in the dark using the silicone oil centrifugation method, with thylakoids stored either in low- or high-salt conditions and assayed for uptake in the phosphorylation assay reaction mixture. Fig. 2 shows composite data from 4 separate (days) experiments, using 5 mM pyridine with tracer amounts of [^{14}C]pyridine. Two points are clear:

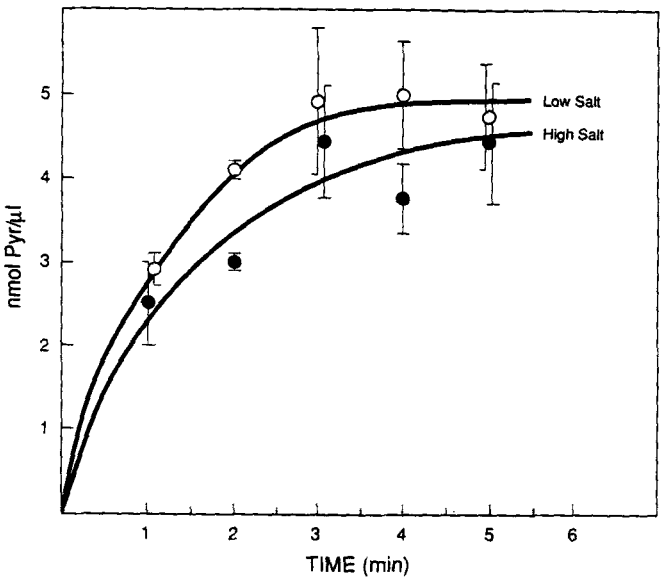

Fig. 2. [^{14}C]Pyridine entry into thylakoids stored in low-salt or high-salt medium. [^{14}C]Pyridine uptake was initiated with the addition of 75 µg Chl/ml into a reaction mixture which contained 10 mM sorbitol, 50 mM Tricine-NaOH (pH 8.0), 3 mM $MgCl_2$, 1 mM KH_2PO_4, 5 mM pyridine, 400 nm nonactin, 10 µM DCMU and 0.5 µCi/ml of [^{14}C]pyridine or 0.5 µCi/ml of [^{14}C]inulin or 2 µCi/ml [^{3}H]H_2O at 10 °C. At various times thylakoids were centrifuged in a Beckman microfuge for 30 s through silicone oil (Versilube F-50 and SF-96 [22]; 2:2 (v/v)) into 10% perchloric acid in 0.4 M NaCl. To determine the radioactivity in the pellet, the tubes were frozen on solid CO_2; then sliced just above the silicone oil/pellet interface and the tip containing the pelleted thylakoid membranes was used for scintillation counting. The internal concentration of [^{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_2O, and the calculated lumen volume. The latter was calculated as the difference between the [^{14}C]inulin permeable space and the [^{3}H]H_2O permeable space.

(a) there is no significant difference in the kinetics of pyridine entry into the thylakoid lumen for low- or high-salt-stored membranes (in agreement with the earlier results [10]); (b) in both cases the concentration of pyridine calculated as entering the lumen after 5 min is very close to the 5 mM external concentration; i.e., complete equilibration occurred in both cases. The conditions used for this experiment were similar to those used for the flash or steady-state phosphorylation experiments except that neither the luciferase-luciferin system nor the diadenosine pentaphosphate (an inhibition of the adenylate kinase enzyme) was present. The thylakoid lumen volumes under those assay conditions for either the low- or high-salt-stored sample were generally in the range of 20–30 µl H_2O per mg Chl, close to that reported previously for similar conditions [10].

Effects of amine on total H^+ uptake measured by external pH changes

An additional test for whether pyridine or HEM readily enter the lumen of either low-salt- or high-salt-stored thylakoids was obtained by measuring H^+ uptake (using the pH electrode method to follow external pH changes) with and without the amines and under both basal and coupled conditions. This assay method, first used by Nelson et al. [23] and extensively studied by Avron [24], is a diagnostic indicator both of (A) whether the energized thylakoids acidify the lumen to a pH approaching the pK_a of the amine buffer, and (B) whether the neutral amine is permeable across the thylakoid. For a given amine such as pyridine or HEM with a $pK_a \approx 2$–3 units below the external assay pH and with the assay conditions kept similar except for ±ADP to give coupled or basal conditions, one can predict that the more acidic the lumen, the greater will be the amine stimulation of total H^+ uptake. For example, Avron (Fig. 6 of Ref. 24) found that the amines analine (pK_a 4.5) and phenylenediamine (pK_a 6.2) stimulated H^+ uptake ($pH_{ext} = 8.0$) nearly linearly with decreasing external pH to $pH_{ext} = 7.0$, and from other work with lettuce chloroplasts from Avron's group [24,25] and with spinach from Hope's group [26], we know that the internal pH drops nearly linearly in the same external pH range. Thus, an increase in H^+ uptake in the presence of permeable amine buffers, such as pyridine (5 mM) and HEM (0.5 to 1.0 mM) which do not stimulate electron transport at the concentrations used (see below, Table III), is an indication that (A) the H^+ uptake had to be into the lumen, and (B) that the amine readily enters the lumen.

