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# Energy-Dependent Changes of the Electrokinetic Properties of Chloroplasts[†]

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ABSTRACT: Free-flow electrophoresis of spinach chloroplasts reveals that illumination causes a marked increase of net negative surface charge. The light stimulation is most pronounced in broken chloroplasts, missing the outer envelope membranes. Broken chloroplasts, prepared in the presence of $MgCl_2$, showed a much larger light stimulation (90–120%) than if $MgCl_2$ was omitted during preparation (40–50%). The effects of mono- and divalent cations on electrophoretic mobility and its stimulation by light are not simply explainable in terms of the Gouy–Chapman theory of the diffuse double layer. In addition to their charge-screening effect, these ions influence the membrane integrity and presumably thereby the energy-linked exposure of negative surface charges. The light stimulation is dependent on photosynthetic electron transfer and sensitive to both anionic and cationic uncouplers, to valinomycin, and to treatment with glutaraldehyde or a short heat shock. The calculated chloroplast charge densities are about one electronic charge per 3500 Å$^2$ in the dark and one electronic charge per 1700 Å$^2$ in the light. The electric potentials derived from the electrostatic interaction of cationic aminoacridine probes with chloroplasts behave qualitatively similar with respect to light stimulation and chemical or physical treatments as the electrokinetic properties, reported here. It is concluded that the light-induced increase of net negative surface charge of chloroplasts is the result of an energy-dependent conformational rearrangement of thylakoid membrane components.

Most biological membranes bear a net negative surface charge in the physiological pH domain (Gitler, 1971, 1972), including chloroplast membranes (Mercer et al., 1955; Dilley & Rothstein, 1967; Gross & Hess, 1974; Berg et al., 1974). The corresponding negative electric surface potential may be considered as an entity that controls the ionic composition in the diffuse double layer adjacent to the membrane surface and thereby intrinsic metabolic and structural membrane properties, including the translocation of ions (Gross & Prasher, 1974; Barber et al., 1977; Theuvenet & Borst-Pauwels, 1976a,b). There is clearly a mutual influence of membrane surface phenomena on the one hand and transmembrane phenomena on the other [see also Rumberg (1977)]. A number of surface-related phenomena, such as the electrostatic adsorption of dye molecules (Montal & Gitler, 1973; Kraayenhof & Arents, 1977; Searle et al., 1977) and uncouplers (Bakker et al., 1975), seem to be in good harmony with the theory of the diffuse double layer developed by Gouy & Chapman [see Davies & Rideal (1963); Aveyard & Haydon (1973)]. McLaughlin (1977) has lucidly reviewed those aspects of this theory and of electrostatic phenomena at mem-

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0006-2960/80/0419-1922$01.00/0

brane surfaces in general that are relevant to biological membranes.

We are particularly interested in the dynamic changes of membrane structure and electrical charges in the course of photosynthetic energy transduction. Already in 1955, Mercer et al., investigating the structural and osmotic properties of *Nitella* chloroplasts, determined the isoelectric point by free-flow electrophoresis. A net increase of negative surface charge of 15% upon illumination of intact spinach chloroplasts has been observed (Nobel & Mel, 1966) by means of several particle electrophoresis techniques. On the other hand, in a recent electrophoretic study on both intact chloroplasts and divalent cation-depleted thylakoid membranes, Nakatani et al. (1978) did not observe any light-induced increase of surface charge density. Earlier experiments with a number of different 9-amino-substituted acridine derivatives, including species with different p$K_a$ values and lipid solubility and with nonpermeant or paramagnetic groups in the 9-amino side chain as well as covalently coupled analogues, led to the conclusion that the cationic compounds interact electrostatically with light-generated negative charges on the chloroplast thylakoid membrane [Kraayenhof, 1973; Kraayenhof & Slater, 1975; for a review, see Kraayenhof et al. (1976)]. From the light-induced apparent p$K_a$ shifts observed with these probes in broken chloroplasts, we inferred a change of the thylakoid "surface" potential from about −40 mV (dark) to about −90 mV (light). Preliminary results from free-flow particle microelectrophoresis indicated that the electrokinetic potential ($\zeta$) changed from about −30 mV (dark) to about −55 mV (light), and a correlation between these two phenomena was suggested (Kraayenhof & Schuurmans, 1977; Kraayenhof, 1977).

In this paper we report on a further characterization of the electrokinetic behavior of intact and broken chloroplasts in response to energization by light.

