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ANALYSIS OF THE INTERACTIONS BETWEEN THE TWO PHOTOSYSTEMS IN ISOLATED CHLOROPLASTS

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SUMMARY

Studies have been made with isolated chloroplasts using a new amperometric method which allows us to measure the activities of Photosystems I and II separately or cooperating in enhancement and chromatic transients.

1. The occurrence of the latter phenomena in isolated chloroplasts could be clearly established.

2. The action spectra of the two photosystems and the ratio between these two have been measured with precision.

3. The action spectrum of System I proved to be independent of the state of the traps of System II; this demonstrated absence of photon transfer from pigments of System II to pigments of System I (spillover).

4. The dependence of the rate upon the concentration of active trapping centers has been studied for each photosystem. In the case of Photosystem II, the same hyperbolic relation was found which was observed earlier with whole Chlorella cells. In contrast, in Photosystem I the relation between the rate and the concentration of active P^-_{700} proved to be strictly linear.

5. The steady-state concentration of open trapping centers of System I and System II (P^- and E^+) has been measured in weak light as a function of wavelength. The results indicated that the overall reaction, in which the active forms of the two photoconverters are regenerated, has a low equilibrium constant $K(K = 3\text{–}10)$. This low value implies: (a) a small loss of free energy in the dark reactions which connect the two photoacts, (b) a relatively weak dependence of the overall quantum yield upon the light distribution between the two photoacts and (c) a restraint of the maximum quantum yield of the overall process.

INTRODUCTION

Basic to the concept of two photoreactions in photosynthesis[1–3] are BLINKS'[5,6] observations of chromatic transients and the enhancement experiments of EMERSON, CHALMERS AND CEDERSTRAND[4], which were both performed with whole algae. Albeit with generally less clear results, similar phenomena have been observed with isolated

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenol-indophenol.

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chloroplasts[7–9]. This paper reports a reinvestigation of the latter material with a modulated amperometric method which allows independent measurement of the rate of each photosystem[11]. The data pertain to the mechanism of quantum flow within each photosystem and to the interaction between the photoacts in terms of quantum and electron flow.

Quantitative analysis of enhancement, first proposed by BANNISTER AND VROOMAN[32], was recently developed by ELEY AND MYERS[39]; our analysis is essentially similar, while one of the basic hypotheses is modified.

The trapping centers of the O_2-evolving Photosystem II are denoted E, for most purposes equivalent to the symbol Q used by DUYSENS for the fluorescence quencher of System II (Fig. 7b in ref. 40). We assume that Photoact II is the reduction of E by a quantum absorbed by System II pigment:

$$E \xrightarrow{h\nu} E^- \qquad (I)$$

and that Photoreaction I is the oxidation of P by a System I quantum:

$$P \xrightarrow{h\nu} P^+ \qquad (II)$$

Eqn. III describes the regeneration in a thermal reaction of the active forms of the two photochemical centers:

$$E^- + P^+ \underset{k_b}{\overset{k_f}{\rightleftharpoons}} E + P \qquad \frac{k_f}{k_b} = K \qquad (III)$$

In fact, several intermediary steps occur between E and P, one of which involves an intermediate electron carrier "A" which occurs in a much larger concentration than that of the trapping centers[15,40]. These and all other dark steps, however, can be neglected in steady-state measurements in weak light as described in this paper, so that only the overall equilibrium constant K and the distribution of light quanta between the two photosystems determine the concentrations of E and P.

MATERIALS AND METHOD

Chloroplasts were isolated from freshly harvested, greenhouse-grown spinach leaves, following the procedure described by SCHWARTZ[10]. Immediately before each experiment they were diluted in a 0.05 M phosphate buffer containing 0.1 M KCl. The final concentration, depending upon the type of experiment, varied between 0.2 and 0.8 mg chlorophyll per ml.

The modulated polarograph and its accessories are described in detail in ref. 11. Since the rate of light conversion is proportional to the quantum flux into the sensitizing pigment and is an increasing function of the concentration of the trapping centers which are in the active state (C), modulated light of intensity $I_\sim$ will induce a rate $V_\sim = kI_\sim f(C)$. We should stress here that conditions can be chosen so that the signal $V_\sim$ indicates the rate of one photosystem exclusively: in negative polarization (O_2 measurement) the rate of System II, in positive polarization (measurement of a low potential oxidant such as viologen), the rate of System I: At high enough modulation frequency, the large pool A and the rate-limiting reaction between the photosystems introduces a filtering effect. This prevents a modulation of the concen-

tration of one trapping center by a modulation of the concentration of the centers of the other photosystem. A significant degree of this undesired modulation will yield a variation of the phase angle of the signal compared to the phase angle measured in very weak light. In the experiments reported, we checked for constancy of the phase angle. We also made sure that $I_\sim$ did not appreciably modulate C, and that the quantity of C destroyed in each light period was a small fraction of the amount of C present in the active form. One consequence of the requirements is that higher intensities $I_\sim$ could demand the use of a faster modulation rate.

With the modulated method one can study $kI_\sim$ and (C) separately. For instance, in the enhancement experiments of Figs. 1 and 2, $kI_\sim$ is kept constant and all variation of $V_\sim$ is due to a variation of (C). In studies of action spectra, (E) and (P) are held constant and the variation of $V_\sim$ is due to the variation of $kI_\sim$ with wavelength.

CHROMATIC TRANSIENTS AND ENHANCEMENT IN CHLOROPLASTS

The experiment of Fig. 1 was made using negative polarization, detecting the rate of O_2 production in the presence of $NADP^+$ and ferredoxin as electron acceptors. Two light beams were used, one 650 mμ the other 700 mμ, adjusted to yield equal steady-state rates of O_2 evolution (V_{s650} and V_{s700}) in the linear range of rate and intensity. Either beam could be modulated (90 cycles/sec) or used unmodulated with a 50% transmission filter to obtain equal average intensity. The following successive observations were made: exposure to a continuous background light of 700 mμ yielded no modulated O_2 response. At time A the continuous 700-mμ beam was replaced by the modulated 650-mμ beam. We observed an initially enhanced rate (V_p) which in a few seconds decayed to a stationary level V_{s650}. This observation is a typical chromatic transient as reported earlier[6]. At time B the continuous 700-mμ beam was switched on again, which resulted in an increase of the rate to level V_{e650}. At time C the 700-mμ beam was removed and the modulated rate returned to the

