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# MEASUREMENT OF CHLOROPLAST INTERNAL PROTONS WITH 9-AMINOACRIDINE

## PROBE BINDING, DARK PROTON GRADIENT, AND SALT EFFECTS *

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## Summary

A defined ratio, $\gamma$, of the total proton uptake to the concentration change of free internal $H^+$ is observed for illuminated envelope-free chloroplasts (Haraux, F. and de Kouchkovsky, Y. (1979) Biochim. Biophys. Acta, 546, 455—471). Proton uptake is measured by the external pH shift, free internal $H^+$ by 9-aminoacridine fluorescence quenching. Extension of this work leads to the following conclusions, which, in the case of 9-aminoacridine behaviour, should apply to any kind of diffusible protonizable $\Delta$pH probe:

1. The $\gamma$ constancy is preserved when the internal volume ($V_i$) is modulated by chlorophyll and osmolarity changes: thus, 9-aminoacridine behaves as expected from the $\Delta$pH distribution of an amine of high p$K$; previous doubts on this point are attributed to the lack of control of the external proton uptake.

2. With variable 9-aminoacridine concentration, however, some variation of $\gamma$ confirms the existence of slight light-induced probe-membrane interactions.

3. According to the diffuse layer theory, salts decrease the negative potential at the 'plane of closest approach' of the thylakoids, thereby releasing the excess 9-aminoacridine in this diffuse layer, which increases its fluorescence. Although of equal valency, $NH_4^+$ is more potent than $K^+$, suggesting competition between amines for specific anionic binding sites.

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* An abstract of this report has been published [16].

Abbreviations and conventional symbolism. Subscripts e, i, and ei: refer to external (e), internal (i) phases, and to their difference; superscripts $^{\bullet,\circ}$: refer to situations in darkness ($^\bullet$) and in light ($^\circ$); H, total protons (bound + free, $H^+$); $\gamma$, ratio of the internal concentration changes of total/free protons; $r$, Donnan ratio $[H_i^+]^\bullet/[H_e^+]^\bullet$; $F$: 9-aminoacridine fluorescence; $V$: volume; Chl, chlorophyll; $\mu H^+$ $\mu$gequiv./l (free or total protons). Tricine, *N*-tris(hydroxymethyl)methylglycine.

4. Two categories of membrane modifications are induced by salts: in addition to the above-mentioned electrical effect, mono- and divalent cations at high concentration increase the chloroplast proton binding capacity. $La^{3+}$ is only able to release the excess dye in the diffuse layer and leaves $\gamma$ unchanged. Therefore the probe-membrane interactions should have limited importance for steady-state $\Delta pH$ measurement.

5. A Donnan-type dark pH difference, which could seriously bias these $\Delta pH$ estimates, is found experimentally to be less than 2 (no significant $\gamma$ change when $V_i$ varies) and even theoretically less than 1 (on the basis of the concentration of the non-diffusible internal protonizable groups). Similarly, the predictable errors of $V_i$ and its possible light-induced variations must have a small effect on $\Delta pH$ under present experimental conditions.

## Introduction

Since its first use by Schuldiner et al. [1], the 9-aminoacridine method for measuring the transmembrane pH difference ($\Delta pH_{ei}$) has been applied widely in various fields. In the case of liposomes, which have a known internal pH, the dye apparently distributes between the internal and the external phases as predicted from their respective pH values [2,3]. With bacterial chromatophores, some authors found that 9-aminoacridine behaves as an ideal probe [4,5], whereas others consider that the fluorescence quenching is due partly to the amine's binding to the membrane [6], in a way similar to that reported by Fiolet et al. [7] for isolated chloroplasts. However, the probe membrane interaction may be cancelled out by addition of salts in darkness [8,9] as well as under light [10].

It is interesting to compare the different methods available for estimation of the pH difference. For instance, $\Delta pH_{ei}$ may be computed from the $NH_4^+$ uptake — detected with an ion-sensitive electrode — under the same conditions as the 9-aminoacridine fluorescence quenching measurement (stirred suspension, continuous and even illumination): similar values are then obtained [11]. However, when radioactive alkylamines are used together with the technique of centrifugation through a silicone fluid layer [12—14] or with the flow-dialysis method [6], lower $\Delta pH_{ei}$ values are always obtained. Only these latter results were claimed valid by the authors concerned, who concluded that the higher figures obtained with 9-aminoacridine were due to the above-mentioned membrane interactions. This implicitly means that no such interactions exist with methyl- (or hexyl-) amine: a rather questionable assumption, since there is no reason to grant a privilege to them against $NH_4^+$ or 9-aminoacridine: indeed, any cation may be attracted in the Gouy 'diffuse layer' [8].

The present work was undertaken to determine the relative importance of the different factors able to alter the pH difference measurements. Amongst these factors, the main ones to be considered were the amine binding to the membrane and the possible existence of a dark proton gradient of the Donnan type. The various parameters which could be changed in this study were the probe and chloroplast concentrations, the medium osmolarity, and the ionic strength. In all experiments, control of the overall proton uptake was obtained

by following the external pH shift simultaneously with the 9-aminoacridine fluorescence quenching [15].

## Methodology

Envelope-free chloroplasts were extracted from spinach leaves as previously described [15]. The external pH measured with a glass electrode and the 9-aminoacridine fluorescence analysed at 528 nm (excitation 420 nm) were determined in a thermostatically controlled and stirred spectroscopic cuvette, with the same arrangement as used before [15]; the red actinic light, devoid of infrared, was approx. 500 $W \cdot m^{-2}$. The experiments were performed under aerobic conditions at 20° C. Each sample, first allowed to relax in darkness, was illuminated for 1 min: therefore the measured signals correspond to an equilibrium in darkness and a steady-state in light. The redox chain included the two systems ($H_2O \rightarrow$ methyl viologen).

The light-induced change of total internal proton concentration — hereafter symbolised by $\Delta[H_i]^\circ$ — is equal, with opposite sign, to the external proton uptake, $\Delta[H_e]^\circ$, multiplied by the external/internal volumetric ratio, $V_e/V_i$:

$$\text{total proton change } \Delta[H_i]^\circ = -\frac{V_e}{V_i}\Delta[H_e]^\circ \qquad (1)$$

$\Delta[H_e]^\circ$ is obtained by multiplying the external pH shift (commonly no greater than 0.1) in light by the external buffer power, essentially that of the slightly buffered medium (generally approx. $10^{-4}$ equiv. $\cdot$ $pH^{-1}$) [15].

