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THE BUFFERING CAPACITY OF THE INTERNAL PHASE OF THYLAKOIDS AND THE MAGNITUDE OF THE pH CHANGES INSIDE UNDER FLASHING LIGHT

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Summary

The buffering capacity inside thylakoids is determined and the magnitude of flash-induced pH changes inside is calibrated in the pH range from 6.4 to 8.1. The work is based on flash-induced absorption changes of neutral red in a chloroplast suspension in which the outer phase is strongly buffered by bovine serum albumin. It is shown that neutral red is bound inside thylakoids. The binding can be described by a simple isotherm with an apparent K_m = 4 μM and saturation at 1 neutral red per 17 chlorophylls. The apparent pK of neutral red is shifted from 6.6 in solution to 7.25 when bound inside. It is demonstrated that neutral red is a clean indicator of pH changes inside, i.e. when properly used it shows no response to other events. Although bound it reports pH changes which occur in the internal osmolar (aqueous) volume of thylakoids. This is obvious from the influence of chemically very different buffers on the magnitude of the absorption changes of neutral red. These act in a manner proportional to their calculated buffering capacity in aqueous solution. The intrinsic buffering capacity of the internal phase is determined with the aid of these buffers, at pH 7.2 it is between 0.8 and 1 mM (at 60 mosM). The absence of large variations in the buffering capacity in the range from pH 6.4 to 8.1 suggests that proteinaceous groups are involved in addition to the lipids which may dominate the buffering capacity at lower pH. The magnitude of the internal pH change is approx. 0.6 (at pH 7.3) under stimulation of both photosystems with a short xenon flash of light.

Introduction

For more than a decade the pathway of energy flow in photophosphorylation and in oxidative phosphorylation has been under debate. After the discussion on whether or not protons are obligatorily involved has faded away (for a review of the various positions, see the multiauthored review by Boyer et al. [1]) in favour of the causality sequence: electrons-protons-ATP postulated by Mitchell [2,3], another controversy has been revived. The question is whether protons drive the ATP synthesis via the osmolar (aqueous) volumes (Mitchell [2—5] in his chemiosmotic hypothesis) or whether they are confined to special conducting subspaces within the respective membrane (Williams [6—9] in his localized proton hypothesis). In the public debate the weight seems to be on the side of the chemiosmotic concept. These is no doubt that in chloroplasts electron transport is coupled to inwardly directed proton pumping (for reviews, see Refs. 10—13) and that protons placed in the internal osmolar volume can be used for ATP-synthesis (for a review, see Jagendorf [14]). However, there are a few observations which seem to shed doubt on the chemiosmotic concept:

1. Ort et al. [15] studied ATP synthesis under illumination of chloroplasts with short light gates. In the presence of valinomycin (supposed to abolish the light induced electric potential under their conditions) and of permeating buffers (supposed to delay the rapid build-up of a sufficiently high acidification inside) they found the time lag for the onset of ATP synthesis hardly affected by the buffers. This led them to suggest that 'the protons produced by electron transport may be used directly for photophosphorylation without ever entering the bulk of the inner aqueous phase of the lamellae system'.

2. Studies on the free energy difference of the proton across the cristae membrane and on the phosphate potential in steadily respiring mitochondria led VanDam et al. [16] to speculate that the protom-motive-force seen by the ATP synthase is not the one existing between the aqueous bulk phases.

3. Ausländer and Junge [17] found that proton binding at the outer side of the thylakoid membrane lagged behind proton consumption by the respective redox reactions, because of a (removeable) diffusion barrier. They concluded that localized proton sinks exist within the outer part of the thylakoid membrane.

The salient point of the controversy on the pathway of protons is whether or not passage of protons through the internal volume of thylakoids is obligatory for their use by the ATP synthase. We have tackled this by using the pH-indicating dye neutral red, which we have introduced previously [18—21]. Our results (preliminary represented in book articles [22,42]) show that protons which are liberated by the water-oxidizing enzyme system equilibrate with the internal osmolar (aqueous) volume (with relaxation times down to 100 μs) before being taken up by the ATPsynthase. This seems to exclude localized protons in photophosphorylation. However, it makes the apparent contradiction between the data of Ort et al. [15] and the chemiosmotic concept even worse. It seems worthwile to reexamine the matter from the energetic point of view. Ort et al. [15] had no access to the electric potential across the thylakoid membrane nor to the magnitude of the flash induced pH changes in the internal

phase. The quantitative measurement of the small flash induced pH changes inside is the subject of this communication.

Again we have used the dye neutral red as an indicator of pH changes inside thylakoids. We demonstrate that this dye is a clean pH indicator when used properly. The we present evidence that it monitors pH changes which occur in the internal aqueous volume and not just somewhere within the thylakoid membrane. We have investigated the intrinsic buffering capacity of the internal phase which is close to 1 mM (at pH 7.2). And we have calibrated the pH change induced inside by a single turnover of both photosystems it is approx. 0.6 units.

Materials and Methods

Chloroplasts were prepared from market spinach according to standard procedures [23]. They were stored under liquid nitrogen until use. (Control experiments with freshly prepared chloroplasts have yielded similar results.) After thawing aliquots were suspended in the follwoing standard reaction medium (deviations from this are given in the figure legends): chlorophyll, 10 μM; KCl, 25 mM; $MgCl_2$, 3 mM; bovine serum albumin, 1.3 mg/ml; benzylviologen, 10 μM. The concentration of neutral red (if present) is given in the figure legends. The pH was adjusted to 7.2—7.3 if not otherwise stated. The medium pH was stable to within —0.1 units during the actual sampling interval (10 min). All experiments were done at room temperature.

Neutral red (3-amino,7-dimethylamino,2-methylphenazine) was purchased from Sigma (95% pure) or from Merck (70% pure according to our measure-

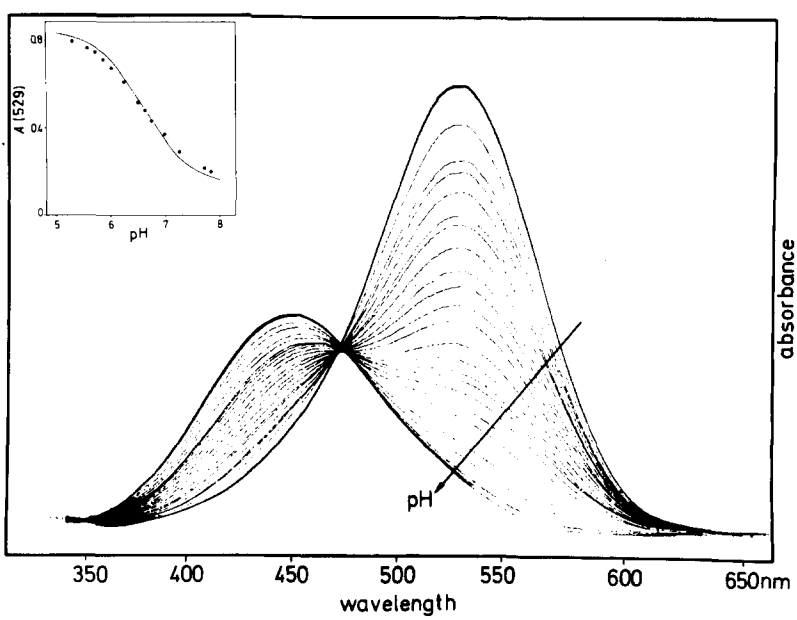

Fig. 1. Titration of neutral red absorption versus pH. Neutral red at 30 μM. The titration behaviour was independent of the salt concentration (in the range of the standard reaction medium). The deviation from a true isosbestic point and a simple one-pK-titration is not caused by experimental error.

ments) and used without further purification. (When a 1 : 0.7 correction was applied to results obtained with the Merck product both batches produced compatible figures.)