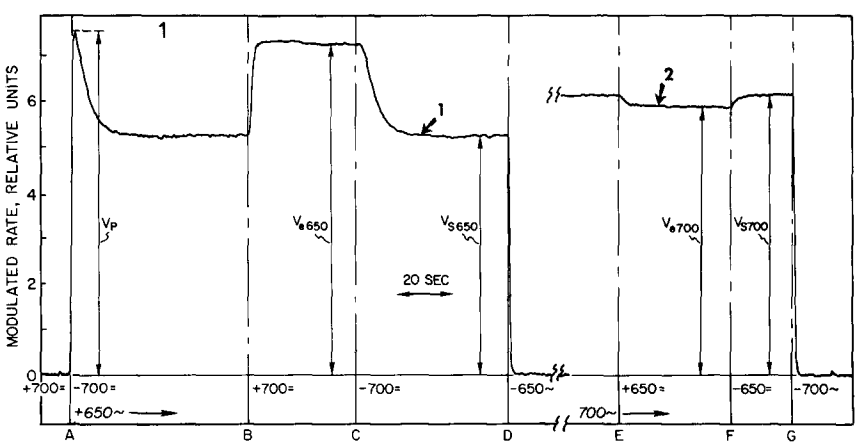


Fig. 1. Chromatic transients and enhancement in chloroplasts. O_2 detection (polarization, −0.6). Acceptor: $NADP^+$ (10^{-3} M)–ferredoxin (catalytic amount). pH = 7.9. Phosphate buffer + KCl + ADP (10^{-3} M) + $MgCl_2$ (10^{-3} M). Room temperature, approx. 23°. Frequency of modulation: 90 cycles/sec. Curve 1: Modulated beam = 650 mμ. Continuous beam = 700 mμ, I = 50%. Curve 2: Modulated beam = 700 mμ. Continuous beam = 650 mμ, I = 50%.

original level. During time BC the 650-mμ modulated response was enhanced by the 700-mμ background light. Note that V_{e650} is slightly lower than the level V_p. This is due to the fact that when the 650-mμ and the 700-mμ beam are combined, the ratio of System I over System II light is not as favorable as during the 700-mμ preilluminination. The level V_p is close to the maximum efficiency with which 650-mμ light can be utilized. Fig. 1 further shows that d.c. light gives no response since the signal before time A equals that after time D when the chloroplasts were in darkness. Linearity of the biological response rate with light intensity was checked by adding a d.c. 650-mμ light. In this case no variation of the signal was observed. If a too weak, modulated 650-mμ beam had been used, we might have seen an increased response upon addition of the non-modulated 650-mμ beam, corresponding to activation of System II[14,15]. Curve 2 in Fig. 1, shows the inverse experiment. First the chloroplasts are illuminated by the modulated 700-mμ beam; after attainment of a steady-state rate V_{s700} the unmodulated 650-mμ beam is added during time E–F. This causes a

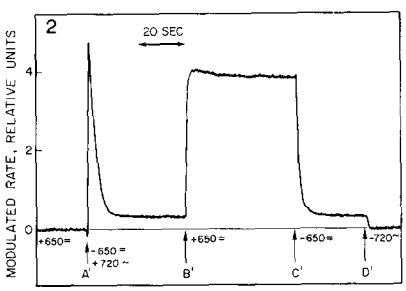


Fig. 2. Chromatic transients in chloroplasts; positive polarization, +0.6 V. Acceptor: methyl viologen, $5 \cdot 10^{-5}$ M. Other conditions same as in Fig. 1.

slight de-enhancement of the rate to the value V_{e700}. Since $V_{e650} + V_{e700}$ represents the total rate which would have been observed if the two beams had been modulated simultaneously, the classical way to express enhancement $V_{650+700}/(V_{650} + V_{700})$ becomes: $(V_{e650} + V_{e700})/(V_{s650} + V_{s700})$. Computed in this way the data of Fig. 10 indeed show an enhancement factor of $(73 + 59.5)/(52.5 + 62) = 1.16$, a very similar value to that observed in whole Chlorella cells[20]. The data confirm the classical concept that System I provides oxidized substrate for System II.

Fig. 2 shows a similar experiment performed with positive polarization to detect the rate of System I activity, methyl viologen being the oxidant. The sequence of observations was the same as that used for the experiment of Fig. 1, only with a reverse combination of a much stronger continuous 650-mμ beam and a weaker modulated 720-mμ light. The experiment thus shows that System II provides reducing power for System I. The greater stimulation (13-fold) seen in Fig. 2 rests upon the fact that a large percentage of long-wave light is absorbed by System I whereas at 650 mμ the two systems are more nearly equally excited.

We should mention here that, contrary to some other reports[16], we could clearly observe similar phenomena with high potential oxidants such as quinone, DCIP and ferricyanide. With ferricyanide, lag periods of 1 to several seconds were seen in some cases when the 650-mμ beam was superimposed upon the 720-mμ modulated beam and it could be shown that during this lag time System II operated independently.

Most experiments described in this article, therefore, were performed with low potential oxidants such as methyl viologen or $NADP^+$, which can accept electrons only from System I. Unlike the high potential oxidants, the latter reagents yielded a perfectly constant phase angle, indicating that only a single process was involved.

OBSERVATIONS OF INTERMEDIATE POOL A *via* SYSTEM I

The amount of O_2 evolved during the initial gush occurring when algae or chloroplasts are illuminated with strong light[14,17,18], is proportional to the concentration of the oxidized form of large pool A, located between the two photosystems[15]. This pool is completely reduced in strong white light and slowly reoxidized in the dark. This reoxidation is considerably accelerated by far red illumination[15,19]. The same oxidation and reduction of pool A has now been observed by measurement of System I activity. In the experiment of Fig. 3 the reduction of methyl viologen was detected.

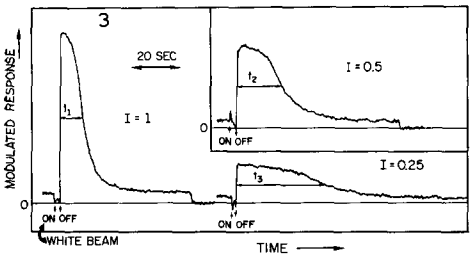


Fig. 3. Observation of pool A *via* System I. Polarization +0.6 V. Acceptor: methyl viologen, $5 \cdot 10^{-5}$ M. Frequency of modulation: 95 cycles/sec. After 2 sec of a strong continuous white beam, one observes an enhancement of the response to modulated 710-mμ light of various intensities (I = 1, 0.5 or 0.25). The arrow ↓ indicates the end of the preillumination by the white beam.

