Effect of Hydrogen Ion Concentration on the Absorption Spectrum and Fluorescence Life Time of Chloroplasts

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Subjecting pea chloroplasts to hydrogen ion gradients results in small but significant changes in the absorption and circular dichroic spectra. The relative intensities of the fast and slow fluorescence decays are modified by the hydrogen ion concentration. The life time of the slow components is only slightly altered.

At pH 11 the circular dichroic peak at 690 nm, assigned to Photosystem I chlorophyll-protein complex, is clearly resolved. Also, there is a splitting of the peak at 650–640 nm, attributed to Photosystem II chlorophyll-protein. At pH 3.9 splitting of the 650–640 nm peak is observed. There is also a positive component at 705 nm which may be associated with an aggregated form of chlorophyll or pheophytin.

The changes in absorption spectra are determined by measuring the difference in spectra between identical chloroplasts suspensions one at pH 7.6 and the other at alkaline or acidic pH. At pH 2 and pH 3.7 there are time dependent increases in absorbance at 430, 445, 520, 538, 670 nm, and a decrease in absorbance at 606 nm. After a delay of 11 min at pH 2, there is also an increase in absorbance at 700 nm. The latter is interpreted as possible formation of aggregated pheophytin. At pH 11.0 there are time dependent increases in absorbance at 638, 454 and 440 nm, and decreases in absorbance at 675, 660, 508 and 472 nm. The changes in absorbance of the pigment around 520 nm (associated with an electrochromic effect) is interpreted as resulting from a leakage of hydrogen ion across the thylakoid membrane. The spectral changes that occur during the first 9 min are reversible. After the chloroplasts have incubated for more than 15 min most of the spectral changes are not reversible.

Introduction

The concentration of ions in chloroplasts has a profound effect on the ultrastructure, fluorescence and phosphorylation. A hydrogen ion concentration gradient is one of the important factors required for phosphorylation. Jagendorf and Uribe [1] have reported that high energy phosphates can be formed by incubating chloroplasts at pH 3.8, then transferring them to a higher pH (8.3). There was no phosphorylation when chloroplasts were subjected to only pH 5. Barber [2] discussed the effects of high concentration of monovalent ions on thylakoid stacking. Changes in the absorption and emission spectra and in the lifetime of fluorescence of chloroplasts, induced by the ionic effects of salts have been observed [3, 4].

The present work describes the effect of different hydrogen ion concentrations on the absorption properties of chloroplasts. It is found that acid or alkaline treatment of chloroplasts results in small but significant modifications in absorption.

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Materials and Methods

Chloroplasts were prepared from pea leaves as described by Nakatani and Barber [5]. Pea leaves were ground in cold medium then filtered through 10 layers of muslin and a layer of cotton wool. The grinding medium was 0.33 M sorbitol, 0.2 mM $MgCl_2$, 20 mM Mes (2-(N-morpholino)-ethanesulfonic acid) adjusted to pH 6.5 with a few drops of 2 M Tris (tris-(hydroxymethyl)-aminomethane). The filtrate was centrifuged at $2600 \times g$ for 30 sec. The supernatant was centrifuged at $5900 \times g$ for 5 min. The pellet was resuspended in low salt buffer (LSB): 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM NaCl, 50 mM Hepes (N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid) adjusted to pH 7.6 with KOH.

Sonicated chloroplasts were used to minimize scattering artifacts and to essential eliminate the problem of chloroplasts settling in the spectral cuvettes during the rather long experiments. That settling

of chloroplasts had no effect on the spectrum was demonstrated by shaking the cuvettes to resuspend any chloroplasts which might have settled during an experiment; it was observed that the shaking had no effect on the spectrum. A change in index of refraction could slightly change the scattering properties of the chloroplasts [6]. Scattering effects are minimized by the use of an opal glass plates immediately after the sample and reference cuvettes, between the cuvettes and the photodector.

Difference spectra were measured with an Aminco DW4 recording spectrophotometer. Two identical 3 ml samples were prepared with an absorbance of 1.4 for the red absorption band. One, at pH 7.6, was used in the reference beam. The pH of the second cuvette was varied and used in the sample beam. The pH was varied by adding 5 µl of concentrated acid or base to the sample cuvette. An equal volume of LSB was added to the reference beam to compensate for any dilution effect. The pH was determined with a standard glass electrode. The difference in absorbance between the two cuvettes at different pH's is indicated as (pH 2.0 − pH 7.6) or (pH 11.0 − pH 7.6).