## Materials and Methods

Chloroplast preparations enriched in "intact" (outer membrane containing) chloroplasts were made from freshly grown or market spinach by homogenization of precut leaves (10 g) with a cavitation disperser (IKA Ultra-Turrax, suited with 18N shaft) for 2 × 1 s at maximal speed, followed by filtration through eight layers of perlon net (56-$\mu$m mesh width) and 1-min centrifugation at 2000*g*. The isolation and resuspension medium (usually 50 and 2 mL, respectively) contained 330 mM sorbitol and 2 mM Tricine[1] buffer, pH 7.2, and, where indicated, 5 mM $MgCl_2$. Broken chloroplasts were obtained by suspending the pellets in 1 mL of $H_2O$ or 10 mM $MgCl_2$ for 1 min, after which 1 mL of double-strength medium was added. The electrophoresis media were as indicated in the legends to the tables and figures. The chlorophyll content was 10 $\mu$g/mL in all experiments. The electrophoretic mobility ($u$) of individual chloroplasts was determined in a thermostated (25 °C) laterally oriented rectangular quartz chamber (depth, 0.7 mm; height, 14 mm) connected to reversible Cu|$CuSO_4$ electrode compartments. The two instruments used in this study were the one described by Fuhrmann et al. (1964) and a modified Rank Brothers (Cambridge, England) particle electrophoresis apparatus, Mark II. Both instruments were equipped with phase-contrast optics (Leitz; large working distance) and a fiber optics actinic illuminator providing red light (610-nm cutoff filter) with an intensity of 22 mW/cm$^2$ at the electrophoresis chamber. Measurements were performed at constant currents (1–5 mA) resulting in electric field strengths between 5 and 35 V/cm. The observation light was filtered by a green (545 nm) interference filter and kept at the lowest possible intensity so as to minimize actinic effects (see under Results the effect of uncouplers on "dark" mobilities). The objective was focused on one of the two predetermined stationary phases. The Rank Brothers instrument was provided with a TV monitor (Philips), facilitating the observation of different types of chloroplasts at low light level and the timing of migration by several (noncommitted) persons. The electrophoretic migration was timed for both forward and backward (reversed field) runs over a known distance (37.5 or 75 $\mu$m). Usually 10 consecutive runs of different chloroplasts of the same type were timed, and the mean values of $u$ are given in the tables and figures; within the same chloroplast preparation, the standard deviations were in the order of 1–3%, never exceeding 8%. The electrical conductance and viscosity of the different media, including chloroplasts, were measured with conductivity bridges (Philips PR 9501 and Radiometer CDM 3) and an Oswald-type viscosimeter, respectively.

Table I: Light-Induced Changes of Electrophoretic Mobility in Different Classes of Chloroplasts[a]

| | intact chloroplasts | | | broken chloroplasts | | |
|---|---|---|---|---|---|---|
| | $-u \times 10^5$ (cm$^2$ V$^{-1}$ s$^{-1}$) | | $\Delta u_{D\to L}$ | $-u \times 10^5$ (cm$^2$ V$^{-1}$ s$^{-1}$) | | $\Delta u_{D\to L}$ |
| expt | dark | light | (%) | dark | light | (%) |
| A | 16.8 | 21.3 | 27 | 19.9 | 28.0 | 41 |
| B | 11.5 | 13.7 | 19 | 12.4 | 27.2 | 119 |

[a] The mobility values are means of 10 runs of different chloroplasts of the refractive (intact) or dark-granular (broken) type, present in the same preparation. Experiment A: chloroplasts isolated in the absence of $MgCl_2$; electrophoresis was carried out in isolation medium *plus* 10 $\mu$M diquat. Experiment B: chloroplasts isolated in the presence of 5 mM $MgCl_2$; the electrophoresis medium was as in experiment A but contained in addition 0.05 mM $MgCl_2$, introduced with the chloroplast suspension.

Glutaraldehyde-treated chloroplasts were obtained by incubating broken chloroplasts for 2 min in 2.5% glutaraldehyde, followed by two washing steps.

ACMA was synthesized according to the procedures described by Albert (1966). S-13 was a donation of Dr. P. C. Hamm of Monsanto Co. (St. Louis).

