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ETHYL RED AS A PROBE INTO THE MECHANISM OF LIGHT-DRIVEN PROTON TRANSLOCATION BY ISOLATED CHLOROPLASTS

I. THE SPECTRAL SHIFT OF ETHYL RED AND MEMBRANE CONFORMATIONAL CHANGES

ROBERT L. HEATH

Departments of Biology and of Biochemistry, University of California, Riverside, Calif. 92502 (U.S.A.)

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SUMMARY

A suspension of isolated chloroplasts partially binds ethyl red, a pH-indicator dye. Using differential spectrophotometry a bathochromic shift in the spectrum of ethyl red, which is indicative of the dye moving from an organic to an aqueous environment, can be detected upon illumination of the suspension. When the light is extinguished, the shift is reversed and the original absorbance level returns. The light-on and light-off responses are first-order processes with half-times of approximately 0.8 and 4 s, respectively. Both half-times as well as the extent of this light-induced ethyl-red response are decreased greatly by uncouplers of photophosphorylation and proton transport, such as ammonium, chloroquine phosphate, carbonyl cyanide *m*-chlorophenylhydrazone, Triton X-100, gramicidin and nigericin (+ K^+). Cations also decrease the light-induced binding change most dramatically, 50% inhibition requires about 25 mM for Na^+. The amines, including ammonia, require only 15 mM for half-inhibition while Tris and choline require higher concentrations of 40–50 mM. It appears that there is a negatively charged site within the chloroplast membrane which binds ethyl red. The equilibrium of binding is altered by the amount of cations, including H^+, which are present in the chloroplast. As the chloroplasts are illuminated, H^+ is transported into the chloroplasts, thereby shifting the equilibrium away from ethyl red binding. The use of ethyl red may promote an understanding of the primary events in the structural alterations of the membrane which are required for efficient photophosphorylation.

INTRODUCTION

Recently many experiments have been designed to observe the dynamic conformational changes within the grana membranes of chloroplasts brought

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ethyl red, (1-ethyl-2-(1-ethyl-4-methenyl)quinolinyl)quinolinium chloride; k_D and k_L, first-order rate coefficients for the light-off and light-on ethyl-red response, respectively; MES, 2-(*N*-morpholino)ethanesulfonic acid; methyl viologen, dimethyldipyridyl chloride; Triton X-100, non-ionic detergent CH_3-$(CH_2)_7C_6H_4(OCH_2CH_2)_{10}OH$.

about by electron and ion transport. For example, Packer and his associates[1–3] have utilized light-scattering measurements to investigate conformational changes within the membrane systems of both mitochondria and chloroplasts. Electron microscopy[4] and optical rotatory dispersion measurements[5] have also been used to examine membrane alterations, but only after attempting to halt any further changes within the membranes by fixation with glutaraldehyde.

Chance and Mela[6] first used pH-indicator dyes, which were bound within mitochondrial membranes, to monitor internal pH changes and alterations of the membrane. In addition, the fluorescent dye (1,8-anilinonaphtol sulfonic acid) has been used for observing changes in nerve membranes during conduction[7] and in mitochondria actively engaged in electron transport[8]. The change in internal pH upon illumination within isolated chloroplasts can be calculated from data obtained using the fluorescent dye aminoacridine[9]. Furthermore, Murata and Sugahara[10] and others[11,12] have found that the variation in the fluorescence yield of the chlorophyll in isolated chloroplasts can measure, in part, changes within the grana stacks connected to ATP synthesis.

Heath and Hind[13], while screening possible indicators of the pH within the internal space of chloroplasts, discovered that (1-ethyl-2-(1-ethyl-4-methenyl)-quinolinyl)quinolinium chloride (ethyl red) is strongly bound to the grana membranes of chloroplasts in a buffer-impermeable region. Ethyl red, in a buffered suspension, monitors changes associated with internal pH and the binding capacity of the membrane for ethyl red. Binding changes were also found to be brought about by illumination of the suspension of chloroplasts; however, the spectrophotometry was difficult to do.

This paper reports a spectrophotometric technique which can separate both ethyl red responses. The light-induced response of ethyl red binding is quite sensitive to membrane changes induced by light-driven proton flow. A working hypothesis is that ethyl red is monitoring sites of negative charge within the chloroplast membrane which are altered by photo-induced proton flow. While Mitchell[14] believes that proton transport is responsible for ATP synthesis only, Dilley[15] believes that the production of these negative charges within the chloroplasts drives photophosphorylation and proton motion. Since ethyl red may be a very sensitive probe of those negative charges, its use may lead into a more complete understanding of the linkage between ion flow and photophosphorylation.

METHODS

Chloroplasts from commercially-grown spinach were prepared as previously described[13] by grinding in an unbuffered sucrose–$MgCl_2$ medium, washing once in hypotonic saline and resuspending in 0.8 M sucrose, 0.03 M NaCl. The absorbance changes were observed using an Amino-Change double beam spectrophotometer which has been equipped with ports into the sample compartment for illumination. The actinic illumination was provided by a 750 W projector using a quartz–iodine lamp and a 645-nm Baird-Atomic filter (half-power band width=6 nm) which provided about 55 kergs$\cdot$cm$^{-2}\cdot$s^{-1} of light energy.

A typical experiment consisted of suspending isolated chloroplasts (25 μg chlorophyll per ml) in 3.0 ml of 0.4 M sucrose, 35 μM phenazine methosulfate

or pyocyanine, 17 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 6.0, and 10 μM ethyl red. After temperature equilibration to 16 °C, the chloroplasts were exposed to actinic illumination. Reference wavelength was always 568 nm and the half-width of the monochromators were set at 2 nm.

