INDUCTION OF FLUORESCENCE IN QUINONE POISONED CHLORELLA CELLS[1,2]

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The efficiency of fluorescence quenchers has been shown to be dependent on the concentration of the fluorescing dye. This effect was attributed to the occurrence of the migration of electronic excitation (6). In view of the special importance of this problem in the theory of the photochemical aspect of photosynthesis, it was found desirable to look at a possible difference between the efficiencies of fluorescence quenching of chlorophyll by quinone in vitro and in vivo. However, our attention was soon diverted toward a very striking change in the induction of fluorescence upon poisoning of Chlorella cells with quinone. Contrary to the impression given by one published observation (9), we found that quinone at moderate concentration did not suppress the induction of fluorescence completely. On the other hand under suitable conditions, the mean fluorescence level is depressed and a new induction course appears, the main feature of it being a rapid rise of fluorescence followed by a slower decay to a steady state.

The general significance of the Hill reaction and related processes is now well established: mechanical separation (isolated chloroplasts) or chemical inhibition (quinone reaction with Chlorella) uncouples the water-photolysis mechanism from the key hydrogen acceptor of the CO_2 reduction cycle. This must bring considerable simplification in the kinetic picture and might support the hope of untangling some of its components more easily. The present report will try to illustrate this point of view.

MATERIAL AND METHODS

Algae (*Chlorella pyrenoidosa*) were grown in Knopp solution at 23° C with 2 % CO_2-air mixture bubbling at the rate of 1.5 l per minute. Light was supplied by 2 banks of 3 fluorescent tubes (16-watt), 1 bank on each side of the culture flasks. For use, cells were centrifuged and resuspended in phosphate buffer 0.1 M, pH 6.4 plus KCl 0.05 M. A stock solution of purified quinone was added to the suspension giving a quinone concentration of 3×10^{-4} M (unless otherwise stated) and chlorophyll concentrations from 0.03 to 0.05 mg per ml. The mixture was incubated for ca. 10 minutes before the measurements were made.

The fluorescence was excited with monochromatic light at 670 mμ (half-band width: 13 mμ) from a Bausch and Lomb grating monochromator operated with a tungsten ribbon lamp. The fluorescent light was passed through a 2nd monochromator of the same type to the detector, an infra-red sensitive photomultiplier tube (Dumont, type K 1292). This 2nd monochromator was set at 725 mμ with a half-band of 26 mμ.

Fluorescence curves were automatically recorded with a galvanometer and a Photodyne recorder (Sefram); in several instances, oscillographic recording has been used. The cell suspension was introduced in a narrow vertical glass tube 1.5 mm inside diameter. A 10 mm section of this tube was illuminated with the exciting light which had a maximum incident intensity of ca. 3500 erg/sec cm^2 which will be referred to as I = 100. Constant temperature was maintained with a water jacket surrounding the glass tube. The fluorescence measurements were corrected for stray light using a correction factor estimated from measurements of the light scattered by light bleached cells.

RESULTS AND DISCUSSION

Figure 1 is a typical fluorescence curve, recorded under high exciting light intensity. Letters O, P and S have been used throughout this report as representing the initial, maximum and stationary levels of fluorescence; they divide the fluorescence curve into 2 main phases: O–P and P–S. We will also make extensive use of the quantities p_M, p and s, as defined on figure 1.

Several basic observations are to be noted first: 1.) Level S lies only slightly above level O, and conditions can be chosen that will maximize the ratio p_M/(light intensity): low temperature and high light intensity. 2.) The illumination can be interrupted for short periods of time during the P–S phase without significantly disturbing the time course of p. 3.) The time curve of p is best described by the equation:

$$1/p = A + Bt \qquad (1)$$

where A and B are constants and t is time. However, the validity of equation 1 is restricted to the end of the P–S phase, the 1st points lying definitely above the straight line (fig 2). 4.) The whole fluorescence curve can be repeated several times if the preparation is allowed to stay in darkness for a minimum period of time. The minimal interval depended on the temperature. Recording the fluorescence peaks at shorter periods of dark rest gives intermediate p_M values, which fall on a smooth curve when plotted as a function of dark time (fig 3). This process will be referred to as the recovery phase.