Fig. 1 shows the titration of neutral red in a Shimadzu MPS-50 spectrophotometer. The solid curve in the inset shows the calculated behaviour of a dye with one single pK = 6.6 and a difference of the respective extinction coefficients of the protonated and the unprotonated form: $\epsilon^{H}(524\ nm) - \epsilon(524\ nm) = 2.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. The difference at 550 nm was $2.2 \cdot 10^4$. The dye does not show an accurate isosbestic point and a deviation from the calculated titration curve (see inset) is also apparent. However, we have not observed the pronounced complex titration behaviour (with two pK values 5.89 and 7.38) which was reported by Bartels [41]. The reason for the simpler behaviour of the dye (in the above standard medium except for bovine serum albumin) has not been investigated.

The internal volume of thylakoids was controlled by the addition of sorbitol which does not penetrate this space [24]. The published figures for the dependence of the internal volume on the osmolarity are greatly at variance (e.g. refs. 15, 24, 25). We will use no absolute figures for the internal volume except where we present semiquantitative controls for quantitative estimates. Therein we will follow the internal volumes obtained by Ort et al. [15] by ^3H_2O and centrifugation of chloroplasts through silicone [26] in order to remove external water.

Various buffers * have been used to probe the accessibility of the space inside thylakoids from which neutral red indicates pH changes. All buffers (see Fig. 3 for a collection) acted on the magnitude of the pH_{in}-indicating absorption changes of neutral red after less than a minute's incubation. Even for notoriously hydrophilic buffers (e.g. phosphate) the observed effect was independent of whether the experiment was performed after 1 or 20 min incubation.

The reaction mixture was used to fill an optical absorption cell (2 × 2 cm) mounted in a kinetic spectrophotometer (see ref. 27). The measuring light was filtered through an interference filter (Al 550 nm, Schott). The measuring

* Buffers

Symbol	Name	pK	Charge of base
Tris	tris(hydroxymethyl)aminomethane	8.3	
Tricine	*N*-tris(hydroxymethyl)methylglycine	8.15	1—
Mops	morpholinopropanesulfonic acid	7.2	1—
P_i	phosphate (inorganic)	6.8	2—
PP_i	pyrophosphate (inorganic)	6.54	3—
Aces	*N*-2-acetamido)-2-aminoethanesulfonic acid	6.9	1—
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid	7.55	1—
Mes	2-(*N*-morpholino)-ethanesulfonic acid	6.15	1—
Pipes	piperazine-*N*,*N'*-Bis(2-ethanesulfonic acid)	6.8	1—
Tes	*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid	7.5	1—
	glycylglycine	8.4	1—
	imidazole	7.1	
	(2-aminoethyl)trimethylammonium chloride hydrochloride : (cholamine chloride)	7.1	1+

light was gated open only during the measuring interval (typical 250 ms) plus an allowance of 200 ms to adapt the photomultiplier to the light level. The energy per pulse of the measuring light was below 18 $\mu J/cm^2$, which excited less than 5% of the reaction centers at the given wavelength. The sample was excited by a short flash of light (Osram, XIE 200, 15 μs FWHM) filtered through a RG2/3 (Schott and Ge.) with a short wavelength cut-off at 625 nm. The photomultiplier cathode was protected against flash burst artefacts by two filters (AL550, Schott and Ge. and 575 nm cut-off, Balzers). The aperture of the photomultiplier relative to the cuvette was 4°. The absence of apparent absorption changes due to light scattering transients was confirmed by measurements at different apertures. The photomultiplier tube (EMI 214 D) was operated at 500 V with 4 or 6 dynodes. Transient absorption changes were digitized and averaged on a Nicolet NIC 1072 computer. The time resolution of the circuit was limited by the time-per-adress setting of the computer (typical 1 ms, but also down to 20 μs, integrating input SD 72IA). The photochemical reactions were initiated repetitively by flashes at a repetition rate of 0.1 and sometimes 0.03 Hz (see figure legends). Up to 120 signals were averaged. Transient signals obtained in the presence and in the absence of neutral red were subtracted using the arithmetic capability of the averaging computer.

As the pH_{in}-indicating absorption changes were obtained as the difference between transient signals measured in the presence and in the absence of the dye (and hence measured with different samples and at different times), special care was taken to avoid artifactual differences due to ageing (synchronous handling) or to pipettage error (chlorophyll determination in the respective samples).

Results and Discussion

Is neutral red a clean indicator of pH changes inside thylakoids?

When a chloroplast suspension is excited with light in the presence of neutral red additional absorption changes appear. These were attributed by Lynn [28] to pH changes inside thylakoids. We have previously shown that this attribution holds only if the external phase is strongly buffered by a non-permeating buffer (e.g. bovine serum albumin) [18]. We will now discriminate the pH_{in}-indicating absorption changes of neutral red from other absorption changes, i.e. the intrinsic absorption changes of chloroplasts and other absorption changes of neutral red (due to photochemical reactions of the dye or to redox reactions with chloroplasts).