## Results

The available data on chloroplast electrophoresis cited in the introduction suggest that chloroplast integrity may be an important factor in determining the electrophoretic mobility ($u$) and the effect of illumination. Therefore, we compared the mobilities of "intact" (outer membrane containing) and broken chloroplasts in the dark and light and under different incubation conditions (Table I). The spinach chloroplast suspension used here was heterogeneous, although enriched in intact organelles (70–80%). The phase-contrast optics enables the discrimination of the refractive envelope containing chloroplasts and the broken ones with dark granular appearance. In such mixed suspensions a bimodality is found in a frequency histogram of the electrophoretic mobilities and of the stimulatory effect by light. Broken chloroplasts showed

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[1] Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; PEA, 9-[[(propylamino)ethyl]amino]-6-chloro-2-methoxyacridine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; $u$, electrophoretic mobility (cm$^2$ V$^{-1}$ s$^{-1}$); $\zeta$, electrokinetic potential at the hydrodynamic plane of shear (mV); $\psi_0$, electric potential at the membrane surface (mV); $\psi_d$, electric potential at distance $d$ from the membrane surface (mV); Tricine, *N*-tris(hydroxymethyl)methylglycine; diquat, *N*,*N*′-ethylene-2,2′-dipyridilium dibromide; PMS, *N*-methylphenazonium methyl sulfate.

Table II: Effect of $MgCl_2$ on the Electrophoretic Mobility of Broken Chloroplasts Prepared in the Absence or Presence of $MgCl_2$[a]

| expt | $MgCl_2$ (mM) | $-u \times 10^5$ ($cm^2$ $V^{-1}$ $s^{-1}$) dark | light | $\Delta u_{D \to L}$ (%) |
|---|---|---|---|---|
| A | 0 | 19.9 | 28.5 | 43 |
| | 0.05 | 19.0 | 24.5 | 29 |
| | 1.0 | 13.0 | 14.6 | 12 |
| B | 0.05[b] | 13.8 | 26.9 | 95 |
| | 1.0 | 13.6 | 18.2 | 34 |

[a] The broken chloroplasts were prepared in the absence (experiment A) or presence (experiment B) of 5 mM $MgCl_2$. In both experiments the electrophoresis medium was as described in Table I. [b] This concentration of $MgCl_2$ was introduced into the medium with the chloroplast suspension.

a slightly larger dark mobility, and the light stimulation ($\Delta u_{D \to L}$) is roughly 2–5 times that of intact ones. In other experiments (not shown) using normal through-light or dark-field optical arrangements for observation, most of the clearly visible particles showed the behavior of intact chloroplasts; the faster moving particles could hardly be observed and are therefore easily overlooked with this observation method.

Dubacq & Kader (1978) submitted chloroplasts to preparative free-flow electrophoresis and obtained pure intact and broken chloroplast fractions. However, they found the intact chloroplasts in the faster moving fraction, using an electrophoresis medium containing sucrose (400 mM) and Tris–citrate (6 mM; pH 7.3) at 5 °C. As yet, we cannot explain this opposite electrokinetic behavior.

We found the light stimulation in both types of chloroplasts to depend on the presence of added cyclic or noncyclic electron transfer mediator [contrast Nobel & Mel (1966)]. No difference in stimulation was noted in the presence of pyocyanine, PMS, $K_3Fe(CN)_6$, methyl viologen, or diquat. The latter compound was routinely used in the reported experiments.

On the basis of the charge-screening effect of bivalent cations, it is expected that the electrophoretic mobility is larger in the case that the chloroplasts were prepared in the absence of added $MgCl_2$. This is indeed found in our experiments (Table I). The light stimulation is largest in the broken chloroplasts, prepared in the presence of $MgCl_2$. Further experiments to be reported here are performed with broken chloroplasts only.

The effect of $MgCl_2$ was further investigated and is summarized in Table II. If the chloroplasts were prepared in the absence of $MgCl_2$, but without drastic depletion of bivalent cations [cf. Nakatani et al. (1978)], one observes a decrease of electrophoretic mobilities and light stimulation at increasing $MgCl_2$ concentration in the electrophoresis medium (Table II, experiment A). On the other hand, if 5 mM $MgCl_2$ was present in the isolation medium, the effect of additional $MgCl_2$ on the electrophoretic mobilities is less and the light stimulation is significantly larger (Table II, experiment B).

