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Regulation of the imbalance in light excitation between Photosystem II and Photosystem I by cations and by the energized state of the thylakoid membrane

Gur Braun and Shmuel Malkin

Biochemistry Department, Weizmann Institute of Science, Rehovot (Israel)

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The imbalance in photoactivity between the two photosystems in broken chloroplasts during steady-state electron transport was investigated using modulated chlorophyll *a* fluorimetry and oxygen evolution. No imbalance in favor of PS II (imbalance term equals zero) was found at low cation concentration (e.g., 10 mM NaCl) where the membranes are unstacked, while some imbalance in favor of PS II (imbalance term about 0.1–0.2) could be observed at 'high' cation medium (e.g., 100 mM for univalent, 5 mM for divalent and 100 μM for trivalent cations) where the membranes are stacked. At the high cation concentration the imbalance was particularly noticeable at a pH range 6–7.5 under conditions where the membranes were non-energized, e.g., in the presence of a range of uncouplers and ionophores (an imbalance term of between 0.44 and 1.1). In the absence of uncouplers or ionophores the imbalance term was initially high, decreasing to a low steady-state value during the light-induced energization of the membranes. The increase in the imbalance measured with the addition of gramicidin D was wavelength-dependent, implying changes in the allocation of excitation energy to the photosystems rather than any other mechanism. This effect was reversed at higher pH: At a pH higher than about 8, the imbalance in absence of uncouplers or ionophores was stronger than in their presence. The relation between the state of imbalance and the cross-membrane proton gradient (ΔpH) was not straightforward or simple, as follows: (i) Imbalance was induced by uncouplers and ionophores also at sufficiently low light intensities which produce only very small ΔpH. (ii) Valinomycin (+KCl) had the same effect as uncouplers like gramicidin D, nigericin, NH_4Cl and others, although it presumably abolishes membrane potential but not ΔpH. (iii) The effect of gramicidin D and NH_4Cl was close to saturation at concentrations which affect ΔpH still minutely. (iv) The same level of large imbalance could be achieved even without uncouplers or ionophores when a much higher cation concentration (approx. 10-fold) than that considered normal to achieve membrane stacking was used. It is therefore concluded that: (a) High cationic levels change the allocation of excitation energy, probably via the screening of negative charges on the thylakoid surface. (b) The effect of uncouplers and ionophores in the presence of high cationic levels most probably reflects, at least in part, the effect on external surface charges, their exposure by membrane energization and their screening by cations.

Introduction

Activity distribution between Photosystem I (PS I) and Photosystem II (PS II) of oxygenic photosynthesis in light-limiting conditions is regulated in response to the ambient conditions [1–7]. At least two 'states' of energy distribution are known in vivo: 'state 1', achieved by adaptation to far-red light ($\lambda > 690$ nm) absorbed mostly by PS I (light 1) and 'state 2', achieved by adaptation to light of shorter wavelengths (light 2). In state 1, such short wavelength light excitation ($\lambda < 690$ nm) is absorbed in favor of PS II, particularly at wavelengths where chlorophyll *b* absorbs most (around 480 and 650 nm). In state 2, light 2 tends to distribute itself more evenly.

In cyanobacteria and red algae, the process of light distribution presumably involves direct Förster-type excitation energy transfer from PS II to PS I, which is also regulated in response to the ambient conditions, al-

Abbreviations: PS, photosystem; ATP, adenosine triphosphate; NADP, nicotinamide dinucleotide phosphate; LHC, light-harvesting chlorophyll *a*/*b*-protein complex; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; GD, gramicidin D; MeV, methylviologen; TEC, tris(ethylenediamine)cobalt(III) chloride; 9-AA, 9(5)-aminoacridine hydrochloride monohydrate.

Correspondence: S. Malkin, Biochemistry Department, Weizmann Institute of Science, Rehovot 76100, Israel.

though the details of this regulation are still not resolved [4]. In higher plants, however, PS I and PS II complexes are considered to be located mostly in different regions of the membrane [8–10], thereby making energy transfer impossible (however, cf. Ref. 11). In this case, state 1 to state 2 transition involves redistribution of pigment-protein complexes between PS II and PS I [9,12].

In vitro, one can further distinguish two other states. Thylakoid membranes in suspension show different characteristics depending on the medium composition: In the so-called 'low-salt' medium, the granal structure of the photosynthetic membranes disintegrates and the membranes become completely unstacked [13]. In a 'high-salt' medium, the thylakoids preserve or reform the granal structures with connections by isolated membrane bridges resembling the stroma lamellae [13,14]. Such a state is also characterized by a high level of the variable fluorescence relative to the non-variable fluorescence [15–17] and thus resembles the in vivo situation. Moreover, it was shown that in this state one can induce phosphorylation of the light-harvesting complex of PS II (LHC II) in vitro and by this induce a range of phenomena similar to those reflecting the in vivo state 1 to state 2 transition [18]. However, in these measurements there was neither quantitation of the activity distribution nor a clear relation about how such a distribution depends upon the ambient conditions. Seemingly, there are contradictory results depending on whether or not salt levels (cations) change the allocation of light energy to PS I and PS II [15,19–21].

The aim of the present work was to quantitate accurately the light activity distribution between the two photosystems in isolated chloroplasts and to examine the changes which occur in it upon a transition from a 'low-salt' to a 'high-salt' medium. The main experimental tool used was modulated fluorimetry [6,18,22], where activity distribution in light limiting conditions is defined by measuring three fluorescence yield parameters: (a) the momentary fluorescence as it varies with time (F) or in the steady-state (F_s); (b) the fluorescence corresponding to fully opened reaction centers of PS II (F_o); (c) the fluorescence corresponding to fully closed reaction centers of PS II (F_m). In such analysis, one distinguishes between changes in F_m alone (which were usually used to characterize the transition from 'low'- to 'high'-salt media) and changes in the steady-state level, F_s, relative to F_o and F_m, which reflect changes in the light distribution. In this paper, we provide a firm evidence that salt levels change the allocation of light energy to the photosystems. Furthermore, a surprising and significant relation between the activity distribution and membrane energization is found. It will be shown that this relation is at least partially established through effects exerted by the 'high-energy' state on the membranal surface.

Materials and Methods

Materials

Hepes, Mes, antimycin A, gramicidin D, valinomycin, nigericin, methylviologen (MeV) and NADP were purchased from Sigma. 9(5)-Aminoacridine hydrochloride monohydrate (9-AA) was purchased from Fluka, Switzerland. TEC was purchased from Alfa Products, Danvers, MA, U.S.A. 2,6-Di-*t*-butyl-4-(2′,2′-dicyanovinyl)phenol (SF6847) was kindly provided by Dr. V. Nishizawa, Sumitomo Chemical Industry, Osaka, Japan. 5-Cl,3-*t*-butyl,2′-Cl,4′-NO_2-salicylanilide (S13) was kindly given by Dr. P. Hamm, Monsanto, St. Louis, MO, U.S.A. Ferredoxin, isolated from *Spirodella*, was kindly given by Dr. Y. Shahak from our department.

Chloroplast preparation

Broken chloroplasts from market lettuce or from greenhouse-grown spinach or tetragonium were prepared and stored in a liquid nitrogen container as described in Ref. 23. The storage buffer contained 0.4 M sucrose, 20 mM Hepes, 10 mM NaCl, 5 mM $MgCl_2$ and 30% (v/v) ethylene glycol (pH 7.3). Total chlorophyll concentration was determined according to Ref. 24.

