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QUANTITATIVE ESTIMATION OF THE PHOTOSYNTHETIC PROTON BINDING INSIDE THE THYLAKOIDS BY CORRELATING INTERNAL ACIDIFICATION TO EXTERNAL ALKALINISATION AND TO OXYGEN EVOLUTION IN CHLOROPLASTS

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Summary

The external alkalinisation ΔpH_e, or the rate of oxygen evolution vO_2, of a suspension of envelope-free chloroplasts was correlated with their internal acidification, estimated from the transmembrane ΔpH_{ei}. Knowing the external buffer value, the concentration of the total protons moved H_i was calculated from the ΔpH_e, measured with a glass electrode ($[H_i]$ was also obtained from vO_2), and the free proton concentration $[H_i^+]$ was determined from ΔpH_{ei}, measured with 9-aminoacridine. This gives a ratio $\gamma_i = \partial[H_i]/\partial[H_i^+]$, which is independent of the thylakoids internal volume. Within a large pH_i range, scanned by varying the light intensity, γ_i was kept reasonably constant; it was hardly sensitive to pH_e. This apparent invariability implies a continuous change of the internal buffer value β_i with pH_i, since $\beta i/\gamma_i = -2.3..... 10^{-pH_i}$, a relationship which includes neither the total concentration of protonizable groups $[A_i]$ nor pK_i. As $\gamma_i \approx K_i[A_i]/(K_i + [H_i^+])^2$, to keep γ_i constant when pH_i drops, pK_i and $[A_i]$ must increase. This may be achieved by a progressive unmasking of anionic functions, initially inaccessible in the membrane. The relative slowness of this process may explain why γ_i calculated from the initial kinetics was sometimes smaller in high than in low light, where it always equalled that measured from the steady-state amplitude at all intensities. A small deficit of $[H_i^+]$ deduced from what could have been expected from ΔpH_e may reflect a limited binding of protons in the membrane itself, about 1 H^+ for 30—130

Abbreviations and conventional symbolism: subscripts e, i, ei, external, internal, external minus internal phase or state of the thylakoid space, respectively; subscripts $^{\circ}$, conditions in darkness $^{\bullet}$ and in light $^{\circ}$; H, total protons (bound + free, H^+); $\beta = \partial[H]/\partial pH$ (buffer capacity, <0); $\gamma = \partial[H]/\partial[H^+]$ (proton binding ratio, >0); μH^+, μequiv./l of protons (free or total); Chl, chlorophyll.

chlorophylls (γ_i could be between 70 and 240, more frequently around 100); these numbers varied depending on the samples, but were constant for a given preparation.

Introduction

It is important to study the proton binding inside the thylakoid for several reasons: (1) the inner buffer capacity or a related value must necessarily be used in any quantitative modelling of the proton movements across the thylakoid membrane; (2) this parameter or any similar one is directly related to the energy storage in chloroplasts (see e.g. Ref. 1), at least in the frame of Mitchell's chemiosmotic hypothesis [2]; (3) any buffering group acts as an osmotic as well as an ionic trap, thereby modulating the magnitude and the direction of water, solutes and cations fluxes (e.g., see Refs. 3, 4); and (4) if the proton binding property is of membranous nature, it will necessarily be involved in the control of the light-induced conformational changes.

Jagendorf and Uribe [5] and Rottenberg et al. [6] have shown that most of the internal protons must be in a bound form; similar problems were treated in mitochondria [7]. Polya and Jagendorf [8], then Walz et al. [9] have measured the overall buffering property of chloroplasts in darkness and under illumination; the latter group attempted to evaluate the inside buffer capacity and estimated that the mean pK_i is close to 5.2. However, in all these works, the outside and the inside proton movements were not measured in a common experiment and, more often, only one of these was studied. This justified to follow simultaneously both events on the same sample, thereby establishing as clear as possible a distinction between the external and internal proton storage properties. A preliminary outline of this work was published [10]. The use of the 9-aminoacridine fluorescence quenching [11] for measuring the light-induced transmembrane pH difference, ΔpH°_{ei}, is debated in Discussion.

Methodology

Material. Envelope-free chloroplasts were extracted from freshly harvested, approximately 2-months-old spinach (*Spinacia oleracea* L.), grown in a regulated chamber (day: 11 h, 22°C, night: 13 h, 12°C), or sometimes from lettuce. About 50 g of washed leaves were chopped twice for 5 s under dim light in a blender with an equal volume of the cold buffer 400 mM sorbitol/10 mM Tricine/10 mM NaCl, pH 7.8, containing fresh 40 mM sodium ascorbate/bovine serum albumin (1 g per l). The extract was filtered through ten layers of cheesecloth and centrifuged for 2 min at 200 × *g*, then again 5 min at 2000 × *g*; the chloroplast pellet was washed once with approx. 100 ml of 10 mM Tricine/10 mM NaCl, pH 7.8, hypotonic buffer and centrifuged for 5 min at 3000 × *g*. The chloroplasts were kept concentrated (2—5 mM Chl) in ice and darkness; they were coupled to at least 80 mmol ATP · mol^{-1} Chl · s^{-1} in the phenazine methosulfate functioning mode and lost only few percent of their activity during the experiment. Chlorophylls *a* + *b* (Chl) were determined in aqueous acetone

with coefficients computed from the data of Mackinney [12].

Measurements. A simple set-up, similar to that previously built [13], was used to measure simultaneously ΔpH°_{ei} and, either ΔpH°_{e} with a glass-calomel combined electrode (kinetic controls being sometimes made with the fluorescein probe [13]), or O_2 evolution with a Clark-type membrane electrode, designed to fit into a 1 × 1 cm temperature-controlled cuvette, magnetically stirred. A weak ($<1\ W \cdot m^{-2}$) blue light at 420 nm (half bandwidth 7 nm), exciting the 9-aminoacridine, was passed through one of the windows, opposite to another through which was passed the strong (approx. $500\ W \cdot m^{-2}$) red light at 650—695 nm (half bandwidth about 95 nm), exciting the chloroplasts. At right angles to these beams, a 10 mm diameter fiber guide conducted the dye fluorescence to the photomultiplier cathode (S-20 type), protected by a set of filters determining a transmission band around 528 nm (half bandwidth about 28 nm). The double channel recorder used has a response time of 1/3 s.

Computation of ΔpH°_{ei} and of ΔpH°_{e} (extent and rate); measurement of the electron flow. It was previously established by one of us that the complete formula relating $[H^{+}_{i}]^{\circ}$ to a monoamine partition across the thylakoid membrane is:

$$[H^{+}_{i}]^{\circ} = (K + [H^{+}_{e}]^{\circ})\left\{\frac{[R_e]^{\bullet}}{[R_e]^{\circ}}\left(\frac{V^{\bullet}_{e}}{V^{\bullet}_{i}} + \frac{K + [H^{+}_{i}]^{\bullet}}{K + [H^{+}_{e}]^{\bullet}}\right)\frac{V^{\bullet}_{i}}{V^{\circ}_{i}} - \frac{V^{\circ}_{e}}{V^{\circ}_{i}}\right\} - K \qquad (1)$$

where K is the amine dissociation constant; $[R_e]$ is the external total amine concentration, measured, for the 9-aminoacridine, by the fluorescence intensity F, the dye concentration being always low; and V_e and V_i are the external and internal volumes of the thylakoids.