Chloroplasts were illuminated by a weak modulated 710-mμ beam which provoked a very low steady-state response. During about 2 sec a strong d.c white beam was superimposed which caused the modulated response to decline almost to zero, *i.e.* a nearly complete oxidation of P. Since at the same time pools E and A are completely reduced in this illumination, we conclude that the rate-limiting step of the overall process must be between A and P, as is presently assumed by most investigators.

At the end of the white preillumination and in a time shorter than can be followed by the recorder (0.5 sec), a strong enhancement of the 710-mμ response is observed. We interpret this enhancement as being due to a rapid reduction of P by A^-. Subsequently the signal slowly decreases to the original steady-state value. If the same experiment is repeated with detecting beams of lower intensity (approx. 0.5, 0.25) the decay time of the signal proves to be inversely proportional to the intensity of the detecting light. The area bounded by the three curves which represent the amount of viologen reduced during the "gush" are nearly identical. Therefore, one can conclude that a definite pool of reductant has been formed during the strong preillumination. The high rate, observed immediately after preillumination with strong light, represents the highest possible rate of System I in a given intensity since at this moment all P is in the active state.

A more complete analysis of this phenomenon will be discussed in a following paper.

ACTION SPECTRA OF SYSTEMS I AND II

As discussed above, conditions can be chosen so that the modulated method responds exclusively to one of the two photosystems. As long as the concentration of the conversion centers (E or P) is held constant, regardless at which value, the rate will be proportional to $k_2 I$ or $k_1 I$ (see METHODS). For constant incident quantum flux, the rate is proportional to k_2 or k_1 and $k_2 f(\lambda)$ or $k_1 f(\lambda)$ represents the true action spectrum of the photosystem studied.

To maintain E or P constant one can, for instance, use a background light of any wavelength in which the traps will attain a certain steady-state concentration. The modulated detecting beam now must be weak enough so as not to disturb this concentration (approx. 2 % of the continuous beam). Absence of transients upon addition of the modulated beam is a check that this condition is fulfilled. We should point out that in our method the background light, theoretically of any wavelength, serves a different purpose than, *e.g.*, in the experiments of FRENCH AND MYERS[20] or DUYSENS[21]. In the latter, the background light served to displace the overall action spectrum towards the spectrum of the rate-limiting photosystem. In our case, a change of background wavelength changes the amplitude only (see Fig. 5).

For measuring the action spectrum of System II (O_2 detection) a continuous 720-mμ background beam yields maximum sensitivity. Fig. 4, Curve 2, shows an action spectrum obtained by this method, in the presence of $NADP^+$ and ferredoxin. It shows a maximum at 677 mμ and a clear shoulder at 650 mμ due to participation of chlorophyll *b*. Curve 1 in Fig. 4 shows the action spectrum of System I (methyl viologen reduction) as measured on a background light of 650 mμ. The maximum is located at 681 mμ, no 650-mμ band can be discerned. The spectra generally agree with earlier ones reported in the literature[15,20,28].

The two spectra of Fig. 4 were measured using different electrode polarization and the rates observed in the two cases differ by an unknown factor, a problem which

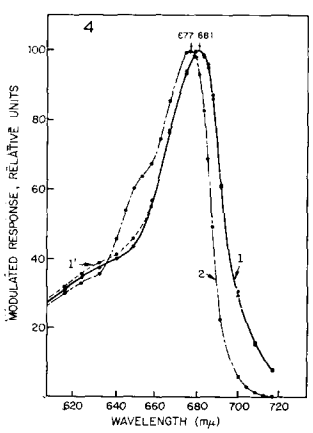


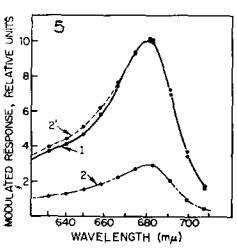
Fig. 4. Action spectra of System I and System II. Curve 2: Action spectrum of System II. O_2 detection (polarization, —0.6 V). Same conditions as Fig. 1. Curve 1: Action spectrum of System I. Polarization, + 0.6 V. Same conditions as Fig. 2. Curve 1': Action spectrum of System I in presence of DCMU, 10^{-5} M. Donor system = diaminodural + excess ascorbate.

will be discussed later in more detail. Thereore, assuming equal active absorption at 680 mμ, the ratio between the action spectra of Systems I and II was plotted in logarithmic units (Fig. 6). The shape of this curve is not affected by the relative amplitude of the two spectra. Results obtained with different chloroplast preparations indeed showed very little variation of this shape. Another interesting aspect of this ratio curve is its relative insensitivity to systematic errors in energy calibrations and to the screening effect of the upper chloroplast layer, which is identical in the two measurements. The maxima and minima in Fig. 6 are in good agreement with those reported earlier in the spectra of chromatic enhancement and transients, which also depend upon this ratio[6,15,30]. We may mention here that we have observed a decrease of the ratio at very long wavelengths ($\lambda > 725$) in agreement with MYERS AND GRAHAM[29] and GOVINDJEE[30] who reported a decrease of enhancement at such long wavelengths.

ABSENCE OF PHOTON TRANSFER BETWEEN SYSTEM II AND SYSTEM I

The question whether in terms of photon transfer the two photosystems are completely isolated ("separate package" hypothesis) or energy absorbed by System II can be transferred to System I ("spillover") is as yet disputed[21,31–33]. Curve 1', Fig. 4, shows the action spectrum obtained in the presence of DCMU to block System II activity; diaminodural and an excess of ascorbate[22] were added to feed electrons to System I. The rates in the second experiment were somewhat higher, but after normalization the two spectra are identical within experimental error. This result proves that the System I action spectrum is independent of the concentration of the trapping centers of System II: In the presence of DCMU these are inactivated, while we calculate (from the data of Fig. 7) that in absence of the poison and with a background of 650 mμ (Curve 1, Fig. 4), System II photons are used with a relative efficiency of about 70 %.