Reaction mixtures

For the fluorescence measurements, chloroplasts were diluted 500–1000 fold in a 2 ml cuvette, so that the concentration of chlorophyll did not exceed the value of 5 μg/ml. The standard reaction mixture contained 20 mM Hepes (pH 7.3–7.4), 10 mM NaCl, 5 mM $MgCl_2$ and 200 μM MeV as an electron acceptor. In some experiments ferredoxin (10 μM) and NADP (1 or 2 mM) replaced MeV. In different experiments $MgCl_2$ was excluded or replaced by 100 mM NaCl. For the study on the pH dependence, the reaction mixtures contained 10 mM Hepes, 10 mM Mes, 10 mM NaCl, 5 mM $MgCl_2$ and 200 μM MeV. Different pH levels were obtained by titration with NaOH.

Chlorophyll a fluorescence measurements.

Modulated chlorophyll *a* fluorescence was measured by a home-built fluorimeter with two exciting light sources powered by d.c. supplies. The light from one source (the measuring modulated light) was chopped at a frequency of about 100 Hz, passed through a 10 nm bandwidth 480 nm interference filter (Baird Atomic) and delivered to the sample by lens optics (max. intensity ≈ 3.5 nE $\cdot$ cm^{-2} $\cdot$ s^{-1}). This intensity was sufficient to exert also actinic effects, but still within the light-limiting range of electron transport. The fluorescence signal from the sample was delivered through a fiber-optic light guide to a photodiode detector (EG&G HUV 4000) and separated by an interference 683 nm filter (Ditric Optics – 10 nm bandwidth) together with

RG 665 long-pass glass filter (Schott) and a 700 nm short-pass interference filter (Ditric Optics) for further spectral purity. The signal from the photodetector was processed by a lock-in amplifier (PAR 128 A) the output of which, corresponding to the amplitude of the modulated fluorescence, was recorded on a strip chart recorder (Houston Instrument, Omniscribe D 5000). The light from the second source was not modulated and served to exert actinic effects (background light). It either passed through a combination of short-pass 580 nm filter (Ditric Optics) and a blue broad band (Corning 4-96 glass) filter to serve as saturating light for electron transport (intensity about 120 $nE \cdot cm^{-2} \cdot s^{-1}$) or alternatively through a 10 nm band-width 730 nm interference filter (Ditric Optics) serving as light 1 (maximum intensity of about 30 $nE \cdot cm^{-2} \cdot s^{-1}$).

Variation of light intensities was achieved by use of neutral-density filters (Balzers). Excitation of the sample with the measuring light or the addition of actinic light was initiated and terminated by the operation of two electronic shutters. Before each measurement, the chloroplasts were suspended and diluted in the dark for at least 2 min. All measurements were done at room temperature (22–25 °C).

Excitation under light-limiting conditions with the weak modulated 480 nm light finally resulted in a certain steady fluorescence signal, F_s. Superimposing the measuring modulated beam with the actinic photosynthetically saturating light yielded a momentary maximal saturated modulated fluorescence signal, F_m, which was also checked occasionally for no further increase by the introduction of DCMU. Superimposing 730 nm light in sufficient (saturating) intensity lowers fluorescence momentarily to a minimum fluorescence level, taken to represent the parameter F_o. This method was described previously [6,22].

Calculation of photo-activity distribution between the photosystems in the modulated light from the modulated fluorimetry

The chlorophyll fluorescence quenching from F_s to F_o by light 1 (far-red light) is an alternative parallel monitor of the Emerson enhancement effect in rate measurements and leads essentially to the same information. The light activity distribution coefficients of PS II and PS I (β and α, respectively) are related to the chlorophyll fluorescence parameters [6]. We define the degree of openness of PS II reaction centers, f, by the experimental value of F_s weighed against F_o and F_m:

$$f = (F_m - F_s)/(F_m - F_o) \quad (1)$$

The need of balance in electron flow between the two photosystems in the steady state, when no additional light is added, requires:

$$f\beta = \alpha \quad (2)$$

Hence, the ratio of photoactivities PS II/PS I is simply:

$$\beta/\alpha = 1/f \quad (3)$$

Eqns. 2 and 3 are valid only for the case when the light distribution is in favour of PS II (i.e., PS I is limiting) or at least in full balance. In the last case $f = 1$ and hence $\beta = \alpha$.

If there is no waste of light energy (i.e., $\alpha + \beta = 1$) it follows also that:

$$\beta = 1/(1+f); \quad \alpha = f/(1+f). \quad (4)$$

Our results were expressed frequently in terms of $(\beta/\alpha) - 1$, i.e., the relative deviation from full balance between the two photosystems, which from Eqn. 3 is equal to $(1-f)/f$. In this case, no assumption with respect to a possible waste of light energy is made, and the contribution of each photosystem alone to the imbalance is actually not attempted.

9-Aminoacridine fluorescence measurements

9-Aminoacridine (9-AA) fluorescence quenching was used to estimate ΔpH across the thylakoid membrane [25,26]. 10 μM of 9-AA incorporated in the chloroplast suspension was excited in parallel to the chlorophyll fluorescence by the introduction of an additional 100 Hz weak modulated excitation beam through a 396 nm interference filter (Ditric Optics). The 9-AA excitation light intensity was adjusted to the lowest level possible for tolerable signal/noise ratio. Light emission was led through additional light guide and a 450 nm interfernece filter to a second photodetector connected to a separate lock-in amplifier. In such experiments the 480 nm excitation of chlorophyll had a different modulation frequency of about 150 Hz so that one excitation did not influence the emission signal of the other, as was also checked independently. Even when the two beams had the same frequency the 9-AA excitation light intensity was very low so as not to influence the 683 nm signal from chlorophyll fluorescence. The proton concentration inside the thylakoids, $[H^+]_i$, was calculated using the formula described in Ref. 26:

$$[H^+]_i/[H^+]_o = [(F_1/F_2) - 1]V_o/V_i$$

where $[H^+]_i$ and $[H^+]_o$ are the proton concentrations in the inside and outside compartments of the thylakoids, respectively, F_1 is the 9-AA fluorescence of non-energized membranes obtained after addition of saturating amounts of gramicidin D or of NH_4Cl. F_2 is the 9-AA fluorescence at any other condition of the measurement. V_o and V_i are the volumes of the outer and inner compartments of the thylakoids, respectively, calculated according to Ref. 26.

$$V_e/V_i = \frac{\text{(osmolarity of the medium)}}{[2(\text{chlorophyll molar concentration})]}$$

It is possible that the ΔpH values are overestimated due to binding of the 9-AA to negatively charged residues, mainly in the internal surface of the thylakoid membrane [27,28].