The instrument used to resolve picosecond fluorescence was described in detail by Tredwell *et al.* [7] and Porter *et al.* [8]. A single 6 ps pulse, wavelength 530 nm, was used to excite fluorescence. Fluorescence from the sample passed through a sharp red cut-off filter into an Imacon streak camera (Model 600, John Hadland P. I. Ltd.). The streak rate was 20 ps/channel. Excitation intensity was less than 2×10^{14} photons cm^{-2} to avoid exciton annihilation. Six to nine fluorescence decays were averaged for each experiment. The fluorescence data were deconvoluted and analyzed by finding the best fit to the expression $y(t) = A_1 \exp\text{-}t/\tau_1 + A_2 \exp\text{-}t/\tau_2$.

Results

Acid conditions: The absorption spectrum of the chloroplast suspension used for this study is shown in Fig. 1. The difference spectrum, (pH 2.0 − pH 7.6), 3 min after shifting the pH from 7.6 to 2.0 is shown in Fig. 1 as a dotted curve. A time dependent change in the difference spectrum is also observed (see Fig. 1). There appear to be isobestic points at 563 and 480 nm. Small shifts in wavelengths are observed for some of the maxima and minima. The main spectral changes are decreases in absorbance

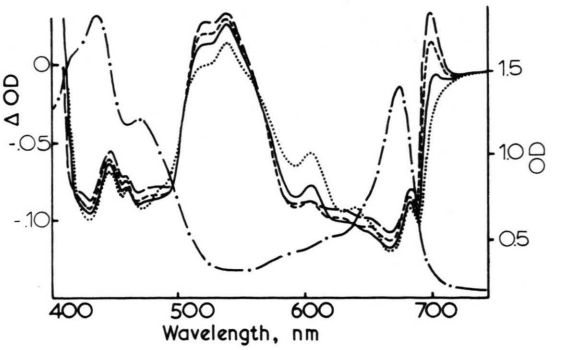

Fig. 1. Difference in absorbance (Δ O.D.) between sonicated pea chloroplast suspensions at different pH's. The difference spectrum (pH 2.0 − pH 7.6) 3 min after the pH of the sample is shifted from pH 7.6 to 2.0 is shown by a dotted line (. . . .); the spectrum at 13 min is shown by a solid line; the spectrum at 16 min is shown by short dashes (- - - -); the spectrum at 25 min is shown by long dashes (— —). The absorption spectrum of pea chloroplasts is shown by alternate dashes and dots (— · — ·); the absorbance is given on the right hand vertical axis.

with peaks at 689, 670, 472 and 436 nm an increase in absorbance around 520 nm. The time dependent increases in absorption at 520 and 538 nm are similar to each other; saturation is reached after 30 min.

The most significant time-dependent spectral changes are shown in Fig. 2. The time-dependent spectral changes fall into several groups. Parallel time dependent increases in absorbance are observed for the peaks at 670, 689, 445 and 430 nm. The increase in absorbance at 472 nm also parallels the latter wavelengths after 15 min; during the first 10 min there is a decrease in absorbance at 472 nm. Thus the change in absorbance at 472 nm, is composed of at least two spectrally different pigments.

Semi-logrithmic plots of the 430, 445, 520, 606, 670, 689 and 700 nm data shown in Fig. 2 yield linear relationships. The most rapid spectral change is at 606 nm with a half time of 5 min. The spectral changes at 520 and 538 nm have a half time of 10 min; the changes at 689 and 670 nm have a half time of 21 min; the changes at 445 and 430 nm have a half time of 27 min.

The time dependent increase at 700 nm is different from the other spectral changes mentioned above. There is an 11 min delay in the onset of change at 700 nm. After the incubation period the absorbance at 700 nm increases with a half time of 6 min; The maximum change in absorbance is about

0.1 compared to a maximum red absorbance of 1.5, the maximum change observed in these experiments is 6%.

Similar, but smaller, spectral changes are observed with chloroplasts at pH 3.7. With the sample at pH 3.7 the increase in absorbtion at 700 nm is not observed (in contrast to the sample at pH 2.0). Even after incubating for 35 min at pH 3.7, no increase in absorption at 700 nm is detected.

