Biochimica et Biophysica Acta, 504 (1978) 265—277

BBA 47576

THE ACCUMULATION OF NEUTRAL RED IN ILLUMINATED THYLAKOIDS

D. SIEFERMANN-HARMS *

Institut für Biologie I, Abteilung Allgemeine Botanik und Pflanzenphysiologie, Universität Tübingen, D-7400 Tübingen (F.R.G.)

(Received April 3rd, 1978)

Summary

Thylakoids isolated from spinach (*Spinacia oleracea* L.) bind only a small fraction of neutral red in the dark whereas they accumulate large amounts of the protonated dye in their inner space under light. Light-induced neutral red uptake depends on the size of the proton gradient across the thylakoid membrane but does not follow the mechanism established for amines. Instead, the correlation between pH gradient and neutral red uptake can be predicted quantitatively assuming that protonated neutral red is accumulated mainly as dimer species.

Under appropriate conditions, accumulation of protonated neutral red in the inner thylakoid space is proportional to an absorbance increase at 520 nm. This 520-nm change can be used for the continuous measurement of pH changes in thylakoids during steady-state illumination.

Introduction

The vital stain neutral red is taken up rapidly by cells and accumulates in large amounts in vacuoles [1] and lysosomes [2]. Binding of neutral red has been observed for mitochondria and submitochondrial particles [3] where it is markedly enhanced under conditions of energization. According to Lynn [4], chloroplasts also bind neutral red and in the presence of the dye exhibit a light-induced absorbance change typical for neutral red protonation. Therefore Lynn suggested that neutral red is an indicator for the internal acidification of thylakoids.

* Present address: Institut für Chemische Pflanzenphysiologie, Universität Tübingen, D-7400 Tübingen, G.F.R.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulphonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Ausländer und Junge [5] and Junge et al. [6] used absorbance changes due to neutral red protonation as a sensitive probe for small pH differences in the internal phase of thylakoids excited by single turnover flashes. Pick and Avron [7] applied this technique for measuring pH changes during steady-state illumination. These authors concluded that under steady-state conditions neutral red is not appropriate for quantitative measurements of ΔpH in thylakoids. Their conclusion was based on observations suggesting that changes in neutral-red absorbance reflect mainly binding of the dye to the inner side of the thylakoid membrane, and furthermore, that absorbance of the internally protonated neutral red is partially masked.

In this paper it will be shown that thylakoids which bind only a small fraction of the neutral red in the dark, accumulate large amounts of the dye in their inner space under light. This uptake of neutral red can be understood assuming that the protonated dye is accumulated mainly as dimer species, a hypothesis that may explain neutral red accumulation also in other acidic compartments of the cell.

Materials and Methods

Spinach (*Spinacia oleracea* L., var. Monopa) was grown in a growth chamber under a daily regime of 10 h illumination with white light at 3 mW · cm^{-2} and 22°C followed by 14 h darkness and 16°C. For chloroplast isolation, 15 g of spinach leaves were homogenized at 2°C for 10 s in a 1-l Braun blendor together with 100 ml of the isolation medium (300 mM NaCl, 50 mM Tricine, 0.5 mM EDTA, 3 mM $MgCl_2$; adjusted to pH 7.8 with NaOH) according to [8], filtered through a layer of 36-μm monofilament nylon cloth and centrifuged for 1 min at 1500 × *g*. The sediment was resuspended in wash medium (300 mM sorbitol, 10 mM NaCl, 50 mM HEPES, 0.1% bovine serum albumin; adjusted to pH 7.2 with NaOH), centrifuged again for 1 min at 1500 × *g*, and suspended in a small volume of wash medium to yield a chlorophyll concentration of approx. 1.5 mg/ml. This concentrated suspension was kept on ice until used, but not longer than 3 h. According to Hall [9], the chloroplasts obtained by this procedure belonged to type C, e.g. naked thylakoids, which was confirmed by the ferricyanide method [10].

Neutral red and 9-aminoacridine uptake were measured by incubating chloroplast suspensions together with these compounds (6 ml) in glass tubes and pelleting them in the swing-out rotor of a Heraeus-Christ refrigerated minifuge (Type 3906). (Glass tubes were prefered to the polyethylene 0.4-ml microtubes of the Beckman 152 microfuge often used for similar studies, since in case of low neutral red concentrations the microtubes adsorbed a significant fraction of the dye.) A 100 W lamp (Osram Krypton) that was placed directly above the rotor allowed to illuminate the tubes during centrifugation. The samples were spun for 1 min at 30 × *g* followed by 5 min at 4000 × *g*; then the upper 2 ml of the supernatant were immediately removed for absorbance measurements, thus minimizing errors due to release of compounds from pelleted thylakoids. Comparison of the neutral red levels in the upper, middle and lower 2-ml fractions of the supernatant showed that such release was indeed small: while the upper and middle fractions agreed in their neutral red

content, the lower fraction contained about 5% more neutral red. Neutral red absorbance was determined at the wavelength of its isosbestic point (472 nm), 9-aminoacridine absorbance at the wavelength of its maximum (398 nm) and the absorbances were corrected for the small absorbance at these wavelengths (<5%) from membrane fragments remaining in the supernatant. Uptake of the compounds by the thylakoids was determined as % loss of absorbance at the indicated wavelengths in relation to the absorbance of controls without thylakoids.