Measurements of MeV reduction

The rate of MeV reduction was determined from oxygen uptake measurements using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). Chlorophyll concentration in each measurement was 50 μM in a 4 ml reaction mixture containing 200 μM MeV, 20 mM Hepes and 10 mM NaCl. The uncoupler gramicidin D was added in several measurements to a final concentration of 1 μM. $MgCl_2$ was added where indicated to a final concentration of 5 mM. Illumination of the sample took place from two perpendicular directions. Light 2 (intensity not exceeding 2 $nE \cdot cm^{-2} \cdot s^{-1}$) was supplied by a light source with a 480 nm interference filter (Baird Atomic). Light 1 (intensity not lower than 20 $nE \cdot cm^{-2} \cdot s^{-1}$) was supplied by a light source with a 720 nm interference filter (Ditric Optics). For the determination of rates we used the initial slopes of the kinetic traces obtained immediately after the onset of illumination. Emerson enhancement in the rate of MeV reduction was calculated as the ratio between the rate obtained when light 2 and light 1 were superimposed and the sum of the rates obtained with each light alone. Practically, the rates obtained with light 1 alone where always negligible or zero. Chlorophyll concentration was 50 μM, about 5-fold the concentration in the fluorescence experiments. Dilution of the chloroplasts in the absence of $MgCl_2$ resulted in a relatively high residual amount of $MgCl_2$ (about 50 μM) which was higher than the residual amount of $MgCl_2$ (about 10 μM) in the fluorescence measurements.

Results

Modulated fluorescence measurements were designed to measure the effect of various factors, such as light intensity, pH, cations and others, on the fluorescence parameters F_o, F_s, F_m and the resulting ratio β/α, in a suspension of thylakoid membranes. In order to ascertain that indeed the system is in light-limiting conditions and to facilitate electron transfer between PS II and PS I, the uncoupler gramicidin D was initially included in the reaction medium without suspecting any special effect. Fig. 1 describes a time-course of the change in fluorescence in a standard reaction mixture upon illumination with weak 480 nm light (light 2) and the effect of adding either far-red (light 1) or strong saturating background lights. As stated above, the maximum extent of fluorescence quenching from F_s to F_o by far-red light reflects the imbalance between the photosystems in the modulated light 2 alone [6,18]. The effect of light 1 is to alleviate the limitation on electron transport by PS I. When light 1 is in excess all PS II reaction centers, which participate in whole electron transport, are presumably maximally open and the fluorescence is at its minimum. On the other hand, with the addition of strong saturating actinic illumination, electron transport is saturated and the fluorescence approaches a maximum level, F_m, which represents the fluorescence obtained when all PS II reaction centers are closed and the quantum yield of photochemistry approaches zero.

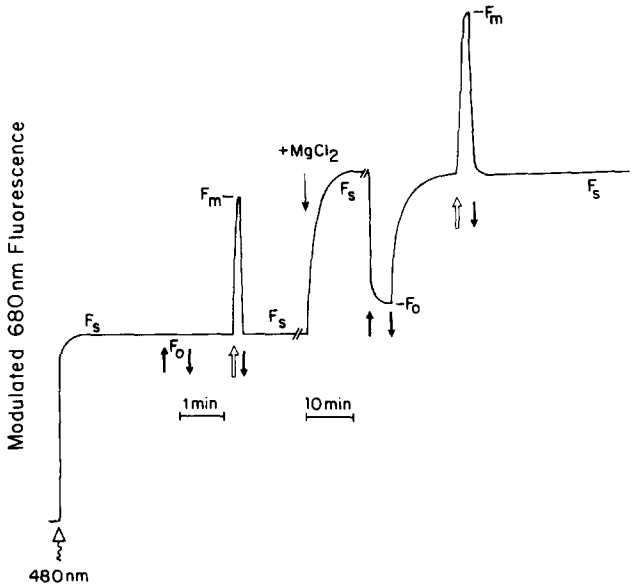

Fig. 1. Time-course of the change in the modulated 680 nm fluorescence, the effect of different illumination conditions and of $MgCl_2$. Fluorescence parameters F_m and F_o were determined as described in Materials and Methods with a weak 480 nm modulated light (approx. 1 $nE \cdot cm^{-2} \cdot s^{-1}$), saturating non-modulated broad-band blue light (approx. 120 $nE \cdot cm^{-2} \cdot s^{-1}$) and saturating 730 nm non-modulated light (approx. 30 $nE \cdot cm^{-2} \cdot s^{-1}$). The reaction mixture contained 20 mM Hepes (pH 7.3), 10 mM NaCl, 1 μM gramicidin D, 200 μM MeV and 5 μM chlorophyll. $MgCl_2$ was added to a final concentration of 5 mM. The upward open arrows denote the turn-on of the saturating light to obtain the F_m. The upward closed arrows denote the turn-on of the far-red light to obtain F_o. Closed downward arrows denote the turn-off of either the saturating or far-red lights. The wavy arrow denotes the turn-on of the modulated light. Other details are as described in Materials and Methods.

The experiment of Fig. 1 started with unstacked thylakoids, i.e., under low cationic environment ('low-salt'). In this case, no over-balance of PS II could be distinguished (i.e., $F_s = F_o$). When cations were added to stack the thylakoids there occurred, besides the previously known large increase in F_m (and slight increase in F_o), also a large deviation of F_s from F_o, indicating an over-balance of PS II relative to PS I. The overbalance is thus a property of stacked thylakoids, which are closer to the in vivo state. This experiment was performed for the usual range of cation concentration sufficient for this purpose ('high salt' – around 100 mM for univalent cations, e.g., NaCl or KCl or 5 mM for

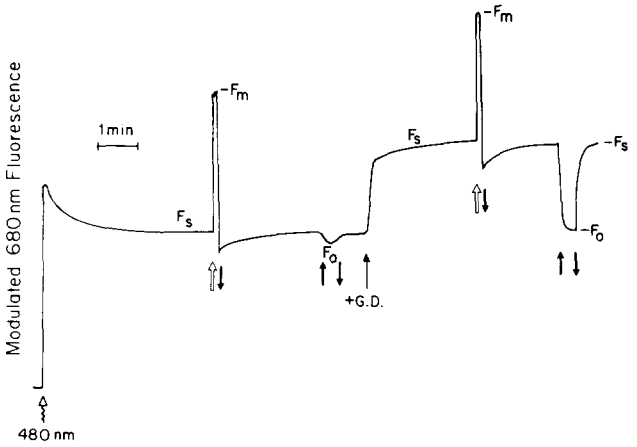

Fig. 2. Time-course of the change in the modulated 680 nm fluorescence, the effect of different illumination conditions and of gramicidin D. The conditions are similar to those in Fig. 1 except that the reaction mixture contained already 5 mM $MgCl_2$ (standard reaction mixture). Gramicidin D was excluded and added afterwards to a final concentration of 1 μM.

divalent cations, e.g., $MgCl_2$ or $CaCl_2$). The above result supports the idea that cations cause a change in the allocation of light energy so that PS II gets more excitation than PS I and therefore there is an imbalance between the photosystems [15,19,21].

It came somewhat as a surprise to discover that gramicidin D plays an essential role in the above phenomenon. Fig. 2 shows that in the absence of gramicidin D in the same 'high-salt' medium there was an initial slow light-induced decreasing transient of F_s due to illumination with the measuring modulated light which was completed within several minutes. Light 1 did not then induce much further quenching, indicating a marginal imbalance in the steady state. Addition of gramicidin D when light 1 is turned off led to a dramatic increase of F_s by about 50% within 1–2 s, followed by an additional increment to about 60–70% after 2 min. Such an increase in F_s led subsequently to a large extent of fluorescence quenching by light 1 of more than 4.8-fold, while F_o and F_m changed only slightly. Seemingly, gramicidin D is an essential factor in getting a maximum imbalance between PS II and PS I. The above experiments were repeated with NADP (plus ferredoxin) as an electron acceptor, giving very similar results (not shown).

