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REACTION BETWEEN PRIMARY AND SECONDARY ELECTRON ACCEPTORS OF PHOTOSYSTEM II OF PHOTOSYNTHESIS

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SUMMARY

We studied some properties and interactions of intermediate 'Q', the fluorescence-quenching trapping center of the O_2-evolving photosystem, and its associated electron acceptor, 'A'.

1. Direct observation of the oxidation in darkness of photoreduced Q by the fully oxidized A pool revealed a half-time of the reaction of approx. 0.6 msec at room temperature.

2. A study of the time course of the reduction of pool A in the light, using the chloroplast's capacity to reduce 2,6-dichlorophenolindophenol in the subsequent darkness as a measure of the pool, showed pool A to be unhomogeneous, $^1/_3$ of it reacting more rapidly with Q^- than the remainder. For this rapidly reacting fraction we estimated a bimolecular rate constant $k_1 = 165$ (pool units)$^{-1} \cdot$sec^{-1}, with one pool unit corresponding to $[Q]_{tot}$.

3. From fluorescence-rise curves, effected by a series of brief flashes (converting Q only), we computed 18 for the ratio of the concentrations of A and Q.

4. The shape of the rise curve in weak light was analyzed in terms of a bimolecular reaction between Q and A, varying the parameters: ratio [A]/[Q] and equilibrium constant K. Good fit with the experiment was obtained by assuming energy transfer between photosynthetic units, an [A]/[Q] ratio of approx. 20, and $K < 10$.

5. It was concluded that the small initial rise of the total rise curve did not reflect the photoreduction of Q, but rather the activation of this trapping center.

INTRODUCTION

The rise curve of fluorescence in isolated chloroplasts (Fig. 1) was previously analyzed in this laboratory by MALKIN AND KOK[1] and MALKIN[2] and explained on the basis of two pools of oxidant: The primary photoreactant (Q) quenches fluorescence in its oxidized state by using the photon to effect its own reduction; after the onset of illumination, fluorescence increases with time due to the photoreduction of Q (ref. 3). A pool of secondary oxidant A, which follows Q in the electron transport chain, accounts for the plateau phase of the rise curve by maintaining Q in the

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

oxidized state for some time. As A is also reduced, it can no longer reoxidize Q, and the final rise occurs.

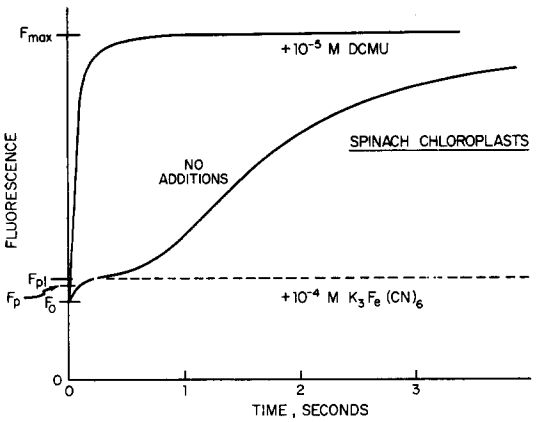

Fig. 1. Diagram showing fluorescence-rise curves observed with dark-adapted chloroplasts, (a) in the absence of external oxidants and in the presence of (b) DCMU and (c) ferricyanide.

In part based on observations at liquid-nitrogen temperature, MALKIN concluded that the ratio of [Q] to [A] was 1:1 and that each is present in the amount of approx. 1/70 chlorophylls. On the other hand, studies of the oxygen gush in algae by JOLIOT[4,5] pointed to a large secondary pool of approx. 1/30 chlorophylls and a small primary photo-oxidant pool of approx. 1/500 chlorophylls which was identified with quencher Q. We have found pool sizes comparable to JOLIOT's; in a study of 2,6-dichlorophenolindophenol (DCIP) reduction after brief and long flashes, we found values of 1/1250 and 1/70 chlorophylls with either spinach chloroplasts or Scenedesmus particles[6]. Other aspects of the system, such as the possible identity of A with the pool of quinone which, according to AMESZ[7] and WITT *et al.*[8], is located in the transport chain between the photoacts are discussed in ref. 9.