RESULTS

A bathochromic shift in the absorption spectrum of ethyl red is produced when ethyl red is bound to the chloroplast membrane structure[13]. This shift resembled that produced by suspending the dye in an organic solvent and is thus interpreted as ethyl red being in an organic environment in the chloroplasts. This change in the spectrum caused by binding can be partially eliminated by actinic illumination and was previously observed on a double-beam spectrophotometer using 610 nm as the reference wavelength. Unfortunately, this wavelength is a poor choice due to the relatively large wavelength span between it and the peak of the spectrum of the ethyl red shift and due to the interference of the actinic light with the reference beam.

However, as Fig. 1 shows, the shift in spectrum or binding induced by actinic illumination can be observed as a change in absorbance with a reference wavelength at the isosbestic of the differential absorption spectrum for ethyl red binding of 568 nm. The upward deflection upon actinic illumination represents an increase in absorption. The slower drift (taking several seconds) at the measuring wavelengths

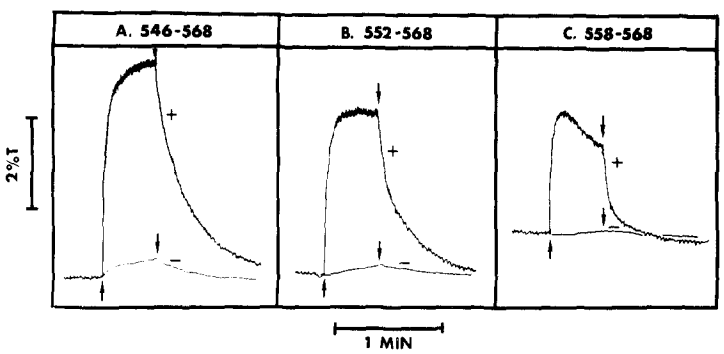

Fig. 1. Spectrophotometric response of ethyl red absorption to actinic illumination. Chloroplasts were suspended as in Methods and, where indicated, 10 μM ethyl red (+) was added. The measuring beam was set at the indicated wavelengths. Light-on at upward arrows; light-off at downward arrows. An upward deflection is an increasing absorbance. The bottom traces (−) in each frame are the controls with no ethyl red present.

of 546 and 558 nm (Figs 1A and 1C, respectively) are due to the bleaching of ethyl red, thought to be caused by an internal acidification[13]. At 552 nm (near the peak of shift in the spectrum due to the ethyl red binding) no slow change is observed and, after several seconds, the light-induced increase in absorbance ceases. This figure (1B) shows there is a rapid on-response (due to illumination) and a slower, but still fast off-response (decrease in absorbance). These responses can be repeated at least five times with minimal changes in the kinetic pattern.

The spectra in Fig. 2 show this light-induced de-binding phenomenon in more detail. The extent of the light-induced shift (given by the maximum absorbance change in Fig. 1) is plotted in Fig. 2A as a function of wavelength with the organic solvent-induced spectral shift (also see ref. 13). At wavelengths below 550 nm, the slow drift due to bleaching of internal ethyl red (see Fig. 1A) makes the extent uncertain and, thus, the spectra do not match. If, however, the rate of absorbance change after the light is extinguished is plotted as a function of wavelength in Fig. 2B, the match in spectra is extremely good even below 550 nm. The internal acidification change is slower than the re-binding of ethyl red[13].

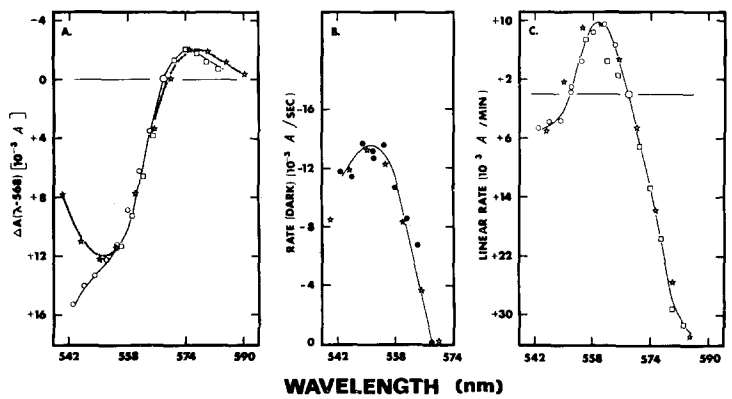

Fig. 2. Spectra of the light-induced change in ethyl red. Chloroplasts were suspended as in Methods with the reference beam at 568 nm. Note inverted absorbance scale. (A) ○ and □, extent of light-induced ethyl red spectral change (for 2 separate experiments); (B) ●, rate of ethyl red light-induced spectral change immediately after the light is extinguished. (A) and (B) ∗, organic solvent induced spectral shift. (C) ○ and □, linear rate of spectral change found after several seconds of illumination (see Figs 1A and 1C) (for 2 separate experiments); ∗, absorption spectrum of ethyl red with added chloroplasts, with normalized zero set at 568 nm (see ref. 13).

The slow drift noted in Figs 1A and 1C is easily explained by Fig. 2C. The spectrum for the rate of the slow drift (after several seconds) matches that of the bleaching of ethyl red (the spectrum of ethyl red with added chloroplast, normalized such that the absorbance at 568 nm is zero). The measuring wavelengths of 552 and 568 nm are on opposite sides of the peak of the ethyl red bleaching spectrum and, thus, the differential absorption (552–568 nm) does not show the bleaching of ethyl red. In addition, the magnitude of the ethyl red spectral shift is relatively large compared with that of the cytochrome changes (see Fig. 1B). Thus, this light-induced shift in the ethyl red spectrum (measured at 552–568 nm) is equivalent to observing membrane bound ethyl red molecules moving towards a more polar solvent.