If there is no dark pH gradient (of Donnan type), $[H^{+}_{i}]^{\bullet} = [H^{+}_{e}]^{\bullet}$; neglecting the possible variation of V_i in light ($V^{\circ}_{i} = V^{\bullet}_{i} = V_i$) and recalling that the total suspension volume $V_t \approx V_e + V_i$ (omission being made of the membrane 'dead' volume), one finally has, since K is negligible here ($pK \approx 10$):

$$\frac{[H^{+}_{i}]^{\circ}}{[H^{+}_{e}]^{\circ}} = \left(\frac{F^{\bullet}}{F^{\circ}} - 1\right)\frac{V_t}{V_i} + 1 \qquad (2)$$

In this case:

$$\Delta pH^{\circ}_{ei} = (pH^{\circ}_{e} - pH^{\circ}_{i}) = \Delta pH^{\circ}_{e} - \Delta pH^{\circ}_{i} \approx -\Delta pH^{\circ}_{i} = (pH^{\bullet}_{i} - pH^{\circ}_{i}), \qquad (3)$$

since $\Delta pH^{\circ}_{e} << \Delta pH^{\circ}_{i}$

Eqn. 2 is almost identical to the equation of Schuldiner et al. [11], generally used, but is valid down to the situation where F° may equal $F^{\bullet}$. Eqn. 1 is used to appreciate the weight of the different parameters (Haraux, F. and de Kouchkovsky, Y., unpublished data).

V_i was estimated by the method [14] of centrifugation of a chloroplast suspension in various osmotic conditions, determining the interstitial volume of the medium in the pellet by colorimetry or fluorimetry of non-permeant markers Blue-dextran or FITC-dextran (de Kouchkovsky, Y., unpublished

data):

$$V_i\ (l \cdot mol^{-1}\ Chl) = k/\text{osmolarity, hence } \frac{V_t}{V_i} = \frac{\text{medium osmolarity}}{k \cdot \text{chlorophyll molarity}} \quad (4)$$

with $k \approx 2.0 \pm 0.3$.

The external pH shift, ΔpH_e°, was measured in a slightly buffered medium, making that its limits never overpassed about 0.1. In this condition, the external buffer capacity β_e was essentially constant and the influence of the CO_2 equilibrium shift and of the possible chloroplast buffering-property change may be considered negligible, minimizing this source of error.

The rates of the protons fluxes were obtained from the initial slope of the ΔpH_e° rise and from the analysis, point by point, of the 9-aminoacridine fluorescence-quenching time course curve; those of O_2 were computed from the slopes before and after turning on or off the actinic light (calibration was made assuming O_2 concentration from air in water at 20°C = 234 μM, the zero O_2 current being negligible).

The graphical error on the fluorescence level was ±0.5%; the 'noise' of the glass electrode, in the presently used slightly buffered medium of sufficient ionic conductivity, was ±0.002 pH unit and that of the O_2 electrode ±0.1% of the air saturated signal. Typical recordings are displayed on Figs. 3 and 6.

Results *

Correlation between external alkalinisation and internal acidification

The basic postulate is the proton conservation: any proton disappearing from one compartment appears in the other (that is, only negligible amount, if any, of protons may be bound by the membrane). Thus, it should be possible to appreciate the internal buffer capacity by comparing in light the total number H_e° of protons taken up from V_e, and the quantity of free protons H_i^+ released in V_i. ΔH_i° must be identical to $-\Delta H_e^\circ$:

$$\Delta H_e^\circ = -V_e \beta_e\ \Delta pH_e^\circ = \Delta H_i^\circ \quad (5)$$

and

$$\Delta[H_i]^\circ = [H_i]^\circ - [H_i]^\bullet = \frac{\Delta H_i^\circ}{V_i} = -\frac{V_e}{V_i}\beta_e\ \Delta pH_e^\circ \quad (6)$$

β_e is equal, for each pH_e, to the slope of the titration curve of the chloroplast suspension at that pH_e. Below pH 7, the titration curves of the suspension and of the medium were superposable, but at alkaline pH, the suspension was more buffered, due to the residual Tricine buffer brought by the chloroplasts added from the stock solution. On the other hand, no significant change of slope was seen when the suspension was titrated in light; yet, the internal buffer capacity should have changed dramatically between the dark initial $pH_i^\bullet$ ($\approx pH_e^\bullet$) and the final steady-state in the light pH_i°, more than three 'units' below. Therefore, the overall buffer capacity of the suspension was essentially that of the external aqueous phase.

The free protons concentration change is:

$$\Delta[H_i^+]^\circ = [H_i^+]^\circ - [H_i^+]^\bullet \approx [H_i^+]^\circ \quad (7)$$

* Care must be taken not to confuse total protons, H, and free protons, H^+.

since $[H_i^+]^\bullet$, assumed = $[H_e^+]^\bullet$, becomes rapidly $<<[H_i^+]^\circ$ (the latter being computed from Eqn. 2, knowing $[H_e^+]^\circ$).

In a first series of experiments, ΔpH_e° and ΔpH_{ei}° were modulated by varying the actinic light intensity. Fig. 1 shows, as already noticed [6,22], that ΔpH_{ei}° saturates at much lower intensities than ΔpH_e°: the relationship between these two functions is therefore not linear (see insert).

From the correlation between ΔpH_e° and ΔpH_{ei}° (i.e. $\Delta[H_i^\circ]$ and $\Delta[H_i^+]^\circ$), a possible molecular mechanism of internal buffering may be imagined. Three simple models were tested: that of a simple equilibrium of a monoacid, that of a constant β_i, and that of a constant γ_i (ratio of change of total protons to that of free protons). In all cases, ΔpH_e° being small, β_e is considered constant.

(a) Monoacid equilibrium

$$AH_i \overset{K_i}{\rightleftharpoons} A_i^- + H_i^+ \tag{8}$$

$$[A_i] = [A_i^-] + [AH_i] \tag{9}$$

$$[H_i] = [H_i^+] + [AH_i] \tag{10}$$

$$K_i = \frac{[A_i^-][H_i^+]}{[AH_i]} \tag{11}$$

$$\frac{1}{[AH_i]} = \frac{K_i}{[A_i]} \cdot \frac{1}{[H_i^+]} + \frac{1}{[A_i]} \tag{12}$$

Consider what happens in light:

$$[AH_i]^\circ = [AH_i]^\bullet + \Delta[AH_i]^\circ = [AH_i]^\bullet + (\Delta[H_i]^\circ - \Delta[H_i^+]^\circ)$$

$$= [AH_i]^\bullet + [H_i^+]^\bullet - [H_i^+]^\circ + \Delta[H_i]^\circ = [H_i]^\bullet - [H_i^+]^\circ + \Delta[H_i]^\circ \tag{13}$$

where total initial $[H_i]^\bullet$ becomes rapidly negligible in light. Indeed, Eqn. 12 may be written: $[AH_i]/[A_i] = [1 + 10^{(pH-pK)}]^{-1}$; taking, for instance pK_i = overall pK given by Walz et al. [9] (5.2), it is clear that $[AH_i]^\bullet/[A_i]$ is very small at $pH_i^\bullet (= pH_e^\bullet) > 6.6$ (lowest value used here). On the other hand, free protons in light are only a small part of the total ones [5,6]: $[H_i^+]^\circ$ may also be neglected. It comes finally, $\Delta[H_i]^\circ$ being given by Eqn. 6:

$$[AH_i]^\circ \approx \Delta[H_i]^\circ = -\frac{V_e}{V_i}\beta_e \, \Delta pH_e^\circ \tag{14}$$

Therefore Eqn. 12 becomes in light:

$$\frac{1}{\Delta pH_e^\circ} = -\frac{V_e\beta_e}{V_i[A_i]} - \frac{V_e\beta_e K_i}{V_i[A_i]} \cdot \frac{1}{[H_i^+]^\circ} \tag{15}$$

(minus signs are due to the negative character of β_e). Thus, by plotting the reciprocal of ΔpH_e° vs. the reciprocal of $[H_i^+]^\circ$, one should obtain a positive straight line, with a positive zero intercept: this is not the case (Fig. 2a) as could have been expected because of the broad ΔpH_{ei}° explored.