In order to examine whether the effect of gramicidin D is related to its property as an uncoupler (i.e., abolition of proton electrochemical-gradient), different uncouplers were tested likewise, indeed causing a similar large increase in F_s and the related quenching by far-red light. Actually, the same effect was detected with a variety of protonophores, cation-proton exchangers and ionophores (Table I). For brevity, for the purpose of this article only, we will refer to all these compounds as 'uncouplers'. The imbalance values in Table I represent typical values obtained in single experiments. Using gramicidin D we could obtain higher values with different samples (up to 1.1).

To further show that the effect of gramicidin D is indeed related to the excitation imbalance between PS II and PS I, we measured the imbalance term for a range of wavelengths of the modulated light, in absence or presence of gramicidin D (Fig. 3). The two spectra described in Fig. 3a and b were measured in two different experiments. In a preliminary experiment (Fig. 3a) the incident light intensity was set to be equal in each wavelength. In the other (Fig. 3b) the light intensity was set so that the absorbed intensity was equal. The spectra in Fig. 3a and particularly those in Fig. 3b are similar in their general features to the spectrum obtained for the Emerson enhancement in vivo [5]. They show three bands with peaks at approx. 480, 550 and 650 nm. From the above similarity of the spectra it is most likely that the effect of gramicidin D indeed reflects changes in the imbalance between PS II and PS I (cf. also Discussion).

In order to gain more direct evidence for the effect of cations and gramicidin D on the photosystem imbalance, measurements of electron transport rate were also performed. Table II demonstrates the effect of added $MgCl_2$, gramicidin D or both on the Emerson enhancement in the rate of MeV reduction, using a Clark-type oxygen electrode. Addition of $MgCl_2$ induced the Emerson enhancement, indicating an imbalance in favor of PS II. Gramicidin D alone also caused an appearance of enhancement, to a lesser extent, probably due to the presence of residual $MgCl_2$ in the reaction mixture which was more concentrated than in fluorescence measurements (see Materials and Methods). The most dominant effect on the enhancement appeared when

TABLE I

Comparison between the effect of several uncouplers and ionophores on the imbalance between PS I and PS II

The imbalance term ($\beta/\alpha - 1$) was calculated from the fluorescence parameters obtained in steady state before and after the addition of the uncoupler/ionophore ('uncoupler'). The reaction mixture and the experimental procedure and conditions were as described in Fig. 2 and in Materials and Methods.

Type of 'uncoupler'	Concentration of 'uncoupler' (M)	Deviation from full balance ($\beta/\alpha - 1$)	
		−'uncoupler'	+'uncoupler'
NH_4Cl	$1 \cdot 10^{-2}$	0.13	0.56
Gramicidin D	$5 \cdot 10^{-7}$	0.13	0.7
S-13	$1 \cdot 10^{-6}$	0.13	0.56
SF6847	$2 \cdot 10^{-8}$	0.13	0.44
Antimycin A	$5 \cdot 10^{-6}$	0.13	0.44
Nigericin	$2 \cdot 10^{-6}$	0.13	0.54
Valinomycin (+30 mM KCl)	$2 \cdot 10^{-6}$	0.13	0.62

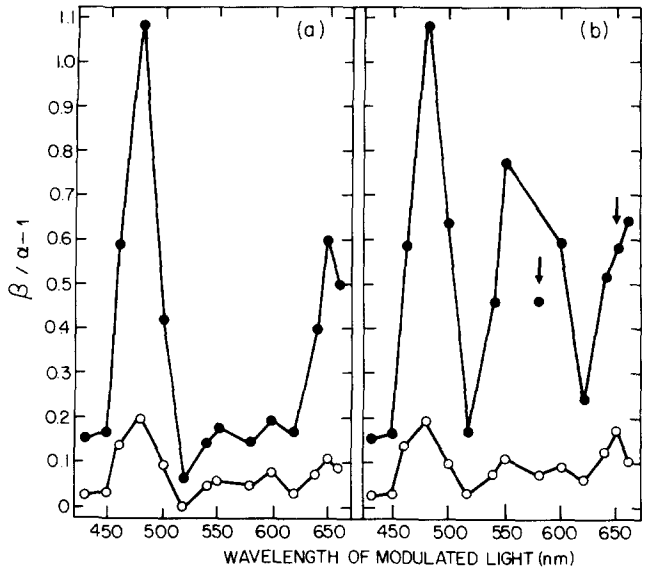

Fig. 3. Dependence of the deviation from full balance, $\beta/\alpha - 1$, on the wavelength of the modulated light. The imbalance term, $\beta/\alpha - 1$ was calculated from the fluorescence parameters obtained in steady state before (open circles) and after (closed circles) the addition of 1 μM gramicidin D to the standard reaction mixture. Other experimental conditions were as described in Materials and Methods except that the wavelength of the modulated light was varied using interference filters (bandwidth 5–10 nm). In (a) the incident light was adjusted by neutral-density filters to the same level at each wavelength. In (b) the incident light was likewise adjusted to equalize the absorbed light intensity, using data from light absorption measurements with an integrating sphere. In both cases, the lowest light intensities (0.35–0.38 $nE \cdot cm^{-2} \cdot s^{-1}$) yielded the maximum effect of the uncoupler at 480 nm. However, 580 nm and 650 nm points (these points are indicated by arrows) were exceptional in that the interference filters did not pass enough light intensity to reach the same absorbed intensity as for the other points.

both high $MgCl_2$ and gramicidin D were applied. These results go in line with the fluorescence measurements and as is shown below, the competence of the 'uncoupler' (gramicidin D in this case) to exert its effect on the imbalance depends strongly on the cation concentration.

TABLE II

Effects of $MgCl_2$ and gramicidin D on the Emerson enhancement in MeV reduction

Calculation of the enhancement in the rate of MeV reduction (E) and the experimental conditions were as described in Materials and Methods.

G.D. (0.5 μM)	$MgCl_2$ (5 mM)	E
−	−	1
−	+	2.3
+	−	1.9
+	+	3.8

The synergism of both high cationic level and 'uncouplers' to establish a remarkable imbalance between the two photosystems led to the question of whether higher levels of the first can replace the other in bringing the same effect. The imbalance term, $\beta/\alpha - 1$, was measured in a wide range of $MgCl_2$ concentrations before and after the addition of gramicidin D. Fig. 4a shows that with increasing concentrations of $MgCl_2$ the imbalance increases until a saturation value is reached. In the presence of the uncoupler this saturation is reached at about 10–20 mM $MgCl_2$. In the absence of uncoupler there is still a tendency to reach the same saturation value but at much higher (roughly 10-fold) $MgCl_2$ concentration. Since the highest $MgCl_2$ concentration used could cause secondary effects, we performed an additional experiment with the trivalent ion TEC, which is known to cause surface charge screening, membrane stacking and grana formation already in the micro-molar range [29]. The results presented in Fig. 4b indeed show a similar pattern of behavior as demonstrated for $MgCl_2$ in Fig. 4a, but at much lower con-

