Bromocresol Purple and Ethyl Red as Indicators of Events Linked to Changes in the Energization of Chloroplast Membranes*

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SUMMARY

1. Two dyes, bromocresol purple and ethyl red, were tested for their ability to register solution and membrane changes resulting from the illumination of suspensions of isolated chloroplasts.

2. Bromocresol purple can measure the external (suspension) pH both rapidly and accurately if the following precautions are observed: (a) since bromocresol purple stimulates electron flow to oxygen, either low concentrations (≤ 2 µM) of bromocresol purple or conditions for noncyclic electron transport must be used; (b) the absorbance shifts must be converted by formula to yield changes in proton concentration.

3. The disappearance of protons from the medium when the light is turned on is bi-exponential with half-times of about 0.7 s and 8 s. The release of protons into the medium when the light is turned off is exponential with a decay half-time of about 30 s.

4. A brief initial lag (<0.2 s) in the uptake of protons follows the onset of illumination, and there is a lag of up to 1 s in the release of protons at the end of illumination. These data suggest the presence of an intermediate pool between electron flow and proton movement.

5. One of the major components of the ethyl red response in buffered suspensions is a band shift, which is thought to represent a change in the position of ethyl red within the membrane resulting from illumination. These responses seem related to the state of coupling of the photosynthetic membrane and could serve as a sensitive indicator thereof.

6. The time relationship between ethyl red and bromocresol purple responses shows that changes in the membrane (ethyl red response) continue after the establishment of the steady state pH shift in the light.

7. Bromocresol purple and ethyl red offer promise as investigative tools for events linked to energy transduction in thylakoids.

The demonstration of light-induced pH changes in unbuffered suspensions of isolated chloroplasts (1, 2), and the apparent relation of this phenomenon to the presence of a high energy intermediate (3, 4), has promoted intensive study of hydrogen ion fluxes in chloroplasts, bacterial chromatophores, and mitochondria. Interest has been heightened by the realization that an understanding of these phenomena is central to determining whether energy transduction in membrane systems occurs by a chemical (5) or a chemi-osmotic (6) mechanism.

Two general methods have been used for the determination of pH changes. In the earliest experiments with chloroplasts, a glass membrane pH electrode was immersed in a stirred suspension and the pH of the medium was monitored by a conventional pH meter. The inherently slow response of this arrangement prohibited study of the initial kinetics of the pH rise at the onset of illumination. Iwao and Hind (7), by use of a constant flow apparatus, showed that the initial rate of the pH rise was faster than had been suspected. More recently, Schwartz (8) has used improved circuitry and a special electrode to study the kinetics with still shorter time scales, possibly to the limit set by charge migration rates across the glass membrane.

An alternative experimental approach which offers the possibility of observing rapid pH transients involves the spectrophotometric determination of absorption changes of a pH-sensitive dye. Chance and Mela (9) attempted to use bromothymol blue as an indicator of pH changes inside mitochondria. However, studies of Mitchell et al. (10), Antonini et al. (11), and Cost and Frenkel (12) suggest strongly that color changes of this indicator are partially due to its redistribution between the solution and the biological membrane, induced by subtle conformational responses to differing energetic states.

Jackson and Crofts (13) have convincingly demonstrated that in chromatophores, bromothymol blue is most responsive to changes in the energetic state of the membranes and only registers internal or external pH changes to a minor degree. However, another dye, bromocresol purple, was largely excluded by the chromatophores and so gave a reliable estimate of pH changes in the external medium. Chance and Mela (9) had earlier shown...
that BCP responds to external pH changes in mitochondrial suspensions.

An external pH indicator for use in suspensions of higher plant chloroplasts must meet the following requirements: it must be (a) weakly bound or not bound; (b) unaltered by oxidation-reduction potentials in the physiological range; (c) unable to inhibit chloroplast reactions; (d) spectrophotometrically measurable with high differential extinction coefficient; and (e) must be most sensitive in the region of largest pH change (pH 6.0 to 7.0). In addition, the wave length at which the indicator is monitored should be in a region where the over-all absorbance of the sample is low, and where major absorbance changes in endogenous pigments are absent.