(b) Buffer capacity $\beta_i = \partial[H_i]/\partial pH_i$ constant. From Eqn. 6:

$$[H_i]^\circ = [H_i]^\bullet - \frac{V_e}{V_i}\beta_e(pH_e^\circ - pH_e^\bullet) \tag{16}$$

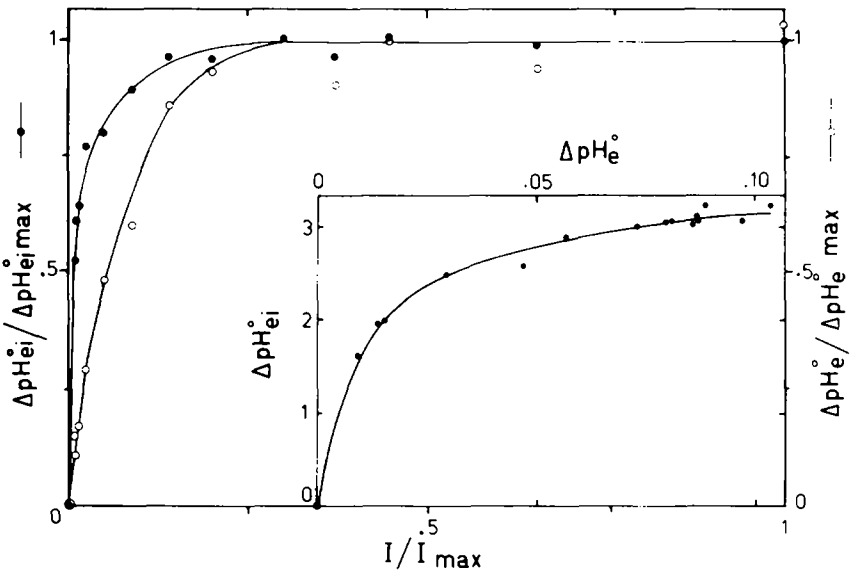

Fig. 1. Variation of ΔpH°_{ei} (approx. ΔpH°_{i}) and of ΔpH°_{e} with the light intensity. •, ΔpH°_{ei}; ○, ΔpH°_{e}. Chloroplast (80 μM Chl) in 100 mM sorbitol/10 mM NaCl + 50 μM methylviologen + 4 μM 9-aminoacridine; $pH^{\bullet}_{e} \approx 7.6$ ($\beta_e \approx -60$ $\mu H^+ \cdot pH^{-1}$). Air, 20°C. $I_{max} \approx 500$ W · m^{-2} red light. Since ΔpH°_{ei} saturates at lower intensities than ΔpH°_{e}, no linear relationship exists between these two numbers (insert): therefore β_i is not constant (see text and Fig. 2b).

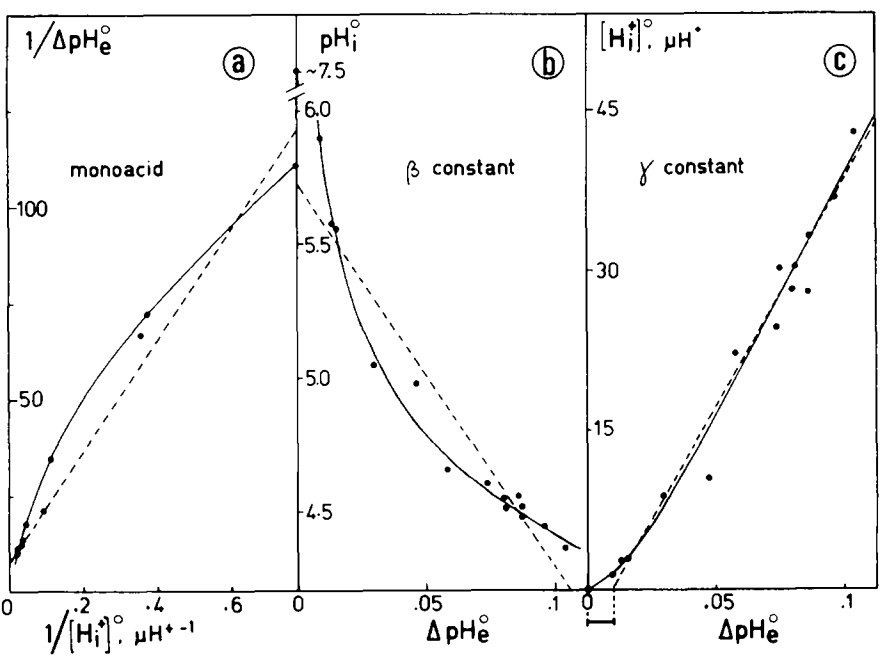

Fig. 2. Three types of correlation between internal acidification and external alkalinisation. Same experiment as Fig. 1. • and ———, experimental points and estimated curve; - - - - - -, theoretical linear regression line if the model tested were exact (see text). Models: (a) simple equilibrium of an internal monoacid; (b) constant internal buffer capacity β_i ($= \partial[H_i]/\partial pH_i$); (c) constant internal proton-binding ratio γ_i ($= \partial[H_i]/\partial[H^+_i]$). In case (c), the regression line intercepts the abscissa at about 0.01 pH unit (small horizontal bar), corresponding to about 0.6 μH^+ (since $\beta_e \approx -60$ $\mu H^+ \cdot pH^{-1}$), that is to an apparent $[H^+_i]^{\circ}$ deficit of approximately 1 H^+/130 Chl.

Thus

$$\beta_i = -\frac{V_e}{V_i}\beta_e \frac{\partial pH_e^\circ}{\partial pH_i^\circ} \tag{17}$$

By integration between $pH_i^\bullet$ and pH_i° it comes:

$$pH_i^\circ = pH_i^\bullet - \frac{V_e}{V_i}\frac{\beta_e}{\beta_i}\Delta pH_e^\circ \tag{18}$$

V_e/V_i is considered invariable and since, experimentally, β_e is constant, as is β_i by the present hypothesis, plotting pH_i° (or ΔpH_{ei}°, since from Eqn. 3, it is $\approx pH_i^\circ - pH_i^\bullet$) vs. ΔpH_e° should give a negative straight line (both β_e and β_i being negative): it is not the case, as shown in Fig. 2b and already deduced from the insert in Fig. 1.

(c) Proton binding ratio $\gamma_i = \partial[H_i]/\partial[H_i^+]$ constant. Replacement of ∂pH_i by $\partial[H_i^+]$ in the above treatment and integration between $[H_i^+]^\bullet$ and $[H_i^+]^\circ$ gives:

$$[H_i^+]^\circ = [H_i^+]^\bullet - \frac{V_e}{V_i}\frac{\beta_e}{\gamma_i}\Delta pH_e^\circ \tag{19}$$

Since, with the exception of the very initial stage of illumination, $[H_i^+]^\bullet << [H_i^+]^\circ$, one may finally write:

$$\gamma_i \approx -\frac{V_e}{V_i}\beta_e \frac{\Delta pH_e^\circ}{[H_i^+]^\circ} \tag{20}$$

The expected linear, positive ($\beta_e < 0$, $\gamma_i > 0$), relationship between $[H_i^+]^\circ$ and ΔpH_e° is acceptably obeyed (Fig. 2c); it was so with all the samples and confirmed by the other methods described below. (An interpretation of the small initial curvature is proposed in Discussion.) Only the resulting γ_i value changed from one day to the other (from 70 to 240), but for a given chloroplast preparation, it was constant. As an average, γ_i was around 100, which means that whatever the pH_i, always about 1% of the total protons coming into the thylakoid remained in the free form.

