# NEUTRAL RED RESPONSE AS A MEASURE OF THE pH GRADIENT ACROSS CHLOROPLAST MEMBRANES IN THE LIGHT

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## 1. Introduction

Neutral red has previously been used to measure the pH gradient formed in the light across chloroplast thylakoid membranes [1]. An internal acidification of up to 1.2 pH units was calculated from the increase in neutral red absorption at 568 nm. Recently the technique was extended and used to measure the pH gradient formed following single turnover flashes resulting in a calculated internal acidification of 0.35 pH units [2].

In this report we demonstrate that the spectral changes in neutral red absorption in the presence of illuminated chloroplasts reflect mainly binding of the indicator to the inside of the thylakoid membrane. Thus, neutral red increases the internal buffering capacity of the chloroplasts but this stimulation is insensitive to the osmolarity of the medium. Evidence is presented for a concentration dependent partial masking of the absorption of internal protonated neutral red.

It is concluded that, at least under steady state illumination, neutral red is not appropriate for quantitative measurements of $\Delta$pH in chloroplasts.

## 2. Materials and methods

Chloroplasts were prepared from lettuce leaves as previously described [3]. The increase in neutral red absorption upon illumination was measured at 550–565 nm in an Aminco-Chance Dual wavelength spectrophotometer. The decrease in absorption of 9-aminoacridine was measured similarly at 420–430 nm. A Corning C.S. 4-96 filter protected the photomultiplier. Proton uptake and photophosphorylation were measured with Radiometer- combined glass electrode type GK-2321 C, a Radiometer pH meter type PHM 64 and a recorder. The difference spectrum of neutral red with illuminated chloroplasts was measured in a Cary 16 spectrophotometer. The photomultiplier was protected by a C.S. 4-96 Corning filter. Illumination was provided by a 24 V halogen lamp projector filtered through a Schott RG 645 filter which provided a saturating light intensity of $4-7 \times 10^5$ ergs $\times$ $cm^{-2} \times sec^{-1}$.

Neutral red binding was measured by incubating the indicator with the chloroplast suspension in polyethylene 0.4 ml microtubes placed inside a Beckman 152 microfuge equipped with a transparent cover. Illumination was provided by a 500 W Philips photoflood lamp and filtered through 10 cm $CuSO_4$ solution. Illumination was during 1 min preincubation and continued for 2 min during centrifugation. The total centrifugation time was 5 min. The supernatant was collected, acidified to pH 4.0 and the absorption of neutral red was measured at 530 nm.

## 3. Results and discussion

Fig.1 demonstrates the absorption changes in neutral red absorption observed in the presence of illuminated chloroplasts. The increase in absorption at 550–565 nm is indicating internal acidification as suggested by its insensitivity to strong buffering of the external medium by tricine, and its inhibition by addition of the uncoupler SF-6847.

Bovine serum albumin which was previously used [2] as an impermeable broad band buffer partly inhi-



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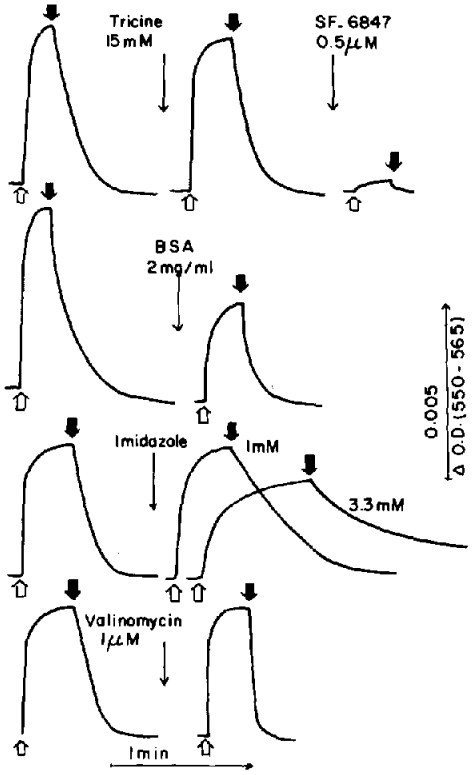


Fig.1. The effect of external buffers, internal buffers and valinomycin on the light induced absorption increase of neutral red. The reaction mixture contained in 3 ml: KCl, 40 mM; tricine, pH 7.9, 1 mM; pyocyanine, 15 μM; neutral red, 3.3 μM and chloroplasts containing 25 μg of chlorophyll. Light on and off are indicated by open and filled arrows, respectively.

bited the signal probably due to some binding of the neutral red to the albumin which decreased the fraction of indicator sensing the internal pH of the chloroplast.