In a survey of possible indicators of pH changes in chloroplast suspensions, two compounds seemed worthy of special attention, ethyl red (ER) \(^a\) and BCP. Of the other indicators already used in chloroplast studies, bromothymol blue (14) suffers from the same uncertainties of interpretation as in other organisms; while neutral red (15) apparently undergoes color changes caused by a reduction-oxidation reaction.

### METHODS

**Chloroplast Preparations**—Chloroplasts were isolated from locally grown spinach. The de-ribbed leaves were washed and then chopped for 10 s in a Waring Blender in an unbuffered solution of 0.2 M sucrose, 0.03 M NaCl, and 5 mM MgCl\(_2\). This solution (pH ~6) was filtered through four layers of cheese cloth and centrifuged at 2500 \(\times\) g for 5 min. The resulting pellet was resuspended in 0.05 M NaCl by gentle agitation. The pellet obtained by recentrifuging was resuspended in 0.8 M sucrose and 0.03 M NaCl.

**Assays**—Oxygen uptake was assayed with a Clark-type oxygen electrode at 20\(^\circ\)C, with actinic illumination by broad band red light (600 to 700 nm) at an intensity of 70 kergs cm\(^{-2}\) s\(^{-1}\).

Changes in pH were measured by a glass combination electrode, type 4858-L60 (A. H. Thomas Co.) connected to a Beckman pH meter. The solution was maintained at 15\(^\circ\)C and the actinic illumination (600 to 700 nm) was at an intensity of 200 kergs cm\(^{-2}\) s\(^{-1}\).

### RESULTS

**Absorbance Changes of Indicators**—Movement of protons by chloroplasts induces an absorbance change in BCP which can be monitored by a double beam spectrophotometer, as in Fig. 1. The wave lengths of reference and measuring beams were selected so that the light scattering is minimal (17). The pH change induced by illumination with actinic light, which corresponds to an uptake of protons from the suspending medium (tested by adding NaOH). The absorbance becomes stationary in about 5 s under saturating illumination. When the light is extinguished, protons are released from the chloroplasts and the absorbance declines to about the initial value. The dark reversion rate is relatively slow; therefore, the recorder speed was changed to dashed line. This on-off cycle can be repeated several times (not shown here). When the BCP is left out of the reaction mixture (Fig. 1B), only a small light scattering response is observed, in the opposite direction (decreasing absorbance) from the +BCP response. An added buffer (5 mM MES at pH 6.0) eliminates nearly all of the light scattering change and totally eliminates the BCP response (Fig. 1C). This shows that BCP is in a buffer-permeable space.

Changes in pH and the absorbance of ER induced by illumination of a suspension of chloroplasts are shown in Fig. 2. The ER kinetics differ from that observed with BCP. Upon illumination with no buffer present the external pH rises in the usual way (Fig. 2A), and this pH rise is eliminated by the addition of a small amount of buffer (Fig. 2A). Unlike the BCP response, the ER response is obviously biphasic in that there

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### Table I

**Dependence of BCP binding on BCP and chlorophyll concentrations**

<table>
<thead>
<tr>
<th>Chlorophyll, mg/ml</th>
<th>BCP, % bound</th>
<th>2/(a)</th>
<th>25</th>
<th>82</th>
<th>109</th>
<th>130</th>
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<tr>
<td>0.00</td>
<td>6.8</td>
<td>3.0</td>
<td>4.5</td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>22.0</td>
<td>15.6</td>
<td>15.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.05</td>
<td>0.66</td>
<td>0.70</td>
<td>0.50</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(a\) [BCP] = 8 \(\mu\)M.