Acid-base reactions are very fast processes [15,16], and in mitochondria, the buffer equilibrium itself is almost instantaneous compared to the rate of protons diffusion [7]. Based on this analogy, one may consider that the protonizable groups inside the thylakoid are always in a quasi-equilibrium state: therefore, it is a reasonable assumption that what was found in steady-state applies also to kinetics, i.e. that a proportionality factor links the rate of $[H_i^+]^\circ$ change to that of pH_e°. Eqn. 20 thus becomes ($v pH_e^\circ = d\Delta pH_e^\circ/dt$ and $v[H_i^+]^\circ = d\Delta[H_i^+]^\circ/dt$):

$$\gamma_i = -\frac{V_e}{V_i}\beta_e \frac{v pH_e^\circ}{v[H_i^+]^\circ} \tag{21}$$

Fig. 3 shows how these rates are computed (see also Methodology). The expected linearity is illustrated in Fig. 4, where the light intensity was changed, and confirmed in Fig. 5c, where the variable parameter was the chlorophyll concentration. Two situations may be described: in low light (giving up to one-half of the maximum ΔpH_e° and three-quarter of ΔpH_{ei}°), the regression line, which now intercepts the origin, has a slope yielding a γ_i equal to that found in

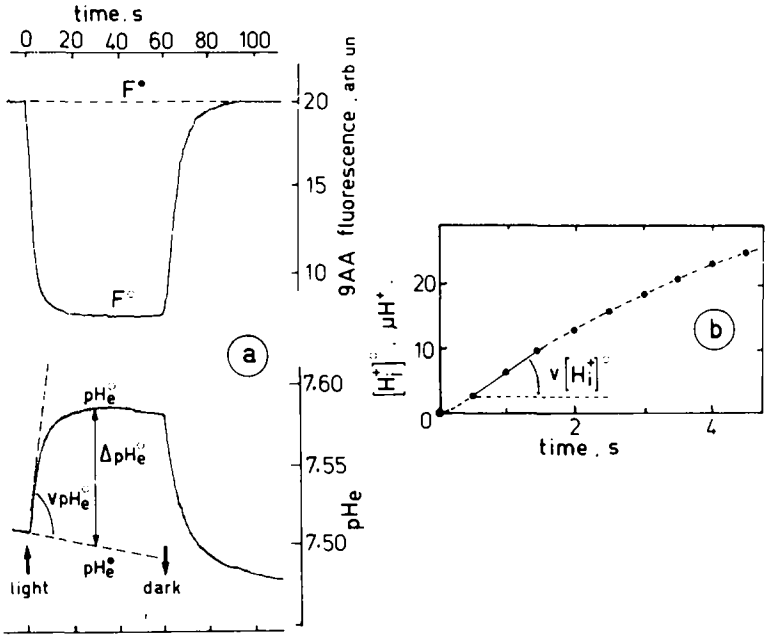

Fig. 3. Simultaneous measurement of external pH_e change and of 9-aminoacridine fluorescence quenching in light. Chloroplasts (80 μM Chl) in 100 mM sorbitol/10 mM NaCl/50 μM methylviologen/4 μM 9-aminoacridine (9 AA); $pH_e^{\bullet} \approx 7.6$. Air, 20°C. Red actinic light ≈45% of I_{max} ($I_{max} \approx 500\ W \cdot m^{-2}$). (a) A recording on a contracted time scale; (b) computation of $v[H_i^+]^\circ$, rate of appearance of free protons in the thylakoid lumen.

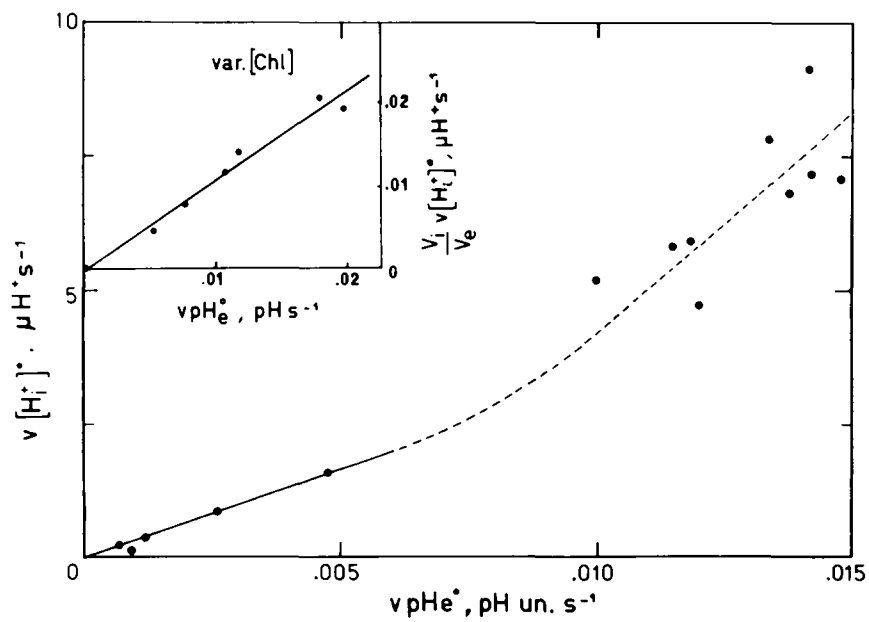

Fig. 4. Correlation between the rates vpH_e° and $v[H_i^+]^\circ$. Same samples as Figs. 1 and 2 (variable light intensity). In low light, the two rates are proportional and no $[H_i^+]^\circ$ deficit is observed (as in Fig. 2c); the slope gives a $\gamma_i \approx 95$, close to that obtained from the steady-state data at all light intensities (about 105). In strong light, the linear regression gives a $\gamma_i \approx 40$. Insert: similar experiment, but with constant maximum light and variable chlorophyll; thus, to normalize the data, the $v[H_i^+]^\circ$ are multiplied by the assumed V_i/V_e ratio, a function of [Chl] as shown by Eqn. 4: the linearity is compatible with a constant γ_i, although here again this kinetically determined parameter was smaller in strong than in low light. (This linearity suggests also that either the glass electrode signal was strictly proportional to the true rate or that its response time limitation had a counterpart in the $v[H_i^+]^\circ$ measurement.)