With that background we can consider the data of Fig. 3 which shows the extent of total H^+ uptake measured at an external pH of 8.0 with the glass electrode technique in either low or high salt-stored thylakoids with or without added pyridine (5 mM) or

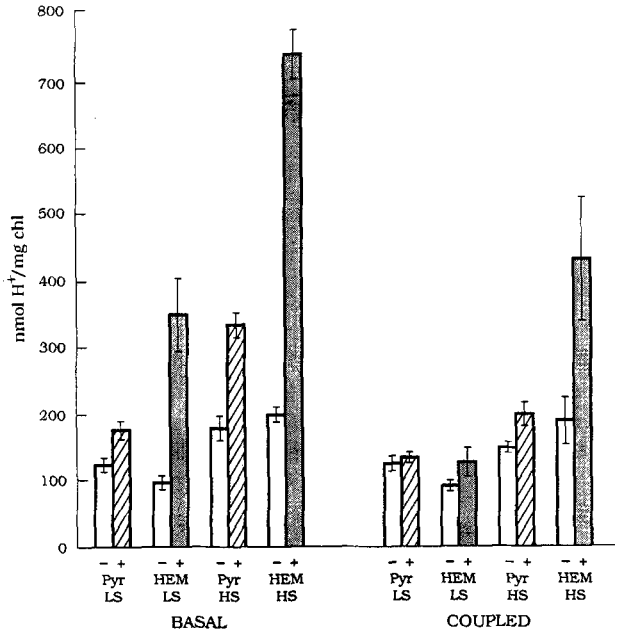

Fig. 3. Proton uptake in low- and high-salt-stored thylakoids as influenced by pyridine and HEM. The reaction medium was as specified in the methods section with the pH adjusted to 8.0. For coupled conditions ADP was at 0.1 mM, P_i at 5 mM; for basal conditions ADP was omitted. Pyridine at 5 mM (cross-hatched) or 0.5 mM HEM (stippled) were present as indicated by the plus sign beneath the bars, with the corresponding control (open bars, minus the amine) indicated by the minus sign. The red light intensity was 39 mW cm^{-2}. The error bars indicate the calculated standard deviations for the measurements (n was usually four trials).

HEM (0.5 mM), in either basal (–ADP) or coupled conditions. Hexokinase and glucose added in the coupled reactions recycled any ATP formed and allowed the pH electrode to record just the H^+ uptake into the thylakoids. As expected from previous work [23,24] both pyridine (pK_a 5.4) and HEM (pK_a 6.3) stimulated, in both types of chloroplast, the basal condition H^+ uptake (Fig. 3, left side) with HEM, having the higher pK_a, giving a considerably greater stimulation just as Avron found with phenylenediamine (pK_a 6.2) compared to analine (pK_a 4.5). This is as expected if the H^+ uptake is into the lumen, which it is predicted by our model [10,27] to be for basal conditions, and if the amines freely penetrate to the lumen and exert their buffering action. The inescapable conclusion, consistent with our previous results [10,27], must be that luminal H^+ uptake occurs for both low- and high-salt-stored thylakoids for basal conditions.

High salt-stored thylakoids under coupling conditions also gave pyridine- and HEM-dependent increased H^+ uptake (right side, Fig. 3), although the magnitudes of the amine effect was less than for the high-salt-stored thylakoids in basal conditions. This is as expected owing to the ATP formation utilizing some of the ΔpH gradient in the coupled condition [28,29].

In striking contrast, the low-salt-stored thylakoids in coupled conditions (right side, Fig. 3) gave no increase in H^+ uptake with pyridine added and very little increase with HEM. The rates of ATP formation (Fig. 1) and electron transport (Table III) were very similar for the low- and high-salt-stored thylakoids (when assayed in medium of similar composition), as was the control level of H^+ uptake. Thus the lack of effect of the amines in the low-salt, coupled case cannot be ascribed to low activity of the energy conversion apparatus, nor can the results be caused by the failure of the amines to reach the lumen. The results are consistent with the notion that the H^+ uptake in the coupled, low-salt case occurs predominantly into localized domains which do not equilibrate with the lumen.

