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Shift from localized to delocalized protonic energy coupling in thylakoids by permeant amines

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We have suggested that in energy-transducing organelles structural constraints may hinder H^+ transport from their sites of active, redox translocation to their sites of passive or phosphorylating escape (microchemiosmosis). We could modulate these constraints first by affecting the physico-chemical properties of the medium, and now by adding permeant amine buffers to a suspension of thylakoids. The following results are obtained. (1) Whether driven by Photosystem I or by Photosystem II, phosphorylation is stimulated by amines (imidazole, hexylamine, NH_4Cl) at concentrations low enough hardly to modify the proton gradient: ΔpH is stable if not reduced, $\Delta\Psi$ is slightly increased but still negligible; this extends the observations made by Giersch. (2) The concentration curves of phosphorylation stimulation by amines exhibit a minor peak before the main one previously reported. (3) ATP synthesis and ΔpH are decreased by amines at higher concentrations, but the dependence of the phosphorylation rate (flow) versus ΔpH (force) is then shifted towards lower ΔpH values. (4) The normally less efficient Photosystem II-driven phosphorylation is comparable to Photosystem I with amines. (5) The flow-force curves, which are distinct when traced by limiting ΔpH by an H^+ influx decrease (light) or efflux increase (nigericin), are much closer with either photosystem when amines are added. The Photosystem I curve produced by increasing nigericin beyond approx. 200 nM becomes insensitive to amines. (6) In general, Photosystem II requires significantly less nigericin or amines than Photosystem I to obtain similar effects. It is proposed that amines have a double effect. By efficiently carrying protons along the membrane, amines lower the protonic resistances and thereby delocalize the proton gradient. At higher amine concentrations, uncoupling occurs, probably by an indirect backflow of protons more than by a buffering effect, as generally admitted. In conclusion, the multiple-resistances microchemiosmotic scheme which we have proposed earlier is strengthened; it predicts that intermediate states may link delocalized (canonical chemiosmosis) and localized coupling modes, which is established here.

Abbreviations: $\Delta\tilde{\mu}_{H^+}$, ΔpH and $\Delta\Psi$, transmembrane difference of proton electrochemical potential, of pH and of electrical potential, respectively; V_e and V_P, rate of electron flow and of ATP formation, respectively; PS I and PS II, Photosystem I and Photosystem II, respectively; CF, coupling factor or ATPase; PYO, pyocyanine; Chl, chlorophyll; DMQ, 2,5-dimethylquinone; P_i, inorganic phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; dAPP, P^1P^5-di(adenosine-5′)pentaphosphate; FeCy, ferricyanide.

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Introduction

Because some of the corollaries of the chemiosmotic theory are not experimentally confirmed, several other interpretations of energy coupling have been proposed (for reviews, see Refs. 2 and 3). However, the basis of Mitchell's model now seems indisputable. Thus, the deviations observed should mainly lie in the complex organization of the energy-transducing systems. This point is considered by hypotheses of localized chemiosmosis, or microchemiosmosis, which would gain in strength if it were possible to modulate the expression of energy coupling by acting on the structural properties of the organelles.

One of the manifestations of energy coupling is the dependence of a given process, such as the phosphorylation flow rate (V_p) as a function of the magnitude of the proton gradient, $\Delta\tilde{\mu}_{H^+}$, or of any other relevant thermodynamic force. In classical chemiosmosis, only a single value of the proton gradient can exist within a vesicle, i.e., $\Delta\tilde{\mu}_{H^+}$ is delocalized and therefore, a one-to-one relation must link the flows to their conjugated forces. On the contrary, in microchemiosmosis, the proton gradient may have local values distinct from the average which is measured. In this situation the flow-force relationship, because it can be established only with this average, may change depending on the variations of local proton gradient with respect to the experimental mean $\Delta\tilde{\mu}_{H^+}$.

With thylakoids illuminated in an isotonic phosphorylating medium, we have obtained flow-force curves of localized character [4–7]. On this basis, and considering also the data given in the literature, we have derived a model [8] in which protonic resistances would cause small, yet significant, potential drops (Fig. 1). That is $\Delta\tilde{\mu}_{H^+}$ would be higher at the H^+ redox-translocating sites (H_2O and PQH_2 oxidation by PS II and PS I, respectively) than at the H^+ backflow ports (membrane leaks and open 'coupling factors' (ATPases)). Two main pathways would exist for the H^+ flow between these different points: along (or in ?) the membrane – the postulated main resistive network – and via the bulk water phase of the lumen, considered non-resistant; the two would be separated by some 'diffusion barriers'.

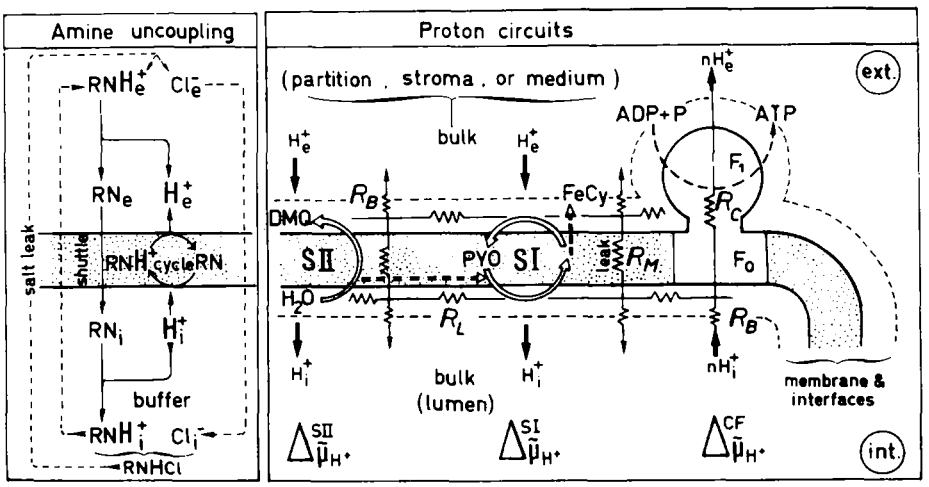

Fig. 1. Sketch of the proton currents (right) and of the mechanisms of amine uncoupling (left). Right. Curved and dashed open arrows: electron transport; plain narrow arrows: proton transport (zigzag = resistances: R_L, lateral; R_B, diffusion barrier; R_M, membrane leak; R_C, coupling factor F_0F_1); bold vertical arrows: H^+ 'translocation' across membrane. The protons liberated by oxidation of water (PS II) or plastoquinol (PS I) first meet a conductive structure on the membrane surface or interface; to reach the further bulk phase, almost absent in shrinked thylakoids, H^+ must go across diffusion barriers which shield the resistive lateral network from the delocalizing lumen space. A symetric situation may exist on the outer membrane face, especially in the granal 'partitions'. Left. To the normal buffering effect of amines, which play no special role in the steady state, would be superimposed a H^+ back-transport as a protonized amine or via an amine cycle in the membrane, two electrical processes, or even through a neutral salt leak. See text for details, especially Discussion.