The light-induced change in free internal proton concentration, $\Delta[H_i^+]^\circ$, is the difference between these concentrations in light, $[H_i^+]^\circ$, and in darkness, $[H_i^+]^\bullet$. Since, rapidly, $[H_i^+]^\circ >> [H_i^+]^\bullet$, $\Delta[H_i^+]^\circ = [H_i^+]^\circ - [H_i^+]^\bullet \approx [H_i^+]^\circ$: the $\Delta[H_i^+]^\bullet$ is symbolized hereafter simply by $[H_i^+]^\circ$. With the restrictions discussed in the Appendix and below, $[H_i^+]^\circ$ may be computed by the equation of Schuldiner et al. [1]:

$$\text{free proton change } \Delta[H_i^+]^\circ \approx \left(\frac{F^\bullet}{F^\circ} - 1\right)\frac{V_e}{V_i}[H_e^+]^\circ \qquad (2)$$

where $F^\bullet$ and $F^\circ$ are the fluorescence intensities in darkness and in light, respectively *. The pH difference is $\Delta pH_{ei}^\circ = \log [H_i^+]^\circ/[H_e^+]^\circ$; with the redox chain used, it was currently above 3.5.

Within the present experimental limits, the chlorophyll-dependent volumetric ratio is proportional to the medium osmolarity, $\Omega$:

$$\frac{V_e}{V_i} = \frac{\Omega}{k[\text{Chl}]} \qquad (k \approx 2.0 \pm 0.3\ [15]) \qquad (3)$$

From Eqns. 1 and 2, the ratio, $\gamma$, of total/free internal proton changes is easily obtained. It was shown to be a better parameter for expressing the thylakoid's internal proton-binding properties than the classical buffering

* $F^\bullet/F^\circ - 1$ is the classical form of the fluorescence quenching (Stern-Volmer equation). However, many authors use it as $Q = 1 - F^\circ/F^\bullet$.

power, to which it is mathematically related [15]:

$$\text{total/free proton ratio } \gamma = \frac{\Delta[H_i]^\circ}{\Delta[H_i^+]^\circ} \approx -\frac{\Delta[H_e]^\circ}{(F^\cdot/F^\circ - 1)[H_e^+]^\circ} \quad (4)$$

Hence, the external/internal volumetric ratio does not interfere in the $\gamma$ computation, at least if there be no dark proton gradient (see below). In the figures, $\Delta[H_i]^\circ$ is therefore expressed by $\Delta[H_e]^\circ$, and $\Delta[H_i^+]^\circ$ by the fluorescence quenching multiplied by the external proton concentration in light (Eqn. 4). Generally, $\gamma$ is around 100 : less than 1% of the incoming total protons remain free in the thylakoid.

## Results

*Effect of the 9-aminoacridine concentration on free and total proton uptake by illuminated chloroplasts*

9-Aminoacridine being a diffusible buffer, one should ensure that the high internal concentrations of the dye in the light does not affect the intrinsic proton-binding properties of the thylakoids. According to Schuldiner et al. [1], only the neutral form of the amine can freely penetrate the membrane. Because of its high p$K$ (approx. 10), the dye is essentially in the protonated form. Thus, when the amine moves across the membrane as a consequence of the internal acidification, it first liberates a proton outside, then binds a proton inside: there is an apparent leak of $H^+$, i.e. uncoupling. Therefore, the correlation between the total and the free protons previously observed [15] should not depend on 9-aminoacridine. With probes of low p$K$, this is no longer valid; indeed, pyridine and aniline increase the internal buffer capacity of chloroplasts, which enhances the external proton uptake [17,18]. Fig. 1, left, shows

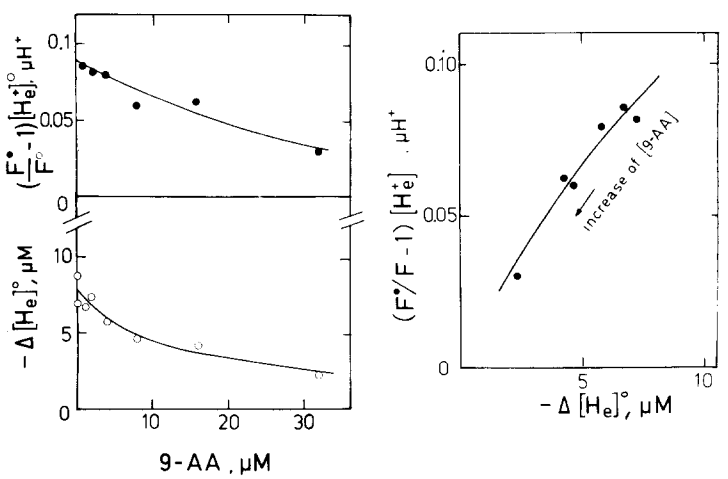


**Fig. 1. 9-Aminoacridine (9-AA) fluorescence quenching and proton uptake as a function of 9-aminoacridine concentration. Chloroplasts 40 µM Chl in sorbitol 100 mM + NaCl 10 mM + methyl viologen 50 µM; $pH_e \approx 7.0$ (slightly buffered suspension); saturating red light. (1) Left: light-induced internal variations of the free protons, expressed by the 9-aminoacridine fluorescence quenching (top), and of the total protons, measured by the external proton uptake (bottom): Eqns. 2 and 1, respectively, with omission of the volumetric ratio $V_e/V_i$ (see text). (2) Right: free proton change vs. total proton uptake.**

that raising the 9-aminoacridine concentration reduces the free proton concentration inside, but has a similar effect on the total, external, proton uptake. (In a control experiment, it was determined that the fluorescence in darkness was proportional to 9-aminoacridine concentration in this range; accordingly, the light-induced quenching was proportional to the amine uptake.) Fig. 1, right, illustrates the correlation between total and free proton changes: it is not strictly linear, as would have been expected from our previous measurements made with a variable light intensity and fixed 9-aminoacridine concentration [15]. Nevertheless, the total/free proton ratio $\gamma$ variation is kept within the limits of ±15% of its average value. It is likely that part of this incurvation may be attributed to a slight probe-membrane light-induced interaction and not to the buffering properties of 9-aminoacridine. Another possibility is that when a low external 9-aminoacridine is used, its concentration inside the thylakoids is not high enough for it to have its fluorescence fully quenched.

*Dark proton gradient and internal volume effects on the measured internal proton concentration in light*

*a. Principle.* The internal total and free protons and the internal volume required for their computation are determined experimentally with some simplifying hypotheses and errors (see Appendix): they are apparent values, which is symbolized below by the prime ($'$). The true values are those which would have been obtained if all the required data were known exactly. In the rest of the text, one assumes that true and apparent quantities are equal, and therefore prime superscripts are not used.