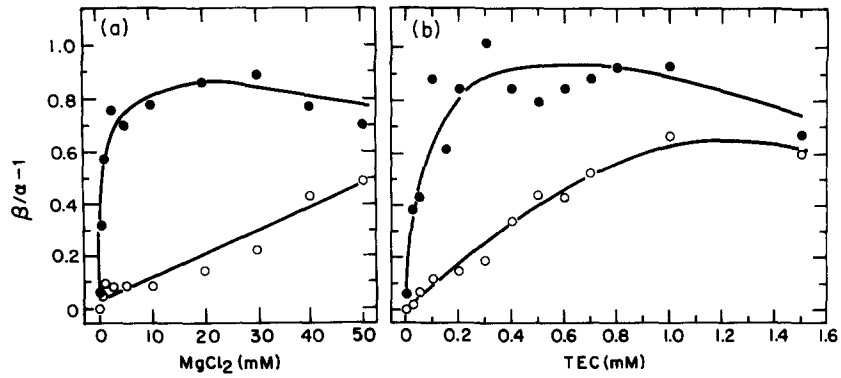

Fig. 4. Effect of cation concentration and of gramicidin D on the deviation from full balance. The imbalance term, $\beta/\alpha - 1$, was calculated from the fluorescence parameters obtained in steady state before (open circles) and after (closed circles) the addition of 1 μM gramicidin D. The reaction medium contained 20 mM Hepes (pH 7.3), 10 mM NaCl, 200 μM MeV and different concentrations of (a) $MgCl_2$ or (b) TEC. In the case of TEC $MgCl_2$ was omitted from the medium and its concentration did not exceed 10 μM. Other details are as described in Fig. 1 and in Materials and Methods.

centrations. Thus, the effect of the 'uncouplers' requires moderately high cationic levels, while extremely high concentrations of cations substitute for the 'uncoupler' for this effect.

If the 'uncoupler' effect on the imbalance is related to the abolition of a proton electrochemical gradient, one expects a high imbalance in the absence of 'uncouplers' at the start of the irradiation, decreasing in time towards a more balanced state, as a proton-gradient is built. Such a transient is indeed observed, as shown already in Fig. 2. The following experiment (Fig. 5a and b) examined this initial transient in more detail. As expected, the transient occurred only when a 'high-salt' medium was used, while, as checked, the same ΔpH was built-up during illumination in either 'low'- or 'high'-salt media [26]. All fluorescence parameters, F_s, F_o and F_m, decreased during the transient time, but while at the end of the transient gramicidin D caused a dramatic increase in F_s, it only partially reversed F_m and hardly affected F_o. Thus, there is only a partial similarity between the state at the initial time of illumination without uncoupler and the state achieved finally with the uncoupler. With regard to energy distribution per se, as calculated from the above parameters, there was initially much more imbalance which decreased towards a steady state, as expected.

The experimental observation of the transient presented some problems, however. The imbalance term, $\beta/\alpha - 1$, obtained after addition of an 'uncoupler', was always higher than the initial transient value following dark adaptation. The extent of the transient at pH 7.3 was not always reproducible. In particular, there were instances where the extent of the transient was quite small, becoming faster and larger when the light intensity increased. Sometimes the transient did not even occur at all, while the effect of the 'uncouplers' occurred as usual. One possible explanation may be the occurrence of a very rapid phase of membrane energization with a concurrent decrease of the imbalance term in a time shorter than the resolution time of our instrument, so that the true initial imbalance value may be indeed higher, close to that obtained with the uncoupler.

In connection with the experiments presented in Fig. 5a and b, one must note that membrane energization affects also the level of F_m, as already mentioned in the literature [30] so that F_m is quenched with time under continuous illumination (energization-dependent quenching) and is reversed upon addition of 'uncouplers'. It seems, however, that there is also a slow and irreversible decrease in F_m which was seen when an 'uncoupler' was present initially (Fig. 5b – level F_{m2}) or after addition of an 'uncoupler' (Fig. 5a – level F_{m3}) relative to the initial level. The extent of the irreversible quenching of F_m (and probably also of F_s) was higher for the case when the membranes were energized (i.e., under illumination in the absence of an 'uncoupler').

The relation between the changes in F_s (which are closely related to the imbalance) and the changes in the membrane energization was examined by simultaneous

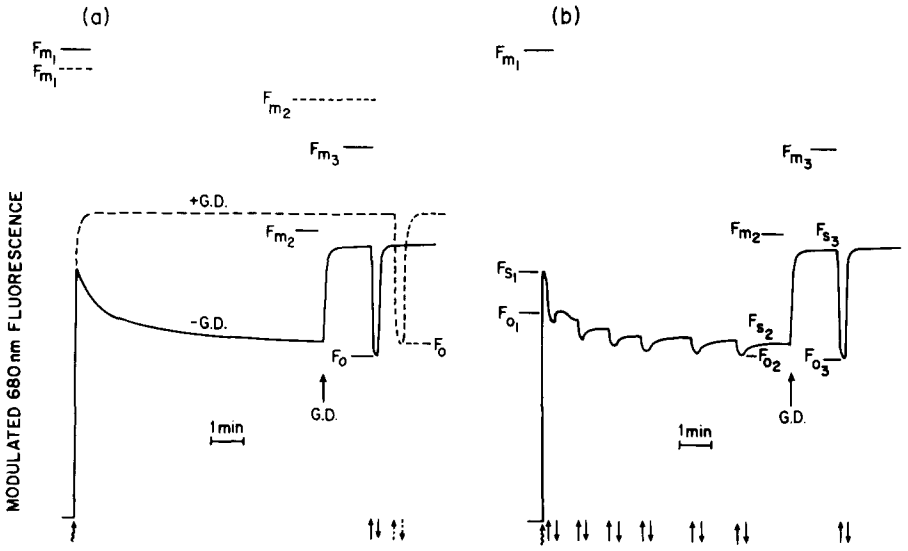

Fig. 5. Time-course of the change in F_o and F_s during illumination; the change in F_m and the effect of addition of gramicidin D. (a) Solid curve, F_s as a function of time from the onset of illumination with gramicidin D added only after a steady state is reached; dashed line, the same with gramicidin D added prior to illumination. Initial and final values for F_m and the steady-state F_o are also shown (horizontal lines). (b) As with the solid curve in (a) but with additional intermittent far-red (730 nm) illumination to measure F_o. The numerical subscripts for the fluorescence parameters, F_m, F_o, F_s denote: 1, the initial levels after dark-adaptation; 2, in the steady state obtained with illumination and 3, after the addition of gramicidin D. Gramicidin D was added to the standard reaction mixture up to a final concentration of 1 μM. The arrows represent turning-on and off the far-red light. The dashed arrows in (a) refer to the sample with pre-added gramicidin D. Other experimental details are as described in Fig. 2. Values of $\beta/\alpha - 1$ corresponding to the dark-adapted state, to the final steady state in the absence of gramicidin D and after its addition were 0.3 and 0.13 and 0.7, respectively.