\(b\) [chlorophyll] = 143 \(mg\) per ml.
is a rapid decrease in absorbance followed by a slower increase (Fig. 2B). When the light is extinguished, an initial slight absorbance decrease is followed by an increase and finally by a slow decrease to the initial level. The controls without ER, Fig. 2C and CC, show only small light-scattering changes. With a buffer present (Fig. 2BB), the ER response is similar to the BCP response, but in the opposite direction. Here the actinic light causes a rapid absorbance decrease, which becomes slower until a steady state level is reached. When the light is extinguished, a slight further decrease in absorbance is followed by a slow increase to the original initial level. Since one of the components of the ER response is not sensitive to external buffering, the unbuffered ER response must be a composite function of this component and of a direct response to the change in external pH.

**Binding of Indicators**—Table I shows the relation between bound and free BCP over a range of BCP and chlorophyll concentrations. The results are consistent with a fixed number of binding sites per chloroplast, per cent of BCP bound is not proportional to chlorophyll added. If the chlorophyll concentration is fixed and the concentration of BCP varied, it can be shown from a double reciprocal plot (data in Table I) that at saturation, occupancy of the membrane is about $10^{-3}$ moles of BCP per mole of chlorophyll.

The binding of BCP markedly increases below pH 6.0 (Table II) but there is little change above this (2 to 3%), and the light-induced pH rise as studied here is not accompanied by significant release of BCP from the membrane.

In contrast to BCP, ER carries a positive charge in the basic and two positive charges in the acidic form, and is much more tightly bound by thylakoid membranes. The amount of ER bound increases with both chlorophyll and ER concentration. Fig. 3 is a Klotz plot (18) from which can be derived the average number of chlorophyll molecules per ER binding site ($\text{intercept on ordinate, } = 7 \pm 1$) and the binding constant ($\text{negative intercept on abscissa, } = 21 \pm 4 \text{ pm}$) by a least squares fit.

**Effects of Binding on Ethyl Red**—Jackson and Crofts (13) observed that the pKₐ of bromothymol blue was shifted from 7.1 to 7.9 upon being bound by chromatophores. Similarly, the pKₐ of ER shifts from 5.70 ± 0.04 to about 5.57 ± 0.05 in the presence of chloroplasts. Since less than one-third of the ER would be bound under these conditions, the true shift experienced by the bound ER is presumably larger. The pKₐ of ER shifts in an opposite direction from that of bromothymol blue because the most strongly charged forms of each have opposite signs, (bromothymol blue)⁻ and ER⁺⁺.

Fig. 4 shows the effect of binding by chloroplasts upon the visible spectrum of ER at pH 8.0. This relatively high pH was used in order to convert nearly all of the ER to the basic form, to increase the binding (see Fig. 6) and to minimize the light scattering (17). A 7% decrease in extinction coefficient results from binding, but of more importance is the peak shift from 556 to 561 nm (compare Traces B and A). Curve C is the

![Fig. 1. Absorbance changes resulting from illumination of chloroplast suspensions containing BCP. Conditions as set out under "Methods." MES buffer (where present, Trace C) 5 mm, pH 6.0. Chart speed changed at dashed vertical line.](image)

![Fig. 2. Absorbance and pH changes resulting from illumination of chloroplast suspensions containing ER. Conditions as under "Methods." MES buffer (where present, Traces AA, BB, CC) 10 mm, pH 5.5. Changes in pH (Traces A and AA) measured as described under "Methods."](image)

<table>
<thead>
<tr>
<th>pH</th>
<th>5.20</th>
<th>5.59</th>
<th>5.90</th>
<th>6.36</th>
<th>6.69</th>
<th>6.90</th>
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<td>BCP, % bound</td>
<td>30</td>
<td>17</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
spectrum of ER in the apolar solvent, dimethylformamide, which shows increased extinction but essentially the same bathochromic shift. This indicates that bound ER occupies a region of low solvent polarity, and thus must dissolve in the membrane. Since ER is charged in acid and basic forms and lacks long side chains, it is unlikely to penetrate more than a few angstroms into the membrane. However, as Chance et al. (19) have demonstrated for mitochondria, it is this zone which best indicates the energy coupling status of the organelle.

It is evident from the traces in Fig. 4 that the peak shift which results from the binding of ER will yield a difference spectrum with a crossover point in the neighborhood of 560 to 565 nm. Such a difference spectrum is shown in Fig. 5 (trace marked (-CHL) - (+CHL)) and corresponds well with the curve predicted from Fig. 4.