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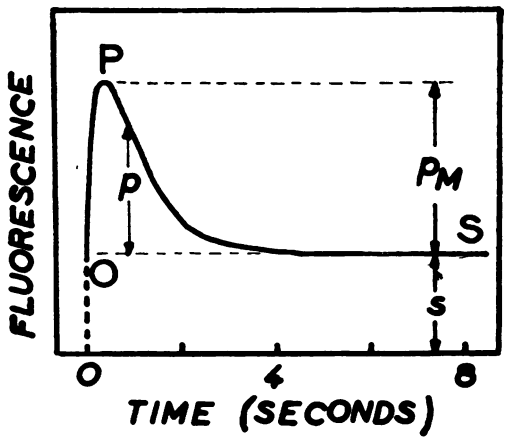
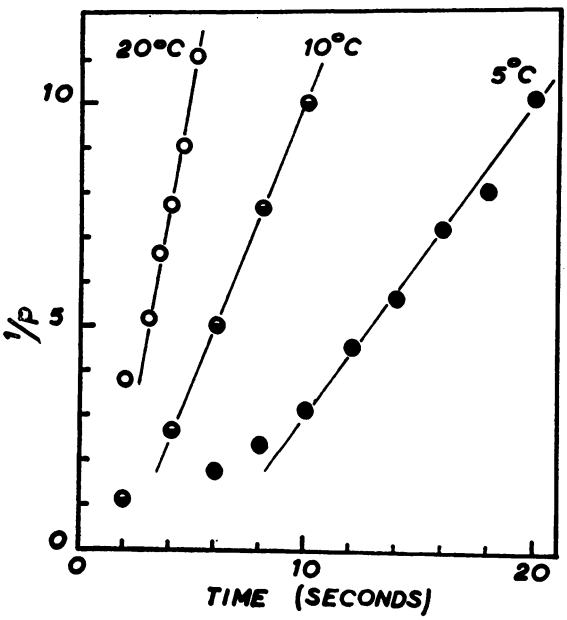
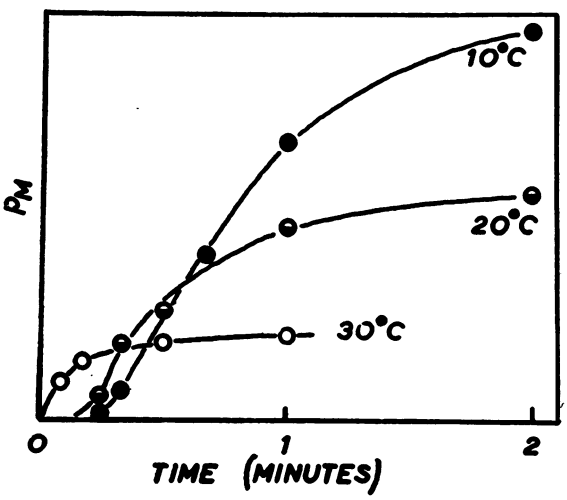
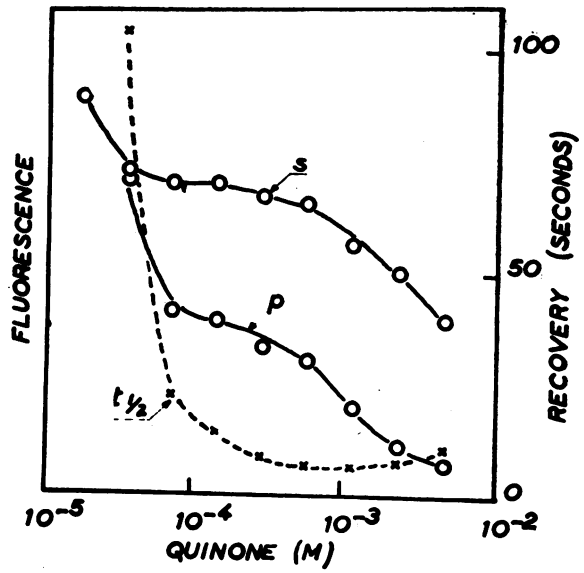