Fig. 2 shows the flash induced absorption changes at 550 nm in a chloroplast suspension containing bovine serum albumin.The three traces in the upper row of Fig. 2 were obtained under conditions where no response of neutral red to a flash induced pH change inside thylakoids could be expected, either because of the absence of neutral red (left and middle) or due to the presence of a permeant buffer (middle and right). The traces below were obtained by subtraction of the traces in the upper row as indicated (Note the twofold expanded ordinate scale.). The left trace in the lower row shows the influence of the added buffer Mops on the intrinsic absorption changes of chloroplasts in the

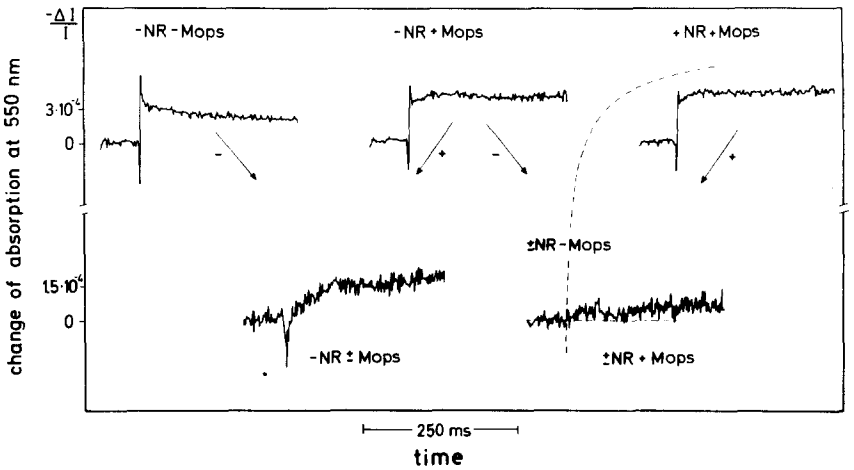

Fig. 2. Flash induced absorption changes at 550 nm which could serve as controls for the pH_{in}-indicating absorption changes of neutral red. Standard medium except for neutral red and Mops. 8 transient signals averaged, repetition rate 0.08 Hz. Time per address of the averaging computer 100 μs. Upper left: no neutral red, no Mops; upper middle: no neutral red, plus Mops, 70 mM; upper right: neutral red, 13 μM, Mops, 70 mM. Lower left: difference upper left minus upper middle; lower right: difference upper middle minus upper right. Lower traces are at 2-fold expanded scale. Broken curve (—Mops ± neutral red).

absence of neutral red. The right trace shows the reponse of neutral red to events other than pH changes inside thylakoids. It is apparent that this 'artefactual response' of neutral red is negligible in comparison with its reponse to pH changes (obtained in the absence of Mops), which is illustrated by a broken line in the lower right of Fig. 2.

We conclude that neutral red is a clean indicator of pH changes inside if the difference of absorption changes in the presence and in the absence of neutral red is recorded. On the other hand, a record of the difference between signals obtained in the absence and in the presence of a permeant buffer produces a small artefact. (We have used this approach in our previous studies.)

Fig. 3 shows the influence of several chemically different buffers on the pH_{in}-indicating absorption changes of neutral red (with or without neutral red). The extent of the flash induced absorption changes is plotted against the buffering capacity, which was calculated for each buffer according to Eqn. 1 (see also Eqn. A3):

$$\beta_b = -2.3B^t HK_b/(H + K_b)^2 \qquad (1)$$

wherein B^t is the total concentration of the added buffer in the suspending medium, K_b is its dissociation constant and H is the proton concentration of the chloroplast suspension (see also Appendix I).

The line in Fig. 3 is the calculated dependence of the flash induced absorption changes of neutral red calculated according to Eqn. A9 in Appendix I under two assumptions: (1) the added buffers are dissolved in the internal aqueous space with their internal concentration equal to their external one, and (2) the internal buffering capacity (intrinsic groups plus neutral red) is 1.15 mM. The experimental points in Fig. 3 show that chemically very different buffers (see full symbols only) with net charges of the respective base

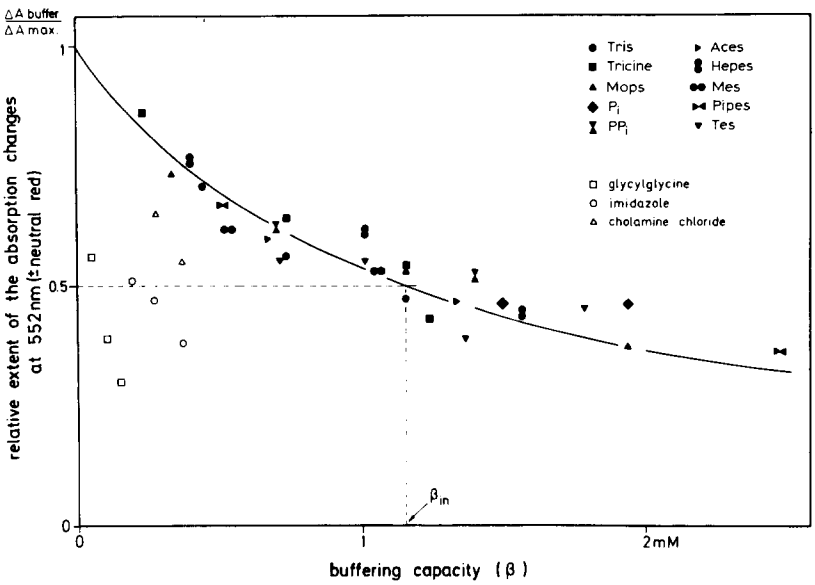

Fig. 3. Extent of the flash induced absorption changes of neutral red (±neutral red) as function of the buffering capacity of added buffers. The line is theoretical, calculated according to Eqn. A9. It was assumed that the buffering capacity of the internal phase ($\beta_{in} = \beta_{intr} + \beta_d$) was 1.2 mM. Neutral red was at a concentration of 13 μM (this contributes about 300 μM to the observed buffering capacity, see below). The pH of the medium was at 7.3.

ranging from minus three (PP_i) to zero (Tris) and with pK values ranging from 6.15 (Mes) to 8.3 (Tris) act almost identically, just as expected if they were dissolved in the internal aqueous volume. The observation that three buffers (cholamine, glycylglycine and imidazole, see open symbols) are more effective may indicate that these buffers are adsorbed or bound to the inner side of the thylakoid membrane and hence enriched in the internal phase (we find that they are slightly uncoupling).

The most probable interpretation of the data represented in Fig. 3 (full symbols only) is: permeant buffers influence the magnitude of the absorption changes of neutral red by buffering pH changes and by nothing else. Otherwise it would be very difficult to imagine why they act proportional to their buffering capacity and not according to their concentration proper or according to their polarity.

Do the pH changes indicated by neutral red occur in the internal aqueous volume?

Pick and Avron [29] and we also [30] have observed that neutral red is enriched (because it is bound or adsorbed inside) in the internal phase of thylakoids. It is worth asking whether neutral red reports pH changes from the internal aqueous (osmolar) volume of from some sub-compartment within the thylakoid membrane. Fig. 3 has already shown that even hydrophilic buffers act on the absorption changes of neutral red according to the buffering capacity they add to an aqueous solution. Further evidence comes from Fig. 4. The extent of the pH-indicating absorption changes of neutral red is plotted vs.