In order to illuminate thylakoid suspensions in the centrifuge with red light of variable intensities, acetate colour filters (Cinemoid No. 6 'primary red' plus No. 8 'deep salmon' in combination with up to 3 filters No. 60 'pale gray'; Rank Strand Electric, Braunschweig) were taped on top of the tube holders. Light intensity was measured in an arrangement with similar geometric properties using an optometer 40 A (United Detector Technology, Calif.); the intensities determined at the top and the bottom of the suspension were averaged, allowing for absorbance by the thylakoids. To keep the temperature in the suspensions at 22 ± 1°C, heating by the lamp had to be balanced by cooling the centrifuge.

Absorbance changes of thylakoid suspensions were determined at 22°C with a Perkin-Elmer Model 356 two-wavelength double-beam spectrophotometer using 3-ml cuvettes with an optical path of 1 cm. An opal-glass plate was placed directly behind the cuvettes and the photomultiplier was shielded from actinic light with a Corning filter CS 4-96. The reference cuvette remained in the dark, while the sample cuvette was illuminated with actinic light from a 100 W lamp (Osram Krypton) filtered through a heat filter (Schott) and either through the same combinations of acetate filters as used for the centrifugation experiments ('red light' > 600 nm) or through a 653-nm interference filter (Schott).

Electron transport was measured as oxygen uptake in the presence of methyl viologen with a Clark-type oxygen electrode (Rank Brothers, Bottisham); chorophyll concentration was determined according to Jeffrey et al. [11].

Inner thylakoid space was determined according to (Neuburger, M. and Douce, R., unpublished results). This method is based on the determination of [^{14}C]glucose-permeable and [^{3}H] water-permeable spaces of the thylakoid suspension; after incubation with the labelled compounds the thylakoid suspension is frozen in liquid nitrogen and $^{3}H_2O$ is separated from [^{14}C]glucose and other residues by sublimation and condensation. The incubation mixtures contained 28 mM KCl, 10 mM HEPES/NaOH buffer at pH 7.0, 0.1% defatted bovine serum albumin, 0.6 mM [^{14}C]glucose (10^6 dpm/ml), $^{3}H_2O$ (10^5 dpm/ml) and thylakoids equivalent 200—600 μg chlorophyll per ml. The inner thylakoid space determined by this method for spinach chloroplasts was similar to values obtained by silicone layer-filtering centrifugation for lettuce chloroplasts [12].

Spectra of concentrated neutral red solutions (up to 2 mM) were measured in the Model 356 spectrophotometer using cuvettes with an optical path of 1 mm.

Nigericin was a gift of Ely Lilly.

Results

1. The light-induced accumulation of neutral red in thylakoids

Thyalkoids that were illuminated in the presence of neutral red bound up to 80% of the dye, whereas dark controls bound only about 10% (Fig. 1). While medium pH did not affect the level of dark-bound neutral red, the light-induced increase of neutral red uptake was pH-dependent with an optimum near pH 7, and was completely inhibited by the uncoupler nigericin. This result suggests that the light-dependent uptake of neutral red required a pH-gradient across the thylakoid membrane.

Fig. 2a shows that the uptake of neutral red and of the amine 9-aminoacridine by illuminated thylakoids was similarely affected by light intensity. This conformity, however, did not indicate that neutral red uptake followed the mechanism established for amines [13]; Fig. 2b (experimental data presented as ○) shows indeed that neutral red uptake occurred at ΔpH values that were approx. 1 pH unit smaller than expected for an amine with a pK_a value similar to neutral red.

Neutral-red uptake by illuminated thylakoids was further studied under saturating light while varying the amount of thylakoids and, in consequence, the size of inner thylakoid space. Fig. 3a shows that neutral red uptake, instead of depending on the size of inner thylakoid space more or less linearely as predicted by the amine mechanism, increased strongly for low amounts of thylakoids before approaching a constant level; it also shows that at any thylakoid level much more neutral red was taken up than predicted by the amine mechanism. Assuming that the neutral red taken up by illuminated thylakoids was dissolved in the inner thylakoid space, the data of Fig. 3a allow to calculate the concentration ratio of neutral red inside and outside the thylakoids as presented in Fig. 3b. Contrary to predictions derived from the amine mechanism, the neutral red ratio was not constant for variable inner thylakoid space but decreased for increasing amounts of thylakoids.