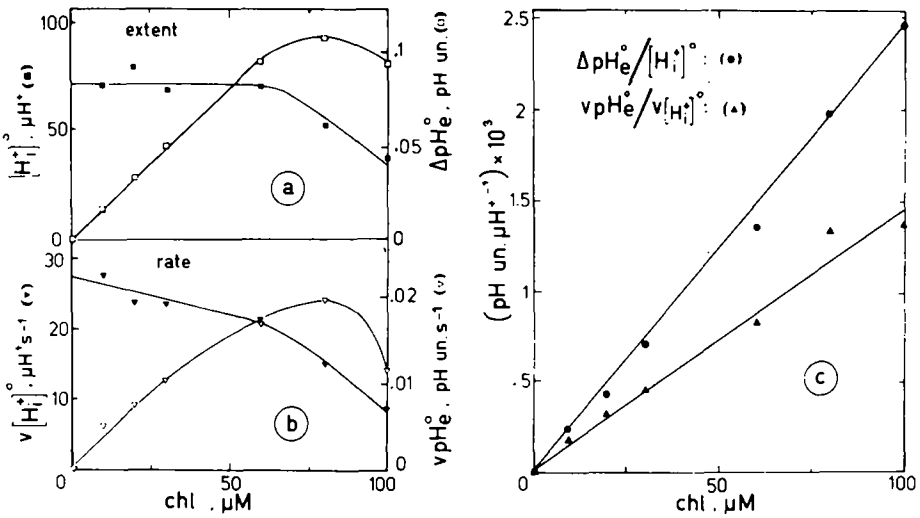

Fig. 5. Variations of ΔpH°_e, $[H^+_i]^\circ$ (extents) and vpH°_e, $v[H^+_i]^\circ$ (rates) with the chlorophyll concentration. Chloroplasts (10—100 µM Chl) in 100 mM sorbitol, 10 mM NaCl, 50 µM methylviologen, and 4 µM 9-aminoacridine; $pH^\circ_e \approx 6.75$. Air, 20° C. Red actinic light ≈500 W · m^{-2}. In (c) $\Delta pH^\circ_e/[H^+_i]^\circ$ is computed from (a) and $vpH^\circ_e/v[H^+_i]^\circ$ is computed from (b). A constant γ_i implies that, since ΔpH°_{ei} is independent on [Chl], ΔpH°_e is proportional to it, and when ΔpH°_{ei} declines, because of the light limitation by screening effect, ΔpH°_e should also decline, in a linearly correlated manner (a). The rate $v[H^+_i]^\circ$, as expected, is more sensitive to the light factor than the extent, measured by $[H^+_i]^\circ$; to keep the linear relationship between vpH°_e and $v[H^+_i]^\circ$ (since γ_i is supposed constant), a linear decrease of $v[H^+_i]^\circ$, seen between 0 and 60 µM Chl, should be accompanied by a parabolic curve for vpH°_e, containing the parameters of the line $v[H^+_i]^\circ$. Such a theoretical parabol was traced and seems in good agreement with the experimental points (case (b), points △): indeed, if $v[H^+_i]^\circ = A - B[\text{Chl}]$, where $A = v[H^+_i]^\circ_{\text{Chl}=0}$, with $\gamma_i = -C\beta_e vpH^\circ_e/[\text{Chl}]v[H^+_i]$, deduced from Eqn. 22 where $C = k/\text{osmolarity}$ and the rates are used in place of the extents, one gets $vpH^\circ_e = -(\gamma_i/C\beta_e)(A[\text{Chl}] - B[\text{Chl}]^2)$.

the steady-state, at low or high light. With stronger illumination, the points are more scattered, the weight of the graphical errors being heavier, but they fall acceptably well near another line which slope is not far from being two times steeper. In some experiments, however, a unique γ_i is obtained in steady-state or by kinetic measurements, in low or in high light.

Four reasons of these differences in γ_i, depending on how it is computed, may exist. The first is purely methodological: in low light intensity, the ΔpH°_e signal rises slowly enough as to be faithfully followed by the glass electrode, of response time ≈1 s, but not in strong light: hence an apparently too slow vpH°_e (note, however, that the ratio $vpH^\circ_e/v[H^+_i]^\circ$ is kept constant when the chlorophyll concentration increases: insert in Fig. 4). The second possibility is that $v[H^+_i]^\circ$ is measurable only after the filling-up of a small hypothetical proton pool in the membrane (see Discussion). The third reason, initially proposed [10], could be the time shift, in strong light, between the plastoquinone reduction by System II and its reoxidation by System I (resulting in the so-called small oxygen burst [17] preceding the sustained O_2 uptake by the Mehler reaction). Although this would indeed change the apparent

initial H/e^- ratio, it should not alter that of H_i/H_e. The final hypothesis is that the proton binding is by the membrane inner face and not by soluble substances in the lumen: therefore, it is dependent on the relative velocity of the pH-induced conformational changes; this is considered in Discussion. It should however be noted that with some samples the straightforward relationship between $v\mathrm{pH}_e^\circ$ and $v[H_i^+]^\circ$ is obeyed with the same slope all along the whole light intensity range.

It is possible to demonstrate the constancy of γ_i in some other way. From Eqns. 20 and 4 one gets:

$$\frac{\Delta\mathrm{pH}_e^\circ}{[H_i^+]^\circ} = -\gamma_i \frac{k}{V_e\beta_e \text{ osmolarity}} [\mathrm{Chl}] \tag{22}$$

That is the ratio of $\Delta\mathrm{pH}_e^\circ$ over $[H_i^+]^\circ$ should linearly depend on the chlorophyll concentration if γ_i is constant, since all the other parameters are experimental data. Fig. 5 shows that, up to 60 μM, the extent of $\Delta\mathrm{pH}_{ei}^\circ$, that is $[H_i^+]^\circ$, is unchanged, as could be expected inasmuch as the internal volume of each thylakoid does not depend on their number; above 60 μM Chl, $\Delta\mathrm{pH}_{ei}^\circ$ starts to decrease, owing to the light limitation due to the mutual screening of the chloroplasts. On the contrary, the amount of H_e taken up, i.e. $\Delta\mathrm{pH}_e^\circ$, must rise with [Chl], up to a concentration where light becomes limiting (Fig. 5a). An apparent less clear-cut picture is obtained if the rates are plotted (Fig. 5b, but see the comments in the corresponding legend). Nevertheless, with both extent and rate, the ratio of $\Delta\mathrm{pH}_e^\circ$ over $[H_i^+]^\circ$ is indeed proportional to the chlorophyll concentration (Fig. 5c). The two lines intercept the origin, but the slope computed from the rate is almost twice as low as that concerning the extent. The latter gives a γ_i almost equal to that found, on this same preparation, when, at constant [Chl], the light intensity was varied: 240 instead of 230, although two different external pH values were used, 6.75 and 7.60, respectively. Thus, at least between these two pH_e values, in fact in a much broader range (not shown), and down to a pH_i which may be as low as 4, γ_i seems constant.

Correlation between the electron flow rate and the rate of protons release in the inner space

If there is a fixed stoichiometry between the light-induced electron and protons fluxes, the correlation between the redox chain velocity (as measured by the O_2 evolution) and that of internal acidification reflects directly the ratio between the total and free protons. That is, replacing in Eqn. 21 $-\beta_e v\mathrm{pH}_e^\circ$, which is $-v[H_e]^\circ$ and therefore $= v[H_i]^\circ$ (total proton flow rate), by $n\ v\mathrm{O}_2$ (where n is the protons/oxygen ratio), one gets:

$$\gamma_i = \frac{V_e}{V_i} n \frac{v\mathrm{O}_2}{v[H_i^+]^\circ} \tag{23}$$

Such writing implies that the passive proton outleak remains negligible, what may be considered true at the onset of illumination: $v[H_i^+]^\circ$, which corresponds

to a balance between the influx and efflux, is quantitatively identified to the influx rate only.

It is a common observation that the electron transport begins, at pH_e near or above neutrality, with an initial fast rate followed by a lower steady-state, a consequence of the feedback effect, by unknown mechanisms, of the building-up of the ΔpH°_{ei} on the redox chain. Addition of an uncoupler maintains the steady-state rate equal to the initial value. Since the membrane-type O_2 electrode used here cannot detect the initial events, it was assumed that the steady-state uncoupled rate is representative of the initial one without added uncoupler. Practically, above 5 mM, NH_4Cl enhances no more the electron flow (at this concentration, however, a ΔpH°_{ei} around 2 may be detected in strong light [18]).

