PHOTOSYNTHETICA 8 (2): 109–117, 1974

Light-Induced pH Changes in Chloroplast Suspensions from a Yellow-green Alga

Kinetic Properties and Wavelength Dependence*

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Abstract

Chloroplasts of the yellow-green alga *Bumilleriopsis filiformis* show a light-induced pH change similar to that described for chloroplasts of higher plants with regard to kinetics, relationship to illuminance, and maximum proton uptake. Upon weak illuminance the pH of the outer medium rises and reaches a plateau; upon darkening the pH decays to the original value, apparently with first order kinetics. Under intense illuminance the pH passes through a maximum; the dark decay shows a transient undershoot. Repeated strong illuminations diminish the extent of the pH shift and accelerate its dark decay. The action spectrum for the initial rate of the pH rise resembles that for NADP reduction with water as electron donor; it is different from the action spectrum for NADP reduction with ascorbate-DCIP as electron donor in the presence of DCMU. A joint action of photosystem I and II in proton translocation is indicated.

Light-induced pH changes in chloroplasts of higher plants have become increasingly interesting over the past years especially in view of the chemiosmotic theory of photophosphorylation (see JAGENDORF and URIBE 1967; WALKER and CROFTS 1970; SCHWARTZ 1971). One goal of this investigation was to find whether isolated algal chloroplasts are suitable for studying this aspect of photosynthesis. Algae can easily be grown under controlled conditions, so that seasonal variations should not affect the results. The organism used in this work is the yellow-green alga *Bumilleriopsis*. Its chloroplasts catalyze light-dependent phosphorylation and Hill reactions at rates comparable to those obtained with spinach (BÖGER 1969a,b). The alga consists of short filaments with up to seven chloroplasts in each cell.

Since the observed pH changes seemed basically similar to those described for chloroplasts of higher plants, a more general question could be raised: Which pigment system is driving the proton pump? Action spectra have been reported indicating a predominant participation of pigment system I in proton translocation (DILLEY 1967; HEATH 1972); furthermore quantum yield determinations corroborated these findings (DILLEY and VERNON 1967; HEATH 1972). On the other hand, evidence has been presented for the involvement of both light reactions (SCHLIEPHAKE *et al.* 1968). A knowledge of the site of the proton pump and of the pigment system(s) sensitizing it gains further relevance if a proton gradient should really be driving photophosphorylation as suggested originally by NEUMANN and JAGENDORF (1964).

* *Received* 20 September 1973; *accepted* 26 November 1973.

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MATERIAL AND METHODS

Bumilleriopsis filiformis was grown as described by BÖGER (1969a), with the omission of vitamins in the growth medium. Chloroplasts were isolated essentially according to his method.

For measurements of pH changes the chloroplasts were suspended in a medium containing 0.35 M sucrose, 10^{-2} M KCl, 10^{-3} M $MgCl_2$ and 2×10^{-4} M MES buffer* pH 6.5. The chlorophyll concentration was 20 µg ml^{-1} except for action spectra measurements, where it was 15 µg. . ml^{-1}. Chlorophyll concentration was determined spectroscopically after MACKINNEY (1941). Pyocyanine was prepared according to JAGENDORF and MARGULIES (1960). The vessel used for the kinetic experiments was a horizontal transparent cylinder 2.5 cm in diameter and 1 cm long with a mirrored surface at one end. The contents of the cylinder were rapidly stirred with a propeller driven by an electromotor. The temperature was held at 20 °C. The glass electrode was plugged in from the top and the reference electrode through a side compartment which was connected to the main compartment by a 1 mm bore. Additional substances were injected through a side port. An Ingold glass electrode type *LoT 205—M5* was used in combination with a Ag/AgC-reference electrode. Differences in potential between the electrodes were amplified and displayed on a strip-chart recorder or on a *Siemens Oscillomink* recorder (band width 1 kHz). The response time of the system was tested by following the acidification accompanying the photoconversion of PMS**. For this purpose the medium normally used (see above) with 5×10^{-3} M PMS was illuminated with a xenon flash lasting 4×10^{-5} s between 50% values; the resulting pH change was followed with the measuring apparatus. 50% response was reached in the first 60 ms and 90% after about 400 ms. pH changes could be related to the number of protons fluxed by adding small amounts (0.5—1 µl) 0.02 N HCl with a metrohm microburette. The buffer capacity was constant for a range of at least 0.1 pH unit. Maximum pH changes caused by illumination of chloroplasts were around 0.05 pH units. Action spectra were measured at room temperature in a non-reflecting transparent cell 1×1 cm in cross section. This vessel was used in order to have the same geometry as in the standard 1 cm cuvette used for measuring the action spectra for NADP reduction.