The present study was undertaken to establish firmly the ratio and the reaction kinetics of the two pools involved in the fluorescence rise curve. The model used is the following:

$$Q \xrightarrow[h\nu]{\lambda} Q^- \tag{1a}$$

$$Q^- + A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} Q + A^- \tag{1b}$$

Q quenches fluorescence, and Q^- does not.

For reasons which will be explained later in this paper, we have concluded that the initial rise of fluorescence from F_0 to the intermediate plateau level F_{p1} (see Fig. 1) is largely due to a photoprocess other than the reduction of Q. We therefore concern ourselves only with the fluorescence rise from F_{p1} to F_{max} in the main part of this paper and leave discussion of the initial rise from F_0 to F_{p1} to the end.

JOLIOT AND JOLIOT[10] explained the linearity of the fluorescence-rise curve observed in very strong light or in the presence of 3(3,4-dichlorophenyl)-1,1-dimethyl-

urea (DCMU) regardless of intensity (Fig. 1) by postulating energy transfer from 1 unit of pigment molecules to another. In whole algae they computed a value of 0.6 for the probability of transfer (P). Our observations with isolated chloroplasts agree with this model and indicate a slightly lower probability: P about 0.5. Conveniently, the value of 0.5 allows simplification of the JOLIOT equations: Without transfer,

$$P = 0 \qquad [Q] = 1-F \qquad \text{Rate} = \lambda[Q] = \lambda(1-F) \tag{2}$$

With transfer,

$$P = 0.5 \qquad [Q] = (1-F)/(1+F) \qquad \text{Rate} = \lambda 2[Q]/(1+[Q]) = \lambda(1-F) \tag{3}$$

where F is the intensity of fluorescence above the plateau level (F_{p1}), [Q] is the amount of oxidized Q. 'Rate' is the rate of photochemical conversion, and λ is a constant which includes light intensity, absorbtivity, and quantum yield for the photochemical conversion. Since one cannot describe reactions between fixed intermediates in terms of molarities, we express concentrations in 'pool units', the total amount of Q being defined as 1 pool unit. Fluorescence was normalized to $F_{max} - F_{p1} = 1$.

It is useful to note that when transfer is assumed, the photochemical rate is still linear with $(1-F)$, although [Q] is no longer linearly related to either rate or F. This means that even though energy transfer was not taken into account, MALKIN's determinations[1] of quantum yield and total pool size are valid, since they were obtained from the area over the fluorescence-rise curve, which is a product of photochemical rate and time.

The linear correspondence of rate and $(1-F)$ rests on the basic assumption that the probability of non-photochemical quenching is constant. Energy transfer between units thus does not disturb the fluorescence–rate correspondence. On the other hand, it must be pointed out that there may be at least one non-photochemical quencher which varies with time[11] (see below), and this could lead to some error.

METHODS

Spinach chloroplasts, prepared according to ref. 12 were diluted to 0.05 mg chlorophyll/ml in 0.05 M phosphate buffer at pH 7.4–8.0, and subsequently remained in darkness for at least 10 min before an experiment. Unless otherwise noted, all experiments were done at room temperature (22–25°).

Two different instruments were used for fluorescence measurements. In both, the blue light (incandescent source with Corning 5-58, 4-96, filters) which was used to excite fluorescence could be turned on in 2 msec with an electromechanical shutter. Fluorescence emission, $\lambda > 700$ mμ selected by Schott RG-8 filters, was detected with an S20 type photomultiplier. After amplification the signal was recorded, either on an oscilloscope memory screen (Tektronix 564) or in a Fabri-Tec computer.