The kinetics of the light-on and light-off responses of ethyl red are shown in Fig. 3, plotted semi-logarithmically. Both processes are apparently first-order with rate constants for the light-on (Fig. 3A) and light-off (Fig. 3B) responses of $k_L = 0.72\,s^{-1}$ and $k_D = 0.17\,s^{-1}$, respectively, with both pyocyanine and phenazine methosulfate as the electron acceptor. The increase in absorbance (light on) takes less than 5 s for completion, while the fall in absorbance (light off) to the original level takes 30 s or more.

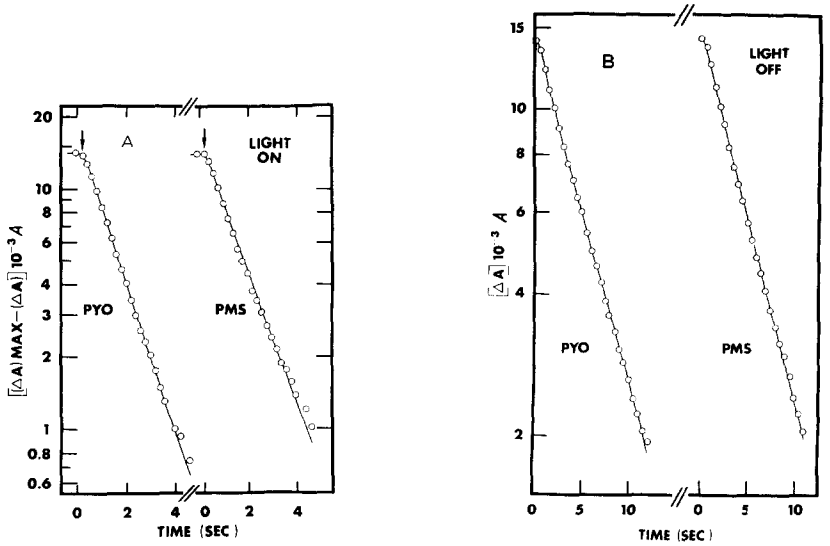

Fig. 3. Kinetics of ethyl red spectral change (552–568 nm). Chloroplasts were suspended as in methods with either phenazine methosulfate (PMS) or pyocyanine (PYO) as the electron acceptor. Note the changes of the ordinate and the logarithmic scale.

Unfortunately, the binding of ethyl red to chloroplasts does not follow a simple linear relationship to the amount of chlorophyll present[13], but rather an equilibrium reaction relation[16]. Fig. 4 documents the light-induced binding change with regards to the concentration of ethyl red and of chloroplasts. The extent of the light-induced ethyl red change shows saturation with respect to a high concentration of chloroplasts (Fig. 4A). As Fig. 4B shows, however, the light-induced response with 25 μM ethyl red is not 3 times that of the response with 8.3 μM ethyl

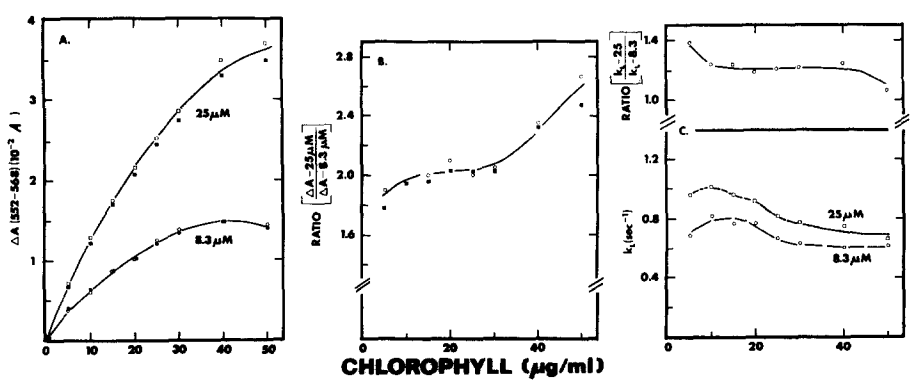

Fig. 4. Effect of varying concentrations of chloroplasts and ethyl red upon the light-induced ethyl red spectral response (552–568 nm). Chloroplasts were suspended at the noted concentration (as μg chlorophyll per ml) with the two concentrations of ethyl red. The solid points represent the first illumination period and the darkened points represent the second (after 60 s of darkness). ΔA is the extent reached after 15 s of illumination, and k_L is the first-order rate constant for the light-on response.

red, but is more nearly 2 times. This ratio (extent for 25 μM ethyl red divided by extent for 8.3 μM ethyl red) varies with the amount of chloroplasts used; although, in the range of 15–30 μg/ml chlorophyll, the ratio is nearly constant at 2.

There is a small amount of interaction between the k_L and the amount of ethyl red and chloroplasts used, as seen in Fig. 4C. The ratio of k_L with 25 μM ethyl red to that with 8.3 μM ethyl red is constant but larger than unity (1.2) between 10 and 40 μg chlorophyll per ml. Thus, the constant, k_L, increases only by 20% for a 3-fold increase in ethyl red. The complexity of the phenomenon of binding precludes easy interpretation or easy manipulation of these relationships. In this study, the experimental design utilizes the region in these diagrams of insensitivity to ethyl red concentration; that is, between 15–30 μg chlorophyll per ml.