The Appendix gives the correct way of computing the true internal free-proton concentration $[H_i^+]^\circ$ (Eqn. A-10) and shows how it depends on the possible light-induced change of the internal volume: $V_i^\bullet \rightarrow V_i^\circ$ and on the possible dark internal/external Donnan ratio, $r$:

$$r = \frac{[H_i^+]^\bullet}{[H_e^+]^\bullet} = 10^{\Delta pH_{ei}^\bullet} \tag{5}$$

Eqn. A-13 of this Appendix establishes the correlation between the apparent $[H_i^+]^{\circ\prime}$ and true $[H_i^+]^\circ$, and Eqn. A-16 does the same for the apparent internal concentration change of total protons, $\Delta[H_i]^{\circ\prime}$, and the true $\Delta[H_i]^\circ$.

By varying the light intensity, a grossly linear relationship was previously found between apparent total and free proton accumulations inside the illuminated chloroplasts [15]:

$$\Delta[H_i]^{\circ\prime} = [H_m]' + \gamma'[H_i^+]^{\circ\prime} \tag{6}$$

where $[H_m]'$ is the apparent 'pool of membrane-bound protons' (a small proportion of protons disappear from outside without being detected inside) and $\gamma'$ is the above-mentioned apparent total/free proton ratio. With the corrected values of free and total protons, a new correlation is thus obtained, containing the dark proton gradient $r$ and the volumetric factors $V_i'/V_i^\bullet$ and $V_i^\bullet/V_i^\circ$:

$$\Delta[H_i]^\circ = \frac{V_i^\bullet}{V_i^\circ}[H_m] + \gamma[H_i^+]^\circ \tag{7}$$

with

$$[H_m] = \frac{V_i'}{V_i^\bullet}[H_m]' + \frac{V_i'/V_i^\bullet - r}{\gamma'/\gamma}[H_e^+]^\circ \tag{8}$$

and

$$\gamma'/\gamma = \frac{V_e/V_i' + rV_i^\bullet/V_i'}{V_e/V_i' + 1} \approx 1 + r\frac{V_i^\bullet}{V_e} \tag{9}$$

($[H_m]$ is the 'true' pool of membrane-bound protons; $V_i'$ is the apparent internal volume as measured with Eqn. 3). Clearly, Eqns. 6 and 7 are formally similar: the existence of a Donnan-type gradient changes only the apparent numerical value of $\gamma$ and $[H_m]$ (the weight of $[H_e^+]^\circ$ on the latter is small). However, note that $[H_m]$ is affected by a possible light-induced variation of the internal volume, whereas $\gamma$ is not. Depending on the conditions, the chloroplasts in light shrink or swell, but the factor altering $V_i$ seems barely to exceed 2 [19]; under our conditions, we were unable to detect any appreciable light-scattering change, which would manifest gross structural variations. Therefore, $V_i^\bullet/V_i^\circ$ was certainly small and, moreover, since this factor is applied to an already small parameter, $[H_m]$, its weight in Eqn. 7 is limited. Thus, only the dark proton gradient may have any significant effect on the thylakoid internal buffering properties, experimentally estimated by $\gamma'$.

*b. Application to the detection of a dark proton gradient.* Eqn. 9 shows that experimental total/free proton ratio should vary linearly with the internal/external volumetric ratio, due to the dark proton gradient. Assuming no error in the estimation of the internal volume (i.e. $V_i^\bullet/V_e$ is the $V_i/V_e$ given by Eqn. 3) one finds:

$$\gamma'/\gamma = 1 + r\frac{k[\mathrm{Chl}]}{\Omega} \tag{10}$$

Actually, Fig. 2 shows a slight increase of $\gamma'$ with chlorophyll concentration (true $\gamma$ is, in principle, invariable), but the theoretical lines calculated from Eqn. 10 for different values of $r$ and drawn on this figure indicate that the dark transmembrane pH difference is certainly less than 2 and probably insignificant. Therefore the true $\gamma$ value is almost identical to the apparent value.

*c. Theoretical estimation of the Donnan-type dark proton gradient.* A theoretical estimation of the Donnan-type $\Delta pH_{ei}^\bullet$ may be attempted. Non-permeant protonizable groups inside the thylakoids must be responsible for ionic gradients across the membrane at equilibrium, in the dark relaxed state. Here will be examined a non-diffusible internal monoacid (total concentration [At], equilibrium constant $K$), the protonizable form of which is either neutral (case 1: $AH \rightleftharpoons A^- + H^+$) or cationic (case 2: $AH^+ \rightleftharpoons A + H^+$); in addition, only the effect of monovalent diffusible salts (cation $Ct^+$) will be considered. Under these conditions, the extension of previous computations [20], with the additional inclusion of the $OH^-$ contribution, gives, after various substitutions:

$$[H_i^+]^3 + (K + \alpha Z)[H_i^+]^2 - ([H_e^+]^2 + \beta Z)[H_i^+] - K[H_e^+]^2 = 0 \tag{11}$$

$$\text{with } Z = [\mathrm{At}]/(1 + [Ct_e^+]/[H_e^+]) \tag{12}$$

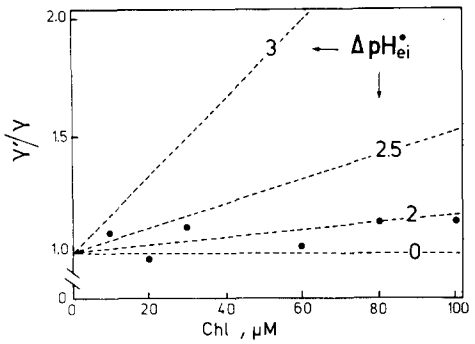


Fig. 2. Estimation of the Donnan-type dark pH difference $\Delta pH^{\bullet}_{ei}$. Chloroplasts 10—100 μM Chl in 100 mM sorbitol + 10 mM NaCl + 50 μM methyl viologen + 4 μM 9-aminoacridine; $pH_e \approx 7.8$ (slightly buffered suspension); saturating red light. •, experimental total/free proton ratio, $\gamma'$ (Eqn. 4), normalized to its extrapolated value at zero Chl; - - - - -, theoretical variations on the apparent/real ratio $\gamma'/\gamma$ for different $\Delta pH^{\bullet}_{ei}$ values (see Eqn. 10 and the text).