The first instrument utilized a spinning disc to select flashes from a continuous 2000-W xenon arc lamp, and a complimentary disc to prevent them from affecting the photomultiplier. The length of these flashes could be varied between 0.2 and 2 msec by changing the size of the hole in the disc. For single flash experiments a xenon flash lamp (G.E.) was used, triggered so as to coincide with the disc opening; the length of this flash was about 1 msec. In this setup, fluorescence was observed

from the surface of the vessel which was illuminated by the flash and by the exciting beam.

In the second apparatus, single flashes of durations from 1 msec to 2 sec were selected from a 1000-W, continuous xenon arc by a two-bladed electromechanical shutter. The leading and trailing edges of these flashes were 0.3 msec long. Another electromechanical shutter was used to protect the photomultiplier, and opened after the flash. Here, fluorescence was observed out of the opposite side of the vessel (3 mm) from the flash and exciting beam.

The second instrument could be modified to measure the photoreduction of DCIP. It was used as a single beam instrument at 585 mμ; interference and color filters were used to prevent the actinic flashes from interfering with the S11-type photomultiplier. The observed changes of absorbance were recorded on both an X–Y recorder and in the memory of a Fabri-Tec computer; in some cases several traces were averaged to improve the signal.

RESULTS AND DISCUSSION

Effect of a single flash, rate constant k_1

Fig. 2A shows the effect of a brief flash on the fluorescence-rise curve with DCMU. Fluorescence is brought to F_{max} by a single flash, indicating that Q is completely reduced during the flash. Since the reaction of Q with A is blocked by DCMU (ref. 3) Q remains reduced and fluorescence remains high. This result was predicted from the work of MORIN, who found that in very strong light Q could be reduced and fluorescence raised in less than 1 msec (ref. 13).

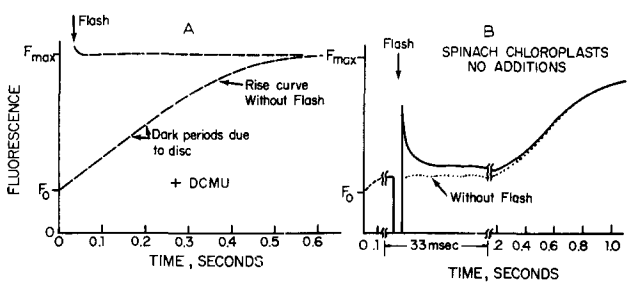

Fig. 2. A. Time course of fluorescence observed with dark-adapted, isolated chloroplasts in the presence of DCMU. The observations were made with the spinning-disc apparatus in which the photocell was darkened approx. 3 msec every 33 msec. The response during the dark periods is not shown. In one of the observations a brief flash was given during the first dark period following the onset of the illumination. B. Same experiments performed in the absence of DCMU, and with an approx. 10-fold stronger fluorescence exciting light. The oscilloscope time base was accelerated during one revolution of the disc; at the beginning of this period a flash was given to the sample while the photocell was darkened. Dotted curve shows a control experiment without flash.

Without DCMU, the flash effect is much different, as shown in Fig. 2B. Although the fluorescence is raised to $^2/_3$ of F_{max} by the flash, it quickly decays to a level only slightly greater than that before the flash. Apparently Q is reduced during the flash, but is rapidly reoxidized by large secondary pool A. If the flash is given at a late time during the rise curve, the level after the flash is F_{max}, and the decay is slower, since there is little oxidized A to react with reduced Q. Analysis of the decay curve yielded an estimate of the half time of reoxidation of approx. 0.6 msec.

An alternate estimate of the rate constant k_1 can be obtained by studying the photoreduction of A *via* Q: By measuring the oxygen evolved in flashes of different lengths, JOLIOT observed in Chlorella a biphasic time course of the gush; the half time of the first phase, which comprised $^1/_3$–$^1/_2$ of the total pool was 0.1 sec at 5°. DE KOUCHKOVSKY AND JOLIOT[14] obtained similar results with isolated chloroplasts. We have done analogous experiments, measuring the DCIP-reducing pool[6] and the pool represented by the area over the fluorescence-rise curve, assuming these phenomena also reflected pool A. Our results are shown in Fig. 3.