Table I shows the effect of a variety of uncouplers on the light-induced ethyl red spectral response, measured at 552–568 nm. Many cations exert an effect upon this ethyl red response. This effect will be discussed more fully later; however, the initial results are seen in Table I. The addition of NH_4^+ to the chloroplast suspension decreases the extent but increases both the rate constant of the light-on and light-off responses (k_L and k_D). These results must be compared with the addition

TABLE I

EFFECT OF UNCOUPLERS UPON THE LIGHT-INDUCED SPECTRAL RESPONSE OF ETHYL RED

Compound	*Extent* (10^{-2} *A units*)	k_L^* (s^{-1})	k_D^* (s^{-1})
1. None**	0.92	0.58	0.14
NH_4Cl (6.7 mM)	0.37	0.87	0.37
(17 mM)	0.19	1.16	0.38
NaCl (6.7 mM)	0.58	0.53	0.11
(17 mM)	0.43	0.58	0.12
Sodium trichloroacetate (6.7 mM)	0.66	0.64	0.21
(17 mM)	0.41	0.63	0.24
2. None*** 1.61	1.61	0.62	0.17
Chloroquine phosphate (175 μM)	1.09	0.79	0.24
(350 μM)	0.87	0.80	0.27
3. None†	1.50	0.62	0.16
Carbonyl cyanide 3-chloro-phenylhydrazone (1 μM)	0.93	0.64	0.39
(3 μM)	0.37	0.63	0.74
(5 μM)	0.19	0.63	1.45
4. None**	1.70	0.69	0.13
Triton X-100 (0.006%)	0.85	1.38, 0.80††	0.26

* k_L and k_D are first-order rate constants for the light-on and light-off response, respectively measured at 552–568 nm.

** Chloroplasts (25 μg chlorophyll per ml) were suspended as in Methods at indicated concentration of NH_4Cl (pH 6) or NaCl or sodium trichloroacteate (pH 6).

*** Chloroplasts (30 μg chlorophyll per ml) were suspended as above.

† Chloroplasts were suspended as above except 0.03% ethanol was added to all trials.

†† The two rate constants were measured from a biphasic curve containing a break at 2.9 s.

of NaCl, where the extent also declines but there is no marked change in eigher k_L or k_D.

Chloroquine phosphate is an uncoupler similar to quinacrine (atebrin)[17], but is colorless and, therefore, does not interfere spectrophotometrically. Chloroquine phosphate affects the ethyl red spectral response in a manner similar to NH_4^+ (Table I). The extent is lowered and both rate constants (k_L and k_D) are increased in the presences of chloroquine phosphate. These results are to be contrasted with the effects of carbonyl cyanide 3-chlorophenylhydrazone, which also decreases the extent and increase k_D, but does not alter k_L at all.

The non-ionic detergent $CH_3(CH_2)_7C_6H_4(OCH_2CH_2)_{10}OH$ (Triton X-100) which can both uncouple and inhibit electron transport depending upon the concentration[18], decreases the extent and increases k_D nearly 2-fold, similar to other uncouplers. The rate constant for light-on, k_L, is altered drastically. Not only is k_L increased by Triton X-100 similar to the other uncouplers, but it also becomes biphasic. Two separate first-order rate constants can be observed with a break in the curve occurring after about 2 s of illumination. The control without ethyl red has no large absorbance change; therefore, this break is not a light-scattering artifact.

Many antibiotics are powerful uncoupling agents of both the light-induced proton translocation and photophosphorylation[19]. These antibiotics also affect the light-induced ethyl red spectral response, as shown in Fig. 5. Only gramicidin,

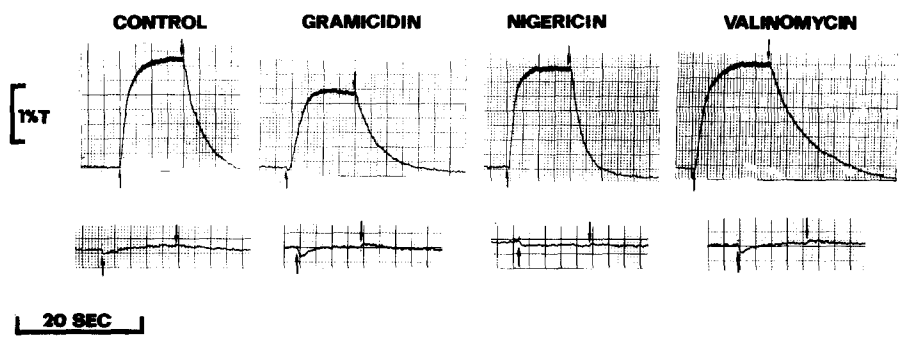

Fig. 5. Effect of antibiotics upon the light-induced ethyl red spectral response. Gramicidine (0.017 μM), nigericin (0.33 μM) or valinomycin (0.67 μM) were added to a chloroplast suspension, as described in Methods, with 2 mM KCl. An upwards deflection represents absorbance increase (552–568 nm). Upward arrows indicate light-on and downward, light-off. Upper trace, in presence of ethyl red. Lower trace, without added ethyl red.

however, completely eliminates the response at a concentration of 0.33 μM. At a 20-fold lower concentration, gramicidin produced initially (at light-on) an increase (downward deflection) in absorbance (Fig. 5B) and a lowered extent. This initial drop is compensated for by a light-scattering artifact (−ethyl red); however, if the +ethyl red response is corrected for the −ethyl red response, a pronounced lag in the initial rise remains (several tenths of seconds). Nigericin (at a concentration of 0.33 μM) lowers the extent slightly and increases both k_L and k_D, similar to chloroquine phosphate and NH_4^+(Fig. 5C). Valinomycin also alters the extent

slightly but decreases both rate constants (k_L and k_D) in constrast to nigericin, chloroquine phosphate and NH_4^+ (compare Figs 5A and 5D).

Table II shows that K^+ is required for most of the effects of valinomycin and nigericin upon the ethyl red spectral response[20]. Without K^+ slight effects are still observed, however. Most probably, these are due to small amounts of K^+ remaining in the chloroplast preparation. Again K^+ as a salt (even at 3 mM) lowers the extent and slightly decreases k_L, similar to the effect of Na^+ (see Table I). Even though this concentration of KCl is not optimum[20], higher concentrations depress the extent too much to be easily used.