Imidazole – a weak amine ($pK$ = 6.9) which stimulates the extent of proton uptake in illuminated chloroplasts by acting as an internal buffer [4] slows down the rate of decay similar to the inhibition of the apparent rate of proton efflux by imidazole, as measured with a pH electrode (fig.5). At concentrations above one milimolar imidazole also inhibits the extent of the absorption signal due to partial uncoupling, as indicated by its inhibition of the rate of photophosphorylation at these concentrations (not shown).

Valinomycin which stimulates the rate of both proton uptake and efflux also stimulates the rates of the absorption changes.

All of these observations are in agreement with the suggestion [1,2] that neutral red absorption changes can be used to measure the rates and extent of the internal acidification in illuminated chloroplasts.

The magnitude of the light induced absorption change increases only at low neutral red concentrations, reaches a maximum (around 10 μM) and decreases at higher concentrations (fig.2) probably indicating partial uncoupling combined with partial masking (see below). The rate of decay is slowed down at the high neutral red concentrations. This may indicate that neutral red increases the internal buffering capacity of the chloroplast similar to imidazole (see fig.1 and below). It should be noted that imidazole and neutral red are both weak amines with similar p$K$ values. Both of the latter conclusions are strengthened by the data of fig.3, which show that high concentrations of neutral red stimulate the extent of proton uptake and inhibit photophosphorylation.

Fig.4 demonstrates that the stimulation of proton uptake by imidazole is markedly inhibited by increasing the osmolarity of the medium by adding sorbitol, as would be expected for any compound which dissolves in the osmotic solution inside the

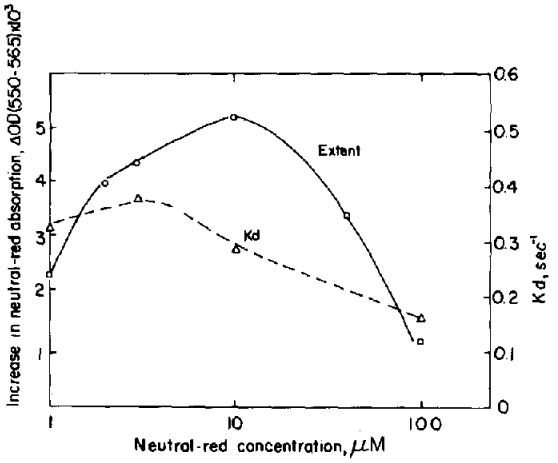


Fig.2. The dependence of the extent of neutral red absorption increase and decay constant on neutral red concentration. The reaction mixture was identical to that described under fig.1 except for the neutral red concentration. The extent represents the absorbance change at 550–565 nm and the kinetic constant $K_d$ is the apparent first-order decay rate constant.

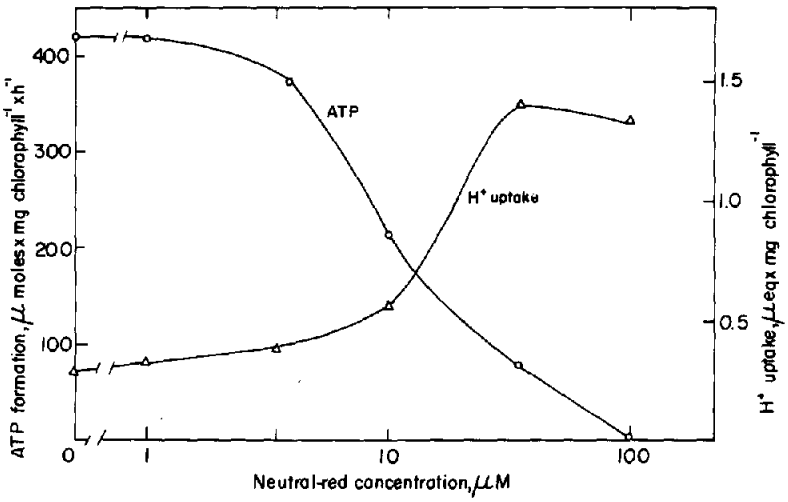


Fig.3. The dependence of proton uptake and photophosphorylation on neutral red concentration. The reaction mixture contained in 3 ml: KCl, 40 mM; K-tricine pH 7.8, 0.5 mM; inorganic phosphate 0.5 mM; $MgCl_2$, 1.0 mM; pyocyanine, 15 μM and chloroplasts containing 35 μg chlorophyll. ADP, 0.2 mM was added when the rate of photophosphorylation was measured. The values represent the extent of $H^+$ uptake and steady state phosphorylation rates.