ΔpH measured by [^{14}C]methylamine distribution

An additional experiment to test whether low-salt-stored thylakoids maintain a significantly less acidic lumen than the high-salt case is the direct determination of the lumen pH in continuous illumination coupled conditions for the low- and high-salt-stored membranes. That was done using the [^{14}C]methylamine distribution technique for the ΔpH determination [22,25], with side-by-side samples used with [^{32}P]P_i to determine rates of ATP formation. It is assumed, following past work [22,25], that the ΔpH determined by this method is between the bulk phases, the lumen and the external phase. Fig. 4 shows the results of such an experiment in which we routinely found that for any rate of ATP formation, the ΔpH for the low-salt-stored thylakoids was about 0.4 units less than that for the high salt case. The ΔpH was varied by varying the light intensity. At a phosphorylation rate of 100 μmol ATP

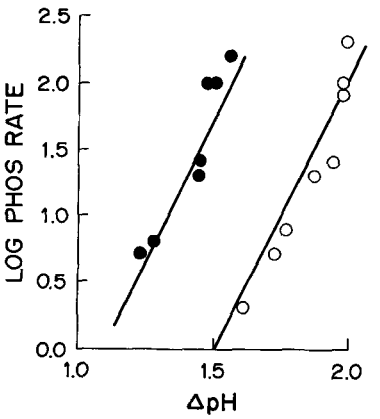

Fig. 4. Transthylakoid membrane ΔpH measured by the [^{14}C]methylamine distribution method versus log rate of ATP formation. The conditions for ATP formation and the [^{32}P]ATP determination were as stated in Materials and Methods, as well as the method for determining, in separate samples, [^{14}C]methylamine distribution. Corrections for trapped external fluid volume used [^{14}C]inulin, and for total H_2O space used [^{3}H]H_2O as stated in Materials and Methods.

(mg Chl h)$^{-1}$ the ΔpH for the high-salt case was close to 2.0 units, similar to that reported by Schuldiner et al. [25] using the methylamine method with 100 mM KCl as the osmoticum in the assay medium and with a 'high-salt-type' thylakoid isolation medium (300 mM NaCl as the osmoticum). Similar results were obtained in many experiments carried out over a several-week period. Even if there were systematic errors in the ΔpH determinations so as to underestimate the true ΔpH (see Hope and Matthews [30] for a discussion of sources of possible error), the large difference in the ΔpH values for the low- and high-salt cases supports the contention that the lumen is differently involved in the two cases, a point already abundantly supported by other data, discussed above and reported earlier [6,7,9,10].

If the ΔpH data of Fig. 4 are taken at face value, the threshold ΔpH observed for the low-salt-stored membranes is far below what would be expected as necessary to energize steady illumination ATP formation, even if one assumes an H^+/ATP ratio of 4 as mentioned in some recent reports [31–33]. For the high-salt-stored case the threshold value of 1.5 ΔpH (Fig. 4) is energetically sufficient for an H^+/ATP ratio of 4 but not if a value of 3 is assumed. Several independent evaluations have supported an H^+/ATP ratio of 3 [28,34,35], but a value of 4 may be correct for certain conditions [33].

More work needs to be done on luminal pH values under various conditions and new methods are becoming available, but the conclusion seems reasonable that the lumen is considerably less acidic for the low-salt-stored (coupled) thylakoid case compared to the high-salt-stored (coupled) case at equal rates of ATP formation. That supports the other results presented in this paper and gives additional evidence favoring our hypothesis for there being a mechanism for having either localized or delocalized coupling mechanisms at work in thylakoids.

Factors involved in amine effects on steady illumination ATP formation

During our efforts reported here to learn whether responses consistent with localized energy coupling occur in low-salt-stored thylakoids in continuous light, we incidentally observed that HEM causes, in the high-salt-stored case only, a greater lag in the onset of the ATP formation signal compared to the control (no HEM) following the beginning of continuous illumination. Table II shows data from such experiments with a minimum of three repeats of each observation, and it is clear that the high-salt-stored membranes showed nearly a 3 s increase in the onset lag period when 0.5 mM HEM was present. The low-salt-stored membranes gave only a slight HEM effect on the onset lags. Typical steady-state rates of ATP formation for these experiments are also shown in the table. The rates were low owing to the reduced light intensity used, but similar to the rates of ATP formation for the experiment shown in Fig. 1, traces C and D. We interpret these results as showing, by the accepted criteria of amine effects on H^+ uptake [23,24], that the low- and high-salt-stored samples used herein did indeed initiate ATP synthesis utilizing either localized or delocalized coupling, respectively. By analogy, we suggest that the transient slowing of the ATP formation rate in high-salt-stored thylakoids upon addition of HEM after the steady-state rate was achieved (Fig. 1) also could be caused by a transient drop in the $\Delta\tilde{\mu}_{H^+}$ owing to the HEM reaching the lumen. Other causes for the transient delay having nothing to do with effects on the $\Delta\tilde{\mu}_{H^+}$ are possible and experiments were done to test for such effects (none was found), as discussed below.