According to this description, a decrease of the surface lateral resistances or a rupture of the diffusion barriers should favour a statistically even, isopotential, distribution of protons. In other words, $\Delta\tilde{\mu}_{H^+}$ would then be delocalized in the bulk phase, in accordance with Mitchell's view. This was achieved by swelling the lumen phase, which barely exists in vivo or in similarly isotonic conditions in vitro, through lowering the medium osmolarity or through raising its ionic strength: the two effects being additive [7].

At variance with our observation of a divergent behaviour of flow-force relationships depending on how the force is produced – PS I vs. PS II or H^+ influx decrease (light reduction) vs. efflux increase (nigericin addition) – McCarty's group consistently reported results in perfect agreement with the classical formulation of delocalized chemiosmosis [9,10]. As stated above, we have shown that the medium composition may determine, at least to some extent, the type of coupling. However, we felt it necessary to extend this work by using another delocalizing agent, and permeant amine buffers seemed appropriate, especially since amines, not always at negligible concentrations, are often used as probes of ΔpH [9,10].

Giersch and coworkers have noticed that low concentrations of amines [11,13] or nigericin [14] enhance the steady-state phosphorylation rate of chloroplasts more than expected from the estimated stimulation of the proton gradient. An unspecified action on a kinetic, rather than a thermodynamic factor, was suggested.

Permeant amines have been used since many years [15,16]. However, one should distinguish between the results obtained in the steady-state, with intact chloroplasts (Giersch) or thylakoids (present work), and during transitory events. Being buffers, amines have necessarily capacitive effects which smooth transient kinetics. Thus, they delay, in repetitive flash experiments, the onset of phosphorylation and prolong the post-illumination ATP formation. Of special interest in this respect are imidazole ($pK \approx 6.9$) [17–21], pyridine ($pK \approx 5.2$) [18,20], aniline ($pK \approx 4,6$) [20], and also aliphatic amines ($pK \approx 10,6$) like methylamine or octylamine [11,22] and even ammonium ($pK \approx 9,3$). It should be noted that short-term assays are difficult to interpret, since a flash-induced ΔpH pumps in turn the amines with times in the order of 2.5–4 s [20]. The published results do not always agree. Thus, whereas Ort et al. [17] did not notice a lag in the onset of ATP synthesis, this was not confirmed [18,21]. However, if a delay separates the end of the flash train from the ATP measurement, the amount of ATP would be overestimated by some post-illumination synthesis. Taking into consideration this point in the discussion of their results, Horner and Moudrianakis concluded that a gated mechanism switches the H^+ flow from an intramembrane circuit, not accessible to amines, to the bulk phase when phosphorylation starts [20]. Because the latter is an open phase, amines may penetrate it and therefore enhance the post-illumination ATP synthesis by their buffering power. This was called capacitive phosphorylation, in contrast with the photosynchrone phosphorylation, which is buffer-insensitive and occurs during the flash train.

The interest of steady-state experiments is to free oneself from such convolution effects; then the penetration time and the buffer capacity of amines do not play a role, allowing a study of their other properties. Thus, because amines diffuse in space and perhaps also interact with membrane components like polypeptides, thought to be involved in H^+ pathways [24], they may tend to distribute randomly H^+ in their respective thylakoid phases: external (partition [25]) and internal (lumen [8]). This should lower the lateral resistance and diffusion barriers depicted in Fig. 1 and therefore delocalize $\Delta\tilde{\mu}_{H^+}$. The present paper presents results in agreement with this prediction. A partial account of this work is given in Ref. 1.

Methodology

Envelope-free (class II) cloroplasts, with an intact thylakoid network, were extracted from the mature soft green parts of lettuce as previously described in Ref. 7. The procedure includes a hypotonic washing of the crude chloroplasts and subsequent resuspension of the pellet in an isotonic buffer (pH 7.8) containing 200 mM sorbitol, 10 mM Tricine, 10 mM Hepes, 10 mM KCl, 6 mM $MgCl_2$ and 2 mM KP_i. The dense preparation, up to 5 mM Chl, was kept on ice in the dark. Just before experimentation, an aliquot was di-

luted to 10 μM Chl with the same buffer in a magnetically stirred open thermostated (20 °C) 1 × 1 cm cuvette. To this medium was also added 50 μM pyocyanine for PS I or 500 μM dimethylquinone (DMQ) for PS II as electron acceptor; 500 μM ADP as phosphorylation substrate and 10 μM P^1P^5-di(adenosine-5′)pentaphosphate (dAPP). The latter inhibits adenylate kinase would could bias ATP analysis. 50 nM valinomycin was present to dissipate the K^+-dependent transmembrane electric potential $\Delta\Psi$. Controls without dAPP or valinomycin (or, with double concentration of ionophore and/or K^+) showed that, if present, adenylate kinase is inactive in thylakoids and that indeed the $\Delta\Psi$ is normally small. Measurement of $\Delta\Psi$ by its electrochromic effect on pigment bands (small absorbance increase around 515 nm) supported this conclusion in the control and even amine-treated chloroplasts (see Discussion).

Thus, solely ΔpH is given on the figures to express the total proton gradient: $\Delta\tilde{\mu}_{H^+} = F\Delta\Psi - 2.3\ RT\ \Delta pH$ ($\approx 5.6\ \Delta pH$ at 20 °C for $\Delta\tilde{\mu}_{H^+}$ in $kJ \cdot mol^{-4}$); the sign depends on which direction the H^+ moves. ΔpH was estimated with 9-aminoacridine (4 μM) which distributes, according to the respective pH, between the medium, where it fluoresces, and the vesicles where it is quenched [26]: $\Delta pH \approx \log (F^\bullet/F^\circ - 1) + \log V_e/V_i$ (pK of the probe, approx. 10, is much above pH). $F^\bullet$ and F° are the fluorescences, excited at 420 nm and analysed at 480–530 nm, in the dark-relaxed and light-energized states, respectively. V_e/V_i is the volumetric ratio of the medium and of the sum of internal vesicular spaces; V_i is indirectly measured – theoretically, it is inversely proportional to the medium osmolarity and so was estimated here – but the knowledge of V_e/V_i is not required for studies based on a comparison of phenomena occurring at the same energized state, i.e., at equal probe signals; indeed, V_e/V_i gives only an additive term in the ΔpH equation above. Nevertheless, to conform with earlier publications, the data are expressed in ΔpH terms, which are marked 'apparent' on the figures to recall their uncertainties. The electron flow rate with ferricyanide as terminal oxidant was measured spectrophotometrically, in the same cuvette as for ΔpH and V_p, by the absorbance decrease at 420 nm ($Fe^{3+} \rightarrow Fe^{2+}$). ATP was determined by luminescence of the complex luciferine-luciferase added to diluted aliquots removed from the sample.

