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CHLOROPHYLL *a* FLUORESCENCE INDUCTION AND CHANGES IN THE ELECTRICAL POTENTIAL OF THE CELLULAR MEMBRANES OF GREEN PLANT CELLS

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SUMMARY

1. The kinetics of light-induced changes in the near infrared chlorophyll *a* fluorescence were measured in intact cells and broken cell preparations of the algae *Nitellopsis obtusa* and *Nitella translucens* and of spinach and tomato leaves.

2. The part of the fluorescence induction which is associated with the transition of the pigment systems from light state 1 to state 2, *i.e.* the long-term decrease in fluorescence yield during the so-called P_2M_3 phase, occurring after an initial increase in the light, is not observed in broken cell preparations.

3. Simultaneous measurements of fluorescence changes and changes in the electrical potential of the two cytoplasmic membranes in *Nitella translucens* show a close association between the transition of the states of the pigment systems and the changes in membrane potential.

4. The results are discussed in terms of a hypothesis which emphasizes that the light and dark transitions of the states of the pigment systems occur in association with regulatory ion transport processes across the cytoplasmic and outer chloroplast membranes.

INTRODUCTION

The fluorescence yield of chlorophyll a_2 (chlorophyll *a* excited by system 2) in algal and green plant cells undergoes transitory changes during illumination[1,2]. The kinetic pattern of the changes is known as the fluorescence induction. It has been discussed[2-7] that the long-term decrease in fluorescence yield, observed in the light after an initial increase, cannot fully be interpreted in terms of the interaction between the two pigment systems. Amongst the several interpretations, which have been given to explain the phenomenon in relation to the efficiency with which light-driven electron transport by both pigment systems proceeds, the ones proposed by BONAVENTURA AND MYERS[7] and by MURATA[6], shared the conclusion that the relatively

Abbreviation: DCMU, 3,4-dichlorophenyl-*N*,*N*-dimethyl urea.

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slow changes in fluorescence yield are associated with a change in mode of excitation transfer between the two pigment systems. In the terminology used by BONAVENTURA AND MYERS[7], the decrease in fluorescence yield during what we call the P_2M_3 phase* represents the transition of the equilibrium state of the pigment systems denoted by light 1 state into another state, called light 2 state. In state 2 a smaller fraction, α, of incident quanta is transferred to system 2 than in state 1, in which a fraction α_{max} is delivered to pigment system 2. PAPAGEORGIOU[4] and PAPAGEORGIOU AND GOVINDJEE[5] suggested that the slow decrease in fluorescence yield during the $M_2(P_3)M_3$ phase (*i.e.* the transition from state 1 to state 2) is associated with conformational changes of the lamellar system, related to the phosphorylation process. VREDENBERG[8,9] has proposed that the state 1 to state 2 transition is associated with intracellular ion transport, and that the state 1 and state 2 of the pigment systems are reflections of specifically alterable energy states of the inner chloroplast membrane. DUYSENS[10] explained the state 1 to state 2 transition in terms of an energy-dependent mutual approach of pigment molecules of system 2 and system 1, which causes a more efficient energy transfer from the pigments of system 2 to those of pigment system 1.

It has been shown[11] that in giant cylindrical algal cells like *Nitellopsis obtusa* and *Nitella translucens* the electrical potential across the three cellular membranes in series (cell wall, plasmalemma and tonoplast) increases reversibly upon illumination with light absorbed by both photochemical systems. The quantum efficiency of the light-induced potential changes in *Nitella translucens* has been reported to be close to 1 (ref. 12) suggesting that these changes are associated with a primary photochemical process. As such a primary translocation of ions, probably protons, at the phase boundaries of the chloroplasts in the cytoplasm has been proposed.

The present experiments will show that the reactions which cause the long-term decline in fluorescence yield in the light and its recovery in the dark (*i.e.* the transition of the pigment systems from state 1 into state 2 and *vice versa*) are closely associated with the reactions that cause the reversible change in membrane potential in the light and in the dark. These results and those which show that the state 1 to state 2 transition is largely absent in broken cell preparations give experimental support for the hypothesis that the transitions occur in association with ionic changes in the cytoplasm and the inner stroma phase of the chloroplasts, due to changes in ionic fluxes across the cytoplasmic membranes and outer chloroplast membrane. The changes in membrane potential are discussed to be indicative for the ion movements across the cytoplasmic membranes. Simultaneous fluorescence and electrical measurements were done mainly with cells of *Nitella translucens*. Other separate fluorescence experiments were done with intact fresh leaves of spinach or tomato and with intact cells of *Nitellopsis obtusa*.