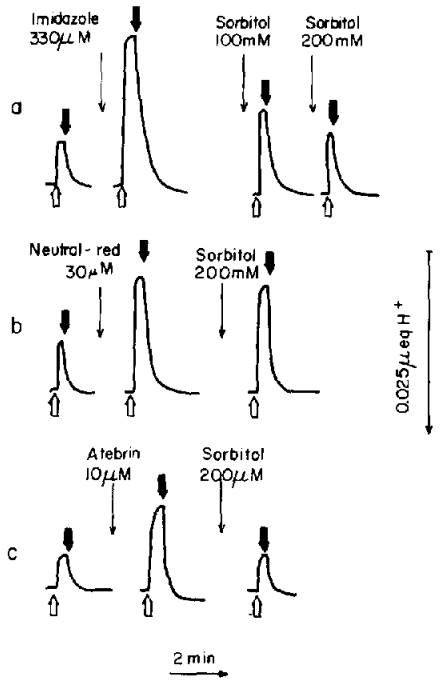


Fig.4. The effect of medium osmolarity on the stimulation of proton uptake in illuminated chloroplasts by imidazole, neutral red and atebrin. The reaction medium contained in 3 ml: KCl, 20 mM; K-tricine, pH 7.9 in traces a, b and pH 8.7 in trace c, 1 mM; pyocyanine, 15 μM and chloroplasts containing 50 μg chlorophyll.

chloroplasts. However, the stimulation of proton uptake by neutral red is essentially insensitive to the osmolarity of the medium (trace b), indicating that neutral red inside the chloroplast is not freely soluble but is either membrane bound or in an aggregated form. Further support for this conclusion comes from the fact that neutral red is about 10 times as effective as imidazole in stimulating proton uptake (although both have nearly the same p$K$) indicating that the distribution of neutral red across the chloroplast membrane is not determined only by the ΔpH and the p$K$ of the amine but also by internal binding.

The stimulation of $H^+$ uptake by atebrin (fig.4, trace c) or 9-aminoacridine (not shown) is markedly inhibited by increasing the osmolarity of the medium, indicating that atebrin and 9-aminoacridine are mostly freely soluble inside the chloroplasts*. These data are relevant to the controversy regarding the mechanism of response of acridines to the pH gradient in illuminated chloroplasts [6,10].

In fig.5, the magnitude of the light-induced neutral red absorption change is plotted as a function of the inner thylakoid pH, which was varied by changing the

*An analysis of the relation between the extent of $H^+$ uptake stimulation, ΔpH, the p$K$ of the amine and the osmotic volume will be given elsewhere (in preparation).

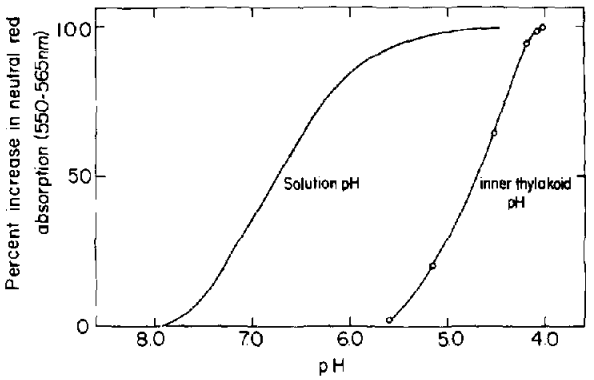


Fig.5. The dependence of neutral red absorption increase on internal pH in illuminated chloroplasts. The reaction mixture was identical to that described under fig.1. The inner thylakoid pH was varied by changing the light intensity ($3 \times 10^2$ – $3 \times 10^5$ ergs $\times$ $cm^{-2}$ $\times$ $sec^{-1}$) and measured from the extent of 9-aminoacridine absorption decrease under identical conditions [6] assuming an internal volume 20 μl/mg Chl. 9-aminoacridine concentration was 2 μM. The 'solution' curve represents the titration of neutral red in solution from pH 7.9 downwards.