The proton gradient ΔpH, and consequently the phosphorylation rate V_p given by ATP synthesis, were modulated by decreasing the H^+ influx (slowing down of the redox chain by light reduction) or by increasing the H^+ efflux (creation of new passive leaks by nigericin addition).

The amines were dissolved in buffer for imidazole and NH_4Cl or in ethanol for hexylamine (no solvent effect). At the used concentrations, they did not affect the medium pH and quenched by only a few percent the fluorescence of the probe, which is taken into account in the ΔpH computation.

Results

General effect of amines on proton gradient, electron flow and phosphorylation

Fig. 2 shows a parallel recording of the light-induced pH change of the medium, its continuous rise measuring the approx. 1/1 scalar proton uptake per ATP formed [27], and of the correlated ΔpH-dependent fluorescence quenching of 9-

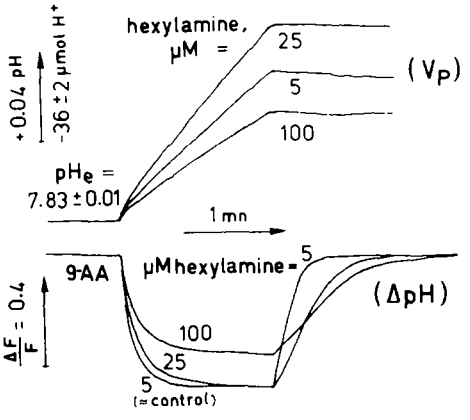

Fig. 2. Recorder traces of external pH shift (top) and of 9-aminoacridine (9-AA) fluorescence quenching (bottom) for three hexylamine concentrations (the control is equivalent to the lowest value). The sustained pH rise is due to the scalar consumption of 1 H^+ per ATP formed, due to acid-base equilibria, and measures phosphorylation rate V_p [27]. ΔpH is computed from probe fluorescence [26]. Chl: 15 μM, slightly buffered medium containing in mM: Tricine 1.5 + KCl 50 + $MgCl_2$ 5 + P_i 0.5 + ADP 0.5. PS I chain (pyocyanine 50 μM). Other details: see Methodology.

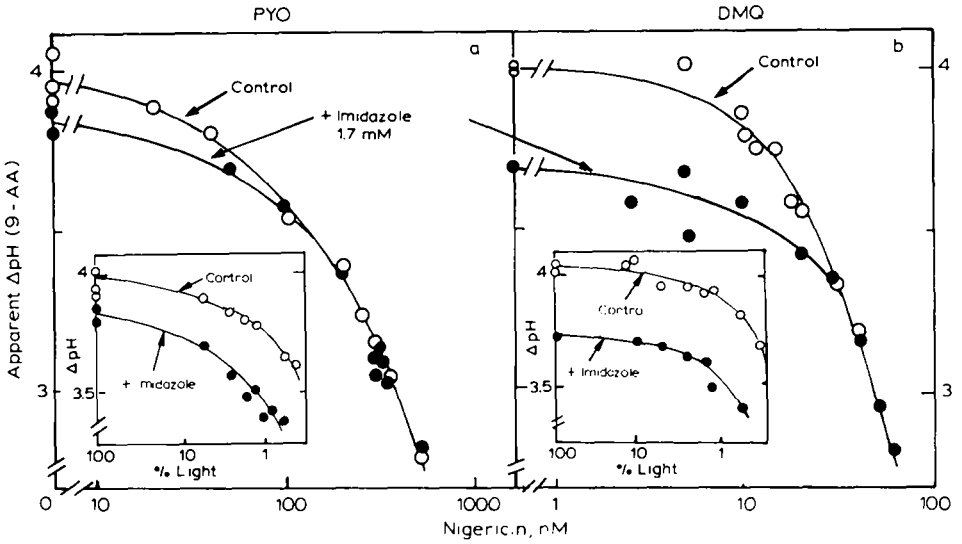

Fig. 3. Differential imidazole sensitivity of ΔpH when the latter is lowered by a H^+ efflux increase (nigericin addition) or by a H^+ influx decrease (light reduction), for PS I (left) and PS II (right) chains. With nigericin, imidazole is ineffective when $\Delta pH \leq 3.5$ for PS I, attained at approx. 200 nM nigericin, and when $\Delta pH \leqslant 3.3$ for PS II, which requires approx. 20 nM nigericin. If light is decreased (insets: log scale, origin on the right), imidazole maintains its effect in all circumstances. Conditions: see Methodology. 9-AA, 9-aminoacridine.

aminoacridine. The control (not shown) was close to the case with 5 μM hexylamine. One should note that, although the steady-state amplitude of ΔpH, i.e., of the fluorescence level, is the same at 25 and at 5 μM, photophosphorylation proceeds at a much faster rate. At 100 μM, both V_p and ΔpH are depressed and, as could be expected from the buffering nature of hexylamine, H^+ influx and H^+ efflux kinetics are affected.

Another result, relevant to the question of an amine effect on the redox chain is shown in Fig. 3 in which PS I (pyocyanine) and PS II (dimethylquinone) -driven electron transfer are compared with and without imidazole at 1.7 mM, i.e., at a concentration used for delocalization. If the ΔpH is sufficiently lowered by nigericin, the proton gradient becomes resistant to imidazole. The lack of amine effect at nigericin concentrations above this critical value, approx. 200 nM for PS I (Fig. 3a) and 20 nM for PS II (Fig. 3b), cannot be due to a limited internal amine concentration, since a significant proton gradient, larger than 3, exists and allows good amine pumping into the lumen [16]. Indeed, similar ΔpH values produced by light