Curves 1 and 2 in Fig. 5 show two action spectra of System I measured in the presence of background lights of 650 and 700 mμ, respectively. Curve 2' was obtained by multiplying spectrum 2 by 3.5 so that its maximum coincided with that of Curve 1.



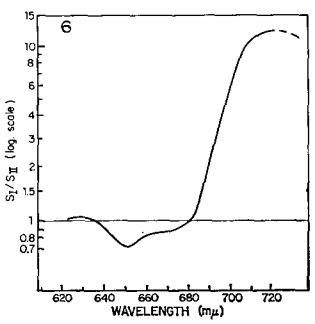


Fig. 5. Action spectra of System I in presence of different background lights. Polarization, + 0.6 V. Acceptor: methyl viologen, $5 \cdot 10^{-5}$ M. Same conditions as Fig. 2. Curve 1: background, 650 mμ. Curve 2: background, 700 mμ. Curve 2': same as Curve 2, but normalized with Curve 1.

Fig. 6. Ratio $R = S_I/S_{II}$ computed from data of Fig. 4, in logarithmic units. 680 mμ has been chosen as the wavelength where Systems I and II absorb equally.

Curves 2′ and 1 are almost identical, which again proves that the spectrum of System I is independent of the state of the System II traps. The small discrepancy observed in the 650-mμ region indicates that, under this extreme condition, separation between the photosystems was not fully complete. Note, however, that the discrepancy is in the direction opposite to that which would be predicted by the assumption of energy transfer between Systems II and I (spillover).

In summary, our data show that photons absorbed by System II pigment, and not used by a System II trapping center, are not available for System I.

DEPENDENCE OF THE RATE OF SYSTEMS I AND II UPON THE CONCENTRATION OF ACTIVE CENTERS

System II

It was reported previously[12,23] that in Chlorella cells the rate of O_2 evolution depends in a non-linear fashion upon the concentration of active (oxidized) center E. The same dependence has now been observed with isolated chloroplasts: The relative amount of O_2, evolved by a brief saturating flash (20 μsec), which indicated the relative concentration of E, was measured as described in the preceding paper[11]. This measurement was made during illumination of the chloroplasts with a weak, modulated 695-mμ beam to measure the rate of O_2 evolution. Since the flash caused only a very brief perturbation of the modulated signal, rate and flash yield could be measured simultaneously. The modulated rate could be varied over a wide range by adding a 650-mμ continuous beam of adjustable intensity. In the absence of an electron acceptor, this addition causes an immediate decrease of the modulated rate, due to a depletion of the E pool which, with high enough intensities, can be complete. On the other hand, maximum rate and maximum concentration of active E was observed, immediately after a preillumination with 720-mμ light. In Fig. 7 we plotted the data obtained with three different chloroplast preparations after normalization of the maximum values of rate and (E). As extensively discussed in the literature[12,24–27] the non-linear relation between V_2, the rate of System II and E shows that the units of System II are not separate entities in respect to photon transfer. The curve drawn in Fig. 7 represents the theoretical function:

$$V_2 = e\frac{1}{1 - a(1 - e)} \qquad (1)$$

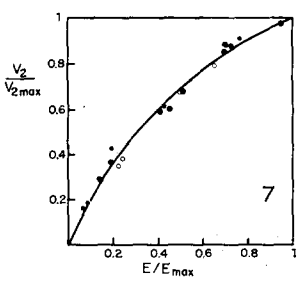


Fig. 7. Function $V_2/V_{2max} = f(E/E_{max})$. O_2 detection. Polarization, −0.6 V. No acceptor, pH = 6.4. V_2 = stationary rate of System II. E = concentration of photoactive traps of System II; detecting beam, 695 mμ modulated at 40 cycles/sec. A continuous 650-mμ background beam of varying intensity was used to vary the concentration of E. The three different symbols correspond to the data obtained with 3 different chloroplast preparations. The curve represents the theoretical function discussed in the text, assuming $a = 0.55$.

in which $e = E/E_{max}$, the fraction of E in the active state and parameter a was chosen equal to 0.55. If we assume the existence of pigment units, parameter a represents the probability of cross transfer between units. An important aspect of the experiment of Fig. 7 is that the entire function $V = f(E)$ was obtained in the absence of inhibitors such as DCMU, which had to be used in earlier experiments with whole cells, for obtaining low values of (E). The results of the experiment of Fig. 7 are almost identical to those obtained with Chlorella[23], in 7 experiments with isolated chloroplasts a varied between 0.55 and 0.6. This value of a thus appears to be an invariable characteristic of photosynthetic material.

System I

With isolated chloroplasts and methyl viologen as electron acceptor, we could perform for System I, the same type of experiment as described above for System II. The function $V(P)$ was determined in two ways: in both cases, chloroplasts were illuminated by a very weak modulated beam of 710 mμ. In the first case, d.c. background lights of different wavelength were added and, when a steady-state response was attained, we measured the (relative) amount of methyl viologen, reduced by a brief saturating flash. In the second case, chloroplasts were preilluminated during 2 sec by a strong white beam which oxidized all P and reduced all A and E (see Fig. 3). A fraction of a second after cessation of the white preillumination, P is completely reduced by the excess A^- and the rate of System I is maximal (Fig. 3). In the course of the 10–20 sec it took the modulated rate to decline to its minimum value (the far red beam depleting pool A^-) 3 to 4 flashes were superimposed at approx. 2-sec intervals. The rate, measured immediately before each flash, is plotted against the relative amount of methyl viologen reduced during the flash.

In Fig. 8 we collected data obtained with 3 different chloroplast preparations by the two experimental approaches. The maximum values of rate and amount of P again were normalized. Fig. 8 shows the unexpected result that, in System I, the relation between V and P is strictly linear.