Table 1
Binding of neutral red to chloroplasts in the light and in the dark

| Neutral red concentration (μM) | Chloroplast concentration (μg/3ml) | Neutral red bound | |
|---|---|---|---|
| | | Dark (% of total) | Light |
| 1 | 9 | 23 | 33 |
| | 50 | 35 | 77 |
| 3.3 | 9 | 23 | 31 |
| | 50 | 28 | 69 |
| 10 | 9 | 15 | 19 |
| | 50 | 22 | 63 |
| 20 | 9 | 14 | 24 |
| | 50 | 19 | 59 |

The reaction mixture contained in 3 ml: KCl, 40 mM; tricine, pH 7.9, 1 mM; $MgCl_2$, 0.5 mM; pyocyanine, 15 μM and chloroplasts and neutral red at the indicated concentrations. The extent of binding was determined in a microcentrifugation procedure as described under Materials and methods.

light intensity, and calculated from the drop in 9-aminoacridine absorption in the light. The titration of neutral red in solution is also plotted for comparison. Clearly the two do not match. This may indicate either (1) that the fraction of neutral red sensing the internal pH of the chloroplast depends on the extent of the ΔpH increasing with an increase in ΔpH or (2) that the apparent p*K* of neutral red inside the chloroplast is shifted to a lower value. Support for the former is provided by the observation that the binding of neutral red is markedly higher during illumination than in the dark (table 1). Neutral red binding following energization by ATP was reported earlier with submitochondrial particles [11].

Assuming that an increase in the extent of proton uptake by one equivalent represents one fully protonated neutral red molecule facing the inside of the chloroplast, the fraction of neutral red facing the inside of the chloroplast out of the total bound indicator can be calculated from the total binding in the light (table 1) and stimulation of proton uptake (fig.4).

Fig.6 shows that nearly all the indicator bound in the light is sensing the inside pH at all concentrations tested. From table 1 it is seen that almost half the amount bound in the light is already bound in the dark. Nevertheless, fig.5 indicates that of the dark bound dye only a very small fraction senses the internal pH since at low light intensities leading to a decrease of the internal pH to 5.5 which is already far below the p*K* of neutral red (6.8) only a slight increase in absorption was observed. Assuming that the p*K* of internal bound dye is identical to the p*K* of the dye in solution this would mean that a reorientation of the bound indicator takes place under the influence of the ΔpH – from a situation where most of the bound dye faces the outside (in the dark) to a situation where most of it faces the inside of the thylakoid vesicles (in the light).

The fraction of neutral red sensing the chloroplast internal phase can also be estimated from the extent of absorption change (fig.1). Fig.6 demonstrates that at the higher neutral red concentrations only a small fraction of the bound indicator is revealed by the absorption change. This probably reflects the inaccuracy of this method due to partial masking of the neutral red inside the illuminated chloroplasts at the higher concentrations (see below). At 1 μM the correlation between all three methods is nearly perfect.

The light minus dark difference spectrum of neutral red in the presence of chloroplasts is compared with the spectra observed in solution at several pHs in fig.7. The increase in absorption at 530 nm indicating appearance of protonated neutral red inside the

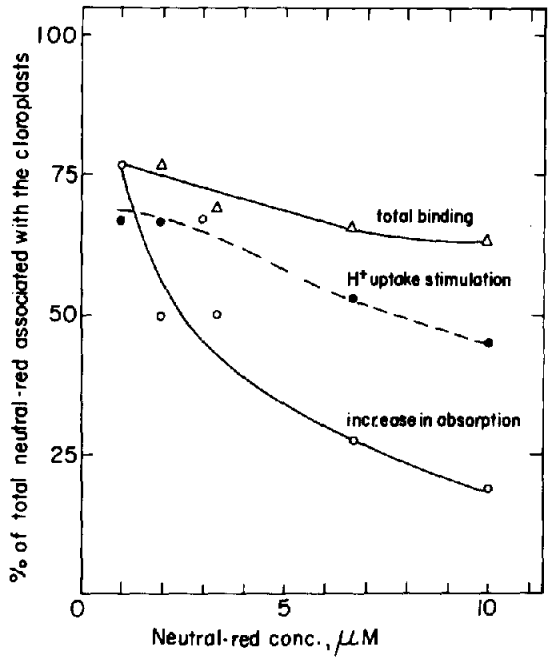


Fig.6. The fraction of neutral red sensing the innerthylakoid pH in illuminated chloroplasts. The reaction mixture was identical to that described in table 1 with chloroplasts containing 50 μg chlorophyll in 3 ml. The total amount of neutral red bound was determined by the microcentrifugation technique. The amount sensing the inner thylakoid pH was estimated from either the stimulation of proton uptake induced by neutral red (fig.4), or from the light induced increase in absorption at 550–565 nm. For the latter, the reaction mixture was acidified to pH 4.0 in the dark (after completing the illumination cycle) to convert all the neutral red to the protonated form from which the total neutral-red content was calculated. Other details as described under Methods.