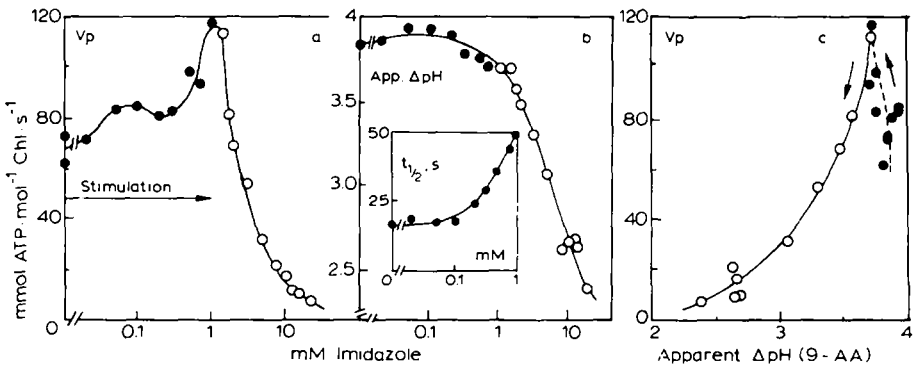

Fig. 4. Concentration-dependent effect of imidazole on phosphorylation rate V_P (a), on ΔpH (b) and on the resulting flow-force relationship (c). Conditions: see Methodology; PS I chain with pyocyanine. 9-AA, 9-aminoacridine.

intensity adjustment instead of nigericin addition still respond to imidazole (inserts in Fig. 3). Thus, if imidazole has no more effect with nigericin, it is certainly because this ionophore is a more efficient uncoupler than the amine, and consequently the H^+ efflux is then negligibly increased by imidazole. Since the ΔpH is unchanged, so is the H^+ influx, i.e., the redox chain. This lack of inhibition was directly checked on the electron flow (V_e-) itself by using the chain $H_2O \rightarrow$ PS II $\rightarrow$ PS I $\rightarrow$ ferricyanide (0.8 mM). The curve of the V_e increase vs. ΔpH decrease was scanned with nigericin. If 2 mM imidazole is added instead of nigericin, the ΔpH is somewhat lowered (from 3.74 to 3.55) but V_e- is increased (from 66 to 85 mmol ATP per mol Chl per s) and the corresponding point is still on the curve traced with nigericin (not shown). Thus, imidazole acts here only as an uncoupler, since if it had inhibited the chain, the point would have been below the curve because V_e would have decreased and not increased.

Fig. 3 shows two additional facts. The first is the different nigericin-sensitivity of the two photosystems: PS I resists more than PS II, likely because the pyocyanine redox loop has a faster turnover than the dimethylquinone chain. The second is that the uncoupling effect of imidazole vanishes at lower nigericin concentrations with PS II than with PS I. The reason must be that the lower ΔpH then attained with PS II decreases more strongly the internal amine concentration, hence reduces its uncoupling ability.

Fig. 4 confirms with imidazole added to thylakoids the earlier reports of a stimulation of phosphorylation by methylamine [11–13]. Moreover, it reveals that very low concentrations of imidazole, up to approx. 0.1 mM, enhance V_p without significantly changing ΔpH. A further increase apparently induces a transient drop, followed by a new rise of V_P. Yet, at the same time the ΔpH starts or continues to decrease, together with a steep lengthening of the half-relaxation time (inset Fig. 4b); a similar effect is given by hexylamine, Fig. 2. Finally, above approx. 1 mM imidazole, both ΔpH and V_P sharply decrease and, as a consequence, the V_P vs. ΔpH curve, Fig. 4c, exhibits two distinct domains of the flow-force relationship.

Fig. 5 extends to other amines (a,b) these observations and shows that similar complex behaviour is also obtained with PS II (c). It must be pointed out that if the concentration curves are variable in shape and amplitude, the overall picture is maintained. Another remark is that the concentration range for hexylamine (pK 10.6) is at least one order of magnitude lower than for imidazole (pK 6.9) or NH_4Cl (pK 9.3). Thus, if the pK of the amines play a role, other parameters, like amine permeability through the membrane, are also important.

Besides the reason for the biphasic concentration curves, discussed below, one main question is why phosphorylation gets higher whilst the proton gradient is unchanged or even diminished ($\Delta\Psi$ is negligibly stimulated: see Discussion). As a working hypothesis, we propose that diffusing amines may rapidly shuttle protons from their redox sources to their points of escape, especially coupling factors; in other words, they lower the lateral resistance R_L, Fig. 1. As a consequence the actual ΔpH at the coupling factors, Δ^cpH, compared to the measured ΔpH (which is a mean value, $\overline{\Delta pH}$) increases, according to the simple equation [28]: $\Delta^c pH/\overline{\Delta pH} = (1 + 2R_B/R_M)/(1 + R_L/R_C)$, where R_B, R_M and R_C are the resistances of the diffusion barriers (between membrane domain and bulk phase), of the membrane leaks, and of the coupling factors, respectively. Since Δ^cpH is raised, so is the phosphorylation rate. On the other hand, the declining part of the V_P and ΔpH vs. the amine concentration curves, hence also their interrelation (open symbols, Fig. 4), obviously results from an uncoupling due to swelling [29] or to an amine-induced backflow of protons (see Fig. 1 left panel and Discussion).

Since before delocalization is complete, uncoupling starts to predominate, the real maximum delocalizing effect is obtained for concentrations above the peak (Figs. 4a and 5). To minimize, nevertheless, the superimposed uncoupling, we have routinely chosen concentrations near to this peak for the next experiments.

Amine-induced equalization of Photosystems I and II effectiveness for phosphorylation

Fig. 6 is an example of the variation of the phosphorylation rate, V_P, and of ΔpH modified by light intensity, from which flow-force relation-

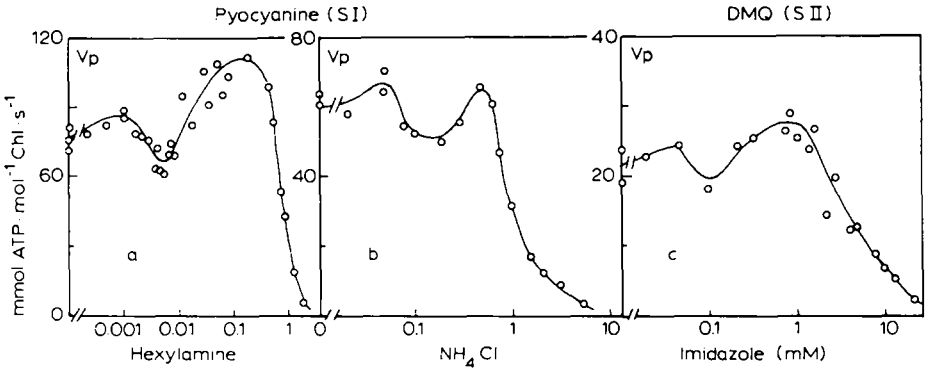

Fig. 5. Concentration curves of amines on PS I (a,b) or PS II (c) chains. Conditions: see Methodology.