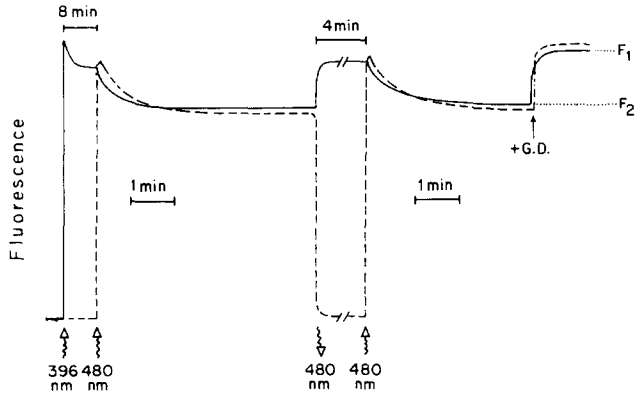

Fig. 6. Changes in the modulated fluorescence of 9-aminoacridine and of chlorophyll *a* due to different illumination conditions and due to gramicidin D addition. Chlorophyll fluorescence parameter F_s (dashed curve) and 9-AA fluorescence (solid curve) were monitored in parallel as described in Materials and Methods with the modulated 480 nm light intensity of 1 nE·cm^{-1}·s^{-1}. ΔpH was calculated from F_1 and F_2 as described in Materials and Methods. Standard reaction mixture was used for the experiments with the addition of 10 μM 9-AA. The wavy arrows upward and downward denote turning-on and turning-off the modulated lights, respectively.

observation of 9-AA fluorescence and of F_s for both the initial transient in the absence of 'uncouplers' and for the effect of the uncoupler. This is shown in Fig. 6. The sensitivities of the measuring instruments were set so that the transients in 9-AA fluorescence and in F_s were normalized to the same initial value. Incidentally, the extents of the changes were also about the same. More importantly, the kinetics of the two phenomena were very similar. The experiment of Fig. 6 was used to calculate the ΔpH as described in Materials and Methods. The final steady-state ΔpH was about 3.1 with modulated light intensity of about 1 nE · cm^{-2} · s^{-1} and 3.5–3.7 with light intensity above 3 nE · cm^{-2} · s^{-1}. Prior to the experiment, there was an initial very slow phase of decline to a steady state in the 9-AA fluorescence by the measuring light used for 9-AA excitation. This is probably related to the equilibrium of the pigment with the membranes and not to any light-induced formation of ΔpH, as this excitation light was very low and also since, as can be seen in the figure, the later addition of saturating amounts of the uncoupler did not cause the fluorescence to rise back to its initial level. In order to examine the question whether the bulk ΔpH is directly involved in the phenomena observed, we checked the correlation between ΔpH and the imbalance extent $\beta/\alpha - 1$ in the steady state by varying the uncoupler concentration. Fig. 7 shows that the relation between the two is not linear and that $\beta/\alpha - 1$ is much more sensitive to the uncoupler than the ΔpH. For example, in the case of gramicidin D, the imbalance is within 10% of the saturation value while ΔpH decreased to about 60% (Fig. 7a). Most of the collapse in the ΔpH occurred, in both cases, after at least 75% deviation from full balance was already reached. In the case of NH_4Cl, the imbalance extent is close to its saturation value while ΔpH decreased only by about 40% (Fig. 7b).

There was a marked difference between the rate and extent of the light-induced transient in F_s obtained in pH 7.3 to those obtained in pH 8.5 so that the rate and extent of the transient were much smaller in the higher pH. It was therefore of interest to look for the effect of pH on the imbalance. Fig. 8 describes the dependence of the imbalance between the photosystems on the external pH of the medium in the presence or the absence of gramicidin D or NH_4Cl. In their absence, there is no essential change in the low imbalance value between pH 5.5 and 7. However, as the pH increases above 7 the imbalance increases significantly, reaching a maximum at a pH around 8.5–9. The presence of the uncouplers resulted in a shift of the above curve to lower pH values, thus obtaining higher values of the imbalance in the range of lower pH, with a maximum at about pH 7. The dependence of the imbalance on pH could therefore be due partly to mixed effects of both internal and external pH with the internal pH playing a dominant role. For example, at an external pH of 8.2,

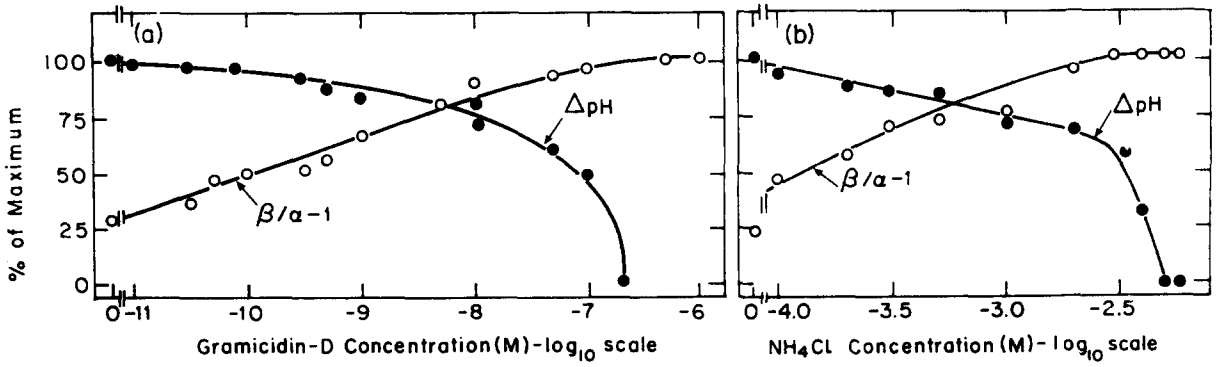

Fig. 7. Effect of the uncoupler concentration on the deviation from full balance and on ΔpH. The imbalance term, $\beta/\alpha - 1$ (open circles) is given as the percent fraction of the maximum obtained with saturating concentration of (a) gramicidin D or (b) NH_4Cl. ΔpH (closed circles) is given as the percent of the maximum obtained without the uncoupler. ΔpH was determined from the 9-AA fluorescence data and its maximal value was about 3.1 in the standard reaction mixture. The other experimental details were as in Fig. 6.

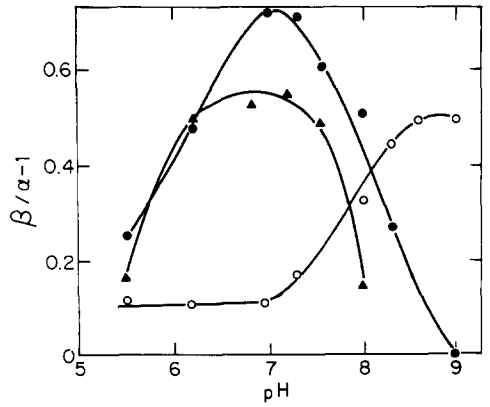

Fig. 8. pH-dependence of the deviation from full balance $\beta/\alpha - 1$ and the effect of gramicidin D and of NH_4Cl. Open circles denote control experiment without uncoupler; closed circles and triangles denote the addition of either 1 μM gramicidin D or 10 mM NH_4Cl respectively. The reaction mixture contained 10 mM Hepes and 10 mM MES (adjusted to different pH values with NaOH), 10 mM NaCl, 5 mM $MgCl_2$, 200 μM MeV and 5–10 μM chlorophyll. Fluorescence parameters were determined as described in Materials and Methods. Other experimental conditions were as described in Fig. 1 except that the modulated 480 nm light intensity was lower (0.3 $nE \cdot cm^{-2} \cdot s^{-1}$).