Interpretation of Light-induced ER Absorbance Shifts—The light-induced absorbance decrease of buffered ER samples (see Trace BB of Fig. 2), measured at 575 - 490 nm in the dual wave length spectrophotometer, could represent either (a) a pH decrease in a compartment accessible to ER but not to buffer, such as perhaps the internal aqueous phase of the thylakoid; or (b) a change in the quality or quantity of ER binding by the membranes. However, absorbance changes in ER which register pH shifts will have the same polarity in differential spectrophotometry regardless of the measuring wave length. An absorbance change which is due to a binding shift will change its sign between 540 and 580 nm.

In differential spectrophotometry, obviously this is only valid for the wave length range in which \( A_1 - A_{ref} \) has a constant sign. For ER and \( A_{ref} = 490 \text{ nm} \), \( A_1 > A_{ref} \) only up to about 583 nm. Since ER has negligible extinction at 610 nm, this was used as reference for the \( L-D \) spectrum in Fig. 5. It is generally less favorable than 490 nm because of proximity to the actinic wave length.

Convincing evidence that part of the ER responses of Fig. 2 indeed represent binding shifts or changes in the hydrophobicity of the membrane at the binding site is given in the trace marked \( L-D \) in Fig. 5. This shows that spectral dependence of the ER response resembles closely the difference curve obtained by adding chloroplasts to ER. Illumination results in a decreased effective chloroplast concentration by reason of either a lessened apparent ability of the membranes to bind ER or a decreased membrane hydrophobicity. Two components of the light-on response can be recognized kinetically, and the spectrum shown is that of the faster component (half-rise time about 1 s). The slower component has a spectrum resembling that of ER. Further work is being carried out on both of these components.

Fig. 3. Binding of ER in suspensions of isolated chloroplasts; dependence on ER concentration. Binding measured at pH 6.0 described as under "Methods." Chlorophyll, 50 \( \mu \text{g} \) per ml.

Fig. 4. Spectra of ER in different solvents. All spectra were recorded in a Cary 14 spectrophotometer using 1-cm square cells and opal glass diffusing plates. Trace A, 8.5 \( \mu \text{M} \) ER in 5 mM Tricine N-tris(hydroxymethyl)methylglucone, pH 8.0, containing 20 \( \mu \text{g} \) per ml chlorophyll (as chloroplasts). Trace B, as Trace A but without chloroplasts. Trace C, 8.5 \( \mu \text{M} \) ER in 1 mM Tricine pH 8.0 and 97% v/v, \( N_N \)-dimethylformamide.
to isolate the pellet from the supernatant. The centrifuge was illuminated from above with white light from a projector filtered through 7 cm of a 5% CuSO₄ solution. After 15 s of illumination (or darkness) the centrifuge was switched on for 60 s, and at the end of the spin, the light was turned off. The ER content of the supernatant was estimated from the absorbance at 500 nm. In eight runs no significant differences (within 1 to 1.5% binding) could be determined between illuminated suspensions and dark controls, even though the light-induced absorbance shift at 575 to 610 nm was normal. The percentage of ER bound at pH 6.5 was 66%.

Dependence of ER Binding on pH—Binding of ER is influenced by pH in the manner shown in Fig. 6. A pH rise from 6.0 to 6.5 in the dark will result in a binding change of about +8%, to a total in excess of 50% under the conditions specified.

Interaction of Indicators with Chloroplast Reactions—The binding or the presence of an indicator may result in functional changes in the electron transport system and associated spurious absorbance shifts in the dye itself. BCP, for example, slightly inhibits the MV-supported electron flow from water (Fig. 7), but is free of uncoupling activity and of effect on the light-induced proton uptake. In the absence of MV (Fig. 7), BCP increases the ammonia-uncoupled electron flow rate apparently by serving as the terminal electron acceptor and gives half-maximal rates at a concentration of about 6 μM. BCP was also found to catalyze oxygen uptake when the indophenol dye-ascorbate couple was used as the electron source (data not shown).

