THE FLUORESCENCE TRANSIENT OF *CHLORELLA PYRENOIDOSA*

BY

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I. INTRODUCTION

A. Foreword

Green plants emit fluorescence when illuminated, most fluorescence coming from the photosynthetic pigment, chlorophyll a (Chl a). If the illumination is sudden and intense, the Chl a fluorescence intensity undergoes a transitory variation. We shall call this variation the fluorescence transient.

The problem is to explain the fluorescence transient in terms of the mechanism of photosynthesis. According to the presently accepted hypothesis, two photochemical reactions participate in photosynthesis -- each energized by its own set of Chl a molecules and accessory pigment molecules. One reaction leads to production of molecular oxygen and the other to reduced nicotinamide adenine dinucleotide phosphate (NADPH). The set of Chl a associated with oxygen production emits the transitory fluorescence. The shape of the transient is believed to conform to changes in the redox state of a molecule called Q associated with this set of Chl a.

The transient excited by intense illumination is modified when a weak illumination precedes or is simultaneous with the intense illumination. In this investigation we study the modifications produced by weak illumination of various wavelengths and intensities. The goal is to understand what causes the changes in the redox state of Q.

All experiments have been done on Chlorella pyrenoidosa, whose growth, photosynthesis, and fluorescence properties are well characterized by previous work in this laboratory and elsewhere.

B. The Use of Fluorescence Analysis in the Study of Photosynthesis

The use of fluorescence analysis has two justifications: one is that Chl a, the primary sensitizer in photosynthesis (89), is fluorescent in its in vivo state (95); the other is that fluorescence rises and decays so rapidly following light absorption that it may be regarded as instantaneous for nearly all experimentation -- the in vivo fluorescence of Chl a in Chlorella has an exponential decay time of only 1.6×10^{-9} seconds (5). Fluorescence represents light energy lost from photosynthetic pigments, and thus it competes with the use of light energy by the photosynthetic reactions. In general, fluorescence varies inversely with the use of light energy by these reactions (69). We may thus regard fluorescence as an instantaneous monitor of the use of light energy by the photochemical reactions of photosynthesis in the living cell.

Much of the interest in fluorescence analysis has been directed at steady-state fluorescence. For example, from its study much has been learned of energy transfer from accessory pigments to Chl b and a (12, 13, 33). The study of fluorescence bands as a function of temperature has contributed to the knowledge of energy transfer (3) and of proposed energy trapping centers (37). The study of polarized fluorescence has shown that some of the chlorophyll molecules are more regularly arranged than the rest (65, 79). These and other varieties of fluorescence yield studies have contributed to all areas of photosynthesis research.

The name "steady-state fluorescence" may be misleading, because large changes in fluorescence yield take place for at least the first half-hour of illumination, and small changes continue for much longer. Recent work by Papageorgiou and Govindjee (81) in this laboratory shows that the changes in yield during the first hour are accompanied by spectral changes, these depend on external conditions during the experiments, and on the wavelengths of excitation. This work warns us that if consistent results are to be obtained, and valid comparisons made with results obtained by other workers, careful attention must be paid to the conditions of measurement. It is necessary to standardize conditions and take measurements at a specific time following the onset of illumination of a specified intensity. In the literature it is often impossible to determine at what time the claimed steady-state measurements were made.

Steady-state fluorescence analysis does not in general make full use of one of the advantageous characteristics of fluorescence, namely its instantaneity as a monitor of light energy utilization. This characteristic has been fully exploited in fluorescence transient analysis.

Historically, the transient has been called fluorescence induction, in keeping with the terminology applying to the gas exchanges which occur upon illumination after a dark period. Rabinowitch (84, p. 1314) in his review of photosynthesis distinguished between "short" and "long" induction of gas exchanges, with the first term applied to events in the first second or two of illumination, and the second applied

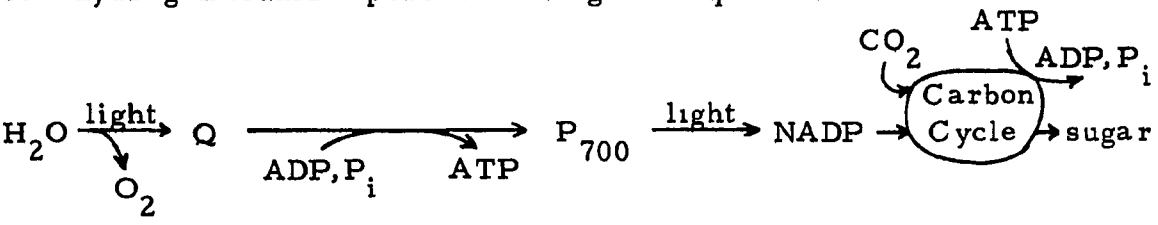
to slow changes lasting perhaps for several hours, and fully developed only after several hours of darkness. It is clear that long induction could be related to the said changes of steady-state fluorescence.

We prefer the name "fluorescence transient" to "fluorescence induction" because "transient" is the familiar term used by physicists, chemists, and electrical engineers for the phenomenon under study. Our use of the term "transient" will apply only to variations a few seconds in duration.

A transient in oxygen exchange was discovered by Warburg (92) in 1920, and thus preceded by many years the 1931 discovery by Kautsky and Hirsch (55) of the fluorescence transient. A transient in carbon dioxide exchange was first observed by McAlister (73) in 1937. McAlister and Myers (74) in 1940 made the first study in which carbon dioxide exchange and fluorescence during the transient period were measured simultaneously. Despite numerous studies of the transients in the 1940s, no clear and detailed explanation of them emerged.

Since the late 1950s, however, it has become fruitful to again study the transients. The reason lies in the discovery by Emerson, Chalmers, and Cederstrand (22) in 1957 of the necessity in photosynthesis for two photochemical reactions rather than one. This discovery has paved the way for the rapid advance in understanding of photosynthesis which has taken place in the 1960s. At the present, there are consistent and widely accepted parts of theory: they include

a serial two-photochemical-reaction scheme and a carbon cycle. The carbon cycle is a system of chemical reactions which incorporates carbon dioxide into simple sugars with the aid of adenosine tri-phosphate (ATP) and reduced NADP. Of the two photochemical reactions, one reduces NADP and oxidizes a specialized chlorophyll molecule called P_{700}, and the second oxidizes water, yielding oxygen, and reduces a substance called Q. Through dark reactions involving intermediates, reduced Q delivers its electron to oxidized P_{700}. Some of the energy made available in the dark reactions produces ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i). Overall, photosynthesis is a hydrogen transfer process having the sequence:



In this general framework, the oxygen transient originates in reactions close to water and Q. The fluorescence transient conforms to changes in Q. Rate constants for some reactions and the concentrations of some reactants have been determined. It may be summarized that since the development of the theory of two photochemical reactions in series, the study of transients has yielded detailed information about the mechanism of photosynthesis.

C. The Literature of Fluorescence Transient Study

The history divides readily into two periods, one before and

one after the 1957 two-light-reaction hypothesis of Emerson et al. (22). For the earlier period, we cover experimental results both important to present-day theory and basic to the experimental work of this thesis. Our emphasis is on the transient despite the fact that the earlier workers concentrated on long induction. Little attention is given to the hypotheses of the earlier period, because they have been overturned or overhauled since the two-light-reaction hypothesis. For the period since 1957, both experimental results and hypotheses are discussed. Emphasis is given to the development of schematic models.

Figure 1 shows oscillographs of the transient in Chlorella pyrenoidosa (obtained by the author). Lavorel (68; 1959) introduced the labels, O, P, and S for its different phases: O for the initial level preceding any changes in fluorescence yield, P for the temporary maximum, and S for a brief steady level following P. We adopt his labels and include three others: one is I, suggested by Joliot and Lavorel (49, 1964) for an intermediate hump between O and P, another is D, which we suggest for an often-observed dip between I and P; the third is t_p, which stands for the time between onset of illumination and P.

1. Prior to 1957

Müller (76, 1874) was the first to notice a transient, but Kautsky and Hirsch (55; 1931) were the first to emphasize the transient. They visually observed a transient in a green leaf. Because of the crude visual technique, only an initial low level, a rise, and a decline could

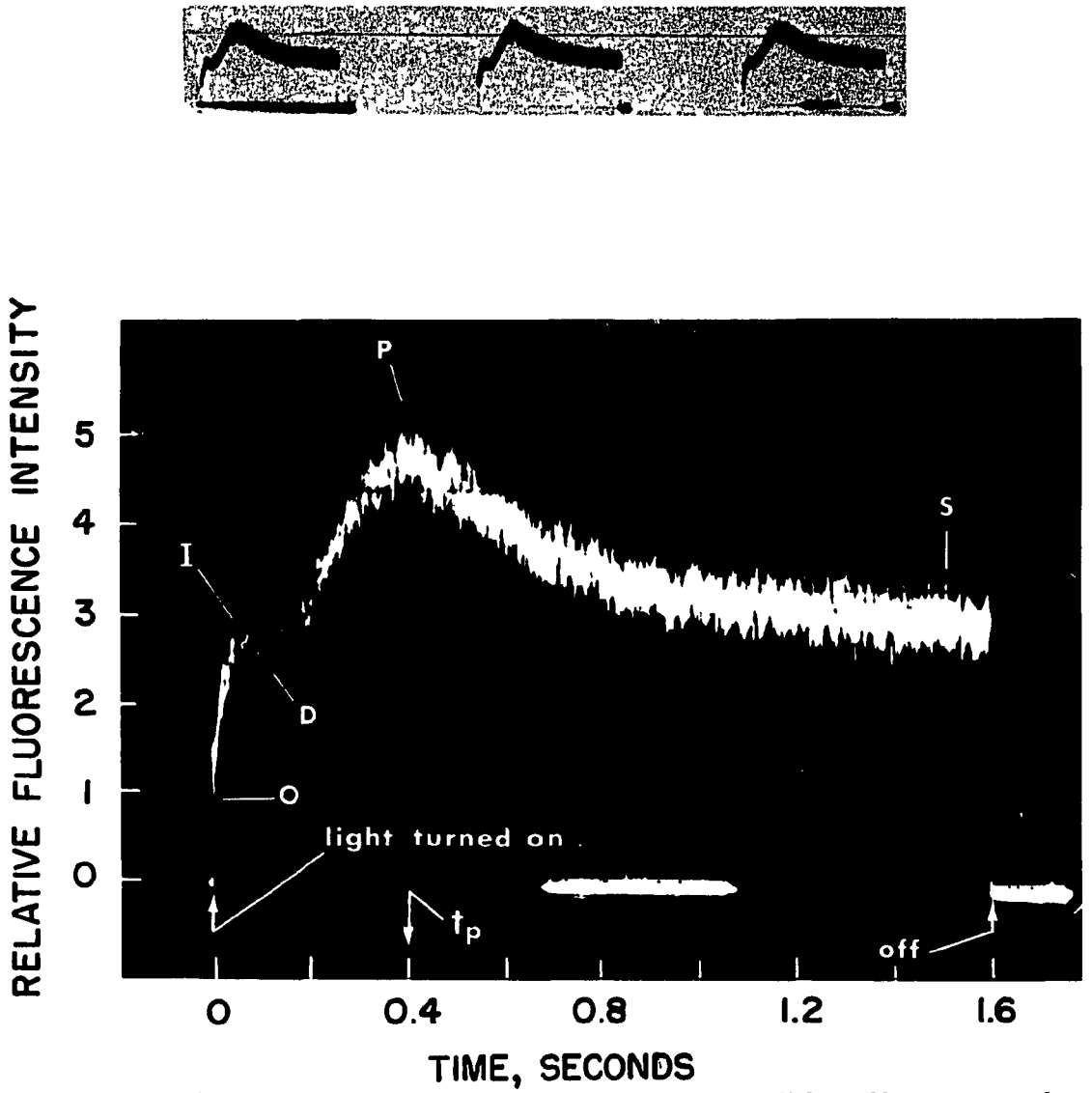


Figure 1. The fluorescence transient in aerobic Chlorella pyrenoidosa. Wavelength of measurement: 685 nm; half-maximum band width (BW), 6.6 nm. Excitation: 500 nm; BW, 120 nm, incident intensity 1.5×10^4 ergs/sec-cm^2. The three photographs at the top are successive oscillographs obtained in one experiment at 4-minute dark intervals, the photograph at the bottom is an enlargement of the top right-hand photograph. The points O, I, D, P, and S are clear in each photograph.

be noticed, corresponding probably to I, P, and S. In their subsequent work, they proved that the transient is common to all plants -- the transient was observed in many species of higher land and water plants, ferns, and algae, Chlorella vulgaris was mentioned in 1935 (53). Among various observations of the behavior of the transient, they reported that the P - S phase was slowed by raising the CO_2 concentration from 1% to 4% (53). In the absence of oxygen, leaves fluoresced more brightly and the initial rise was not observed (54).

J. Franck and Wood (30; 1936) compared in vivo fluorescence changes with a slow fluorescence transient in organic solutions of chlorophyll. The transient in solution was explained in terms of an oxidation of chlorophyll. They hypothesized that the in vivo transient involved the formation of an adsorption complex between chlorophyll and a reducing substance, and the photochemical oxidation of this complex.

J. Franck and Wood (30) were the first to observe the O level. By the ingenious method of illuminating a spot near the edge of a rotating disc covered with sections of green leaves, they were able to illuminate each small area for just a few msec (milliseconds), yet obtain a steady and thus measurable fluorescence. At the intensities used by Franck and Wood, the fluorescence yield does not change greatly in the first few msec (milliseconds) of illumination. Consequently, their determination of O was an accurate measurement of the fluorescence level preceding any changes in fluorescence yield.

In our experiments, O is the level obtained when a shutter has completely opened. The shutter opens in 2 msec, before any significant change in fluorescence yield takes place. Our determination of O is thus an accurate measurement of the initial fluorescence level. We remark that there is a rise of fluorescence amplitude (but not of fluorescence yield) occurring as the shutter opens. The rise will be simultaneous with the shutter opening, unless the fluorescence is emitted by a pigment other than the absorbing pigment, in which case the fluorescence will be delayed because of the finite time required for energy transfer from one pigment to the other. Brody and Rabinowitch (5) were the first to measure the delay caused by transfer from phycoerythrin to Chl a in vivo; the delay is 0.5×10^{-9} seconds, a vanishingly short time with respect to our experiments.

Wassink and Katz (94, 1939) carried out the first detailed study of the transient in a Chlorella species, Chlorella vulgaris. The technique involved monitoring fluorescence with a photocell, galvanometer, and amplifier, rapid enough to measure fluorescence before P was reached, but not to distinguish O or I. Their study therefore concentrated on levels at P, S, and later.

They were the first to report a large second maximum of fluorescence following S. It is called the "second wave." The second wave may occur between 30 seconds and a minute or more after the first maximum, P, and is by comparison quite drawn-out. Its presence depends on the plant or alga, and on other conditions such as age, and carbon dioxide

supply during growth and during measurement (for a review see Rabinowitch (84), pp. 1378-1382). We emphasize the fact that its presence depends on conditions during measurement. In our laboratory, Papageorgiou (80; 1968) has demonstrated the presence of the second wave in cultures of Chlorella pyrenoidosa, while for the same cultures but different measurement conditions, we have observed the lack of the second wave.

A basic study of the influence of various factors on P and the second wave was performed by McAlister and Myers (74; 1940). Observations were made in wheat and Chlorella. This was the first study involving simultaneous measurement of variations in fluorescence and carbon dioxide uptake. Upon onset of high intensity illumination, they were inversely related for at least one minute. In Chlorella the behavior was greatly influenced by the culture condition. A second wave appeared in Chlorella grown in air and studied either in air or carbon dioxide-enriched air. For cells grown in enriched air, the second wave was lacking, this conclusion now must be discarded, because Papageorgiou (80) has observed a second wave in Chlorella grown in air containing 5% CO_2.

J. Franck, French, and Puck (28, 1941) studied the transient in Chlorella and Hydrangea. They found that the rise to P was faster in higher light intensity, but it was not affected by temperature, P and the I - P rise were independent of carbon dioxide concentration. In contrast, the P - S decline was retarded by high carbon dioxide concentration, by

low temperature (0° C compared to 23°) and by hydrogen cyanide. The reactions causing the P - S decline (in normal cells) occurred even in the dark, and had a half-life of 1.7 seconds.

The claim that I - P is temperature independent was shown to be incorrect by the work of Kautsky and U. Franck (52; 1943). Fluorescence time curves of Ulva lactuca at different temperatures clearly showed that an increase of temperature from 0°C to 10°, and to 20° speeded the rise to P.

Kautsky and U. Franck also restudied the effect of oxygen concentration on the transient. In 1935 when the effect of oxygen-lack had first been studied, the visual measuring technique had failed to reveal any detail. But because of improvements in technique made by Kautsky and Eberlein (51; 1939), the new study showed that under complete anaerobiosis, fluorescence began at a high level (I), declined steeply (D), and then rose to a high steady level (P and S). Even 0.1% oxygen concentration produced a high I, although D was not as deep. Less complete anaerobiosis produced smaller effects. 1.25% oxygen barely affected I but raised P. At 5% oxygen an effect on P was just noticeable. Kautsky and U. Franck studied Ulva lactuca, the anaerobic transient in Chlorella pyrenoidosa is similar -- Figure 2 shows oscillographs of the transient in Chlorella as air is replaced by 2.6% CO_2 in argon (oscillographs obtained by the author).

The reason for the anaerobic effects must be discussed for a moment, because the anaerobic condition has been utilized in the research

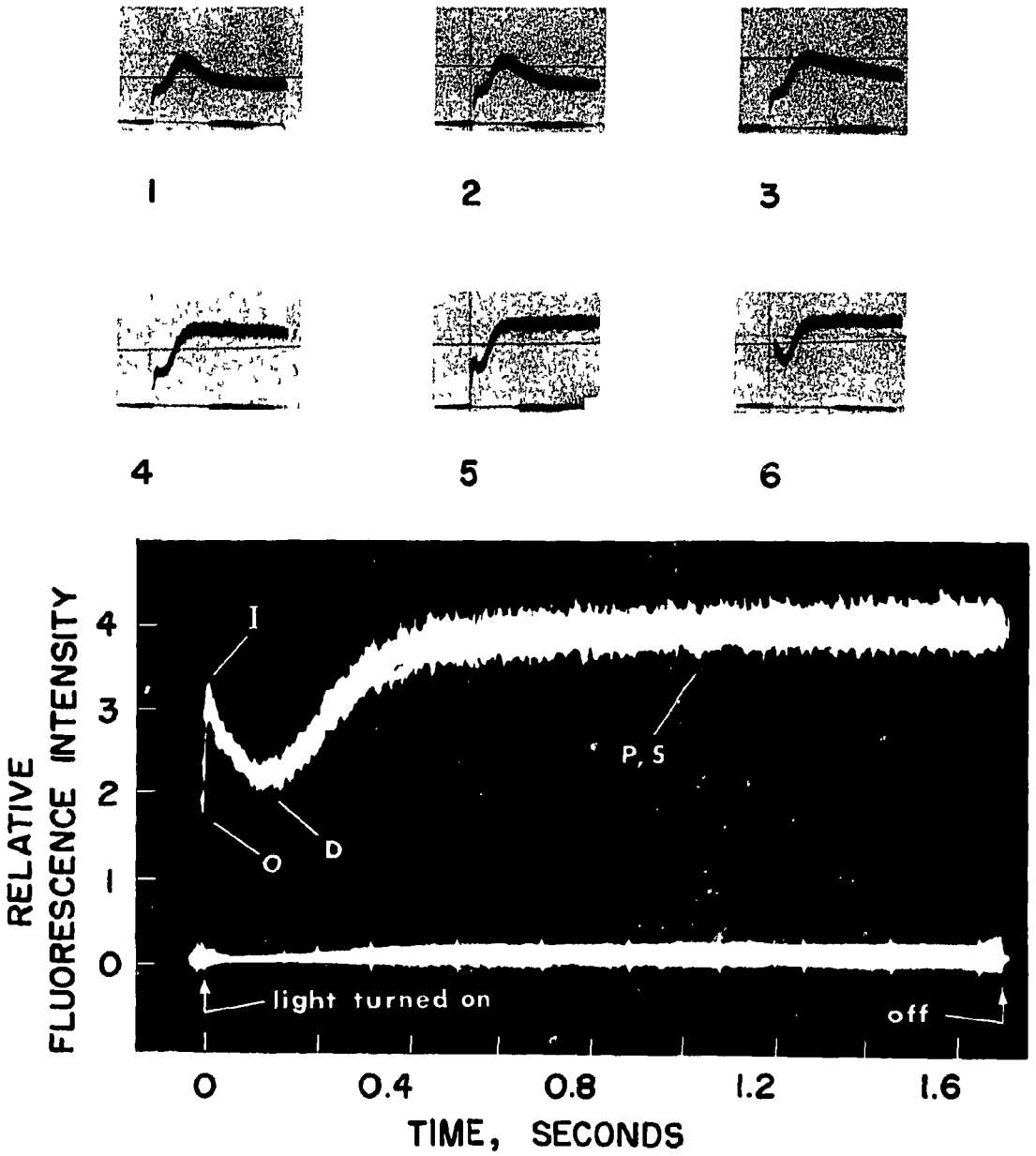


Figure 2. The fluorescence transient in anaerobic Chlorella pyrenoidosa. The small photographs: 2.0% CO_2 in air is replaced by 2.6% CO_2 in argon. Replacement began a few seconds after picture 1 and was complete by picture 6. Subsequent pictures were identical to picture 6. The pictures were taken at four-minute dark intervals. The bottom photograph, an enlargement from a different anaerobic experiment, shows the distinction between O and I.

reported in this thesis. Kautsky and Eberlein (51) believed that low-oxygen effects were not due to anaerobic incubation. The reasons: the length of exposure to low-oxygen was only 60-90 minutes, the effects were dependent on oxygen concentration, and the effects were reversed within one minute after admission of normal air. Rabinowitch (84, p. 1400) objected that in some species after-effects of anaerobic incubation are seen after only an hour of incubation. We shall rephrase the argument in more specific terms: do the low-oxygen effects result from low oxygen at the site of photosynthesis?, or changes in glycolytic and Krebs cycle pathways with after-effects on photosynthesis? We find that changes in the transient progress rapidly as oxygen is swept out. Our procedure involves exciting the transient every four minutes; small changes are noticed in the first transient, that is, four minutes after starting to sweep out oxygen. These observations suggest that the anaerobic effects are not due to long-term metabolic changes.