MATERIALS AND METHODS

Nitellopsis obtusa and *Nitella translucens* were collected from fresh water pools in The Netherlands. The cells were bathed in large tanks filled with artificial pond

* The long-term decrease in fluorescence yield will be termed the P_2M_3 phase, according to the nomenclature given by BANNISTER AND RICE[3]. In cases where this phase consists of a fast and a slow component, the fast and slow components are termed the P_2M_2 and M_2M_3 phase, respectively. In a previous paper[8] we called the long-term decrease in fluorescence yield in Porphyra the P_2M_2 phase instead of the P_2M_3 phase.

water containing 1.0 mM NaCl, 0.1 mM KCl and 0.1 mM $CaCl_2$, and were kept growing in the laboratory in shadowed northern daylight.

Spinach and tomato leaves were collected, just before they were transferred to the measuring apparatus, from growth cultures in the laboratory green house.

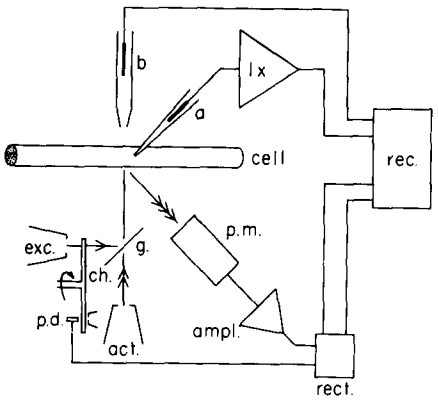

Fig. 1. Schematic diagram of the apparatus for measuring light-induced changes in fluorescence yield and in membrane potential. exc. and act. indicate light sources for excitation and actinic light, respectively; ch. is a chopper, p.d. is a photodiode; g. is a glass plate; p. m. is a photomultiplier; ampl. is an a.c. amplifier; rect. is a phase sensitive rectifier; rec. is a multi-channel recorder. a and b are the internal and external Ag/AgCl electrodes, respectively, the signal of which is amplified by a unity gain, high input amplifier (1 ×). Electrode a was inserted into the vacuole of the cell at its midpoint. Illumination spots of actinic and excitation light were also at the midpoint of the cell and of about 2 cm length and 0.5 cm width. Excitation and actinic light beams are marked with one and two arrows, respectively. The fluorescence light beam is marked with tree arrows. Lenses and filters have been omitted in the diagram. Further explanations are in the text.

The measurements were carried out in a fluorescence apparatus, in which light-induced changes in fluorescence yield and in membrane potential could be measured simultaneously. A schematic diagram of the arrangement is shown in Fig. 1. Fluorescence is excited by a 400-Hz modulated monochromatic light beam of weak intensity. This fluorescence (F_0) is detected by a photomultiplier, the output of which is connected to an amplifier and phase-sensitive rectifier. The 400-Hz reference signal for the rectifier is of an illuminated photodiode mounted on the support of the chopper. The output of the rectifier is connected to a multi-channel galvanometer recorder Non-modulated monochromatic actinic light causes changes in the amount of modulated fluorescence (F) (*i.e.* changes in the relative fluorescence yield F/F_0), and changes in the membrane potential. The detection system does not respond to the (continuous) strong fluorescence emitted by the actinic light. The response time for the changes in fluorescence signal was about 60 msec. The membrane potential was measured by an electrode assembly similar to that described elsewhere[11,12]. a is a 3 M KCl-filled glass microcapillary, usually inserted transversely into the vacuole of a *Nitella translucens* cell at its midpoint, in contact with a Ag/AgCl wire *via* an agar–KCl bridge, b is an Ag/AgCl–agar 3 M KCl electrode, bathed in the external medium. In the experiments in which the light response of the two inner cellular membranes were traced, b was substituted by an a-type electrode which was inserted into the cell just across the cell wall. Upon insertion, the potential difference between electrode a and b changed by about +40 to +70 mV which, consistent with the results of others[13–15], is due to the

cell wall potential. The response time for the change in potential was limited by the response time of the recorder, which was about 15 msec.