TABLE II

ATP formation onset lags in continuous illumination for low-salt or high-salt-stored thylakoids

Thylakoids (1 μg/ml) were incubated in medium as specified in Materials and Methods and in the legend to Fig. 1, with or without 0.5 mM HEM. Incubation was for 3.0 min and then sub-saturating light was turned on. Onset lags were determined by measuring the time back-extrapolated from the steady-state rate to the horizontal line coincident with the signal which resulted after the light-on jump in the signal

Thylakoid storage	HEM (mM)	ATP formation onset lag (s)	Steady-state rate μmol ATP (mg Chl h)$^{-1}$
Low-salt	–	2.0 ± 0.3	91 ± 7
Low-salt	+0.5	2.6 ± 0.3	79 ± 8
High-salt	–	2.8 ± 0.6	89 ± 17
High-salt	+0.5	6.7 ± 0.3	91 ± 11

At first thought, it is perplexing why the HEM-dependent transient delay in ATP formation rate recovered at all, for it is expected that neutral HEM would continue to enter the lumen in response to the protonation of the base. We considered that the rate recovery may be associated with the electric field effects. As the HEM entering the lumen is protonated, it begins accumulating as a net positive charge concentration with the lumen pH rising in proportion (this allows more H^+ ions to enter the lumen, see below). The increased positive HEM^+ charges would initially be compensated by Val-K^+ efflux, but the K^+ supply is limited, so one can question whether there is sufficient K^+ to maintain the low $\Delta\Psi$ state. The luminal K^+ supply is estimated to be roughly 0.50 μmol K^+ (mg Chl)$^{-1}$ (20 μl luminal H_2O/mg Chl × 0.025 μmol K^+/μl = 0.50 μmol K^+/mg Chl). At a rate of H^+ influx near 300–400 μmol H^+ (mg Chl h)$^{-1}$ it will require about 5 s to deplete the K^+ pool if [K^+] is 25 mM, and 10 s if [K^+] is 50 mM. At the lower light

intensity used in Fig. 1 the time required to exchange out the internal K^+ stores would be longer. The transient delay in the ATP formation rate was between 6 s (Fig. 1B) to 12 s (Fig. 1D) at the high and lower intensities, respectively. Thus, depending on the rate of ATP formation (H^+ pumping), after perhaps 5–10 s, as HEM^+ cation continues to accumulate, there could develop a positive $\Delta\Psi$ contribution to the total protonmotive force. In our medium, with low Cl^- content (6 mM), the possibility exists that insufficient anions are present to influx into the lumen and balance the HEM^+ cation. If that were the case, and if a positive $\Delta\Psi$ builds up, we may expect the ATP formation rate to return to near normal as HEM^+ accumulation occurs.

One way to test the above notion is to add a second aliquot of HEM. If a positive $\Delta\Psi$ were forming owing to (protonated) HEM^+ accumulation uncompensated by K^+ efflux, then the second addition of HEM would show little or no slowing of the ATP formation rate. Experiments to test this suggestion gave exactly that pattern (Fig. 5). The top trace shows high-salt-stored thylakoids given 1 mM HEM after 24 s of steady illumination and 12 s later a second 1 mM HEM addition was given. The second HEM addition had very little effect on the time course of the ATP formation, and a third addition at 56 s likewise gave only a slight effect. The control low-salt-stored thylakoids, as usual, showed very little effect of adding 1 mM HEM (lower trace, Fig. 5). These results are consistent with the notion that the recovery of the ATP formation rate after the transient slowing may be caused by a positive $\Delta\psi$ which develops as HEM^+ cation accumulates beyond the capacity of the internal K^+ stores to compensate by efflux.

Another way to test the above point is to have present an additional rapidly permeating anion, such as NO_3^- or SCN^-, and observe whether that causes the rate of ATP formation to remain low after HEM addition. Such an experiment was done with up to 5 mM SCN^- (as KSCN) or up to 10 mM KNO_3 (data not shown), but the results showed no effect (in high-salt-stored membranes) on the HEM-induced transient delay and the recovery of the phosphorylation rate. This does not necessarily rule out the proposed effect, if, for instance, the permeability of NO_3^- or SCN^- were slow enough to not dissipate a putative positive $\Delta\psi$ (not as fast as the formation rate of HEM^+ cation). Higher concentrations of KSCN or KNO_3 could not be used owing to general inhibition of the (initial) phosphorylation rate.