New study of the transient by Shiau and J. Franck (87; 1947) confirmed previous reports. The effect of anaerobiosis was a high P and S. Quinone (an oxidant) lowered S, while o-phenanthroline (a poison) raised it. These observations (with others of steady-state fluorescence behavior) were interpreted as supporting the hypothesis that plants are able to produce natural narcotics upon illumination. Narcotics supposedly cover the surface of the chlorophyll, raise the fluorescence yield, and suppress photochemical activity. The rise of fluorescence during the transient was explained by the effect of the photochemically produced narcotics.

2. After 1957

The two-light reaction theory was first given prominence by Emerson, Chalmers, and Cederstrand (22; 1957). Their paper had two important precedents, which we summarize first. In 1943, Emerson and Lewis (24) reported a steep drop in the quantum yield of oxygen evolution for exciting light wavelengths beyond 685 nm, a puzzling finding since Chl a still absorbed strongly in this wavelength region. In 1956, Emerson, Chalmers, Cederstrand, and Brody (23) made the startling discovery that the low quantum yield of light beyond 685 nm could be improved by supplementary light of shorter wavelengths. In the 1957 paper, Emerson et al. (22) reported that wavelengths absorbed by Chl b were the most effective for this improvement. They offered the explanation that perhaps photosynthesis involved two light reactions, one sensitized by Chl a absorption, and the other by Chl b or other pigments. The improvement-phenomenon discovered by Emerson and coworkers is now known as the Emerson Enhancement Effect.

A fluorescence phenomenon analogous to the Emerson Enhancement Effect was discovered by Govindjee, Ichimura, Cederstrand and Rabinowitch (38; 1960). They showed that fluorescence yield of Chlorella illuminated simultaneously by short and long wavelengths of light was lower than the yield for either light given alone.

The suggestion of two light reactions was a catalyst for other theoretical developments. Hill and Bendall (41; 1960) hypothesized that the reactions cooperate serially. Their scheme centered on a

proposed function for two cytochromes which had been identified in chloroplasts, cytochromes $\underline{b}_6$ and $\underline{f}$. It was supposed that one light reaction transfers an electron from water or a substance reduced by water, at a redox potential close to that of the H_2O/O_2 couple, +0.88 electron volts, to cytochrome $\underline{b}_6$, which has a redox potential of 0.0 volts. This reaction allows cytochrome reduction and release of molecular oxygen. The second light reaction oxidizes the $\underline{f}$ cytochrome at +0.4 volts, and reduces an intermediate used in NADP reduction, at least as negative as -0.4 volts. The serial connection between the two light reactions was completed by a dark reaction, in which reduced cytochrome $\underline{b}_6$ gave its electron to cytochrome $\underline{f}$. This scheme explained the finding by Duysens (15, 1954, 16, 1955) of the oxidation of cytochrome $\underline{f}$ (or $\underline{c}$, Duysens' technique did not distinguish between the two) by light, and its reduction during a subsequent dark period.

We shall outline a few subsequent developments. Although they are not centered on the fluorescence transient, they contribute greatly to its explanation.

Duysens, Amesz, and Kamp (18, 1961) suggested that each light reaction was carried out by its own set of pigments. They named the pigments responsible for oxygen production, system II, and those responsible for cytochrome $\underline{f}$ oxidation, system I. Each system presumably acted as a photosynthetic unit, a concept introduced by Emerson and Arnold (20, 1932), in which an assembly of molecules transfers absorbed light quanta to a photochemical reaction center.

In a short time, various studies showed that the two systems have different absorption spectra (17, 25, 26, 32, 35, 40). It is now generally accepted that fluorescence is emitted primarily by system II. Butler (7, 1962) showed that in anaerobic cells a short exposure of system I light leads to a prolonged decrease of system II fluorescence, while a short exposure of system II light increases system II fluorescence.

These developments were paralleled by those concerned with the transient. Kautsky, Appel, and Amann (50, 1960) made a thorough study of the transient in aerobic and anaerobic Chlorella pyrenoidosa: different phases of the transient were analyzed one by one. In 1943, Kautsky and U. Franck (52) had shown that under phenylurethane (a poison) the transient is smoothed out and the amplitude is high, in 1960, with faster measuring apparatus, Kautsky et al. could see that under phenylurethane, fluorescence at O was nearly normal, and rapidly rose to the high level in a smooth manner. An appropriate plot of points from the smooth rise revealed that the rise was apparently exponential (new work, discussed later, shows that the rise is S-shaped). Kausky et al. proposed that the exponential rise indicates a first-order photochemical deactivation of a fluorescence quencher. We shall call the quencher "Q", Kautsky et al. gave it a different label, but "Q" is now the popular one. From the rate of absorption of quanta, and the assumption that each quantum was utilized for deactivation of one Q molecule, the ratio chlorophyll:Q was calculated to be at least 400:1. This ratio is good evidence supporting the concept of the photosynthetic unit. Q presumably resides at the

reaction center of the system II unit.

In normal cells, not treated with phenylurethane, Kautsky et al. observed that if the light exposure were short enough to allow only the O - I rise, then after a similarly short dark period, the O - I rise could be repeated. This observation suggested that a rapid dark step reactivated Q. Under phenylurethane treatment the dark reactivation did not occur; Kautsky et al. supposed that phenylurethane interfered with a substance required for reactivation of Q. They suspected that this second substance was not present at the beginning of an exposure, instead that it was photochemically produced, after exposure began.

This idea was tested in a study of anaerobic cells. Kautsky et al. found that under a flashing exciting light, the I - D phase of the anaerobic transient was prolonged, moreover, the I - D decline required a constant number of light flashes, even though the dark interval between the flashes was varied. The decline therefore does not progress in the dark; it requires light.

The easiest explanation was that the substance assisting reactivation of Q was formed in a light reaction and did not already exist before the exposure. We shall call this substance "A", the now-popular label. Having explained the O - I rise as the result of one photoreaction, they suggested that the I - D decline indicated a second photoreaction, the production of A.

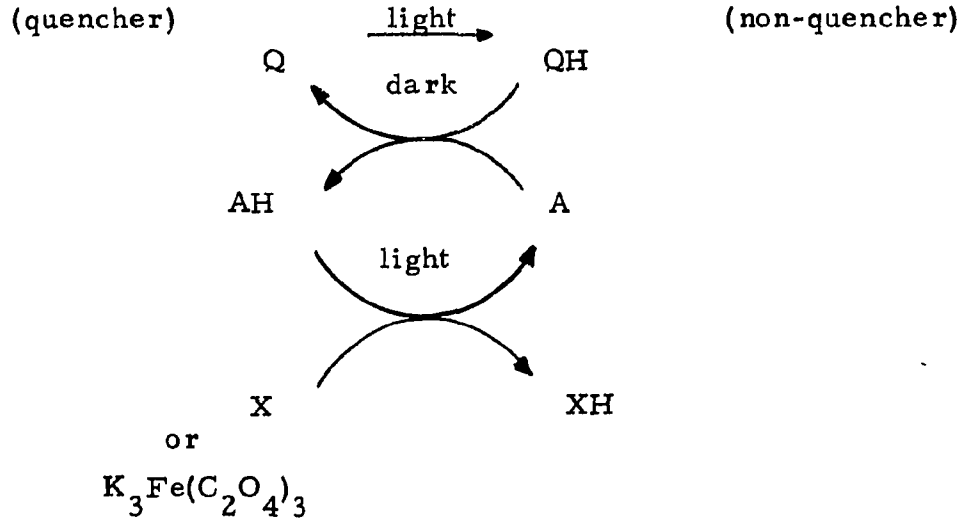
Kautsky et al. went on to analyze the D - P rise. The arguments are simple but numerous and lengthy -- we shall mention only the

conclusion of the analysis, that the D - P rise indicates exhaustion of a substance called X which is consumed in the photoproduction of A.

The last of the experimental work concerned the effect on the anaerobic transient in Chlorella of $K_3Fe(C_2O_4)_3$. This substance is known as a Hill-oxidant because it is reduced in the Hill-reaction. (The Hill-reaction is the production of oxygen under the condition that an experimentally-introduced substance, instead of CO_2, acts as the terminal electron acceptor of photosynthesis.) The effect of $K_3Fe(C_2O_4)_3$ was the elimination of the D - P rise. Kautsky et al. concluded that $K_3Fe(C_2O_4)_3$ was replacing X in the photoproduction of A.

Today we know that the effect of $K_3Fe(C_2O_4)_3$ on D - P cannot be used to prove which intermediate is exhausted. $Fe(C_2O_4)_3^{---}$ has a reduction potential of about +0.4 v., more positive than X, A, or Q, which means it may accept electrons from all of them. It is arbitrary to presume it will accept electrons only from X.

The review of the work by Kautsky et al. closes with a scheme we have drawn to summarize their conclusions. Although based only on their experimental results and their interpretation, it is quite different from the scheme they drew, because it excludes details of their scheme which, according to more recent developments, are probably incorrect.



Q is reduced by one light reaction, A oxidizes QH by a dark reaction, and X oxidizes AH by the second light reaction. The scheme is horomorphic with the Hill and Bendall (41) scheme.

It has been shown several times that the fluorescence spectrum changes during the transient. Of the studies by Virgin (90, 1954), Lavorel (64; 1962), Duysens and Sweers (19; 1963), and Rosenberg, Bigat, and DeJaegere (86; 1964), the most detailed is by Lavorel, who used a superior technique. Lavorel pumped dark-adapted Chlorella through a tube which was opaque throughout its length except for a small segment. Here, fluorescence was excited and measured. The pumping apparatus utilized a piston and valve assembly to give the pumping two phases: flow, and rest. In the flow phase the cells traversed the section in a few msec, the fluorescence measured was thus the O level. In the rest phase, the cells lingered until the level P was reached and measured. Fluorescence was recorded continuously, as the pumping action proceeded and the wavelength of measurement was

automatically advanced through the spectrum. The results were that the main fluorescence band at 686 nm was relatively higher at P than at O, compared to fluorescence beyond 700 nm, and in addition red-shifted by 2 nm to 688 nm. There was a pronounced band at 716 nm in the O spectrum which was conspicuously lower in the P spectrum. Because 685 nm fluorescence is assumed to be from system II, the finding that P is richer than O in 686 nm fluorescence indicates that the transient originates in system II.

Other research supports the association of the transient with system II. In 3-(3,4-dichlorophenyl)-1,1-dimethylurea (i.e., DCMU)-treated Chlorella, Duysens and Sweers (19, 1963) found that the transient fluorescence begins at O as usual, but rises to a high P and does not decline. The scheme on page 19 suggests that DCMU blocks the dark reaction between A and QH. It is known that DCMU inhibits oxygen production (46) and inhibits cytochrome f reduction, the functions of system II, but does not stop cytochrome f oxidation, the function of system I. It is generally accepted now that DCMU impedes electron flow from system II to system I, and that the transient fluorescence originates in system II.

Lavorel, Joliot and Delosme (69, 1959) showed that oxygen production and fluorescence are anti-parallel for the first few seconds of illumination. Measurement at short time by Joliot (47; 1965), with an amperometric technique for measuring oxygen production faster than 0.1 seconds, showed that the rate of oxygen production is parallel

with fluorescence from O to I, and anti-parallel as fluorescence rises from I to P. These observations are most easily explained by the idea that the transient is produced by system II, the oxygen-producing system.

The popular label Q for the system II fluorescence quencher was introduced by Duysens and Sweers (19; 1963). They suggested that Q acts as both a fluorescence quencher and an electron acceptor. It drains excitation energy collected by the photosynthetic unit and utilizes the energy to oxidize a donor molecule. In this reaction, Q is reduced to the form QH. QH is unable to drain excitation energy; with the drain blocked, the excitation energy is more likely to be emitted as fluorescence. With this hypothesis in mind, Duysens and Sweers conceived of P of the transient as a temporary maximum in the concentration of QH.

Morin (75; 1964) studied the transient in Chlorella pyrenoidosa under very intense exciting light, so intense that the O - I rise was completed in one msec rather than 50 msec. At such high intensity, the rate of the rise was dependent only on intensity. It was not affected, for example, by the inhibitor o-phenanthroline which usually accelerates and modifies the fluorescence transient. Because of the intensity dependence, Morin called the O-I rise "purely photochemical." The I - P phase at high intensity proceeded at the same rate despite any increase of intensity. The I - P phase was consequently called "thermal." Morin explained the photochemical and thermal phases in terms of a scheme equivalent to that of Q and A presented above. But, he conceived of the maximum of fluorescence during the transient in terms of exhaustion

not of both X and A, but only of A. He also omitted any consideration of the two-light-reaction theory. We have seen that Kautsky et al. (50) thought P was due to exhaustion of first X and then A, and came to this belief after recognizing two light reactions instead of one. The divergence of views regarding P has not been reconciled in the work of other authors reporting since 1964, and is investigated in this thesis.

Morin (75) was the first to examine the effect of preillumination on the fluorescence transient. However, the examination was incomplete because the spectral content of the preilluminating light was not considered. We expect a difference in the effects of system II and system I preillumination. Our results show that there are differences and that they can be explained in terms of the Hill and Bendall (41) scheme.

Up to this point, we have mentioned schemes involving only two forms of Q. There have been suggestions that Q has three forms. Duysens and Sweers (19; 1963) came to this conclusion in the following way: they found that system I is rate-limiting for light of 560 nm in Porphyridium cruentum; from investigations of Duysens and Amesz (17, 1962), system I was estimated to be 30% less active than system II at 560 nm. For the steady-state, they presumed a stoichiometric balancing between the two systems, which would require the activity of system II to be reduced by 30%, this is the case when 30% of all Q is reduced. Because steady-state fluorescence was lower than expected, Duysens and Sweers postulated that QH can be converted by a dark reaction in the presence of light into a form Q', which can quench

fluorescence below the expected level. Q' was thought to revert to Q by a slow dark reaction.

This argument involves hidden assumptions. One is that the S level chosen is indeed a steady level. S was determined after 30 seconds of illumination. From work of Papageorgiou (81, 1967; 80, 1968) in our laboratory on Chlorella and Anacystis, fluorescence is still undergoing large changes at that time. Another assumption involves the O level. The calculation about QH requires the assumption that the O level indicates a basic fluorescence which remains constant during 30 seconds of illumination. This assumption cannot be tested because there is no way to measure the basic fluorescence at S.

Another suggestion that Q has three forms has come from Sybesma and Duysens (88, 1966). Changes of fluorescence yield upon illumination were found to be greater when system I background light had been applied before exposure. The interpretation was that an inactive form of Q was formed in a dark reaction between exposures. Activation required light energy, which was supplied by the system I background light.

P. Joliot (46, 47; 1965) has also hypothesized that the system II reaction center involves a substance with three forms. The scheme is based on a careful study of the kinetics of both oxygen production and fluorescence, we shall not review the experimental work here. In the scheme a hypothetical substance E is reduced by system II. The question has arisen whether or not Q and E are identical.

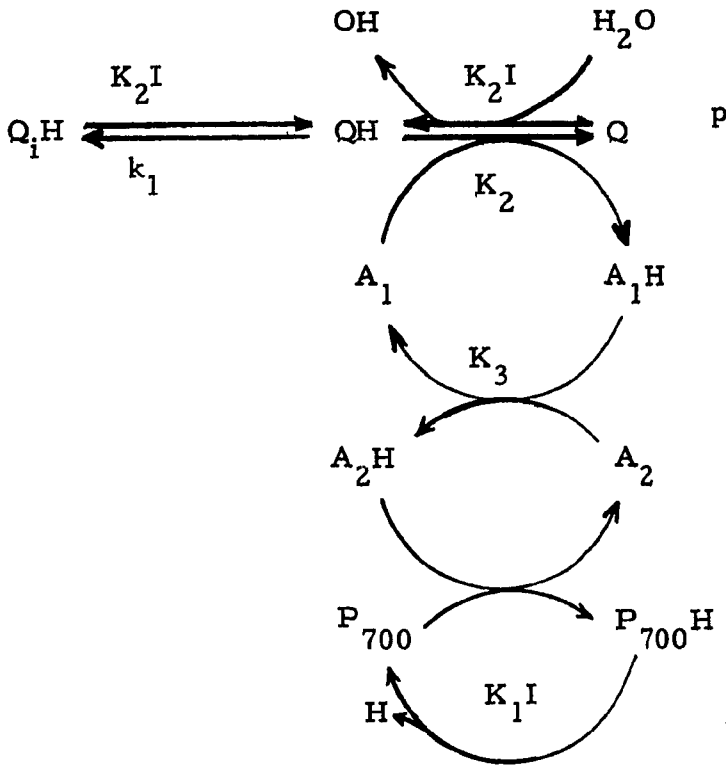
In a review, Avron (2; 1967) has distinguished between E and Q

according to concentration, since the ratio Chl:Q from Duysens and Sweers (19) is 150:1 and at first look the ratio chl:E from P. Joliot (46), and from P. Joliot, Delosme, and A. Joliot (48; 1966) is 1500:1. However, P. Joliot and co-workers mention quantity of oxygen produced rather than amount of E. Since E is presumed to accept one electron, and four electrons must be transferred from water for release of each oxygen molecule, the ratio chl:E is 375:1. At the present time, we cannot regard the difference between 375 and 150 as significant, and therefore Avron's reason for believing E and Q are different breaks down.

Believing that E and Q are different, Avron has proposed a scheme for the photosynthetic apparatus in which E is oxidized rather than reduced by light, while Q is reduced. This novel possibility was also proposed by Malkin (71; 1966).

P. Joliot et al. (48; 1966) have come to believe that E and Q are identical. A new paper by deKouchkovsky and P. Joliot (60; 1967) reaffirms this belief, but points out the lack of clear proof. A. Joliot (44; 1966) suggests that E is the system II reaction center complex, and that Q is one part of the complex. We shall assume throughout this thesis that E and Q are identical, and use the label Q.

In P. Joliot's scheme Q takes three forms: Q_iH -- an inactive form produced by a slow dark reaction, QH -- a reduced form, and Q -- an oxidized form. The scheme:



photoreactions of system II

photoreaction of system I

Calvin cycle

$$X + H \longrightarrow XH \longrightarrow X + H$$

This scheme is similar to our drawing based on work of Kautsky et al. (50). P. Joliot adds the inactive form of Q_i and the substance P_{700}— a chlorophyll molecule functioning as the reaction center of system I, investigated by Kok (56, 1960)— and instead of a single intermediate between Q and P_{700} labeled A, two intermediates, labeled A_1 and A_2.

Murata, Nishimura, and Takamiya (78; 1966) studied the fluorescence transient in spinach chloroplasts; chloroplasts treated with 3.2×10^{-7} molar DCMU showed a three-phase rise from O to S without a temporary P. The three phases were modified differently as the

DCMU concentration was varied; Murata et al. concluded that there are three substances sequentially reduced in the course of the transient. In P. Joliot's model, the corresponding substances would be Q, A_1, and A_2.

Murata et al. (78) utilized an integration of the area bounded by the curve of the transient to calculate the number of quanta used in the reduction of Q, A_1, and A_2. This integral they called a "work integral," because it evaluates reductive work. From the work integrals for normal chloroplasts and for DCMU-treated chloroplasts, the ratio of chlorophyll to $(Q + A_1 + A_2)$ was found to be 13:1, and the ratio of chlorophyll to Q was found to be 100:1. From these ratios we calculate the ratio of $A_1 + A_2$ to Q to be 6.7:1.

Malkin and Kok (72, 1966) have independently used the same method for measuring reductive work; their study of chloroplasts revealed, however, only a two-phase rise from O to S, and thus not three but two substances sequentially reduced in the course of the transient. The two substances are present in a ratio of 1:1, and the ratio of chlorophyll to each is 70:1. Malkin and Kok assigned to the two substances the labels Q and P (where P is equivalent to A). Theoretical analysis of the transient by Malkin (71; 1966) yielded curves which successfully matched the experimental transient. Malkin presumed that each Q can interact with just a single A; because of the success of his analysis, he feels that Q and A form linear chains between system II and system I.

Malkin and Kok (72) studied also the oxidation of reduced Q and A by system I light. This oxidation permits restoration of the capability for a transient, because the transient can only occur when Q and A are oxidized. In 720 nm light complete restoration occurred at all intensities; at shorter wavelengths, however, the degree of restoration decreased with higher intensity. 690 nm was the shortest wavelength causing restoration. The explanation was that system II is excited to a diminishing degree past 680 nm, and that at 720 nm only system I is excited. At low intensities the quantum yield of system I is high, and restoration proceeds easily, but in intensities beyond saturation of system I, system II opposes the restoration by system I.