In the first experiment (full line), we monitored the concentration of reduced DCIP. Flashes varying in length from 1 msec to 2 sec were given to a suspension of chloroplasts which contained 0.1 mM DCIP. We measured the amount of DCIP which was reduced in the dark after the flash and assumed this to represent the amount of reduced A which had been built up in the light. The biphasic curve reveals a small pool (A_1) amounting to approx. $^1/_3$ of the total which is filled with a half time of approx. 4 msec and a larger pool (A_2) which fills up with a half time of approx. 0.1 sec. Using a value of 2.0 for the Q_{10} of the Q–A reaction the curve of Fig. 3 agrees well with those reported for oxygen evolution[4,14]. As will be discussed later, the total amount of A exceeds that of Q (1 pool unit) by some 20-fold. Thus the rapidly reacting third of A amounts to 7 pool units.

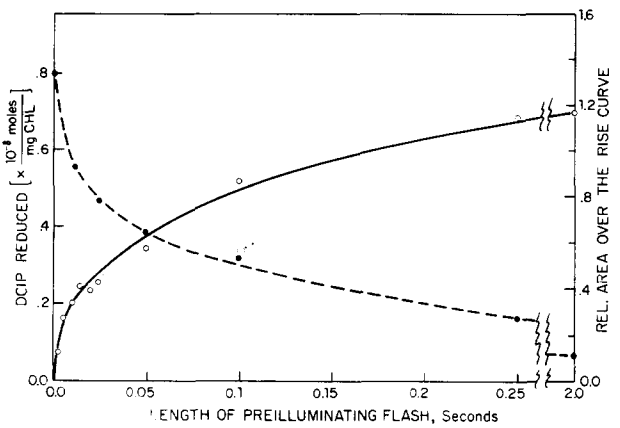

Fig. 3. Full line: Amount of DCIP reduced in darkness following a flash of saturating intensity of indicated duration. Chloroplast concentration, 50 μg chlorophyll/ml. DCIP, 0.1 mM. Optical path length, 3 mm. Broken line: Area over fluorescence-rise curves, observed 0.2 sec after similar flashes as used for above experiment. Chloroplast concentration, 20 μg chlorophyll/ml, no additions.

In the fluorescence experiment of Fig. 2B, we viewed the oxidation of Q^- by fully oxidized A; the half time reflected $k_1[A]_{tot}$. In the DCIP experiment we followed the reduction of A by Q^- maintained in the reduced form by the strong light; the half time reflected $k_1[Q]_{tot}$. Thus we expect a ratio between the two observed half times $[A_1]/[Q] = 7$, which was indeed found. Both of these half times yield a bimolecular rate constant k_1 of approx. 165 (pool units)$^{-1}\cdot$sec^{-1}.

In the second experiment of Fig. 3 (broken line), flashes of different lengths were followed by 0.2 sec of darkness and then by the observation of a fluorescence-rise curve. The area over each rise curve was used as a measure of oxidized A and plotted

vs. flash length. Despite some difficulty in evaluating the area over the slow, final phase of the fluorescence rise, the curve is qualitatively like that seen in DCIP reduction and oxygen evolution. This is furher evidence that all three types of measurement reflect the same intermediate.

Effect of successive flashes, ratio Q_{tot} to A_{tot}

In order to determine the ratio of Q_{tot} to A_{tot}, an experiment was designed to count the number of times Q has to be filled in order to fill A. Bright flashes of red light were given repetitively, with 33-msec intervals between them; these were essentially dark intervals, since the fluorescence exciting beam was so weak as to exert negligible photochemical action. Since each flash completely reduced Q and the dark periods allowed time for reoxidation by A, the number of flashes necessary to complete the rise curve was a reflection of the pool size of Q compared to that of A.

