Maintenance of Photophosphorylation Despite Inhibition of the Transthylakoid pH Gradient by Tetracaine

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Z. Naturforsch. **47c,** 717–725 (1992); received June 26, 1991/February 17, 1992

Photophosphorylation, Energy Coupling, Local Anesthetic, Tetracaine, Salt Concentrations, Localized Protons

The inhibition of the transthylakoid pH gradient, ΔpH, and of photophosphorylation by the local anesthetic tetracaine was investigated with isolated chloroplasts from *Spinacia oleracea* L. Tetracaine strongly inhibited ΔpH in the presence of low salt concentrations. In the presence of high salt concentrations, the inhibition of ΔpH was much smaller. This effect of salt concentration was observed only when both, cation and anion were easily membrane permeable. It was concluded that the effect of salts on ΔpH inhibition was excerted on the inside of the thylakoid membrane. The rate of photophosphorylation, V_P, driven by the PS I-dependent artificial proton carrier phenazine methosulfate decreased with ΔpH in the presence of both, high and low salt concentrations. In contrast, V_P driven by the endogenous proton pumps of PS II + I-dependent linear electron flow was largely independent of ΔpH changes in the presence of low salt concentration. It appeared that energy coupling during linear electron transport, in contrast to artificially produced PS I-dependent coupling, may be localized to membrane-bound proton domains which are not accessible to the employed indicators of ΔpH. The data were discussed with respect to recent hypotheses on localized energy coupling in chloroplasts.

Introduction

During illumination of chloroplasts, an electrochemical gradient of proton potential, $\Delta\tilde{\mu}H^+$, is generated across the thylakoid membrane. There is general concurrence that $\Delta\tilde{\mu}H^+$ is a link of energy between electron transport and photophosphorylation, as suggested by the hypothesis of chemiosmotic coupling [1, 2]. In chloroplasts under steady-state illumination, the contribution of the pH gradient, ΔpH, to $\Delta\tilde{\mu}H^+$ largely predominates that of the electrical potential gradient, $\Delta\psi$ [3]. However, the stationary $\Delta\psi$ is dependent on the concentration of electrolyte and may significantly increase in thylakoid membranes stored in low salt media [4].

Most of the basic postulates of the chemiosmotic hypothesis of energy coupling in chloroplasts are generally accepted today. However, there is a continuous debate on the extent to which $\Delta\tilde{\mu}H^+$ is delocalized over the bulk spaces on both sides of the thylakoid membrane. Considerable evidence has accumulated meanwhile that proton currents between light-driven proton pumps and the thylakoid ATPase may be localized to domains on the surface or in the interior of membranes (for review see ref. [5–7]). The assumption of localized proton currents repeatedly derived from a variability in the relationship between velocity of photophosphorylation, V_p and the magnitude of $\Delta\tilde{\mu}H^+$ [8–10].

A recent line of evidence for the existence of membrane-bound proton pathways in energy coupling was based on the unconventional effects of local anesthetic amines in chloroplasts [11–13]. These amines inhibited ΔpH and photophosphorylation, but had almost no effect on pH-dependent electron flow control [12]. The latter is known to be under control of the lumenal pH and, therefore, supposed to decay along with ΔpH [14]. Since local anesthetic amines are no inhibitors of the capacity of electron transport, it was assumed that electron flow control or photophosphorylation, or both, were associated with localized proton domains. A proton domain was defined as a space, containing potentially mobile protons which are not in energetic equilibrium with protons in the bulk phases [7].

Abbreviations: AA, 9-aminoacridine; CCCP, carbonylcyanide-chlorophenylhydrazone; Chl, chlorophyll; MV, methylviologen; ΔpH, proton concentration gradient; $\Delta\tilde{\mu}H^+$, electrochemical potential gradient of protons; PMS, phenazine methosulfate; PS, photosystem; V_I, V_E, volumes of the intra- and thylakoid spaces; ψ_o, surface electrical potential; $\Delta\psi$, transmembrane electrical potential gradient.

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D-W-7400 Tübingen
0939–5075/92/0900–0717 $ 01.30/0

It was the object of this study to support the hypothesis of localized energy coupling in isolated chloroplasts. We intended to show that the relationship between V_P and $\Delta\tilde{\mu}H^+$ may be severely influenced by the pathway of proton translocation to the inside of the thylakoid membrane. For this purpose the build-up of $\Delta\tilde{\mu}H^+$ was either driven by photosystem (PS) II + I or solely by PS I. $\Delta\tilde{\mu}H^+$ was then inhibited with the local anesthetic 4-butyl-aminobenzoic acid-(2-dimethylaminoethyl)-ester, tetracaine and determined together with the velocity of photophosphorylation, V_P. Since the concentration of electrolyte had a major effect on the efficacy of ΔpH inhibition by tetracaine, these investigations were carried out in high and low salt media.