For measuring NADP reduction a reaction mixture was used containing 0.35 M sucrose, 10^{-2} M KCl, 10^{-3} M $MgCl_2$, 10^{-2} M Tricine buffer pH 8.0, 5×10^{-4} M NADP, 4×10^{-6} M ferredoxin and chloroplasts yielding a final concentration of 15 µg chlorophyll ml^{-1}; if wanted, the medium contained, in addition, 2×10^{-6} M DCMU, 10^{-5} M DCIP and 4×10^{-2} M ascorbic acid as an artificial electron donor system. For the preparation of ferredoxin the procedure of BÖGER (1969b) was followed through the first two purification steps on diethylaminoethyl cellulose, then the ferredoxin containing eluate was dialyzed against 0.01 M Tricine pH 8.0 and concentrated by ultrafiltration. The concentration of ferredoxin was determined spectroscopically at 420 nm using a molar extinction coefficient of 10 000 cm^{-1} M^{-1} (BÖGER 1969b). NADP reduction was followed spectroscopically as the increase in absorption at 340 nm. The measuring beam traversed the 3 cm length (top to bottom) of a 1×1 cm cuvette and fell on a *Maurer Vp 12 bK* photomultiplier protected from actinic stray light by a *BG 12* filter (*Schott*).

A slide projector with an appropriate lens system served as the light source. Heat was removed by an infrared-reflecting filter and a 6 cm layer of water. Additional filters were introduced as needed into the light path. Unless indicated otherwise, a red glass *RG 630* (*Schott*) was used for the kinetic experiments. For the action spectra a *Bausch & Lomb* 500 mm grating monochromator was used in conjunction with a 420 W tungsten lamp, a 2 cm water flushed cuvette, a lens, a heat reflecting glass *T6* and a cut-off filter *RG 590* (both from *Schott*). The slits were set for a half band width of 6.5 nm. Illuminances were measured with a calibrated silicon cell.

Acknowledgements: I am obliged to Dr. Tanner for stimulating discussions.

* *Abbreviations used*: MES, 2-(N-morpholino)ethanesulphonic acid; PMS, N-methylphenazonium methosulphate; Tricine, tris-(hydroxymethyl)-methylglycine; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

** Suggested by Dr. Schwartz.

RESULTS

Kinetics

After turning on the light the pH in the outer medium increases, reaches a plateau after several seconds and sinks to its original level upon darkening (for an example of weak illuminance effects see Fig. 1, top). The decay follows apparently first order kinetics with rate constants ranging between 0.05 and 0.15 s^{-1} for different chloroplast preparations. In a few cases the pH rise induced by illuminating was conspicuously biphasic with a rapid alkaline gush. Such behaviour has been noted previously (IZAWA and HIND 1967; SCHWARTZ 1968; HEATH and HIND 1972); the phenomenon was observed however, only rarely here.

Under strong illuminance (>100 W m^{-2}) the pH builds up to a maximum, then gradually declines, eventually coming to a steady state level (Fig. 1, bottom). After switching off the light,