TABLE II

EFFECTS OF ANTIBIOTICS ON THE LIGHT-INDUCED ETHYL RED SPECTRAL RESPONSE

The relative percentage change is measured with respect to the controls (100%) without antibiotics but either in the presences or absence of KCl measured at 552–568 nm. The controls were similar to those in Table I. Conditions: The chloroplasts were suspended as in Methods with, where indicated, 3 mM KCl, 0.33 μM nigericin, 0.33 μM valinomycin or 0.017 μM gramicidin in 0.1% ethanol.

Antibiotics	K^+	*Percentage change*		
		Extent	k_L	k_D
Nigericin	—	98	106	134
	+	79	132	225
Valinomycin	—	100	96	90
	+	97	74	50
Gramicidin	—	51	103	44
	+	46	121	39

The effect of added K^+ is most pronounced upon the action of nigericin on the ethyl red spectral response, especially, with regards to lowering the extent and increasing the rate constants (k_L and k_D). The action of valinomycin is affected by K^+ only for the rate constants; decreasing both k_L and k_D. On the other hand, gramicidin decreases the k_D; but does not affect the k_L (after the initial lag). K^+ has no apparent effect upon the action of gramicidin

The type of electron acceptor used to support electron flow has little effect upon the ethyl red spectral response (Table III). Even without an electron acceptor the Mehler reaction causes electron flow to proceed at a sizable rate[13]. Noncyclic cofactors cause a depression of the extent, which in the case of potassium ferricyanide is considerable (40%), compared with the cyclic cofactor (pyocyanine). The rate constant for the on-response (k_L) is not altered except in the case of dimethyldipyridyl chloride (methyl viologen) (35% increase). The rate constant for the light-off response is lowered for all cofactors compared with that for pyocyanine (30%).

Table III also documents the effect of the inhibitor of non-cyclic electron flow, 3-(3,4-dichlorophenyl-1,1-dimethylurea (DCMU)[21]. DCMU greatly lowers the k_L rate constant and extent, while increasing the k_D rate constant. Although

not shown here the lowering of the extent by DCMU is constant over repeated cycles of light. This is to be compared with a progressively increasing inhibition (with cycles of illumination) observed for H^+ transport itself with DCMU addition[22].

As previously mentioned cations affect the light-induced ethyl red spectral response by lowering the extent with little change in the rate constants (k_D or k_L). As can be seen in Table IV, the anion has little effect upon this phenomenon. The use of a relatively large, weak acid anion, such as acetate, does not change the high degree of inhibition of the light-induced ethyl red response by Na^+. However, large cations, such as choline or Tris, do not cause as great of inhibition as that caused by added Na^+.

The extent of the ethyl red response is lowered in a very characteristic manner

TABLE III

EFFECT OF ELECTRON-TRANSPORT REAGENTS UPON THE LIGHT-INDUCED ETHYL RED SPECTRAL RESPONSE

Chloroplasts were suspended as in Methods with indicated amounts of reagent. The errors of the mean were ±0.01 for the *Extent*, ±0.03 for the k_L and ±0.02 for the k_D. See Table I for symbols. The electron flow was measured (coupled) as O_2 evolution (for ferricyanide) or O_2 uptake (for none and methyl viologen) using Clark-type O_2 electrode[13] under similar conditions except chlorophyll concentration was 40 μg/ml. Electron flow for pyocyanine-mediate rate cannot be measured.

Compound	*Light on*		*Light off*	
	Extent (10^{-2} A units)	k_L (s^{-1})	k_D (s^{-1})	*Electron flow (μequiv/mg per h)*
1. None	0.61	0.55	0.23	410
Methyl viologen (0.1 mM)	0.57	0.71	0.19	440
Pyocyanine (0.04 mM)	0.78	0.58	0.28	—
Ferricyanide (0.3 mM)	0.47	0.53	0.18	380
2. None	0.69	0.69	0.30	
DCMU (7 μM)	0.56	0.39	0.38	

TABLE IV

THE INHIBITION OF THE LIGHT-INDUCED ETHYL RED RESPONSE BY ADDED SALTS

The % inhibition is tabulated from a control with no additions, for two separate experiments, measured at 552–568 nm. The chloroplasts were suspended as in Methods with 25 mM of the indicated salt added to the suspension. Choline chloride and Tris acetate were used (see Table V).

Cation	*% Inhibition*	
Anion:	*Chloride*	*Acetate*
Na^+	62; 66	56; 54
Choline/Tris	27; 29	24; 27

by an added cation, as shown in Fig. 6 for NaCl. The inverse of the light-induced extent is linearly related to the concentrations of the cation, similar to an equilibrium-type reaction between ethyl red and the NaCl for a common site. It is important to note here that ethyl red is also a cation with at least 1 or 2 positive charges[13]. The equilibrium is more clearly seen with a higher concentration of ethyl

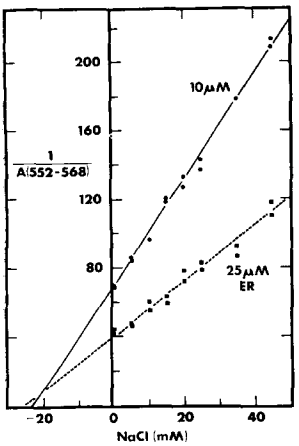

Fig. 6. Effect of added cation upon the extent of the light-induced ethyl red spectral response (552–568 nm). Chloroplasts were suspended as in Methods with varying concentration of NaCl at two concentrations of ethyl red (ER).

red. There is a characteristic constant (k_I) for this reaction given by the intercept of the line on the abscissa; in this case, about 23 mM. Since the lines for the two concentrations of ethyl red seem to intercept on the axis, a non-competitive type of reaction is indicated.