Materials and Methods

Chloroplast preparation and reaction medium

Intact chloroplasts were isolated from secondary leaves of 3–5 week-old plants of *Spinacia oleracea* L. *cv.* Monatol. The leaves were grinded in a medium containing 300 mM sorbitol, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 10 mM KCl and 25 mM morpholinoethanesulfonic acid, adjusted to pH 6.1 with KOH. The homogenate was filtered for removal of large-sized cell material and the remaining chloroplast containing fraction was sedimented by centrifugation. The chloroplasts were washed twice in storage medium containing 310 mM sorbitol, 0.5 mM EDTA, 0.5 mM $MnCl_2$ and 10 mM hydroxyethyl-1-piperazineethanesulfonic acid (Hepes), adjusted to pH 7 with KOH. The suspension of intact chloroplasts in storage medium was kept in the dark on ice until use. The initial integrity of chloroplasts was between 80 and 95%. Osmotical rupture of the chloroplast envelope was performed immediately before use. All experiments were carried out in a medium containing 100 mM sorbitol, 10 mM Hepes/KOH, pH 8 and additions of salt as individually described. 50 nM valinomycin was added to all samples to accelerate the equilibration of potassium concentrations between the medium and thylakoid lumen.

Determination of ΔpH

The light-induced ΔpH was calculated either from AA fluorescence quenching [15] or from NH_3 uptake of osmotically shocked chloroplasts [16]. I) AA fluorescence quenching: Fluorescence was excited with light of a peak wavelength of 400 nm and was detected at 460 nm wavelength. The AA concentration was 5 μM. ΔpH was calculated from fluorescence quenching following the equation

$$\Delta pH = \log(\Delta F \cdot F^{-1} \cdot V_E \cdot V_I^{-1}). \quad (1)$$

(ΔF and F: fluorescence quenched and remained after 120 s in the light. V_E and V_I: volumes of external and lumenal spaces.)

II) NH_3 uptake: The uptake of NH_3 was measured with an ammonia-sensing electrode (Colora, Lorch, F.R.G.) at 20 °C. In a stirred cuvette, 60 μM of NH_4Cl was added to osmotically shocked chloroplasts. The actinic light was thoroughly filtered from heat, since the NH_3 electrode was sensitive to temperature changes. A calibration of NH_3 concentration was performed in each sample by stepwise increase of the NH_4Cl concentration from 40 to 60 μM. ΔpH was calculated from the equation

$$\Delta pH = \log([NH_4^+]_I \cdot [NH_4^+]_o^{-1} \cdot V_E \cdot V_I^{-1}). \quad (2)$$

($[NH_4^+]_i$ and $[NH_4^+]_o$ denote the amounts of NH_4^+ bound by thylakoids and free in the medium.)

Determination of intrathylakoid volume

Osmotically shocked chloroplasts were incubated with 21 kBq ^3H_2O and 11 kBq [^{14}C]sorbitol for 120 s. The chloroplasts were then sedimented, still in the light, through a 5 mm layer of silicon oils AP 150 and AR 20 (Wacker, München, F.R.G.) into a compartment containing 3 M $HClO_4$. V_i was calculated from the radioactivity in the $HClO_4$ compartment following ref. [17].

Photophosphorylation

Light-driven ATP formation by osmotically shocked chloroplasts was allowed for 120 s after “light on” at room temperature, in the presence of 0.5 mM ADP, 2 mM K_2HPO_4 and 2 mM $MgCl_2$. Electron transport was driven either by PS II + I or solely by PS I. Amounts of ATP synthesized during PS II + I-dependent electron flow were determined enzymatically, using hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). Amounts of ATP synthesized during PS I-dependent electron transport were determined with the luciferin/luciferase (EC 1.14.14.3) method.

Electron transport

PS II + I-dependent, linear electron flow from H_2O to oxygen was catalyzed by 50 µM methylviologen, MV, and was excited by red light (filters: RG 630, Schott and Oriel 3-57400, Lot). Cyclic electron flow around PS I was mediated by 50 µM phenazine-methosulfate, PMS, in the presence of 100 nM dichlorophenyl-dimethylurea and was excited by far-red light (filters: RG 695, Schott and Oriel 3-57400, Lot). The light flux density was 2500 $\mu E \cdot m^{-2} \cdot s^{-1}$, photosynthetically active radiation in both cases.