Fig. 7 extends the illustration given in Fig. 6 that NH_4Cl in low light does not enhance the O_2 evolution (it even slightly inhibits). Thus, the coupled steady-state rate is saturated already at 25% of maximum intensity, whereas the maximum uncoupled rate may well be still unattained: the initial (= uncoupled)/coupled ratio is over 5 (hyperbolic regression analysis of the light curve suggests that it may be as high as 10).

The expected correlation between $v[H^+_i]^{\circ}$ and steady-state vO_2 is linear only in coupled condition (Fig. 8). Therefore, of the two possible relationships between vO_2 and $v[H^+_i]^{\circ}$, that implying a sigmoidal kinetics of internal acidification, real or apparent (due to instrumental reasons or to time migration of 9-aminoacridine), seems more probable than that which supposes an instantaneous maximum rate (Fig. 9 and see also Fig. 3b). Recalling that $e^-/O_2 = 4$

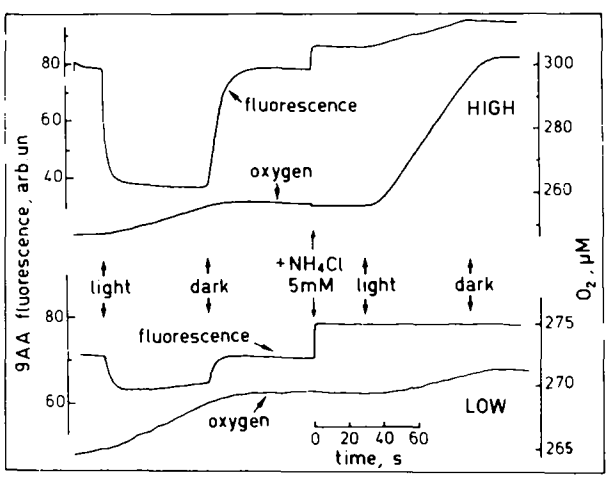

Fig. 6. Simultaneous measurement of O_2 evolution and 9-aminoacridine fluorescence quenching during ferricyanide reduction in light. Chloroplasts (20 µM) in 400 mM sorbitol, 10 mM Tricine, 10 mM NaCl, 1 mM $K_3Fe(CN)_6$, and 4 µM 9-aminoacridine (9 AA); $pH^{\bullet}_e = pH^{\circ}_e = 7.6$ (buffered medium). Air, 20°C. High maximum red actinic light ($\approx$500 W · m^{-2}); low = 1.5% of 'high'. Addition of NH_4Cl (5 mM) enhances the 9-aminoacridine fluorescence in the presence of chloroplasts (not in their absence), probably in consequence of the removal of the surface-bound amine [28—30]. The 9-aminoacridine fluorescence increase in strong light after NH_4Cl addition may be attributed to the abolishment of the ferricyanide quenching and light-absorption effects.

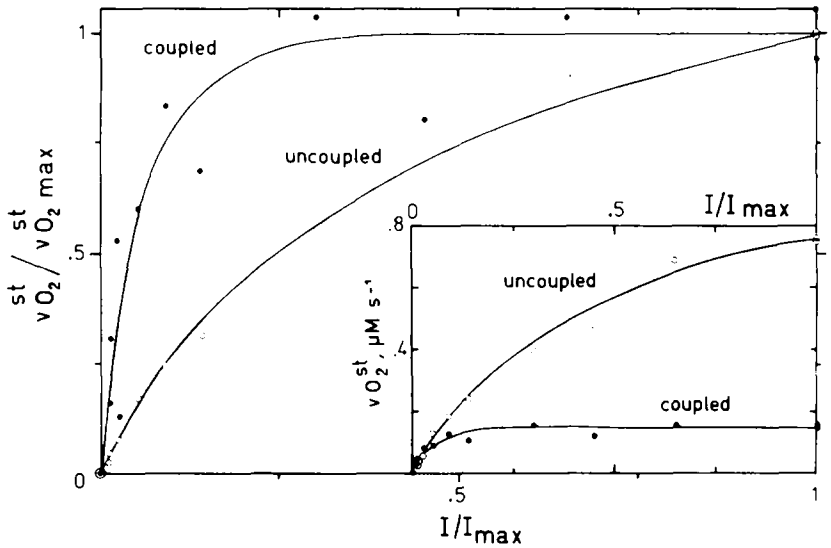

Fig. 7. Variation of the coupled and uncoupled steady-state rates of O_2 evolution in ferricyanide-type Hill reaction with the light intensity. Same conditions as in Fig. 6. •, coupled; ○, uncoupled (+NH_4Cl, 5 mM).

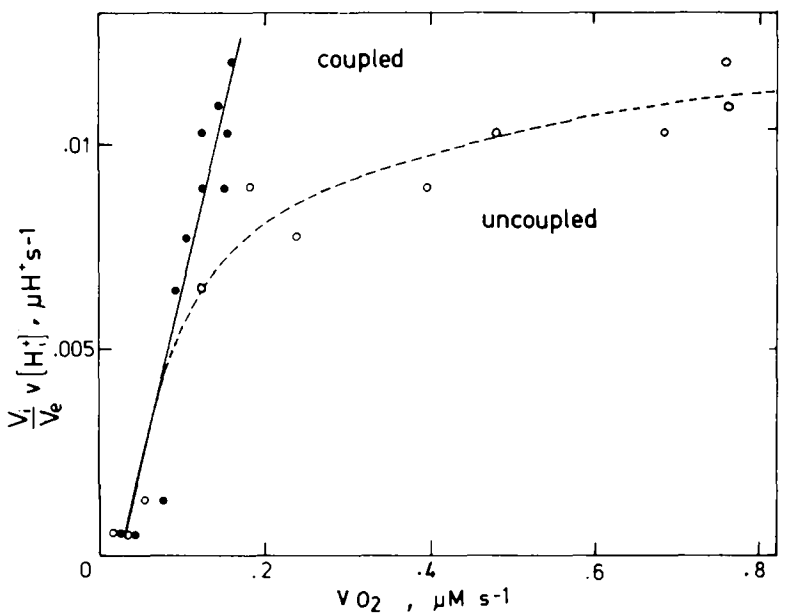

Fig. 8. Correlation between the rates of internal acidification, $v[H_i^+]^\circ$, and of O_2 evolution, vO_2. Experiment of Fig. 7. At each light intensity, the sample is illuminated 1 min a first time without NH_4Cl (giving $v[H_i^+]^\circ$ and steady-state coupled vO_2) (•); then, after NH_4Cl addition and 1 min dark interval, 1 min again (giving vO_2 steady-state uncoupled) (○). $v[H_i^+]^\circ$ is multiplied by V_i/V_e as in the Fig. 4 (insert), and therefore if vO_2 is multiplied by the postulated ratio $n = H_i/O_2$ (giving $v[H_i]^\circ$), the slope-reciprocal represents γ_i: $\gamma_i = [(V_e/V_i)] \cdot [(n \cdot vO_2)/(v[H_i^+]^\circ)]$; if $n = H_{i(=e)}/O_2 = 8$, $\gamma_i \approx 90$, a value which compares well with the general γ_i values found by direct $[H_i^+]^\circ$ and ΔpH_e° correlation.