In case 1, $\alpha = 0$ and $\beta = K$, in case 2, $\alpha = 1$ and $\beta = 0$; for both, only one real root exists and, when $[At] \to 0$ or $[Ct^+_e] \to \infty$, $[H^+_i] \to [H^+_e]$; this also occurs if $K \to 0$ (case 1) or $\infty$ (case 2) i.e. when the non-diffusible groups are totally in their neutral form. In case 1, more likely to occur here, the protons are attracted by $A^-$ and therefore $\Delta pH^{\bullet}_{ei} > 0$: the interior is more acidic than the exterior; in case 2, it is the opposite, since protons are repelled by $AH^+$. Given reasonable values for the concentration and p$K$ of the buffering groups [15,21], a monovalent salt at an external concentration of at least 10 mM, routinely used, should reduce $\Delta pH^{\bullet}_{ei}$ below 1, at any external pH (Fig. 3). Consequently, the importance of $\Delta pH^{\bullet}_{ei}$ in the determination of $[H^+_i]^\circ$ becomes negligible.

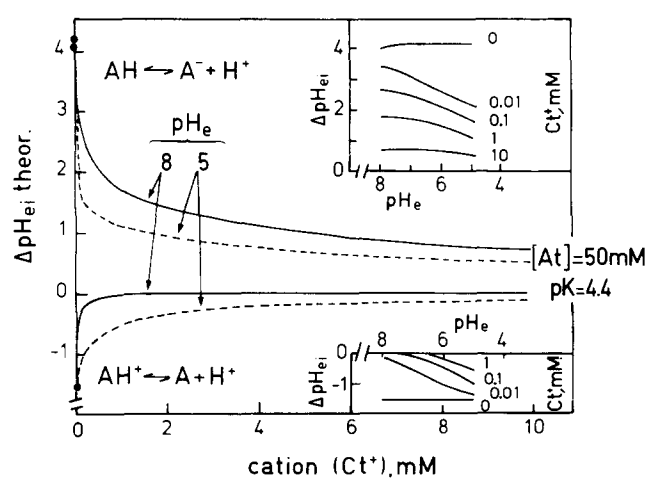


Fig. 3. Theoretical variation of the Donnan-type dark transmembrane pH difference, $\Delta pH^{\bullet}_{ei}$, with the external pH ($pH_e$) and the external concentration of a diffusible monovalent salt (cation $Ct^+$) (see text). The total concentration, [At] = 50 mM, and the p$K$ (= 4.4) of the internal non-diffusible monoprotic groups are the extreme values previously estimated [15]. Thes groups may be either neutral (AH) or charged ($AH^+$) in their protonated state.

*Salt effect on the 9-aminoacridine fluorescence with chloroplasts in darkness*

In addition to a trivial optical effect, the injection of chloroplasts into a solution of 9-aminoacridine in darkness causes a slow quenching of its fluorescence, which can be suppressed by a salt addition. Searle et al. [8] have interpreted these results as a cation-reversible dye interaction with the membranes. The higher the cation valency, the lower the concentration required for a given effect. These results are in agreement with electrostatic attraction of the 9-aminoacridine cation within the membrane Gouy-type diffuse layer. Fig. 4, top, compares the action of KCl and $NH_4Cl$ in that respect. The concomitant increase in osmolarity played a minor role in the fluorescence increase, as was demonstrated in a control sample — not shown — to which ethylene glycol was added. Although $NH_4Cl$ is more potent than KCl at low

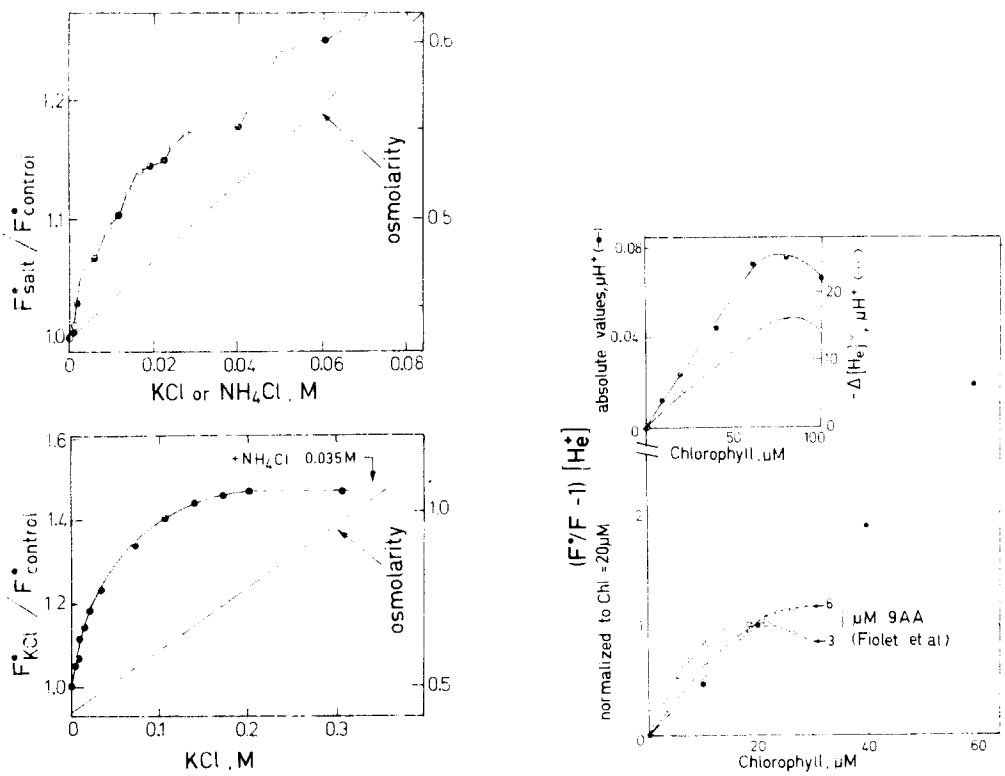


Fig. 4. Comparative effectiveness of $K^+$ and $NH_4^+$ in increasing the 9-aminoacridine fluorescence within a chloroplast suspension in darkness. Chloroplasts (20 μM Chl) in 400 mM sorbitol + 30 mM Tricine + 10 mM NaCl + 4 μM 9-aminoacridine; $pH_e = 7.8$ (strongly buffered suspension). Top: alternate and successive addition of KCl (●) and $NH_4Cl$ (○). Bottom: dark fluorescence increase saturation curve. In all cases, fluorescence and concentrations were corrected for the dilution factor; the sloping lines indicate the osmolarity, Ω, increase (initial Ω, 0.45 osmol · $l^{-1}$; initial cation concentration, 0.01 M).