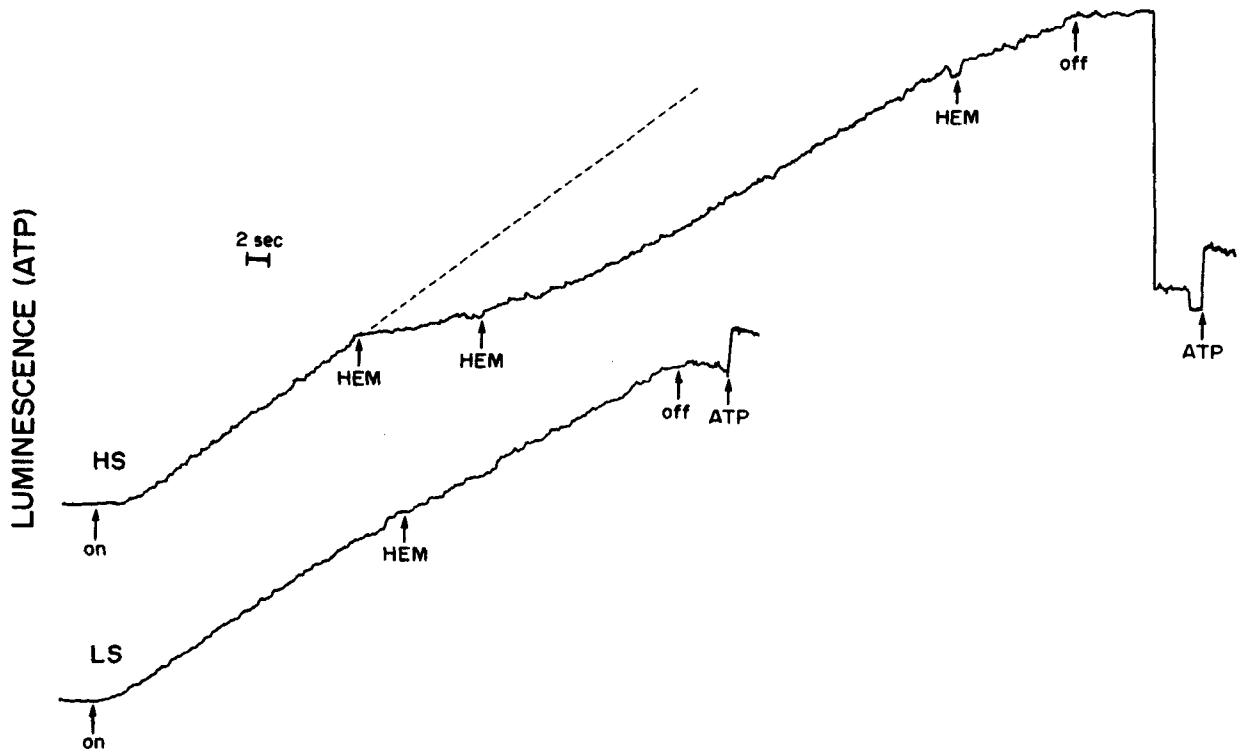

Fig. 5. Effect of a second and third addition of HEM on the rate of ATP formation. Conditions were similar to those of Fig. 1, with the low actinic light intensity (about 30 mW cm^{-2}). At 24 s the first addition of 1 mM HEM was given to the high-salt-stored sample (HS, top trace) and about 12 s later the second addition was made, and the third addition 48 s later. The second and third HEM additions did not result in a significant transient rate decline as did the first HEM addition. The lower trace, Using low-salt-stored thylakoids (LS), shows a typical (negligible) response to addition of 1 mM HEM.

Luminal H^+ accumulation, a predicted corollary to accumulation of HEM cation

As mentioned above (see Fig. 3), measuring the effect of permeable buffers on the extent of H^+ uptake is a direct test for proton accumulation into the thylakoid lumen driven by uptake of permeable buffers [23,24]. If, as implied by the data of Fig. 1, high-salt-stored thylakoids have significantly more luminal acidification during steady-state ATP formation than low-salt-stored membranes, then there should be a greater HEM-dependent H^+ uptake in the sample having more luminal acidification. We measured H^+ uptake $\pm$HEM in the steady illumination protocol of Fig. 1, using the same thylakoid preparation as used for the ATP formation assays (similar to the experiment reported in Fig. 3) in both types of thylakoid. The H^+ uptake experiment was done in two ways; having HEM present before turning on the light (as in Fig. 3), or adding HEM at 40 s after illumination began. Results of the H^+ uptake measurement (with the amine added before illumination began) very similar to those of Fig. 3 were observed routinely for both pyridine and HEM. The thylakoids used for the H^+ uptake experiment were tested for the HEM effect on the rate of ATP formation using the luciferase method, and data similar to those of Fig. 1 (data not shown) were obtained. Where HEM caused the large increase in the H^+ uptake assay in coupled conditions (high-salt-stored case) there was also the transient delay in the steady-state ATP formation rate upon adding the HEM after 40 s of illumination. For the low-salt-stored, coupling conditions case HEM addition at 40 s gave essentially no effect on the steady-state ATP formation rate, and very little, if any, effect on H^+ uptake, in agreement with the data of Fig. 3.

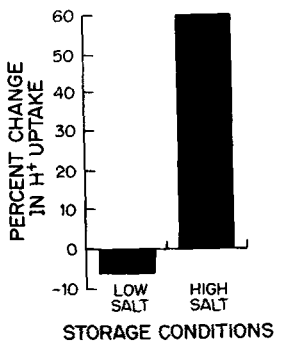

Fig. 6. Effect of 0.5 mM HEM addition after 40 s of illumination on H^+ uptake in low- and high-salt-stored thylakoids. Thylakoids (40 μg chlorophyll/ml) were incubated in a medium as in Fig. 3 and illuminated until steady-state H^+ uptake was achieved. At 40 s, 0.5 mM HEM was added and additional H^+ uptake was measured. H^+ uptake was calibrated with HCl standard. The addition of the amine buffer caused an alkaline shift owing to absorption of H^+ ions by the base. This reproducible effect was subtracted from the total signal, yielding a calculated estimate of the net proton uptake.