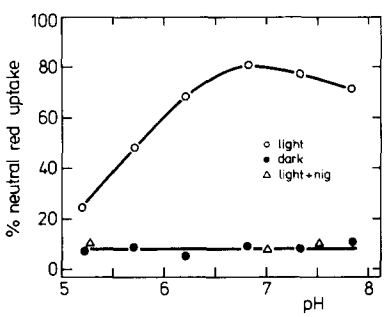

Fig. 1. The effect of medium pH on the uptake of neutral red by the thylakoids in the dark, in the light, and under conditions of light plus nigericin (nig). The reaction mixtures contained 28 mM KCl, 10 mM succinate/NaOH buffer at pH 5.2, 5.7 and 6.2, or HEPES/NaOH buffer at pH 6.8, 7.4, and 7.8, 0.1% defatted bovine serum albumin, 5 μM neutral red and thylakoids equivalent to 30 μg chlorophyll per ml. Nigericin was added in ethanolic solution at final concentrations of 0.1 μM nigericin and 0.2% ethanol. Actinic white light was at 3 mW · cm^{-2}.

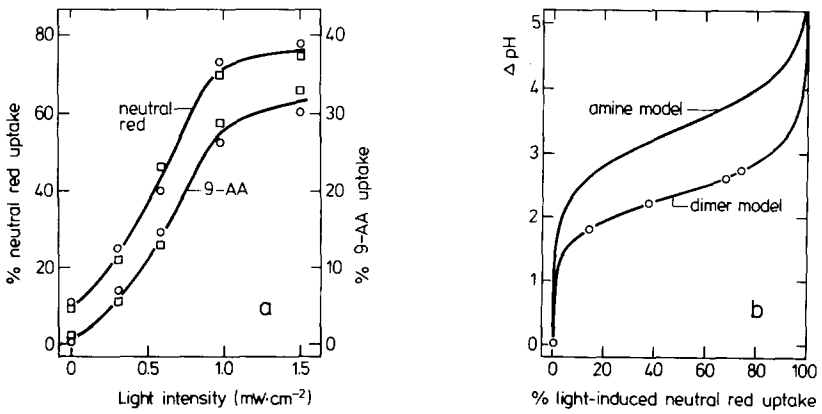

Fig. 2. The effect of light intensity (a) and light-induced proton gradient (b) on the uptake of neutral red by thylakoids. The reaction mixtures contained 28 mM KCl, 10 mM HEPES/NaOH buffer at pH 7.0, 0.1% defatted bovine serum albumin, 5.5 μM neutral red or 9-aminoacridine (9-AA) and thylakoids equivalent to 30 μg chlorophyll per ml. (a) Uptake of neutral red and 9-AA by thylakoids illuminated with red light (>600 nm) of the indicated intensities. Different symbols represent different thylakoid preparations. (b) Comparison of pH-dependent uptake of neutral red and of a hypothetical amine with the same pK_a value as neutral red. The experimental data (○) were obtained from (a) by calculating the ΔpH at various light intensities by means of the 9-AA method [12], and by correcting the percentage of neutral red uptake shown in (a) for light-dependent uptake only, thus eliminating the amount of neutral red bound to the membranes in the dark. The theoretical curve for the 'dimer model' was calculated from Eqn. 6 of Appendix using $pH_o = 7.0$, $pK_a = 6.8$ [19], $K = 45$ μM, $[R_T] = 5$ μM, and $V/v = 1000$ based on an inner thylakoid space of 34 μl per mg chlorophyll. Using these values, the theoretical curve for the 'amine model' was calculated from Eqn. 7.

2. *Correlation between light-induced neutral-red uptake and absorbance change*

As has been reported before [7], thylakoids incubated with neutral red exhibit an absorbance change in the light that is maximal near 520 nm indicat-