ships such as those shown in Fig. 7 are traced (different experiments). In Fig. 6, it is obvious that for an equal ΔpH in low light, i.e., identical quenching of 9-aminoacridine fluorescence, ATP synthesis proceeds at a higher rate with pyocyanine (PS I loop) than with dimethylquinone (PS II chain). According to our hypothesis, this would indicate a lateral ΔpH drop between H^+ redox sources and H^+ phosphorylating sinks [6,8]. Indeed, the greater the distance between these two points, the more significant is the drop and consequently the more pronounced is the local deficit of $\Delta\tilde{\mu}_{H^+}$ across ATPases relative to the mean measured $\Delta\tilde{\mu}_{H^+}$, restricted here to ΔpH. This is expressed by the variation of the $\Delta^c pH/\overline{\Delta pH}$ ratio with the lateral resistance R_L, according to the relation given just above. R_L would be higher with PS II than PS I, because the main location of PS II is in the appressed granal regions while that of PS I is in the exposed membrane areas – essentially stromal lamellae – the only place where the ATPases are found [30].

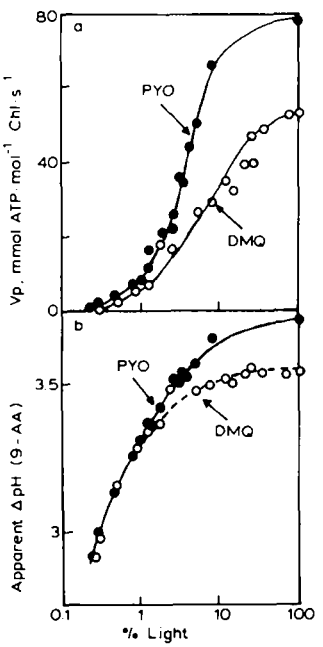

Fig. 6. Light-intensity curves of phosphorylation rate and ΔpH for PS I (pyocyanine) and PS II (dimethylquinone) redox chains. Notice distinct V_P but similar ΔpH in low light (log scale). Conditions: see Methodology. 9-AA, 9-aminoacridine.

However, besides confirming our previous reports [6,8], these experiments reveal the intrinsic ΔpH-delocalizing property of amines. Fig. 7a, right, shows that, as expected from the proposed lateral shuttle mechanism, imidazole at an optimum concentration (Fig. 4a) superimposes the pyocyanine and dimethylquinone curves of V_P vs. ΔpH. In other words, imidazole reduces the resistance between PS II – and PS I – and the ATPases, through an R_L and/or an R_B decrease (see above). Fig. 7b extends this conclusion to hexylamine in a hypotonic saline buffer, a combination similar to that used in Refs. 9 and 10. Without amine (control Fig. 7b, left), delocalization by this medium was only partial, although generally it is complete [7] because swelling of the lumen and change of its ionicity is normally sufficient to decrease the lateral resistance. But if hexylamine is added (Fig. 7b, right), PS I and PS II curves are again superimposed. Notice that the concentration needed to obtain this result is much lower than in the

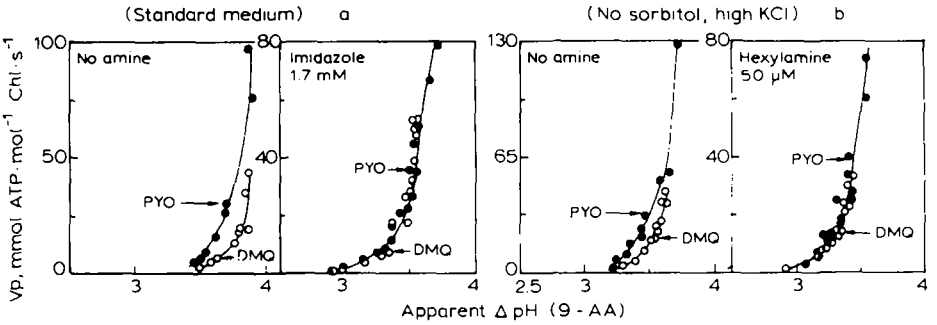

Fig. 7. Delocalizing effect of amines expressed by merged PS I and PS II flow-force curves. Notice the different concentrations used for imidazole and for hexylamine, more lipophilic and of higher pK. Conditions: see Methodology; different experiments.

imidazole case: 50 µM vs. 1700 µM. Incidently, this concentration is within the range of hexylamine used as a radioactive probe.

Combined ΔpH-delocalizing effect of nigericin and imidazole

It has been reported, first with chromatophores [31] and then with thylakoids [7,32,33], that ATP synthesis is more depressed when the ΔpH is lowered by a H^+ influx decrease (light) than by an efflux increase (nigericin). A classical picture of this is given in Figs. 8 and 9 by solid and dashed traces, respectively. This was taken as a good argument in favour of the existence of local proton gradients, albeit the fact that the mechanisms differ according to the investigators. Fig. 8 also shows that the nature of the redox chain, PS I or PS II, does not matter.

The presence of imidazole has several consequences revealed by analysing Fig. 8. First, all curves are moved towards smaller ΔpH values which means that imidazole greatly enhances phosphorylation for a given ΔpH. The difference between the curves of V_P vs. ΔpH with PS I or PS

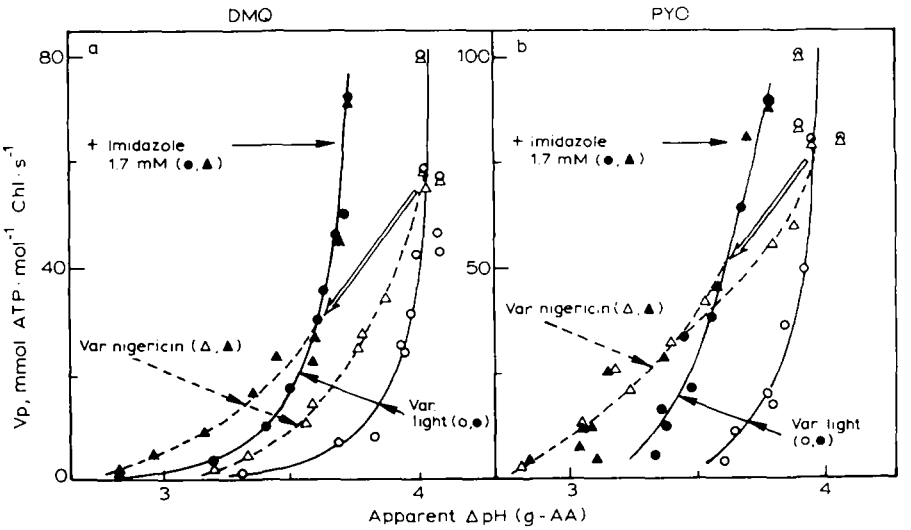

Fig. 8. Combined nigericin and imidazole effects on the flow-force relationships of PS II (a) and PS I (b) chains. The ΔpH shift induced by imidazole (●, ▲) is less pronounced (a), if not abolished (b), when nigericin addition (Δ, ▲) instead of light reduction (○, ●) is used to lower ΔpH; also, the point of departure of nigericin (------) and light (———) curves is shifted by imidazole towards lower phosphorylation rates, V_p, and lower ΔpH: see the white oblique arrows. Conditions: see Methodology; nigericin range for PS I: 100–500 nM, for PS II: 5–60 nM (see text for comments). 9-AA, 9-aminoacridine.