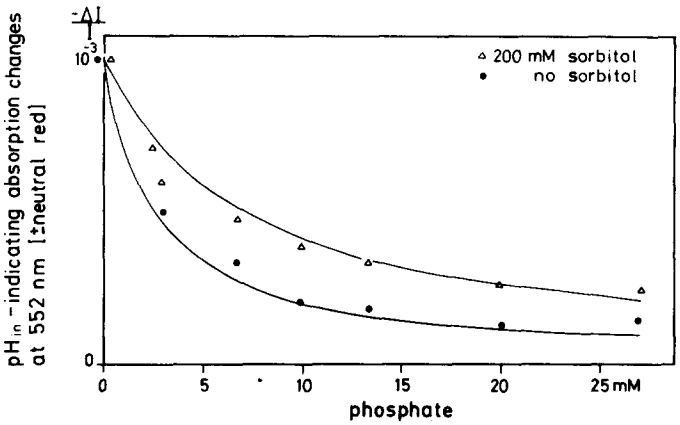

Fig. 4. Extent of the flash induced absorptioh changes of neutral red (±neutral red) as function of added phosphate for two different osmolar states of thylakoids. Standard medium except for neutral red, 13 μM and the presence of sorbitol (upper set of points only), 200 mM. The solid lines show the calculated behaviour according to Eqn. A11 under the assumption that the internal volumes at the two osmolarities are related as 3 : 1.

the concentration of added phosphate buffer for two different osmolar states of thylakoids. The osmolarity is dominated by salts (25 mM KCl plus 3 mM $MgCl_2$) and by 200 mM sorbitol plus these salts, respectively.

Two features are evident from inspection of the experimental points in Fig. 4. (1) The extent of the absorption changes of neutral red in the absence of added buffer is independent of the internal volume of thylakoids. (2) The efficiency of phosphate in buffering the absorption changes of neutral red is higher for the larger internal osmolar volume. These features can be qualitatively interpreted as follows: (1) It is reasonable to assume that the number of active proton pumps and the number of intrinsic buffering groups inside is independent of the osmolar state of thylakoids (in the van't Hoff range). As the buffering capacity of these groups exceeds the one of internal water (see below) the flash induced pH change should be independent of the internal volume. This in turn would produce absorption changes independent of the internal volume if neutral red was enriched (bound or adsorbed to the membrane) inside, so that the total amount 'seeing' the pH-changes was unaltered. (2) The lower efficiency of phosphate for smaller internal volume is consistent with the notion that higher osmolarity 'squeezes out' water and therewith permeant water soluble buffers.

The curves in Fig. 4 were calculated according to Eqn. A11. All under the assumption that the ratio of the internal volumes at the osmolarities 60 mosM and 260 mosM is 3. This is less than the expected ratio for an ideal osmometer (4.3) under these conditions, however, it is the same order of deviation from ideality that Ort et al. [15] have observed in their determinations of the internal volume. To fit the data we have assumed that the internal buffering capacity contributed by the intrinsic groups and by neutral red, $\beta_{intr} + \beta_d$ = 1.15 mM. The buffering capacity contributed by phosphate was calculated according to Eqn. 1.

The approximate fit of the data by the calculated curves confirms that

neutral red 'sees' pH changes in a compartment which, at least, rapidly exchanges protons with the internal aqueous volume of thylakoids. Hence practically it monitors pH changes in this volume proper. In other communications we have reported on the time required for the intercommunication (in terms of protons) between the neutral red space and the internal aqueous volume [22,42]. The half-time is at less than 100 μs.

The apparent pK of inside located neutral red molecules

As mentioned previously, neutral red is bound inside thylakoids. How much is the apparent pK of the dye changed upon the binding to the inner side of the membrane? Fig. 5 shows the extent of the flash induced absorption changes of neutral red as a function of the pH of the suspending medium. The repetition rate of the periodical flash was very low (0.03 Hz) and the measuring light was also very low (the time averaged intensity of the gated light was less than 1 μW/cm^2) to avoid any pH difference except the one induced by the flash just fired. The experimental points belong to three different chloroplast preparations. The curve is the calculated differential sensitivity of a dye with a pK = 7.25 according to Eqn. A6 (see Appendix I). (It is worth mentionning that the same profile of the empirical sensitivity of neutral red was observed at different osmolarities and at different ionic strength. However, the absolute magnitude

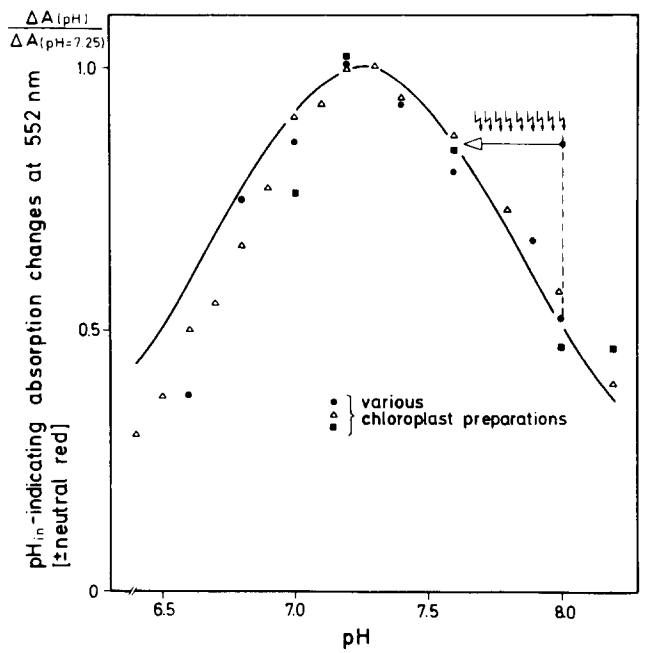

Fig. 5. Extent of the flash induced absorption changes of neutral red as function of the medium pH. Standard medium with 9 μM neutral red. Repetitive flash excitation at 0.03 Hz. Time averaged intensity of the gated measuring light less than 1 μW/cm^2. The data points were obtained with three different chloroplast preparations. They were independent of the presence of sorbitol. The curve shows the theoretically expected differential sensitivity of an indicator dye with a pK of 7.25 (calculated according to Eqn. A6).

of the absorption changes was lower at high salt. This will be discussed in more detail elsewhere.)

Broadly speaking, the experimentally observed behaviour of inside located neutral red molecules conforms to a pK of 7.25. A shift of the apparent pK from the value in solution (6.6) towards alkalinity parallels the behaviour of neutral indicator dyes bound to negatively charged lipid micelles known from the model studies by Fernandez and Fromherz [31]. Lacking independent measure of how much the variation of the medium pH acts on the binding equilibrium of neutral red inside (and thereby on the shape of the sensitivity curve) we will use the experimental points of Fig. 5 rather than overemphasizing the theoretical fit. We note that the empirical sensitivity curve is rather flat in the pH range from 7.0 to 7.5. It can be expected that the small flash induced pH changes inside are linearly related to the absorption changes of neutral red in this pH range. This is advantageous for kinetic studies.