Such a multiple-flash experiment is illustrated in Fig. 4. This figure should be given some further explanation. To prevent the 'measuring' beam from exerting a significant photochemical action, it was necessary to keep its intensity very low, and therefore to use a highly, sensitive means of detection. As a result the luminescence signal masked the rapid return of Q from the reduced state, although it decayed rapidly enough to permit observation of the final level of Q at the end of each dark period. The 'grass' on top of the curve is due to this luminescence, which is not faithfully recorded because of attenuation on the oscilloscope memory screen. The useful information, therefore, is only the level of Q seen after the decay of luminescence. In the single flash experiment of Fig. 2B the measuring beam was brighter, so we were able to use a lower sensitivity and to watch the rapid return of Q without interference of luminescence.

Fig. 4 shows that after the first flash fluorescence does not return to F_0 but to the level F_{pl}. After later flashes, the return level begins to rise in the manner of a fluorescence-rise curve in continuous light. Q, in reaching equilibrium with partially reduced A, is not completely reoxidized; *i.e.*, whereas the first flash results in an

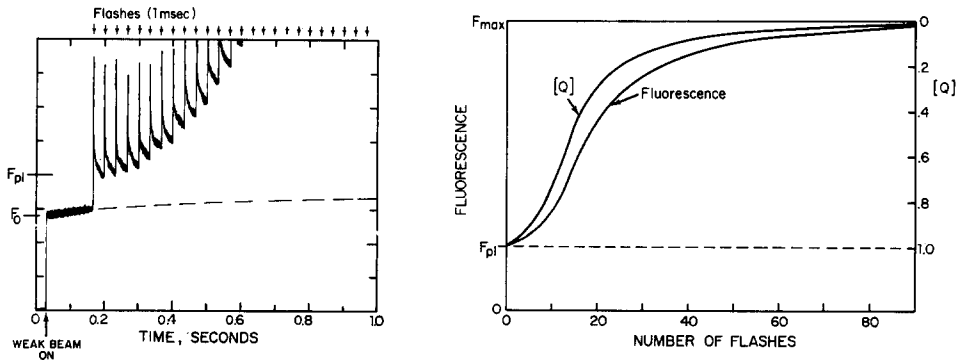

Fig. 4. Rise of fluorescence yield observed with dark-adapted chloroplasts upon exposure to a sequence of bright flashes. A weak exciting light was used to monitor the fluorescence yield; this beam by itself caused the yield to rise according to the dashed curve.

Fig. 5. Envelope of the fluorescence rise in flashing light (0.25-msec flashes spaced 33 msec apart) obtained in experiments as shown in Fig. 4. Also shown is the time course of [Q] derived from *F via* Eqn. 3.

amount of photochemical conversion equivalent to $[Q]_{tot}$, subsequent flashes convert less and less, Q becoming more and more reduced. The amount of conversion by each flash is the amount of oxidized Q available before the flash. The total amount of photochemical conversion is found by summing the amounts for the individual flashes.

In practice the ratio A_{tot}/Q_{tot} was computed as follows: The envelope of the rise curve in flashing light was drawn through the fluorescence levels at the endpoints of each cycle (*cf.* Fig. 5). From this fluorescence curve we computed the state of Q as a function of the number of flashes using Eqn. 3. The area bounded by the latter curve ($[Q] \times$ number of flashes) was used as a summation of the amounts of conversion achieved by each flash. This area divided by the area over the first flash (when $[Q] = 1$) yields the total conversion A_{tot}.

From the experiments in Fig. 4 we obtained a total pool size of 19 for $Q_{tot} + A_{tot}$, or an A_{tot}/Q_{tot} ratio of 18. This value is in fair agreement with the value of 15 obtained by FORBUSH from measurements of pool sizes in DCIP reduction, and the value of approx. 20 generally observed as the ratio between the area over the fluorescence-rise curve with and without DCMU (Fig. 1). DE KOUCHKOVSKY AND JOLIOT[14] derived a similar ratio from oxygen-gush experiments. Pool sizes are known to vary depending upon the condition of the chloroplasts; both Q_{tot} and A_{tot} decrease with time after preparation. The smaller, rapidly reacting fraction of the A pool seems to be identical to the plastoquinone pool described by WITT *et al.*[8].