The pH dependency of the dark and light mobilities is shown in Figure 1. Both mobility curves sharply decline below pH 6 and indicate an isoelectric point at pH 4.8, roughly confirming the results obtained by Mercer et al. (1955) and by Nakatani et al. (1978) on nonenergized chloroplasts or chloroplast particles, respectively. It appears that illumination causes exposure of additional negative groups with identical $pK_a$ values as those of the groups already exposed in the dark.

The effect of monovalent salt on the electrophoretic mobility is illustrated in Figure 2. Higher KCl concentrations lower the mobilities, as expected from the Gouy–Chapman theory, and the light stimulation decreases. Dark and light mobilities are slightly stimulated between 5 and 15 mM KCl; although this observation was unexpected, it seems to be in line with the stimulatory effect of 0.05 mM $MgCl_2$ (Table I, experiment B, and Table II, experiment B). Since the KCl optimum is shifted toward higher concentration by illumination, one observes a rather complicated KCl dependency of the light stimulation.

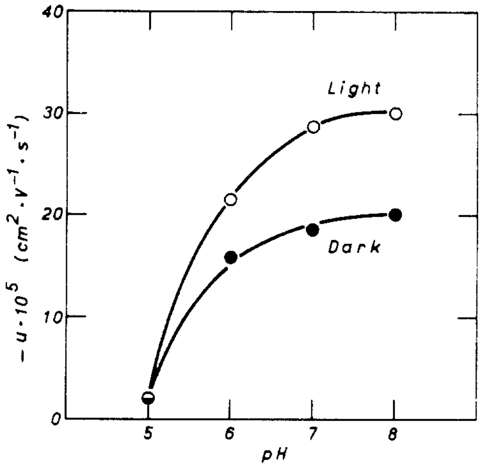

FIGURE 1: pH dependency of the electrophoretic mobility of broken chloroplasts in the dark and light. In the isolation and electrophoresis media, $MgCl_2$ was omitted. Electrophoresis conditions were as described in Table I, experiment A.

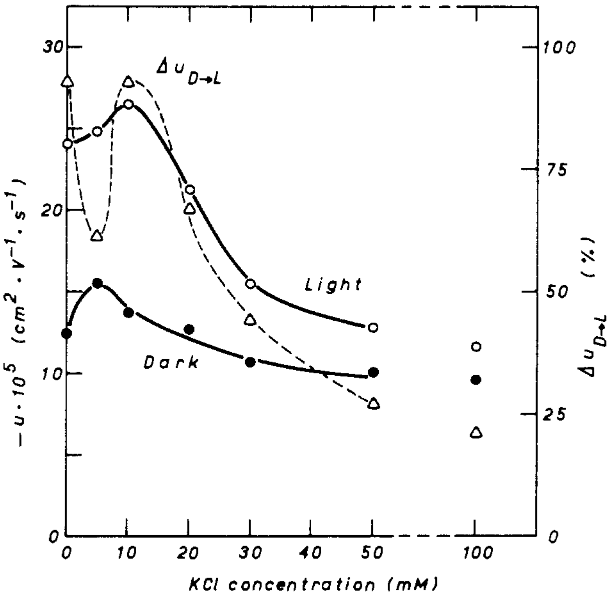

FIGURE 2: Effect of a monovalent cation on the electrophoretic mobility of broken chloroplasts in the dark and light. The chloroplasts were prepared in the presence of 5 mM $MgCl_2$, and electrophoresis was carried out as described in Table I, experiment B.

Table III lists the effects of a number of additions and physical treatments on the electrophoretic mobility of broken chloroplasts. The electron transfer inhibitor DCMU does not affect the dark mobility but prevents the light stimulation; additional PMS (cyclic electron transfer) restores the light effect. Nonsaturating actinic light intensities also lowered the percentage of stimulation (not shown). Different types of uncouplers are found to prevent the light stimulation, in contrast to the observations by Nobel & Mel (1966). Higher concentrations of lipid-soluble anionic uncouplers (CCCP in Table III) also show the tendency to enhance the electro-