Fig. 5 Variation of the light-induced 9-aminoacridine (9-AA) fluorescence quenching with the chloroplast population increase as measured by chlorophyll concentration [Chl]. Conditions of Fig. 2. ●——●: our data, normalized to 20 μM Chl; □- - - - -□ and △- - - - -△: Fiolet et al. [7] data, same normalization. Insert: absolute values of the free proton increase expressed as the fluorescence quenching (●) and of the total proton change measured by the proton uptake (○): both are proportional to [Chl] up to 60 μM, then decline in parallel (which keeps their ratio, γ, constant over the whole range).

concentrations, it becomes inefficient when added in the presence of a saturating amount of KCl (Fig. 4, bottom). These salt effects might be interpreted a priori as being due to the dissipation of a Donnan-type $\Delta pH^{\bullet}_{ei}$, which should pump in 9-aminoacridine as does $\Delta pH^{\circ}_{ei}$ in light, and $NH_4^+$ would add to its cation property that of a diffusible buffer. However, if Eqn. 2 is rewritten by replacing $F^{\circ}$ with the fluorescence at given salt concentration and $F^{\bullet}$ with the maximal fluorescence at saturation, it is easy to compute, from Fig. 4, bottom, the corresponding $[H_i^+]^{\bullet}$ and, thence, $\Delta pH^{\bullet}_{ei}$. It would be approx. 3.65 for $[Na^+] = 10$ mM and $[K^+] = 0$ (initial conditions) and approx. 3.50 for $[Na^+] + [K^+] = 20$ mM. As discussed above, these high numbers are unrealistic (indeed, with a $pK = 4.4$, the internal concentration of buffering groups should be 150 molar!). Therefore, the cancellation of a $\Delta pH^{\bullet}_{ei}$ plays but a secondary role in the observed salt effects. Besides, the nonpermeant $La^{3+}$ is very effective in displacing the dye from the diffuse layer [8], which we have also observed. Thus, the 9-aminoacridine trapping, revealed by its fluorescence quenching in darkness, is mainly at the charged surface. However, more specific mechanisms may also exist, as suggested by the differential behaviour of small additions of $NH_4^+$ and $K^+$ (selective binding sites for $NH_4^+$, such as amino acid carboxyl groups?).

*Light-induced 9-aminoacridine fluorescence quenching as a function of the chloroplast internal volume*

For a given proton gradient, the light-induced fluorescence quenching must be proportional to the internal volume, $V_i$. More precisely, Eqns. 2 and 3 point out that one should have:

$$\left(\frac{F^{\bullet}}{F^{\circ}} - 1\right) [H_e^+]^{\circ} = [H_i^+]^{\circ} k \frac{[Chl]}{\Omega} \qquad (13)$$

Fiolet et al. [7] could not confirm this prediction. However, they did not ensure that the chloroplast proton uptake was not altered by the changes they imposed in their experimental conditions: they implicitely assumed that the proton gradient was kept constant.

We followed on the same sample the 9-aminoacridine quenching and the external proton uptake, $\Delta[H_e]^{\circ}$. Fig. 5 shows that the light-induced quenching is proportional to [Chl] up to about 60 $\mu$M. The deviation from linearity observed by Fiolet et al. [7] at much lower concentrations is probably due to sub-saturating light, which caused a decrease in $[H_i^+]^{\circ}$ (their curves were redrawn on our Fig. 5). Under our conditions, this did not occur below 60 $\mu$M chlorophyll and when the fluorescence quenching started to decline, so did the external proton uptake.

In Fig. 6, the internal volume was decreased by raising the medium osmolarity through sorbitol addition. The ideal hyperbolic Boyle-van't Hoff law, suggested by the dashed line on Fig. 6, top, is not obeyed for osmolarities under 0.2 M, but the control of the total proton uptake, $\Delta[H_e]^{\circ}$, (Fig. 6, bottom) exhibits a similar behaviour. Hypotonicity may have caused the most fragile thylakoids of the heterogeneous population used to burst and/or may have altered the topological and functional properties of the redox chain [22]. This similarity in the variation of the free and total proton concentration

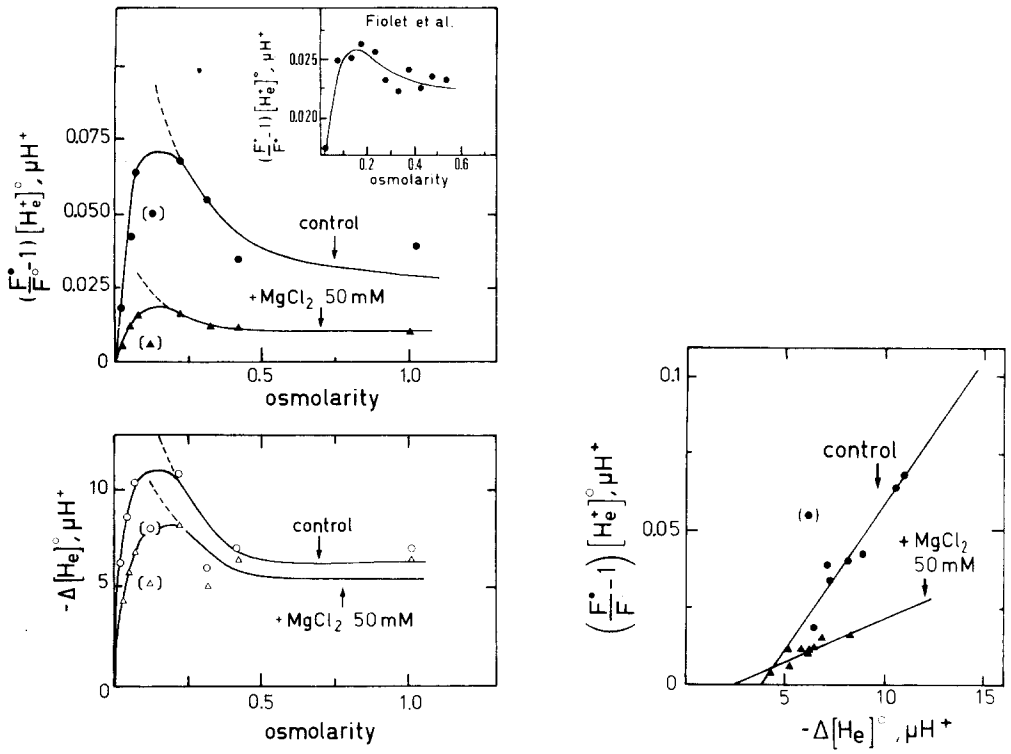


Fig. 6. 9-Aminoacridine fluorescence quenching and total proton uptake as a function of osmolarity (variable sorbitol), and effect of $Mg^{2+}$. Chloroplasts (40 $\mu$M Chl) in 10 mM NaCl + 50 $\mu$M methyl viologen + 4 $\mu$M 9-aminoacridine; different samples for each sorbitol concentration (10—1000 mM); $pH_e \approx 7.0$ (slightly buffered suspension); saturating red light. Each sample was illuminated 1 min without $MgCl_2$ (•, ○), left in darkness for 1 min before addition of $MgCl_2$ (50 mM final), and, 1 min later, illuminated 1 min again (▲, △). Top: free internal proton increase, estimated by the fluorescence quenching (•, ▲). Bottom: total internal proton increase, measured with a glass electrode (○, △). The theoretical hyperbolic curve is suggested by the dashed lines (see text). Insert: Fiolet et al. [7] data (their Fig. 4), redrawn on our ordinate scale.