When chloroplasts are subjected to acid (or alkaline) conditions for only 9 min, then returned to pH 7 there is a time dependent reversal of most of the spectral changes. On the other, when chloroplasts are subjected to acid (or alkaline) conditions for more than 15 min, most of the spectral changes are not reversible only the spectral change at around 520 nm is reversible.

For comparison purposes the effect of acid on chlorophyll a in 80% aqueous acetone solution was tested. Upon addition of acid to the solution to give an indicated pH of 3.5 the spectrum of chlorophyll was completely modified to that of pheophytin in less than a minute.

Alkaline conditions: The difference spectrum between a chloroplast suspension at pH 11.0 and

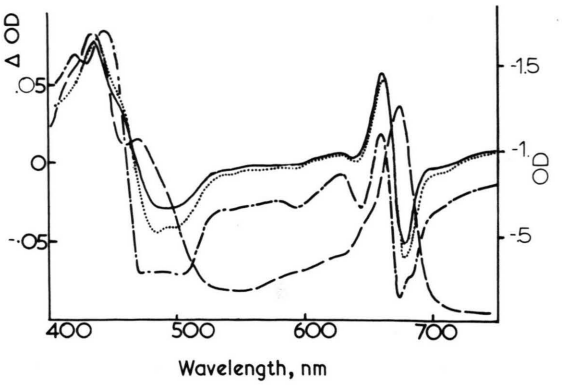

Fig. 3. Difference in absorbance between a sonicated pea chloroplast suspension at pH 11.0 and at pH 7.6. The difference spectrum 2 min after shifting the pH of the sample from 7.6 to 11.0 is shown by a solid line; the spectrum at 4 min is shown by a dotted line (. . . .); the spectrum at 30 min is shown by long and short dashes (— — —). The absorption spectrum of the chloroplasts is shown by long dashes (— —).

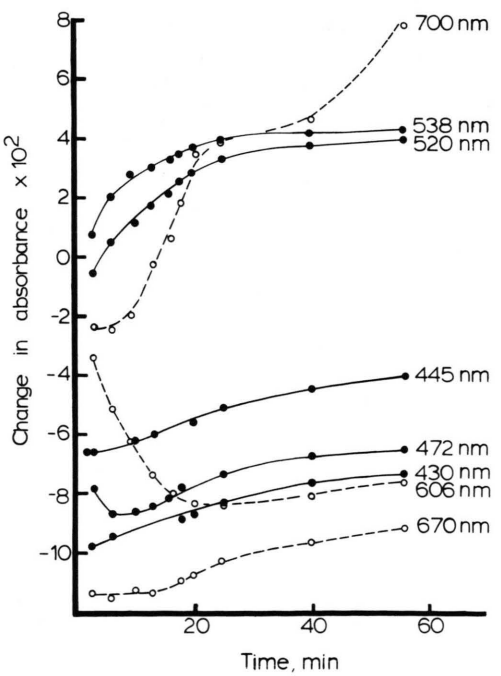

Fig. 2. Time dependent change in the difference in absorbance between sonicated chloroplasts at pH 7.6 and pH 2.0. The wavelength for each curve is indicated.

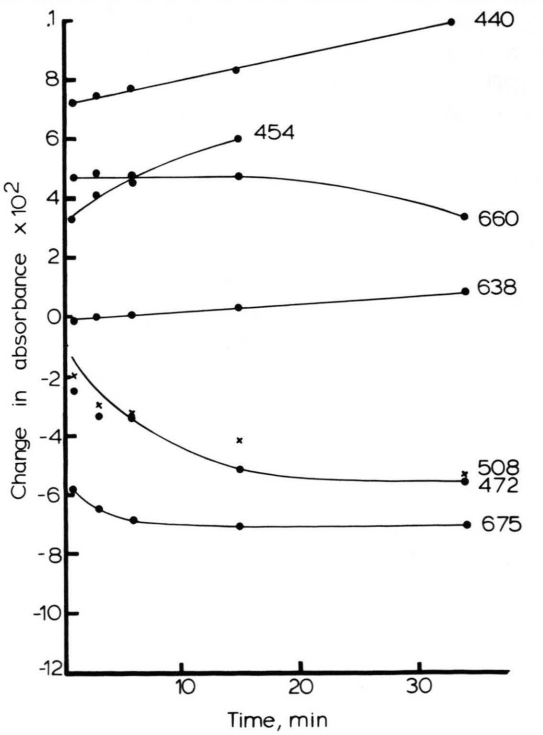

Fig. 4. Time dependent change in the difference in absorbance between chloroplasts at pH 7.6 and pH 11.0. The wavelength for each curve is indicated.