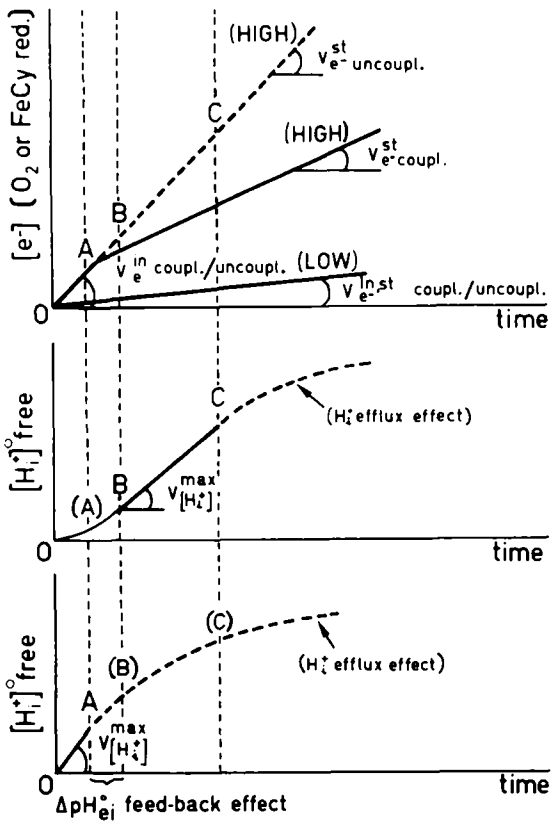

Fig. 9. Theoretical time dependence between the electron transfer rate and that of the free protons release in the thylakoid lumen. Top curve: the biphasicity of the electron transfer rate, seen in high but not in low light, leaves place to a sustained high rate (- - - - -) in uncoupled conditions, thought to represent the initial rate without uncoupler added; in coupled conditions, the change of the initial, in., (segment OA) to the stationary, st., (segment BC) rate is classically interpreted as the feed-back effect, on the redox chain, of the ΔpH°_{ei} building-up, which is represented by the H^+_i release inside the thylakoid. Middle curve: in a first hypothesis, $v[H^+_i]^\circ_{max}$ corresponds to vO_2st. (segment BC): the lag-time in the proton kinetics may be instrumental (time migration of 9-aminoacridine) or due to the filling up to an intermediary pool, such as the membrane (see text). Bottom curve: in a second hypothesis, $v[H^+_i]^\circ_{max}$ corresponds to vO_2in. (segment OA). Fig. 8 shows that the linear correlation holds only for vO_2st. coupled: therefore, the second hypothesis may be ruled out. In both situations, the bending down of the curve of the $[H^+_i]^\circ$ variation (- - - - -) may reflect the progressive increase of the proton efflux (dependent on the ΔpH°_{ei} magnitude and on the membrane permeability [18]).

and assuming an overall $H_e/e^- = 2$ [19], γ_i computed from Eqn. 23 gives 90 (if a 'protonmotive-type Q cycle' [20] may operate [18], giving $H_e/e^- = 3$, $\gamma_i \approx 130$). This value agrees well with that found by direct measurement of ΔpH°_e and ΔpH°_{ei}.

Discussion

The use of 9-aminoacridine is based on some postulates which were challenged to various extent by some authors [21—24] but considered essentially valid by others [11,25—27]. One main point is that the quenching could be due not to the amine distribution between two compartments of different pH, but

to some membraneous effects, probably related to a surface electrical double layer [28—30]. Actually, an acridine fixation may well be proportional to ΔpH°_{ei}, through a mechanism similar to that suggested by Dilley and Giaquinta [31]. It should also be remarked that none of the other probes thought to be more faithful, such as [^{14}C]methylamine [23], was used in identical conditions as 9-aminoacridine, making a quantitative comparison difficult; on the other hand, some of the reported anomalies refer to intact chloroplasts [24]. Nevertheless, it is clear that an amine binding always occurs, which may be released by cations addition [28—30]. But we found that if this affects the light-induced dye quenching, ΔpH°_{e} is similarly changed and, independently of the actual γ_i value, the same type of H^+ binding as that described here is still observable (Haraux, F. and de Kouchkovsky, Y., unpublished data). Thus, a strict correlation seems well to exist between the total number of external protons taken up in light (= internally released) and the change of the protons remaining free in the thylakoid lumen. The resulting proportionality ratio γ_i is illustrated by the linear relationship linking the internal acidification to the external alkalinisation or to the electron flow (O_2 evolution). However, the straight line joining the points of $[H^+_i]^{\circ}$ vs. ΔpH°_{e} never passes through the origin (Fig. 2c): some H_e disappear without having their counterpart appearing inside the thylakoid or this counterpart escapes the measure because the corresponding protons are completely bound to protonizable groups. The binding may be in the membrane itself, which could be related to the Williams' hypothesis of membrane anhydrous protons [32] or to the suggestion of Dilley and Prochaska [33] that the protons released by the water-splitting system may be temporarily trapped inside the membrane. Knowing β_e, the intercept ΔpH°_{e} of Fig. 2c gives this reservoir capacity of the membrane: about 8 H^+/1000 Chl here, but this value may be up to ten times higher, depending on the samples and, seemingly, the external pH.

It must be emphasized here that the concept of a constant γ_i should not be considered in a dogmatic way. Many factors govern this parameter and are discussed in a forthcoming paper. One may think that one of these could be the erroneous estimation of the internal volume V_i. This should indeed alter the calculation of free $[H^+_i]^{\circ}$, as shown by the Eqn. 1, but on the other hand, the concentration change of total $[H_i]^{\circ}$ also depends on V_i. Eqns. 2 and 6 give, in effect (since $V_t/V_i \approx V_e/V_i$ and +1 becomes quasi immediately negligible):

$$\gamma_i = \frac{\partial [H_i]^{\circ}}{\partial [H^+_i]^{\circ}} \approx \frac{\Delta [H_i]^{\circ}}{[H^+_i]^{\circ}} = \frac{-\frac{V_e}{V_i}\beta_e \Delta pH^{\circ}_{e}}{\left(\frac{V_t}{V_i}\left(\frac{F^{\bullet}}{F^{\circ}} - 1\right) + 1\right)[H^+_e]^{\circ}} \approx \frac{-\beta_e \Delta pH^{\circ}_{e}}{\left(\frac{F^{\bullet}}{F^{\circ}} - 1\right)[H^+_e]^{\circ}} \tag{24}$$

Therefore γ_i is independent of V_i.

The parameters γ_i and β_i are mathematically related, since $\gamma_i = \partial [H_i]/\partial [H^+_i]$ and $\beta_i = \partial [H_i]/\partial pH_i$. It is easy to show, recalling that $d[H^+_i] = d(10^{-pH_i})$, that:

$$\beta_i/\gamma_i = (-\ln 10)\, 10^{-pH_i} \tag{25}$$

This means that, if γ_i is constant, β_i should increase in absolute value when pH_i decreases. For instance, with $\gamma_i = 100$, $\beta_i = -23$ $\mu H^+ \cdot pH^{-1}$ at $pH_i = 7$, and -2300 $\mu H^+ \cdot pH^{-1}$ at $pH_i = 5$. It is interesting to note that the relationship

between β_i and γ_i depends neither on the concentration of the concerned acid nor on its pK.