Fig. 7. Correlation between light-induced 9-aminoacridine fluorescence quenching and total proton uptake at variable osmolarity. Data of Fig. 6. The lines are computed by least-squares regression analysis; their slopes give the total/free internal proton ratio, $\gamma$, which is trebled by $MgCl_2$ additions: $\gamma$ no $Mg^{2+}$ (•) $\approx 70$; $\gamma + Mg^{2+}$ (▲) $\approx 190$.

changes with the osmolarity is better expressed by Fig. 7, which shows that, at all sorbitol concentrations, the light-induced quenching ($\rightarrow [H_i^+]^\circ$) increases linearly with $\Delta[H_e]^\circ$, as expected from the above-mentioned relationship between total and free protons. The ratio $\gamma$ is 105, a result in good agreement with our previous estimations [15].

Addition of concentration of $MgCl_2$ high enough to release all the dye from the diffuse layer reduces slightly the total proton uptake $\Delta[H_e]^\circ$ but more dramatically the free proton accumulation measured by the fluorescence quenching (Figs. 6 (top and bottom) and 7). A plot of $(F^\bullet/F^\circ - 1)[H_e^+]^\circ$ vs. $\Delta[H_e]^\circ$ shows a decrease in the slope, the total/free proton ratio, $\gamma$, being trebled (Fig. 7), a point discussed below. In the insert of Fig. 6, top, a redrawing of Fiolet et al. data [7] with the same ordinate scale as ours shows a good similarity with our results.

In conclusion, as in the preceding case of variable chlorophyll, the departure from linearity of the quenching versus the internal volume is due to the variations of the proton uptake and not to an artifactual response of the probe.

*Salt effect on the 9-aminoacridine fluorescence quenching with illuminated chloroplasts*

The internal acidification was studied in conditions under which 9-aminoacridine interaction with membrane was suppressed by addition of a salt. As

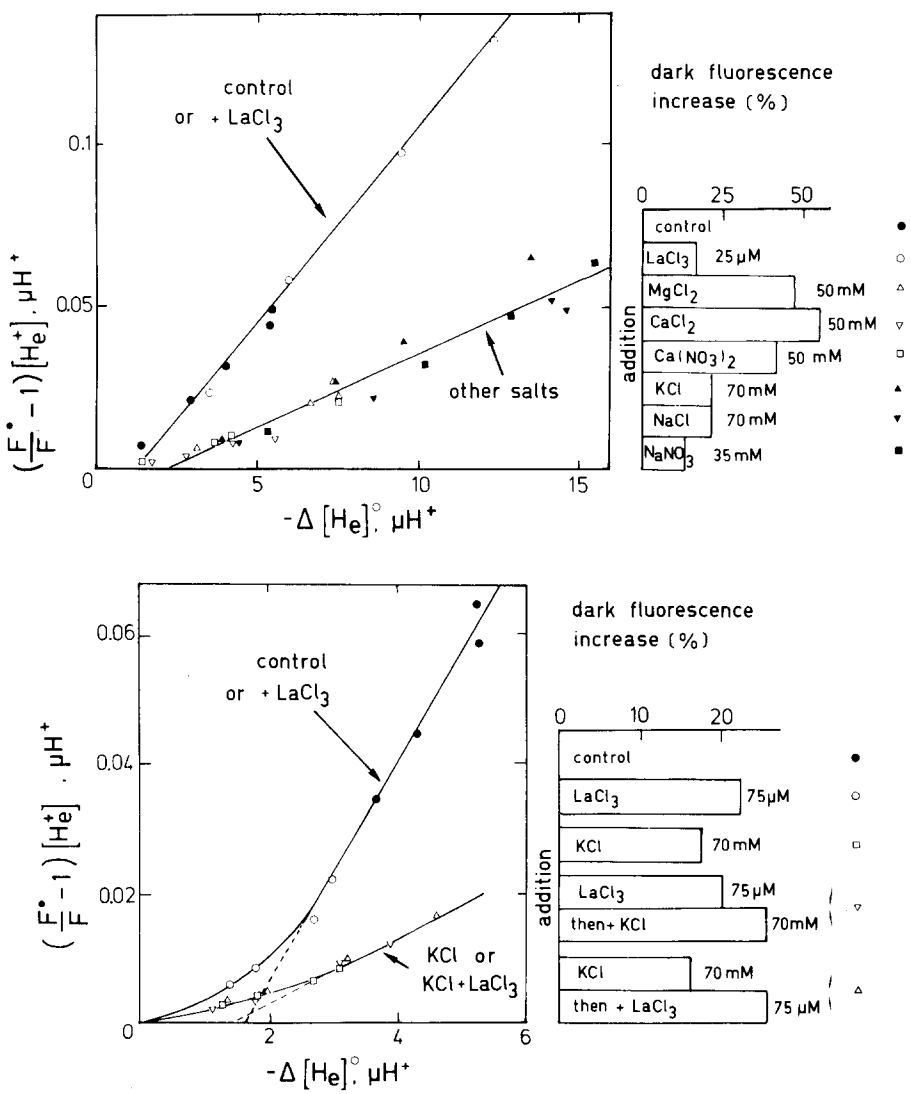


**Fig. 8. Salt effects on the correlation between the 9-aminoacridine fluorescence quenching and the proton uptake. Chloroplasts 40 μM Chl in 200 mM sorbitol + 10 mM NaCl + 50 μM methyl viologen + 4 μM 9-aminoacridine; $pH_e \approx 7.0$ (slightly buffered suspension); each sample was illuminated 4—5 times with an increased intensity of the red actinic light (approx. 5 → approx. 500 W · $m^{-2}$). Right-hand part: diagrams showing the salt effectiveness on the 9-aminoacridine fluorescence with chloroplasts in darkness. Top: $La^{3+}$ and other cations tested separately. Bottom: effect of $La^{3+}$ with and without KCl.**

usual, the proton uptake was also controlled. To obtain the data shown in the Fig. 8, top, mono-, di- and trivalent cations (with different anions) were used, at variable light intensity. Only $La^{3+}$ does not change the correlation between the light-induced quenching and the total proton uptake. With all the other salts, $\gamma$ is little less than trebled, as in the experiments described above made at variable osmolarities. This is still observed in the presence of additional $La^{3+}$ (Fig. 8, bottom). Yet $LaCl_3$ is as effective as KCl, NaCl or $NaNO_3$ in releasing the dye excess from the diffuse layer. It may be that the change in $\gamma$ is mainly due to a salt-increase of the chloroplast buffering power, similar to that previously reported [23]. Mono- and divalent cations, by the significant ionic strength increase, will indeed induce protein conformational changes (cf. Ref. 24). This will not happen with $La^{3+}$, which may be used only at much lower concentrations (above approx. 100 $\mu$M, the chloroplasts aggregate); besides, $La^{3+}$ probably does not cross the membrane, contrary to the (slow) permeation of the other ions.