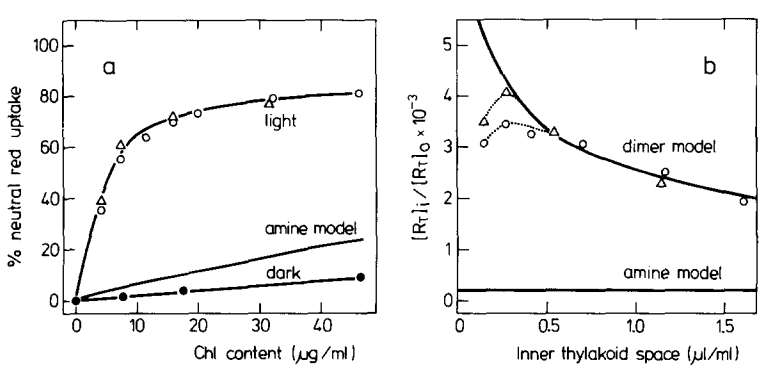

Fig. 3. The effect of thylakoid concentration on the uptake of neutral red. The reaction mixtures were the same as the neutral red-containing mixture of Fig. 2, except for chlorophyll content which was varied as indicated. Actinic white light of saturating intensity (3 mW · cm^{-2}) was used. (a) The uptake of neutral red by variable amounts of thylakoids in light and dark, in comparison to the uptake of a hypothetical amine with the same pK_a value as neutral red. Chl, chlorophyll. (b) Comparison of the ratios of neutral red and amine concentrations inside and outside the thylakoids under conditions of variable inner thylakoid space. The experimental values (○, △) were derived from the data of (a) and an inner thylakoid space of 34 μl per mg chlorophyll. The theoretical curves were calculated using Eqns. 6 and 7 of Appendix for the 'dimer model' and the 'amine model', respectively, and $pH_o = 7.0$, $pH_i = 4.3$ (determined for saturating light intensity as in Fig. 2), $pK_a = 6.8$, $K = 45$ μM, and $[R_T]$ decreasing from 5.5 to 5 μM for increasing amounts of thylakoids.

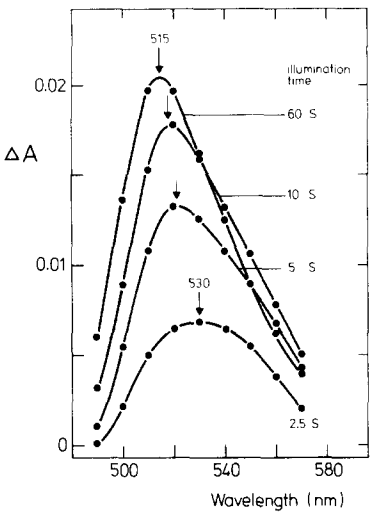

Fig. 4. Difference spectra of neutral red incubated thylakoids after various illumination times. The reaction mixture was described in Fig. 2, except for neutral red concentration being 3 μM. The spectra were constructed using the kinetics of the light-induced absorbance increase of thylakoids as monitored at the indicated wavelengths. The difference spectrum obtained after 60 s of illumination agreed with difference spectra measured directly during an illumination period between 1 to 15 min. Actinic light at 653 nm and saturating intensity (1.8 mW · cm^{-2}) was used.

ing the occurrence of protonated neutral red. Fig. 4 shows the difference spectra of this absorbance change after various illumination times. The absorbance change was complete within 60 s with a half-rise time of approx. 5 s, remained stable for all tested light periods (up to 15 min) and was reversible in the dark. Most important, with increasing illumination times the maximum of the difference spectra was shifted to shorter wavelengths reaching a final value at about 515 nm.

The final extent of the light-induced absorbance increase at 520 nm of thylakoids incubated with neutral red showed the same correlation with light intensity as the size of the neutral red fraction taken up by the thylakoids (Fig. 5a). This suggests that the size of the absorbance change was a direct measure for the amount of neutral red taken up in the light, and furthermore, that the dye was accumulated in its protonated form. Fig. 5b shows that the relationship between neutral red uptake and 520-nm change was linear; at 520 nm the difference extinction coefficient for neutral-red protonation in thylakoids was determined to be $\Delta\epsilon$ = 8.7 $mM^{-1} \cdot cm^{-1}$, if the concentration of protonated neutral red was expressed in relation to the total suspension. This value is in the same range as the difference extinction coefficient at 520 nm of pure neutral red solutions at pH 4.80 versus 7.15, the latter being calculated from ref. 14 to be 11.5 $mM^{-1} \cdot cm^{-1}$. Although both extinction coefficients have been determined for rather different conditions, their relative close agreement indicates that the neutral red taken up by the thylakoids was not masked to a higher extent.