The concentration for various cations required for a 50% inhibition of

TABLE V

APPARENT INHIBITION OF LIGHT-INDUCED ETHYL RED RESPONSE BY CATIONS

The point of half-inhibition (K_I) was tabulated from a reciprocal plot as shown in Fig. 6 for the extent of the light-induced response with the standard errors of a least squares fit of the data. Two cycles of 15 s of light were presented to the chloroplasts separated by a dark time of 45 s. Chloroplasts were suspended as in Methods. The cations (as chloride salts, neutralized to pH 6.0) were added to the chloroplast suspension in concentrations from 0 to 50 mM.

Cation	K_I (*mM*)	
	Light cycle 1	*Light cycle 2*
Na^+	27 ±2	23 ±2
NH_4^+	13.5±0.7	8.8±0.9
Methylamine	17.6±0.9	16.9±0.5
Diethylamine	19 ±2	11.3±0.9
Trimethylamine	17 ±4	14.7±0.9
Tris	44 ±3	36 ±1
Choline	53.0±0.3	44 ±2

the light-induced ethyl red response seems to fall into three categories (Table V). The amines, including NH_4^+, require a lower concentration to half-inhibit the ethyl red response (between 10–20 mM) compared with that for Na^+. The larger cations such as Tris and choline require a concentration of more than twice that for sodium (40–50 mM) for the same half-inhibition point. An interesting point for NH_4^+ and diethylamine ion is that the K_i during the second light cycle is noticeably lower than that of the first light cycle. A slight uncoupling can be observed (using potassium ferricyanide as an electron acceptor) only with 40–50 mM diethylamine and trimethylamine.

The rate constants (k_L and k_D) are also altered by the suspending cation; however, to a much less extent except for NH_4^+, as shown in Fig. 7. The rate

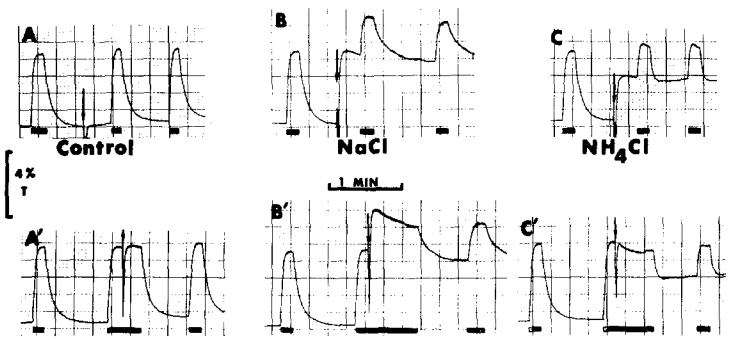

Fig. 7. The effect on the rate constant of the light-on ethyl red spectral response (552–568 nm). Conditions were as in Table V, with the k_L figured by methods of least-squares fit of the exponential on-response (see Fig. 3). ○, Na^+; □, choline; ●, NH_4^+; ■, methylamine.

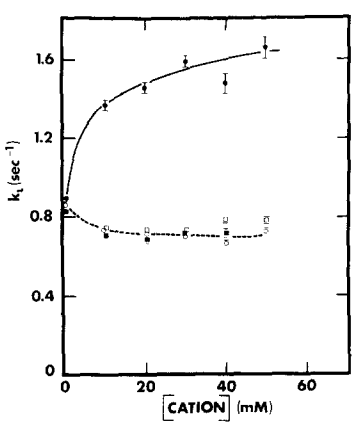

Fig. 8. The kinetic change of the ethyl red spectral response (552–568 nm) by added cations, Chloroplasts were suspended as in Methods with an addition (25 μl in 3 ml total volume) of water, NH_4Cl (final concentration, 10 mM) or NaCl (final concentration, 30 mM) in the light or dark (shown by arrow). Dark bar represents light-on. Top trace (unprimed) represents additions in dark. Bottom traces (primed) represent addition in light.

constant for the light-on response (k_L) drops about 17% for all cations tested except NH_4^+, which causes a rise (100%). The rate constant for the light-off response (k_D) also shows a slight decline except in the case of NH_4^+ where k_D again rises considerably (see Table I).

Cations cause ethyl red to be bound less strongly in the dark as shown from the kinetic spectral changes in Fig. 8. In Figs 8A and 8A', the addition of water in the light and dark causes only a slight artifact due to dilution. The addition of NaCl (Figs 8B and 8B') and NH_4^+ (Figs 8C and 8C') causes a sizable increase (upward trace) in the absorbance for an addition in both the light and dark. The addition of NaCl and NH_4Cl shifts the baseline for the light-induced response in addition to lowering the net response. In both cases, after the cation is added in the light, the new steady state takes at least 10 s to be established; indicating a relatively slow process is occurring.

DISCUSSION

The first measurements of light-induced proton translocation by isolated chloroplasts used the slowly responding pH electrode making initial kinetics impossible to investigate[23]. The use of faster responding electrodes[24] and flow techniques[25] as well as spectrophotometrically-responding indicator dyes[13,24–26], has established that there exists at least two kinetically-separable flows of protons. The initial flow is rapid with a $t_{1/2}$ of about 0.8 s, while thesecond is the slower flow, usually measured by the pH electrode, with a $t_{1/2}$ of about 8–10 s (ref. 13). Both half-times depend somewhat upon the experimental conditions. The existance of a still faster flow[26] has been shown but not yet repeated. The two flows cited above can be demonstrated with the external pH indicator, bromocresol purple[13] and with the dye, ethyl red ($t_{1/2}=1$ s). The second ethyl red response will be more completely covered in the next paper of this series, but seems to show the flow of protons internal to chloroplasts with a $t_{1/2}$ of about 8 s.