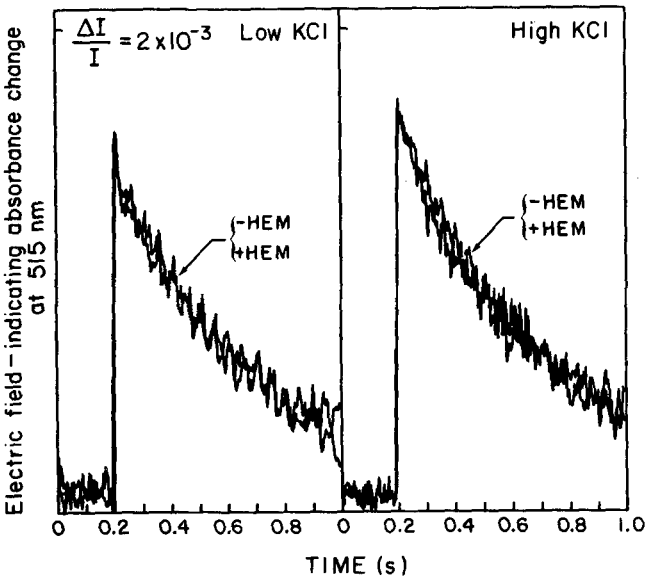

Fig. 7. The effect of HEM on the ionic permeability of thylakoid membranes in low-salt- and high-salt-treated samples. Thylakoids were suspended in and medium similar to that for Fig. 1 except that diadenosine pentaphosphate and the luciferase-luciferin reagent were not added. Traces show the electric field-indicating electrochromic absorption change at 515 nm; the rate of decay reflects the rate of ion (including proton) movement across the membrane. The signals from eight flashes fired at 1/8 Hz were averaged. Thylakoids were added to 19 μg Chl/ml, and when present, the concentration of HEM was 0.5 mM.

In another series of H^+ uptake experiments, the HEM was added to the thylakoid suspension after 40 s of illumination, and protocol more closely matching that of the Fig. 1 experiments. Despite a (purely chemical, non-biological) pH change induced by addition of the base to the aqueous medium, the net H^+ uptake into the thylakoids could be measured. Just as in the case when the HEM was present prior to illumination, for low-salt-stored thylakoids under coupled conditions there was very little effect of 0.5 mM HEM addition on the steady-state H^+ uptake (Fig. 6). However, with high-salt-stored membranes, adding HEM after 40 s of illumination resulted in a rapid, additional H^+ uptake, reaching about 1.6-fold the control value.

HEM does not affect the ionic permeability of the thylakoid membrane

The possibility was examined that HEM acts to increase the proton permeability of the thylakoid membrane in the high-salt-, but not low-salt-stored samples by measuring the decay of the flash-induced absorption change at 515 nm [3]. This signal reports the electric field produced across the membrane by the primary charge separation in the photosynthetic reaction centers, and its decay is controlled by the ionic permeability of the membrane. If HEM facilitates a leak of protons across the membrane, it would cause a faster decay of the 515 nm signal. Fig. 7 shows that no such

TABLE III

The rates of coupled electron transport in high- and low-salt-stored thylakoids

A. Methyl purple as electron acceptor, and rates were measured spectrophotometrically. The assay medium was as in Fig. 1, except that luciferin/luciferase and methylviologen were omitted, 20 μM methyl purple was present and chloroplasts were added to 1.25 μg Chl/ml. Samples were incubated in the dark for 2 min, then illuminated with red light (Corning CS2-58 filter) for 30 s. The sub-saturating light intensity used in this experiment was the same as that used for the experiment of Fig. 4. The transmittance change of methyl purple was monitored during the illumination period. Traces were calibrated by measuring the change in methyl purple transmittance in response to dilution of the dye by injections of a known amount of water into the cuvette. The subsequent dilution of the dye provides a calculated Δ absorbance.

B. Methylviologen as electron acceptor. In this assay an oxygen electrode cuvette was used. The assay system was similar to that in Part A above, except methylviologen at 0.1 mM was the electron acceptor and the chlorophyll content was 20 μg ml^{-1}. White light at saturating intensity was used. In this experiment 1.0 mM HEM was added after 40 s of illumination and the steady rate was measured prior to and after HEM addition.