The linear relationship between neutral red concentration inside the thylakoids and ΔA_{520} (Fig. 5b) indicates that the extinction coefficient at this wavelength is not affected by high local neutral red concentrations. Since no

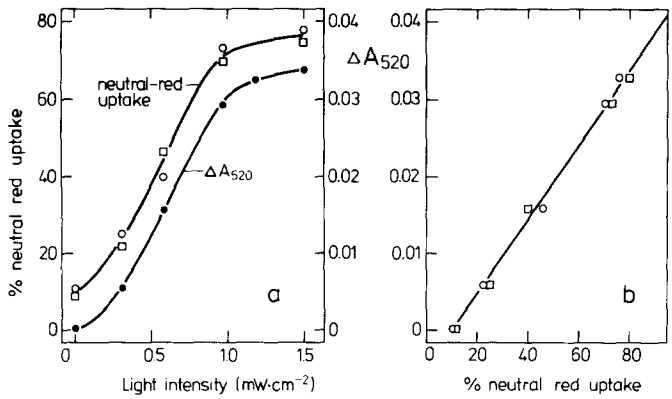

Fig. 5. Correlation between light-induced neutral-red uptake and absorbance increase at 520 nm. The reaction mixtures were the same as the neutral red containing mixtures of Fig. 2; controls run without neutral red were used to correct the 520-nm change for neutral red independent changes. (a) Comparison of neutral red uptake and final extend of the absorbance increase under the indicated intensities of red light (>600 nm). (b) Relationship between the amount of neutral red taken up by the thyalkoids and the extend of the absorbance change.

linearity was observed for absorbance changes at longer wavelength (data not shown), the linearity at 520 nm suggests the existence of an isosbestic point for protonated neutral red dissolved in the inner thylakoid phase at this wavelength. Consistent with this suggestion are studies on the optical properties of concentrated neutral red solutions which revealed an isosbestic point on the short-wavelength side of the absorbance maximum obtained for diluted solutions [15].

The correlation between absorbance change and light-induced neutral red binding was further studied when the pH gradient across the thylakoid membrane was decreased [16] under phosphorylating conditions (Fig. 6a) and when the inner thylakoid space was diminished [12] in the presence of sorbitol (Fig. 6b). While the extent of the absorbance change as well as the size of the bound neutral red fraction were smaller in the presence than in the absence of ADP, sorbitol reduced the absorbance change but did not affect neutral red binding. The smaller absorbance change in the presence of ADP or sorbitol was not caused by their direct interaction with neutral red since the spectra of neutral red solutions at pH 7 or 5 were unaffected by these compounds.

Finally, it should be mentioned that the presented data were obtained in the absence of exogenous electron acceptors, and that DCMU inhibited neutral red uptake and absorbance change (data not shown). These findings suggest that neutral red uptake and protonation occured under conditions of linear electron transport from water to oxygen in a Mehler-type reaction. Addition of 100 μM methyl viologen caused an increase of the absorbance change by about 10%, in accordance with enhanced electron transport and proton pumping in presence of the acceptor.

3. A model for the accumulation of neutral red in illuminated thylakoids

The fact that light-induced neutral red uptake was much larger than predicted by the amine mechanism suggests that the neutral red species accumu-

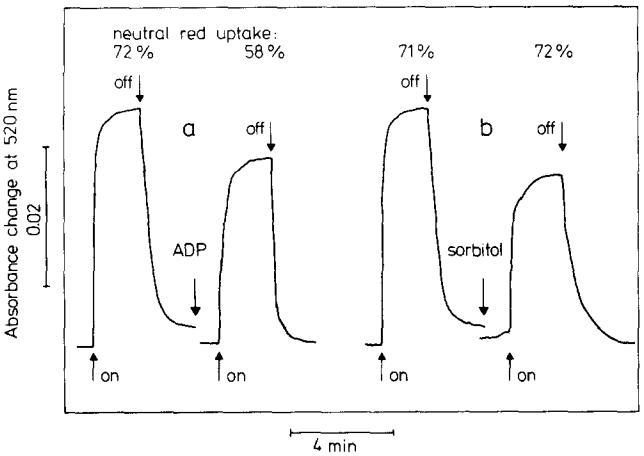

Fig. 6. Effect of ADP and sorbitol on the light-induced absorbance change at 520 nm in neutral red incubated thylakoids and on the uptake of neutral red by thylakoids. The reaction mixtures were the same as the neutral red containing mixture of Fig. 2, but in (a) 5 mM sodium phosphate and 2 mM $MgCl_2$ were also present. Where indicated, 0.5 mM ADP (25 μl of a 60 mM stock solution at pH 7.0) or 200 mM sorbitol (200 μl of a 3 M stock solution at pH 7.0) were added to the reaction mixture. Actinic red light (>600 nm) of 1.5 mW · cm^{-2} was used. The light-induced absorbance increase of controls without neutral red was 10 to 15% of the signal observed in presence of the dye and was little affected by addition of sorbitol.

lated in the thylakoids was not simply the protonated dye in its monomer form. Under the experimental conditions of Fig. 3, neutral red concentrations in the inner thylakoid space were calculated to be in the range of 2—10 mM for 1.5—0.3 μl of inner space per ml of thylakoid suspension, that is at concentrations at which protonated neutral red is known to aggregate. According to Bartels [15], protonated neutral red at concentrations up to 1 mM aggregates to dimers only, whereas at concentrations higher than 1 mM the dimers start aggregating to tetramers; and these processes produce a shift of the absorbance maximum from 533 nm for the monomer to less than 495 nm for the aggregates [15]. Since an absorbance shift to shorter wavelengths was observed during the accumulation of protonated neutral red inside the thylakoids as well (Fig. 4), it seemed reasonable to consider the possibility that neutral red forms aggregates also when concentrated inside the thylakoids.