The bathochromic shift in the ethyl red spectrum (Fig. 2) when the chloroplasts are illuminated indicates a movement of some of the ethyl red from a non-aqueous to an aqueous solution[13]. When the light is extinguished, the ethyl red molecule, presumably, returns to its previous position. Under the conditions used here and in the previous publication[13], approximately 40% of the ethyl red in solution is bound to the chloroplasts (*e.g.* moves with the chloroplasts in a centrifugal field). Comparing the total spectral shift when chloroplasts are added to an ethyl red solution ($A_{554\,nm}-A_{568\,nm}=0.12$) to the same spectral shift induced by light ($A_{554\,nm}-A_{568\,nm}=0.012$), one can conclude that approximately 4% of the total ethyl red is taking part in the light-induced spectral shift. Since no light-induced de-binding into the external media, as measured by the centrifugation method[13], takes place (within 1–1.5%), one must also conclude that the spectral shift is a de-binding of the ethyl red molecules within the chloroplast internal granal space.

From the extent in Fig. 4A, and the amount of binding of ethyl red to chloroplast[13], the binding and the de-binding seems to be an equilibrium between a binding site (S) and the concentration of ethyl red as given below.

$$\text{Ethyl red} + S \rightleftharpoons (\text{ethyl red}\cdot S)_{\text{bound}} \quad \text{and} \quad K_B = \frac{(\text{ethyl red}\cdot S)_{\text{bound}}}{(\text{ethyl red})(S)} \tag{1}$$

It is the (ethyl red·S) bound which possesses the spectrum with a bathochromic shift. Thus, illumination of the chloroplasts can be thought to cause either (*i*) a change in the number of available sites (S), (ii) a shift in the binding constant (K_B), or (iii) a combination of both. In all cases, the imprecision of the model and the complexity of the mathematics is such that a mathematical discussion, at this time, would be of little value. It is sufficient to say that the relationships between the light-induced extent and the concentrations of chloroplasts and of ethyl red is non-linear and, under a high concentration of one or the other, the extent can be saturated.

All the uncouplers of photophosphorylation and proton transport which were tested (Tables I and II, Fig. 5) decrease the extent of the light-induced ethyl red response. Uncouplers have been found to lower the amount of H^+ translocated[1,13]. The rate coefficient, k_D, is increased by uncouplers, except in the case of gramicidin and valinomycin. In other words, the rate of change back to the original binding state (in the dark) is more rapid in the presence of uncouplers, which cause an increased rate of H^+ leakage in the dark. The rate coefficient, k_L, is increased by the addition of most of the uncouplers tested, *i.e.* NH_4^+, chloroquine phosphate, Triton, nigericin and gramidicin. The action of uncouplers on the initial rate of H^+ transport unfortunately has not been systematically studied. Only valinomycin decreases k_L. No change in k_L is noted for the uncoupler, carbonyl cyanide 3-chlorophenylhydrazone, which slows H^+ translocation[13].

The action of antibiotics deserves special mention (Fig. 5, Table III). Gramicidin completely inhibits the light-induced ethyl red response at a concentration at which for valinomycin and nigericin does little. From studies of model systems[27], the basic difference between gramicidin and valinomycin is that gramicidin induces a large permeability of the chloroplast membrane to H^+. Thus, one can argue that the ethyl red response is primarily a reaction to H^+ movement with a small dependence upon the K^+ concentration. Nigericin, by speeding K^+ and H^+ exchange[19] would increase both k_D and k_L and its action would be dependent upon K^+ in the medium (Table II). The effect of valinomycin on the ethyl red response, also dependent upon K^+, indicates the possible collapse of a small potential gradient without the concurrent increase in H^+ transport[26,28]. Thus, valinomycin could lower the initial H^+ influx in the light (driven by K^+ gradient) and the efflux of the H^+ from the interior in the dark (exchange with K^+).

In this regard, cations do not alter the k_L or the k_D by much (Fig. 7) however; they do lower the extent (Table V) and cause a lowering of the amount of total binding (Fig. 8). If the site to which ethyl red was binding is a negative site (a plausible explanation since ethyl red is a positively charged molecule), then cations would be bind to the site instead of ethyl red and, thus, lower the extent. Furthermore, involvement of H^+ transport would indicate that H^+ is also binding to the same site. Hence, the transport of both cations and H^+ would change the net ethyl red binding. This site is similar to that proposed by Dilley[15] which is involved in photophosphorylation. It is clear from the nature of the ethyl red spectral shift that this site may be in a lipophilic environment.

This site-hypothesis is summarized as follows: The site is normally negatively charged. As ethyl red (+1), cation$^+$ or H^+ is added to the chloroplast, the negative charge is neutralized. The pH is 6.0; therefore, one would guess that protonation is the preferred reaction with a equilibrium constant of less than 10 μM. The equilibrium constant for ethyl red binding is about 25 μM (ref. 13). The least preferred equilibrium is for cation (equilibrium constant of 10 mM or greater, see Table V). The shift of equilibrium depends upon the relative concentrations of H^+, cations and ethyl red. The protons (to protonate the site) are provided by the initial light-driven translocation[2,13]; thus, explaining the light-induced ethyl red spectral shift (driving off the ethyl red bound to the site). The protonation of the site shifts the equilibrium away from site·ethyl red. Uncouplers lower the H^+ build-up within the granal space and thus lower the extent of the ethyl red response. Since DCMU blocks non-cyclic electron flow[22] and methyl viologen, potassium ferricyanide or no electron acceptor[20] keep the electron flow slow (compared with pyocyanine), the build-up of the H^+ gradient would likewise be slowed (Table III). The amines by competing for the site and internal H^+ (ref. 29) would be expected to be more effective than other cations (Table V). In this respect, the biphasic amine uptake response observed by Crofts[29] may be caused by this membrane protonation.