Light sources for the excitation and actinic light were modified lamp house assemblies of a 24-V, 250-W, d.c. slide projector. Monochromatic light was obtained by placing combinations of color and interference filters with half-band widths of 15–20 nm, in the light beams. Intensities were measured with a calibrated thermopile and are given in nEinstein·cm^{-2}·sec^{-1}. The fluorescence emission was detected in the wavelength region above 670 nm by placing color filters, transmitting above this wavelength, in front of the photomultiplier.

Excitation and actinic light was of a wavelength band around 430 nm, which for the cells used in these experiments is light absorbed by both pigment systems. All experiments were carried out at room temperature.

RESULTS AND INTERPRETATIONS

(1) *Fluorescence changes in intact cells and chloroplast preparations*

Figs. 2 and 3 show the kinetics of the changes in fluorescence yield F/F_0, occurring upon illumination of a single cell of *Nitellopsis obtusa*. Several distinct phases in the kinetics of the so-called fluorescence induction can be distinguished. A biphasic increase in yield from F_0 at level o to the peak at P_2, with an intermediate inflection at P_1 (=M_1), is completed within 0.7 sec, and is followed by a decrease towards a lower level M_3, which is reached after 0.5–2 min. The P_2M_3 phase is biphasic with a first rapid (P_2M_2) phase which takes about 1 sec for completion. The rapid decrease occurs also in the absence of actinic illumination, as can be concluded from a

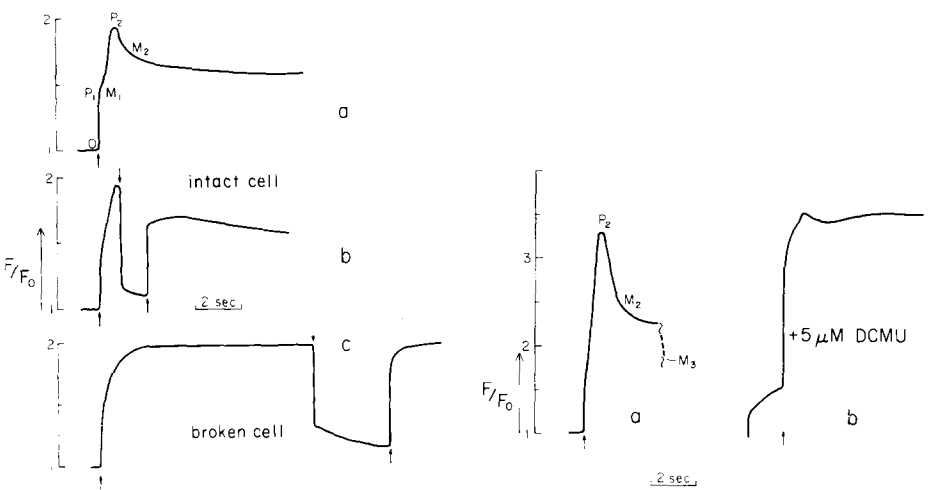

Fig. 2. Kinetics of the light-induced changes in chlorophyll *a* fluorescence yield, F/F_0, in an intact (a, b) and broken cell (c) of *Nitellopsis obtusa*. Intensities of 430-nm excitation and actinic light were of the order of 0.01 and 1 nEinstein·cm^{-2}·sec^{-1}, respectively. Upward and downward pointing arrows mark the beginning and the end of an actinic illumination period, respectively.

Fig. 3. Kinetics of the light-induced changes in chlorophyll *a* fluorescence yield in an intact cell of *Nitellopsis obtusa*, in the absence (a) and presence of 5 μM DCMU. M_3 is the final steady-state fluorescence yield reached after 1–2 min of actinic illumination. Further explanations are in the legend of Fig. 2.

comparison of the experiments plotted in Figs. 2a and 2b. When the actinic light was shut off at P_2 and turned on after 1 sec darkness (Fig. 2b) the fluorescence yield was approximately at the same level as the one reached in the same time in continuous actinic illumination (Fig. 2a). Fig. 2c shows the kinetics of the fluorescence changes, after the cell has been broken. A usual procedure for this was to puncture the cell at one end with a needle, sufficiently large to make a hole in the cell wall such that the cell lost its turgor. Care was taken that no external medium streamed into the cell interior during and after puncturing, in order to prevent mixing of cytoplasm and vacuolar sap with external medium. Thus, the chloroplasts were kept in their physiological environment but vacuolar sap may have been mixed with cytoplasm. It appears (Fig. 2c) that for the chloroplasts in the broken cell, the fluorescence induction during the P_2M_3 phase has disappeared. The $O(P_1M_1)P_2$ phase is not changed appreciably.