## Discussion

The first problem raised by the use of 9-aminoacridine is due to its optical detection. Although not just one simple mechanism should be involved, it is universally admitted that this dye does not fluoresce inside the vesicles. Also, the eventual light-induced variations of the chloroplast optical properties have insignificant effect on the fluorescence of an external probe (fluorescein test, Ref. 25), which was confirmed here. Finally, when the fluorescence quenching of 9-aminoacridine is measured directly or by flow-dialysis, similar proton gradients are found [6]. Thus the optical artifacts may be neglected.

The second problem concerns the comparative behaviour of 9-aminoacridine and of the other probes. As shown by Figs. 5—7, the criticisms of Fiolet et al. [7] against 9-aminoacridine may be largely overcome if the proton uptake be controlled on the sample used for the fluorescence determination. This remark applies also to the experiments of Tillberg et al. [10] who, in addition, used intact chloroplasts while testing the $NH_4Cl$ effect. Unfortunately, a similar type of measurement could not be made on our envelope-free chloroplasts: the minimum $NH_4Cl$ concentration required to displace significantly 9-aminoacridine from the diffuse layer inhibited to a great extent their protonmotive activity.

Authors using radioactive alkylamines [12—14,18] always observed $\Delta pH^{\circ}_{ei}$ values higher than those reported with 9-aminoacridine. This could be expected, since unstirred centrifuge tubes were illuminated during the organelle sedimentation through the silicone fluid layer: not only was light unevenly distributed — and may have been limiting — but also centrifugation may have allowed some relaxation of the proton gradient. Besides, when 9-aminoacridine and $NH_4Cl$ are used in comparable conditions [1,11], similar $\Delta pH^{\circ}_{ei}$ values are obtained. However, a different picture was described when, with the same flow-dialysis technique, radioactive methylamine and 9-aminoacridine were compared [6]. But this may be understood if one considers that he bacterial chromatophore concentration used in the first case was more than 100-fold above that in the second: the light factor was certainly determinant here again.

The other points to discuss are, in our opinion, not specific to 9-aminoacridine but must be extended to all the amines and even to any kind of protonizable probe. Among them is the importance of the internal volume, $V_i$, in the determination of the internal proton concentrations. Even though the true total and free proton concentration changes inside are affected by a light-induced variation of $V_i$ (Eqns. A-16 and A-13 of the Appendix), their ratio, $\gamma$, used in the present work, eliminates this volumetric factor; furthermore, the absolute error in $V_i$ has only a limited influence on the true $\gamma$ (Eqn. 9). On the other hand, one should notice that the possible permeation of the amine cation [18] may only reduce the fluorescence quenching as a dynamic change in $V_i$ would have done: yet it is an overestimation of the $\Delta pH^{\circ}_{ei}$ which is suspected by some authors [6,10,14].

One of the main questions is that of the probe-membrane interaction [8—10]: 9-aminoacridine is attracted, as any other cation, to the diffuse layer of the thylakoid membranes. The resulting ion excess depends on the potential $\Psi$ existing at the 'plane of closest approach': $\Psi$ decreases when the surface charge density, $\sigma$, decreases or the salt concentration in the bulk solution increases. Whereas mono- and divalent cations reduce the potential via this second mechanism, $La^{3+}$ probably reduces it via the first one. This is suggested by the strong binding of $La^{3+}$ and $Tb^{3+}$ to the membrane, illustrated by the chlorophyll fluorescence studies [24,26], and the required concentration for a significant effect is quite low. The dye release from the Gouy diffuse layer, which results from this potential drop, does not alter the free proton measurement, as shown by the $\gamma$ constancy. On the other hand, functioning of the redox chain may also induce a change of $\sigma$ and therefore of $\Psi$. But the electrical field is much stronger at the onset of illumination than at the steady-state [27,28]. Therefore, the related 9-aminoacridine fluorescence quenching should finally be limited and its importance on the stationary proton gradient estimation small.

Lastly, one must discuss the existence of a Donnan-type pH difference in darkness ($\Delta pH^{\bullet}_{ei}$). Because it occurs at equilibrium, i.e without energy input, it cannot be dissipated by a protonophore such as gramicidine, as was expected [9]. However, our theoretical and experimental study (Figs. 2 and 3) showed that such a $\Delta pH^{\bullet}_{ei}$ is certainly small under the current ionic strength conditions (see Chow and Hope [29] for a detailed modeling of the ionic distribution, especially in light). At the same time, the added salts lower the $\Delta pH^{\bullet}_{ei}$, they also reduce the pH difference between the bulk solution and near the surface, which is correlated to the membrane potential. At 10 mM KCl, this difference may be approx. 1 pH unit [30]. Although such residual pH differences would not significantly bias our proton-gradient measurements in the light, they should place the membrane reactive components in an environment distinct from that prevailing in the suspending medium.

## Appendix

*Internal proton concentration measurement with diffusible amines*

$R$ symbolizes the protonizable monoamine. The basic assumption [1] are: the charged form is completely impermeant and the neutral form freely equilib-

rates in all conditions. However, because we ignore the solvent state inside the thylakoid, a partition coefficient, $p$, ($\gtrless 1$) may govern the distribution of the neutral $R$ between the external and internal compartments of volumes $V_e$ and $V_i$, respectively ($V_e + V_i = V_t$, constant).