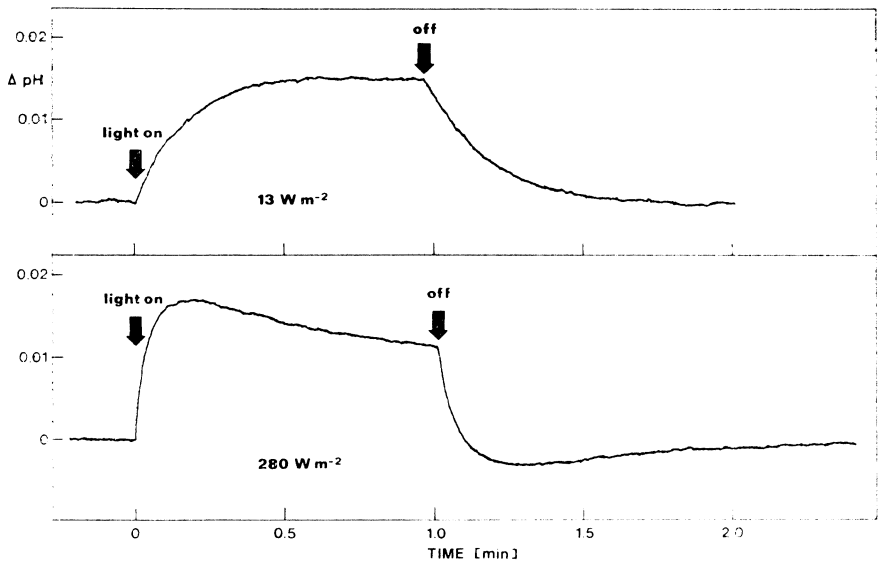

Fig. 1. Kinetics of pH changes induced by illuminating *Bumilleriopsis* chloroplasts.

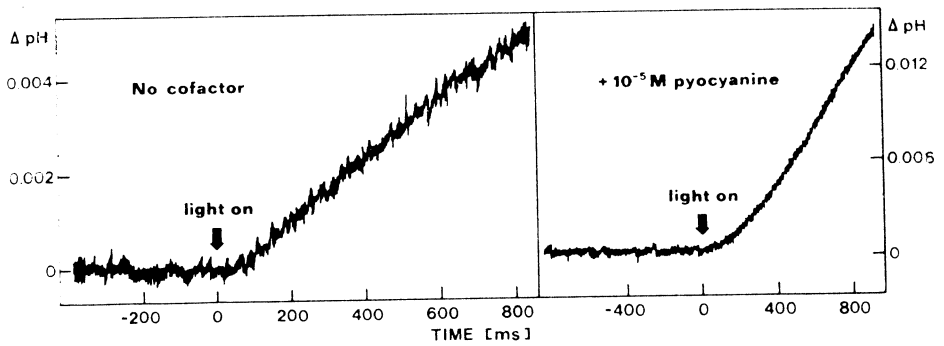

Fig. 2. Initial kinetics of light-induced pH rise at 280 W m^{-2}.

there is a decay with a significant undershoot and recovery to the original value. The decline during the light phase might be due to some photodamage to the thylakoid membrane leading to an increased permeability for protons. The data shown in Table 1 provide some support for this idea:

Table 1

Extent of proton uptake reached after 1 min (A_{1min}) and dark decay rate constant (k_d) after successive cycles of 1 min light - 2 min dark. "White light" with filter *GG 420 (Schott)*; illuminance 810 W m^{-2}.

Exposure	A_{1min}, rel.	k_d [s^{-1}]	A_{1min}, rel.	k_d [s^{-1}]
1	28	0.075	31.5	0.11
2	24	0.10		
3	19	0.175	dark	dark
4	12.5	0.19		
5	7	0.25	27.5	0.17

Several successive strong illuminances cause a considerable increase of the dark decay rate constant with a concomitant depression of the extent of the proton uptake. To exclude that the effect is due merely to the aging of the chloroplasts, in a parallel experiment only two exposures were given, corresponding in time to the first and last dark cycle. The decay rate constant rose somewhat but far less than in the case of repeated exposures.