Shape of the rise curve, equilibrium constant K

The shape of the fluorescence-rise curve from F_{pl} to F_{max} should be compatible with reaction scheme 1, energy transfer (Eqn. 3) and the observed A_{tot}/Q_{tot} ratio. In order to demonstrate this we have computed curves based on these predictions. Even though Q and A are probably in a fixed moiety, we assume that the kinetics of reaction 1 are those of a bimolecular reaction in solution. This is reasonable since their ratio is large.

Eqns. 1 and 3 yield the following differential equations:

$$d[Q]/dt = \lambda 2[Q]/(1+[Q]) + k_1([Q]_{tot}-[Q])\,[A] - k_{-1}[Q]\,([A]_{tot}-[A]) \quad (4a)$$

$$d[A]/dt = -k_1\,([Q]_{tot}-[Q])\,[A] + k_{-1}[Q]\,([A]_{tot}-[A]) \quad (4b)$$

To compute the theoretical rise curve, we solved simultaneously the two differential equations describing the two reactions, taking $[Q]_0 = [Q]_{tot} = 1$ and $[A]_0 = [A]_{tot}$ as initial conditions. Because of the complicated term introduced by energy transfer, the equations could not be solved explicitly and numerical solution by digital computer was used. Solutions were found for values of A_{tot}/Q_{tot} from 10 to 30; for k_1/k_{-1} from 1 to 60, and for λ from $\lambda \to 0$ to $\lambda = 100 \times k_1$.

We usually measure the fluorescence-rise curve at exciting light intensities so low that $\lambda \ll k_1$; in Fig. 1 for instance, $\lambda < 0.02 \times k_1$. Under these conditions, the reaction (1b, 4b) is effectively at equilibrium, and the problem can be reduced to that of one differential equation *plus* equilibrium restrictions. In this paper we will discuss only curves in which λ approaches 0. For intermediate light intensities we have made only a cursory comparison of theory and experiment, and no striking disparity was observed. DELOSME[11] has already considered the case where $\lambda \gg k_1$, *e.g.* $\lambda = 1000 \times k_1$.

Fig. 6 shows three theoretical rise curves computed for $A_{tot}/Q_{tot} = 25$ and $K = k_1/k_{-1} = 1$, 3 and 6, respectively. It also shows the range of observed experimental curves, plotted without the small initial rise. To allow easy comparison, the curves have been normalized on the time axis so that their half-rise points fall at the same time. The experimental curve does not exactly fit either the theoretical curve with $K = 1$ or 3 but fits $K = 3$ for the first half and $K = 1$ for the second half.

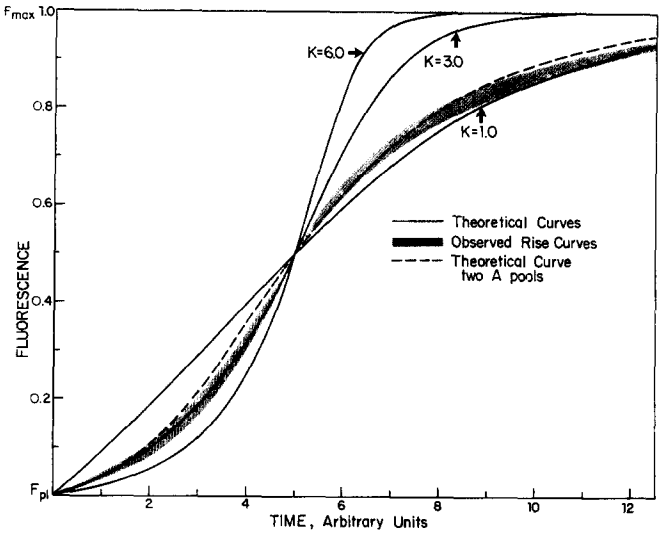

Fig. 6. Comparison of observed rise curves (dotted band) with theoretical ones, computed assuming energy transfer between units ($P = 0.5$) for $A_{tot}/Q_{tot} = 25$ and $K = 1$, 3 and 6, respectively (solid lines). The dashed line is based on the assumption of two equal fractions of A (each equal to 12 $[Q]_{tot}$), having equilibrium constants of 0.5 and 6, respectively.