Tetracaine fluorescence

Spectra of fluorescence excitation and emission of 10^{-4} M tetracaine were measured in the absence of chloroplasts, in reaction medium at pH 8 and 20 °C. The salt concentrations were varied as described below. Tetracaine fluorescence was excited with light of 279 nm wavelength and detected at 371 nm. The bandpass was 10 nm.

Results

Tetracaine and salt effects on ΔpH

Tetracaine inhibited the build-up of the light-induced ΔpH, driven by linear, PS II + I-dependent electron transport (Fig. 1). The strength of inhibition was essentially dependent on the electrolyte concentration in the reaction medium. In the presence of only 6 mM KCl, inhibition was distinctly stronger than in the presence of 156 mM KCl. Apparently, high concentrations of KCl partly protected ΔpH from tetracaine effects. The mere increase of KCl concentration affected ΔpH only slightly. The maximum effect of 156 mM KCl occurred after less than 1 min incubation time with chloroplasts, when 30 nM valinomycin were present in the assay. A comparable experiment using the uncoupler carbonylcyanide-chlorophenylhydrazone, CCCP, for inhibition of ΔpH did not reveal a comparable effect of KCl (not shown).

The salt effect described was not specific for KCl. Also $MgCl_2$ (Fig. 2) and $BaCl_2$ (Table I) were effective. The effect of $MgCl_2$ was saturated already at about 30 mM of salt added. When SO_4^{2-} was added as the anion of Mg^{2+}, no effect on uncoupling by tetracaine occurred as compared to samples in the presence of only 6 mM KCl (Table I). The trivalent salt tris-(ethylenediamine)cobalt-III-chloride, TEC, effective for screening electrical charges on thylakoid outer surface [18], was also without effect on ΔpH inhibition (Table I). The data suggest that the salt effects observed are not

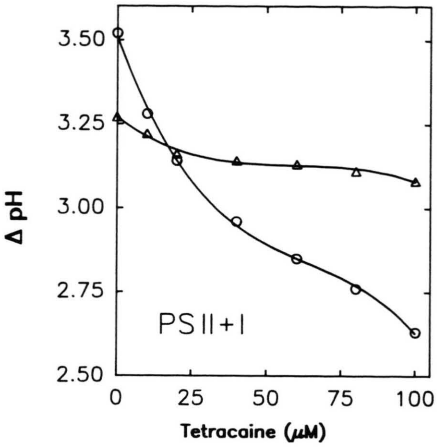

Fig. 1. Inhibition of light-induced ΔpH by tetracaine in the presence of either 6 mM (○) or 156 mM KCl (△). Thylakoid energization was driven by PS II + I-dependent electron transport from H_2O to MV, in the presence of ADP and P_I, in saturating light. ΔpH was calculated from AA fluorescence quenching. The Chl concentration was 20 $\mu g \cdot ml^{-1}$

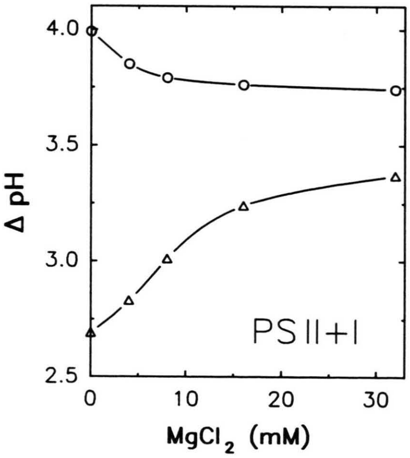

Fig. 2. Effects of $MgCl_2$ concentrations on light-induced ΔpH in the presence (△) and absence (○) of 100 µM tetracaine. Electron transport from H_2O to MV was driven by PS II + I in saturating light. ΔpH was determined from AA fluorescence quenching. The Chl concentration was 10 $\mu g \cdot ml^{-1}$.

Table I. Effect of salt concentrations on inhibition of PS II + I- and PS I-driven ΔpH by tetracaine. PS II + I-dependent ΔpH was driven by electron transport from H_2O to MV, PS I-dependent ΔpH by cyclic electron transport, as mediated by PMS. ΔpH was determined in the absence of ADP, P_I and valinomycin and was calculated from AA fluorescence quenching. The salts were added 5 min prior to tetracaine. The Chl concentration was 10 μg·ml^{-1}. ΔpH values indicated represent the mean from $n \geq 5$ measurements, SD $< \pm 0.15$.