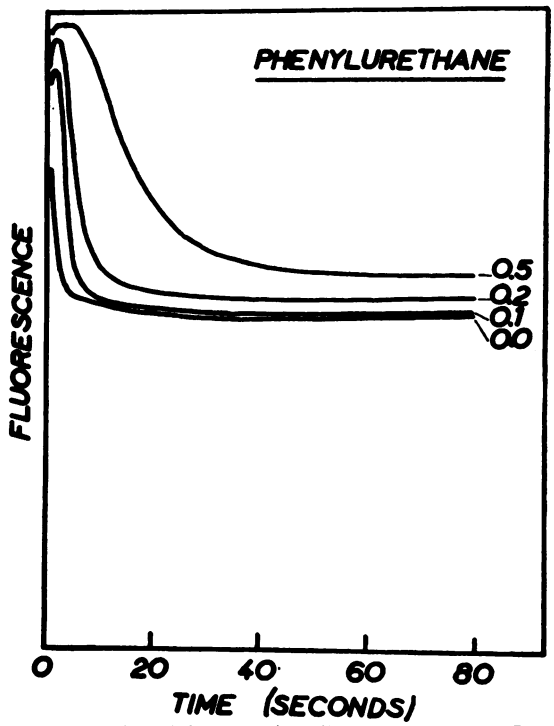


FIG. 1 (*top left*). Typical fluorescence curve. I = 100, 18° C (redrawn from oscillographic recording; symbols in text).

FIG. 2 (*center left*). Variation of 1/p during the P–S phase at different temperatures. I = 100.

FIG. 3 (*bottom left*). Recovery phase at different temperatures. I = 100. Note increase of final p_M value with decreasing temperature.

FIG. 4 (*top right*). p_M, s and half-time of recovery ($t\frac{1}{2}$) as a function of quinone concentration. I = 100, 18° C.

FIG. 5 (*bottom right*). Effect of phenylurethane on fluorescence induction with quinone. I = 100, 18° C. Numbers are volumes in ml of aqueous saturated phenylurethane solution per ml of mixture.

The above mentioned observations will serve as the basis of our working hypothesis. One important, although somewhat independent assumption, is that the fluorescence emission can be split into 2 distinct parts: a constant back-ground of magnitude s, and a part subject to induction of variable magnitude p. The latter, which is all that concerns us here, is supposed to arise from a chlorophyll complex whose change in composition is partly reflected in the fluorescence change during the induction period. Specifically, 1 form of this complex, labelled ChlP, is fluorescent and non-photoactive: p is a measure of its relative concentration. Two other forms of the complex are needed to complete the picture: a non-fluorescent photoactive form, ChlO and a non-fluorescent non-photoactive form, ChlS. The 3 forms appear in the following sequence. Before illumination, the total amount of the complex is present in the state ChlO. When light is turned on, ChlO is photochemically transformed in ChlP: this accounts for the rapid fluorescence rise O–P. The next step P–S is essentially a thermal bimolecular process whereby ChlP combines with a substance X′ whose concentration at first lags behind, then equals that of ChlP near the end of the decay. This is in agreement with the 2nd observation and the asymptotic behavior of the P–S phase towards equation 1. This assumption in turn implies that, during the photochemical phase O–P, ChlO dissociates into ChlP plus a substance X which is then transformed in X′, an intermediate reaction that will at first delay the disappearance of ChlP by the bimolecular reaction. The end product of the latter reaction is the 3rd form of the complex, ChlS. A further reaction will transform ChlS in ChlO, thus closing the cycle. This is a slow process which, according to the 4th observation, gradually rebuilds ChlO during the recovery phase.

Summarizing, the chlorophyll complex undergoes the following sequence of reactions:

$$\text{O–P phase: ChlO} \xrightarrow{k^*I} \text{ChlP} + \text{X} \qquad (2\,a)$$