Delosme (11; 1967) has reported a study of the transient in Chlorella under extremely high light intensity. The intensity is the same as that utilized by Morin (75, 1964) to show that at high intensity the O - I phase is photochemical and the I - P phase is thermal. Delosme concentrated on the shape of the O - I rise: from the shape he has calculated that the probability of energy transfer between the chlorophyll molecules within system II equals 0.995. The probability for transfer from the trapping center chlorophyll to a bulk molecule when Q is reduced is 0.66. When Q in one unit is reduced, the probability of transfer from the trapping center of that unit to the trapping center of another unit is 0.45.

Delosme (11) has decided that there are two fluorescence quenchers operating simultaneously in Chlorella. His reasoning is that if only

one quencher were present, extremely bright light could reduce all of this quencher before any dark oxidation could occur. In that case, fluorescence would rise smoothly from O to P, without the intermediate hump I. He observed that the light intensity could be made high enough for the fluorescence yield during the phases O - I and I - P to be constant despite further intensity increases, but that I did not disappear. The hypothesized quencher was named R and concluded to be among the intermediates located between system II and system I. Delosme speculated that R may be identical with A.

D. The Problem

When our research began in 1965, the available literature suggested that P was due to a temporary maximum in the concentration of QH. To test this point of view we decided to see if system I preillumination would oxidize QH and thereby lower P.

P was lowered as expected. However, the preillumination lowered I as well, and raised S. Thinking that the I, P, and S effects could be separated according to wavelength, we started a detailed study of preillumination as a function of wavelength. We then discovered another complication, that the preillumination effects were not linear with preillumination intensity. Our study was thereupon broadened to include the problem of intensity. As results of the study were gathered, it became clear that a simple view of P in terms of QH was not adequate.

We noticed that between I and P there frequently appeared a dip.

This dip had not been seen before in aerobic cells, although Kautsky et al. (50) had seen it in aerobic cells. We have named the dip, D. Observation of D stimulated us to examine the kinetic behavior of models based on Q, to see which ones could allow D.

In one suitable model, D is explained by the production of A by system I and the resulting oxidation of QH. In another model, D is explained by the interaction of three forms of Q. The Malkin model has been found unsuitable because it does not allow D.

We began to question whether the QH maximum at P was caused by an exhaustion only of A, or of a different intermediate as well. Several experiments were designed to give the answer. System I background light added during the transient of anaerobic cells was found to be just as effective as system II light in hastening D. This result means that production of A by system I is occurring by the time of D. Direct exhaustion of A by system II later on is therefore unlikely. A short exposure of system I light during the phase D - P hastens P; since system I activity produces A, P should have been delayed if it is caused by exhaustion only of A. Finally, addition to a cell suspension of an artificial electron acceptor interacting only with system I (methyl viologen, $E_o' = -0.445$ v.) causes the disappearance of P. These results indicate that P is caused by a limitation in the oxidation of XH, the reduced form of the system I primary oxidant. This view of P makes more understandable the preillumination results, and they are discussed with this view in mind.

II. BIOLOGICAL MATERIAL, APPARATUS, AND TECHNIQUE

A. Selection of Material

A fluorescence transient is displayed by all types of photosynthetic tissue. The unicellular algae are among the most convenient to study, because they can be grown indoors the year around under completely controlled conditions, and they are easily prepared for experimentation. For example, we can prepare a sample of algal cells for experiments which is one cell-layer deep and which has a particular optical density. This is not possible with leaves. With chloroplasts extracted from leaves, such a sample can be prepared if we are careful to select only whole and not broken chloroplasts. But chloroplasts quickly decline in ability to carry on photosynthesis.

Of well-characterized algae most often used in our laboratory, blue-green Anacystis nidulans has a small fluorescence transient (O - P - S) and for this or other reasons has rarely been used for transient study. Both the red Porphyridium cruentum and the green Chlorella pyrenoidosa have a large transient. From these two algae, we chose Chlorella pyrenoidosa; it is the alga whose transient has been most often studied by different investigators.

Chlorella pyrenoidosa (Emerson's strain 3) is unicellular with a diameter of 5 microns (35). The cellular shape is spherical. A large green chloroplast shaped like a thick hemispherical bowl occupies the greater part of one-half of the cell, which we are able to see easily under a light microscope. The chloroplast contains the pigments --

Chl a, Chl b, and carotenoids (35).

B. Culturing

Chlorella was grown in completely inorganic medium prepared from reagent grade (A.C.S. specifications) chemicals and distilled water. The composition of the medium is shown in table I (see also ref. 35). The culture flasks were 300 ml Erlenmeyer flasks modified by the addition of side arms for intake and exit of gas. The intake arm protruded to the bottom of each flask to insure that gas bubbles passed through the medium. The bubbling rate was sufficient to circulate the cells and medium and keep cells from clumping. The gas was 5% CO_2 in air mixed in the laboratory from gas cylinders supplied by Linde Division, Union Carbide Corporation.

To establish a culture: 200 ml of medium was put in a flask, and cotton plugs inserted into the side arms and mouth. The flask was autoclaved at 15 lbs/in^2 for 20 minutes and left to cool. 20 ml of cell suspension obtained from a stock culture one to two weeks old was inserted under sterile conditions into the flask. It was attached to the gas supply and placed in a temperature-controlled water bath maintained at 20°C by tap water. This bath had a glass bottom suspended 8 inches above an incandescent light bulb. The bulb had a rating of 40 watts; it remained on continuously. The intensity of the light from this bulb above the water bath was 3750 ergs/cm^2-sec. The intensity incident on the cells was slightly different, however,

TABLE I

CULTURE MEDIUM FOR *CHLORELLA PYRENOIDOSA*

MAIN CONSTITUENTS

CHEMICAL	CONCENTRATION g/l
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	1.875
Na_2HPO_4	0.025
KNO_3	1.25
$CaCO_3$	0.016
KCl	0.025
$FeSO_4 \cdot 7H_2O$	0.0057

MICRONUTRIENTS

Soln A_2 — 1 ml/l

ABC 0.5 ml/l*

A_2

$MnCl_2 \cdot 4H_2O$	1.81 g/l
H_3BO_3	2.86 g/l

ABC

5 ml A_3 + 5 ml B_9 + 5 ml C_{10}
made up to 100 ml

A_3

$ZnSO_4 \cdot 7H_2O$	0.22 g/l
$CuSO_4 \cdot 5H_2O$	0.079 g/l
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.20 g/l

MICRONUTRIENTS, contd.

B_9

	mg/l
$Al_2(SO_4)_3Na_2SO_4 \cdot 24H_2O$	0.786
KBr	0.119
KI	0.083
$Cd(NO_3)_2 \cdot 4H_2O$	0.154
$Co(NO_3)_2 \cdot 6H_2O$	0.145
$NiSO_4 \cdot 6H_2O$	0.131
$Cr(NO_3)_3 \cdot 9H_2O$	0.040
$Na_3VO_4 \cdot 16H_2O$	0.041
$Na_2WO_4 \cdot 2H_2O$	0.033

C_{10}

	mg/l
As_2O_3	6.61
$SrSO_4$	10.49
$HgCl_2$	6.77
$PbCl_2$	6.71
LiCl	30.55
RbCl	14.20
$K_2TiF_6 \cdot H_2O$	5.00
$NaSeO_4$	11.96
$Be(NO_3)_2 \cdot 3H_2O$	103.7
Uranyl nitrate	10.0

*This value is ten times the value used by earlier workers (35).

because the flask sat partially under water, the intensity from the bulb varied with position in the bath, there was some background room light, and the flask contents by their light absorption created an intensity gradient with intensity highest at the bottom of the flask. The circulation caused by bubbling of gas should have averaged out the effect of the intensity gradient.

C. Harvesting and Preparation

A flask was disconnected from the gas supply and removed from the water bath two to five days after innoculation. The cotton plug in one side arm was removed; about 20 ml of cell suspension was poured through the side arm into a centrifuge tube, and the suspension was centrifuged for 10 minutes at 5000 x g. The supernatant was discarded and replaced by a small volume of buffer (described below). The cells were resuspended by gently swirling the buffer. The new suspension was diluted with buffer to an optical density at 678 nm, the "red" absorption peak of Chl a, of 0.4 for 1 cm path length. Figure 3 is an absorption spectrum of one sample of Chlorella pyrenoidosa.

Infrequently, over a two year period, there were accidental changes in culture conditions. On most of these occasions, the change was in light intensity. Intensity changes caused up to 20% changes in the ratio of absorbance at 678 nm (the peak of Chl a in vivo) to that at 650 nm (the peak of Chl b in vivo). Nearly always there was also a change in the P - S decline of the transient. We accepted for study only

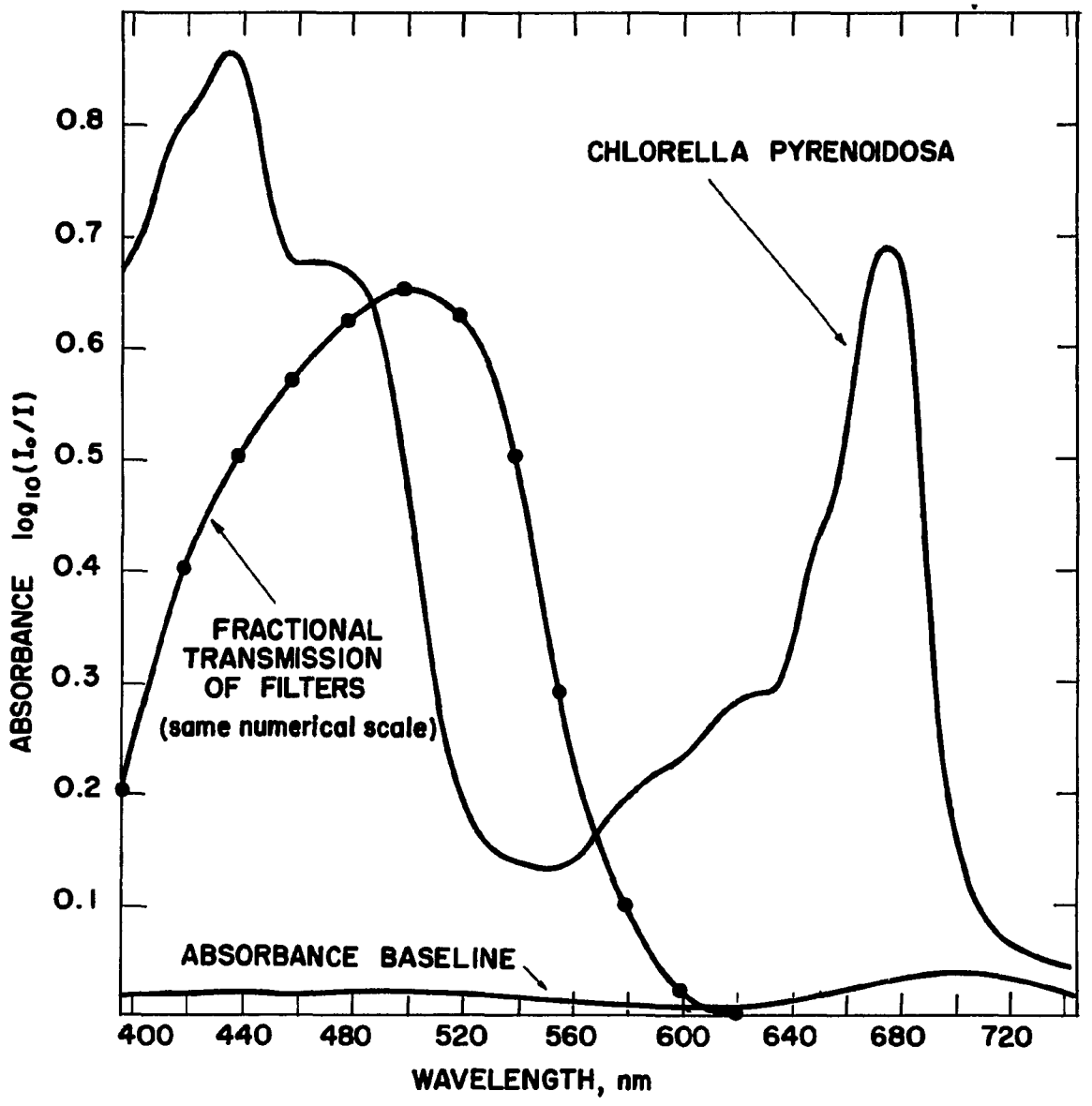


Figure 3. Absorption spectrum of Chlorella pyrenoidosa: absorbance ($\log_{10} I_o/I$) versus wavelength, pathlength 1.0 cm. For experiments, samples were diluted to obtain an absorbance of 0.15 for 0.374 cm. pathlength. The dotted line is the fractional transmission versus wavelength of the filters used with a tungsten projection lamp to excite fluorescence during experiments.

those cultures displaying a "normal" P - S decline. In normal cultures, the P to S ratio was 1.5. All cultures having a markedly lower ratio were rejected.

Three ml of the properly diluted buffer suspension was placed in a Dewar flask (H. S. Martin & Co., Evanston, Ill.) with a clear flat bottom. With the Dewar flask placed in the spectrofluorometer (described in section E), the excitation light beam and the preillumination light beam strike the flat bottom from below, and fluorescence emitted downward is captured by a collector lens and directed (through a monochromator) toward a phototube. This arrangement avoids a problem encountered when illumination and fluorescence collection are from the side; in that case, as cells settle, the density of the suspension changes and consequently the fluorescence intensity changes. In our arrangement the cells in the shallow liquid settle on the bottom of the flask within a few minutes.

By a microscopic examination of the cells after settling, we have observed that they do not overlap, except for the occurrence of some clumping as time goes on, which is facilitated by Brownian motion. The number of illuminated cells was calculated from a cell-count made by microscopic examination of a hemacytometer -- the number is (very roughly) one-half million. Because of this large number we believe that fluorescence effects due to cellular differences have been averaged out. The similarity of thousands of transients observed over a two year period indicates that the belief is valid.

The depth of 3 ml, the standard volume of suspension, when

placed in the particular Dewar flask used in this study is 0.374. Our Dewar flask had black electrician's tape on the inner surface of the bottom. The tape surrounded a small area on the bottom, thus acting as an optical stop for each beam of light. As a result, the same cells were illuminated by both beams. The absorbance during measurement was calculated from the depth of 3 ml and the absorbance for 1 cm. For example, absorption at 678 nm during measurement was $0.374 \times 0.4 = 0.15$.

For the bathing medium during measurement we chose a buffer made from $NaHCO_3$ and K_2CO_3 (an 85/15 mixture of 0.1 molar stock, pH 9.2). This choice was based on several considerations. The carbonate-bicarbonate mixture maintains the CO_2 concentration at 91×10^{-6} moles/liter (93), above the limiting value for photosynthesis at the light intensities used here; without buffering, solubility of CO_2 from air would produce in water a CO_2 concentration ten times less, which may become limiting. The buffer lacks inorganic nutrients necessary for growth, a desirable feature because the life cycle of Chlorella is short enough (10 to 20 hours depending on conditions) to allow considerable growth during an experiment several hours long.

The buffer is in other ways quite different from the growth medium. The growth medium and buffer have osmotic pressures of 1.44 and 5.3 (the first number is always for the growth medium); ionic strengths of 0.038 and 0.13; K^+ -- 0.026 M and 0.03 M; Na^+ -- 0.0004 M

and 0.083 M; and K^+/Na^+ -- 65 and 0.35.

Although stopping growth and being quite different from the growth medium, the buffer does not impair ability to carry on photosynthesis: Pratt (83) showed that for Chlorella grown in a medium containing mainly KNO_3, $MgSO_4$, and KH_2PO_4 (82), and transferred to a buffer containing 0.035 M $KHCO_3$ and 0.065 M $NaHCO_3$, the rate of oxygen evolution was constant for over 15 hours. In a buffer containing 0.025 N $KHCO_3$ and 0.075 M $NaHCO_3$, the rate declined to 95% of the initial rate within 3 hours. The buffer used here has concentrations close to the above and can be expected to maintain nearly constant oxygen evolution for at least 3 hours, perhaps 6 or 8. Our experiments usually lasted about 6 or 8 hours.

The result obtained by Pratt (83) in a solution containing only 0.1 M $NaHCO_3$ is startling: although the rate of oxygen evolution declined from the start and after 10 hours was 45% of the initial rate, it was constant thereafter for the next 14 hours. This result indicates how much "stamina" the photosynthetic apparatus of Chlorella has in the face of extreme and unusual cellular environments.

The buffer we used was chosen for one other major reason. It is similar to the $NaHCO_3$ - Na_2CO_3 (85/15 of 0.1 Molar stock, pH 9.2) buffer described by Warburg (93) in 1919 and since known as buffer #9. In our buffer, Na_2CO_3 is replaced by K_2CO_3 in order to provide potassium for the maintenance of membrane potentials. Buffer #9 has become a tradition in the study of Chlorella -- our use

of a similar buffer makes comparison of our results with those of the literature more trustworthy.

In the course of the work, we experimented with media to see what differences they would cause in the transient. We found that the transient was similar for cells in growth medium at pH 5.5, and $NaHCO_3$ - K_2CO_3 buffers mixed to obtain three different pH values -- 9.2, 10.2, and 10.7. The long-term effects of growth medium and the buffer at pH 9.2 were similar -- P shifted to a later time, and in some experiments, I and D became more pronounced and noticeable.

D. Measurement of Absorption

Absorbances were measured with a Bausch and Lomb, Inc. (Rochester, New York) recording spectrophotometer, Spectronic 505, equipped with an integrating sphere to reduce errors due to light scattering. The half-maximum band width of measurement was 5 nm. Absorbances were not corrected for sieve and detour effects (see ref. 84, p. 672).

The pigment in Chlorella which absorbs furthest into the near-infrared is Chl a. In a solution of Chl a in diethyl ether the red absorption peak is at 660 nm, and measurable absorption persists to nearly 700 nm. In the cell a minor portion of the Chl a molecules have peak absorption at 700 nm. If the absorption band widths in solution and in the cell are not too different, there is measurable absorption in the cell as far into the near-infra-red as 740 nm.

Despite the integrating sphere, and despite the lack of pigments absorbing beyond 740 nm, Chlorella suspensions showed absorption between 740 nm and 760 nm. Most of this absorption must be due to scattering or non-uniformity of the coating of the integrating sphere rather than actual absorption. To correct for this absorption, the base line for wavelengths of interest was raised by an amount equal to the amount of this absorption at 740 nm. The magnitude of the correction was about 5% of peak absorption at 678 nm. This correction procedure is valid as long as the wavelength dependence of the scattering is not severe. The scattering in Chlorella pyrenoidosa, according to Latimer (62), has two components: one component is scattering from the cell walls and colorless structure within the cell, which is nearly wavelength independent in the red wavelength region. The other component is scattering by the pigments, called selective scattering, which is strongly wavelength dependent. Selective scattering is maximal at wavelengths to the longer wavelength side of absorption peaks. But it contributes only a small amount to total scattering. Latimer (63) reported that total scattering varies with wavelength by only a few percent.

E. The Spectrofluorometer

The basic components of the spectrofluorometer include lights for exciting fluorescence, monochromators for isolating narrow wavelength bands of exciting light and fluorescence, and a photomultiplier tube for detection of fluorescence. These basic components were

assembled by J. Spencer and Govindjee. For measurement of fluorescence transients, some modifications have been necessary -- shutters and fast recording apparatus. The modified instrument is diagrammed in figure 4.

The main exciting light came from a Radiant Lamp Corporation (Newark, New Jersey) DDY 750 watt projection lamp. The lamp voltage was supplied by a General Radio Co. (Concord, Massachusetts) variac set at 120 volts, and connected to a Sola Electric Co. (Elk Grove Village, Illinois) line voltage regulator. A five centimeter water bath absorbed heat. Filters selected a small band of wavelengths from the broad wavelength band emitted by the lamp; the filters and wavelengths selected are described in section F.

A secondary exciting light was used for preillumination and background illumination. This light was a General Electric Co. (Cleveland, Ohio) microscope illuminating lamp (tungsten ribbon filament, 18a/TID/1-6V) run at 20 amperes. The power supply was built in the laboratory by N. Kalra; it was connected to the Sola line voltage regulator. A Bausch and Lomb monochromator (600 grooves/mm; dispersion -- 3.3 nm half-maximum bandwidth per mm slit; blazed at 300 nm) selected the desired wavelength band.

The two lights were arranged on opposite sides of the holder for the Dewar flask. The illuminations were directed horizontally toward the flask by lens systems. Underneath the flask were two mirrors which deflected the illuminations upward to the bottom.