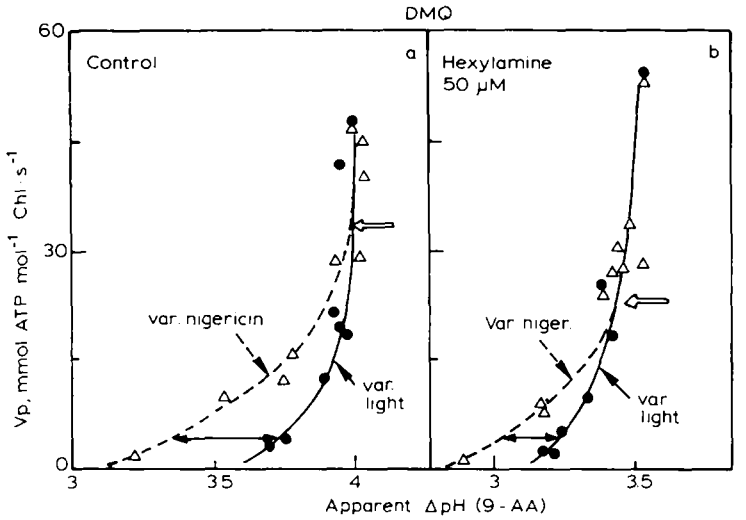

Fig. 9. Combined nigericin and hexylamine effect on flow-force relationships with PS II chain. ΔpH lowering by nigericin addition (△) or light reduction (●); notice the reduced ΔpH distance between the curves (↔) in presence of hexylamine and the shift of nigericin (------) and light (———) curves, indicated by the white arrow. Conditions: see Methodology. 9-AA, 9-aminoacridine.

II is also made smaller and even abolished, as in the right-hand parts of Fig. 7a and b. Second, the divergence between nigericin and light curves is reduced. This is seen by comparing the ΔpH distance between the dashed and solid lines of imidazole and control curves at a given V_P, for instance 25 mmol ATP per mol Chl per s. Notice also that the starting point of the separation between the nigericin and light traces is shifted downward to the left on the graph, which is indicated by open oblique arrows. Third, in the pyocyanine case, the nigericin curves with and without imidazole merge below a critical ΔpH (approx. 3.3). Fourth, as can be seen in Fig. 8 and was already apparent from Fig. 4, PS I requires much higher nigericin concentrations than PS II to produce a similar sized effect.

The replacement of imidazole by hexylamine does not alter these changes, except that about 30 times less amine is sufficient (Fig. 9). Thus, the different amines act here in a similar fashion.

A plausible interpretation of the crossed effect of imidazole and nigericin is that the latter, in addition ot being the well-known transmembrane ionophore, can also serve as a fast lateral H^+ carrier along the membrane surface, if not inside it. The final result is a homogenization of the proton gradient within the vesicle. It would therefore be the same to that obtained with amines, even though the actual targets and molecular mechanisms may differ. As a consequence, when the nigericin concentration is high enough, its effect would overcome that of the amines which would only function as capacitive buffers, concealed in the steady state. This explains the merging of the PS I flow-force curves with and without amine in the presence of nigericin. A similar merging cannot be observed with PS II because if the concentration of nigericin used was the same as for PS I, 100–200 nM, it would fully inhibit PS II-driven phosphorylation. Finally, one should recall that no special attention should be paid to the values given for ΔpH on the figures as they have only a qualitative significance and were computed and marked on the scales as apparent ΔpH, only to allow comparison with earlier data.

The insets of Fig. 3 show that if ΔpH is lowered by decreasing H^+ influx through light dimming, its amplitude and, of course, kinetics remain sensitive to amine. This confirms the synergetic uncoupling by nigericin and imidazole, proposed above. In the absence of nigericin, as in the insets, this amine effect is no longer masked by the ionophore.

Discussion

Overall effect of amines on phosphorylation

A first point is the complexity of the concentration curves presented in Figs. 4 and 5. Because the lowest concentrations were not previously explored in detail [11,12], the first shoulder has not been reported before. Besides the complex physico-chemical effects of amines on the proton-conducting structures, one may suspect that this biphasicity reflects the existence of two types of energy-transducing system: one minor subgroup or compartment would be highly sensitive to amines, the other significantly less. Such a heterogeneity may have different origins, but we can exclude the presence of distinct chloroplast populations, according to our flow cytometry and microscopic (several modes) controls [35]. An obvious cause of heterogeneity could be that appressed and exposed thylakoids, rich in PS II or in PS I, have their own sensitivity to amines; however, the two-stepped concentration curves are observed with either of these photosystems (Fig. 5). Thus the heterogeneity, if any exists, more likely results from a compartmentation within the thylakoids themselves and could even result from different H^+ pathways [20,34], which would be but a special version of localized chemiosmosis...

The stimulation of V_P would be a consequence of an increase of the proton gradient available to the coupling factors relative to the measured average ΔpH. The cause of this would be a lessening of the proton potential drop between the H^+ redox sources and the phosphorylating enzymes. That is, the lateral resistance R_L linking these two points and/or the diffusion barriers R_B, Fig. 1, would be reduced by the proton-carrier properties of the amines.

In agreement with Rottenberg [36], we consider that the uncoupling by amines is not simply a buffer effect [29]. This would only slow down the formation or decay of the ΔpH, not decrease its amplitude. It rather implies a backflow of protons by an amine cycling of H^+ in the membrane, a shuttle of RNH^+ across the membrane, or a salt leak (Fig. 1). The two latter cases would limit the massive amine uptake proposed by Crofts [29] and therefore the thylakoid swelling. The shuttle will also compensate the $\Delta\Psi$ increase, since RNH^+/H^+ exchange is neutral.