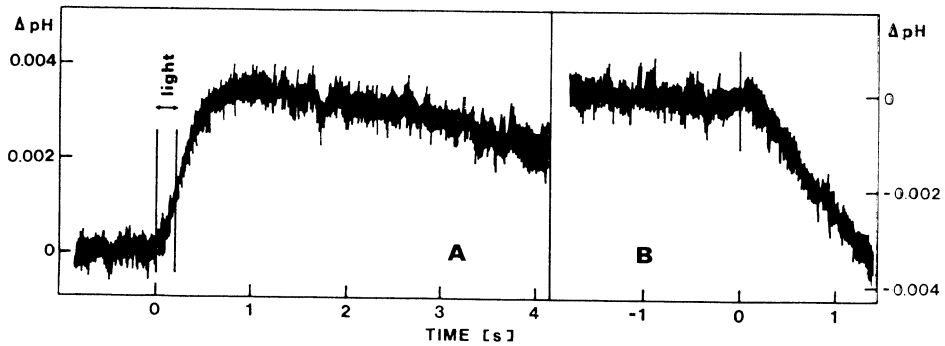

Fig. 3. *A*: pH rise following an 0.2 s illuminance with 280 W m^{-2}.
B: initial kinetics of pH decay after an 1 min exposure to 280 W m^{-2}.

The initial rate of proton influx was much less affected by strong illuminance. Interestingly enough, it could be stimulated slightly. This reminds one of the effect of Triton X-100 and chlorpromazine, which accelerate both proton influx and efflux in spinach chloroplasts (JAGENDORF and NEUMANN 1965).

The initial phase of response to illuminance (Fig. 2A) begins with a 40—80 ms lag until a steady rate of proton uptake is reached. By adding 10^{-5} M pyocyanine or methyl viologen to the reaction mixture the lag is extended to 150—200 ms and the steady rate is increased several times (Fig. 2*B*).

The kinetics of the pH response to switching off the light was recorded for two different cases. After a short illumination period of 0.2 s (Fig. 3*A*) when the pH in the medium had not yet

reached a constant value, the signal rose for 0.8 s upon darkening. After a longer exposure (Fig. 3*B*) when the pH had come to a steady state level, maximum rate of proton efflux upon darkening also appeared only with a lag of *ca.* 0.4 s.

Relation to illuminance

Different illuminances were employed 1 min each with 2 min intervals of darkness. The maximum values of ΔpH as a function of illuminance (Fig. 4) are reached after an approximately linear section saturation at about 30 W m^{-2}. Under strong illuminance ΔpH reaches its maximum value well before 1 min of illumination (*cf.* Fig. 1).

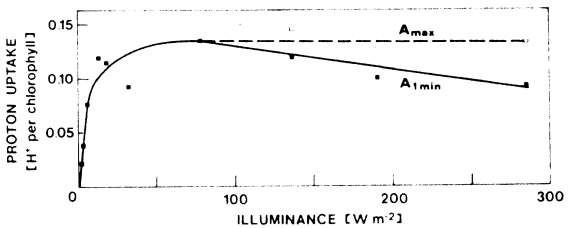

Fig. 4. Relationship of illuminance to values of ΔpH reached after 1 min (A_{1min}) and to the maximum values (A_{max}) reached in the course of a 1 min illumination period (*cf.* Fig. 1).

As the dark decay follows first order kinetics — neglecting the noticeable undershoot at strong illuminances —, the initial decay rate should be proportional to the extent of the pH shift (with the assumption of an invariable rate constant); one would therefore expect both maximum proton uptake and the dark decay rate to yield curves with the same shape when plotted against illuminance. As Fig. 5 shows, however, saturation for the decay rate is hardly reached even at strong illuminances. Obviously the values at the greater illuminances turn out to be so high because of the stimulating effect of strong illuminance on the dark decay rate (*cf.* Table 1).

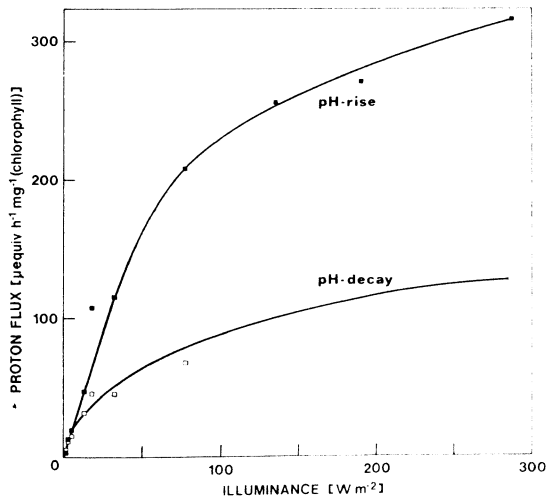

Fig. 5. Initial velocity of the light-induced pH rise and of the dark decay rate versus illuminance.