This suggests that pool A is non-homogeneous, which was also indicated by the biphasic reduction of Q by A (Fig. 3).

The dashed curve in Fig. 5 demonstrates that, given this extra variable, one can achieve good agreement between theoretical and experimental rise curves: it shows a solution assuming two equal pools of A each amounting to 12 units; each in equilibrium with Q, but with K values of 0.5 and 6, respectively. There is some evidence that the low K pool is the rapidly reacting plastoquinone pool A_1, and the high K pool is A_2.

In weak light the residual rate of electron transport which, in the absence of added oxidant, is sustained by molecular oxygen prevents the experimental rise curve from reaching the true F_{max} value. This yields an uncertainty in the evaluation of the lower of the two equilibration constants and, together with the variability of the material, limits the precision attainable.

From the examples given, however, it is obvious that the rise curve reflects a low equilibrium constant between Q and A. This agrees with other available evidence concerning these intermediates[4,6].

It was of interest to determine the dependence of the theoretical rise curve on the A_{tot}/Q_{tot} ratio, because this ratio seems to vary with different chloroplast preparations as pointed out above. For $K < 10$ there proved to be almost no difference in the theoretical curves for $A_{tot}/Q_{tot} = 10$ and 30; for higher values of K there was

some difference. Since experiment shows $K \leqslant 5$, it is safe to conclude that the shape of the fluorescence-rise curve is insensitive to the A_{tot}/Q_{tot} ratio.

Another point of interest is the effect of energy transfer on the shape of the rise curve. In Fig. 7 two theoretical curves are plotted for $K = 3$, one with (1) and one without (2) the assumption of energy transfer between units. Qualitatively the curves are the same, but it is evident that we would have arrived at a slightly higher value for K (approx. 8) if we had not assumed transfer.

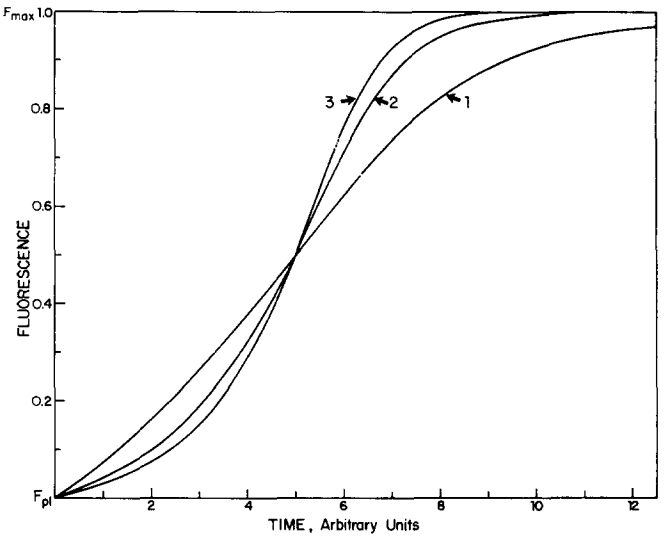

Fig. 7. Curve 1: Theoretical rise curve computed for $A_{tot}/Q_{tot} = 25$, $K = 3$ and assuming absence of energy transfer between units. For Curve 2 a transfer probability $P = 0.5$ was assumed. Curve 3 incorporates the additional assumption of a non-photochemical quenching process, proportional to [A] according to DELOSME[11].

Finally, in Curve 3 we considered the possible effect upon the shape of the rise curve of the non-photochemical quencher observed by DELOSME[11]. The shape of the rise curve is hardly altered by such a quencher.