chloroplast is much smaller than that expected from the drop in absorption at 450 nm due to the disappearance of the unprotonated indicator from the external medium, and the absorption peak at 530 nm is shifted to 510 nm. Both may be the result of the partial masking of the internal neutral red, as surmised from the data of fig.6. This apparent masking of internal neutral red may be due to internal aggregation (concentration effect) or internal binding to fixed negative charges, as has been previously described [12].

In summary, we suggest that the absorption changes with neutral red in illuminated chloroplasts reflect mainly accumulation of neutral red inside the chloroplasts similar to the other amines in addition to reorientation of the prebound dye. Unlike most hydrophilic amines neutral red seems to be mostly bound to the membrane. The probability of the charged protonated neutral red to flip from one face to the membrane to the other may be expected to be much lower than the probability of the unprotonated

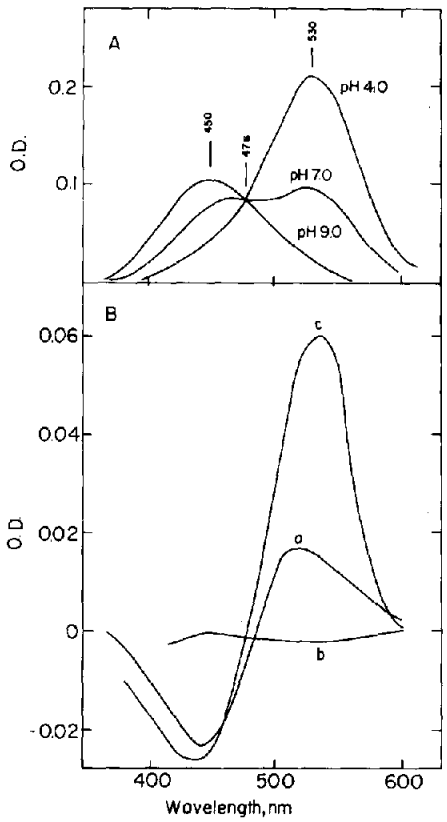


Fig.7. Comparison of light minus dark difference spectrum of neutral red in the presence of chloroplasts, to its absorption in solution. (A) Absorption spectra of 15 μM neutral red in KCl 30 mM and 10 mM buffer at the indicated pH. The buffers were Na-glycine at pH 9.0, Na-tricine at pH 7.0 and Na-succinate at pH 4.0. (B) Light minus dark difference spectrum of neutral red in the presence of illuminated chloroplasts (trace a) in a reaction mixture identical to that described under fig.1, except for the chlorophyll content which was 30 μg and the neutral red concentration which was 10 μM. The reference couvette was kept in the dark. Trace b – same, but without neutral red. Trace c – Stimulated expected difference spectrum. The sample couvette had two components (each with 5 mm optical path); one contained 10 μM neutral red at pH 4, and the other 10 μM neutral red at pH 7.9. The reference couvette (10 mm optical path) contained 10 μM neutral red at pH 7.9.

neutral red to do the same leading to a response by the latter to the internal pH while being mostly membrane bound. This mechanism can explain the accumulation of neutral red by chloroplasts in response to internal acidification in the light without assuming an increase in the negative surface of the inner face of the membrane [12].

The spectral changes in neutral red absorption under steady state illumination cannot be directly correlated with the proton concentration gradient across the chloroplast membrane since (a) the indicator is mostly membrane bound and reorients itself across the membrane in response to the ΔpH (b) the response is dependent upon the neutral red and chlorophyll concentration and (c) the interpretation is further complicated by partial masking of the neutral red absorption when bound to the chloroplast membrane. Nevertheless, with proper precautions the technique may be used as a very sensitive, simple and rapid way to follow qualitatively internal pH changes in chloroplasts, particularly with the single flash technique [2] where many of these difficulties may not be as serious because of the rapid measuring time.

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