$$\text{P–S phase: X} \xrightarrow{k_1} \text{X}' \qquad (2\,b)$$

$$\text{ChlP} + \text{X}' \xrightarrow{k_2} \text{ChlS} \qquad (2\,c)$$

$$\text{Recovery phase: ChlS} \xrightarrow{k_3} \text{ChlO} \qquad (2\,d)$$

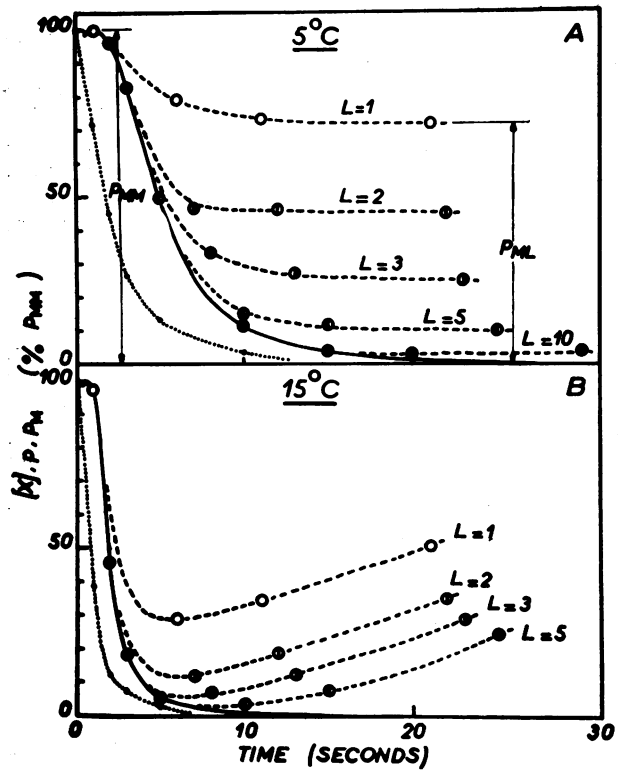
It is to be noted that points O, P and S in figure 1 correspond to the chlorophyll complex present in the state ChlO, ChlP and ChlS respectively. This, as well as attributing a specific reaction or group of reactions in scheme 2 to a given phase of the induction curve, is only legitimate in so far as the rate constants fall in the order: $k^* I \gg k_1$, $k_2 \gg k_3$. High light intensity, low temperature and medium quinone concentration are conditions typically fulfilling these requirements. Under different conditions (as will be seen below), one cannot draw such a sharp distinction between the successive parts of the induction curve.

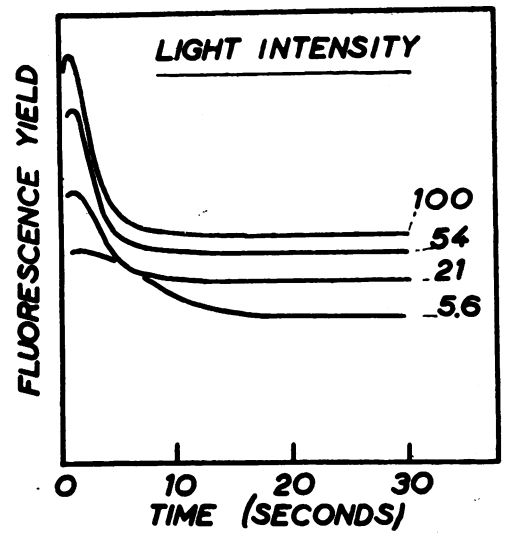
Quinone Concentration: The characteristic modifications of fluorescence induction here described make their appearance at quinone concentrations as low as 10^{-5} M. The action of quinone at such low concentrations is indicative of a primary inhibition of some important catalyst of the CO_2 pathway. On the other hand, we believe that true fluorescence quenching is not effective until the concentration is above 3×10^{-4} M (fig 4). According to Livingston and Ke (7), half-quenching concentrations in solution range from 8.1 to 9.6×10^{-3} M, depending on solvent. The rate of recovery, as measured by its half-time, is strongly dependant on quinone concentration (fig 4). Such a dependance can also be seen with Chlorella cells first exposed to the action of quinone, and then washed several times. The modification of fluorescence induction is not reversed—a fact reminiscent of the well known irreversible loss of photosynthetic ability—but the rate of recovery decreases with increasing number of washings. This points to attributing reaction 2 d to the reduction of quinone; if so, ChlS would be the reduced form of the complex.