The initial rate of proton uptake increases up to considerable illuminances (Fig. 5) showing half saturation at *ca.* 50 W m^{-2}. Whereas at higher illuminances, the initial rate is more than twice the corresponding decay rate, at weaker illuminances both tend to become equal.

The maximum rates of initial proton uptake were in the range from 200—300 μequiv. h^{-1} mg^{-1} (chlorophyll), the maximum extent ranged from 0.1—0.25 H^+ per chlorophyll. By adding pyocyanine the initial rate was increased 2 to 6 times, the extent of proton uptake 1.5 to 2 times.

Action spectra

Action spectra were measured for the initial rate of proton uptake. Maximum ΔpH and dark decay rate are less direct indicators of the proton pump activity than the initial rate of proton uptake is, as they depend also on the thylakoids' buffer capacity and permeability which may vary in the course of an experiment (see above).

The illuminances were kept in a range where the rates were proportional to illuminance, *e.g.* below 1.4 W m^{-2} at 680 nm. In order to have comparable conditions for each wavelength given, a rhythm of 10 s light — 80 s dark was used; furthermore, the illuminances at the different wavelengths were adjusted so as to keep variations in rates down to a factor 3 or lower. The average from two action spectra obtained in this way (Fig. 6, full line, ■) shows a maximum around 670 nm and a minor peak around 625 nm.

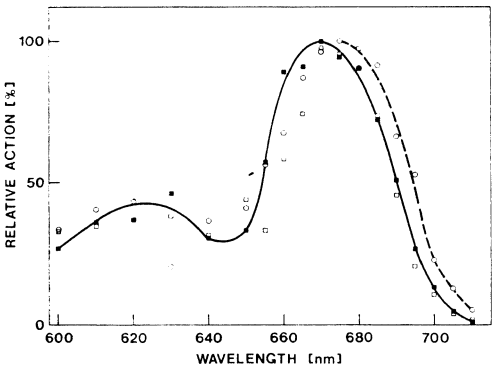

Fig. 6. Action spectra of initial rate of pH rise (■, full line), NADP reduction with water as electron donor (□) and with DCIP-ascorbate in the presence of DCMU (○, broken line).

For comparison action spectra were also measured for NADP reduction,

(*a*) with water as electron donor, a reaction, in which both photosystems have to participate,

(*b*) with the ascorbate-DCIP couple as electron donor in the presence of DCMU; in this case only photosystem I should be active (HOCH and MARTIN 1963).

For these measurements the same light-dark schedule was employed as for the pH action spectra, similarly appropriate illuminances were chosen to stay well below saturation with radiant energy and to yield similar rates at the different wavelengths. In the case with water as electron donor the action spectrum (Fig. 6, □) coincides fairly well with that for the initial rate of proton uptake. The reality of the disagreement around 660 nm is uncertain, as it was not observed in another set of spectra and a third experiment showed too much scatter at that particular wavelength. The action spectrum for NADP reduction with the artificial electron donor system (Fig. 6, ○, broken line) shows a marked red-shift of the long wavelength flank. Between 695 and 705 nm the relative action is about twice that as with water as electron donor or as for the proton uptake. The data from Fig. 6 were obtained on one day with the same chloroplast preparation. From these experiments it is concluded that under the conditions used here the proton pump is not driven preferentially by photosystem I; it rather seems to be sensitized by both pigment systems as is NADP reduction with water as electron donor.