Thus:

$$[R_i] = p[R_e] \tag{A-1}$$

$$RH^+ \overset{K}{\leftrightarrow} R + H^+ ; \qquad K = \frac{[R][H^+]}{[RH^+]} \tag{A-2}$$

$$\text{total amine } [Rt] = [RH^+] + [R] \tag{A-3}$$

Therefore (in $V_e$ or $V_i$):

$$\frac{[Rt]}{[R]} = 1 + \frac{[H^+]}{K} = q \tag{A-4}$$

Since $[H^+]$, $p$, and even $K$ are, or may be, different in the relaxed (dark$^\bullet$) and energized (light$^\circ$) states, and in $V_e$ and $V_i$, one has: $q_e^\bullet$, $q_i^\bullet$, $q_e^\circ$ and $q_i^\circ$. Replacing in Eqn. A-4 $[R_i]^\bullet$ and $[R_i]^\circ$ by $p^\bullet[R_e]^\circ$ and $p^\circ[R_e]^\circ$ from Eqn. A-3, one has, after division.

$$[Rt_i]^\bullet = p^\bullet \frac{q_i^\bullet}{q_e^\bullet} [Rt_e]^\bullet \tag{A-5}$$

$$[Rt_i]^\circ = p^\circ \frac{q_i^\circ}{q_e^\circ} [Rt_e]^\circ \tag{A-6}$$

The conservation law is:

$$V_e^\bullet[Rt_e]^\bullet + V_i^\bullet[Rt_i]^\bullet = V_e^\circ[Rt_e]^\circ + V_i^\circ[Rt_i]^\circ \tag{A-7}$$

The replacement of $[Rt_i]^{\bullet,\circ}$ by their values from Eqns. A-5, 6 and of $q_{i,e}^{\bullet,\circ}$ by their values from Eqn. A-4 gives finally the true $[H_i^+]^\circ$ in the most general form:

$$[H_i^+]^\circ = (K_e^\circ + [H_e^+]^\circ) \frac{g^\bullet V_i^\bullet}{g^\circ V_i^\circ} \left\{ \frac{[Rt_e]^\bullet}{[Rt_e]^\circ} \left( \frac{V_e^\bullet}{g^\bullet V_i^\bullet} + \frac{K_i^\bullet + [H_i^+]^\bullet}{K_e^\bullet + [H_e^+]^\bullet} \right) - \frac{V_e^\circ}{g^\bullet V_i^\bullet} \right\} - K_i^\circ \tag{A-8}$$

with

$$g^\bullet = p^\bullet K_e^\bullet / K_i^\bullet \text{ and } g^\circ = p^\circ K_e^\circ / K_i^\circ \tag{A-9}$$

Thus the partition coefficients and the dissociation constants ratios may apparently change the true internal volume. At the present time, for lack of information, one has to assume that $p^\circ = p^\bullet = 1$ and that all the $K$ values are equal. Also, when $[H^+] >> K$ (p$K$ of ammonium $\approx 9.24$ and of the 9-amino-acridine N-ring $\approx 9.99$), $K$ may be omitted. At last, since $V_e >> V_i$, it may be considered invariable: $V_e^\circ = V_e^\bullet = V_e$. Therefore:

$$[H_i^+]^\circ \approx [H_e^+]^\circ \left\{ \frac{[Rt_e]^\bullet}{[Rt_e]^\circ} \left( \frac{V_e}{V_i^\bullet} + \frac{[H_i^+]^\bullet}{[H_e^+]^\bullet} \right) - \frac{V_e}{V_i^\bullet} \right\} \frac{V_i^\bullet}{V_i^\circ} \tag{A-10}$$

which is similar to the equation previously published [15] and is used here to

appreciate the weight of the dark proton gradient and of the light induced internal volume change.

One would admit, with the other authors, that 9-aminoacridine does not fluoresce inside $V_i$, and is therefore detected only outside, in $V_e$. Since the protonated form predominates much above the other, even though the external pH may change in light, the complex parameter, $b$, used below may be considered invariable; in addition, because $[Rt_e]$ is kept low, the proportionality approximation applies. Thus ($a$ is constant in any given experimental optical condition):

$$F = a(1 - e^{-b[Rt]}) \approx ab[Rt] \tag{A-11}$$

that is, the total amine external concentration ratio is replaceable by the fluorescence ratio $F^\bullet/F^\circ$. In case $[H_i^+]^\bullet = [H_e^+]^\bullet$ and $V_i^\circ = V_i^\bullet = V_i$, one finally has

$$10^{\Delta pH_{ei}^\circ} = \frac{[H_i^+]^\circ}{[H_e^+]^\circ} \approx \left(\frac{F^\bullet}{F^\circ} - 1\right) \frac{V_t}{V_i} + 1 \approx \left(\frac{F^\bullet}{F^\circ} - 1\right) \frac{V_e}{V_i} \text{ (with } V_e \approx V_t\text{):} \tag{A-12}$$

the form with $V_e/V_i$ being identical to the formulation of Schuldiner et al. [1] and that with $V_t/V_i$ being valid even when $F^\circ \to F^\bullet$.

*Relationship between the apparent, experimental, data (symbol') and their true counterparts*

Experimentally, only Eqn. A-12 is easily utilisable, but it is based on the above-mentioned assumptions and the internal volume which it uses is that given by Eqn. 3 (which is emphasized by writing it $V_i'$ below). The internal free proton concentration computed in this way is therefore apparent and shall be written here $[H_i^+]^{\circ\prime}$, whereas the true $[H_i^+]^\circ$ is that of Eqn. A-10. Substituting $F^\bullet/F^\circ$ in Eqn. A-12 by its value from Eqn. A-10, where $[Rt_e]^\bullet/[Rt_e]^\circ = F^\bullet/F^\circ$, the following relationship is found between apparent and true free internal protons ($r = [H_i^+]^\bullet/[H_e^+]^\bullet$):

$$[H_i^+]^{\circ\prime} = \frac{(V_i^\circ/V_i^\bullet)(V_e/V_i' + 1)[H_i^+]^\circ + (V_e/V_i^\bullet - rV_e/V_i')[H_e^+]^\circ}{V_e/V_i^\bullet + r} \tag{A-13}$$

A similar situation exists for total proton concentration change:

$$\text{true } \Delta[H_i]^\circ = -\frac{V_e}{V_i^\circ} \Delta[H_e]^\circ \tag{A-14}$$

$$\text{apparent } \Delta[H_i]^{\circ\prime} = -\frac{V_e}{V_i'} \Delta[H_e]^\circ \tag{A-15}$$

$$\text{Hence } \Delta[H_i]^{\circ\prime} = \frac{V_i^\circ}{V_i'} \Delta[H_i]^\circ \tag{A-16}$$

(In Eqns. A-13 and A-16, free $[H_e^+]^{\bullet,\circ}$ and total $\Delta[H_e]^\circ$ external protons are considered exactly known, since no special assumptions are needed here.)

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