The effect of 3,4-dichlorophenyl-*N*,*N*-dimethyl urea (DCMU) is shown in Fig. 3b. In the presence of DCMU, in addition to the inhibition of the P_2M_3 phase, the $P_1(M_1)$ P_2 phase is enhanced. This is consistent with the fact that DCMU inhibits electron transport between the acceptor Q of pigment system 2 and a pool, probably plastoquinone. In the absence of DCMU, the pool withdraws electrons from reduced Q at a high rate during the initial period of illumination after darkness, because in the dark, or after illumination with light of system 1, the pool is in a more oxidized state[16, 19].

Fig. 4 shows results with intact leaves of spinach and tomato (a) and of broken cells of these materials (b). Broken cells were rapidly prepared by softly scratching a small area of the leaf at the place where it is illuminated in the fluorescence experiments. The treated part of the leaf can be considered as a crude chloroplast preparation. Before and during preparation, a small amount of 0.4 M sucrose, 0.05 M phosphate buffer (pH 7.2) was placed upon the leaf. The preparative procedure took about 30–60 sec. The results indicate (Fig. 4b) that the P_2M_3 phase is inhibited in the broken

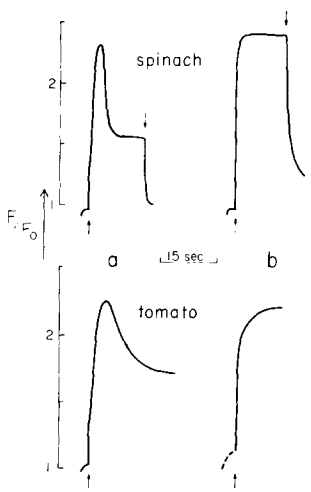

Fig. 4. Kinetics of the light-induced changes in chlorophyll *a* fluorescence yield in intact leaves of spinach and tomato (a) and in a broken cell preparation of these leaves (b). A 15 mm × 15 mm surface area of each leaf which was illuminated by the actinic light, was covered by a few droplets of a 0.05 M phosphate buffer, 0.4 M sucrose solution (pH 7.2). Broken cells were prepared by softly scratching this area with a small flat-end spatula. Further details are in the legend of Fig. 2.

cells. These observations (Figs. 2 and 4) demonstrate that the reaction which causes the decline in fluorescence yield during the P_2M_3 phase (*i.e.* the transition from state 1 into state 2 (ref. 7)), proceeds only when the photosynthetic apparatus, contained in the chloroplasts, is an interaction with an environment as exists in whole cells.

(2) *Fluorescence changes and changes in membrane potential*

Fig. 5 shows the results of simultaneous measurements of the kinetics of changes in fluorescence yield and in potential across plasmalemma and tonoplast in different cells of *Nitella translucens*. The fluorescence induction usually shows the fast initial decline in yield (P_2M_2 phase), followed by the slower decline to the steady-state level M_3 (M_2M_3 phase). As has been reported before[11], the increase in membrane potential upon illumination starts after a short lag time of about 1–2 sec. Sometimes a small initial decrease in potential was observed during this lag time. From a number of experiments similar to those shown in these figures, done with different cells, it was concluded that the end of the lag time observed in the potential change coincides with the start of the M_2M_3 phase. The measurements show that the reaction(s) which cause(s) the change in fluorescence yield during the M_2M_3 phase of the induction, and the change in membrane potential take the same time for completion. As Fig. 5 shows, the reaction times were different for different cells. The kinetics of the changes are not always completely iden-