DISCUSSION

Chloroplasts of *Bumilleriopsis* are a suitable material for studying pH changes induced by illuminance. They offer an alternative to chloroplasts of higher plants since they have in common with them several characteristics of the pH changes (and also other reactions, *cf.* BÖGER 1969b). The overall shape of the kinetics – rise, plateau and dark decay – is similar. Reasonable agreement exists for the values of the decay rate constant, the maximum extent of proton uptake and the stimulation by pyocyanine; an earlier saturation with radiant energy for the extent of the proton uptake than for the initial rate is also seen in the illuminance curves of other authors (NEUMANN and JAGENDORF 1964; KARLISH and AVRON 1968). It has been found here (Fig. 5) that the rates of initial pH rise and dark decay (from the steady state level) seem to become equal at low illuminances; equal rates at low illuminance have been predicted in a theoretical treatment (SCHWARTZ 1971). Only partial agreement with the literature exists on other points. The lag of 40–80 ms at the onset of illumination (Fig. 2*A*) is similar to that found by SCHWARTZ and EBERT (see SCHWARTZ 1971); in the case here, however, it may just reflect the response time of the measuring system.

HEATH and HIND (1972) encountered a delay of *ca.* 150 ms although they employed an indicator for pH measurements, with a much shorter reaction time. They had, however, included pyocyanine in their reaction mixture, which might have lengthened the lag, as was the case here (Fig. 2*B*). The postillumination pH rise (Fig. 3*A*) – observed also by JAGENDORF and NEUMANN (1965) and by HEATH and HIND (1972) – and the delay of the dark decay of the steady state pH gradient (Fig. 3*B*) should be real as they exceed the response time of the measuring system. These data are difficult to reconcile with the results of IZAWA and HIND (1967) who neither found a significant pH overshoot in the dark nor any time lag at the beginning of the illumination.

The increased lag of the response to illuminance in the presence of pyocyanine or methyl viologen may represent the time needed for the reduction of these cofactors to a level at which they are most active in catalyzing electron transport and an associated proton pump. This idea is supported by the observation that pyocyanine has to be reduced before it becomes an effective catalyst of photophosphorylation (HAUSKA *et al.* 1970).

The action spectrum for the initial pH rise is different from that for a photosystem I sensitized reaction (Fig. 6). This is contrary to the results of DILLEY and VERNON (1967) and HEATH (1972). These authors however had obtained their spectra in the presence of pyocyanine; this dye probably specifically catalyzes proton uptake driven by photosystem I, similar to its effect in photophosphorylation (AVRON and BEN-HAYYIM 1969). The action spectrum reported here resembles that for NADP reduction with water as electron donor, suggesting a joint action of both pigment systems; proton translocation may then be envisaged in a pseudocyclic electron flow. It cannot be ruled out, however, that the pH rise spectrum is that of a system II

116 reaction only, as differences to the NADP reduction spectrum might exist within the scatter of the points. An involvement – be it only partial – of photosystem II in the proton pump should be expected from the chemiosmotic theory since energy conserving steps seem to be localized around photosystem II (BÖHME and TREBST 1969; GIMMLER 1973; IZAWA *et al.* 1973, TREBST and REIMER 1973). SCHLIEPHAKE *et al.* (1968) presented evidence that each of the photosystems caused half of the pH-change induced by a saturating flash. Their data do not necessitate a cooperation of the two light reactions. If a sole or at least predominant action of photosystem II can be excluded, a cooperation has to be assumed here, for otherwise the proton pump should work with considerable efficiency also in the far red region; this however, is not the case.

REFERENCES

AVRON, M., BEN-HAYYIM, G.: Interaction between two photochemical systems in photoreactions of isolated chloroplasts. — In: METZNER, H. (ed.): Progress in Photosynthesis Research. Vol. III. Pp. 1185—1196. Tübingen 1969.

BÖGER, P.: Photophosphorylierung mit Chloroplasten aus *Bumilleriopsis filiformis* VISCHER. — Z. Pflanzenphysiol. **61** : 85—97, 1969a.

BÖGER, P.: Ferredoxin-katalysierte Reaktionen im zellfreien System der Alge *Bumilleriopsis filiformis* VISCHER. — Z. Pflanzenphysiol. **61** : 447—461, 1969b.

BÖHME, H., TREBST, A.: On the properties of ascorbate photooxidation in isolated chloroplasts. Evidence for two ATP sites in noncyclic photophosphorylation. — Biochim. biophys. Acta **180** : 137—148, 1969.