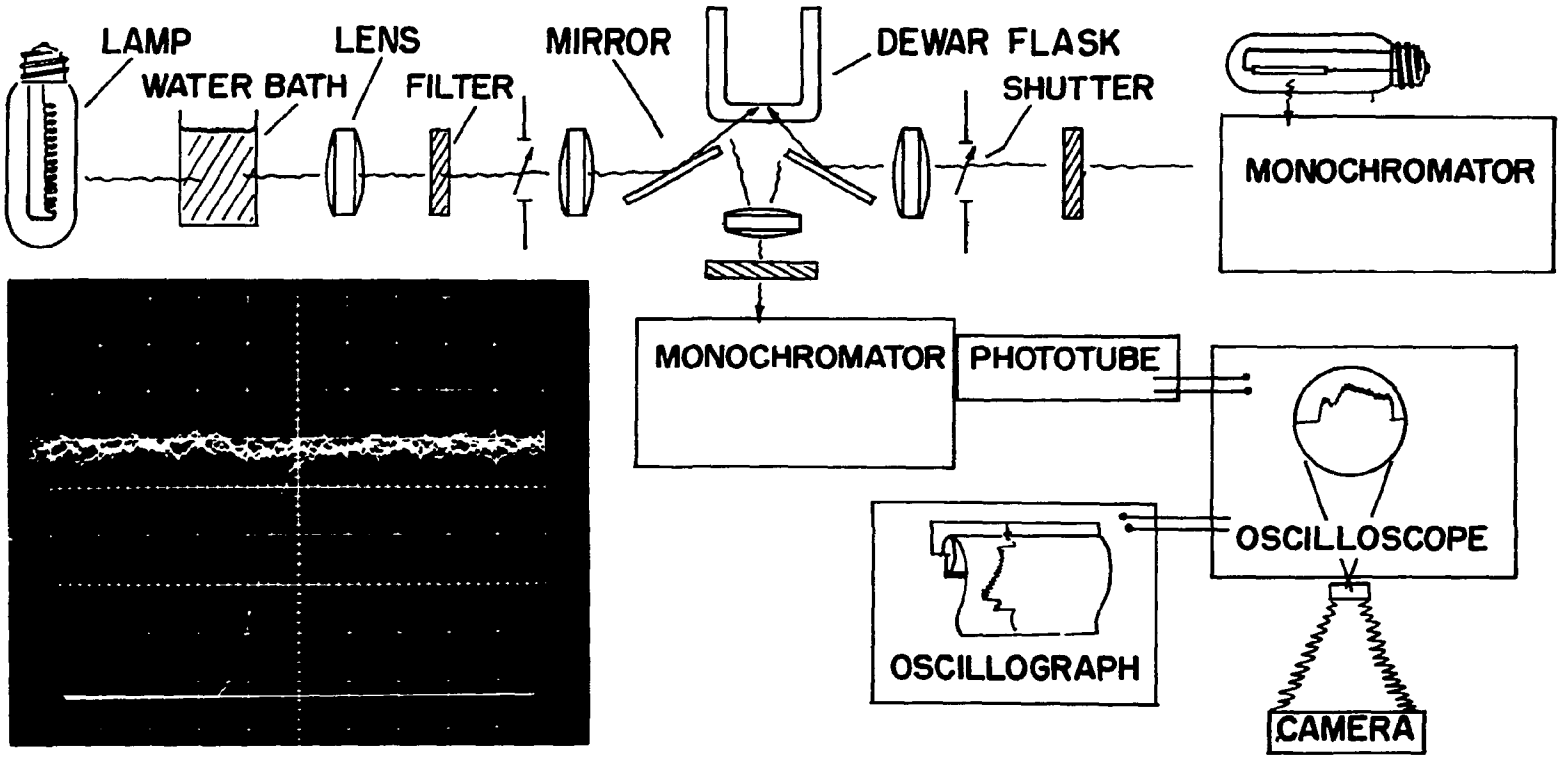


Figure 4. Block diagram of the spectrofluorometer. Other apparatus used included a thermopile and micro-voltammeter for measuring light intensity, and a spectrophotometer for measuring absorption spectra.

Figure 5. (bottom left). An oscillograph of the phototube signal during a shutter opening. Horizontal scale· 1 msec/cm. The sloping vertical line denotes the opening. The opening is complete in 2 msec.

Compur shutters (Burke and James, Inc., Chicago, Illinois) were mounted to intercept each beam of illumination. The f-stop on the shutter intercepting the main exciting light was closed down to keep opening time to less than 2 msec, allowing measure of O. Figure 5 shows an oscilloscope photograph of the photomultiplier tube signal during a shutter opening. For this photograph, magnesium oxide in the flask reflected illumination toward the phototube.

Fluorescence which passed vertically downward was collected by a lens and deflected by a mirror into a second Bausch and Lomb monochromator (blazed at 750 nm). A small wavelength band passed by the monochromator struck an Electra Megadyne, Inc. (New York) 9558 B photomultiplier tube. The power supply for the tube was by NJE Corporation (Kenilworth, New Jersey). Output from the photomultiplier tube was amplified and displayed by a Tektronix, Inc. (Portland, Oregon) 502 A oscilloscope.

Two methods were used to record the amplified signal. In one, the oscilloscope trace was photographed on Eastman Kodak Co. (Rochester, New York) 35 mm linagraph ortho film by a Cossor Instruments Ltd. (London, England) oscillograph camera, model 1458. This method was used to record nearly all the data presented in the thesis. The method required time-consuming photographic development and data processing after an experiment was completed, too late to allow minor adjustments of procedure. Near the end of the experimentation we obtained the means for a second method. In the second method, the amplified signal from

the oscilloscope was fed to a Midwestern Instruments, Inc. (Tulsa, Oklahoma) 810 B oscillograph. In this instrument the signal is projected by means of a galvanometer onto moving Eastman Kodak Linagraph Direct Print photographic paper. The paper develops in under two seconds upon exposure to sky light or fluorescent lighting. This method allowed faster and on-the-spot data processing.

In order to vary gas conditions during experiments, the Dewar flask was capped by a rubber stopper, and various gas mixtures were introduced through a hole in the stopper. The mixtures were made from gas cylinders supplied by Linde Division, Union Carbide Corporation. The flow conditions were such as to exponentially flush out old gas; the time constant (for the old gas concentration to drop to 1/e its original value) was roughly four minutes.

The surface to volume ratio of the liquid in the Dewar flask was seven; this high ratio permitted rapid equilibration of gases between the liquid and the air above. We observed that for cells left in buffer under stagnant air for a half-hour, the transient remains the same upon starting a flow of 2.6% CO_2 in air. In some experiments cells remained in buffer under stagnant air for several hours, and the small changes which occurred in the transient were opposite to those caused in other experiments by removing O_2. Cells in buffer, where CO_2 is buffered, and growth medium, where it is not buffered, showed the same changes. We conclude from these results that CO_2 and O_2 are not limiting for several hours. This conclusion validates the assumption that proper gas

conditions prevailed in this and previous work done with the Dewar flask technique.

Temperature within the Dewar flask was measured by a Tri-R Instruments (Jamaica, New York) electronic thermometer. The temperature was 25°C, on a few days the temperature rose three degrees over a several hour period because air conditioning in the building was not operating. On these days the room temperature rose seven degrees; the smaller rise in the Dewar flask indicates its effectiveness in maintaining constant temperature.

Some experiments required that the exciting illumination consist of flashes. This was accomplished by means of a rotating sectored disk mounted on a small 3 volt DC motor powered by a dry cell. The disk was sectored to provide two flashes and two dark periods, all of equal length, for each rotation. The speed of rotation was about 6 Hz, making the length of each of the light flashes and dark periods about 40 msec.

F. Wavelengths of Illumination and Fluorescence

Because the fluorescence transient is emitted by system II, which has the greater share of Chl b (32, 40), we chose wavelengths for the exciting light which are preferentially absorbed by Chl b. Although a narrow wavelength band would have been desirable in order to limit excitation to Chl b, the need for high intensity demanded a wide wavelength band. The band was provided by a combination of

Corning Glass Works (Corning, New York) filters, C.S. 3-75 and 4-96. The band provided by the filters had a maximum at 500 nm and a half-maximum bandwidth of 120 nm. The spectral output of the lamp, in the 500 nm region, is rising slightly with wavelength. The band of light reaching the cells therefore had a peak at a wavelength slightly greater than 500 nm. This band encompasses the Soret band of Chl b in vivo, the peak of which is located at 480 nm. On figure 3 there is a plot of the % transmission of the combination of filters as a function of wavelength.

We wanted to compare effects of light absorbed by systems II and I In Chlorella, system I can be preferentially excited to an increasing degree as the wavelength of excitation is increased beyond 690 nm (32, 35). At 725 nm only system I is excited (72). Although no wavelength excites only system II, it can be preferentially excited by wavelengths between 680 nm and 640 nm (32). Therefore, the range chosen for study was 640 nm to 725 nm. Different wavelengths in this range were selected by the preillumination-lamp monochromator. 2 mm slits were used, permitting a half-maximum band-width at each wavelength setting of 6.6 nm, and a full bandwidth of 10 nm. Some scattered second-order blue light was noticed in the monochromator output, it was eliminated by a Corning C.S. 3-69 red cut-off filter.

The fluorescence spectrum of Chlorella excited by system II light has a maximum at 685 nm at room temperature. Consequently, the measuring monochromator was set at 685 nm with 2 mm slits. Despite the setting and bandwidth, a small amount of exciting light at first leaked

through the measuring monochromator and was included in the fluorescence measured by the phototube. This leak was reduced by placing a Corning C.S. 2-60 red cut-off filter in front of the measuring monochromator. With this filter in place, and the Dewar flask containing a one-quarter inch depth of magnesium oxide, the leak was about 2% of the signal normally measured from Chlorella. Because the reflectance of magnesium oxide is much greater than that of Chlorella, the leak during measurement of fluorescence from Chlorella was much lower than 2%.

G. Measurement of Light Intensity

Light intensity was measured with an Eppley Laboratory, Inc. (Newport, Rhode Island) Bi/Ag/lampblack-soot thermopile and a Keithley Instruments, Inc. (Cleveland, Ohio) 605 A Microvolt-ammeter. The Eppley Laboratory had calibrated the thermopile against a standard lamp supplied by the National Bureau of Standards. The light intensities measured were always within the calibration range. It was not possible to place the thermopile at the same location as the Dewar flask. Consequently, during intensity measurements the illumination beam was deflected by a mirror to a different location. The thermopile was adjusted at this location so that the spot of light normally focused on the inner surface of the Dewar bottom was focused on the thermocouple elements. The light spot traversed two layers of glass in the Dewar flask but traversed only one quartz window in the thermopile. Consequently,

the intensity values we report are about 4% high.

Light intensity was varied by the use of Balzers (Geraetebauanstalt, Balzers, Fürstentum, Liechtenstein) neutral density filters. The transmission values prescribed by Balzers were checked against measurements made with the photomultiplier tube.

H. Intensity of Illumination

In the literature on the fluorescence transient there is mention of the requirement of high exciting light intensity, but no mention of an objective intensity standard. We decided that it would be useful to compare the intensity required to produce the transient, to the light intensity which produces high fluorescence yield in the steady-state. The investigation produced interesting results and is discussed in detail in chapter IV.

For this section it suffices to state that as intensity increases, the steady-state yield changes from a constant value at low intensity to twice that value at high intensity. The change occurs in the intensity region where oxygen evolution becomes saturated (27, 28). We found that the transient is hardly observable in the low-intensity region, and that it becomes easily observable and prominent in the high-intensity region.

The fluorescence yield and oxygen evolution vs. intensity curves allow establishment of a biologically standardized intensity for photosynthetic organisms: the transition intensity in these curves. The

transition intensity in the case of fluorescence can be defined as the intensity at the mid-point between low and high yields. By use of the standard we can give a meaningful description of our illumination intensity: it was twice the transition intensity. For our Chlorella, this intensity was 1.5×10^4 ergs/sec-cm^2 (incident). The absorbed intensity was 4×10^{14} quanta/sec-cm^2, an estimate based on the shape of the absorption spectrum and the shape of the broad exciting light spectrum.

There is a feature of the transient itself which indicates the exciting light intensity, and that is the time interval between onset of illumination and the peak, P. We call this interval, t_p. A graph of t_p versus light intensity is shown in figure 6. As intensity increases, t_p becomes smaller but approaches a limiting value. The shape of the curve suggests that t_p and I obey the relation, $It_p = k$, where k is a constant. If so, then a plot of $\frac{1}{t_p}$ versus I should be linear. The plot is shown in figure 6; it is linear except at the highest intensities.

Because It_p is proportional to the number of quanta absorbed between onset of illumination and the peak, the validity of $It_p = k$ shows that the peak requires a constant number of quanta for its development. At the highest intensities in figure 6, where $\frac{1}{t_p}$ versus I deviates from linearity, some factor besides intensity must become limiting. We can presume that at low intensities a photochemical reaction is limiting, and that the highest intensities a thermochemical reaction is limiting. When Morin (75) distinguished I - P as thermal and O - I

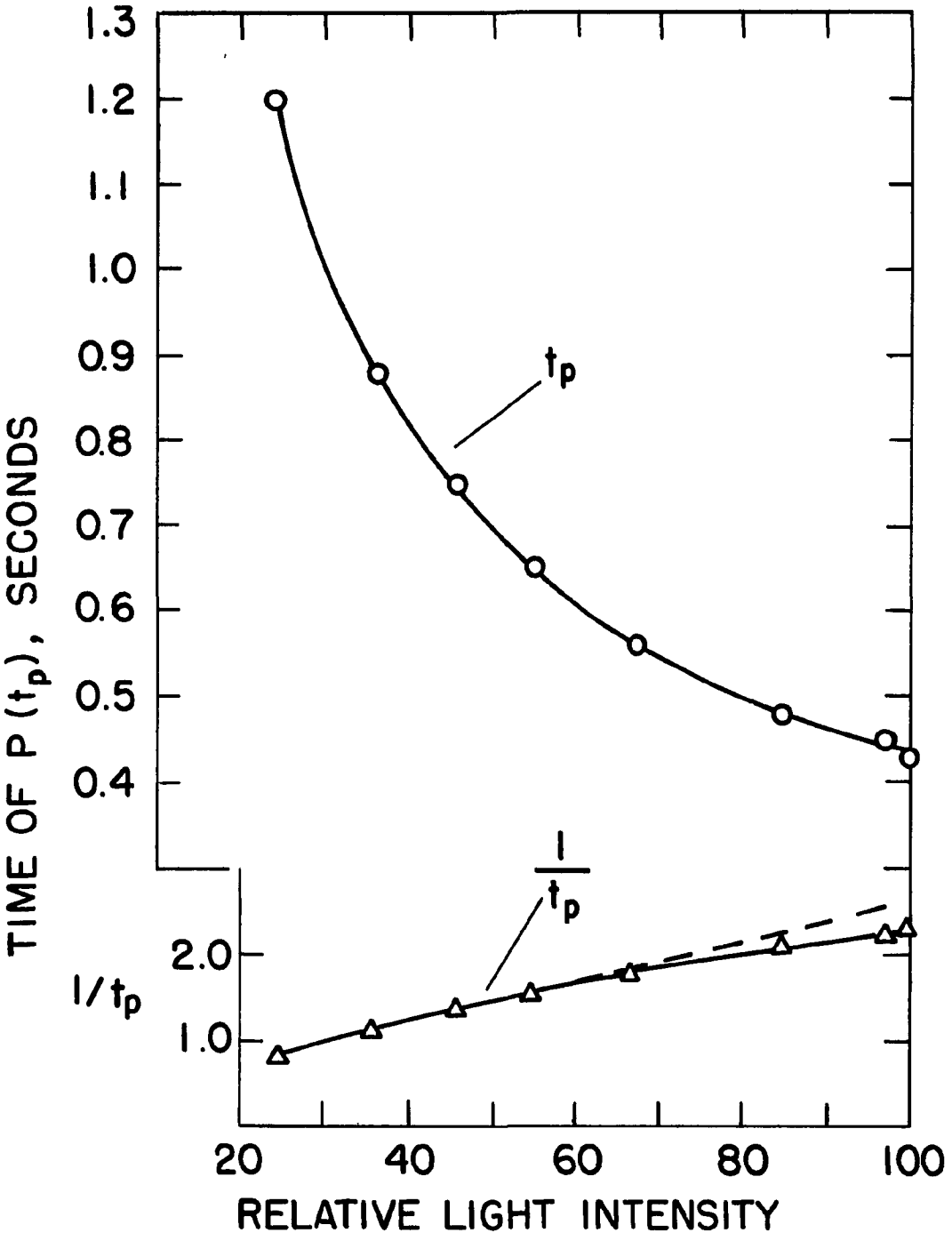


Figure 6. Time of the peak, t_p, versus light intensity. The highest intensity (100) is 1.5×10^4 ergs/sec-cm^2 incident. The plot of $1/t_p$ is linear at low intensity, indicating that the product of intensity times t_p is a constant.

as photochemical, his intensity was undoubtedly higher than the highest used here. Our curve allows a precise determination of the degree to which I - P has become thermal.

Because of occasional variations in Chlorella cultures, t_p sometimes changed from day to day despite a constant exciting light intensity. When t_p varied, the exciting light intensity was adjusted to restore t_p to 400 - 450 msec.

The preillumination light intensity was much lower than the exciting light intensity. A range of preillumination intensities was used because it was found that the preillumination effects varied with intensity. The upper limit of the range was 750 ergs/sec-cm^2 (incident). In order to compare effects of different preillumination wavelengths it was necessary to calculate absorbed quanta/sec-cm^2, a typical value was 5×10^{11} quanta/sec-cm^2. This value is 1000 times smaller than absorbed exciting light intensity, 4×10^{14} quanta/sec-cm^2.

I. Experimental Procedure

The height of P excited by light exposure depends on the length of the preceding dark period. The dark period requirement depends in turn on the length of the light exposure prior to the dark period. In initial experimentation a 30-second light exposure was employed, with this long exposure, a 5-minute wait permitted 75% restoration of maximum P, and complete restoration took over 10 minutes. In most experimentation a 2-second light exposure was employed. This shorter

exposure permitted faster restoration. Figure 7 shows the development of a higher P as the dark period increases. Restoration is complete in 6 minutes.

The dark requirement necessitates long experiments. They can be avoided if fresh dark-adapted cells are introduced anew for each exposure. We decided against this alternative, and in favor of repetitive exposure of one sample of cells, for several reasons: first, the constancy of cell behavior is reconfirmed in each exposure. Second, results requiring many exposures are obtained from just one sample. Third, damage to the photosynthetic apparatus by extreme light intensity can be noticed in successive exposures and henceforth avoided. Fourth, the effect of noise can be determined by examining successive pictures from cells under constant conditions; the percentage variations in the primary data determined in this way have been found to be smaller than two or three percent.

Figure 7 shows that 4 minutes after a 2-second exposure, P is more than 95% restored. On that basis we chose a cyclic procedure, involving 2-second exposures and 4-minute dark periods, repeated as many times as necessary to carry out an experiment. In the preillumination experiments, a dark period was replaced by preillumination. The effect of the preillumination sometimes persisted through two subsequent cycles, making necessary a wait of three cycles before another application of preillumination. In some experiments the secondary

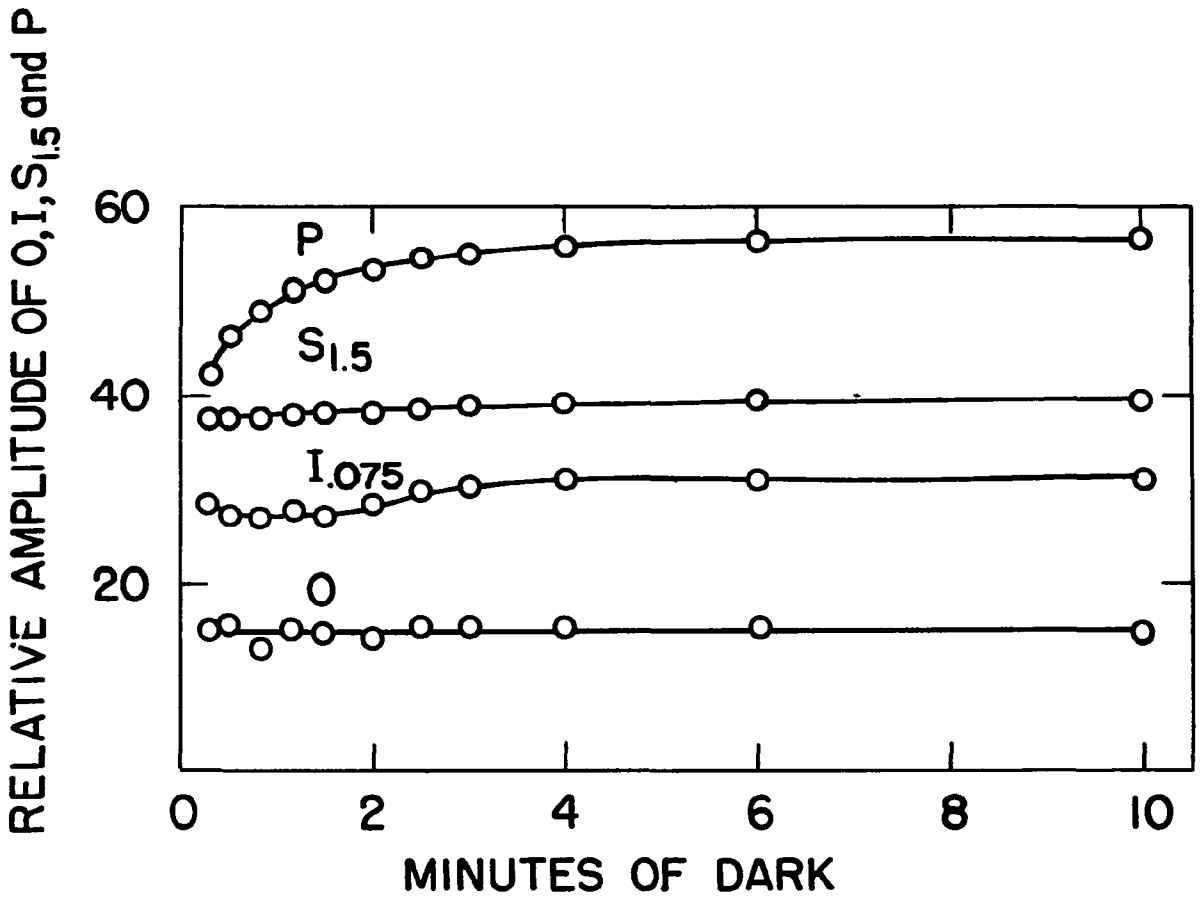


Figure 7. Amplitude of points of the transient as the length of the preceding dark period increases. After a four-minute interval, which we employed in most experimentation, P is more than 95% of its maximum, dark-adapted value. $S_{1.5}$: S measured at 1.5 seconds. $I_{.075}$: I measured at 0.075 sec (75 msec).

light was applied not as preillumination but as background illumination simultaneous with the exciting illumination, because the background effect persisted only a few minutes, a wait of only one cycle was needed. The variation of gases whenever employed required a wait of several cycles for complete flushing out of old gas. In every experiment, an initial wait of at least 8 cycles was necessary to allow the sample to settle, and to adjust to the cyclic procedure and to the local conditions such as temperature in the Dewar flask.