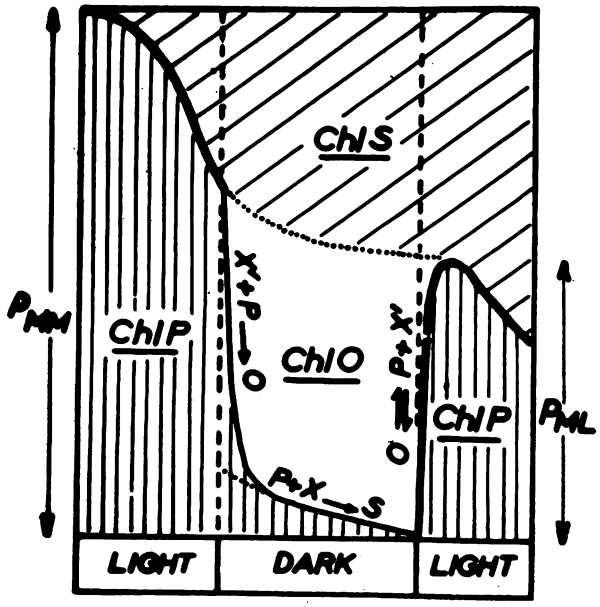
Inhibitors: Among inhibitors known to affect the Hill reaction, we have found that phenylurethane and *o*-phenanthroline had similar strong effects, whereas the only action of hydroxylamine was the lengthening of the recovery phase. Upon adding increasing amounts of one of the active inhibitors at room temperature, it is found that p_M increases up to a maximum value and that the P–S phase starts with a plateau and gets progressively slower (fig 5). These results can be understood on the basis of a specific inhibition of reaction 2 b. In particular, a maximum value of p_M means that the balance between production and consumption of ChlP, which determines the concentration of this substance at point P, is shifted toward maximum accumulation. Low temperature (see below) obviously must have similar effect.

Another effect of phenylurethane which would require a special explanation is the increase in the rate of recovery. As this accelerates the cycle, the stationary level S tends to rise slightly, as can be seen on figure 5.

P–S Phase in Darkness: Scheme 2 suggests that, providing the recovery reaction 2 d can be kept slow enough and sufficient light has been given to terminate the photochemical reaction 2 a, the P–S phase should not depend on sustained illumination and should proceed to completion in darkness (not only for short periods of time, as specified in the 2nd observation). Accordingly, the preparation was pre-illuminated during a time L and the peak of fluorescence p_M was recorded after a dark period D. Figure 6 is a plot of p_M as a function of L + D for several durations of pre-illumination at 2 temperatures. The P–S curve in continuous illumination is also given for comparison. It is seen that the dark evolution approximates the light one for only short periods of time following the end of pre-illumination and that there is accumulated a limited amount of ChlS—as measured by the difference $p_{M_M} - p_{M_L}$ (see fig 6 A)—







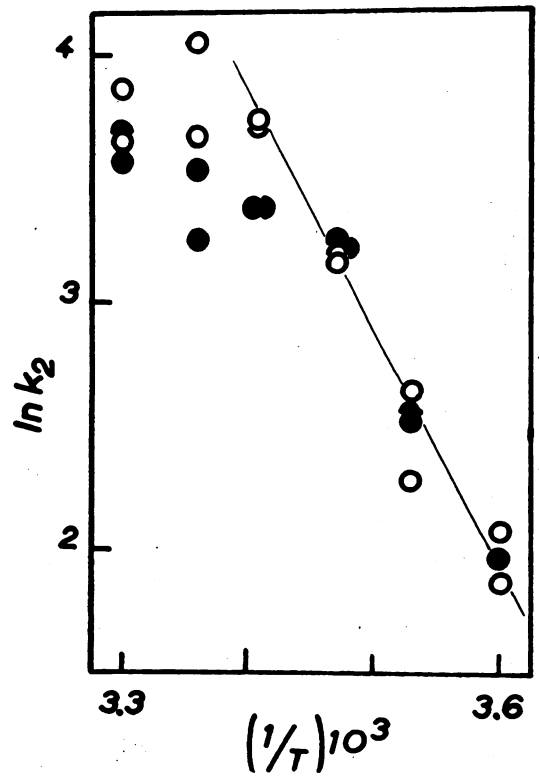


FIG. 6 (*top left*). P–S phase in darkness at two temperatures. (———) P–S in continuous illumination, (- - - -) p_M curve following pre-illumination L (final p_M value at 5° C is p_{ML}), (. . . . .) time curve of X.

FIG. 7 (*bottom left*). A sketch of the kinetic evolution of the system upon interrupting light during the P–S phase. Heavy inked line corresponds to the fluorescence curve.