Thylakoid storage	Light intensity	Rate (μequiv. (mg Chl h)$^{-1}$)
A. High salt	saturating	292
	sub-saturating	263
Low-salt	saturating	271
	sub-saturating	236
	Rate (μequiv. (mg Chl h)$^{-1}$)	
	Before HEM	After HEM
B. High salt	230 ± 20	248 ± 17
Low salt	267 ± 8	266 ± 20

acceleration of the decay of the electric field was observed upon HEM addition to either the high-salt- or low-salt-stored chloroplasts. The possibility that HEM acts as a non-electrogenic proton/cation antiporter is not consistent with the proposed mechanism of action of other uncoupling amines, i.e., NH_3^+, methylamine, etc. [23].

Electron transport rates

Fig. 1 showed that the rates of ATP synthesis were approximately the same (for identical light intensities) in both chloroplast types, and Table III, part A, demonstrates that electron transport rates, using methyl purple as a PSI electron acceptor [21], were also approximately the same. It is noteworthy that these experiments were performed in the same instrument using the same cuvette geometry as for the steady-state ATP formation measurements, eliminating any artifacts introduced by physical changes demanded by the different measurements.

Part B of Table III shows that adding 1.0 mM HEM at 40 s after illumination began had little or no significant effect on electron transport rates (using the methylviologen oxygen uptake system as the assay). In all the assays with high-salt-stored thylakoids there was, on average, about a 10% increase in the rate after HEM addition, as indicated by the average values shown in the Table. Low salt-stored thylakoids did not show that trend.

Concluding remarks

This work broadens the experimental basis underlying the still-unsettled question in chloroplast bioenergetics concerning whether thylakoids can be energized for ATP formation by a localized as well as by a delocalized $\Delta\tilde{\mu}_{H^+}$ gradient. In particular, we have studied this question under steady illumination conditions in order to compare the results with those found for the more fully-studied initial light-on transient. Just as permeable amine buffers have predictable effects on the flash-initiated ATP formation onset lag when the $\Delta\tilde{\mu}_{H^+}$ which drives ATP formation is delocalized (i.e., increasing the flash lag) as in high-salt-stored thylakoids [9,10,14], so the addition of the amine HEM after 40 s of continuous illumination caused a sudden but transient inhibition of ATP formation (Fig. 1, B and D). Low-salt-stored thylakoids, used by our group [9–11] and by two other groups [6–8], do not show amine effects in extending the ATP formation onset lag in flash trains. Nor did they, in the present study, respond to amine addition after 40 s of illumination (Fig. 1, A and C). We can conclude that, to the extent that we and the other groups mentioned above have correctly interpreted the amine effects in flash energization experiments, low-salt-stored thylakoids can maintain localized $\Delta\tilde{\mu}_{H^+}$ coupling in continuous illumination.

Borchard and Junge [4] have raised questions on the use of pyridine by suggesting that, for reasons not clear, pyridine may not enter the lumen of low-salt thylakoids, or if there, not exert its buffering action. We believe this is not a valid point, but we took the possibility into serious consideration. Accordingly, we have tested this rather tenuous but intriguing suggestion by (A) measuring [^{14}C]pyridine entry into the lumen volume in both low- or high-salt-stored thylakoids (and under strictly comparable assay conditions used in our ATP formation experiment; and (B) to measure total H^+ uptake with and without the amines pyridine and HEM present in both types of chloroplast under coupled compared to basal conditions. Not only does pyridine (and by analogy HEM) equilibrate freely in both types of thylakoid, reaching the external 5 mM concentration in both cases with the lumen volumes being similar at about 25 μl H_2O per mg Chl for the low-salt-stored and about 18 μl H_2O per mg Chl for the high salt-stored samples (Fig. 2), but both amines readily exert their buffering potential in the lumen and caused the predictable increase in

H^+ uptake under basal conditions (Fig. 3). The coupled condition did not give a significant increase in H^+ uptake when pyridine was added and gave a slightly greater effect with HEM. The point to be emphasized here is that the only difference between the basal and coupled conditions was the addition of ADP, which did not significantly change the internal volume. Hence, there is no conceivable explanation that we can imagine which would explain away, as Borchard and Junge [4] seek to do, the capacity of pyridine or HEM, present in the lumen, to exert its buffering power toward protons in the lumen in the coupled, low salt-stored case, if they in fact were deposited there. Rather, we conclude that the data better support our hypothesis that energy coupling protons in that case are kept predominantly in localized domains (cf. Refs. 2, 37 for further discussion of domains).

This notion is further supported by the observation that the transmembrane ΔpH, measured by the [^{14}C]methylamine method under coupling conditions was about 0.4 pH units less, for all rates of ATP formation measured, in the low-salt-stored sample compared to the high-salt-stored case (Fig. 4).

Acknowledgements

The authors thank Ms. Janet Hollister for help in the manuscript preparation and Mr. Mark O'Neil for illustrations. The work was supported in part by a grant from the U.S. Department of Energy.