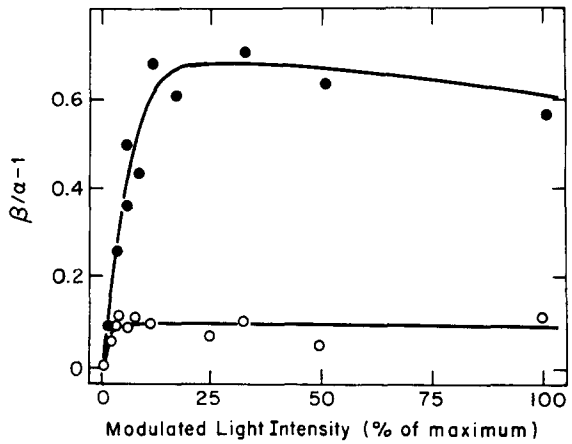

Fig. 10. Effect of modulated light intensity and of gramicidin D on the deviation from full balance. The imbalance term $\beta/\alpha - 1$ was calculated from the measured fluorescence parameters in steady state before (open circles) and after (closed circles) the addition of 1 μM gramicidin D to a standard reaction mixture with different intensities of the 480 nm modulated light. The maximum light intensity (100%) was about 3.5 $nE \cdot cm^{-2} \cdot s^{-1}$. Other details are as described in Fig. 1 and in Materials and Methods.

where the internal pH in the absence of 'uncouplers' is expected to be around 6 [31], the same level of imbalance is obtained as that at pH 6 in the presence of uncoupler where the internal pH is expected to be 6 as well. There is a certain similarly between the curves in Fig. 10 and those presented for the saturation rates of electron transfer [32], which again points to the control by the internal pH. The increase obtained in the maximum rate of electron transport at pH 8.5 is presumably due to the collapse of ΔpH by proton efflux through the coupling factor (Evron and Avron, BBA, in press) and it might be that this is also the case for the imbalance. The shift in the pH profiles for the two cases in the presence of uncoupler might arise from different relations to the external pH.

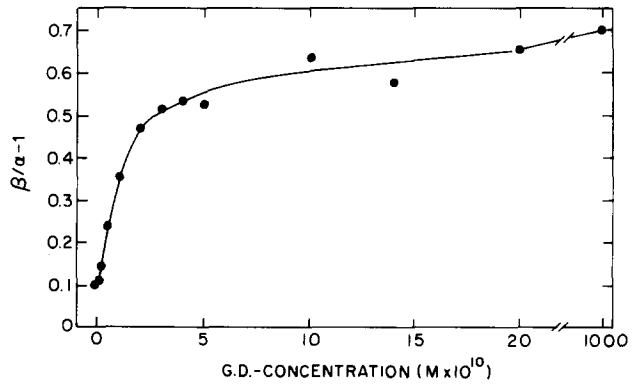

Fig. 9. Effect of gramicidin D concentration on $\beta/\alpha - 1$ at low 480 nm modulated light intensity. The experimental conditions were similar to those described in Fig. 1 except that the 480 nm modulated light intensity was lowered to 0.35 $nE \cdot cm^{-2} \cdot s^{-1}$ and with 5 mM $MgCl_2$ present.

The sensitivity of the imbalance to 'uncouplers' was markedly dependent on the modulated light intensity. While at the normal intensity used (i.e., 1–2 $nE \cdot cm^{-2} \cdot s^{-1}$), the effect of gramicidin D on β occurred at the micro-molar concentration range, at lower intensities (< 0.5 $nE \cdot cm^{-2} \cdot s^{-1}$) the effect of gramicidin D was stronger, reaching saturation at nanomolar concentrations (Fig. 9). A more detailed study was made by measuring the change in the imbalance caused by adding saturation amounts of gramicidin D as a function of the modulated light intensity. The effect of the un-

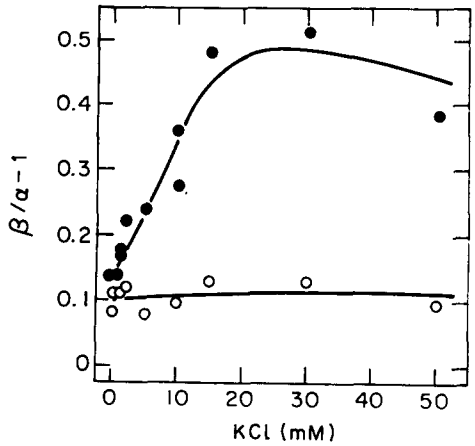

Fig. 11. The effect of KCl concentration and of valinomycin on the deviation from full balance. The imbalance term, $\beta/\alpha - 1$, was calculated from the measured fluorescence parameters in steady state before (open circles) and after (closed circles) the addition of 2 μM valinomycin to chloroplasts in a standard reaction mixture containing different KCl concentrations. Other details were the same as in Fig. 1. A control experiment using increasing NaCl concentrations in the absence of KCl was performed showing no effect of valinomycin with up to 200 mM NaCl.

coupler on the imbalance reached its maximum already at a light intensity of about 10% of maximum (approx. 0.35 $nE \cdot cm^{-2} \cdot s^{-1}$) (Fig. 10). One may suspect that it is not the bulk ΔpH that dominates directly the changes in the imbalance, since we could not notice any ΔpH as measured by the 9-AA fluorescence change, at the above light intensity. In this context, Fig. 11 demonstrates that the component of membrane potential in the total membrane energization is a significant factor in affecting the imbalance. This is seen from the effect of valinomycin on the deviation from full balance which depended on added KCl but not on NaCl. The effects on the imbalance of a ΔpH dissipator such as nigericin and that of membrane potential dissipator such as valinomycin (+KCl) were each maximum and therefore not additive when using both chemicals in saturating amounts.

Discussion

The present work demonstrates a significant imbalance in the photoactivities of the two photosystems in favor of PS II under the following circumstances: (a) at cation concentrations just sufficient to form stacked membranes but in the presence of 'uncouplers' in the steady-state or only at the first moment of illumination; (b) at much higher cation concentration, even in the absence of uncouplers. The requirement for cations complements other studies [7–17,33] which support the possible effect of cations on grana formation [13,14]. According to these studies, high cationic levels may lead to segregation between PS II and PS I [8], and hence abolish the possibility of energy transfer ('spillover') from PS II to PS I [33]. Sinclair has also observed, using the oxygen rate electrode, Emerson enhancement in chloroplasts, emphasizing its dependence upon the presence of suitable concentrations of cations [21]. For broken chloroplasts, the Emerson enhancement ($=\beta/\alpha$) was measured at one pH only (8.2), at which it was around 1.5 with 5 mM $MgCl_2$. This result agrees with our measurements under similar conditions (Fig. 8). Sinclair did not consider, however, the effect of membrane energization.

One has to consider that imbalance in photoactivities could result from either imbalance in excitation allocation to the two photosystems, from diversion of the photoactivity to other type of activity in one of the photosystems (e.g., from linear to cyclic electron flow), or from unequal quantum yields of primary electron transport in each one of the photosystems.