On the non-linear dependence of the flash induced absorption changes of neutral red on the dye concentration

Fig. 6 shows the magnitude of the flash induced absorption changes of neutral red as function of the dye concentration in the suspending medium. The solid curve is a fit of the data by a binding isotherm (Eqn. 3):

$$D^{t}_{in}(\text{bound}) = D^{t}_{in,max}(K_{in}/D^{t}_{out} + 1)^{-1} \quad (D_{bound} >> D_{free}) \tag{2}$$

wherein D^t is the total dye concentration inside and outside, respectively, and K_m is the dissociation constant of the binding equilibrium. (We understand the concentration of a species, which is bound to the membrane, such as if it were homogeneously distributed over the internal aqueous volume.)

To fit the data we have chosen K_m = 4 μM and the constant $D^{t}_{in,max}$ such that

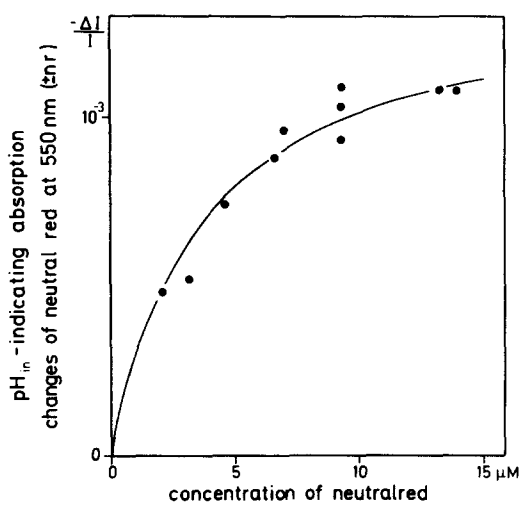

Fig. 6. Saturation of the magnitude of the flash induced absorption changes of neutral red as function of the neutral red concentration in the medium. Standard medium, pH 7.3. The curve is a fit by a binding isotherm with a dissociation constant K = 4 μM and a maximum at $-\Delta I/I = 1.5 \cdot 10^{-3}$.

the maximum corresponds to a relative change in transmitted intensity $-\Delta I/I = 1.4 \cdot 10^{-3}$. The fit just shows that saturation of a binding equilibrium is one possible reason for the nonlinear dependence of the absorption changes on the dye concentration. (We will later show that the increasing contribution of neutral red to the internal buffering capacity does not account for the curvature in Fig. 6. However, dimerization of the dye, which we found to occur at the millimolar range (in aqua), might also reduce the amount of sensitive neutral red molecules.)

The internal buffering capacity of thylakoids

The total buffering capacity of the internal phase of thylakoids is the sum of the capacities contributed by intrinsic groups, by inside located neutral red and by added buffer (see Appendix I). The buffering capacity of internal water is negligible. According to Eqn. A9 the magnitude of the pH_{in}-indicating absorption changes of neutral red are inversely proportional to the total buffering capacity inside. When buffer is added at a concentration such that the buffering capacity of the added buffer (β_b) is equal to the buffering capacity of the intrinsic groups plus the dye ($\beta_{intr} + \beta_d$) the magnitude of the flash induced pH changes is halved. Hence curves as in Fig. 3 can be used to read out the internal buffering capacity ($\beta_{intr} + \beta_d$) (see vertical and horizontal lines in Fig. 3).

Experiments as documented in Fig. 3 were run under variation of the medium pH. The result is shown in Fig. 7. Experimental points are given by full symbols. Each point represents the average of experiments with at least two different buffers (out of the set of ten marked by full symbols in Fig. 3)

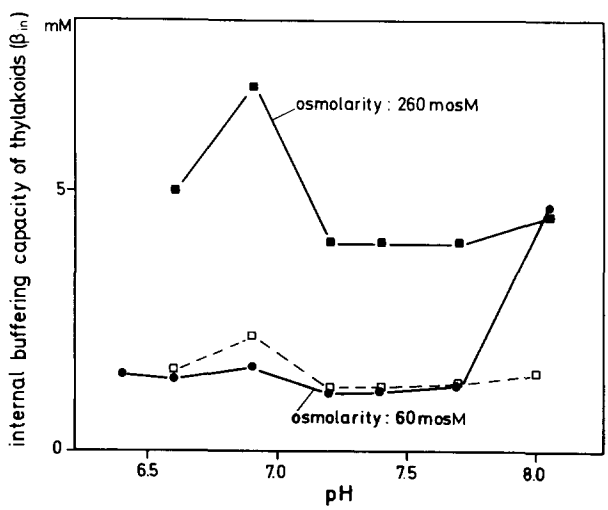

Fig. 7. Intrinsic buffering capacity of thylakoids as function of medium pH. Standard medium, neutral red, 13 μM. Each point represents the average of results obtained with different preparations and at least two of the following buffers: Hepes, Mops, PP_i, Tricine and Tris. Solid lines connect measured points at two different osmolarities as given in the figure. The broken line connects points calculated from the full square points (at 260 mosM) by multiplication with a factor 22/70. This accounts for the influence of the internal volume on the intrinsic buffering capacity as given in Eqns. 1 and A10. The expectation is that the open squares should coincide with the full circles (for the deviation at pH 8, see text).

and with different chloroplast preparations. The internal buffering capacity was determined for two different osmolar states of chloroplasts as indicated. The experimental points at higher osmolarity were transformed to lower osmolarity with the aid of Eqn. A11 by setting the ratio of the internal volumes at the two osmolarities (60 mosM : 260 mosM) $v_0 : v = 3.2$ (as in the discussion of Fig. 4). The transformed points are indicated by open squares. The agreement between the experimental points for low osmolarity and the calculated ones (based on experiments at high osmolarity) is satisfactory. (There is one drop-out point at pH 8.1 which was experimentally reproducible, but has found no explanation yet.) The agreement further strengthens the argument of foregoing sections that neutral red indicates pH changes in the internal osmolar volume.

We asked for the contribution of neutral red to the internal buffering capacity. The determination of the buffering capacity at pH 7.2 and 60 mosM but now at a neutral red concentration of only 3 μM yielded a figure of 1 mM in contrast to the 1.13 mM of Fig. 7 and 1.15 mM of Fig. 3 obtained at 13 μM. According to arguments forwarded in Appendix II we conclude that the buffering capacity contributed by neutral red at an outer concentration of 13 μM is 260 μM. This implies that the intrinsic buffering capacity at this pH (7.2) and at the given osmolarity (60 mosM) is 0.8 mM = β_{intr}. The contribution of neutral red is largest at pH values near its pK. Hence the data in Fig. 7 are a good approximation of the intrinsic buffering capacity of the internal phase (except for a −20% correction around pH 7.25). The data in Fig. 7 show that the intrinsic buffering capacity is not very variable in the pH range from 6.4 to 8. This implies that several groups with different pK values contribute to the buffering capacity. The data furthermore show that the buffering capacity at a given osmolarity (say 60 mosM) can be used to calculate the intrinsic buffering capacity at another osmolarity via the following relation:

$$\beta_{intr}(y) = \beta_{intr}(60)\, v_{in}(60)/v_{in}(y) \qquad (3)$$

The magnitude of the flash induced pH change inside

We determined the magnitude of the flash induced pH changes under excitation with a series of flashes starting from an initial pH 8 in the medium. As successive flashes make the internal phase more and more acidic the operational point on the sensitivity curve of neutral red as function of the pH is shifted to the left (this is illustrated in Fig. 5). In consequence one expects that the magnitude of the flash induced absorption changes increases with increasing flash number. This expected behaviour is in fact observed as evident from the absorption changes of neutral red at excitation with a group of flashes documented in Fig. 8. On the other hand when this experiment is performed at an initial pH 7.3, the extent of the absorption changes decreases with increasing flash number (time course of absorption changes not documented, but the extent numerically represented in Table I). This behaviour has been used to calibrate the absorption changes into pH changes with the aid of the empirical dye sensitivity shown in Fig. 7. We took advantage of the fact that the internal buffering capacity is practically constant in the relevant pH range as well as the number of protons released inside. One example for reading out the pH changes (from Fig. 8 and from a corresponding experiment starting at pH 7.2) is

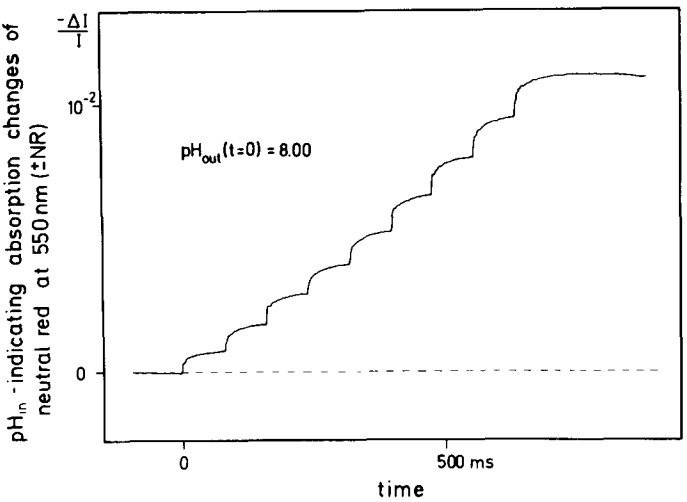

Fig. 8. The pH_{in}-indicating absorption changes of neutral red at 552 nm under excitation of chloroplasts with a series of flashes at 75 ms intervals. The pH of the medium was 8.0. An increase of the magnitude with increasing flash number is obvious. (Measuring light intensity less than 1 $\mu W/cm^2$ and repetition rate of flash groups 0.03 Hz).

TABLE I

CALIBRATION OF THE MAGNITUDE OF THE pH CHANGE INSIDE THYLAKOIDS INDUCED BY STIMULATION OF BOTH PHOTOSYSTEMS FOR A SINGLE TURNOVER

The evaluation is based on the experiment shown in Fig. 8 and on another experiment with a medium pH 7.3 (the downward run of the sensitivity curve shown in Fig. 5). The magnitude of the flash induced absorption change (as function of flash number) in a given run was related to the sensitivity curve (similar to Fig. 5) which was measured in a parallel experiment. The measured magnitude, via the sensitivity curve, yields the value of the internal pH before the firing of the respective flash. The difference between the pH-values associated with successive flashes id the magnitude of the flash-induced pH change.

Flash No.	Initial pH 8.0		Initial pH 7.25	
	$\Delta A_{rel.}$	$\Delta pH \times 10^2$	$\Delta A_{rel.}$	$\Delta pH \times 10^2$
1	0.47		1.0	
		0.0		
2	0.47		0.92	
		4.5		7.0
3	0.51		0.83	
		4.0		5.0
4	0.54		0.77	
		1.5		8.0
5	0.55		0.67	
		10.0		2.0
6	0.62		0.64	
		0.0		4.0
7	0.62		0.59	
		5.5		2.0
8	0.67		0.57	
		2.0		−2.0
9	0.68		0.59	
		3.44		3.71

illustrated in Table I. Repeated experiments with four different preparations of chloroplasts (an empirical sensitivity curve as in Fig. 5 was determined for each preparation) and with two runs starting at pH 8 and two at pH 7.3 yielded: The pH change induced by stimulation of both photosystems for a single turnover (medium pH around 7.3) is —0.05 units. The variance of a total of 16 runs was 0.017. Taking into account that this figure was determined in experiments in the presence of neutral red (outer concentration: 13 μM, equivalent to a 20% contribution to the internal buffering capacity in the relevant pH range), it follows that the pH change in the absence of this dye is —0.065 units.

There is an independent way to estimate the magnitude of the internal pH change per flash. According to Eqns. A1 and A8 the internal pH change is related to the internal buffering capacity (β_{in}), the specific internal volume (v_{in}) and the specific proton release (dh^t_{in}) as follows:

$$dpH_{in} = -\beta_{in}^{-1} \cdot v_{in}^{-1} \cdot dh^t_{in} \qquad (4)$$

Taking reasonable figures for these parameters: β_{in} = 1.15 mM (see above), v_{in} = 58 l/mol chlorophyll (compatible with e.g. ref. 15) and dh^t_{in} = 2/600 per chlorophyll we obtain a pH change of —0.05 units. Hence our previous calibration is consistent with the independently obtained results on the internal buffering capacity.

It might be argued that the method to determine the magnitude of the flash induced pH changes under excitation of chloroplasts with a series of flashes is complicated by the redistribution of neutral red in response to the then relatively large pH difference across the thylakoid membrane. Such a redistribution is the basis for determinations of larger pH differences by fluorescent or radiatively labeled amines [34—37]. First experimental evidence that this redistribution does not interfere severely with our experiments comes from the observation that the magnitude of the absorption changes decreases with increasing flash number when starting from pH 7.2. However, we present more detailed arguments in Appendix II.

It is worth noting that the smallness of the flash induced pH changes justifies the use of the differential equations for the absorption changes (Appendix I) instead of integrated expressions.

Synopsis and General Discussion

The first to use neutral red with chloroplasts was Lynn [28] who claimed that it indicated pH changes in the internal phase of thylakoids. We have previously demonstrated that neutral red indicates pH changes in both aqueous phases, the outer and the inner one, unless the outer phase is strongly buffered (e.g. by bovine serum albumin) [18]. The dye was later used to time resolve proton release inside thylakoids [18—22,38,42] which has helped to locate the water oxidation at the internal side of the membrane (for a review, see ref. 12). As neutral red is bound to the internal side of the thylakoid membrane there has been some doubt whether it can be used for quantitative studies (see Pick and Avron [29] or Saphon and Crofts [38]). The data presented in this communication clearly show that neutral red, if properly used, is a clean indicator of pH changes inside thylakoids. Moreover, although bound, it reports from a

subcompartment which rapidly exchanges protons with the internal aqueous phase, hence, in practice, it reports from this volume proper. The evidence relies largely on the observation that ten chemically very different buffers (among which there are notoriously hydrophilic ones) influence the magnitude of the pH-indicating absorption changes of neutral red in the same way, proportional to their buffering capacity in aqueous solution. The argument is strengthened by the observation that the efficiency of added buffers is decreased if the internal aqueous volume is decreased. (It is noteworthy that even hydrophilic buffers as pyrophosphate seem to permeate the thylakoid membrane at around 10 s.)