	ΔpH			
	PS II + I		PS I	
Addition of salt	Controls	+100 μM tetracaine	Controls	+100 μM tetracaine
6 mM KCl	4.1	2.6	3.7	2.7
150 mM KCl	3.9	3.2	4.0	3.5
30 mM $BaCl_2$	3.7	3.1	4.1	3.6
30 mM $MgSO_4$	3.9	2.7	4.4	3.4
100 μM TEC	3.9	2.5	3.4	2.5

related to a charge screening by cations on the outer membrane surface. Rather, reactions occurring on the inside of the membrane appear to be affected.

The inhibition of ΔpH by tetracaine was also influenced by an increase of salt concentrations when ΔpH was driven by cyclic electron flow of PS I (Table I, Fig. 3). Also in this case, KCl, $MgCl_2$ and $BaCl_2$ diminished the inhibitory effect of tetracaine. As described for PS II + I-dependent ΔpH, a mere increase of salt concentration only had a small effect on PS I-dependent ΔpH. The values of ΔpH pictured in Fig. 1 to 3 were calculated from AA fluorescence quenching. A comparison of these values with those calculated from light-induced NH_3 uptake showed that both methods delivered comparable results on salt and tetracaine effects (Table II). However, ΔpH calculated from NH_3 uptake was generally smaller than that determined from AA fluorescence.

The uptake of ammonium by energized thylakoid vesicles was assumed to be related to proton uptake according to the equation

$$[H^+]_I \cdot [H^+]_o^{-1} = [NH_4^+]_I \cdot [NH_4^+]_o^{-1} \qquad (3)$$

(subscripts I and o: in- and outside of the thylakoid membrane) [16]. By measuring the concentration of NH_3 dissolved in the external space (reaction medium) at pH 8, pH_i and thus ΔpH could be calculated from the intrathylakoid space, V_I, and the amount of NH_3 taken up in the light, assuming a constant pk_α-value of NH_3 in and outside the thylakoid vesicles. This method was derived from that in ref. [16], but had the advantage of being independent on the present concentration of monovalent cations.

The effects of salt on uncoupling by tetracaine are not due to a specific interaction of salts and tetracaine, *e.g.* salt-dependent dimer-excimer for-

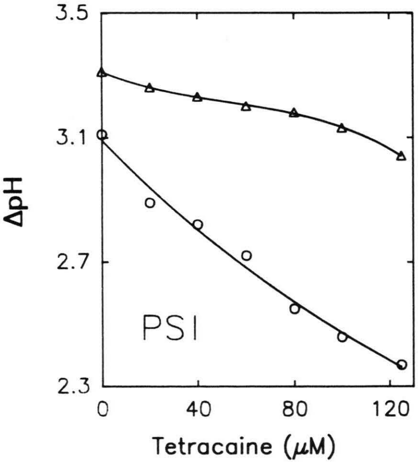

Fig. 3. Inhibition of PS I-dependent light-induced ΔpH by tetracaine, in the presence of 6 mM (○) or 156 mM (△) KCl. Cyclic electron flow was mediated by PMS in the presence of ADP and P_I. The light flux density was saturating for ATP formation. ΔpH was determined from AA fluorescence quenching. The Chl concentration was 20 μg·ml^{-1}.

Table II. Effect of salt concentrations on inhibition of PS II + I- and PS I-driven ΔpH by tetracaine. Determination of ΔpH from NH_3 uptake. ΔpH was driven by linear PS II + I- or PS I-dependent electron transport. The salt concentrations indicated were added 5 min prior to tetracaine. ΔpH was determined using an NH_3-sensing electrode. ΔpH determination was carried out in the absence of ADP, P_I and valinomycin. ΔpH values indicated represent the mean from $n \geq 6$ measurements, $SD \leq 0.09$.

	ΔpH			
	PS II + I		PS I	
Addition of salt	Controls	+100 µM tetracaine	Controls	+100 µM tetracaine
6 mM KCl	2.8	1.7	3.0	1.7
150 mM KCl	2.7	2.4	3.3	3.0
15 mM $MgCl_2$	2.8	2.4	3.3	3.1

mation in the reaction medium, in the absence of chloroplasts [19]. The fluorescence excitation and emission spectra of the amine were not influenced in peak wavelengths or intensity by the ionic strength of the solvent at least up to 400 mM $MgCl_2$ and 800 mM KCl (data not shown). This, however, does not exclude excimer formation during tetracaine binding to the thylakoid membrane.