FIG. 8 (*top right*). Effect of light intensity on fluorescence induction with quinone. 5° C.

FIG. 9 (*botom right*). k_2 (arbitrary unit) as a function of absolute temperature T in the range 5 to 30° C. Cells grown in normal (O) and 70 % normal light (●).

which increases with L up to the maximum possible value. At higher temperature (fig 6 B) reaction 2 d proceeds at an appreciable rate and prevents the accumulation of ChlS from reaching a stable level, as the ascending curves of p_M show. The more likely explanation of this dependance is that reaction 2 a is in fact a photochemical equilibrium:

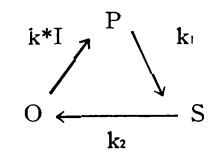
$$\text{ChlO} \xrightarrow[k']{k^*I} \text{ChlP} + \text{X} \qquad (2\,a')$$

As long as equilibrium 2 a′ is shifted far to the right by high light intensity (pre-illumination), X disappears by reaction 2 b, as was assumed above. As soon as light is turned off, equilibrium 2 a′ quickly shifts to the left, thus returning part of ChlP to ChlO and bringing reaction 2 b to a standstill. The remainder of ChlP keeps following the "normal" path 2 c to the extent of the amount of X′ produced during the preceding light period. When light is turned on again, the maximum amount of ChlP compatible with the instantaneous state of the kinetic system is quickly formed through equilibrium 2 a′ and appears as p_{ML}. This complex behavior is schematically depicted on figure 7. Thus, the maximum amount of ChlS produced following pre-illumination L can be used as a measure of the amount of X consumed during time L; the time course of X consumption by reaction 2 b has also been plotted on figure 6.

Light Intensity: The effect of this variable is illustrated by figure 8; time curves of relative fluorescence yield have been used, enabling direct estimation and comparison of the amount of ChlP present under different light conditions. The increase of ChlP concentration with light intensity at point P is self explanatory. The lengthening of the P–S phase when light intensity decreases results from the above given considerations: at low light intensity, equilibrium 2 a′ lies in the middle of its maximum excursion, the more to the left the lower the intensity. This keeps the concentrations of ChlP and X low, thus slowing down the subsequent dark reactions. That the S level rises when light intensity increases is evidence that reaction 2 d is not so slow in this instance as to prevent the cycle from running at a steady rate; hence there is still a small amount of ChlP when the stationary level S obtains. Similarly, at low light intensity, ChlO is likely to become a non-negligible form of accumulation of the complex. This tends to depress the concentration of ChlP. It must be noted that in such a case the S level lies appreciably above the O level and that, since for practical reasons ChlP concentrations are measured with reference to the S level, equation 1 should not be strictly valid. Physiological factors affecting the difference between the 2 levels have not yet been investigated. However, we infer from the general validity of equation 1 that the difference is usually small.

Temperature: The effects of this variable on the thermal processes included in scheme 2 bear some similarity with those described under the section Inhibitors and do not need special explanations. Lowering the temperature increases p_M, depresses the rate of the P–S evolution and of the recovery reaction (see fig 6 and 3). The activation energy of reaction 2 c can be computed from the variation of k_2 (equation 1, where $B = k_2$) with temperature (see fig 2). Figure 9 is the corresponding Arrhenius plot. A deviation from the linear relation can be noticed at high temperature. We have already emphasized that exact correspondence between each step of the cycle and successive phases of the induction curve was not to be expected with every set of experimental conditions. At high temperature, each step tends to affect the following ones more or less and the rate of fluorescence change can no longer be a function of a single rate constant (e.g., k_2 for the end of P–S phase). It can be seen that the sign of the discrepancy is what the above analysis would predict. From the straight part of the curve in figure 9 an activation energy of ca. 20 kcal can be computed.