At a medium pH around 7 the binding of the dye to the internal side of the membrane follows a simple isotherm with K_m = 4 μM and saturation at about 1 neutral red per 17 chlorophylls.

We have calibrated the sensitivity of inside located neutral red to flash induced pH changes as function of the medium pH. The apparent pK of neutral red came out as 7.25, shifted by 0.65 units towards alkalinity in comparison with the apparent pK of neutral red in aqueous solution. Both the direction and the magnitude of this shift are compatible with what Fernandez and Fromherz [31] have observed for a neutral indicator bound to negatively charged lipids in well controlled model studies.

The saturation of neutral red binding is the reason why the redistribution of this dye in reponse to a pH difference between the two aqueous phases does not prevent a quantitative evaluation of the results (for a thorough discussion of the complications with amines, see ref. 39).

We have used the flash induced absorption changes of neutral red to determine the intrinsic buffering capacity of the internal phase of thylakoids. At an osmolarity of 60 mosM at a medium pH 7.2 we found 900 μM. This figure is already corrected for the contribution of neutral red to the buffer capacity of the internal phase. We did not observe dramatic variations of the buffering capacities in the pH range from 6.4 to 8.1. Little variation in the buffering capacity as function of the pH is characteristic for proteins with several titratable groups with scattered pK values. The intrinsic buffering capacity of the internal phase has been previously determined in two laboratories (Rottenberg et al. [24] and Reinwald [25]). It was attributed to groups with a uniform pK near 5.5. However, the respective experiments were restricted to the more acid range of the internal pH. Whe extrapolating Reinwald's [25] data to the pH-range of our experiments we obtain the following figures for the specific buffering capacity (in mmol per chlorophyll) at a given pH inside (in parentheses): 2.4 (8), 7.2 (7.5), 22 (7), 60 (6.5) and 127 (6). Under the assumption of an internal volume of 58 l/mol chlorophyll our data (at 60 mosM) yield: 127 (6.4) and 68 (7.2). This is compatible with the former figures at the acid end, however, we have to conclude that in addition to the (probably) lipids governing the buffering capacity at lower pH, proteins take over at neutral and alkaline pH.

We have determined the magnitude of the flash induced pH changes inside thylakoids. At pH 7.2 we find a pH change of —0.06 units when both photosystems are stimulated for a single turnover by a short flash of light. It is noteworthy that we obtain the same figure from the measured intrinsic buffering

capacity inside under a reasonable assumption for the internal volume. As the number of protons pumped and the number of intrinsic buffering groups are independent of the internal volume the magnitude of the flash induced pH change inside also is independent. The magnitude of the flash-induced pH change at other values of the medium pH can be inferred from the above figure under consideration of the pH dependence of the internal buffering capacity. We have previously published another estimate for the magnitude of the pH change inside (0.15 units in ref. 21). This was based on experiments with the permeant buffer imidazole. Imidazole is one of those buffers which (since probably binding to the membrane) acts more effectively than the majority of buffers (among which are the very hydrophilic ones). This leads to underestimate the internal buffering capacity by a factor of 3—4.

We have left out the influence of surface potentials on neutral red binding and on the magnitude of the flash induced pH changes inside. All experiments were carried out at rather higher salt concentrations. Salt effects will by the subject of another communication.

Appendix I

On the extent of pH-indicating absorption changes of a dye located inside a biological vesicle

We derive an expression for the extent of pH-indicating absorption changes of a dye located inside thylakoids as function of the pH, the presence of buffers and under variation of the internal volume. To simplify the discussion we assume only two relevant phases, the outer and the inner one. Buffering groups bound to the membrane are attributed to the phase from which they are most accessible to protons. We assume that the delocalization of protons in both aqueous phases is rapid enough to guarantee a constant electrochemical potential of the proton throughout each phase in the time domain of interest. The outer phase should be strongly buffered so that stimulation of the proton pump in the membrane causes pH changes only in the inner phase. The treatment is restricted to very small pH changes inside (complications arising from larger changes are given in Appendix II).

The following symbols are used:

x	a buffering species,
K_x	the dissociation constant of x (acid),
X, X^H and X^t	the concentration of x in the unprotonated form, the protonated form and total (unprotonated plus protonated),
β_x	the differential buffering capacity of x,
β_{intr}	the buffering capacity by intrinsic groups inside,
D (and subscript d)	the concentration of the pH-indicating dye inside,
B (and subscript b)	the concentration of added buffer inside,
V_{in} and V_{out}	the volumes of the respective phases,
dH_{in} and dH^t_{in}	a differential change of the internal concentration of free protons and of all protons (bound plus free), respectively.

When thylakoids are considered in particular the following symbols are used:

C_{chl} the concentration of chlorophyll in a chloroplast suspension,

dh^t_{in} the differential amount of protons released inside (bound plus free) related to the number of chlorophyll molecules (in mol H^+/mol chlorophyll), 'specific proton release'.

We define the differential buffering capacity of a given species x:

$$dH^t = -\beta_x \, dpH \tag{A1}$$

If several buffering groups are present in a given phase a similar relationship holds with the total buffering capacity additive in the particular ones. We take the buffering capacity of the internal phase of thylakoids as due to intrinsic groups, the pH-indicating dye and added buffer so that:

$$\beta^t_{in} = \beta_{intr} + \beta_d + \beta_b \tag{A2}$$

The buffering capacity contributed by a particular species x is:

$$\beta_x = 2.3 \, X^t \cdot K_x \cdot H/(H + K_x)^2 \tag{A3}$$

where H is the concentration of free H^+. The intrinsic buffering capacity of the internal phase is due to several groups with different pK values and in addition to inside located water molecules:

$$\beta_{intr} = 2.3 \left(\sum_{n=1}^{N} C^t_n K_n/(H + K_n)^2 + a \right) H \tag{A4}$$

where a acounts for the buffering power of water (a is the order of 1 and negligible relative to the other terms!).

The contribution of the inside located dye molecules to the total absorbance of a chloroplast suspension is:

$$A_{d,in} = (\epsilon^H(\lambda) D^H_{in} + \epsilon(\lambda) D_{in}) \cdot l \cdot V_{in}/V_{out} \text{ (with } V_{in} \ll V_{out}) \tag{A5}$$

where ϵ are the decadic extinction coefficients of the acid and the base, λ is the wavelength and l is the optical pathlength of the cuvette. (We assume that the sieve effect (due to a non-homogeneous distribution of the dye in the cuvette, see Duysens [40]) is negligible. Since the inside concentration of the dye is always below 1 mM it does not produce sieving by itself and the sieving by intrinsic pigments is also negligible at a wavelength of 550 nm where most of the experiments were carried out.)