Many experiments probed the effect of a change in exciting light or preillumination intensity. The order of presentation of different intensities had a slight effect on the results. Minimizing and standardizing these effects required:

i) in fluorescence yield studies, the use of a monotonic sequence of intensities, first decreasing to a minimum intensity and then increasing;

ii) in preillumination experiments, the use of monotonic sequence of increasing light intensities (a subsequent increase was omitted because the length of experiments would have become excessive).

In experiments several hours long, we noticed some changes in the transient: t_p increased, P and S decreased, and sometimes I and D became more pronounced. As much as possible, extremely long experiments were avoided because of the small possibility that changes would affect results. However, 6 to 8 hour experiments were

the general rule.

J. Data Extraction and Processing

Exposed film was developed, fixed, and dried. One photograph in each series was of the illuminated graticule of the oscilloscope; by means of a photographic enlarger, an image of this photograph was projected onto graph paper and the magnification adjusted until the 1 cm. markings of the graticule corresponded to 1 inch of the graph paper. Time and amplitude scales according to the oscilloscope sweep speed and sensitivity were then drawn on the graph paper. Images of the photographs of the transient were aligned on the graph paper, then, the amplitude and time of points on the images were recorded in list form or converted directly to graphical form.

The amplitude of points O and P of the transient by nature were easy to determine. S was determined at 1.5 seconds (or at 1.0 seconds and 30 seconds, in some older experiments). I was determined consistently in the photographs of each experiment; the determination was made at usually 50 msec.

Oscillograph paper required less treatment than the film. The oscillograph by its operation puts time and amplitude scales on the paper, furthermore, photodevelopment is so brief and easy that the paper was photo-developed during experiments only seconds after recording, and was analyzed immediately.

Graphs of O, I, P, S, and t_p as a function of elapsed time were

based directly on the photographic images of the transient. Other graphs were constructed from these graphs and required only simple calculations such as percent changes.

K. Discussion of Errors

A characteristic of Chlorella cells (and of all living biological material) which imposes a limitation on error analysis is their vital dynamism. Despite constant environmental conditions during an experiment, there are continuous internal changes. Transients obtained in successive light exposures are not from exactly identical cells. Consequently, a rigorous determination of the size of errors in each experiment is not possible, and we have to be satisfied with those determinations which are less than rigorous.

The limitation, it turns out, is not bothersome. Each Chlorella sample contained one-half million cells, enough to average out cellular differences. Variations in successive pictures from cells under constant conditions were found to be smaller than two or three percent.

Variations in the effects of preillumination were sometimes as much as 10%. When such large variations were too frequent, the experiment was repeated an extra time. The cause of the larger variations was never discovered. The figures in chapter V are typical results of single experiments and show that the larger variations do not seriously interfere with the drawing of smooth curves.

Slightly more serious were the over-all variations encountered

from one experiment to the next. For example, the ratio P/S might vary 10% from day to day, and the effect of preillumination might vary 10%. Maintenance of constant cultural conditions, especially growth light intensity, helped to minimize variations. We coped with them by repetition of experiments.

Bias during inspection of photographs was determined to have negligible effect on results. On several occasions we re-inspected old photographs and extracted once again the numerical data. The old and new data agreed within one percent. On many occasions other members of the laboratory looked at records and confirmed the basic observations.

One potential source of error more significant to our conclusions than any other is the possible inaccuracy in absorption values in the region beyond 700 nm. The absorption decreases so rapidly with wavelength that by 720 nm, the baseline correction is several times as large as the calculated absorption. We believe that this source of error by its nature should not produce systematic (one-sided) variations, but random variations in absorption values. Because all of the experiments involving spectral comparison gave consistent results, we feel confident that possible random variations in absorption values have not invalidated our results.

Some sources of error have not been investigated beyond brief consideration and adjustments of procedure. They include parallax between the graticule and oscilloscope screen, parallax during enlargement of the photographs, and small variations in the timing of the

shutter opening.

The reliability of the photographs and figures included here is high. About 5000 photographs were taken. Each experiment was repeated several times, and each experiment included many trials.

L. Calibration and Alignment of Apparatus

The monochromators were periodically aligned by means of a mercury vapor lamp. The band pass per mm slit width was measured. The optics were aligned nearly every week (necessary because other persons used the apparatus in different optical alignments). The oscilloscope (new), although not calibrated against independent standards, was internally consistent. It was tested twice by laboratory technicians. The oscillograph (new) was obtained near the end of the research and was used for only a few experiments. The oscillograph and the oscilloscope gave similar results. The (new) galvanometer of the oscillograph was not tested for linearity in our laboratory. However, the data obtained with the oscillograph were not affected by linearity because they involved time rather than amplitude. The timing circuitry, being purely electronic, was considered reliable. The galvanometer was shortly damaged by excessive current, and then failed a test of linearity, we then returned to photography. The microvolt-ammeter (used with the thermopile) was calibrated against a standard cell; on several occasions a check showed that scale factors were consistent, and noise and drift were negligible for more than 15 minutes. The

photomultiplier tube had to be replaced once. The second tube was not tested for linearity by means of independent standards but it gave results similar to the first.

III. STANDARDIZATION

Standardization required that we study the effects of various factors on the fluorescence transient. Although work of this kind has been done before (see chapter I, and the review by Rabinowitch, ref. 84, pp. 1375-1406), new and interesting results emerged which deserve special discussion.

A. Adaptation

A change in the amplitude of the transient occurred during the initial cycles of each experiment. This change necessitated a half-hour wait before trials began.

Figure 8 shows the rise of O, I, P, and S which occurred initially in one experiment. Evidently, O is not constant. We cannot be certain whether the change occurs during illumination or during the dark period; however, we feel that O changes slowly. O becomes steady if external conditions are constant for over a half-hour. Another feature is that the changes in I, P, and S are similar to the change in O. In some experiments, the changes in I, P, S, and O were not only similar but also strictly proportional. These features suggest that the O change is caused by conditions underlying fluorescence as a whole, rather than by events involved directly in the transient. Perhaps the O change involves changes in intermolecular distances and the orientation of molecules. Such changes would affect the probability of energy transfer between chlorophyll molecules, and, consequently, the fluorescence

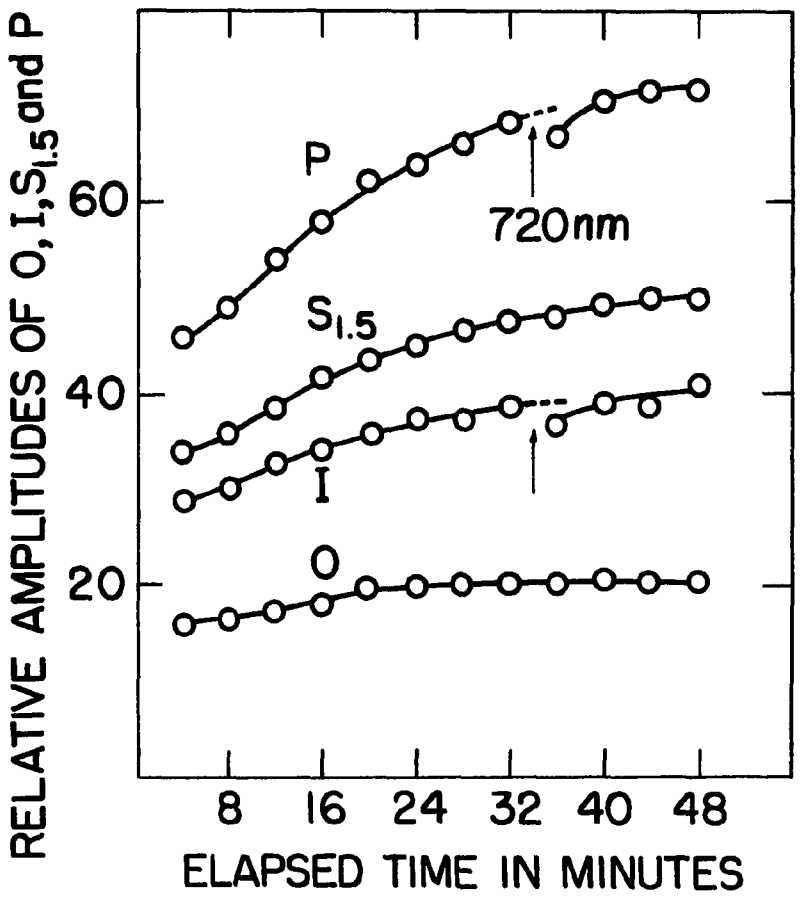


Figure 8. Amplitude of points on the transient during an adaptation period at the beginning of one experiment. Evidently, O changes with time. I measured at 50 msec. 720 nm preillumination depressed P and I. It usually raised S as well, but in this culture the effect appeared only at higher 720 nm intensity.

yield. Papageorgiou and Govindjee (81) have suggested that such changes may control the second wave of fluorescence in Anacystis nidulans.

The question is which system produces the O fluorescence. Lavorel (64) has determined the spectrum of the fluorescence at O and P· the O spectrum contains a prominent band at 717 nm which is not as prominent in P. The spectral difference between O and P suggests that much of O originates in system I, because the majority of P originates in system II. But Lavorel also observed that the fluorescence yield at 717 nm varies a small amount with exciting light intensity. Vredenburg and Duysens (91) studied the 720 nm fluorescence band in Schizothrix, and also found that its yield was variable by a small amount. Although the band was excited by system I light, the variation in yield was not correlated with the redox changes of P_{700}, the trap for system I. These variations of fluorescence at 720 nm may be understood as deriving from a system II component of fluorescence at 720 nm. It is not likely that the variations derive from system I, because Krey and Govindjee (61) have shown in Porphyridium cruentum that while fluorescence excited by system II light is nonlinear with intensity, the fluorescence excited by system I is linear.

We emphasize that fluorescence measured at any wavelength, be it 685 nm or 720 nm, contains both system II and system I contributions. As the fluorescence wavelength changes, so does the ratio of contributions. Detailed understanding of the O level is not possible until we know the proportion of system II fluorescence at 720 nm.

However, it is clear that fluorescence at 720 nm is variable, and that fluorescence at O is variable. Because variations in O seem slow, and cease after a half-hour wait, and probably are caused by conditions underlying all of fluorescence, we believe that they do not interfere with our experiments.

B. Effects of the Gaseous Environment

Raising the CO_2 concentration from 1% to 4% was found by Kautsky and Hirsch (54) to retard the P - S decline. We confirm their finding: we observed that a change from 2.6% to 5% causes the retardation.

Kautsky and U. Franck (52) studied the effect on the fluorescence transient of different O_2 concentrations, but in their study, cells were swept with each concentration for 60 to 90 minutes. The question arose whether the results were due directly to the O_2 concentration or to a long-term effect of O_2 depletion on metabolic intermediates. Long-term effects have been demonstrated. Noack, Pirson, and Michels, according to Rabinowitch (ref. 84, p. 1366) demonstrated the presence of a diffusible inhibitor in Chlorella after thirteen hours of anaerobiosis. However, some anaerobic effects develop rapidly. Van der Veen (ref. 84), p. 1370) found that 30 minutes of incubation of Holcus lanatus with nitrogen containing 0.3% oxygen did not substantially affect oxygen evolution induction curves, but when oxygen was removed altogether from the nitrogen, an effect was noticeable after one minute of

anaerobiosis in darkness. Hill and Whittingham (ref. 84, p. 1371) also noticed an effect on oxygen induction in Chlorella after anaerobic incubation of a few minutes.

In our experiments, where new gas exponentially replaced the old, the effects of oxygen depletion were noticed within four minutes. Figure 9 shows the effect on the phases of the fluorescence transient when stagnant air is replaced by 2% CO_2 in air, and then by 2.6% CO_2 in argon. The argon was applied twice in order to test reversibility and repeatability of its effects. The amplitudes of P and $S_{1.0}$ show a clear jump upward after the flow of argon is begun. Because the points are spaced four minutes apart, P and $S_{1.0}$ obviously were affected within four minutes. Kautsky and U. Franck (52) claimed that effects of anaerobiosis were reversed within one minute of admission of air; our figure shows that the reversal is, at the least, faster than four minutes.

P is lower after anaerobiosis than before, indicating a residual effect; however, anaerobiosis is not responsible for all of the change because P usually declined even in the course of an aerobic experiment.

According to Kautsky and U. Franck (52), the O and I levels are affected at much lower oxygen concentrations than P and S, and only when the oxygen concentration is below 0.3%. The O and I levels were affected in our experiments later than the P and S levels because of the time required to diminish the oxygen concentration to this low level. Although the oxygen concentration was not monitored, we

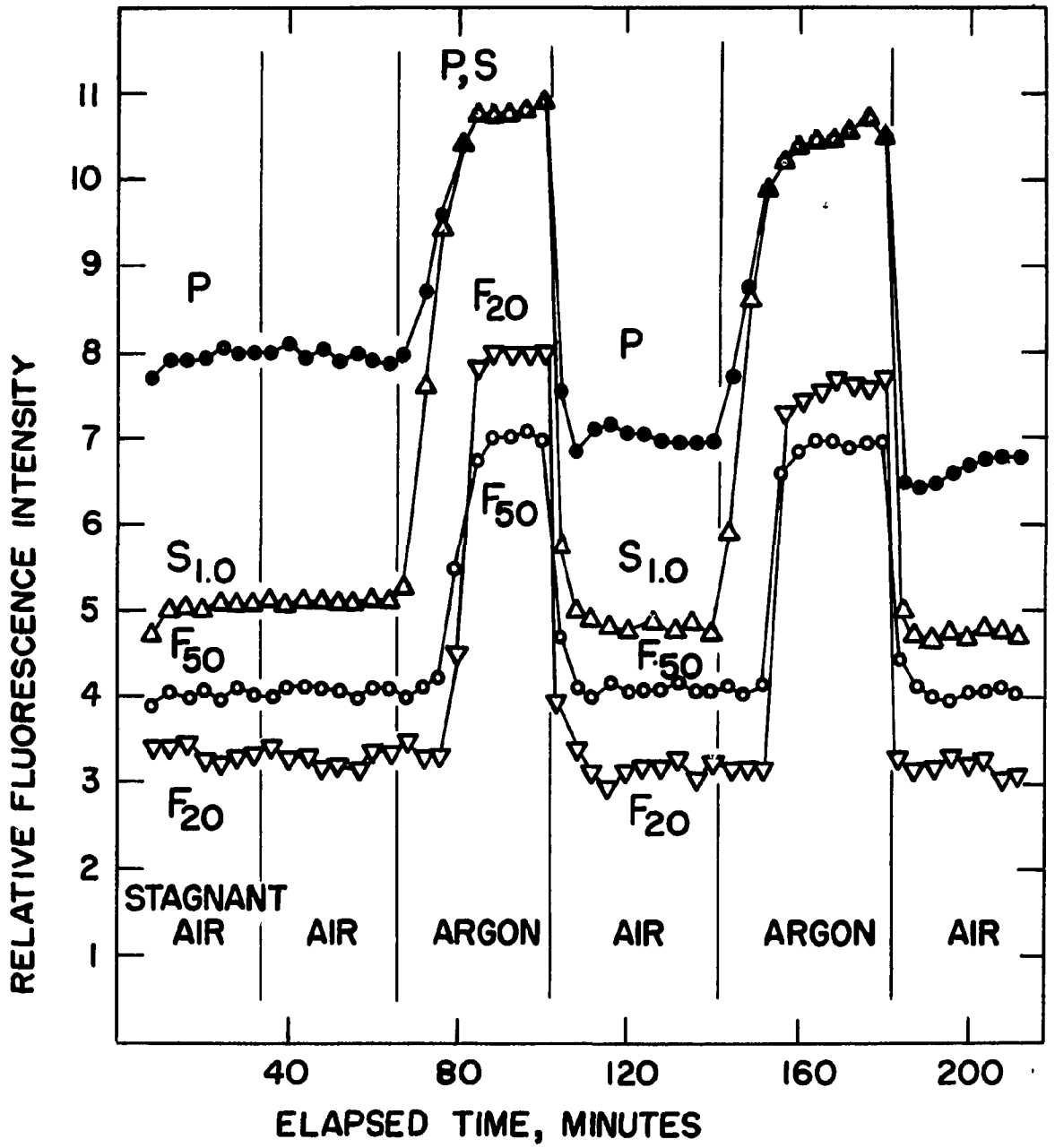


Figure 9. The effect of replacing air by argon on points of the transient. F_{20} and F_{50}: fluorescence at 20 msec and 50 msec after onset of illumination. Stagnant air was changed to flowing 2% CO_2 in air, then to flowing 2.6% CO_2 in argon. P and $S_{1.0}$ are affected within four minutes after introduction of argon. The argon effect is reversible and repeatable. This experiment produced the small photographs of figure 2.

calculate from an estimated argon flow rate, the volume of gaseous space flushed out, and the length of time before O and I were affected, that they were affected when the oxygen concentration dropped below 0.4%. The calculation is based on an exponential replacement of old gas by new. Despite possible error, the agreement between this figure and that of Kaustky and U. Franck is good.

Figure 2 on page 12 includes a strip of photographs from the experiment graphically presented in figure 9. The photographs show the marked changes in the fluorescence transient as air is replaced by argon. They also demonstrate clearly the development of I of the aerobic transient into the spike of the anaerobic transient. This fact will be basic to discussion in later chapters.

The rapid development and reversal of anaerobic effects on the fluorescence transient permit a general conclusion: the effects are not due to long-term metabolic changes. If any disarrangement of the photosynthetic apparatus is involved, it is evidently mild and easily repaired. These conclusions allow the confidence that results obtained from anaerobic cells are from physiologically healthy cells.

C. Cell History

In section C of chapter II, we mentioned that accidental light intensity changes during growth affected the absorption spectrum and the P - S decline. When growth light intensity was intentionally varied, the effects were large. In a special experiment, an intensity during

growth of twice the normal was obtained from 6 cool-white 15 watt fluorescent lamps. The normal intensity we call "low", and twice the normal, "high," despite the fact that the high intensity was only half the saturation intensity of photosynthesis i.e., the transition intensity (defined on page 47). The absorption spectra of high and low light cells were different. In the low light culture, the 680 nm to 650 nm absorbance ratio was 1.4, while in the high light culture, the ratio was 1.8. Similar spectral differences resulting from changed intensity during growth have been reported (4, 34, 61); therefore, these are not surprising.

Figure 10 shows the transients obtained from the pair of high and low light cultures. Since we recorded the transients but did not measure absolute fluorescence yields, no comparison between amplitudes in the high and low light cases is possible, and the transients have been arbitrarily normalized at 0. However, we know that the high light cells should produce more fluorescence, since Bedell (unpublished) has observed that high compared to low light grown Chlorella show lower photosynthetic yield, and higher fluorescence yield by a factor of about 1.5.

It is apparent from figure 10 that the high light cells display a retarded P - S decline, and that the ratios, $\frac{P}{O}$ and $\frac{P}{I}$ are 1 1/2 times the normal.