This linear dependence can be interpreted in two ways: (1) Each P is associated with a certain number of harvesting chlorophylls as a completely isolated unit. (2) The photochemical centers of System I always trap an incoming quantum, regardless of whether they are in the active or inactive state. In either case an arriving photon

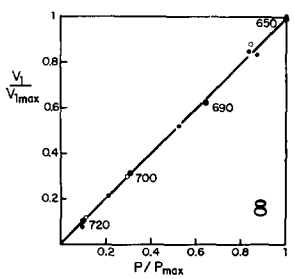


Fig. 8. Function $V_1/V_{1\,max} = f(P/P_{max})$. Positive polarization, +0.6 V. Acceptor: methyl viologen, $5 \cdot 10^{-5}$ M. Frequency of modulation: 40 cycles/sec. Large dots: The detecting beam was a very weak, modulated 710-mμ beam to which continuous beams of different wavelengths were added. Open circles, small dots: 3 or 4 flashes were superimposed upon the detecting beam at approx. 2-sec intervals during the depletion of A^- following a 2-sec preilluminaton in strong white light (see text and Fig. 3).

is either converted into chemical energy (active center) or converted into heat (inactive center). A similar situation has been described for System II after the trapping centers were inhibited by hydroxylamin[35].

Our second hypothesis meets a difficulty due to the fact that P700 bleaches after absorption of a photon[13]. However, P700 appears to be associated with a minor long-wave chlorophyll component, C700[36,37]. While the probability of transfer from the shorter wavelength pigment toward this long-wavelength chlorophyll is high, in the reverse direction it is low. Consequently, the energy is always trapped in the long wavelength pigment group. The fact that no variable fluorescence component has been observed which originates from Photosystem I also indicates that all energy not trapped by P700 is converted into heat.

VREDENBERG AND DUYSENS[38] reported in the case of photosynthetic bacteria a non-linear relation between fluorescence and the concentration of B890. In this aspect System I seems to differ from the bacterial system.

CONCENTRATION OF E AND P AS A FUNCTION OF WAVELENGTH

Rate transients as a function of wavelength

In these experiments the steady-state modulated rate (v_s, Figs. 1 and 2) has been compared to the initial rate (v_p), obtained immediately after a d.c. preillumination. The transient response of oxygen evolution was measured in the presence of $NADP^+$ and ferredoxin while that of System I was observed using methyl viologen.

Experiments were performed at pH 7.9 in the presence of ADP and Mg^{2+} to obtain a high level of the saturating rate of photosynthesis. The ratio v_s/v_p was studied by using a constant wavelength of preillumination and varying the wavelength of the modulated detecting beam. The half band of the monochromatic detecting beam was about 7 mμ to obtain a sufficiently high intensity, which was in the range of linearity between rate and intensity.

In O_2 measurements the chloroplasts were preilluminated by a 720-mμ, d.c. light within the linear rate *vs.* intensity range. After about 30 sec (E) reaches its maximum value, so that rate V_{p2}, observed immediately after preillumination, represents the maximum possible rate of System II obtainable with a detecting beam of given intensity and wavelength. In the methyl viologen measurements, made with the same batch of chloroplasts as used for the O_2 measurements, v_{p1} was measured immediately after 2 sec of a strong white preillumination (see Fig. 3). As discussed before, a fraction of a second after cessation of this preillumination, (P) is maximal, so that v_{p1} represents the maximum rate of System I obtainable with a detecting beam of given intensity and wavelength.

For reasons explained below, the data plotted in Fig. 9 were measured 4–7 min after the chloroplast were placed on the electrode. This time is generally too short to obtain complete settling and stable response. For this reason we made two measurements of v_p at each wavelength, one before, the other after measurement of v_s and obtained the v_p value corresponding to v_s by interpolation. In the course of the experiment, the v_s/v_p ratio's increased systematically. The tips of the arrows in Fig. 9 show the final values of v_s/v_p, attained some 20 min after introducing the chloroplasts. Interestingly, however, no such increase of the ratio v_{s1}/v_{p1} occurred in detecting wavelengths longer than 695 mμ. High values of v_s/v_p were also obtained when

chloroplast had been resuspended in the phosphate –KCl buffer more than a few minutes before the experiment.

Fig. 9 shows the ratio v_{s1}/v_{p1} and v_{s2}/v_{p2} as a function of wavelength for both photosystems. The two curves show a cross-over point at 678 mμ. As discussed later, this wavelength represents the point of equal active absorption in both photosystems. Because of the linear relation between v_{s1}/v_{p1} and P, Curve 1 directly shows the variation of P/P_{max} with wavelength. In contrast, the relation E/E_{max} had to be computed from Curve 2 using Eqn. 1 and the observed value of $a = 0.6$.

Different batches of chloroplasts yielded quite similar spectra but a significant variability (665–677 mμ) of the crossing point between the V_{s1}/V_{p1} and V_{s2}/V_{p2} curves.

Wavelength dependence of E and P as measured by flashes

In our second approach we directly measured the amount of O_2 evolved, or methyl viologen reduced, by a single saturating flash given on background lights of various wavelengths. We obtained results very similar to the ones described above, despite the fact that a different batch of chloroplasts was used. Table I shows a few values of E/E_{max} and P/P_{max} observed in different wavelengths. The value of the constant K given in line 3 will be discussed later.

Effect of pH, uncoupler, ADP and ATP on the functions $V_{s1}/V_{p1} = f(\lambda)$

In the experiments reproduced in Fig. 10 we measured the rate of methyl viologen reduction at 2 pH values, in the presence or absence of 10^{-3} M NH_4Cl. This experiment clearly shows a significant increase of the ratio v_s/v_p at the lower pH while at 650 mμ, P attains the value P_{max}. The data of Fig. 10 further show that the

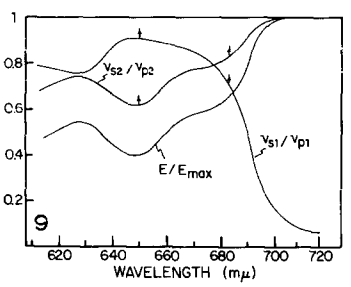


Fig. 9. Concentration of E and rate transients as a function of wavelength. Polarization, –0.6. Acceptor: $NADP^+$ (10^{-3} M) + ferredoxin. Frequency of modulation: 90 cycles/sec. Curve 1: $V_{s2}/V_{p2} = f(\lambda)$. V_{s2} = steady-state modulated rate. V_{p2} = initial rate after preillumination by a 720-mμ continuous beam. Curve 2: $e = E/E_{max} = f(\lambda)$, computed from Eqn. 1, assuming $a = 0.55$, and taking $V = V_{s2}/V_{p2}$ from Curve 1. Curve 3: Polarization, +0.6. Acceptor: methyl viologen, $5 \cdot 10^{-5}$ M. Frequency of modulation: 90 cycles/sec. $V_{s1}/V_{p1} = f(\lambda)$ with V_{s1} = steady-state modulated rate of System I, V_{p1} = initial rate after 2 sec of strong white preillumination. The tips of the arrows show the final values of V_s/V_p, attained some 20 min after introducing the chloroplasts.