The dye molecules located inside change their absorbance in response to a very small pH change in the internal phase:

$$dA_{d,in} = -(\epsilon^H - \epsilon)\beta_d \, V_{in}/V_{out} \cdot l \cdot dpH_{in} \tag{A6}$$

In experiments with thylakoids it is convenient to relate the pH change to the number of protons released inside. Moreover it is convenient to relate the

proton release as well as the respective volumes to the number of chlorophyll molecules. The specific internal volume v_{in} is:

$$v_{in} = V_{in}/(V_{out} \cdot C_{chl}) \tag{A7}$$

The specific proton release is:

$$dh^t_{in} = dH^t_{in} v_{in} \tag{A8}$$

Taking Eqn. A1 into account Eqn. A6 then becomes:

$$dA_{d,in} = -(\epsilon^H - \epsilon) \cdot \frac{\beta_d}{\beta_{intr} + \beta_d + \beta_b} \cdot C_{chl} \cdot l \cdot dh^t_{in} \tag{A9}$$

The explicit dependence on the internal proton concentration is given by Eqns. A3 and A4. Whe the internal volume is changed under variation of the osmotic pressure the total concentration of buffering groups which are bound to the membrabe is also changed:

$$X^t = X^t_0 \cdot v_0/v \tag{A10}$$

where v denotes the actual specific volume of the internal phase and v_0 a reference volume. In consequence the contribution of bound groups to the buffering capacity is changed. We assume tha this holds for the intrinsic groups and for the dye (see above). Hence $\beta_d = \beta_{d,0} \cdot v_0/v$ and $\beta_{intr} = \beta_{intr,0} \cdot v_0/v$. however the concentration of an added water soluble buffer remains unaffected and therefore $\beta_b = \beta_{b,0}$. The specific proton release is also unaffected. From these considerations the influence of volume changes on the absorption changes of the inside located pH-indicating due is as follows:

$$dA_{d,in} = -(\epsilon^H - \epsilon) \frac{\beta_{d,0}}{(\beta_{intr,0} + \beta_{d,0} + \beta_b \cdot v/v_0)} \cdot C_{chl} \cdot l \cdot dh^t_{in} \tag{A11}$$

Eqns. 10 and 11 are differential equations which describe the absorption changes correctly if the pH changes in the internal phase are small. The error made by using these equations instead of their integrated forms to calculate the absorption changes caused by a pH change of 0.05 units is 5% at a pH which is 1 unit more acidic than the pK of the dye and it is —5% if the pH is by one unit more alkaline. Hence we have used the differential equations without corrections.

Complications like activity coefficients, surface potentials and the redistribution of the permeant species in response to a pH difference (or an electric potential difference) across the membrane have been neglected (but see Appendix II).

Appendix II

Neutral red binding inside and the effect of its redistribution under a pH difference

Two different experiments give information on the extent of neutral red binding inside. We have determined the internal buffering capacity at pH 7.2 for two neutral red concentrations in the medium: while 1.15 mM at 13 μM neutral red the buffering capacity became 1.00 mM at 3 μM neutral red. Taking the saturation curve (Fig. 6) as a measure of the amount of neutral red inside

we find that the difference between 13 μM and 3 μM is 44% of the amount at 13 μM. Hence we describe the difference of the measured internal buffering capacities, 150 μM, to 44% of the neutral red present at an outer concentration of 13 μM. This then becomes 341 μM. At the pK of inside located neutral red the concentration is by a factor 4/2.3 greater than the buffering capacity (see Eqn. 1): 593 μM. In comparison with 13 μM this is an 'enrichment' by a factor 46. At an external concentration of 3 μM the internal concentration is 0.56 × 593 μM which corresponds to an enrichment by a factor 110.

Another independent way to calculate the neutral red concentration inside is from the absolute magnitude of the absorption changes under consideration that the flash induced pH change is 0.05 units. From Fig. 6 we take an relative change in transmitted intensity of $\Delta I/I = -0.63 \cdot 10^{-3}$ (or $\Delta A = 2.8 \cdot 10^{-4}$) at a neutral red concentration of 3.2 μM. Under consideration of Eqn. A4 and with the following data: $\Delta\epsilon(550\text{ nm}) = 2.2 \cdot 10^4\ M^{-1} \cdot cm^{-1}$, $l = 2$ cm and $V_{in}/V_{out} = 7 \cdot 10^{-4}$ one obtains a total concentration of neutral red inside of: $D^t_{in} = 309$ μM at $D^t_{out} = 3.2$ μM. This is an enrichment by a factor 100. Taking the fit of the saturation curve in Fig. 6 by an adsorption isoltherm one obtains an internal concentration of 640 μM at 13 μM outside (enrichment 50). The respective contributions of neutral red to the internal buffering capacities are 178 μM (at 3 μM outside) and 351 μM (at 13 μM). Saturation occurs at 850 μM corresponding to one neutral red per 17 chlorophylls.

The above two estimates for the concentration of neutral red in the internal phase are not too accurate, the first because the contribution of neutral red to the internal buffering capacity is only small and the second because it involves an assumption of the internal volume. However, it is apparent that the measured values for the intrinsic buffering capacity of the internal phase (see Fig. 7) have to be corrected by less than −30% only (to eliminate the contribution of neutral red). It is furthermore evident that neutral red is highly enriched in the internal phase — in the range of external concentrations used in our experiments (up to 13 μM) by a factor of at least 50.

In the above experiments the pH difference induced across the thylakoid membrane was usually very small (−0.05 units). However, in Fig. 8 firing of a series of 9 flashes induced a larger pH difference. A weak acid as neutral red with the neutral form very probably more readily permeating across the membrane is redistributed between the aqueous phases in response to a pH difference as follows:

$$D^{free}_{in}/D^{free}_{out} = (K_d + H_{in})/(K_d + H_{out})\ (D^{free} = \text{total concn. unbound}) \qquad \text{(A12)}$$

For a given pH difference the effect is more pronounced towards acid pH. How does the redistribution of neutral red affect the magnitude of the absorption changes under excitation with a series of short flashes (see Fig. 8). Taking 7.2 for the initial pH and 0.5 for the pH difference (acid inside) and the pK of neutral red solved in the internal aqueous volume as 6.6 we obtain an increase of the internal concentration of free neutral red in both forms (DH and D) by a factor 1.43. Taking the binding isotherm (Fig. 6) at a working concentration of 13 μM we find that this changes the concentration of bound neutral red by only 8%. This, at the accuracy of our experiments, is negligible. The dominance

of bound neutral red (enrichment greater than 50) also explains, why only one pK, the one of the bound dye is seen in our experiments.

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