The reaction causing the P - S decline seems to be both delayed and slower in the high light cells. Brody and Brody (4) and Ghosh and

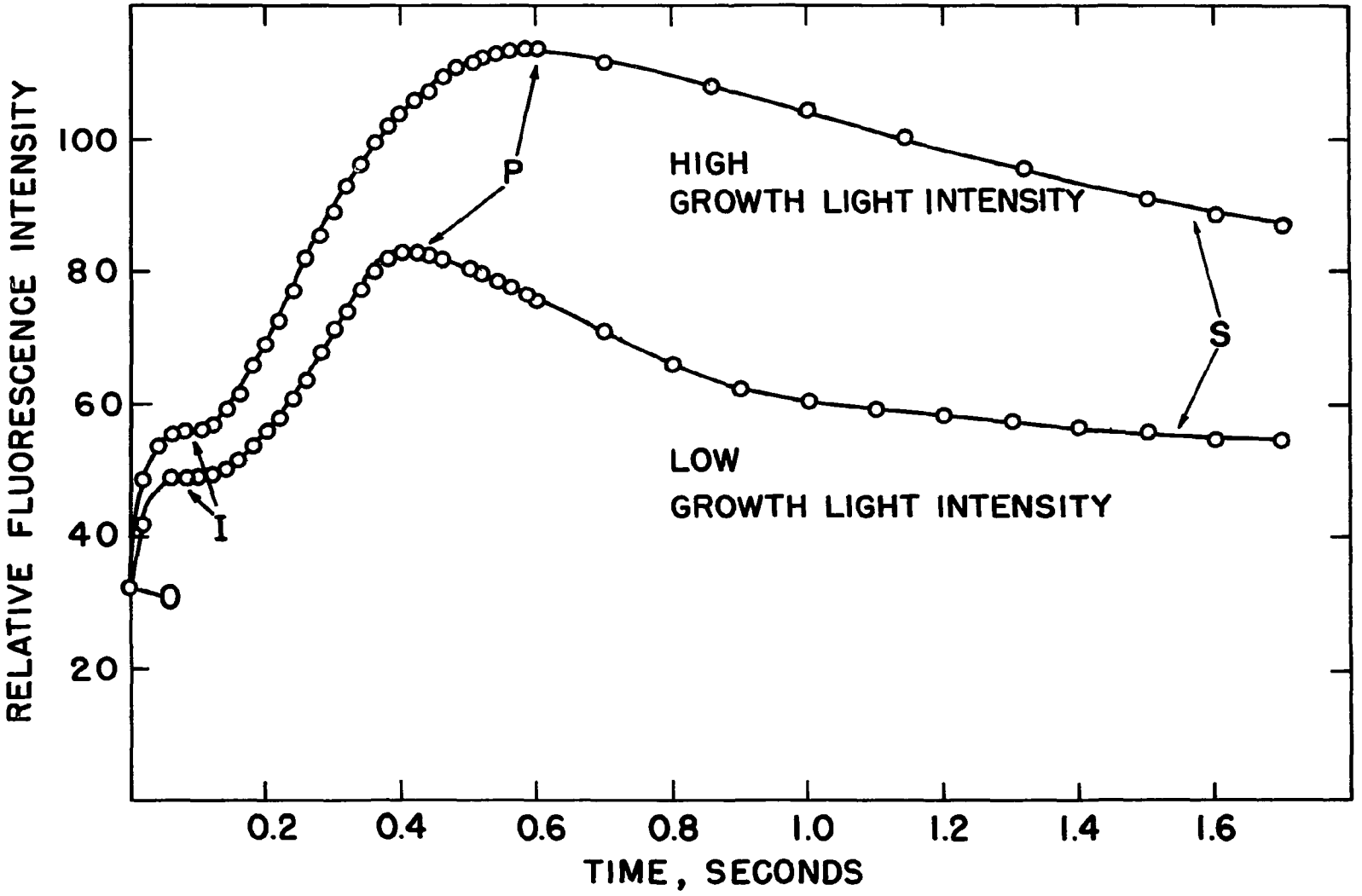


Figure 10. The fluorescence transients in "high"-light grown and "low"-light grown Chlorella pyrenoidosa. "High" intensity: 7500 ergs/sec-cm^2, from 15-watt fluorescent lamps. "Low" intensity: 3750 ergs/sec-cm^2, from a 40-watt tungsten lamp. The spectral quality of the "high" light was more blue than that of the "low" light. The transients were normalized at O.

Govindjee (34) found that variation in growth light intensity modifies efficiencies of energy transfer between photosynthetic pigments, and modifies the relative absorptions of systems I and II. These changes should modify the shape and timing of the transient. Because we felt that cells showing a retardation of the P - S decline had an "abnormal" history, we did not use them. (It might be interesting to deliberately prepare such cells and compare their behavior with respect to the effects of preillumination, and the addition of exogenous electron acceptors and poisons.)

IV. LIGHT INTENSITY AND THE FLUORESCENCE TRANSIENT

McAlister and Myers (74) were the first to notice that the steady-state fluorescence yield increases at high light intensities. Franck, French, and Puck (28) showed that the transition from low to high yields occurred in the intensity region in which photosynthesis becomes saturated. Subsequent research by Brugger (6) confirmed the finding of a yield increase at high intensity. Butler and Bishop (8) in bean leaves and in Scenedesmus, and Krey and Govindjee (61) in Porphyridium cruentum have shown that the increased fluorescence is excited by light absorbed in system II.

The fluorescence transient is also responsive to changes in light intensity. Low intensity produces a slow O - I phase but fails to evoke P. High intensity causes a rapid development of O - I and evokes a large P. Franck, French, and Puck (28) showed in Hydrangea leaves that P and the ratio P/S increased and that P was attained sooner as light intensity increased.

We decided that the light intensity required to produce P should be similar to the intensity required to produce the higher steady-state fluorescence yield. Our reasoning was that both phenomena represent excessive loss of light energy, and that their causes might be the same.

We have measured fluorescence intensity versus light intensity for the points of the transient: O, I, P, S at 1.5 seconds ($S_{1.5}$) and S

between 5 and 50 seconds (S_5). The results are shown in figure 11 (we have previously published a similar figure in a preliminary report; see ref. 39). Figure 11 presents an average of two experiments performed consecutively on one sample. (Figure 6 of chapter II, obtained from the same experiment, shows the dependence of t_p on light intensity.)

Franck et al.(28) showed that for intensities sufficiently beyond the transition intensity, the steady-state fluorescence intensity once again becomes linear with a steeper slope. It is evident that our curves do not achieve linearity for S_5. We purposefully avoided higher intensity while obtaining data for figure 11 because in several other experiments we noticed a decrease in P and S during a series of higher intensity exposures.

The O curve of figure 11 reveals that there is no increase of yield for O. This feature of O was first observed by Lavorel (67). We have discussed in chapter III that O may have contributions from both systems I and II. We also mentioned that the yield of system II fluorescence increases as light intensity increases. The constancy of the yield at O means that if O includes a contribution from system II, this contribution must be a special constant-yield component of system II fluorescence. Delosme (11) and Malkin (71) have assumed that all of the O fluorescence comes from system II, and that O represents the special condition that all Q is oxidized.

P becomes distinguishable from S_5 and $S_{1.5}$ as their yields begin to change. The increase of yield for P is greater than for I,

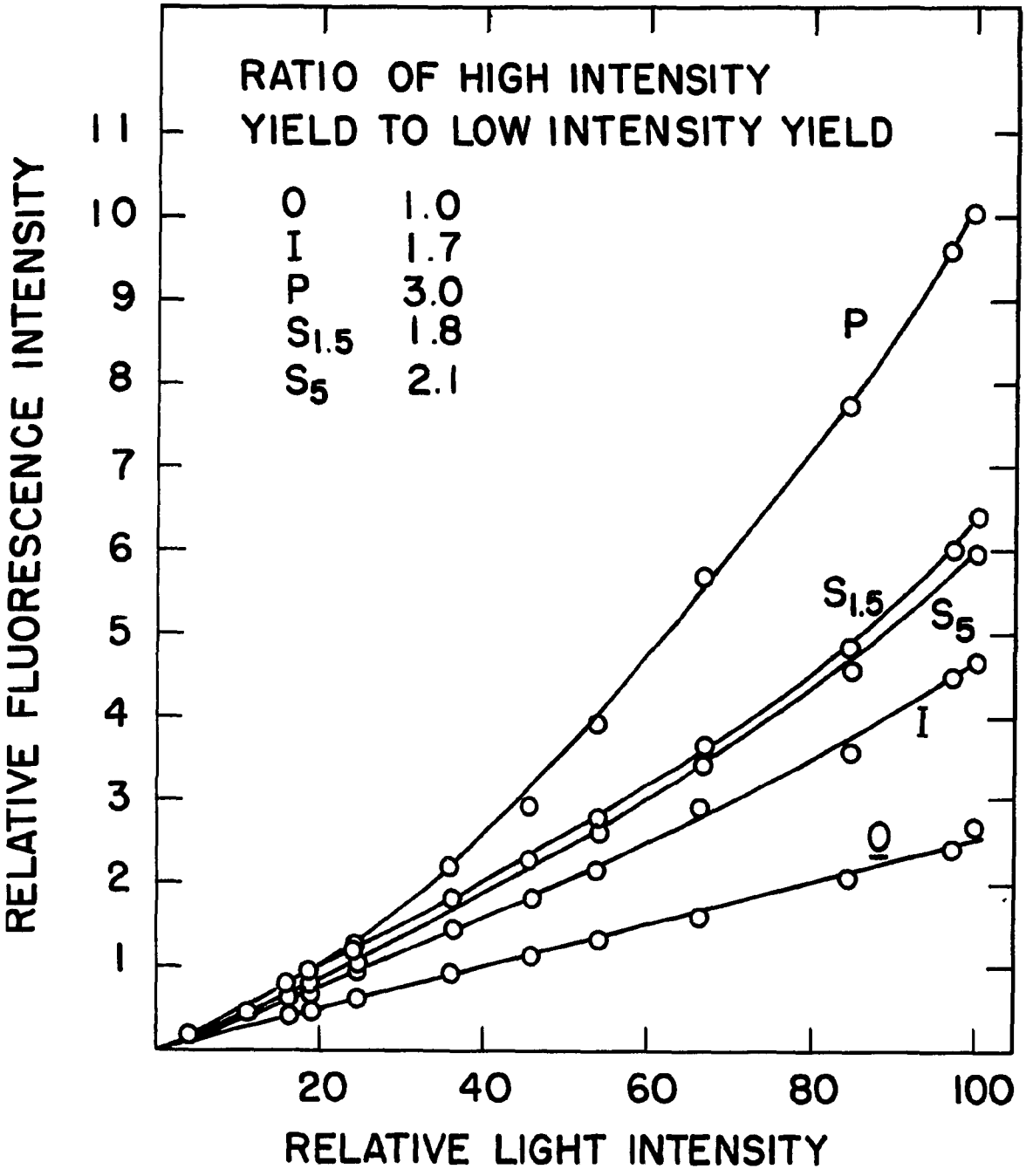


Figure 11. Fluorescence intensity versus exciting light intensity for different points of the transient. O, I, P, and $S_{1.5}$ for each intensity were determined in successive 2-second light exposures (with 4-minute dark intervals) at increasing then decreasing light intensity. S_5 (S between 5 and 30 seconds) was determined by rapid changes of intensity during one subsequent 30 second light exposure. The yield ratios were determined from the slopes at high and low exciting light intensity.

$S_{1.5}$, or S_5. The greater increase for P, because it was measured at 685 nm where system II emission predominates, is new evidence that the extra fluorescence at P is mainly from system II.

Relative fluorescence yields for the phases at both low and high intensities were determined from the slopes of the curves. The ratios of the high intensity yields to the low intensity yields are, for O -- 1.0, for I -- 1.7, for $S_{1.5}$ -- 1.8, for S_5 -- 2.1, and for P -- 3.0.

Several features of these curves and the yield ratio for P impose restrictions on the Franck and Rosenberg (29) model of photosynthesis. This model has been widely discussed as an alternative to the series scheme. Its main details are as follows: the two photochemical reactions of photosynthesis alternate at a single reaction center, rather than taking place at separate centers arranged in series. One reaction utilizes chlorophyll singlet-state energy to reduce a cytochrome (similar to the system II reaction in the series scheme), while the other reaction utilizes chlorophyll triplet-state energy to oxidize cytochrome (similar to system I). The normally observed fluorescence is in competition with triplet state formation, which must precede the triplet reaction. The singlet reaction, however, is so fast it allows no fluorescence. During optimal photosynthesis, half the energy proceeds toward the triplet reaction, with a chance for fluorescence. If some substance required by either reaction becomes lacking, then cytochrome turnover ceases, and energy can no longer be utilized. All energy rather than half then becomes subject to triplet formation and fluorescence.

In this event, fluorescence must double.

Franck and Rosenberg said that the doubling would occur in three cases: 1) algae deprived of oxygen and carbon dioxide, 2) algae incubated with cyanide, and 3) leaves exhibiting the fluorescence transient, where the peak fluorescence is to be compared to the steady-state fluorescence. In this case, the doubling occurs because the reservoir of oxidants is exhausted at the peak. The reservoir is replenished by the Calvin cycle as the steady-state is approached. Franck and Rosenberg pointed out that the test of fluorescence doubling during the transient cannot be applied to algae because Calvin cycle intermediates are too quickly restored to be exhausted at the fluorescence peak. This implies that in algae the ratio of P to S will be less than two. Such is observed: P/S is normally 1.5. However, the point is not convincing, because S is not the level of fluorescence corresponding to optimal use of excitation energy. The level corresponding to optimal use is O, and the ratio of P to O is 4.0 for light intensities not much above saturation of oxygen evolution. The ratio of P in the anaerobic condition to O in the aerobic condition is 6.0. In addition, from figure 11, the yield increase for P as light intensity increases is by a factor of 3.0. In all instances the fluorescence increase is significantly greater than the predicted doubling.

Franck and Rosenberg recognized that fluorescence might more than double if high intensity caused photo-oxidation of chlorophyll with molecular oxygen. They presumed that this photo-oxidation would

become more probable at intensities above saturation. If the model is correct, our results require that such photo-oxidation become dominant above saturation.

If photo-oxidation is dominant, then removal of oxygen at the high intensity should result in a decrease of fluorescence. Figure 9 on page 64 shows that removal of oxygen at the high intensity instead results in a 50% increase of fluorescence at P.

In addition, the fluorescence yield at high light intensity is constant. Krey and Govindjee (61) made careful measurements in Porphyridium and found that the yield was constant for light intensities twice the saturation intensity. If photo-oxidation were involved at these intensities, the yield should be constantly increasing with light intensity.

These observations require that the model be modified with new and appropriate assumptions. If such assumptions could easily be found, and would explain both our observations and a variety of others, we would feel encouraged to regard with equal favor the Franck and Rosenberg model and the series scheme.

V. THE EFFECT OF PREILLUMINATION ON THE TRANSIENT

Our preliminary report of the effect of system I preillumination (henceforth called preillumination 1) was that it delays and decreases I and P, and increases $S_{1.5}$ (39). We expected further study to show that system II preillumination (preillumination 2) would have different effects, because of the antagonistic effects of system I and system II light on steady-state fluorescence yield (7, 18, 19, 38).

Differences in effects of preilluminations 1 and 2 have been found.

These differences are general support for the series scheme. But the effects are not consistent with the widely held view that the high QH level at P is due directly to reduction of A, an intermediate between the two systems. We feel that the preillumination effects suggest a different view of P, that it is due not only to reduction of A and Q but also of X, the hypothetical electron acceptor for system I.

A. The Aerobic Case

The complete study of the effect of preillumination encompassed eight preillumination wavelengths between 650 nm and 725 nm. In Chlorella, this wavelength region includes absorption peaks for both systems. Because of the way in which the absorption spectra for the two systems overlap and cross each other, a predominance of system II effects can be expected only for wavelengths equal to or shorter than 680 nm, and a predominance of system I effects can be expected only

for wavelengths equal to or longer than 690 nm (9). By 720 nm the effect of system II is negligible and clear system I effects can be observed (72).

1. The Effect of Preillumination as a Function of Intensity

We discovered that the effect of preillumination depended not only on wavelength but also on intensity. This discovery necessitated, for every preillumination wavelength, a study of effect as a function of intensity. The time required to study each wavelength was more than 6 hours. In 6 hours, a long-term decline of P and S of the transient became quite noticeable, and to carry on experiments for longer times would have risked the possibility that this decline would affect results. To avoid this risk, each experiment was terminated as soon as study of one preillumination wavelength was complete.

Consequently, it was impossible to study the intensity effects of all the preillumination wavelengths on one sample. We could at best study several wavelengths on a single culture, using different samples obtained on successive days. Slight changes with time in a single culture were inevitable. More than one culture, moreover, was used to obtain the results. The results, however, are reliable since we performed 25 preliminary experiments at various wavelengths, and 3 careful experiments at each of the 8 wavelengths (except for one only at 725 nm).

From the data for each wavelength we have constructed a graph of the effects versus intensity. In order to make possible the quantitative comparison of the effects at the different wavelengths, we have

expressed the intensity of preillumination in terms of absorbed quanta per $sec\text{-}cm^2$. The scales of the axes of abscissas have been adjusted so that similar features of different graphs cover the same distance. The effects themselves are expressed as percentage changes in I (determined at 50 msec), P, $S_{1.5}$ (S determined at 1.5 seconds), and percentage changes in the variable t_p (the time of the peak).

The presentation of results in this section (1) has two parts. The first is an examination of results at each wavelength of preillumination. For each wavelength there is a figure showing results from a single experiment. The second part is a discussion where results are generalized for each point of the transient, and hypotheses are woven into a coherent picture.

a. The Effects of Preillumination at Separate Wavelengths

The effects of preillumination at 725 nm are shown in figure 12. Since at 725 nm, absorption by system II is negligible (72), the results for 725 nm show the effect of preillumination absorbed only by system I. The effect on P is a decrease, the curve having two segments: a steeply sloping segment at very low intensity and a gently sloping segment over a wide range of higher intensities. The effect on S is an increase, the curve also having two segments: an incline at low intensity and a horizontal segment at higher intensities. For intensities along the horizontal segment, the S effect is saturated. The effect on I is a decrease, and the curve has two segments, one of which represents saturation. The time of the peak, t_p, is increased, and again the curve

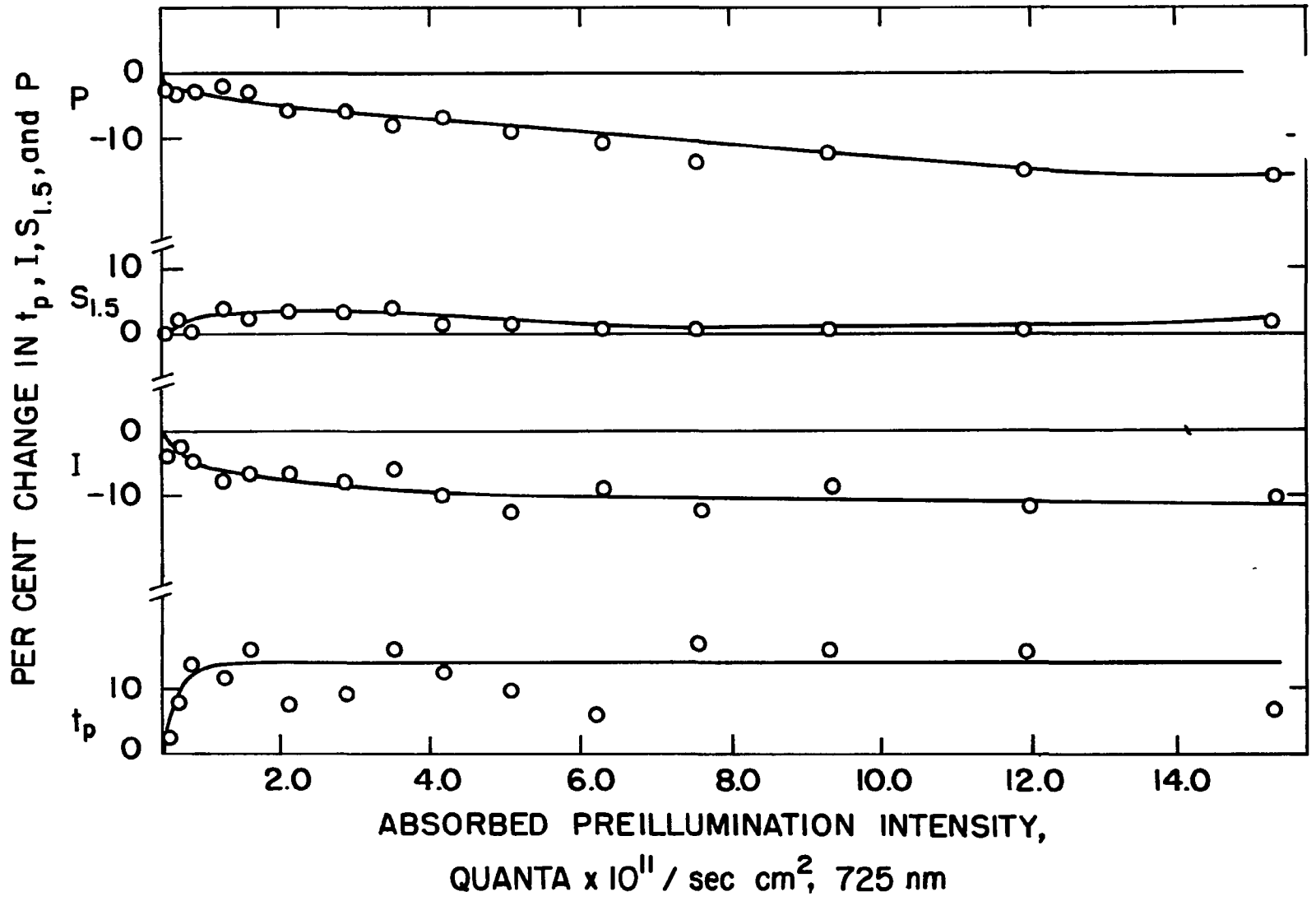


Figure 12. The effects on points of the transient caused by different intensities of preillumination at 725 nm. The preillumination replaced the usual four-minute dark period. The effects are expressed as percentage changes in P, S at 1.5 seconds, I at 50 msec, and t_p. A straight line for t_p at the higher intensities is indicated by other experiments, although noise obscures this feature in this experiment.

has two segments, one of which represents saturation. The saturation intensities are similar and roughly equal to 5×10^{10} quanta absorbed per sec-cm^2. This intensity is roughly 0.1% of the transition intensity in the curve of fluorescence yield versus light intensity.

At first glance, the results seem compatible with the widely held view that P is due directly to reduction of A, an intermediate between the systems. According to this view, preillumination 1 should oxidize intermediates between the systems and thus insure a higher than normal ratio of A to AH. The higher level of A should delay and decrease I and P. This prediction is confirmed by the results.