Table III: Effects of Electron Transfer Inhibitor, Uncouplers, and Physical Treatments on the Electrophoretic Mobility of Broken Chloroplasts[a]

| | $-u \times 10^5$ (cm$^2$ V$^{-1}$ s$^{-1}$) | | |
|---|---|---|---|
| addition or treatment | dark | light | $\Delta u_{D \to L}$ (%) |
| none | 14.0 | 27.0 | 93 |
| DCMU (0.5 μM) | 14.0 | 15.0 | 7 |
| DCMU (0.5 μM) plus PMS (6 μM) minus diquat | 14.9 | 27.1 | 82 |
| CCCP (5 μM) | 17.4 | 14.8 | −15 |
| S-13 (1 μM) | 13.5 | 14.1 | 4 |
| ACMA (15 μM) | 12.1 | 14.0 | 16 |
| 1.5 min, 60 °C | 5.3 | 5.1 | −4 |
| 2 min, 2.5% glutaraldehyde | 6.7 | 6.9 | 3 |
| valinomycin (1 μM) | 12.6 | 20.7 | 64 |
| valinomycin (5 μM) | 12.1 | 12.5 | 3 |

[a] The broken chloroplasts were prepared in the presence of 5 mM $MgCl_2$. The electrophoresis medium contained 330 mM sorbitol, 10 mM KCl, 2 mM Tricine buffer, pH 7.2, and 10 μM diquat (0.05 mM $MgCl_2$ was introduced with the chloroplast suspension).

phoretic mobility (dark) that may be the result of their ability to introduce additional negative surface charges into the membrane components (McLaughlin, 1977; Bakker et al., 1975). The cationic aminoacridine uncouplers ACMA (Table III), 9-aminoacridine, and atebrin (quinacrine) decrease the dark mobilities. This effect, at the used concentrations, is most likely caused by electrostatic binding to negative surface charges (Kraayenhof, 1973, 1977).

A short heat treatment (1.5 min at 60 °C) of a chloroplast suspension drastically lowers the dark mobility and abolishes the light effect. This treatment was earlier shown to result in an increased adsorption of aminoacridine probes (in the dark) which suggested a denaturation-linked exposure of negative charges (Kraayenhof, 1973). However, the electrophoresis experiment rather suggests a concealment of charges, leaving this point questionable. We found that aging of the chloroplasts for 3 h at 25 °C did not affect the dark mobility significantly but gradually diminished the light stimulation (not shown). Treatment of the chloroplasts for 2 min with 2.5% glutaraldehyde results in a decreased dark mobility and light stimulation. It was earlier found that under these conditions ATP synthesis and atebrin binding activities were lost, whereas electron transfer and $H^+$ uptake activities were still present [R. Kraayenhof and L. Packer, unpublished; see also West & Packer (1970)]. It seems, therefore, that $H^+$ uptake or binding is not as closely linked to the generation of negative surface charge as is the binding of aminoacridine probes.

The effect of valinomycin was tested under a variety of conditions. It was found that this antibiotic invariably eliminates the light stimulation both in the presence (Table III) and in the absence (not shown) of added $K^+$ ions.

## Discussion

The presented experiments show that illumination of chloroplasts in the presence of electron transfer mediator causes a marked enhancement of particle migration toward the anode, the effect being most pronounced in broken (envelope-free) chloroplasts. The smaller light stimulation (15–30%) in "intact" chloroplasts confirms the earlier observations by Nobel & Mel (1966). Possibly, a more or less intact outer membrane and stroma phase partially mimic the thylakoid surface charge effects and changes thereof experienced in the plane of shear, explaining the larger light stimulation in stripped chloroplasts exposing their thylakoid membranes. On the other hand, the apparently anomalous effects of $Mg^{2+}$ and $K^+$ ions suggest that the structural and functional integrity of the thylakoid membranes themselves is a prerequisite for the light effect. Extensive depletion of divalent cations and $Tris^+$ washing prior to the electrophoresis experiments (Nakatani et al., 1978) may lead to a modification of gross chloroplast structure (grana stacking) as well as of the molecular structure of the thylakoid membrane; the lipid bilayer is expected to become less ordered and membrane-bound proteins, including the ATPase, are removed. Earlier work on divalent cation binding (Gross et al., 1969; Gross, 1972) shows that divalent cation depletion leads to uncoupling of energy-linked functions. This may explain the lack of light stimulation of electrophoretic mobility in the thylakoid preparations used by Nakatani et al. (1978). The obvious advantage of using stripped membranes, free of cations, in these experiments is that they provide a less complicated model membrane, more suitable for testing the cation effects predicted by the Gouy–Chapman theory. The results of these tests were indeed in good harmony with the theory (Searle et al., 1977; Nakatani et al., 1978). If chloroplast membranes are kept under more physiological conditions during isolation by the addition of $Mg^{2+}$ ions, they show a smaller electrophoretic mobility in the dark, but the stimulatory effect of light is considerably increased. Under these conditions additional $Mg^{2+}$ ions seem to have less influence on charge density and its light-induced increase. This behavior of $Mg^{2+}$ is not simply explainable in terms of models describing cation effects at charged interfaces. A charge-screening effect of $Mg^{2+}$ is obvious (Tables I and II), but in avoiding extensive depletion during the preparation of the membranes, the in situ orientation of membrane components may be more properly conserved, resulting in a decreased accessibility for external $Mg^{2+}$ and also in an enhanced energy-driven generation of negative surface charges. An analogous interpretation of the $K^+$-enhanced mobilities (Figure 2) on top of its charge-screening effect seems to be obvious. Monovalent salts were also found to stimulate the light-induced aminoacridine binding (Kraayenhof & Fiolet, 1974) and to increase the apparent negative charge density on *Nitella translucens* plasma membranes (A. H. C. M. Schapendonk and W. J. Vredenberg, unpublished experiments).