A model for the mechanism of light-induced neutral red accumulation in thylakoids using the assumption of dimer formation is shown in Fig. 7. Based on this model, the correlation between the size of the neutral red fraction taken up by the thylakoids and the pH gradient across the thylakoid membrane was derived as presented in the Appendix. Using a best-fit value for the dissociation constant K for neutral red dimerisation of $K = [RH^+]^2/[(RH^+)_2] = 45\ \mu M$, the dimer mechanism allowed to predict the amount of neutral red taken up at different ΔpH values, as shown in Fig. 2b. The relationship between inner thylakoid space and concentration ratio of neutral red inside and outside the thylakoids was also calculated based on the dimer model. Fig. 3b compares the theoretical curve with the experimental data and shows good agreement for inner space larger than 0.5 μl/ml suspension. Below this value, neutral red uptake was smaller than predicted by the model. This finding seems

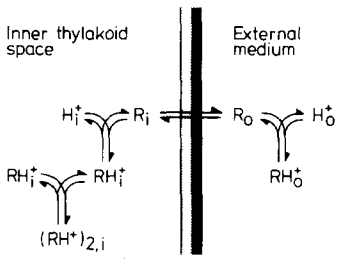

Fig. 7. Dimer model for the uptake of neutral red by illuminated thylakoids. R stands for the unprotonated, RH^+ for the protonated and $(RH^+)_2$ for the dimer species of neutral red. The subscripts i and o indicate the location of the species inside or outside the thylakoid membrane. For mathematical treatment of the model see Appendix.

to reflect the fact that below 0.5 μl inner space per ml suspension electron transport was inhibited due to the high neutral red concentration inside the thylakoids, as confirmed by electron transport measurements in the presence of 0.1 mM methyl viologen and variable amounts of neutral red (data not shown). It should be mentioned that the theoretical inner neutral red concentration increases from 6.25 to 18 mM for thylakoid suspensions with inner thylakoid space decreasing from 0.5 to 0.1 μl/ml suspension.

Whereas the dissociation constant for neutral red dimerisation inside the thylakoids was determined to be 45 μM; Bartels [15] reported a value of 206 μM for neutral red solutions in 15 mM phosphate buffer at pH 2. To check whether such difference was due to different solvent systems, the value of the dissociation constant was determined for neutral red solutions at pH 5 and various buffer concentrations, using the method described [15]. For citrate buffer, the dissociation constant changed from 200 to 100 μM when the buffer concentration was increased from 10 to 100 mM. Thus, the value of the dissociation constant depends on the solvent and may be as low as 45 μM inside the thylakoids.

Since the shift of the absorbance maximum of protonated neutral red due to increasing neutral red concentration was smaller in illuminated thylakoids (Fig. 4) than in pure neutral red solutions [15], absorbance shifts were studied for neutral red dissolved in various buffer solutions. The maxima of 1 mM neutral red solutions at pH 5, buffered with 10 mM citrate or succinate, were at 497 or 504 nm, respectively, whereas the maxima of 2 μM neutral red solutions were at 535 nm for both buffers. Thus, the concentration-dependent shift of the absorbance maximum of protonated neutral red is also affected by the solvent and may be smaller for neutral red dissolved in the hydrophylic phase of inner thylakoid space than in the tested buffers.

Discussion

The light-induced accumulation of neutral red in thylakoids is far too high to be explained by the same uptake mechanism as established for amines (Figs. 2a and 3a); instead, the high accumulation can be explained assuming the formation of neutral red dimers inside the thylakoids (Figs. 2b and 3b). The dimer model for neutral red uptake as presented in Fig. 7 is supported by the fact

that dimer formation has been observed in concentrated neutral red solutions [15]; in addition, protonated neutral red that has been accumulated in thylakoids (Fig. 4) and protonated neutral red that is dissolved in highly concentrated pure solution [15] have similar optical properties.

Another mechanism for neutral red accumulation has been suggested by Pick and Avron [7], based on the assumption that protonated neutral red is bound to the inner side of the thylakoid membranes under light. This hypothesis is questioned by the experimental data presented in this paper, namely, under conditions of such a 'binding-site mechanism' the concentration ratio of neutral red inside to outside of the thylakoid membrane is expected to increase when the amount of hypothetical binding sites is increased; instead, a decrease of the ratio has been observed when the amount of membranes was raised in the suspension (Fig. 3b).