Concluding Remarks: It seems premature to draw conclusions from a comparison between our results and what is known of the induction phenomena of fluorescence in normal algae. We have still to investigate the significance of variations in parameters of the induction curves (for example, absolute and relative variations of p_M and s with culturing conditions, especially light); also, keeping in mind that any photochemical cycle has to operate a light-sensitized hydrogen transfer, we have to locate the point at which O_2 evolution, water re-loading and hydrogen unloading to the acceptor will occur. However, we believe that a discussion of the basic kinetic concepts here postulated, as compared with other theoretical approaches, might be of some relevance for the general problem of induction. Rabinowitch (10) has given a thorough analysis of the two possible mechanisms of induction, namely the building up of intermediates and the activation of catalysts. He also pointed out the weight of experimental evidences in favor of the second mechanism, specifically as postulated in the Gaffron-Franck theory (3, 5). One of the points that Rabinowitch made was that the first mechanism could not explain the characteristic waves of induction, giving only a gradual approach to the steady state. In fact, it is possible to find kinetic systems that will respond with transient waves to a sudden change of condition. It can be demonstrated that even a simple cycle such as:



—a simplified version of our cycle—will yield monotonous induction curves as well as one or several maxima before the establishment of a steady state, depending on the choice of a suitable set of reaction-

rate constants. A salient postulate in the Gaffron-Franck theory is that the O_2-liberating catalyst C is de-activated in the dark and becomes autocatalytically re-activated in light; this triggers a mechanism of photoperoxide accumulation and narcotic production which slowly subsides as catalyst C starts to operate. Using a very fast amperometric technique (time resolution of the order of 0.5 sec) for the O_2 determination, Joliot (unpublished) has found that, after not too long dark periods, the O_2 evolution begins with a burst followed by a characteristic induction period. The maximum rate at the start of the O_2 burst equals the steady photosynthetic rate, when the experiment is carried out at low light intensity. This shows that inhibition of the O_2-liberating catalyst (which Joliot also observed) need not be considered as the only source of induction.

As pointed out by Rabinowitch, one difficult problem in connection with the Gaffron-Franck theory is to understand how a small number of narcotic molecules produced at the beginning of illumination can affect the fluorescence of the totality of chlorophyll molecules. This question obviously cannot be asked as such in our theory, but it has a very interesting counterpart: how is the photochemical reaction 2 a to proceed so rapidly? Indeed, we have compared (observations to be published) the experimental rate constant of the fluorescence initial rise O–P, which we attribute to the photochemical transformation of the photoactive form ChlO, with a calculated rate constant assuming that reaction 2 a proceeds with unit quantum yield and that there is negligible mutual shading between the chlorophyll molecules; this "optimum" calculated value depends on the light intensity and the absorption coefficient, which we took to be of the same magnitude as in chlorophyll solutions. We found that the measured rate constant is from 100 to 200 times larger than the calculated one. In other words, this would mean that the absorption coefficient of ChlO stands in this ratio with the absorption coefficient of chlorophyll in solution. This is very unlikely. The alternative to explain how the ChlO molecules receive more energy than permitted by their own photic "cross section" is that an average number of 100 to 200 chlorophyll molecules in a special state, distinct from the complex state, supply their absorbed energy to a single molecule of this complex in its ChlO form. This would not only be in agreement with the Emerson-Arnold concept of "photosynthetic unit" (2), but would also be in favor of one of its possible interpretations, namely the electronic excitation migration to the "reaction center" (4), as opposed to the material diffusion of the photochemical products or even of the center itself. To us, this would also provide a strong support for the assumption we made as to the heterogeneity of the fluorescence emission. The constant background would arise from the bulk of the chlorophyll acting as photic acceptor and the variable fluorescence from the few molecules of chlorophyll complex in charge of the photochemical transformation of the transferred energy. A similar hypothesis as to the state of chlorophyll in vivo has been repeatedly made since the original elaboration of Seybold and Egle (8). Recently, Franck (1) has proposed a very detailed model—based on the same concept of heterogeneity—in which the 2 functions of chlorophyll (light absorption and photochemical transformation) are likewise attributed to distinct fractions of the pigment.

Summary

The kinetics of the induction phenomena of fluorescence in quinone-poisoned Chlorella cells has been studied. The observations are consistant with the following assumptions.

1) Part of the chlorophyll is involved in a photochemical cycle as a complex that can assume 3 different forms.

2) Successively, there is a non-fluorescent photoactive form, a fluorescent non-photoactive form and a non-fluorescent non-photoactive form.

3) This photochemically participating fraction of chlorophyll is responsible for the variable part of the fluorescence emission, whereas the constant fluorescence background arises from a different fraction which only acts as a photic acceptor.

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