Although the catalytic activity of BCP may be ignored in

**Fig. 5.** Difference spectra resulting from changes in the environment of ER. The spectrum of 8.5 μM ER was measured at pH 6.0 with and without chloroplasts present in both the sample and reference cuvette in a Cary 15 spectrophotometer equipped with opal glass diffusion plates to minimize scattering. Traces O—O and △—△ represent the measured absorbance for ER (−chloroplasts) minus the measured absorbance for ER (+chloroplasts) for two preparations of chloroplasts. Trace ▽—▽, difference spectrum obtained upon illumination of a single sample of buffered chloroplast suspension containing 8.5 μM ER in the double beam spectrophotometer. Other conditions as under "Methods" except chlorophyll 20 μg per ml and reference wave length 600 nm.

**Fig. 6.** Dependence of ER binding on pH. Conditions as under "Methods," with 20 μM ER and 10 mM MES throughout. Two determinations from different chloroplast preparations are shown.

**Fig. 7.** Effect of BCP on electron transport to MV and O₂. Chloroplasts (33 μg chlorophyll per ml) were suspended in 0.8 M sucrose, 0.03 M NaCl, 0.03 M NH₄Cl, 0.01 M MES at pH 6.0 and where shown, 133 μM MV. Electron flow monitored as net O₂ uptake by means of an oxygen electrode.
the presence of excess MV or at low concentrations of BCP, these observations suggest that BCP may be reversibly reduced, and hence exhibit light-induced absorbance shifts unrelated to changes in pH. In an aerobic sample, the size of such transients would be negligible if the oxidation-reduction potential of BCP were sufficiently negative. In fact, BCP is bleached only in the presence of a large excess of dithionite and regains color when shaken in the air; therefore, we estimate its midpoint potential to be more negative than −200 millivolts.

Fig. 1 shows that in the presence of a buffer the BCP response is eliminated; therefore, oxidation-reduction shifts do not contribute to the light-induced absorbance changes.

Effect of Uncouplers—Uncouplers should abolish those light-induced changes of BCP absorbance which result from the pH rise phenomenon (1-4). For example, ammonium ion at uncoupling concentrations (22) inhibits the BCP response and leaves only the small buffer-insensitive responses resulting from scattering changes. The extent and the initial rate of the BCP response diminish in parallel with increasing concentrations of NH₄⁺, and the dark reversion is accelerated. The BCP response is 90% suppressed with 10 mM NH₄⁺, which corresponds to 2.7 mM NH₄⁺ at pH 6.0 and 15°C.

Carbonyl cyanide 3-chlorophenyl hydrazone, an uncoupler, also suppresses the BCP response (Fig. 8). Both the initial rate and extent decline with increasing concentrations of mCl-CCP (half-inhibition at 8 μM). Fig. 8 also shows that the P518 response (which according to Witt (16) measures the electrochemical potential responsible for the proton gradient) and the BCP response decline in parallel with increasing concentrations of mCl-CCP.

Ammonia uncoupling also modifies the ER response (Fig. 9, Traces A and B), in that the extent is lowered and the dark reversion rate is increased. Unlike the situation with BCP, the initial on-response rate is nearly unchanged. The lag in the light-off transient (Fig. 2, Trace BB) is eliminated by ammonia (Fig. 9, Trace B).

Both mCl-CCP, at an uncoupling concentration, and DCMU at an inhibitory concentration, eliminate the net absorbance change due to the ER response (Fig. 9, Traces C and D). The inhibition by DCMU of both of the BCP and ER responses can be totally reversed upon inclusion of a cofactor of cyclic electron flow, 1,4-diaminodurene (data not shown). The light-induced changes in the thylakoid which are registered as ER absorbance shifts are hence related to energy transduction rather than to oxygen evolution.