One may try to imagine a simple pH equilibrium model which would explain the existence of a constant γ, using reasonable numerical values. For instance, the monoacid Eqns. 8—11 give, after derivation:

$$\gamma_i = \frac{\partial[H_i]}{\partial[H_i^+]} = 1 + \frac{K_i[A_i]}{(K_i + [H_i^+])^2} \approx \frac{K_i[A_i]}{(K_i + [H_i^+])^2} \quad (26)$$

To keep γ_i constant, $[H_i^+]$ must always be small compared to K_i: in this case $\gamma_i \approx [A_i]/K_i$. As pH_i° may drop in the vicinity of 4, pK_i must be about 3 in order to maintain the γ_i variation less than 10% (see below and Table I); this implies that for a current $\gamma_i = 100$, $[A_i]$ should be ≈ 0.1 M with respect to the internal volume V_i. This high concentration of A_i would give, considering the value of V_i given by Eqn. 4 for isotonic conditions (osmolarity around 0.2—0.4 M), an amount of protonizable groups comparable to that of chlorophyll molecules, what seems unrealistic; besides, a $pK_i \leqslant 3$ is about two units below the

TABLE I

THEORETICAL VARIATIONS OF γ (PROTON-BINDING RATIO) OF A MONOACID WITH pK AND pH

From Eqn. 26 and for a species j of monoacid at an internal proton concentration $[H_i^+]$:

$$\gamma_j(\text{real}) = 1 + \frac{K_j[A_j]}{(K_j + [H_i^+])^2} \text{ and } \gamma_j(\text{approx.}) = \frac{K_j[A_j]}{(K_j + [H_j^+])^2}$$

For two different $[H_i^+]$ concentrations q < p, one may define a ratio γ_r between the corresponding γ_j at different pK, which measures therefore the variation of γ_j:

$$\gamma_r = \frac{\gamma_{j,q}}{\gamma_{j,p}}; \text{ thus } \gamma_r(\text{approx.}) = \left(\frac{K_j + [H_i^+]_p}{K_j + [H_i^+]_q}\right)^2$$

and, taking $\gamma_{j,p}$ real for reference:

$$\gamma_r(\text{real}) = \gamma_r(\text{approx.}) + (1 - \gamma_r(\text{approx.}))/\gamma_{j,p}(\text{real})$$

(1) Variation of γ_r(approx.) with pK_j and at two couples of pH_i commonly found in the experiments: $[H_i^+]_q = 5\ \mu H^+$, $[H_i^+]_p = 30\ \mu H^+$; $[H_i^+]_q = 30\ \mu H^+$, $[H_i^+]_p = 70\ \mu H^+$

pK_j	γ_r(approx.) = γ_j, 5 $\mu H^+/\gamma_j$, 30 μH^+	γ_r(approx.) = γ_j, 30 $\mu H^+/\gamma_j$, 70 μH^+
3.5	1.16	1.24
4.0	1.53	1.71
4.5	2.83	2.72
5.0	7.11	4.00
5.5	16.31	4.87

The experimental variation of a given sample between these extreme $[H_i^+]$ is much smaller than those given here.

(2) Comparison of γ_r(real) and γ_r(approx.) for three pK_j values and various γ_j(real) (at $[H_i^+] = 30\ \mu H^+$)

γ_j(real)	pK_j = 3.5 (γ_r approx. = 1.16)	pK_j = 4.5 (γ_r approx. = 2.83)	pK_j = 5.5 (γ_r approx. = 16.31)
1	1.00	1.00	1.00
5	1.13	2.46	13.25
10	1.14	2.65	14.78
50	1.16	2.79	16.00
100	1.16	2.81	16.16

published estimates [6,11,14]. Furthermore, if the single monoacid model was really valid, a linear relationship would have existed in Fig. 2a: this is not the case even for the smallest ΔpH°_{ei}.

It could be that the γ_i constancy corresponds to the titration of a mixture of preexistent monoacids (a polyacid may be treated as a sum of monoacids, with the restriction that the same concentration should be used for each of its titrable group). Let be m monoacids; the overall γ_i is necessarily equal to the sum of the inidividual γ_j of all these monoacids taken apart, since γ like β, is an additive expression *. Therefore:

$$\gamma_i = \sum_1^m \gamma_j \quad \text{and} \quad \frac{d\gamma_i}{d[H_i^+]} = \sum_1^m \frac{d\gamma_j}{d[H_i^+]} \tag{27}$$

The relative variation of γ_j with the $[H_i^+]$ may be expressed by a parameter ω_j which, contrarily to γ_j, is not an additive function:

$$\omega_j = \frac{d\gamma_j}{d[H_i^+]} \cdot \frac{1}{\gamma_j} = \frac{d \ln \gamma_j}{d[H_i^+]} \tag{28}$$

and

$$\text{overall } \omega_i = \frac{d\gamma_i}{d[H_i^+]} \cdot \frac{1}{\gamma_i} = \frac{\sum_1^m \frac{d\gamma_j}{d[H_i^+]}}{\sum_1^m \gamma_j} \neq \sum_1^m \frac{d\gamma_i}{d[H_i^+]} \cdot \frac{1}{\gamma_j} \tag{29}$$

Considering absolute values (ω is a negative number, since, see Eqn. 26, γ_i decreases when $[H_i^+]$ increases), total $|\omega_i|$ must be at least equal to the smallest individual $|\omega_j|$ (in a series of ratios x/y, $\Sigma x/\Sigma y$ is necessarily greater than the smallest x/y, if all the x/y are positive). Therefore:

$$\left\{ |\omega_i| = \left| \frac{d \ln \gamma_i}{d[H_i^+]} \right| \right\} > \left\{ |\omega_j|_{min} = \left| \frac{d \ln \gamma_j}{d[H_i^+]} \right|_{min} \right\} \tag{30}$$

Integration of this equation between $[H_i^+]_p$ and $[H_i^+]_q$ ($[H_i^+]_p > [H_i^+]_q$) gives:

$$\frac{\gamma_i, [H_i^+]_q}{\gamma_i, [H_i^+]_p} > \gamma_r \qquad \gamma_r = \frac{\gamma_j, [H_i^+]_q}{\gamma_j, [H_i^+]_p} \min \tag{31}$$

Table I shows how γ_r varies for $[H_i^+]_q$ and $[H_i^+]_p$ = 5 μH^+ and 30 μH^+ or 30 μH^+ and 70 μH^+ in case of monoacids of pK_j between 3.5 and 5.5, a range broad enough to include all the reasonable experimental situations. Unless one accepts the improbable case where the majority of the acids have $pK_j \leqslant 3.5$, the existence of a fixed mixture of monoacids seems excluded: experimentally, γ_i appears almost constant when $[H_i^+]^{\circ}$ varies between 5 and 70 μH^+.

Therefore, another model should be considered, in which the γ_i stability is insured by a K_i and $[A_i]$ continuous variation with pH_i°. For instance, inclusion of the data of Fig. 2 into Eqn. 26 would result in a variation of $[A_i]$ from 7 to 50 mM, with the pK_i shifting from 4.4 to 4.7. A good support to this proposal is its similarity with the protein titration, which progressively reveals, in a time-dependent manner, new titratable groups (see e.g. Tanford [34], especially pp.

* More precisely, as shown by the left-hand part of Eqn. 26, it is $\gamma - 1$ which is additive.

90–95): thus, the buffering molecules may be some proteins at the inner membrane face. In this case, the neutralization, at a particular pH_i°, of the available negatively charged groups may relax the membrane constraint due to the electrostatic repulsion, thereby allowing new anionic functions to be accessible to the new protons incoming. Although this is somewhat an ad hoc hypothesis, based nevertheless on the experimental evidences of thylakoid conformational changes [35], it may also explain why γ_i, kinetically determined, is greater in low light than in high light, and equals γ_i determined in the steady-state at all illuminations. The conformational changes of proteins [34] and of membranes [35] are relatively slow processes; they are completed in the steady-state and may have enough time to follow the ΔpH°_{ei} build-up in low light, but not during the initial step of rapid internal acidification under strong light. This would result in a temporary excess of unbound protons, hence in an underestimation of γ_i.

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