pH 7.0 is shown in Fig. 3. There are increases in absorption at 436, 638 and 660 nm, plus decreases in absorption at 472, 508 and 675 nm. After about 30 min the band at 676 nm is resolved into two bands at 674 and 683 nm; after 60 min the two bands are at 671 and 688 nm. There appears to be an isobestic point at 464 nm. The spectral maxima shift from 436 to 444 and from 662 to 659 nm. The time dependent change in absorbance at some wavelengths is shown in Fig. 4. The time dependent spectral changes of chloroplasts in alkaline conditions fall into several groups. In Fig. 4 it can be seen that the spectral changes at 440 and 638 nm are parallel to each other. The spectral changes at 454, 660 and 675 nm are neither parallel to each other nor to the other spectral changes mentioned above.

Fluorescence life times: Chloroplasts used for the fluorescence life time measurements were in the dark adapted (F_0) state. The fluorescence life times of the slow component at pH 3.0 and 10.0 are 420 ± 10 and 560 ± 10 ps, respectively (Fig. 5). These differences in life times, for "open traps", are small. The main effect of pH seems to be a change in the relative intensities (A_1 and A_2) of the fast and slow fluorescence components (see Table I). In the presence of 10^{-4} M DCMU (3-(3,4-dichlorophenyl)-1,1 dimethylurea) the fluorescence life times for the "closed traps" (F_m) are considerably longer. At pH 3.0 and 10.0 the life times are 900 ± 30 and 840 ± 20 ps, respectively (Fig. 6). Wong *et al.* (1980) measured fluorescence life times for the "P" level in chloroplasts over the pH range 5 to 9. They reported that the life times for the "P" level varied from 1 to 2 ns.

Table I. Fluorescence life times. Fluorescence data were analyzed by finding the best fit to the equation $y(t) = A_1 \exp\text{-}t/\tau_1 + A_2 \exp\text{-}t/\tau_2$.

pH	DCMU	A_1	τ_1, ps	A_2	τ_2, ps
3	–	.079	400 ± 10	.800	15 ± 1
10	–	.030	560 ± 20	.130	115 ± 10
3	yes	.038	918 ± 32	.102	246 ± 10
10	yes	.078	840 ± 20	.902	14 ± 1

Circular dichroism: Circular dichroism of sonicated chloroplasts were measured at three different pH's (*i.e.*, pH 3.9, 7.0, and 11.0, see Fig. 7). At pH 11 the structural resolution is improved. One can clearly resolve the peak assigned to Photosystem I chlorophyll-protein complex at 690 nm; at pH 7 this only appears as a shoulder. At pH 11, there is also a splitting of the peak at 650–640 nm attributed to Photosystem II chlorophyll-protein. The improvement in resolution might arise from a shrinkage of the main peak at 680 nm.

At pH 3.9 a splitting of the peak at 650–640 nm is also observed. The positive component at 665 nm disappears. A clearly defined positive component appears at 705 nm, which might be associated with

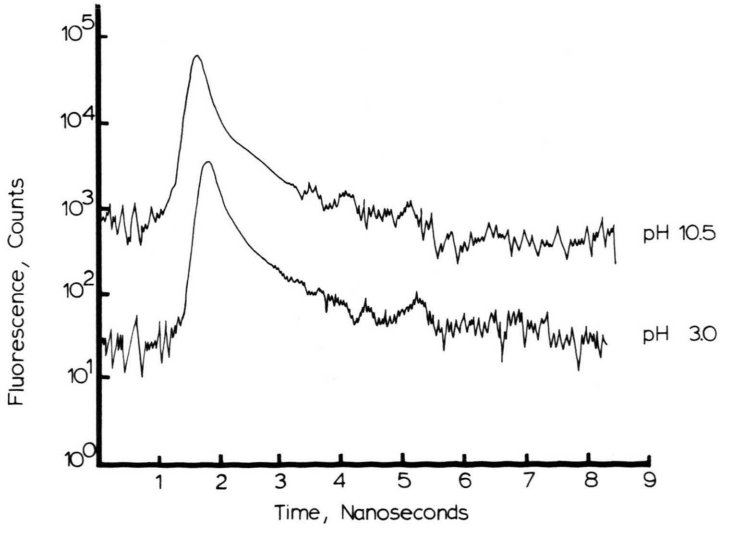

Fig. 5. The time dependent emission from pea chloroplasts at pH 3.0 and pH 11.0.