It is implicitely suggested here that the lumen phase restricts H^+ movement and therefore is at the origin of distinct local proton gradients in the energized state. Although the partition, the narrow layer between adjacent external membrane faces within appressed areas of the granum, can also play a role [25], it is not affected by amine accumulation. Finally, it should be noted that the V_P stimulation with PS II is always smaller than with PS I and that the corresponding amine concentrations in the medium are lower. This is understandable because the internal amine concentration is also lower and, inasmuch as PS II is relatively far from the coupling factors, the H^+ route linking these two sites may be more frequently punctuated with multiple amine-induced leaks. This would mean that uncoupling predominates delocalization well before the latter is complete. A similar picture is given when considering nigericin instead of amines (Fig. 4).

ΔpH and amines

Firstly, it is necessary to comment on how the ΔpH is measured with 9-aminoacridine and, more generally, with amines used as probes. It is certain that 9-aminoacridine overestimates the ΔpH [37,38], but absolute values are not required in comparative experiments. It is even not important to determine exactly the origin of the ΔpH axis or to have its scale obeying a linear law. What we are interested in is to establish if, for a given energized state, viz. probe signal, a ΔpH-dependent phenomenon has a single value for any situation as delocalized chemiosmosis requires: PS I vs. PS II, H^+ influx vs. efflux, etc. To suspect, as in Refs. 9 and 10, that ΔpH may be different for identical probe signals – for example, with different redox chains – is nothing more than to admit that the proton gradient may be heterogeneous, i.e., localized, unless unproven distinct artifacts exist in one situation but not in another. Thus, one could think that the probe-membrane interactions or the internal volume V_i may depend on the way the ΔpH is achieved. Several controls, such as a comparison of glass electrode and 9-aminoacridine signals [7], with and without imidazole [39], have

shown in fact that a unique relation, though not linear, links the total proton uptake from the medium to internal acidification, i.e., to the computed ΔpH. Thus, in the absence of delocalizing factors, PS I and PS II do indeed have distinct phosphorylating efficiencies for identical ΔpH. The probe response, even if submitted to side effects, is by itself independent of the type of redox chain. On the other hand, there is no reason to believe that V_i changes with PS I or PS II, unless these two systems belong to separate compartments with specific V_i variations or, in disagreement with classical chemiosmosis, if their energized state differ for a same global ΔpH.

Concerning the proper effect of amines on ΔpH evaluation, it is certain that amine pumping inside thylakoids raises their osmotic pressure and thereby increases their internal volume V_i (swelling). But, as already discussed by Giersch [11], a larger V_i would overestimate the ΔpH, and not the reverse, since more probe would then be trapped inside and therefore quenched. Consequently, the amine and control flow-force curves, Figs. 8 and 9, could only become more separated if the ΔpH values were corrected for this effect. In fact, below (hexylamine) or around (imidazole) millimolar range, amines do not much affect V_i, in light, judging from the limited scattering change (not shown). To become significant, such effects require higher amine concentrations.

Besides V_i, amines could also alter 9-aminoacridine interaction with the membrane. Again this significantly occurs only at a much higher concentration than used for delocalization. Thus, Figs. 8b (dashed curves) and 3 indicate that when the amine uncoupling effect is overcome by sufficient nigericin, identical ΔpH values are obtained with and without amines. That is, at delocalizing concentrations, amines do not bias ΔpH measurements relative to the control conditions.

In conclusion, provided ΔpH values are taken for what they are, that is indicative, which is sufficient for the present aim, no special restrictions alter the conclusion drawn for the results displayed in Figs. 3 and 7–9.

Finally, the steady-state ΔpH in strong light is lowered by amines at sufficient concentrations. A lower H^+ influx would imply an inhibition of the redox chain or a decrease of the H^+/e^- ratio (a disconnection of a Q_b cycle ?). This alteration of the redox chain has been excluded here, both in uncoupled and in coupled conditions. A higher H^+ efflux is thus more probable, even though it is hidden by the capacitive effect of amines during the dark decay of the ΔpH.

$\Delta\Psi$ and amines

The capture of a proton by a neutral amine preserves the free positive charge. Therefore, one always may suspect that $\Delta\Psi$ increases in parallel with amine pumping, a point which did not escape Giersch's attention. He estimated that methylamine raises the $\Delta\Psi$ of chloroplasts by about 4 mV [11], this effect being minimized in low light [12], which could be expected, since less amine is then translocated. In the steady state, however, ΔpH should not be changed, unless, as shown above, amines act as protonophores.

We have reinvestigated on thylakoids how $\Delta\Psi$ is affected by imidazole and hexylamine by measuring the electrochromic shift at 515–520 nm (amplitude, kinetics and difference spectrum: to be shown elsewhere). As previously discussed [37], the fast $\Delta\Psi$ decay, after steady-state illumination, mainly expresses the potential difference between the internal and external bulk phases, whereas its slow decay represents the slow deprotonation of the internal membrane face accompanying the H^+ return to the medium, i.e., the ΔpH dissipation. When amines are present, this slow phase is combined with a scattering signal rendering its quantitative estimation delicate. Nevertheless, by refering these changes to the (pseudo-)isobestic point at 490 nm, we could compute the $\Delta\Psi$ value using the calibration by Schapendonk and Vredenberg [40]. In our conditions and for amines near peak stimulation of phosphorylation rate, such as in Figs. 4 and 5, $\Delta\Psi$ is augmented by at most 1 mV over the approx. 7 mV of the control. Thus, we consider that a $\Delta\Psi$ increase cannot compensate the larger computed ΔpH decrease induced by amines. This does not rule out that more dramatic changes may not be observed at higher amines concentrations.

One may question why $\Delta\Psi$ changes so little. One reason, suggested by Rottenberg [36], is that the molecule RN, once protonized inside, may leave the lumen with its charge, RNH^+ (see Fig.

1). However, this does not take into account the extra H^+ uptake induced by amines of sufficiently low pK such as imidazole [15,16]. We would prefer a charge neutralization by anion imports, especially Cl^- [29]. However, the resulting increase of internal osmotic pressure, hence lumen swelling which leads to a scattering enhancement, may be limited by a backflow of the neutral salt RNHCl, as already proposed above and sketched in Fig. 1.

Conclusion

From this work is appears that permeant buffers such as amines can efficiently carry protons from their points of active translocation to their points of escape: passive leaks or phosphorylating channels. In addition, they may interact with membrane components or with the solvent (water), and eventually suppress the resistances which may hinder proton currents (Fig. 1). Thus, an initially microchemiosmotic type of coupling can take a delocalizing character.