One possibility to consider is based on the suggestion by Fernyhough et al. [34] and Horton and Lee [35]. Using whole chloroplasts they showed that the imbalance in the photosystems activities for linear electron flow depends on the type of the final electron acceptor and that it was higher when there was an extra demand for ATP in order to reduce the electron acceptor. It was suggested that for ATP such a requirement is satisfied by ATP production in the cyclic electron path around PS I. Considering regulation of the ratio between cyclic and linear electron transport, it is clear that in PS I there would be a diversion of photoactivities and hence an imbalance in linear electron transport which appears to be strongly in favor of PS II, even if light excitation is distributed evenly. Horton and Lee [35] suggested that the regulation of the diversion of photoactivities in PS I is coupled to alterations in the light-induced ΔpH and in the ATP consumption. A recent report [36] shows the existence of a cyclic electron flow around PS I even in washed thylakoids (i.e., in the absence of soluble ferredoxin), which is coupled to the formation of transmembrane electric field and proton gradient. Therefore, it could be possible that here also there is a direct control of membrane energization on the cyclic electron flow around PS I. When membrane energization decreases, the cyclic path competes for more excitation in PS I and hence reduces PS I efficiency towards electrons from PS II, thus giving rise to an imbalance in favor of PS II in the linear electron flow.

In the above possibility, however, one expects that the ratio between the surplus activity in PS II with and without 'uncoupler' would be similar in the entire wavelength range, which is clearly not the case, as shown in Fig. 3. To show this argument in a quantitative way we rewrite the balance equation for the two photosystems taking into account a cyclic electron flow around PS I, as was also considered by Myers [1]. This yields:

$$\beta f = \alpha(1-k)$$

where k is the wavelength-independent diversion ratio of PS I to cyclic electron flow. This yields:

$$f = (\beta/\alpha)(1-k)$$

Denoting the f values with and without 'uncoupler' as f_+ and f_- respectively and taking the ratio, assuming that only k changes due to the 'uncoupler' addition (from k_- to k_+), gives:

$$f_+/f_- = (1-k_+)/(1-k_-)$$

Thus, one expects f_+/f_- to be wavelength-independent. Fig. 12 shows that this is clearly not the case with gramicidin D. It seems, therefore, that there must be genuine changes in the pigment composition of the two photosystems, induced by the effect of cations and the 'uncouplers'. Interestingly, such changes occurred here in a very short time-scale (seconds), while changes in pigment composition in vivo as shown, for example, in state 1 to state 2 transitions usually occur on a much slower scale. Also, unlike the in vivo state 1 to state 2 transitions [6], no proportional changes in F_o and F_m are observed here.

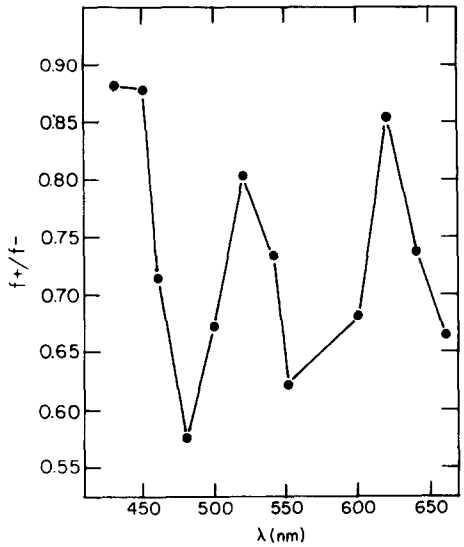

Fig. 12. Dependence of the calculated ratio between f values obtained with and without gramicidin D (f_+/f_-) on the wavelength of the modulated light. f_+/f_- data were calculated from the results obtained in the experiment described in Fig. 3b excluding the points in 580 and 650 nm (see legend to Fig. 3).

Another issue is the mechanism which leads to the changes in the imbalance and the interplay between the effect of the cations and the 'uncouplers'. The fact that membrane energization per se does not lead to changes in the photosystems imbalance (i.e., at 'low salt') and that extremely high cationic concentrations oppose successfully the effect of membrane energization and lead to a high imbalance favors a model based on surface charge effects. In this connection, the work of Kraayenhof et al. [37] is of utmost importance. By measurements of electrophoretic mobility they have shown that illumination of chloroplasts induces an increase of negative charge density in the outer surface of the thylakoid membranes, which was abolished by a line of uncouplers and ionophores, similarly to our case. They suggested that a local proton gradient near the membranal surface is formed by light-induced proton translocations which in turn affect conformational changes leading to exposure of (previously buried) ionizable groups (probably carboxyls) to the aqueous phase leading to increased dissociation and to an increase in the negative surface charge density. In our case, this increase of negative charge density might have been the cause for the changes in the relative activities and possibly in the mutual interaction of the two photosystems in a way leading to less imbalance (see below). Screening of the extra negative charges by further increasing the cation concentration to an extreme limit as shown in Fig. 4a and b restores the imbalance. In this effect, the concentration dependence for uni-, di- and trivalent is as implied from the Poisson-Boltzmann approach adopted in the Gouy-Chapman theory [38]. Uncouplers and ionophores abolish the formation of the proton gradients at the surface, rendering the extra ionizable groups the capacity to become buried again. From this, it appears that there are two related but distinct phenomena which are connected to surface charges. One is the well-known increase of F_m when external negative charges are screened and the membranes become stacked [15–17]. The second phenomenon is the one reported here of the increase in imbalance as the light-created additional negative charges are screened.

It has been postulated in a number of studies [39–41] that the high-energy state involves the light-induced efflux of Mg^{2+} from the lumen to the stroma. This assumption could explain the above observation that very high concentration of $MgCl_2$ may substitute for the 'uncoupler' effect. However, the fact that high concentrations of TEC (which cannot permeate through the membrane like $MgCl_2$) could also substitute for the 'uncoupler' effect (Fig. 4b), indicates that probably the interactions took place on the outer surface of the thylakoid membrane.

Extra exposure of negative charges on the outer surfaces of the thylakoids due to illumination might result in an increased electrostatic repulsion between adjacent membranes. With residues of LHC of PS II (LHC II) probably serving as mediators for the stacking of membranes to form grana [42,43], it is possible that minor changes in the conformation of LHC II units, caused by the membrane energization, might cause microscopic changes in the strength of membrane stacking leading to a more balanced state, while not necessarily change the degree of stacking [13].

Conformational changes in membrane complexes might occur not only as a result of minor fluctuations in the surface charge but also as a result of the transmembrane electric field which is formed within a short time due to illumination. Former studies by Schliephake et al. [44] and by Junge and Witt [45] have shown that gramicidin D concentrations in the nanomolar range are sufficient to dissipate the light-induced transmembranal electric field. In our case, the effect of gramicidin D on the imbalance occurred already with nanomolar concentrations of gramicidin D, provided that a sufficiently low light intensity was applied (Fig. 9). Under these conditions, very low concentrations of gramicidin D may prove to be sufficient in order to dissipate a transmembrane electric field which is probably formed within a very short time as a result of proton displacement from the outer to the inner surface, without forming a noticeable ΔpH between the bulk media. This goes in line with the notion that part of the light-induced transient in F_s was too fast to be monitored by our instrument. This may also explain the similarity between effects of valinomycin and of nigericin which might act to collapse the transmembrane electric field. Since the surface negative charge density is higher in-

side the thylakoid than outside [46], it is possible that under the low light intensity and conditions there is already a considerable amount of proton binding to the inner surface, compared to the dark state. Such binding might be sufficient to cause a conformational change which would lead then to changes in the photosystem excitation balance.

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