The binding-site hypothesis for neutral red accumulation has been proposed to explain the observation that stimulation of proton uptake by neutral red is essentially insensitive to the osmolarity of the medium [7]. Consistent with this observation it could be shown (Fig. 6b) that neutral red uptake is not affected by addition of sorbitol (200 mM) to the reaction mixture. This behaviour is in agreement with the dimer model: as shown in Fig. 3a, neutral red uptake is much less affected by changes in size of inner thylakoid space than uptake of amines, provided the inner space does not become too small. Even a small inner space available for neutral red uptake does not necessarely reduce the uptake: according to [12], addition of 200 mM sorbitol (as in Fig. 6b) results in shrinkage of the inner space by a factor of 10, but yields also an increase of the light-induced pH gradient by approx. 0.4 units. The decrease of inner space alone would cause a decrease in neutral red uptake (Fig. 3a), whereas the increase of pH gradient leads to a large uptake of the dye (Fig. 2b). Since both changes affect neutral red uptake in opposite directions, they may compensate each other.

According to Bartels [15], protonated neutral red, when dissolved at concentrations higher than 1 mM, is aggregated not only to form dimers, but part of the dimers are further associated to tetramers. In 5 mM neutral red solutions approx. 4.8% of the neutral red is present as tetramer species, whereas in 10 mM solutions the tetramer fraction represents already 16%. This additional aggregation has not been considered in the dimer model (Fig. 7) since accumulation of internal neutral red at levels higher than 5 mM (as is the case in Fig. 2 for thylakoid suspensions containing less than 14 μg chlorophyll per ml equivalent to 0.5 μl inner space per ml) causes secondary effects on the membrane like inhibition of electron transport. In addition, excess accumulation of neutral red may change the optical properties of the system; this was probably the case under the experimental conditions of Fig. 6b where the inner space was decreased by the additon of sorbitol. Under these conditions neutral red uptake was unaffected, but the absorbance change was significantly decreased. A similar 'masking' of the absorbance has been reported by Pick and Avron [7] who studied the light-minus-dark difference spectrum in a thylakoid suspension containing 30 μg chlorophyll per 3 ml and 10 μM neutral red.

Considering experimental evidence presented, neutral red uptake by illuminated thylakoids can be explained by the dimer model, as long as appropriate

ratios between neutral red concentration and inner thyalkoid space available for neutral red accumulation are maintained. If appropriate ratios for these parameters are used (under my experimental conditions of 3—5 μM neutral red and chlorophyll concentrations equivalent to 1 μl inner thylakoid space per ml suspension), the light-induced uptake of neutral red by thylakoids is proportional to the light-induced absorbance change at 520 nm (Fig. 5b), and the neutral red uptake is controlled by the pH gradient across the thylakoid membrane (Fig. 2b). Consequently, the absorbance change at 520 nm of neutral red incubated thylakoids provides a measure for ΔpH similar to the fluorescence quenching that occurs in thylakoids incubated with fluorescent amines [13].

Compared to other techniques for ΔpH determination, the neutral red technique has the advantage to allow simultaneous studies of pH change, electron transport and phosphorylation by thylakoids under the same experimental conditions; different from direct pH measurements in the external medium [17], the neutral red containing reaction mixture can be buffered; different from ΔpH determinations based on centrifugation techniques [18], the neutral red method, by monitoring ΔA_{520}, allows continuous and short-time measurements. Finally, the neutral red technique represents a sensitive and simple method for monitoring pH changes in thylakoids under steady-state illumination.

Appendix

Derivation of the relationship between $[H^+]_i/[H^+]_o$ and the fraction x of neutral red taken up by the thylakoids in case of the dimer model

Let R be the unprotonated, RH^+ the protonated and $(RH^+)_2$ the dimer species of neutral red, and $[R_T]$ the overall concentration of all neutral red not bound to the thylakoid membrane, with one dimer molecule being regarded like two neutral red molecules. Let the subscripts i and o indicate concentrations in the inner thylakoid space and outside the thylakoid membrane (Fig. 7), and may $[R_T]_o$ be small enough (<10 μM according to ref. 15) not to form dimers. Then the distribution of neutral red inside and outside the thylakoids can be expressed as shown in Eq. 1:

$$\frac{[R_T]_i}{[R_T]_o} = \frac{[R]_i + [RH^+]_i + 2[(RH^+)_2]_i}{[R]_o + [RH^+]_o} . \tag{1}$$