The light-induced increase of negative surface charge density on the thylakoid membrane is shown to be uncoupler sensitive (Table III) and seems to go hand in hand with the light-induced electrostatic interaction of chloroplasts with cationic probes, such as aminoacridines (Kraayenhof, 1973), ethyl red (Heath, 1973), and PMS (Homann, 1976). We will now compare both phenomena in terms of the electric potentials derived from electrophoretic mobility and aminoacridine adsorption data. Because of the fact that this theoretical approach has serious shortcomings if applied to complicated biological membranes, we do not wish to place much emphasis on the quantitative aspects, but rather to underline the qualitative similarity of both phenomena. If one takes the more simple case of broken chloroplasts, incubated in 330 mM sorbitol, 10 mM KCl, and 2 mM Tricine (pH 7.2) as in Figure 2, and one considers the chloroplasts as smooth spherical particles with a radius much larger than the thickness of the ionic double layer, then the electrokinetic or zeta potential ($\zeta$) at the hydrodynamic plane of shear can be calculated according to the equation developed by Helmholtz & von Smoluchowski [cf. Brinton & Lauffer (1959) and Aveyard & Haydon (1973)]:

$$\zeta = \frac{4\pi\eta u}{\epsilon} \tag{1}$$

where $\eta$ is the medium viscosity and $\epsilon$ is the dielectric constant of water. For this particular case $u$ was found to be $13.5 \times 10^{-5}$ cm$^2$ V$^{-1}$ s$^{-1}$ in the dark and $26.5 \times 10^{-5}$ cm$^2$ V$^{-1}$ s$^{-1}$ in the light (96% stimulation); $\eta$ was found to be 9.59 mP and $\epsilon$ is taken to be 78.54 ($H_2O$; 25 °C). If we make the conventional assumptions that the values of $\eta$ and $\epsilon$ in the double layer are equal to their bulk values, eq 1 becomes

$$\zeta = 13.81 \times 10^4 u \text{ mV} \qquad (2)$$

This results in $\zeta = -18.8$ mV in the dark and $-36.6$ mV in the light. $\zeta$ can be converted into average net surface charge densities (at the hydrodynamic plane of shear), by using a simplified Gouy equation (Bull, 1971; McLaughlin, 1977), of $-0.46$ $\mu$C/cm$^2$ ($-1400$ esu/cm$^2$), corresponding to one electronic charge per $\sim$3500 Å$^2$ in the dark, and $-0.95$ $\mu$C/cm$^2$ (2850 esu/cm$^2$), corresponding to one electronic charge per $\sim$1700 Å$^2$ in the light. These values for $\zeta$ and charge densitiy are of the order of magnitude of those calculated by Nobel & Mel (1966) and Nakatani et al. (1978).

Let us now consider the light-induced interaction between the thylakoid membrane and aminoacridine probes under similar incubation conditions. The electrostatic adsorption of aminoacridine probes to negatively charged polymers and liposomes and to dark and illuminated chloroplasts results in apparent upward shifts of the acridine ring nitrogen p$K_a$ (Kraayenhof & Arents, 1977). This apparent p$K_a$ shift can be considered as a measure of the proximity between the centers of the countercharges on membrane and probe molecules. If the average distance of the adsorbed acridine molecules to the membrane surface is $d$, the local potential ($\psi_d$) can be related to the difference between bulk pH (pH$_b$) and local pH at distance $d$ (pH$_d$) according to [cf. Davies & Rideal (1963) and Bakker et al. (1975)]

$$\psi_d = \frac{2.3RT}{F}(\text{pH}_b - \text{pH}_d) \qquad (3)$$

where $RT/F$ is 25.3 mV at 22 °C; pH$_d$ can be calculated from the measured p$K_a$ shift.