However, the failure of the P decrease to saturate when I and t_p effects saturate is a complication. It appears that preillumination 1 has two effects on P, one of which is independent of the I and t_p effects.

Another complication is indicated by the increase in S. This increase is quite unexpected from the view that P is due to reduction of A. preillumination 1 should raise the ratio of A to AH, and the higher A concentration should permit a lower S level. In our preliminary report (39) we suggested that the increase in S might be explained by a back reaction of A with reduced X (X is the oxidant of system I). Such a reaction would decrease the amount of A available to accept electrons from QH, with the result that the level of QH and fluorescence would be higher than otherwise. We must presume, since S is increased by preillumination 1, that preillumination 1 increases the rate of the back reaction. This is plausible because preillumination 1 has increased the

concentrations of reduced X and subsequent intermediates, and reactions are faster when the reactants have higher concentrations.

A similar view of the S increase is that it reflects a "traffic jam" of electrons at X, preventing P_{700}, AH, and therefore QH from being oxidized. If either of these views is correct, then short wavelengths should have the same effect as 725 nm, because the short wavelengths also are absorbed by system I and drive electrons to X.

The effects of preillumination at 710 nm are shown in figure 13. The effects are similar to those for 725 nm preillumination. P and I are decreased, and S and t_p are increased. All of the curves have two parts, one of which represents saturation. The intensities at saturation are approximately 2×10^{10} quanta absorbed per sec-cm.^{2} The P decrease does not saturate completely.

The effects of 700 nm preillumination are shown in figure 14. The effects are qualitatively similar to those for 710 nm and 725 nm; however, the saturation intensity is greater, being 8×10^{10} quanta absorbed per sec-cm^2.

The effects of 690 nm preillumination are shown in figure 15. The saturation intensity is (roughly) the same as for 700 nm. Otherwise similar, the effects show a new feature, in that the increase of t_p diminishes at the higher preillumination intensities. The highest intensity on this figure is off scale for the previous figures.

The effects of 680 nm preillumination are shown in figure 16. The saturation intensity is (roughly) 10×10^{10} quanta absorbed per

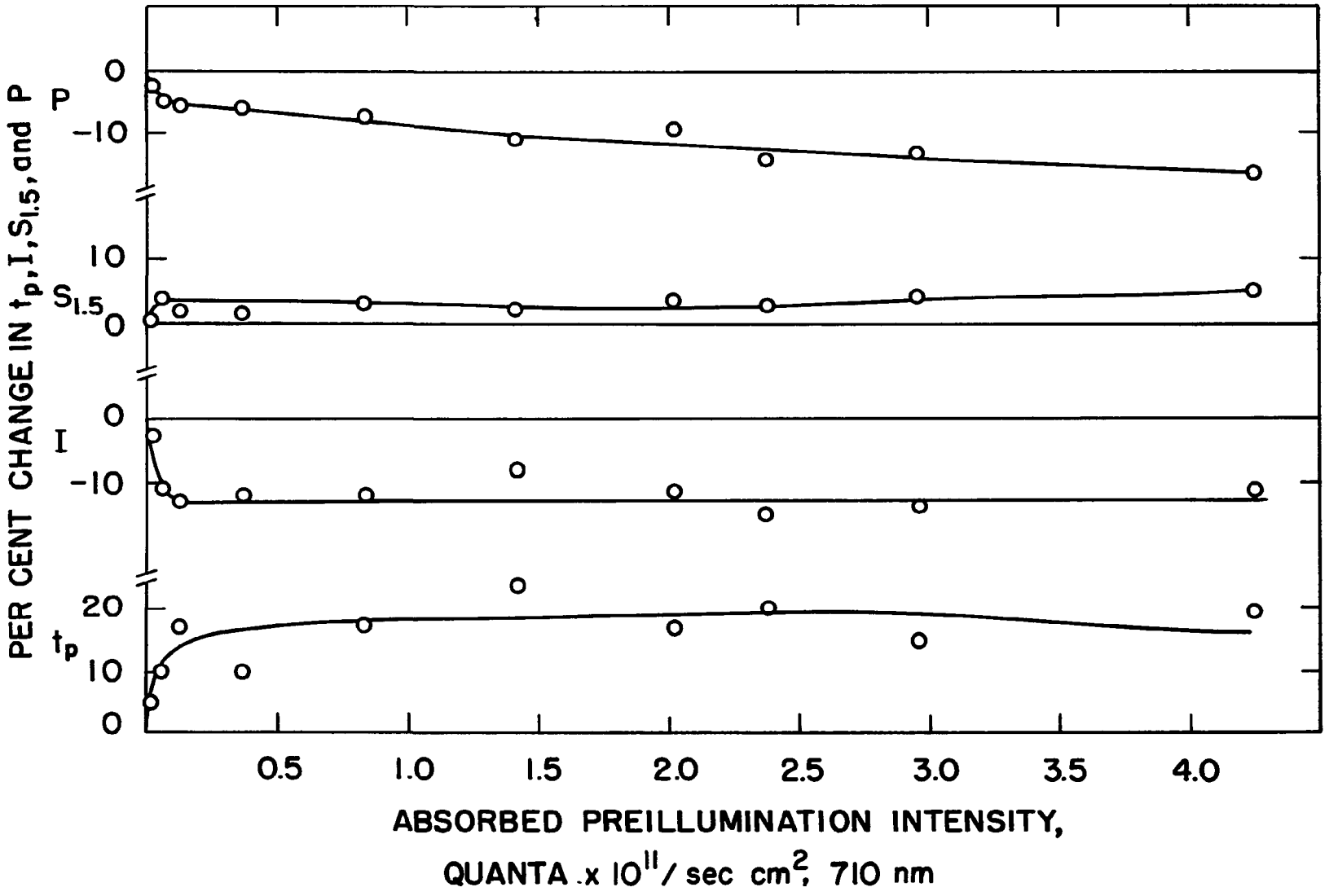


Figure 13. The effect of preillumination at 710 nm; see legend of fig. 12.

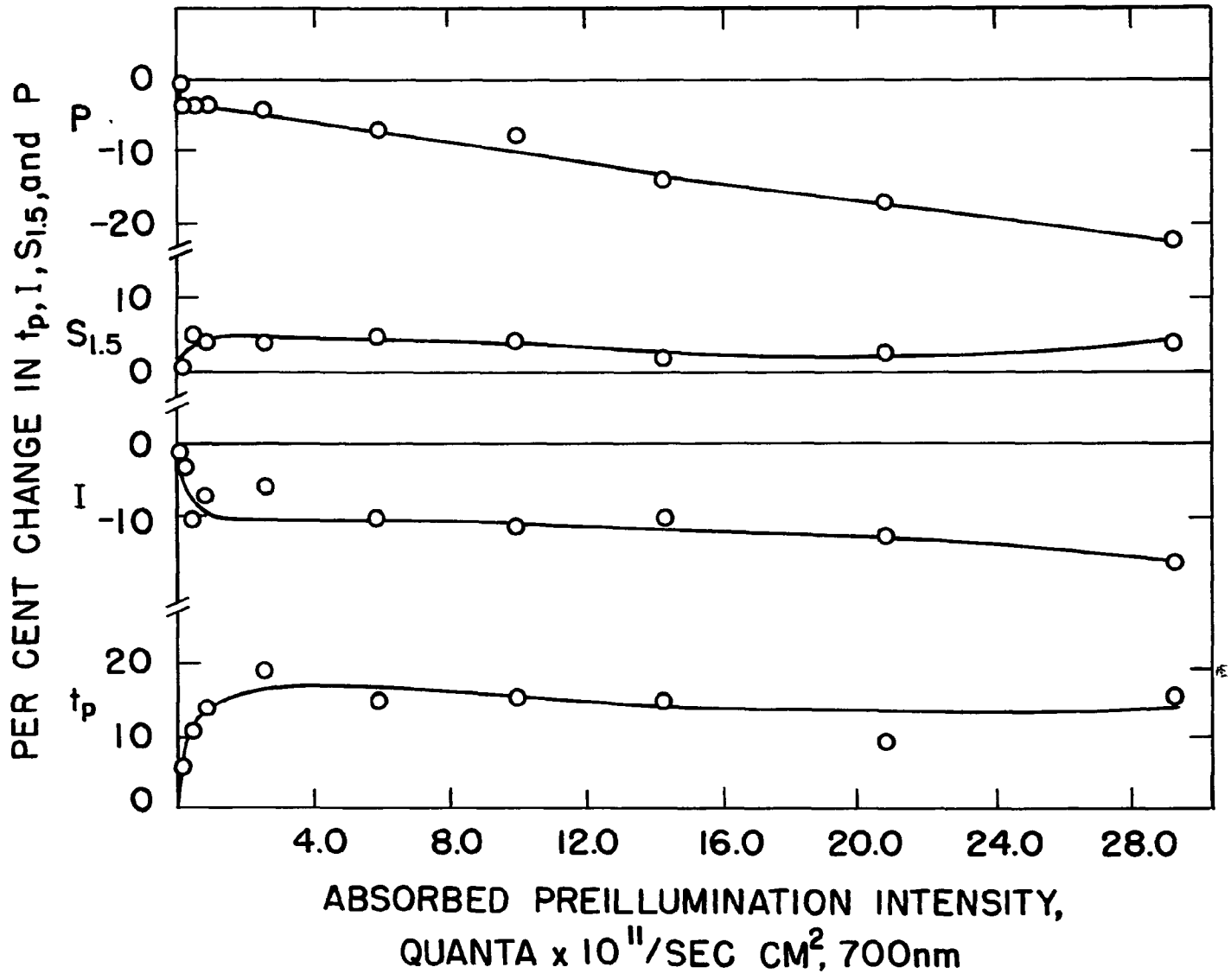


Figure 14. The effect of preillumination at 700 nm; see legend of fig. 12.

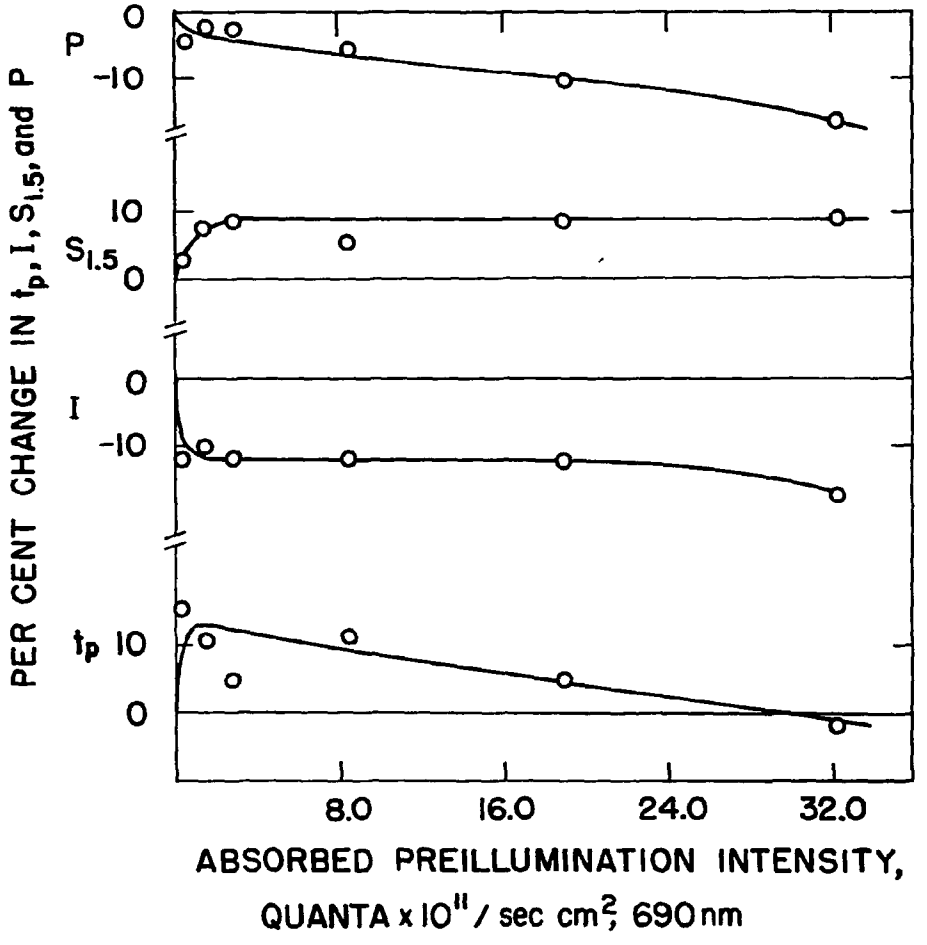


Figure 15. The effect of preillumination at 690 nm; see legend of fig. 12. The percent increase of t_p diminishes at higher preillumination intensity.

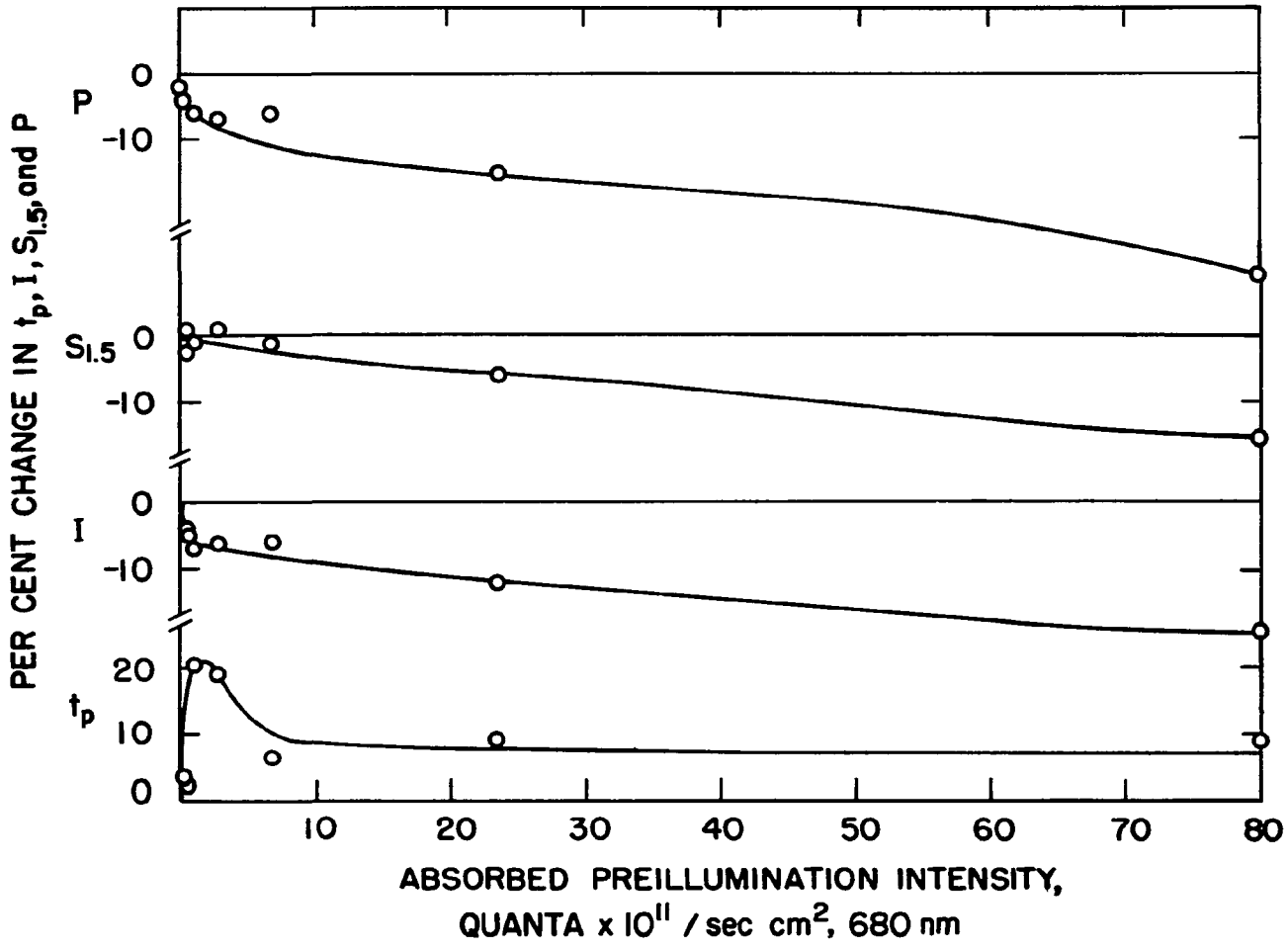


Figure 16. The effect of preillumination at 680 nm; see legend of fig. 12. At this wavelength, S decreases; see text for discussion of t_p.

sec-cm^2. This value establishes that there is a trend toward higher saturation intensities as the wavelength changes from 710 nm to 680 nm (we exclude the value for 725 nm, where absorption is low and hard to measure). The trend signifies that the shorter wavelengths are less effective than the longer wavelengths. As with 690 nm, the increase of t_p for 680 nm diminishes at higher intensities, without the effects on I or P becoming smaller. The t_p curve does not cross the zero line as it did for 690 nm, but we conclude from other experiments not shown here that in some cultures, the t_p curve crosses the zero line for wavelengths below 690 nm, while for other cultures, the crossing occurs only for wavelengths below 680 nm. The experiments shown here for 680 nm and 690 nm were performed on different cultures.

A new feature at 680 nm is that S is decreased rather than increased. This feature does not fit into the explanations offered for the S increase at longer wavelengths, a back reaction and/or a "traffic jam". 680 nm light is absorbed by system I as well as by system II, and therefore drives electrons to X. Consequently, S should have increased rather than decreased. The situation will be discussed more fully later.

The effects of 670 nm preillumination are shown in figure 17. t_p is increased at low intensity, but decreased at high intensity. S is at all intensities decreased except for an infrequently observed increase at the lowest intensities. The saturation intensity is again (roughly) 10×10^{10} quanta per sec-cm^2.

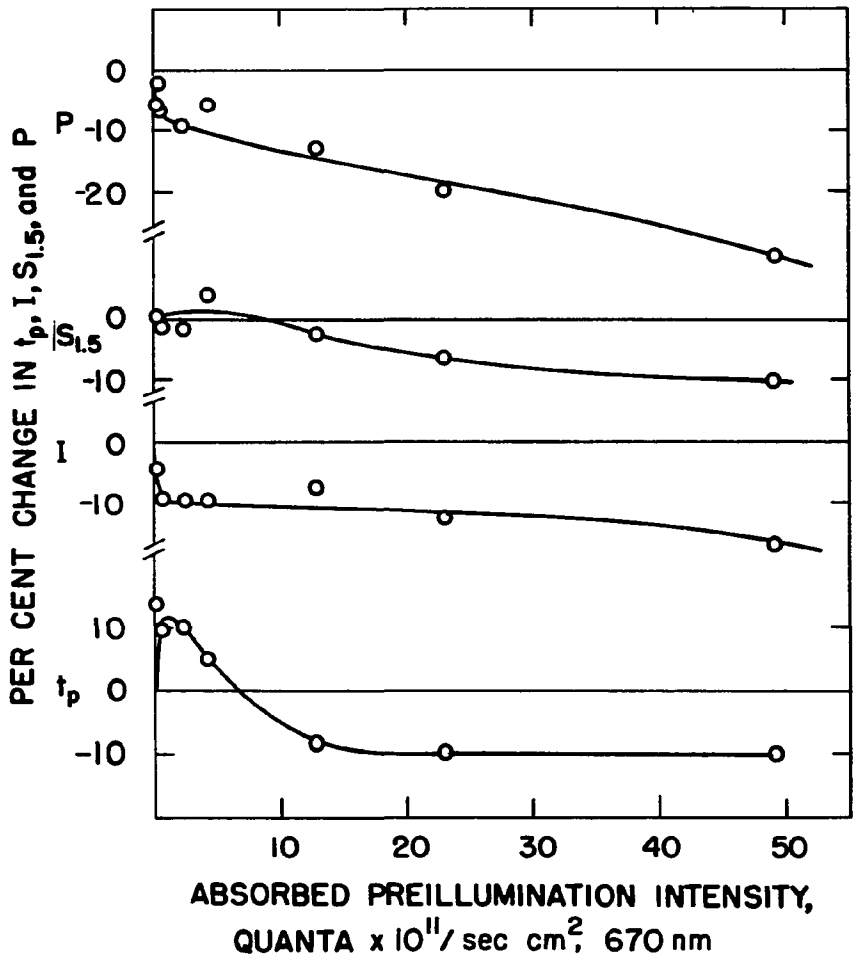


Figure 17. The effect of preillumination at 670 nm, see legend of fig. 12. The change of t_p is negative at higher intensity.