Tetracaine and salt effects on photophosphorylation

The observed effects of high salt concentration on inhibition of ΔpH by tetracaine should be reflected in the velocity of photophosphorylation, V_P. The inhibition of PS II + I-driven ΔpH by tetracaine in the presence of 6 and 156 mM KCl was portrayed in Fig. 1. ADP and P_I were present in this experiment. A simultaneous determination of V_P reveiled that ATP formation did not follow the indicated changes of ΔpH (Fig. 4). In the presence of 6 mM KCl, ATP formation was hardly affected by tetracaine concentrations up to 100 µM. The related ΔpH, however, was severely inhibited (Fig. 1). ATP formation in the presence of 156 mM KCl, on the other side, was largely inhibited by tetracaine although ΔpH was hardly affected (Fig. 4). This strange effect was further illustrated in Fig. 5, where the velocity of ATP formation was plotted *versus* ΔpH. Under the low salt condition a decrease of about 0.8 unit of ΔpH was almost without effect on ATP formation. In contrast, ATP formation sharply declined with ΔpH in the presence of 156 mM KCl.

A significant Δψ may be built-up in the presence of low electrolyte concentrations [4]. Therefore, we had to consider that Δψ may substitute for ΔpH in ATP formation under the low salt condition. To exclude this effect, ΔpH and ATP formation were assayed in the presence of 6 mM KCl and 100 nM gramicidin. Gramicidin may inhibit Δψ in the steady-state of thylakoid energization and also in the presence of low potassium concentration [20]. As expected, V_P was smaller in the presence of gramicidin although ΔpH was not much de-

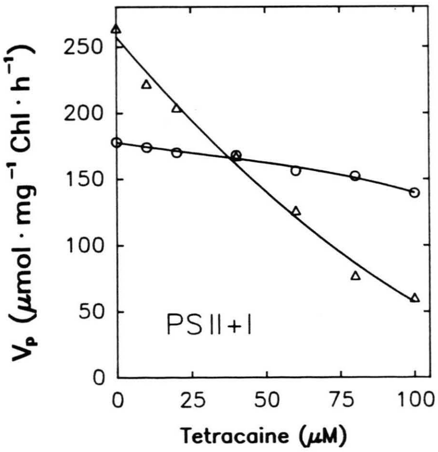

Fig. 4. Inhibition of the rates of PS II + I-dependent photophosphorylation, V_P, by tetracaine, in the presence of 6 mM (○) or 156 mM KCl (△). ATP formation was assayed simultaneously with the ΔpH values shown in Fig. 1. The experimental conditions were as described for Fig. 1.

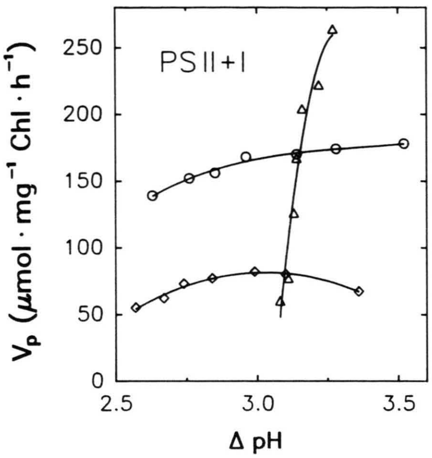

Fig. 5. "Flow force" relationship of the rates of photophosphorylation, V_P, and ΔpH during PS II + I-dependent electron transport. The values of ΔpH and V_P were adopted from Fig. 1 and 4, respectively. ΔpH and V_P were influenced by tetracaine in the presence of 6 mM (○) or 156 mM KCl (△). Additionally, the effect of tetracaine on the relationship of ΔpH and V_P in the presence of 100 nM gramicidin and 6 mM KCl is pictured (◇).

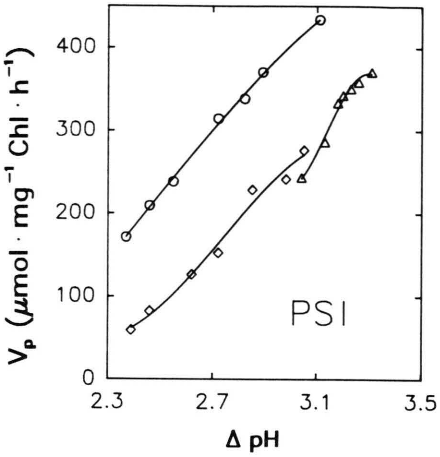

Fig. 7. "Flow-force" relationship of the rates of photophosphorylation, V_P, and ΔpH during PS I-dependent cyclic electron transport. The values of ΔpH and V_P in the presence of 6 mM (○) or 156 mM KCl (△) were taken from Fig. 4 and 6, respectively. Additionally, the relationship of V_P and ΔpH in the presence of 100 nM gramicidin and 6 mM KCl, as influenced by tetracaine, is shown (◇).

creased. This may indicate a significant contribution of $\Delta\psi$ to $\Delta\tilde{\mu}H^+$ under the low salt condition. However, there was still little effect of ΔpH inhibition on V_p (Fig. 5).