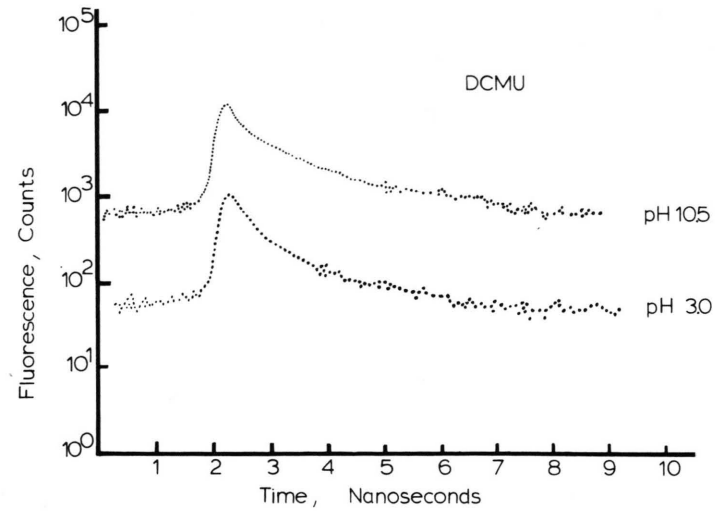

Fig. 6. The time dependent emission from pea chloroplasts treated with 10^{-4} M DCMU, at pH 3.0 and pH 10.5.

P 700 or an aggregated form of chlorophyll or pheophytin. This peak is probably related to the 700 absorbance band shown in Fig. 1.

Discussion

The spectral changes are complicated by the possibility that more than one phenomena is occurring simultaneously. Since the time dependent changes in absorbance for the peaks at 670, 689, 445 and 430 nm are rather parallel to each other (689 nm is not shown in Fig. 2), it appears that these maxima may be associated with a single species. The spectral changes at 520 and 538 nm are parallel to one another and not parallel to the changes at the wavelengths listed above. Therefore, it appears that the changes at 520 and 538 nm arise from a single species, which is different from the "670, 689, 445, 430" nm pigment. Since the time dependent changes at 700 and 606 nm are different from each other as well as the other spectral changes shown in Fig. 2, it appears that as many as 4 different pigments systems might be modified by the acid conditions.

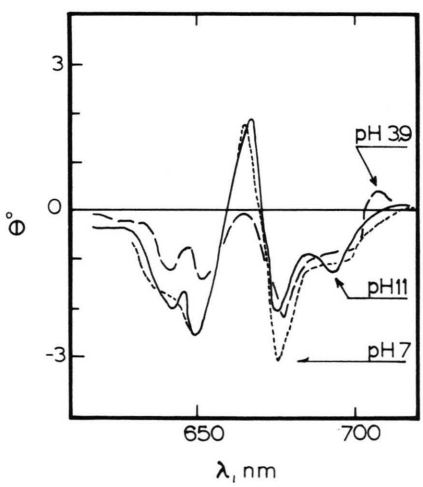

Fig. 7. Circular dichroic spectrum of sonicated pea chloroplasts at pH 11.0 is shown by a solid line; the spectrum at pH 7.0 is shown by a dotted line (. . . .); the spectrum at pH 3.9 is shown by a dashed line (- - - -).

The spectral change at 700 nm (Fig. 1) might be associated with reaction center P 700 or a pheophytinization of an aggregated form of chlorophyll (*i. e.* resulting in the formation of aggregated pheophytin). It is interesting to consider the possibility that an aggregated form of chlorophyll is converted into pheophytin. While aggregation of chlorophyll a results in rather small bathochromic shifts [9, 10] aggregation of pheophytin a in a compressed monomolecular film results in a large spectral shift to give an absorption maximum at about 705 nm [11, 12]. If there is a conversion of some aggregated chlorophyll into aggregated pheophytin, characteristic spectral changes are to be expected. One would expect decreases in absorption at about 685 and 430 nm as the concentration of aggregated chlorophyll is decreased; accompanied by increases in absorption at about 471, 505, 534 and 615 nm, characteristic of the minor absorption bands of pheophytin. Indeed, such increases and decreases in the spectra are observed (Fig. 1).