DILLEY, R. A.: Ion and water transport processes in spinach chloroplasts. — In: Energy Conversion by the Photosynthetic Apparatus. Brookhaven Symp. Biol. **19** : 258—280, 1967.

DILLEY, R. A., VERNON, L. P.: Quantum requirement of the light-induced proton uptake by spinach chloroplasts. — Proc. nat. Acad. Sci. U.S.A. **57** : 395—400, 1967.

GIMMLER, H.: Correlations between photophosphorylation and light-induced conformational changes of chloroplasts in whole cells of the halophilic green alga *Dunaliella parva*. — Z. Pflanzenphysiol. **68** : 289—307, 1973.

HAUSKA, G. A., MCCARTY, R. E., RACKER, E.: The site of phosphorylation associated with photosystem I. — Biochim. biophys. Acta **197** : 206—218, 1970.

HEATH, R. L.: Light requirements for proton movement by isolated chloroplasts as measured by the bromocresol purple indicator. — Biochim. biophys. Acta **256** : 645—655, 1972.

HEATH, R. L., HIND, G.: Bromocresol purple and ethyl red as indicators of events linked to changes in the energization of chloroplast membranes. — J. biol. Chem. **247** : 2917—2925, 1972.

HOCH, G., MARTIN, I.: Two light reactions in TPN reduction by spinach chloroplasts. — Arch. Biochem. Biophys. **102** : 430—438, 1963.

IZAWA, S., HIND, G.: The kinetics of the pH rise in illuminated chloroplast suspensions. — Biochim. biophys. Acta **143** : 377—390, 1967.

IZAWA, S., GOULD, J. M., ORT, D. R., FELKER, P., GOOD, N. E.: Electron transport and photophosphorylation in chloroplasts as a function of the electron acceptor. III. A dibromothymoquinone-insensitive phosphorylation reaction associated with photosystem II. — Biochim. Biophys. Acta **305** : 119—128, 1973.

JAGENDORF, A. T., NEUMANN, J.: Effect of uncouplers on the light-induced pH rise with spinach chloroplasts. — J. biol. Chem. **240** : 3210—3214, 1965.

JAGENDORF, A. T., MARGULIES, M.: Inhibition of spinach chloroplast photosynthetic reactions by p-chlorophenyl-1,1-dimethylurea. — Arch. Biochem. Biophys. **90** : 184—195, 1960.

JAGENDORF, A. T., URIBE, E.: Photophosphorylation and the chemi-osmotic hypothesis. — In: Energy Conversion by the Photosynthetic Apparatus. Brookhaven Symp. Biol. **19** : 215—245, 1967.

KARLISH, S. J. D., AVRON, M.: Analysis of light-induced proton uptake in isolated chloroplasts. — Biochim. biophys. Acta **153** : 878—888, 1968.

MACKINNEY, G.: Absorption of light by chlorophyll solutions. — J. biol. Chem. **140** : 315—322, 1941.

NEUMANN, J., JAGENDORF, A. T.: Light-induced pH changes related to phosphorylation by chloroplasts. — Arch. Biochem. Biophys. **107** : 109—119, 1964.

SCHLIEPHAKE, W., JUNGE, W., WITT, H. T.: Correlation between field formation, proton translocation, and the light reactions in photosynthesis. — Z. Naturforsch. **23b** : 1571—1578, 1968.

SCHWARTZ, M.: Light induced proton gradient links electron transport and phosphorylation. — Nature **219** : 915—919, 1968.

SCHWARTZ, M.: The relation of ion transport to phosphorylation. — Annu. Rev. Plant Physiol. **22** : 469—484, 1971.

TREBST, A., REIMER, S.: Properties of photoreductions by photosystem II in isolated chloroplasts. An energy-conserving step in the photoreduction of benzoquinones by photosystem II in the presence of dibromothymoquinone. — Biochim. biophys. Acta **305** : 129—139, 1973.

WALKER, D. A., CROFTS, A. R.: Photosynthesis. — Annu. Rev. Biochem. **39** : 389—428, 1970.