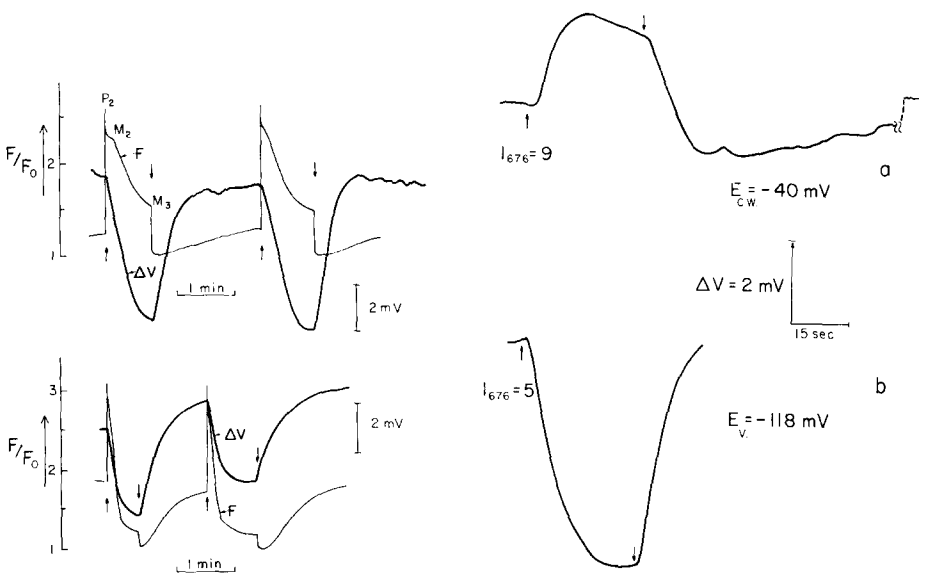

Fig. 5. Kinetics of light-induced changes in chlorophyll *a* fluorescence yield and in potential across plasmalemma and tonoplast in two different cells of *Nitella translucens*. Intensity of fluorescence exciting light was of the order of 0.01 nEinstein·cm^{-2}·sec^{-1} intensity. Intensity of actinic light was approx. 3 nEinstein·cm^{-2}·sec^{-1}. The potential difference between vacuole and internal cell wall phase in the dark was about −70 mV. A downward movement of the potential recording means an increase in potential (less negative).

Fig. 6. Kinetics of light-induced changes in potential across cell wall, E_{CW}, (a), and across cell wall, plasmalemma and tonoplast, E_V, (b) in *Nitella translucens*. E_V means the potential difference between vacuole and external medium. Actinic light was of 676 nm. Intensities are given in nEinstein·cm^{-2}·sec^{-1}. A downward movement of the potential recording means an increase in potential.

tical (Fig. 5a); in some cases the M_2M_3 phase of the fluorescence change is somewhat retarded at the beginning. Note that the fluorescence yield F_0 in the weak exciting light after actinic illumination is lower than after a dark time during which the dark conditions are restored. The rate and the extent of this effect on F_0 were the same when the intensity of the exciting beam was decreased by a factor of about 5. This indicates that the effect was not caused by an actinic effect of the exciting light.

The kinetics of the change in fluorescence yield were compared with those of the change in potential across the plasmalemma and tonoplast, rather than with those of the change occurring across the cell wall in series with these two cytoplasmic membranes. The reason for this is illustrated by experimental results which show that the potential across the cell wall decreases upon illumination, whereas the potential across the cell wall in series with the cytoplasmic membranes increases (Fig. 6). The changes in potential across the two cytoplasmic membranes therefore is expected to be higher than the potential change across the whole membrane system, including the cell wall. This was verified by measuring both changes for the same cell. In some experiments the kinetics of the potential change across the cell wall were not identical with those of the change across the three potential barriers. This may be due to the specific property of the cell wall to act as a cation-exchange barrier[20]. Thus in order to discriminate for any possible cell wall effect, the fluorescence changes were compared with the light response of the two inner cytoplasmic membranes.

DISCUSSION

The results are not inconsistent with an earlier proposed hypothesis[8,9], that the *in vivo* transitions of the states of the pigment systems (state 1 and state 2 (ref. 7)) are associated with cellular processes in which ion movements are involved. The fact that the state 1 to state 2 transition does not occur in broken cell preparations indicates that this transition occurs in response to those changes in the cytoplasm and presumably in the inner stroma phase of the chloroplasts, which (as far as ions are concerned) are controlled by the potential and ionic permeability coefficients of the cytoplasmic membranes[21] and the outer chloroplast membrane[22].