With chlorophyll in acetone solution we observe that the addition of acid "immediately" produces large spectral changes at all wavelengths. Pheophytin in ethyl ether has characteristic minor absorption maxima at 471, 505, 534 and 615 nm. The spectral changes observed in Fig. 1 seem to have small maxima in the vicinity of these characteristic wavelengths. In diethyl ether the red absorption maximum of pheophytin a is at a longer (5 nm) wavelength than the maximum of chlorophyll a, while the blue absorption maximum of the pheophytin is at a shorter (22 nm) wavelength than chlorophyll a. If the spectral properties of pheophytin *in vivo* bear the same relationship to chlorophyll a as in solution, then formation of pheophytin in pea chloroplasts would result in increases in absorption at about 414 and 690 nm. Such increases are not observed in the chloroplasts, (Fig. 1) thus strongly indicating that *monomeric* pheophytin is not formed by subjecting the chloroplasts to an acid environment.

If all chlorophyll were exposed to an aqueous environment, then, adjusting the pH to acid conditions would produce a large population of pheophytin. The spectral changes (Fig. 1) indicate that only a small population of chlorophyll is converted to pheophytin. Since there is so little pheophytin formed, it appears that the bulk of the chlorophyll must be protected by a nonaqueous (lipoprotein) environment or surrounded by protein as observed in the case of the bacteriochlorophyll protein crystal [13].

The 11 min delay in the onset of spectral change at 700 nm suggests there may be a sequential reaction(s) induced by the pH gradient. It appears that before this molecular species undergoes any change the membrane must first undergo some transformation. Perhaps the molecular architecture of the membrane in the "normal" state protects the 700 nm species from the aqueous environment.

In the case of the alkaline treated samples, the symmetric increase in absorption at 660 nm and decrease at 675 nm may result from a hypsochromic shift of the red absorption maximum of a species of chlorophyll. The isobestic point at 464 nm might be associated with transformations of carotene. It probably is not associated with chlorophyll since there are no bands of chlorophyll at about 480 nm.

In contrast to the relatively large spectral changes induced by acid and alkaline conditions there are rather small changes in the (F_0) chlorophyll a fluorescence life times in pea chloroplasts. There are, however, changes in the relative fluorescence contributions of the two fluorescence life times. This would suggest that, for the most part, the fluorescence centers may not be exposed to the aqueous environment. Changes in the state of aggregated chlorophyll (or pheophytin) would have no significant effect on the fluorescence life time since aggregates are weakly fluorescent at room temperature. The relative intensities and the fluorescence life times would be altered, however, if there were a change in the transfer probability from the light harvesting chlorophyll to the fluorescence centers or to an aggregated, non-fluorescent form of pheophytin or chlorophyll.

It is well established that setting up a pH gradient across a membrane can sensitize phosphorylation, presumably via the chemi-osmotic mechanism [1, 14]. Considering the photosynthetic pigments as endogenous probes it is apparent that the pH treatment results in modification of the membrane and pigments. The question arises as to what extent, if any, these modifications in the membrane, or modifications in the photosynthetic pigments, are related to the process of phosphorylation.

By subjecting chloroplasts to hydrogen ion gradients for a long period of time one may be amplifying small spectral changes that occur at specific reaction sites in the normal course of photosynthesis. Similar changes in pigment and membrane may be occuring on a smaller scale when ambient chemistry becomes rich or deficient in hydrogen ion.

The circular dichroic spectra indicate that the pH gradients do not significantly modify the molecular integrity of the chlorophyll-protein complexes. Configurational changes of the complexes could result in a loss of dichroic activity. The main effect of the hydrogen ion gradients appears to be on the ambient chemistry of the complexes resulting in spectral shifts of the optically active pigments.

Witt [15] has shown that the electrical potential difference, across the thylakoid membrane is proportional to the change in absorption at 515 nm. The change in absorption around 520 nm may be reflecting the change in electrical potential across the membrane. On the basis of the present experiments it appears that under acid conditions there is a time dependent increase in the difference in electrical potential across the thylakoid. Under alkaline conditions the opposite results. The consequence of these potential changes is the subject of further study.

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