TABLE I

CONCENTRATION OF THE PHOTOSUBSTRATES OF SYSTEMS I AND II AT DIFFERENT WAVELENGTHS

	650 mμ	*676 mμ*	*686 mμ*
P/P_{max}	0.80	0.74	57
E/E_{max}	0.42	0.52	75
K	3	3.2	4

presence of the uncoupling agent did not modify the results. That the chloroplasts were not uncoupled to begin with was proven by the observation of the expected increase of the rate in strong light, upon addition of 10^{-3} M ammonia. Other experiments have been performed at pH 7.9 in which ADP and Mg^{2+} were omitted from the phosphate–KCl buffer or replaced by ATP. Again, no significant modification of the ratio v_s/v_p was observed.

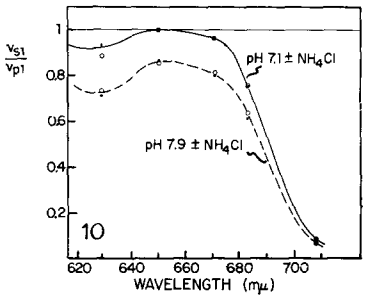


Fig. 10. Effect of pH and uncoupler on $V_{s1}/V_{p1} = f(\lambda)$. Polarization, +0.6 V. Acceptor: methyl viologen, $5 \cdot 10^{-5}$ M. Frequency of modulation: 90 cycles/sec. Full line: pH = 7.1; ADP (10^{-3} M) + $MgCl_2$ (10^{-3} M); open circles, no NH_4Cl; dots, + NH_4Cl (10^{-3} M). Dashed line: pH 7.9; open circles, no NH_4Cl; dots, + NH_4Cl (10^{-3} M).

EQUILIBRIUM CONSTANT OF THE DARK REACTIONS CONNECTING THE TWO PHOTOACTS

Theoretical

We confirmed experimentally two of ELEY AND MYERS' assumptions: the absence of spillover, and the linear dependence of the rate of System I upon the concentration of reduced P. Thus, if p is the fraction of P which is in the active (reduced) state the rate of photoact I is:

$$V_1 = k_1 p \tag{2}$$

As was shown in the experiment in Fig. 7, however, the rate of System II (V_2) does not linearly depend upon e, but instead follows Eqn. 1, for $a = 0.6$. This will be accounted for in our following analysis:

We define k_1 and k_2 as the fractions of the incident photons which would be trapped if p = 1 and e = 1. k_1 and k_2 represent, respectively, $\alpha_1\Phi_1$ and $\alpha_2\Phi_2$, α being the fractional absorption and Φ, the quantum efficiency of the photoacts. Φ varies with the efficiency of energy transfer from sensitizing pigments to the traps, and the presence of pigments and traps destroyed during preparation. Equilibrium III (p. 636) yields the relation:

$$ep = K(1 - e)(1 - p) \tag{3}$$

When a steady-state rate is obtained, $V_1 = V_2$, and Eqns. 1 and 2 yield the following relation in which $R = k_1/k_2$:

$$Rp = e\frac{1}{1 - a(1 - e)} \tag{4}$$

From Eqns. 3 and 4 we derive the two implicit functions: R = f(e) and R = f(p):

$$R = \frac{Ke + (1 - K)e^2}{K(2ae - ae^2 - e - a + 1)} \tag{5}$$

$$R = \frac{K(p - 1)}{p^2(K + a - 1) - pK} \tag{6}$$

Writing $V_1/V_{1max} = v_1$, we obtain $v_1 = p$; writing $v_2 = V_2/V_{2max}$ we obtain:

$$v_2 = \frac{V_2}{k_2} = \frac{V_1}{k_2} = Rp \tag{7}$$

Eqns. 6 and 7 yield the implicit function $R = f(v_2)$ which is the reciprocal of Eqn. 6:

$$R = \frac{v_2^2(K + a - 1) + Kv_2}{K(v_2 - 1)} \tag{8}$$

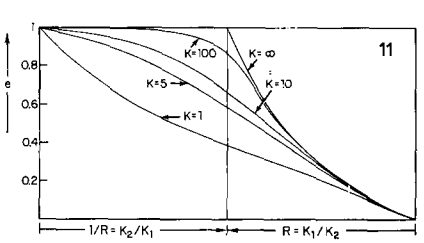
Figs. 11 and 12 present plots of Eqns. 5, 6 and 7 assuming parameter $a = 0.6$ and various values of the constant K (1, 5, 10, 100 and ∞). Note that the functions v_1 and v_2 are symmetric with respect to the vertical axis R = 1, and that the crossing point ($k_1 = k_2$) is always at R = 1.

Fig. 13 gives the values of the ratio Φ/Φ_{max} as a function of R computed according to:

$$\Phi/\Phi_{max} = \frac{2v_1k_1}{k_1 + k_2} = 2v_1\frac{R}{1 + R} \tag{9}$$

in which Φ is the actual quantum yield and Φ_{max} the maximum value attained in case both e and p equal 1. Φ_{max} thus would be equal to 0.5 (2 *hν*/electron) if energy losses during transfer and absorption by inactive pigment were negligible.

In Table I, line 3, we present the values of K, computed from the experimental values of $P/P_{max} = p$ and $E/E_{max} = e$ obtained at different wavelengths. Table II gives the values of K computed from the data of Fig. 9.



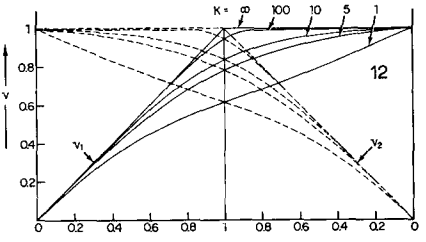


Fig. 11. Theoretical function e = f(R) for different values of K. e = concentration of photosubstrate of System II. R = ratio S_I/S_{II}, from Eqn. 5. $a = 0.6$.