The molecular basis of H^+ conduction, thus of the amine effect, may be multiple. We shall here restrict ourselves to concentrations below or near to the main peak of phosphorylation stimulation (Figs. 4 and 5).

Several hypotheses have been proposed to account for the diverse peculiarities of energy transduction. Thus, to explain the K_m variability for ADP [41–43] or of the catalytic mechanism [44] of phosphorylation, some authors have suggested an interaction between electron-transport chains and coupling factors. This is similar to extreme models of local coupling, like 'mosaic chemiosmosis' [45] or 'collisional' mechanism [46]. Such a privileged connection could explain why PS II and PS I chains have different phosphorylating capabilities, which perhaps may be affected by amines. However, the proposed interaction between coupling factors and PS I [43] would make this chain less, and not more, efficient than PS II, contrary to what is reported here. Moreover, K_m experiments have been recently criticized with good reason [47]. To exclude the classical redox modulation of the coupling factors, we have established that thiol reduction of ATPase with DTT (dithiothreitol) does not change the difference between the flow-force curves traced by varying light or by adding nigericin [1].

Concerning direct amine effects, Giersch and coworkers [11–14] have discussed the removal of an unspecified 'kinetic barrier', the coupling factor possibly being the sensitive target. We rather think that amines may interact with the H^+ network, whatever it is made of: membrane-bound water [5], polar lipid heads [48] or polypeptide chains [24], in addition to bulk water, if any exists when the free space is restricted as in the lumen or the partition. Although the first three conductors are potentially more efficient than free water, they may in fact exist only as small patches of low-resistance H^+-conducting structures separated by some gaps. Thus, water is organized quite differently around apolar and polar groups, and in the latter case, depending on the charge. Around or below their pK – a situation reached inside the thylakoid when it becomes acidic – the charge imbalance is strongly reduced (the global pI of the inner membrane face is around 4 [49]). At the same time, the entering amines are protonized. So, in the energized state, the physico-chemical situation in the lumen is completely different from what it is in the relaxed state and this would certainly affect H^+ conduction (notice that then $[RNH^+] \gg [H_3O^+]$). Thus, amines may be able to fill the gaps suggested above, and would therefore decrease the global lateral resistance R_L shown in Fig. 1. In addition, the diffusion barriers R_B shown on the same figure, perhaps of peptidic nature considering the high amount of proteins in the lumen [50], may be altered by amine interaction: cf. the well-known 'denaturating effect' of ammonium sulfate. This would lower R_B and thereby allow bulk water to shunt R_L. All this would work towards an equalization of the proton electrochemical potential, i.e., would delocalize $\Delta\tilde{\mu}_H$.

Nigericin may also be able to homogenize proton gradients by carrying protons along (or in ?) the membrane in addition to do that, as classically known, across it. The lipophilic character of the ionophore facilitates such a lateral shuttle, and, in some respect, the long aliphatic chain of hexylamine should also favour a similar mechanism. Of course, the longer the distance between H^+ producers (PS II vs. PS I) and consumers (ATPases), the more transmembrane uncoupling will operate,

TABLE I

SHIFT FROM LOCALIZED TO DELOCALIZED PROTON GRADIENTS

In standard conditions (isotonic media of average ionicity containing impermeant – in the time scale of measurement – buffers), the actual proton gradient for a given $\Delta\tilde{\mu}_{H^+}$ (experimentally, for an equal normalized quenching of 9-aminoacridine fluorescence) would be lower at CF than at PS I and PS II proton-translocating sites. This would be due to a small $\Delta\mu_{H^+}$, i.e., ΔpH, drop along some lateral resistance (see Fig. 1) between the H^+ sources and sinks [8,28]. In the absence of important passive leaks (no protonophores), one may propose this sequence of magnitudes of actual $\Delta\tilde{\mu}_{H^+}$ relative to a given mean value: PS II > PS I > mean > CF or leak, no a priori order existing between leaks and CF. Mean $\Delta\tilde{\mu}_{H^+}$ = bulk $\Delta\tilde{\mu}_{H^+}$ when bulk phase exists. Notice that whereas ΔpH and $\Delta\Psi$ vary in opposite ways from surface to bulk in the Stern-Gouy layer [8], as long as internal pH is different from membrane p*I* (approx. 4 inside [49]), $\Delta\tilde{\mu}_{H^+}$ is constant in this direction, even though, as just explained, it may vary laterally. The described treatments, which may be combined for a higher efficiency, tend to equalize all these singularities through an overall increase of the proton conductance between the H^+ input and output ports. See Fig. 1.

Delocalizing factor	Change of transversal resistance	Change of lateral resistance	Change of diffusion barriers	Change of lumen volume
Low osmolarity	no effect	some effect? [d]	decrease	large increase
High ionicity	no effect	some effect [a]	decrease	decrease? [b]
Permeant buffers (amines)	decrease	decrease	decrease	increase
Protonophores (nigericin)	large decrease	decrease	decrease	~ no effect
[Membrane homogeneization] [c]	[no effect]	[increase or decrease] [c]	[no effect]	[no effect]

[a] 'Some effect' means that the effect may be indirect and thus, especially in the case of high salt, depends also on other factors.

[b] A decrease of lumen volume could result from the decrease of the negative surface potential and, thereby, of the repulsive forces which otherwise push away the two opposing inner thylakoid faces.

[c] This would correspond to an even distribution of membrane components, as yet not achieved: attempts to do that by lipid incorporation into the membrane or by lowering the pH to reduce electrostatic repulsion have led to partial inactivation (data not shown); one may think that through changes of the mutual distances, the lateral resistance between PS I and CF would increase, but that between PS II and CF would decrease, not mentioning a possible overall effect on the resistivity itself.

and this is also delocalizing by nature, since it is equivalent to decrease R_B. Notice that the lateral shuttle effect attributed here to nigericin explains some previous results [7,31,33]. Thus, we had until now interpreted the difference between the flow-force curves established by varying H^+ influx (light) or efflux (nigericin) in terms of a restricted access of the ionophore to the H^+ pathway, going from PS II – or PS I – to the coupling factors. Although accounted for by our multiple-resistance microchemiosmotic scheme, this could also be explained by non-resistant direct coupling mechanisms [31,45]. The proposal of a fast lateral transport of H^+ by nigericin reinforces the concept of a lateral resistance.

From this work and the preceding report [7], a quite open range of factors governing the energy-coupling mechanism and yield is now available (Table I). The image which emerges is of a rather flexible system, able by shifting the coupling mechanism from a localized to a delocalized mode, and vice-versa, to adjust the energy supply by the organelles to the energy requirement of the cell.

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