Not all aminoacridines have the same affinity to the membrane and this will probably lead to different values for $d$ and $\psi_d$. For the probe PEA $\psi_d$ was found to be $-44$ mV in the dark and $-96$ mV in the light. From the finding that $|\psi_d| > |\zeta|$, we conclude that the aminoacridine molecules come closer to the surface than the hydrodynamic plane of shear. Interestingly, the discrepancy between $\psi_d$ and $\zeta$ becomes larger upon illumination. This could be due to an increased membrane surface ruffling (i.e., membrane particle density or curvature increase) under energized conditions.

The real thylakoid surface potential ($\psi_0$) will be higher than $\psi_d$ and $\zeta$ that are measured at some distance from the surface. We have compared $\psi_0$ and $\psi_d$ in differently charged liposomes with known charge density. Although these artificial lipid vesicles probably have a smoother membrane than thylakoids, in the negative liposomes $|\psi_d|$ was considerably lower than the calculated $|\psi_0|$ (Kraayenhof & Arents, 1977). We must also remember that the $\zeta$ (and possibly also $\psi_d$) measurements give information on the peripheral layer around the chloroplasts, consisting of closely packed thylakoids and not of the separate thylakoid vesicles.

From the above considerations it is obvious that the assumptions, made on the thylakoid membrane structure in order to allow the application of eq 1 for the calculation of $\zeta$, need revision. The outer thylakoid surface is scattered with protein molecules with a radius (say, 50 Å) comparable to the double-layer thickness. If the electrophoretic mobility is determined by the surface contour rather than by gross particle dimensions, Henry's correction of eq 1 seems to become necessary (Overbeek & Lijklema, 1959; Brinton & Lauffer, 1959). This would result in a roughly twofold increase of $\zeta$. However, we feel that these calculations and appropriate corrections have more significance as soon as we have data on more simple and homogeneous preparations of thylakoid membrane vesicles (having retained their structural and functional integrity) and more detailed knowledge on the thylakoid surface structure is available.

Considering the magnitude of the light-induced increase of surface charge density, it seems very unlikely that this phenomenon is a direct result of charge reorientations at the photosynthetic reaction centers, expected for a chemiosmotic type of electron transfer mechanism (Witt, 1979) since these charge changes are expected to be undetectably small by electrophoresis (Nakatani et al., 1978). A more likely mechanism involves energy-dependent structural rearrangements of membrane components. From chemical modification studies Nakatani et al. (1978) plausibly deduced that the thylakoid charge is mainly determined by carboxyl groups, so that membrane proteins are the most likely sites of the charge generation.

It was recently proposed that the induction of a slow component of the 515-nm (carotenoid) absorbance change is associated with the occurrence of an energy-linked rearrangement of fixed charges in the vicinity of the cartenoid–chlorophyll $b$ pigment complex (Schapendonk et al., 1979; Schapendonk & Vredenberg, 1979). This phenomenon shows similar sensitivities toward chemical and physical treatments as the light-stimulated electrophoretic mobility. The fact that the light-stimulated generation of fixed negative charges can be catalyzed by cyclic electron transfer (Table III) suggests that this phenomenon could indeed be entirely dependent on structural changes in the photosystem I domain.

If the aminoacridine binding is believed to report on the same charge generation as that of the electrophoresis experiments, then the kinetics of the former process show that this charge generation is much faster than the energy-dependent increases of chloroplast light scattering and bulk ion translocation but has a similar half-time ($\approx$50 ms) as a light-induced conformational change in the membrane-bound ATPase, monitored with a covalent fluorescent label, and the significance of this correlation is discussed elsewhere (Kraayenhof, 1977).

Finally, important influences on the apparent change of charge density upon illumination could come from fast short-range redistributions of protons and other ions and by changes of the local dielectric constant and viscosity in the interface and ionic double layer. The latter two entities are assumed to remain constant in the course of membrane energization, but the validity of this assumption is questionable (Brinton & Lauffer, 1959; Aveyard & Haydon, 1973).

Further experiments are designed to clarify the possible role of the light-induced generation of negative surface charges in the energy-transduction process.

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