The protonation of a site on the membrane is not a new proposal for H^+ transport[1,15]; however, ethyl red may well be the first direct measurement of it. The rapid influx of H^+ required to protonate the site would explain the rapid initial rate of H^+ movement, as measured by Izawa and Hind[25] and Heath and Hind[13], which is not explained by the Mitchell chemiosmotic hypothesis[14]. The existance of these two, presumably interrelated, proton flows (binding reaction and internal pH, ref. 13) may have a direct bearing upon the problem of quantum yield of translocation. The Mitchell hypothesis[14] requires a yield of no more than two. However, both Dilley and Vernon[30] (and Heath[22] found that the initial external proton flow yield was about 3–4 ($H^+/h\nu$) in red light. However, if the steady-state proton flow is identified as equal to the efflux of protons after the light is extinguished, a quantum yield of nearly 2 is found in red light. If the initial H^+ flow is made up of protons which, ultimately, have two separate destinations, this quandary might be eliminated.

The question of what this ethyl red binding site is still remains; however, the use of ethyl red to probe its nature is an obvious experimental course. Furthermore, it is not yet clear from these experiments whether or not cations displace protons or protons can displace cations from the (ethyl red) binding site. One might speculate that under normal circumstances the site is neutralized by a cation and as light-driven proton translocation produces an excess of protons within the intergrana space these cations are displaced by protons. This would lead to a light-induced efflux of cations as observed by Dilley and Vernon[30]. The role of the now-protonated site in the control of electron transport and photophosphorylation is likewise speculative at this point. However, given the control of electron flow and coupling that pH has[32] and the role of Mg^{2+} and Na^+ in the control of electron flow proton excitation energy transfer between the photosystems[10,32], the ethyl red binding site may well be that control site. Kinetically ethyl red may allow understanding the primary events in structural alteration which primes or "makes

ready" the photosynthetic mechanisms for efficient electron transport and photophosphorylation[33].

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REFERENCES

1 Packer, L., Murakami, S. and Mehard, C. W. (1970) *Annu. Rev. Plant Physiol.* 21, 271–304
2 Crofts, A., Deamer, D. and Packer, L. (1967) *Biochim. Biophys. Acta* 131, 97–118
3 Packer, L., Utsumi, K. and Mustafa, M. (1966) *Arch. Biochem. Biophys.* 117, 381–393
4 Deamer, D. W. and Crofts, A. J. (1967) *J. Cell Biol.* 33, 395–410
5 Wrigglesworth, J. M. and Packer, L. (1968) *Arch. Biochem. Biophys.* 128, 790–801
6 Chance, B. and Mela, L. (1967) *J. Biol. Chem.* 242, 830–844
7 Tasaki, I., Watanabe, A., Sandlin, R. and Carney, L. (1968) *Proc. Natl. Acad. Sci. U.S.* 61, 883–888
8 Harris, R. A. (1971) *Arch. Biochem. Biophys.* 147, 436–445
9 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) *Eur. J. Biochem.* 25, 64–70
10 Murata, N. and Sugahara, K. (1969) *Biochim. Biophys. Acta* 189, 182–192
11 Heath, R. L. and Packer, L. (1968) *Arch. Biochem. Biophys.* 125, 1019–1021
12 Wraight, C. A. and Crofts, A. R. (1970) *Eur. J. Biochem.* 17, 319–237
13 Heath, R. L. and Hind, G. (1972) *J. Biol. Chem.* 247, 2917–2925
14 Mitchell, P. (1961) *Nature* 191, 144–148
15 Dilley, R. A. (1970) *Arch. Biochem. Biophys.* 137, 270–283
16 Klotz, I. M., Walker, F. M. and Pivan, R. B. (1946) *J. Am. Chem. Soc.* 68, 1486–1490
17 Hind, G., Nakatani, H. Y. and Izawa, S. (1969) *Biochim. Biophys. Acta* 172, 277–289
18 Neumann, J. and Jagendorf, A. (1965) *Biochim. Biophys. Acta* 172, 277–289
19 Jackson, J. B., Crofts, A. R. and Von-Stedingk, L. V. (1968) *Eur. J. Biochem.* 6, 41–54
20 Shavit, N., Degani, H. and San Pietro, A. (1970) *Biochim. Biophys. Acta* 216, 208
21 Gingras, G. and Lemasson, C. (196) *Biochim. Biophys. Acta* 109, 67–78
22 Heath, R. L. (1972) *Biochim. Biophys. Acta* 256, 645–655
23 Neuman, J. and Jagendorf, A. T. (1964) *Arch. Biochem. Biophys.* 107, 109–119
24 Schwarz, M. (1968) *Nature* 219, 915–919
25 Izawa, S. and Hind, G. (1967) *Biochim. Biophys. Acta* 143, 377–390
26 Schliephake, W., Junge, W. and Witt, H. T. (1968) *Z. Naturforsch.* 23B, 1571–1578
27 Pressman, B. C., Harris, E. J., Jagger, W. S. and Johnson, J. H. (1967) *Proc. Natl. Acad. Acad. Sci. U.S.* 58, 1949–1956
28 Barber, J. (1972) *FEBS Lett.* 21, 251–254
29 Crofts, A. R. (1967) *J. Biol. Chem.* 242, 3552–3559
30 Dilley, R. A. and Vernon, L. P. (1 67) *Proc. Natl. Acad. Sci. U.S.* 3, 395–399
31 Dilley, R. A. and Vernon, L. P. (1965) *Arch. Biochem. Biophys.* 11, 365–375
32 Gross, E., Dilley, R. A. and San Pietro, A. (1969) *Arch. Biochem. Biophys.* 134, 450–462
33 Myer, J. (1971) *Annu. Rev. Plant Physiol.* 22, 289–312