The initial fluorescence rise, activation

So far we have ignored the first small rise of fluorescence from F_0 to F_{pl} (Fig. 1), because we could not explain it in terms of Q and A. We predict such a rise at moderate and high light intensities where the photoreduction of Q exceeds the rate of oxidation by A, enough to substantially displace the Q–A equilibrium. However, at low intensity ($\lambda \ll k_1$) the rapid reaction Q → A initially keeps Q completely oxidized, in equilibrium with A. We therefore assumed that the initial rise observed even at low intensities was due to some other photoprocess.

The assumption is strengthened by two observations: (1) As depicted in Fig. 4, a single flash (which can be as short as 5 μsec) is sufficient to effect the initial rise. (2) Even in the presence of a high concentration of ferricyanide to maintain A and Q fully in their oxidized state, the initial rise is still observed in very weak light, or after a single flash (Fig. 1).

The most likely explanation of this other photochemical reaction has been proposed by JOLIOT[5] in terms of the activation process. In the dark, Q reverts to an

'inactive' state Q_I with a half time of about 30 sec in chloroplasts at room temperature[14]. The first reaction after the light goes on is $Q_I \rightarrow Q^-$, quickly followed by $Q^- + A \rightleftharpoons Q + A^-$; from here on the reactions are those of Eqn. 1, since the rate of deactivation is small.

To explain the initial rise, we must assume that Q_I is a quencher like Q, but with a slightly higher efficiency of quenching. Fluorescence is therefore slightly higher when Q is in the active state Q (level F_p) than when it is inactive Q_I (level F_0). The initial rise represents the conversion of inactive Q_I to Q by way of Q^-, a process which occurs in a single flash either with or without ferricyanide. With DCMU, Q^- cannot be reoxidized by A, and we observe only the reaction $Q_I \rightarrow Q^-$. Actually, the moderate light ordinarily used to measure fluorescence-rise curves is sufficient to keep a small amount of Q reduced, even near the beginning of the rise curve or with Hill acceptor present. Thus the rise to F_{pI} is due both to activation and to reduction of a small part of Q.

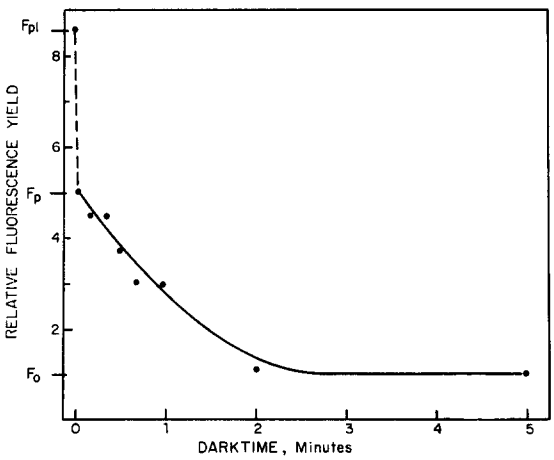

Fig. 8. Time course of the dark restoration of the fluorescence yield from F_{pI} to F_0 observed with isolated chloroplasts in the presence of 5 mM ferricyanide.

To further support this theory, we show the experiment of Fig. 8 which was performed in this laboratory by OWENS AND JOLIOT. The experiment of Fig. 8, made with isolated chloroplasts in the presence of ferricyanide, shows the time course of the fluorescence yield ('F') in darkness after an illumination had brought F to the level F_{pI}. The restoration from F_{pI} to F_0 is definitely biphasic. The fast phase, $F_{pI} \rightarrow F_p$, is over in less than (2 sec) and presumably reflects the reoxidation of the small amount of Q^- to Q. The slow phase, $F_p \rightarrow F_0$, has a half time of about 30 sec which is in fact the half time for the deactivation reaction $Q \rightarrow Q_I$. JOLIOT earlier observed the same phenomenon in whole algae[5], but was not as certain of the oxidizing conditions as in the experiment described with chloroplasts and ferricyanide.

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