Kinetics of BCP Response-Light On—The absorbance of BCP is not linearly related to the H⁺ concentration. The amount of protonated BCP (BCP·H⁺) is dependent upon the H⁺ concentration, as given by the equilibrium expression in Equation 1.

\[ K = \frac{[\text{H}^+][\text{BCP}]}{[\text{BCP} \cdot \text{H}^+]} \]  

where \( K \) is the equilibrium constant. Differences in H⁺ concentrations can be expressed with the aid of Equation 1 in terms of absorbance of BCP

\[ \Delta H^+ = -\frac{K A_1 \beta}{A_0} \frac{\Delta A}{A_0 + \Delta A} \]  

where \( \Delta H^+ \) is the net increase in H⁺ concentration, \( \beta \) is the buffer capacity of the solution (= ΔH⁺ added/ΔH⁺ observed), \( A_1 \) is the absorbance of indicator totally converted to the alkaline form, \( A_0 \) is the absorbance of the BCP present at the initial \( [\text{H}^+] \) in the basic (colored) form, and \( \Delta A \) is the net absorbance change. Only for small absorbance changes is \( \Delta A \) linearly proportional to \( \Delta H^+ \).
Fig. 10. Kinetics of the BCP light-on response. Conditions as for Fig. 1. Ordinate = \( \Delta[H^+] \) calculated at 15 s minus \( \Delta[H^+] \) calculated for times shown. The curve (O---O) was resolved graphically into two exponential components, \( \Delta \) being the faster and the slow component being the other straight line. Inset, time course of the BCP response in pulsed light. Illumination regime and other conditions are given under “Methods.” The ordinate (same scale as main figure) denotes the peak value of the \( \Delta[H^+] \) (resulting in a flash of a given duration) subtracted from \( \Delta[H^+] \) attained in 15 s of continuous light.

The determination of the kinetics of proton movement is complicated by the large buffer capacity (\( p \)) of chloroplasts (1, 2), which varies with pH and is altered by the actinic light (20). If the buffering capacity is assumed to be that titrated in the dark, the kinetics of the proton movement can be examined after converting \( \Delta A \) to \( \Delta[H^+] \) by the use of Equation 2. The time course of a BCP light-on response after correction for light scattering is plotted semilogarithmically in Fig. 10, and illustrates that the calculated proton response is comprised of two pseudo-first order components with rate constants of 0.087 s\(^{-1}\) and 0.92 s\(^{-1}\). Over 90% of the proton shift is accounted for by the faster transient.

A short lag of about 150 ms is noteworthy in the initial phase of the on-response (Fig. 10). Such a lag could result from (a) an induction process in the state of the membrane; (b) the filling of a pool of an intermediate between quantum absorption and proton uptake; or (c) the time constant of the measuring device. This last can be discounted since the over-all instrumentation time constant could be decreased to 10 ms without diminishing the lag. In an attempt to distinguish between (a) and (b) above, light pulses of varying lengths were given to a sample, and the maximum absorbance change was measured for each pulse. Fig. 11 shows a typical absorbance shift during and following a 0.1-s pulse. A marked overshoot is evident, which lasts for at least 10 times the pulse duration and has a half-rise time of 0.3 s. If the maximum change in absorbance induced by the light pulse is plotted against pulse duration, a curve is obtained (Fig. 10, inset) which is linear for short illumination times and extrapolates to zero time without evidence of a lag. This ballistic method has been used to overcome instrumental lags in pH measurement (7). In the present instance, however, it shows that a considerable pool of intermediates occurs between the quantum absorption and proton uptake reactions.

The decay of the proton gradient has been shown by studies with the glass electrode to be exponential (7, 21). Fig. 12 shows the absorbance change of BCP for 24 s after the light is cut off. If the calculated proton change is plotted semilogarithmically (Fig. 12), a straight line is obtained giving a decay rate constant of 0.026 s\(^{-1}\). Thus, the BCP response also has exponential decay kinetics, although unlike the on-response there is only one component observed in the first 30 s.