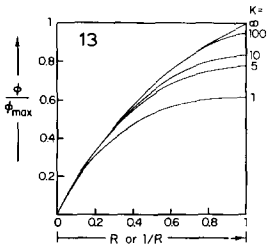
Fig. 12. Theroretical functions $V_1 = f(R)$ and $V_2 = f(R)$ from Eqns. 6 and 8, for different values of K. $a = 0.6$

TABLE II

COMPUTED VALUES OF K FROM FIG. 9

	630 mμ	650 mμ	670 mμ	680 mμ	690 mμ
K	3.7	6.3	7.5	5.0	4.5

The variation of the numbers whithin each experiment is probably insignificant. The important aspect is that K has a low value in all instances. The validity and significance of this low equilibrium constant will be considered further in the remainder of this paper.



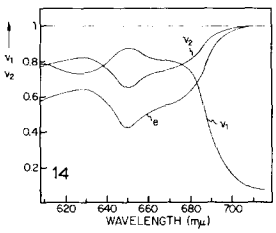


Fig. 13. Theoretical function $\Phi/\Phi_{max} = f(R)$. Φ = quantum yield. Φ_{max} = maximum quantum yield, when both e and p are equal to 1.

Fig. 14. Theoretical variation of V_1, V_2 and e with λ for $K = 5$. The value of R is computed from Fig. 6; 677 mμ has been chosen as the point of equal active absorption by the two photosystems.

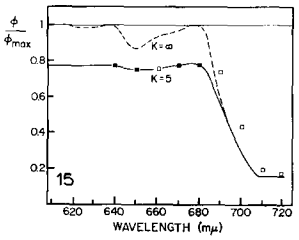
2. Predicted wavelength dependence of v_1, v_2, e and Φ

In the experiment of Fig. 9 the plots of v_1 and v_2 as a function of wavelength cross over at $\lambda = 677$ mμ. As demonstrated above, this wavelength represents the point of equal active absorption by the two photosystems. Different chloroplast preparations showed a variation of the exact position of this crossing point; the extreme values observed in four preparations were 665 and 677 mμ. We assume that a displacement towards shorter wavelengths indicates a selective inactivation of System II. Despite this variation of the crossing point, the value of K was always between 3 and 8. Knowing the wavelength for which R = 1 (Fig. 9) and the relative variation of R with wavelength (Fig. 6) we can compute the true value of R as a function of wavelength. These R values were used for the plots of Fig. 14 which show the wavelength dependence of v_1, v_2 and e computed with the aid of Eqns. 5, 6 and 8 (Figs. 11 and 12), and assuming $K = 5$ (average value of Table II). Agreement between the computed curves (Fig. 14) and the experimentally observed ones (Fig. 9) is excellent in the region of long wavelengths ($\lambda > 675$ mμ) and reasonable in the region of shorter wavelengths. Considering the fact that the function R was measured with another batch of chloroplast, and a narrower band width of the monochromatic light, the discrepancy between experiment and theory appears insignificant.

Another aspect illustrated by the arrows in Fig. 9 is the earlier mentioned slow increase of v_{s1}/v_{p1} and v_{s2}/v_{p2} in the course of the experiments (see p. 644). This variation can be interpreted as a gradual increase of K. The highest values of K, which were computed for data obtained approx. 20 min after the beginning of the experiment were 20–30 for System II measurements and 10–20 for System I determinations. As predicted by the theory, the curve v_{s1}/v_{p1} for $\lambda > 695$ mμ appears to be independent of K.

Using the same assumptions that were used in the case of Fig. 14, we have computed the predicted variation of the relative quantum yield Φ/Φ_{max} with wavelength, using the theoretical curve $\Phi/\Phi_{max} = f(R, K)$, of Fig. 13. Curve 1, Fig. 15 (full line), represents the theoretical quantum yield spectrum for $K = 5$. It is almost flat (± 3 %)

between 610 and 683 mμ, whereas at longer wavelengths it shows a rapid decline. On the other hand, the maximum value predicted for the quantum yield is $\Phi/\Phi_{max} \simeq 0.8$ (*i.e.*, 10.3 quanta per O_2 evolved, if we assume $\Phi_{max} = 0.125$ $O_2/h\nu$ or $1/\Phi_{max} = 8$ $h\nu/O_2$).



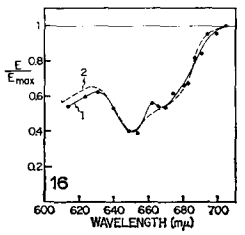


Fig. 15. Theoretical function $\Phi/\Phi_{max} = f(\lambda)$ for $K = 5$ and $K = \infty$. Squares: two sets of experimental data of SCHWARTZ, partly from ref. 43.

Fig. 16. Function $E/E_{max} = f(\lambda)$. Dots and full line: experimental curve obtained with Chlorella. The value of E was measured by a 10^{-4}-sec saturating flash after a preillumination in weak light. Dashed line: theoretical curve, using 681 mμ as the point of equal active absorption, $a = 0.6$, $K = 8$, R computed from Fig. 6.

For comparison we also calculated the quantum yield spectrum predicted for the case $K = \infty$ (dotted curve in Fig. 15). Note that in this case the yield varies considerably with wavelength in the region 610–680 mμ and shows a conspicuous maximum around 680 mμ (where $R \simeq 1$). This curve is very similar to one once computed by MYERS[31] on the basis of enhancement data. Note that in this case for all wavelengths were $R = 1$, $\Phi/\Phi_{max} = 1$, *i.e.*, the minimum theoretical quantum requirement is predicted.

Equilibrium constant in Chlorella

Fig. 16 illustrates that low values of K are not restricted to isolated chloroplasts. The data for Curve 1 were obtained with weak-light grown Chlorella cells and represent O_2 measurements with the concentration polarograph described in ref. 15. Plotted as the ratio E/E_{max} (e) is the amount of O_2 (E) liberated by a 10^{-4}-sec flash after preilluminations with low intensities of various wavelengths.

Curve 2 in Fig. 16 shows the wavelength dependence of e computed for $K = 8$ using the function $R(\lambda)$ measured with isolated chloroplasts (Fig. 6). We compared the absorption spectrum of the whole cells with the sum of the action spectra of Fig. 4 and estimated the point of equal absorption in Chlorella to be at 681 mμ, slightly beyond the longest λ value observed for $R = 1$ with chloroplasts. There is good agreement between the computed and measured curves, even better than obtained with isolated chloroplasts. The deviation in the region of 670 mμ can be ascribed to the use of a better wavelength resolution (< 1 mμ) in the measurement of $e = f(\lambda)$. Had we, like ELEY AND MYERS, assumed $\lambda = 685$ mμ for $R = 1$, then a K value of 10–14 would have resulted—still quite a low value.