References

1 Ferguson, S.J. (1985) Biochim. Biophys. Acta 811, 47–95.
2 Dilley, R.A., Theg, S.M. and Beard, W.A. (1987) Ann. Rev. Plant Physiol. 38, 347–389.
3 Junge, W. (1987) Proc. Natl. Acad. Sci. USA, 84, 7084–7088.
4 Borchard, A. and Junge, W. (1990) in Current Research Photosynthesis (Proc. 8th Int. Congr. Photosynthesis, Stockholm), Vol. III, pp. 97–100, Kluwer, Dordrecht.
5 Ort, D.R., Dilley, R.A. and Good, N.E. (1976) Biochim. Biophys. Acta 449, 108–124.
6 Graan, T., Flores, S. and Ort, D.R. (1981) in Energy Coupling in Photosynthesis. (Selman, B.R. and Selman-Reimer, S., eds.), Developments in Biochemistry, Vol. 20, pp. 25–34, Elsevier/North-Holland, New York.
7 Horner, R.D. and Moudrianakis, E.N. (1983) J. Biol. Chem. 258, 11643–11647.
8 Horner, R.D. and Moudrianakis, E.N. (1986) J. Biol. Chem. 261, 13408–13414.
9 Beard, W.A. and Dilley, R.A. (1986) FEBS Lett. 201, 57–62.
10 Beard, W.A. and Dilley, R.A. (1988) J. Bioenerg. Biomembr. 20, 129–154.
11 Chiang, G. and Dilley, R.A. (1987) Biochemistry 26, 4911–4916.
12 Dilley, R.A. and Chiang, G.G. (1989) N.Y. Acad. Sci. 574, 246–267.
13 Davenport, J.W. and McCarty, R.E. (1980) Biochim. Biophys. Acta 589, 353–357.
14 Vinkler, C., Avron, M. and Boyer, P.D. (1980) J. Biol. Chem. 255, 2263–2266.
15 Sigalat, C., Haraux, F., De Kouchkovsky, F., Hung, S.P.N. and De Kouchkovsky, Y. (1985) Biochim. Biophys. Acta 809, 403–413.
16 Pick, U., Weiss, M. and Rottenberg, H. (1987) Biochemistry 26, 8295–8302.
17 Opanasenko, V.K., Red'ko, T.P., Kuz'mina, V.P. and Yaguzhinsky, L.E. (1985) FEBS Lett. 187, 257–269.
18 Gräber, P. (1982) Curr. Topics in Membr. Transp. 16, 215–245.
19 Beard, W.A. and Dilley, R.A. (1988) J. Bioenerg. Biomembr. 20, 85–106.
20 Allnutt, F.C.T., Dilley,R.A. and Kelly, T. (1989) Photosyn. Res. 20, 161–172.
21 Graan, T. and Ort, D.R. (1984) J. Biol. Chem. 259, 14003–14010.
22 Gaensslen, R.E. and McCarty, R.E. (1972) Anal. Biochem. 48, 504–514.
23 Nelson, N., Nelson, H., Naim, Y. and Neumann, J. (1971) Arch. Biochem. Biophys. 145, 263–267.
24 Avron, M. (1971) in Second Internatl. Congr. on Photosynthesis (Forti, G., Avron, M. and Melandri, A., eds.), Vol. II, pp. 861–871, Dr. W. Junk, The Hague.
25 Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–63.
26 Chow, W.S. and Hope, A.B. (1976) Austr. J. Plant Physiol. 3, 141–152.
27 Beard, W.A., Chiang, G. and Dilley, R.A. (1988) J. Bioenerg. Biomembr. 20, 107–128.
28 Portis, A.R. and McCarty, R.E. (1974) J. Biol. Chem. 249, 6250–6254.
29 Pick, U., Rottenberg, H. and Avron, M. (1973) FEBS Lett. 32, 91–94.
30 Hope, A.B. and Matthews, D.B. (1985) Austr. J. Plant Physiol. 12, 9–19.
31 Bogdanoff, P. and Gräber, P. (1990) in Current Research Photosynthesis (Proceedings of the 8th Int. Congr. Photosynthesis, Stockholm), Vol. III, pp. 217–220, Kluwer, Dordrecht.
32 Rumberg, B., Schubert, K., Strelow, F. and Tuan, T.A. (1990) in Current Research in Photosynthesis (Proc. 8th Int. Congr. Photosynthesis, Stockholm), Vol. III, 125–128, Kluwer, Dordrecht.
33 Van Walraven, H.S., Scholts, M.J.C., Koppenaal, F., Bakels, R.H.A. and Krab, K. (1990) Biochim. Biophys. Acta 1015, 425–434.
34 Hangarter, R. and Good, N.E. (1982) Biochim. Biophys. Acta 681, 397–404.
35 Strotmann, H. and Lohse, D. (1988) FEBS Lett. 229, 308–312.
36 Walz, D., Goldstein, L. and Avron, M. (1974) Eur. J. Biochem. 47, 403–407.
37 Dilley, R.A. (1991) Curr. Topics Bioenerg., in press.