Comparison of Ethyl Red and Bromocresol Purple Response Kinetics—A comparative study of the ER and BCP responses under identical experimental conditions is not possible owing to the necessity of buffering the suspending medium in the one case (ER) and not the other. However, it is instructive to plot the ER response (+RES) versus the BCP response (-MES) with each point on the graph representing a certain time after the light has been turned on or off. These curves (Fig. 13) show a hysteretic relationship and can be divided into the following regions:
DISCUSSION

BCP can be reliably used to monitor the external pH of a chloroplast suspension by absorption spectrophotometry with certain restrictions as outlined under “Results.” Umbelliferone, which undergoes pH-dependent changes in fluorescence yield, has been used by Grünhagen and Witt (22) for the same purpose. These indicators permit measurement of the initial kinetics of the light-induced pH shift at very short reaction times, and overcome the limitations set by slowly responding glass electrodes and electrometers. We have not attempted in this paper to report on the early kinetics of the pH rise phenomenon, but certain conclusions may nonetheless be drawn.

The bi-exponential kinetics (t1 = 0.75 s and 8.0 s) of the BCP on-response (Fig. 10) recalls those previously described for the pH rise by Izawa and Hind (t1 = <0.05 s and 10.0 s) (7), and the flash-induced pH rise (rise time, 20 to 30 ms) of Grünhagen and Witt (22). Karlish and Avron (21) detected only a single exponential component (t1 = 4 to 5 s), probably because of the slow response of their measuring system.

The on-response is further characterized by an initial lag of about 70 ms, while the BCP response decreases; E, after 4 s—the ER and BCP responses are linearly related.

Light-on response: A, the initial 0.1 s—ER response is faster than the BCP response; B, from 0.2 to 3 s—ER and BCP responses are linearly related; C, after 3 s the BCP response becomes saturated while the ER response continues.

Light-off response: D, for the first 4 s—the ER response lags while the BCP response decreases; E, after 4 s—the ER and BCP responses are linearly related.

Fig. 13. Comparison of the time courses of the ER and BCP responses. Chloroplasts suspended as under “Methods” with 10 mM MES buffer pH 6.0 present only in ER samples. Each point represents a certain time after the light is turned on (O——O) or off ( ●—●). Total illumination time, 15 s, total dark time 60 s.

Light-on response: A, the initial 0.1 s—ER response is faster than the BCP response; B, from 0.2 to 3 s—ER and BCP responses are linearly related; C, after 3 s the BCP response becomes saturated while the ER response continues.

Light-off response: D, for the first 4 s—the ER response lags while the BCP response decreases; E, after 4 s—the ER and BCP responses are linearly related.

In this paper, the authors report on the early kinetics of the pH rise phenomenon, but certain conclusions may nonetheless be drawn.
and the establishment of a pH gradient are associated with an increased or decreased hydrophobicity is not known.

If changes in the loading of the membrane by ER account for the ER response (possibility (b) noted above) it follows that the energized condition gives decreased ER binding, presumably due to decreased fixed negative charge density on the surface. Nobel and Mel (28) reported a light-induced increase in fixed negative charge at pH 8, but it is difficult to assess from their data what might occur at pH 6.0 under the conditions of our experiments with ER.

In any event, the inability to observe changes in percentage of ER binding upon illumination (see text) can only mean that the major absorbance changes (see Figs. 2 and 5) result from alteration in the environment of ER which is already bound, and that the ER loading of the membrane is virtually unaltered.

The temporal relationship between the BCP and ER responses shown in Fig. 13 (Section C) indicates that changes in the membrane (ER response) continue after the steady state pH shift has been established. These slower events have the same kinetics, relative to the pH shift, as the light-scattering responses observed previously (17), and are probably related to gross changes in thylakoid conformation. They may also be correlated with changes in membrane buffering capacity (20).

In conclusion, ER offers promise as an exogenous indicator of energization in the chloroplast thylakoid membranes. It has a significant advantage over fluorescence quenching studies on endogenous chlorophyll in that a wider range of experimental conditions and tests can be applied. BCP should find use as an external indicator of the pH of a chloroplast suspension for rapid kinetic studies, and as a means of comparing the ER response with the pH shift under equivalent experimental conditions.

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