Fig. 3 pictured the effect of high and low KCl concentrations on inhibition of PS I-dependent

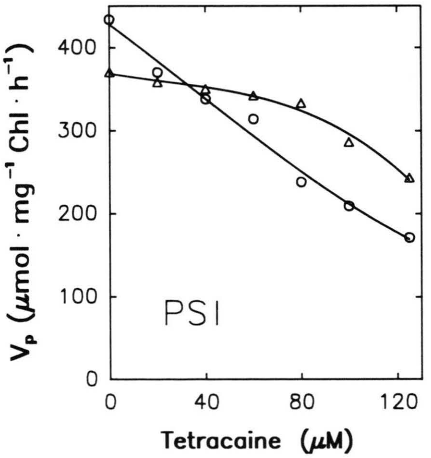

Fig. 6. Inhibition of PS I-dependent photophosphorylation by tetracaine in the presence of 6 mM (○) or 156 mM KCl (△). The ATP formation was assayed simultaneous with ΔpH values, as pictured in Fig. 3. The experimental conditions were as described for Fig. 3.

ΔpH by tetracaine under phosphorylating conditions. The concomitantly measured rates of ATP formation are pictured in Fig. 6. The inhibition of V_P by tetracaine was stronger under the low salt than under the high salt condition. Having in mind the data from Fig. 3, this result may have been expected. When V_P values from Fig. 6 were plotted *versus* related ΔpH values from Fig. 3, a comparable dependence of flow (V_P) and force (ΔpH) appeared under both salt conditions (Fig. 6). No effect of low salt concentration on V_P comparable to that during PS II + I-dependent ATP formation occurred. The influence of 100 nM gramicidin on ATP formation under the low salt condition was tested as for PS II + I-dependent ATP formation. Again, V_P was decreased without a major effect on ΔpH (Fig. 7). Obviously, $\Delta\psi$ contributed to $\Delta\tilde{\mu}H^+$ also during PS I-dependent photophosphorylation.

Discussion

Inhibition of ΔpH by tetracaine

The competence of tetracaine for inhibition of ΔpH was largely reduced by an increase of salt concentrations, *e.g.*, from 6 to 156 mM KCl. This observation was made irrespective of the pathway of electrons involved in the creation of ΔpH (Fig. 1, 3). It may be suggested that for the dissipation of ΔpH by tetracaine protons had to be channelled from the lumenal to the stroma side of the membranes. A limiting step for this process may have been either tetracaine sorption to membrane surfaces or the transport of protonated (monocation) tetracaine across the lipid phase of the membranes.

In particular tetracaine sorption to membrane surfaces appeared to be affected by salt conditions. Thylakoids carry a net negative electrical charge and surface potential, ψ_o [18]. Cations are attracted by ψ_o and give rise to a screening of surface charges [21]. Under the low salt condition applied here, ψ_o may approach −100 mV and lead to a pH decrease in the membrane interphase by attraction of protons. pH in the membrane interphase at $\psi_o = -100$ mV was calculated from the equation

$$\mathrm{pH_i} = \mathrm{pH_b} + F \cdot \psi_o/(2.3 \cdot R \cdot T) \qquad (4)$$

($\mathrm{pH_i}$, $\mathrm{pH_b}$: pH in the interphase and bulk space, respectively; *F, R, T:* Faraday constant, gas constant and absolute temperature) to be 1.7 units below pH in the bulk phase. It is noteworthy here, that $\Delta\tilde{\mu}H^+$ is not affected by ψ_o [22]. Tetracaine located in the thylakoid lumen, especially in the membrane interphase in the light, will exist in its protonated form. In aqueous environment, pk_α of tetracaine is equal to 8.5 [23]. It was suggestive that the concentration of tetracaine in the interphase is decreased and $\mathrm{pH_i}$ is increased by additions of charge-screening cations. Since the lipid permeability of protonated tetracaine is probably lower than that of the uncharged amine [24], it was reasonable to assume that a reduction of the concentration of protonated tetracaine near the membrane surface will decrease the inhibitory potential of the amine.