Let K_a and K be the dissociation constants for the protonation of neutral red (K_a) and the dimerisation of the protonated form (K), i.e.,

$$K_a = \frac{[R]_i \cdot [H^+]_i}{[RH^+]_i} = \frac{[R]_o \cdot [H^+]_o}{[RH^+]_o} \tag{2}$$

where $[R]_i = [R]_o$, as consequence of the assumption that the permeability through the thylakoid membrane of the uncharged species R is high as compared to the protonated species RH^+ (ref. 12), and

$$K = \frac{[RH^+]_i^2}{[(RH^+)_2]_i} \tag{3}$$

and

$$\alpha = \frac{K_a}{[H^+]_o} \tag{4}$$

By means of Eqns. 2—4 the neutral-red concentration terms on the right side of Eqn. 1 can be replaced in part by proton concentration terms:

$$\frac{[R_T]_i}{[R_T]_o} = \frac{1}{\alpha + 1} + \frac{1}{(\alpha + 1)} \frac{[H^+]_i}{[H^+]_o} + \frac{2 \cdot [R_T]_o}{(\alpha + 1)^2 \cdot K} \left(\frac{[H^+]_i}{[H^+]_o}\right)^2 . \tag{5}$$

Replacing the terms $[R_T]_i$ and $[R_T]_o$ in Eqn. 5 by

$$[R_T]_i = x \cdot [R_T] \cdot \frac{V}{v}$$

and

$$[R_T]_o = (1 - x)[R_T]$$

where V = suspension volume, v = volume of the inner thylakoid space, and $x = ([R_T] - [R_T]_o)/[R_T]$, i.e. the fraction of neutral red taken up by the thylakoids in the light, and rearranging the obtained expression gives the following relationship:

$$\frac{2 \cdot [R_T]}{(\alpha + 1)K} \cdot \left(\frac{[H^+]_i}{[H^+]_o}\right)^2 \cdot (1 - x)^2 + \frac{[H^+]_i}{[H^+]_o} \cdot (1 - x) + \left(\alpha + \frac{V}{v}(\alpha + 1)\right) \cdot (1 - x) -$$

$$- \frac{V}{v}(\alpha + 1) = 0 \tag{6}$$

For finite values of K, this equation describes the relationship between size of the proton gradient $[H^+]_i/[H^+]_o$ and fraction x of the neutral red taken up by the thylakoids in case of the dimer model; for infinite K, Eqn. 6 changes to equation

$$\frac{[H^+]_i}{[H^+]_o}(1 - x) + \left(\alpha + \frac{V}{v}(\alpha + 1)\right) \cdot (1 - x) - \frac{V}{v}(\alpha + 1) = 0 \tag{7}$$

valid for the simple amine model and equivalent to other equations derived for the amine model (for example Eqn. 1 in ref. 13).

Acknowedgements

The author is very grateful to Drs. M. Neuburger and R. Douce for the introduction to their unpublished method of thylakoid space determination. She thanks Mrs. V. Uhle for drawing the graphs and Mr. S. Lelke for growing the spinach. This work has been supported by a grant from the Deutsche Forschungsgemeinschaft.

References

1 Strugger, S. (1949) Praktikum der Zell- und Gewebephysiologie der Pflanze, pp. 146—157, Springer, Berlin

2 Bulychev, A., Trouet, A. and Tulkens, P. (1977) Arch. Int. Physiol. Biochem. (Liège) 84, 1055—1056
3 Dell'Antone, P., Colonna, R. and Azzone, G.F. (1972) Eur. J. Biochem. 24, 553—565
4 Lynn, W.S. (1968) J. Biol. Chem. 243, 1060—1064
5 Ausländer, W. and Junge, W. (1975) FEBS Lett. 59, 310—315
6 Junge, W., Renger, G. and Ausländer, W. (1977) FEBS Lett. 79, 155—159
7 Pick, U. and Avron, M. (1976) FEBS Lett. 65, 348—353
8 Gould, J.M. and Izawa, S. (1973) Biochim. Biophys. Acta 314, 211—223
9 Hall, D.O. (1972) Nature New Biol. 235, 125—126
10 Heber, U. and Santarius, K.A. (1970) Z. Naturforsch. 25b, 718—728
11 Jeffrey, S.W., Douce, R. and Benson, A.A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 807—810
12 Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54—63
13 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64—70
14 Bartels, P. (1956) Z. Phys. Chem. (Neue Folge) 9, 74—94
15 Bartels, P. (1956) Z. Phys. Chem. (Neue Folge) 9, 95—105
16 Pick, U., Rottenberg, H. and Avron, M. (1973) FEBS Lett. 32, 91—94
17 Pick, U. and Avron, M. (1976) Eur. J. Biochem. 70, 569—576
18 Portis, A.R. Jr. and McCarty, R.E. (1974) J. Biol. Chem. 249, 6250—6254
19 Bishop, E. (1972) Indicators, p. 473, Pergamon Press, Braunschweig