This similarity of the results obtained with Chlorella and isolated chloroplast shows that (a) the distribution of pigment between the two photosystems is almost identical in the two materials; (b) in whole algae also, "spillover" does not occur at all, or with very low efficiency; if "spillover" were significant the "dip" at 650 mμ would have been much less pronounced; (c) the value of constant K is about the same in Chlorella as in isolated chloroplasts.

DISCUSSION

Other results were discussed earlier in the paper. Here we restrict our attention to the low equilibrium constant.

1. Comparison with ELEY AND MYERS' *results.* The low value of K computed in this paper, is at variance with the high equilibrium constant ELEY AND MYERS[39] arrived at for Chlorella. One aspect which might at least partly underlie this discrepancy is ELEY AND MYERS' assumption of a linear relation between v_2 and E. In our computations this assumption leads to a 3-fold increase of K, which, however, still remains < 50. A further consequence of the non-linearity is that the direct measurement of E, as in our experiment with Chlorella, is more accurate than the indirect measurement of v_2 by enhancement, as performed by ELEY AND MYERS. The precision with which K can be determined decreases with increasing values of K, so that it becomes difficult to discriminate between, *e.g.*, K values of 100 and 1000, particularly by means of rate measurements (see Fig. 13).

We recall that with isolated chloroplasts an increase of K was observed with time (Fig. 9), similar or other physiological variations could possibly occur in whole cells.

2. Consequence of the low K value on wavelength dependence and maximum value of quantum yield. One of the main difficulties encountered by the theory of two series connected photosystems is the invariant quantum yield generally reported in the range between 600 and 680 mμ. The computed low value of K appears to solve this difficulty, while at the same time it puts a (approx. 20 %) restraint on the maximum obtainable quantum yield. In Fig. 15 actual quantum yield numbers obtained in this laboratory by SCHWARTZ[41] are plotted after normalization for $\lambda = 680$ mμ. The agreement with the theoretical curve for $K = 5$ is very good. The use of rather wide band interference filters in the yield measurements accounts for the decline of the observed yields at slightly longer wavelengths, the region where absorption and activity drop sharply.

Using the theoretical curve of Fig. 13 and the average ratio of 4.3 between the yields observed at 720 mμ and 640 mμ we computed $K = 6$, in good agreement with our own determination. The data of HOCH AND MARTIN[33] imply a similar low K value.

Examination of the region 600–680 mμ in the classical quantum yield spectrum obtained with Chlorella by EMERSON AND LEWIS[42], yields an estimate of approx. 682 mμ as the wavelength of equal absorption. The observed average decrease of the yield at 650 mμ (approx. 7 %) then indicates a K value between 3 and 15. Also the yields obtained with high cell densities at wavelengths > 700 mμ are only compatible with a low constant. This range of K corresponds to minimum quantum requirements between 9 and 11 $h\nu/O_2$, slightly better than were actually reported in this paper. We feel this predicted lower limit of the absolute quantum requirement withstands scrutiny in the framework of the literature concerning this subject[34].

3. Biochemical significance of the low value of K. The low equilibrium constant K of the dark reaction chain which connects the two photosystems:

$$E^- + P^+ \underset{k_b}{\overset{k_f}{\rightleftarrows}} E + P \qquad K \simeq 5\text{–}10 \qquad \Delta E = 40\text{–}60 \text{ mV}$$

signifies a small change of free energy and a potential drop corresponding to only 40–60 mV. This small potential drop is in apparent conflict with the midpoint poten-

tials reported for the intermediates which might be involved. The assumption that P700 (0.43 V)[43] and cytochrome *b* (0.0 V)[44] are the endpoints of the two photoacts yields a maximal estimate of the potential drop: ≥ 400 mV. A minimal estimate is a drop of approx. 120 mV if we assume +360 mV as the endpoint of System I (cytochrome *f*[45]) and < +240 mV for System II (the fluorescence titration of pool A[40] assuming E and A have equal potentials).

Our finding of 50 mV disagrees with either estimate, to the extent of 350 mV with the first and 70 mV with the second one. The first estimate easily allows a coupling with ATP formation, which requires a drop of ≥ 200 mV (Eqns. 10, 11) whereas the second estimate falls short of this requirement.

$$E^- + P^+ + X \underset{k'_b}{\overset{k'_f}{\rightleftharpoons}} E + P + X\sim \tag{10}$$

$$ADP + X\sim \rightleftharpoons X + ATP \tag{11}$$

Since in Eqns. 10, and 11 we are dealing with the overall reaction only, the exact location in the reaction chain of X ~, a precursor of ATP, is immaterial. This assumption of energy coupling, however, meets an insurmountable difficulty since it implies that the measured constant K must depend upon the ratio X/X ~:

$$K = k'_f X/k'_b X\sim$$

That is the ratio X/X ~, and thus K, must depend upon the presence of uncouplers, ADP or ATP concentrations, as well as upon time and intensity of illumination: In very weak light one would expect $X \gg X\sim$ and thus a very large constant. The observed lack of an effect on K by uncouplers or ATP would imply irreversibility of Reaction 10. The conflict becomes even more serious if we identify X ~ with X_E of HIND AND JAGENDORF[46] (a proton gradient): build-up of X_E is most noticeable at low pH, which would imply a decrease of K, whereas actually we observed an increase. In summary, a large potential gap between the end products of the photoacts and an energy coupling step within the E → P chain appear incompatible with our data.

On the other hand, they can be more easily reconciled with the small (70 mV) potential discrepancy afforded by our second estimate above. For instance, one can assume some variance between potential values *in vivo* and *in vitro* and indirect effects of H^+ concentration as suggested by the effect of pH upon K. Rejection of a coupling site between E and P yields the interesting situation that, despite our low K value, we arrive at the same conclusion as that drawn by ELEY AND MYERS on the basis of their high estimate of K. As will be discussed in a subsequent paper, we presently feel that the energy for photophosphorylation must originate more or less directly in a photoact, as was proposed in ref. 40.

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