In a recent study on binding of local anesthetic amines to thylakoid membranes, electrostatic attraction of amine by membrane charges, indeed, was found to be strongly decreased by salts [25]. The mechanism of salt effects proposed here was further supported by the finding that only salt effects on the inside of thylakoid vesicles were able to affect ΔpH inhibition (Table I). With regard to uncoupling, it was this side of the membrane where protonated tetracaine had to enter the hydrophobic environment of membrane lipids.

Relationship of photophosphorylation and ΔpH

Assuming that ΔpH is delocalized over the bulk spaces on each side of the membrane and that photophosphorylation is limited only by the magnitude of $\Delta\tilde{\mu}H^+$, a continual decrease of V_P with decreasing ΔpH should be expected. This assumption was apparently confirmed by the following data: when ΔpH was varied by the uncoupler CCCP or by light flux, about 90% decrease of V_P occurred after a reduction of ΔpH by about 0.35 units [26]. However, in the present and recent reports [8−10], amine effects on photophosphorylation were presented which caused considerable deviation from the relationship cited above [26].

When photophosphorylation was driven by linear electron flow from H_2O to methylviologen, a decrease of salt concentration led to a rather strange relationship of V_P and ΔpH. ΔpH was inhibited almost by 0.9 units, V_P, however, only by 20% (Fig. 5). In contrast, under the high salt condition, or irrespective of salt condition when ATP formation was driven by PS I alone, any decrease of ΔpH was followed by a strong decrease of V_P (Fig. 5, 7). The data presented suggest that the driving force of PS II + I-dependent photophosphorylation in the presence of low salt concentrations is not related to the magnitude of ΔpH. This, of course, would be in contradiction to the postulate of chemiosmotic coupling, requesting a fully delocalized $\Delta\tilde{\mu}H^+$ [1].

Seeking to explain this apparent contradiction, it may be assumed that the AA method [15, 27] is no reliable indicator of ΔpH under the low salt condition. A calculation of ΔpH from NH_3 uptake by illuminated thylakoids may refute this assumption (Table II). Both methods, AA fluorescence quenching and NH_3 uptake, unveiled comparable effects of salt on ΔpH inhibition. A further argument for the reliability of the AA method was that comparable data were obtained for ΔpH driven by PS I or PS II + I (Fig. 1, 4).

A factor which may be misleading in the interpretation of flow force relationship is the contribution of Δψ to $\Delta\tilde{\mu}H^+$. In the presence of only 6 mM KCl, a stationary Δψ of about 60 mV (inside positive) was observed in thylakoid vesicles isolated from spinach [4]. This Δψ may substitute for approximately 1 unit of ΔpH in ATP formation [28]. Valinomycin which was added here to accelerate the equilibration of potassium across the thylakoid membrane, was unsuitable to inhibit stationary $\Delta\psi$.

A comparison of V_P values, as derived from either PS II + I- or PS I-dependent electron flow, gave reliable evidence that PS II + I-dependent ATP formation was not limited by the activity of thylakoid ATPase (Fig. 5, 7). Both types of experiments were carried out under comparable conditions, with the exception of electron transport mediators. Accordingly, it was concluded that $\Delta\tilde{\mu}H^+$ in the presence of 6 mM KCl, was not saturating for ATP formation (Fig. 5). Hangarter and Good [28] demonstrated that ΔpH and $\Delta\psi$ contribute to $\Delta\tilde{\mu}H^+$ in a precisely additive way. Therefore, a further increase of $\Delta\psi$ to at least 100 mV under the influence of 100 μM tetracaine would have been required for maintenance of V_P despite of decreased ΔpH. In the first instance, this was excluded by probing V_P in the presence of the protonophor gramicidin. As expected, gramicidin inhibited V_P in the presence of 6 mM KCl (Fig. 5, 7). It was shown recently that $\Delta\psi$ was largely inhibited by 100 nM gramicidin [20]. However, V_P was still rather independent of changes in ΔpH. Furtheron, the different appearance of the flow-force relationships of photophosphorylation originating from either PS II + I or PS I alone, strongly suggested that $\Delta\psi$ may not dissemble the dependence of V_p on ΔpH selectively for PS II.

Hypothesis on tetracaine effects

Localized proton currents in PS II-dependent photophosphorylation have been postulated recently by Dilley and coworkers (for review, see ref. [7]). The authors supposed a pathway of PS II-dependent, localized (membrane-bound) proton current in the coupling between PS II-dependent electron flow and photophosphorylation [29]. This localized pathway was supposed to be operative only under low salt conditions. Under high salt conditions, protonic coupling was assumed to be delocalized. Therefore, the data presented here, may be compatible with the hypothesis forwarded by Dilley [7].