Light-induced changes in membrane potential in Nitellopsis and Nitella and other characean cells have been demonstrated[11,23–25]. Fig. 5 shows that the changes across plasmalemma and tonoplast in the light and in the dark are completed within time periods equal to those of the transition from state 1 into state 2 and *vice versa*. It seems reasonable to assume[11,12] that the efficient light-induced potential changes are primarily associated with electron transport coupled H^+ uptake by the chloroplasts. The ionic concentrations in the intracellular spaces are maintained at dynamic equilibrium in the dark by passive and active ion fluxes across the cellular membranes (*cf.* refs. 21, 26–29). According to the theorem of USSING[31] AND TEORELL[30], the ratio of influx and efflux for each specific ion should change exponentially with a change in the membrane potential. It has been shown[32] that in *Nitella translucens* the dark fluxes of Na^+ and K^+ change when the membrane is clamped at a potential different from the steady-state dark potential. Thus the change in membrane potential brought about by the light will be associated with changes in the ionic flux ratios across the membranes. The changes in flux ratios will cause changes in cytoplasmic ion concentrations, in addition to those caused by the primary light-induced ion uptake at the thylakoid

membranes of the chloroplasts. The potential-linked ion movements across the cytoplasmic membranes of course are absent in the broken cell preparations. We have no experimental justification whether the outer chloroplast membrane has remained intact in our broken cell preparations (Figs. 2 and 4), or not. Thus a possible separate effect of ion movements across the outer (intact) membrane of isolated chloroplasts on the chlorophyll *a* fluorescence yield cannot be ruled out. However, recent experiments (P. MOHANTY, GOVINDJEE AND W. J. VREDENBERG, unpublished) done with intact tomato, corn and escarole leaves and rapidly prepared[33] class I chloroplasts thereof (*i.e.* chloroplasts with intact outer membranes) have shown that this effect cannot completely account for the fluorescence induction observed in the intact cell. The fluorescence induction in these class I chloroplasts was found not to be markedly different from what was observed in our broken cell preparations (Figs. 2 and 4). However, a relatively small and comparatively long lasting irreversible fluorescence decline was observed during actinic illumination.

It might be that, amongst others, the alkali ions and Cl^-, which are known to be involved in the light-activated transport processes in intact green plant cells[34–37], contribute to structural changes at the thylakoid membrane[5–7,10] which are suggested to cause the transitory effect of states 1 and 2 of the pigment systems[7]. There is as yet no experimental proof for such a hypothesis. However, experiments with isolated chloroplasts[38–40], showing that the fluorescence yield is largely dependent on the concentrations of cations and of anions, are not inconsistent with this. MURATA[39], following a similar line of reasoning, interprets the *in vivo* fluorescence phenomena as being caused by electron transport-coupled light-induced efflux of bivalent cations across thylakoid membranes of the chloroplasts. However, if this would be the cause, the absence of the transition from one state into another in a cell-free preparation, cannot be explained. In our interpretation changes in ion fluxes across the cellular and outer chloroplast membrane rather than those at the thylakoid membrane are suggested to be primarily involved. Justification for the assumption that changes in cation concentration induce a change in excitation energy transfer between the two pigment systems awaits further refined experiments. It has been reported[41] that the thylakoid membranes of chloroplasts *in vivo* undergo light-induced conformational and configurational changes. These have been explained in terms of correlated changes in the ionic environment and osmotic properties of the chloroplasts. Results of experiments on light-induced shrinkage of chloroplasts *in vivo* have been suggested by HEBER[42] to indicate that changes in configuration and conformation of the thylakoids are causing changes in the rate of electron flow through the two photochemical systems.

In accordance with what would be predicted from the hypothesis on transitory changes in excitation energy transfer *in vivo*[7], the dark fluorescence yield of chlorophyll a_1 and chlorophyll a_2 (F_0) undergoes changes during the transitions from one state into another (Fig. 5). It is known that the transition from state 2 into state 1 is enhanced by preillumination with light mainly absorbed by system 1 (refs. 2, 7, 8), and in the dark in the presence of specific membrane modifying agents[8]. It has been suggested[8] that the enhancement by system 1 illumination is associated with light-induced cation transport across the cellular membranes. Recent experiments, which will be published in a forthcoming communication, have shown that there are light-induced changes in the membrane potential in *Nitella translucens*, which are associated with cyclic electron transport in system 1.

In summary, we feel that our results are demonstrations of the significant fact that in intact cells light energy conversion occurs by an apparatus which is in interaction with an environment, under control of transport processes across the membranes. The control mechanism, reflected by changes in fluorescence yield of chlorophyll *a*, probably is powered primarily by electron transport-coupled ion transport at the chloroplast thylakoid membrane, and regulated by changes in ion fluxes across the cellular and outer chloroplast membranes. PUNNETT[43] studying the light-induced transition of chloroplasts forms in higher plant cells, simultaneously with photosynthetic enhancement, came to a similar conclusion.

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