As a working model, we propose the following mechanism of tetracaine effects. Tetracaine is an inhibitor of the delocalized ΔpH. It may not inhibit a membrane-bound localized pathway of protons from PS II to the thylakoid ATPase. AA fluorescence quenching and NH_3 uptake in the light are indicators for the delocalized ΔpH only. Under low salt conditions, PS II-dependent energy coupling is localized, under high salt conditions, it is delocalized. PS I-dependent coupling is always delocalized, probably because an artificial proton pump was operative, here. The activity of tetracaine is influenced by the salt conditions. High concentrations of salt on the inside of thylakoids inhibit tetracaine effects, *i.e.,* lead to a maintenance of ΔpH. A connection of independent salt effects on inhibition of ΔpH by tetracaine and on the localization of energy coupling may then account for the experimental data shown in this study.

Acknowledgements

Skillful technical assistance was contributed by Margarete Warmus. A critical reading of the manuscript by Gabriele Günther is gratefully acknowledged. This study was financially supported by the Deutsche Forschungsgemeinschaft.

[1] P. Mitchell, FEBS Lett. **43,** 189–194 (1974).

[2] A. T. Jagendorf, in: Encyclopedia of Plant Physiology, New Series, **Vol. 5,** Photosynthesis I (A. Trebst and M. Avron, eds.), pp. 307–337, Springer, Berlin, New York 1977.

[3] W. Junge and J. B. Jackson, in: Photosynthesis, Energy Conversion by Plants and Bacteria, **Vol. I,** pp. 589–646, Academic Press, New York 1982.

[4] H.-L. Huber, B. Rumberg, and U. Siggel, Ber. Bunsenges. Phys. Chem. **84,** 1050–1055 (1980).

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

[6] H. Rottenberg, Biochim. Biophys. Acta **1018,** 1–17 (1990).

[7] R. A. Dilley, S. M. Theg, and W. A. Beard, Annu. Rev. Plant Physiol. **37,** 347–389 (1987).

[8] C. Giersch, Z. Naturforsch. **37c,** 242–250 (1981).

[9] C. Sigalat, Y. de Kouchkovsky, F. Haraux, and F. de Kouchkovsky, Biochim. Biophys. Acta **934,** 375–388 (1988).

[10] U. Pick and M. Weis, Biochim. Biophys. Acta **934,** 22–31 (1988).

[11] H. Laasch and E. Weis, Biochim. Biophys. Acta **936,** 99–107 (1988).

[12] H. Laasch and E. Weis, Photosynth. Res. **22,** 137–146 (1989).

[13] H. Laasch, J. Schumann, and G. Günther, Planta **183,** 567–574 (1991).

[14] U. Siggel, in: Proc. 3rd Int. Congr. on Photosynth., Rehovot (M. Avron, ed.), pp. 645–654, Elsevier, The Hague 1974.

[15] S. Schuldiner, H. Rottenberg, and M. Avron, Eur. J. Biochem. **25,** 64–70 (1972).

[16] H. Rottenberg, T. Grunwald, and M. Avron, Eur. J. Biochem. **25,** 54–63 (1972).

[17] H. W. Heldt, Methods Enzymol. **69,** 604–613 (1980).

[18] J. Barber, Annu. Rev. Plant Physiol. **33,** 261–295 (1982).

[19] M. G. Palmgren, Analyt. Biochem. **192,** 316–321 (1991).

[20] J. N. Nishio and J. Whitmarsh, Plant Physiol. **95,** 522–528 (1991).

[21] D. J. Shaw, in: Electrophoresis, pp. 4–26, Academic Press, London 1969.

[22] D. B. Kell, in: Bacterial Energy Transduction (C. Anthony, ed.), pp. 429–490, Academic Press, London 1988.

[23] A. G. Lee, Biochim. Biophys. Acta **514,** 95–104 (1978).

[24] J. Westman, Y. Boulanger, A. Ehrenberg, and J. C. P. Smith, Biochim. Biophys. Acta **685,** 315–328 (1982).

[25] G. Günther and H. Laasch, Z. Naturforsch. **46c,** 79–86 (1991).

[26] J. W. Davenport and R. E. McCarty, Biochim. Biophys. Acta **851,** 136–145 (1986).

[27] R. Kraayenhof, F. A. de Wolf, H. S. van Walraven, and K. Krab, Bioelectrochem. Bioenerg. **16,** 273–285 (1986).

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

[29] G. Chiang and R. A. Dilley, Biochemistry **26,** 4911–4916 (1987).