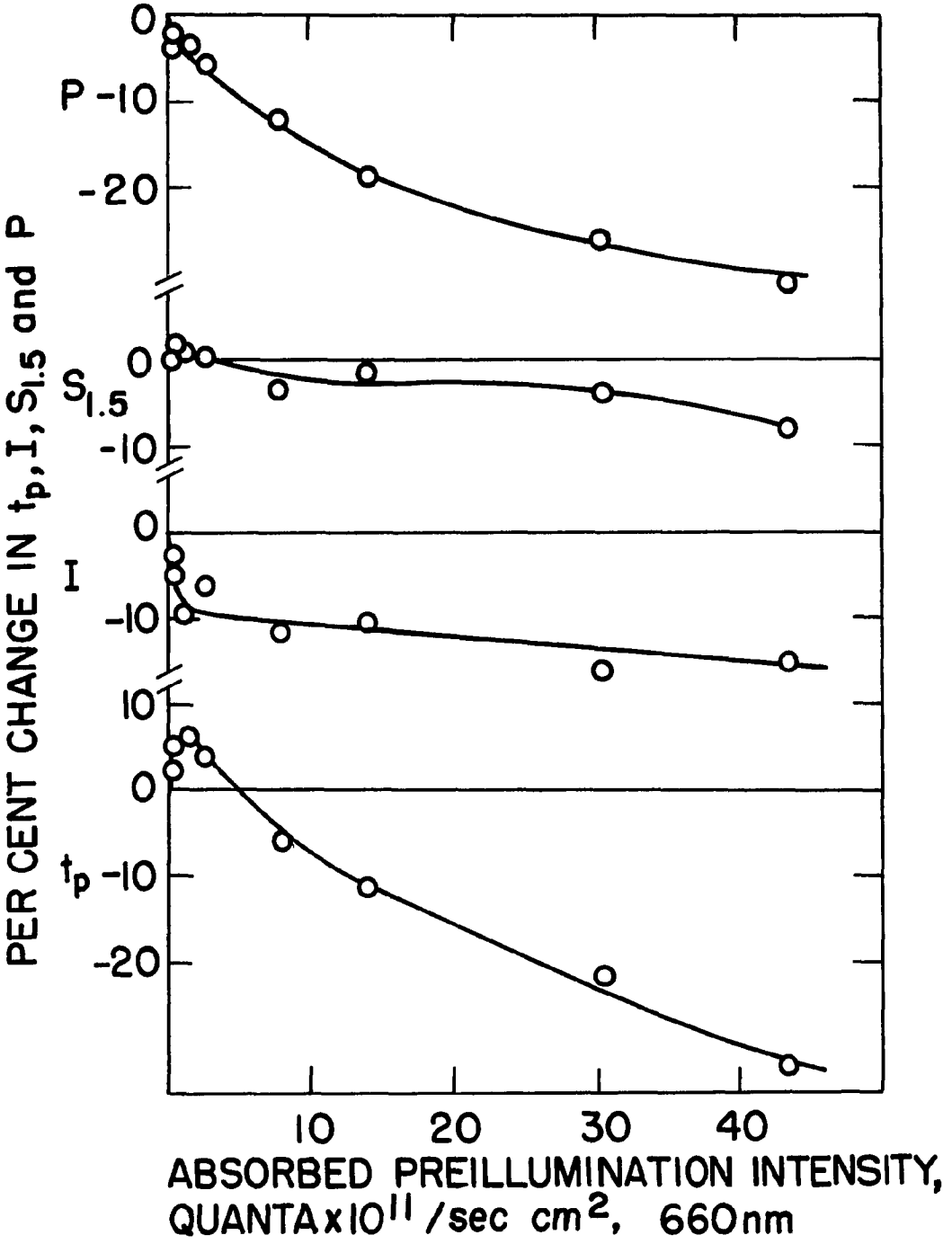


Figure 18. The effect of preillumination at 660 nm; see legend of fig. 12.

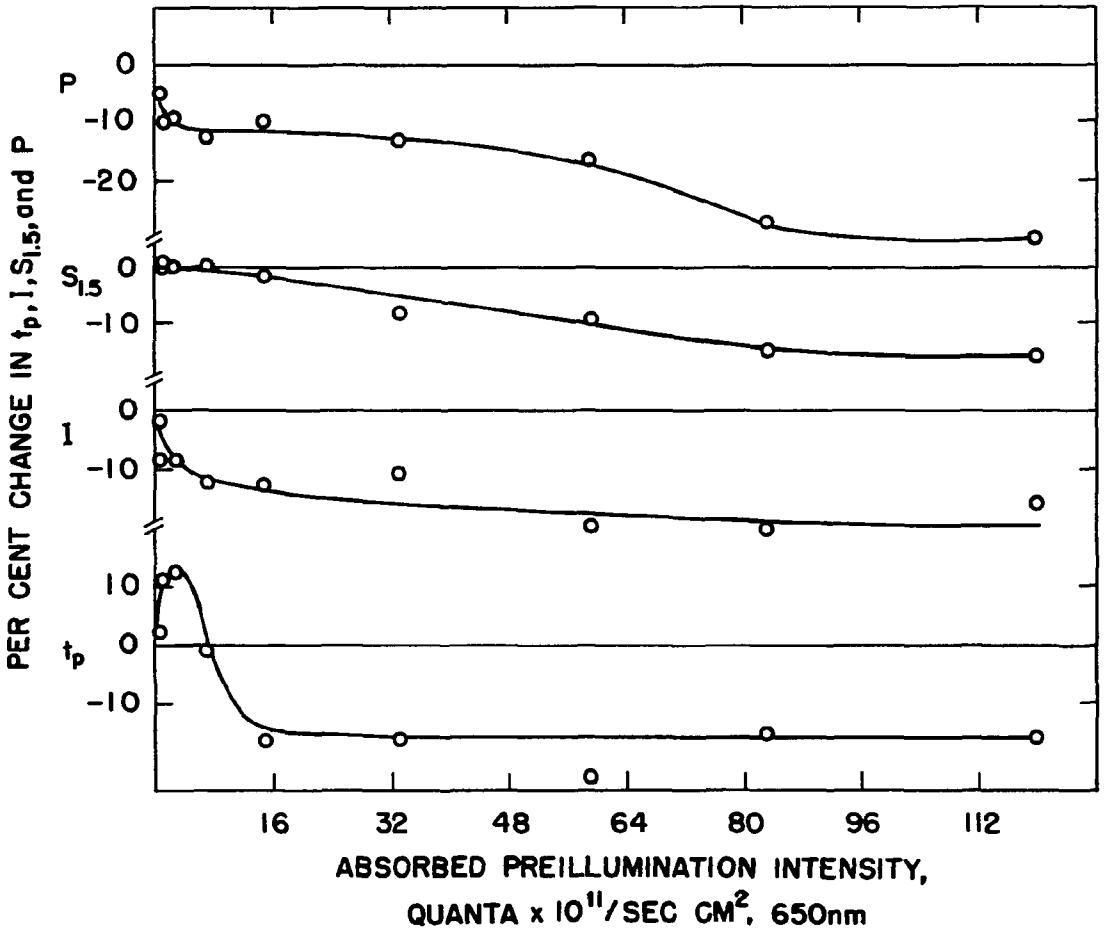


Figure 19. The effect of preillumination at 650 nm; see legend of fig. 12.

At 660 nm, in figure 18, the effects are similar. At 650 nm, in figure 19, they are again similar except that the decrease in S is larger.

b. Comparison of the Results

Comparing all of the results, we arrive at several generalizations. We shall analyze their significance in terms of each point of the transient. We remark, at the outset of this discussion, that the complicated effects of preillumination add to the complexity of the fluorescence transient in Chlorella. The effects are not easily explained. What we have been able to do, however, is briefly discuss alternative explanations which are in accord with the accepted features of the series scheme.

i) The Point O

O is not affected by preillumination (except at extremely high intensity, where it is increased). We have not included O on the graphs because of its constant behavior. At O, all Q is oxidized.

ii) The Point I

I is decreased by preillumination 1. Because preillumination 1 oxidizes AH to A, I must be affected by the concentration of A. The decrease in I may be understood as follows: After preillumination 1 has oxidized AH to A, QH can be oxidized by A more readily. When the exciting light is turned on, and photoreduction of Q to QH begins, the faster oxidation of QH keeps the level of QH below normal.

Consequently, the fluorescence level at I is also below normal. At the intensity which saturates the I decrease, it is likely that AH has been completely oxidized by the preillumination.

The level I is decreased not only by preillumination 1, but also by preillumination 2. This result is unexpected because system II reduces Q and A. We feel that the quanta reaching system II when the preillumination intensity is low are probably wasted, and that the decrease in I is due to quanta of preillumination 2 which reach system I.

Intensities which saturate the preillumination effects are higher for preillumination 2 than for preillumination 1, yet the saturating effects for both preilluminations are similar. Again, the quanta reaching system II are probably being wasted.

Some other workers have decided that system II may waste dim light. Murata, Nishimura, and Takamiya (77) have studied the steady-state fluorescence yield of isolated chloroplasts at very low exciting light intensities. (The intensities are the same as used in our preillumination experiments, and are 0.5% of the transition intensity in the fluorescence yield curve, the intensity which saturates photosynthesis. See pages 47 and 69). It was first observed by Brugger (6) that at the very low light intensities there is a second transition intensity, indicated by a second change in slope of the fluorescence intensity versus light intensity curve. Murata et al. have studied this second transition intensity, and have decided that it can be explained in terms of a back reaction between QH and an intermediate on the water-side of system II.

The transition intensity is the intensity which saturates the back reaction, and thus a small number of quanta per second are involved, compared to the number per second required to saturate photosynthesis. Such a back reaction would be an obvious waste of quanta absorbed by system II.

Joliot (47) believes that the change in slope can be explained in terms of the deactivation of QH to Q_iH. Such a deactivation would also be a waste of quanta absorbed by system II. In either case, a deactivation or a back reaction, absorption in system II at very low light intensities would be ineffective in causing a reduction of Q and A.

iii) The Point D

Following I in a normal curve is the dip, D. It (presumably) indicates an oxidation of QH. Kautsky, Appel, and Amann (50) showed that the dip in anaerobic Chlorella would not develop in the dark, in other words, that its development required light. We supposed, in accord with the series scheme, that the required light is utilized by system I to oxidize AH to A. (We have tested this idea experimentally; see chapter VI.) The newly-oxidized A causes the dip by oxidizing QH.

We shall presume on this basis that the O - I - D phase reflects development of a dynamic balance between systems II and I concerning their effect on Q and A. System II is reducing Q, and system I is supplying oxidized A for the oxidation of QH. Because AH is already being oxidized by the time of the dip, we disagree with the view that P, which follows the dip, is due only to reduction of A and Q. P more

likely involves, in addition to A and Q, a different intermediate, which is reduced in the system I reaction. (We have tested this idea experimentally; see chapter VI.) We presume arbitrarily that it is X, the primary electron acceptor for system I. Reduction of X brings to a halt the oxidation of AH by system I. With regeneration of A from AH halted, the remaining A is quickly reduced in oxidizing QH. When nearly all A is reduced, reduction of Q proceeds unopposed and fluorescence rises to the peak, P.

iv) The Point P

P increases as the dark period increases, while there is little change in I (see figure 7, page 52). The difference is more evidence that, if I involves A, P may involve another intermediate as well.

There are several possible reasons why P increases as the dark period increases. One possible reason is that dark reactions deplete intermediates in the carbon cycle and the severity of the depletion depends on the duration of the dark period. We speculate that the following may be true: the concentration of a dark-depleted intermediate may be dependent via feedback on the concentration of subsequent intermediates in the cycle. Upon illumination, this depleted intermediate causes a bottleneck in the reaction sequence. A "traffic jam" of electrons occurs, halting oxidation of XH, and allowing the peak, P, of the transient. After a second of illumination, the concentrations of subsequent intermediates increase and raise the

concentration of the depleted intermediate, thereby removing the bottle-neck.

We have emphasized that some responses of P are different from those of I, and that whereas I involves the intermediate A, P involves X as well. Nevertheless, some responses of P and I are similar. Pre-illumination decreases them both, and the saturation intensities are the same. We can explain these similar responses in terms of the relation between A and X. We suggest that the decrease in P in the first segment of the P versus preillumination intensity curve (figures 12 through 19) is due to the effect of preillumination in oxidizing AH. The preillumination transfers electrons from AH to X. Because the preillumination is weak, the rate of transfer to X is slow, and dark regeneration reactions easily remove electrons from XH without being overwhelmed. Meanwhile, AH is oxidized. During the next exciting light exposure, extra A must be reduced before the reduction of X can become substantial. The resulting delay in reduction of X is accompanied by a prolongation and depression of P.

The second segment of the P decrease (figures 12 through 19) should be explained without mention of A, because I, which is responsive to changes in A, is not further affected by the higher preillumination intensities. We believe that the key to the explanation of this second decrease is the following: the decrease grows with intensity until P has disappeared. Then, only a long dark rest will restore P. The decrease thus behaves as the converse of the restoration of P in a dark

period. We believe that strong preillumination simply prevents the dark restoration.

A speculative view of the interesting behavior of P is that there may be energy dissipating forms of X or Q which do not participate in serial electron transfer. Duysens and Sweers (19), in an analysis of the S level in Porphyridium, suggested that excess energy may be dissipated in a fluorescence quenching non-photoactive form (Q') of Q. Q' was thought to be formed from QH by a dark reaction, and converted to Q by a slow dark reaction. We have in this idea the concept of a "safety valve." If such forms of Q or X exist, and revert by slow dark reactions to the normal forms, then we may understand P in the following way. We shall explain the possibilities in terms only of Q and its different forms, for the sake of convenience. After a long dark period, all Q' has been converted to Q and illumination may reduce a great deal of Q to QH, resulting in a high P. As Q' is formed from QH, excitation energy may once again be quenched, and fluorescence declines from P. If the dark period is short rather than long, Q' is still abundant, and illumination cannot cause as much reduction of Q to QH. P consequently is low. In the case of intense preillumination, Q' is again still abundant, and P again is low.

The difficulty with these explanations is that fluorescence and oxygen production are in general assumed to be complementary in the phase P - S, whereas if a form Q' quenches fluorescence at S but does not perform chemistry, the relationship between fluorescence and oxygen

production should be non-complementary.

v) The Variable t_p

An increase in t_p occurs for all low intensity preillumination. This increase, we have suggested (in part iv), results from the effect of oxidation of AH on the rapidity of exhaustion of X.

In the case of preillumination 2, t_p increases at low intensity, but decreases at high intensity. This decrease does not represent a reduction of A, because I is still decreased at the high intensity, indicating that AH has been oxidized.

In experiments to be described in chapter VI, P gradually disappeared during a sequence of light exposures after addition of an exogenous electron acceptor; t_p in this sequence decreased slightly as P disappeared. These experiments, where a t_p decrease corresponded with disappearance of P, suggests that the decrease in t_p caused by preillumination may be simply an obligatory coincidence with the near elimination of P.

Some evidence seems to conflict with this view: high intensity preillumination 1 nearly eliminates P, but does not decrease t_p. However, we have not tested the effect of intensities of preillumination 1 high enough to completely eliminate P. Perhaps intensities of this range will cause a t_p decrease.

vi) The Point S

Fluorescence is higher at S than at O, but not maximal, therefore Q and A are moderately but not completely reduced. In a subsequent dark period, QH and AH become oxidized, because upon

turning on the light, fluorescence is low. It is plausible to expect that a sufficiently long dark period will allow oxidation of QH and AH to be complete. This is probably the case with QH, but it is not so with AH: decrease of I by preillumination indicates that AH is not completely oxidized during the usual four-minute dark period. Even ten to thirty minutes of dark fail to decrease I, although it is decreased by preillumination. We conclude that A in the steady-state is kept moderately reduced, and that AH is oxidized slowly during a dark period following light exposure, but that oxidation is not complete even after thirty minutes. Perhaps the oxidation cannot reach completion because of a slow return of electrons to A, from reduced substances such as XH at a more negative redox potential.

Preillumination 1 increases S, while preillumination 2 decreases it. We have already pointed out (in section 1) that if changes in the concentration of A caused the S changes, then they would be in directions opposite to those observed. Therefore, changes in A are not the cause. We have also pointed out that a back reaction of reduced X with A, or a traffic jam at X and beyond, can explain the preillumination 1 effect, but not the preillumination 2 effect.

It is possible that the effects of preilluminations 1 and 2 are not the result of antagonism of systems I and II, even though the series scheme suggests antagonism. In keeping with this possibility, we suggest the following point of view. It is based on an observation made in the course of long experiments, in which P and S increase in

the first half-hour, but decrease in the second or third hour and afterward. The observation is that the decreases are accelerated by very intense preillumination 2. The preillumination-induced decreases of P and S are similar to the light-induced decrease during the "second wave" of fluorescence in continuous light in Chlorella (see Papageorgiou, ref. 80). Most likely the two types of long-term changes have the same cause. Papageorgiou believes the long-term changes are due partly to light-induced alterations in the spatial relations of pigment molecules. We suggest that the decrease in S after preillumination 2 is caused by these light-induced alterations.

It may be asked why preillumination 1 does not also cause an S decrease. One answer is that, according to Papageorgiou, the light-induced alterations require in Chlorella the operation of system II. Another answer is that the preillumination 1 intensities used in our investigations, being always lower than intensities of preillumination 2, were too small to cause the decrease.

Preillumination 1 instead causes an increase. We may explain this effect as before, by a back reaction or a traffic jam of electrons at X and beyond. It is also possible that the system I - induced increase in S simply reverses spatial alterations caused by system II (this is, once again, a view involving antagonism between the two systems).

In the preillumination results presented above, the effect of preillumination 1 on S was small. In experiments performed six to twelve months earlier, we found a much larger effect on S. The

larger effect might have been caused by cultural differences in the Chlorella cells, but we have not been able to "pin down" a reason for cultural differences. (However, some may have been caused by a change in growth conditions resulting from a move of the laboratory and a change in the assistant who grows the Chlorella.) The earlier experiments were performed with cells in growth medium instead of buffer, but recent experiments showed that preillumination results in buffer and growth medium are identical. The most plausible cause of the large S increase is that in the earlier experiments, the duration of each exciting light exposure was 30 seconds rather than 2 seconds. At our exciting light intensities, S declines between 1.0 and 30 seconds. Preillumination 1 may have had a larger effect on S simply because S was lower after 30 seconds than after 2 seconds.

Along with larger S effects, there are other interesting differences in the older results. Figure 20 shows the effect of 690 nm preillumination, which excited mainly system I. The effect of P is a surprising increase at low intensity, and the usual decrease at high intensity. The S increase is 10% rather than 5%. The I decrease is 5% rather than 10%. The t_p change is gradual rather than sudden and easily saturated.

Figure 21 shows the effect on the same culture of 640 nm preillumination, which excites mainly system II. The P increase at low intensity is smaller. There is a small S increase at low intensity, and the usual preillumination 2 decrease at higher intensity. The I decrease is small. The t_p increase is smaller than for 690 nm; however,

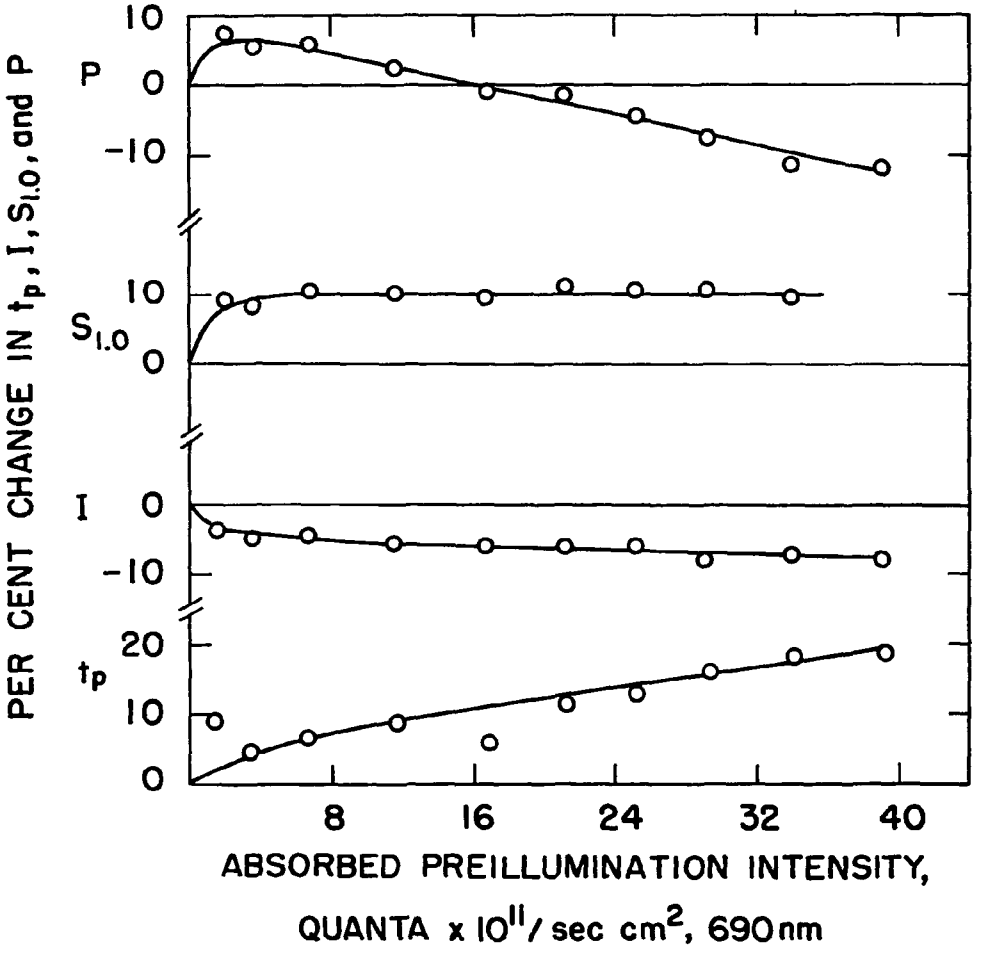


Figure 20. The effect of preillumination at 690 nm in a Chlorella culture where the S increase is large, and P increases at low intensity. In the experiments of figures 20, 21 and 22, the exciting light exposure was for 30 rather than 2 seconds, and the dark periods were 4 1/2 rather than 4 minutes.

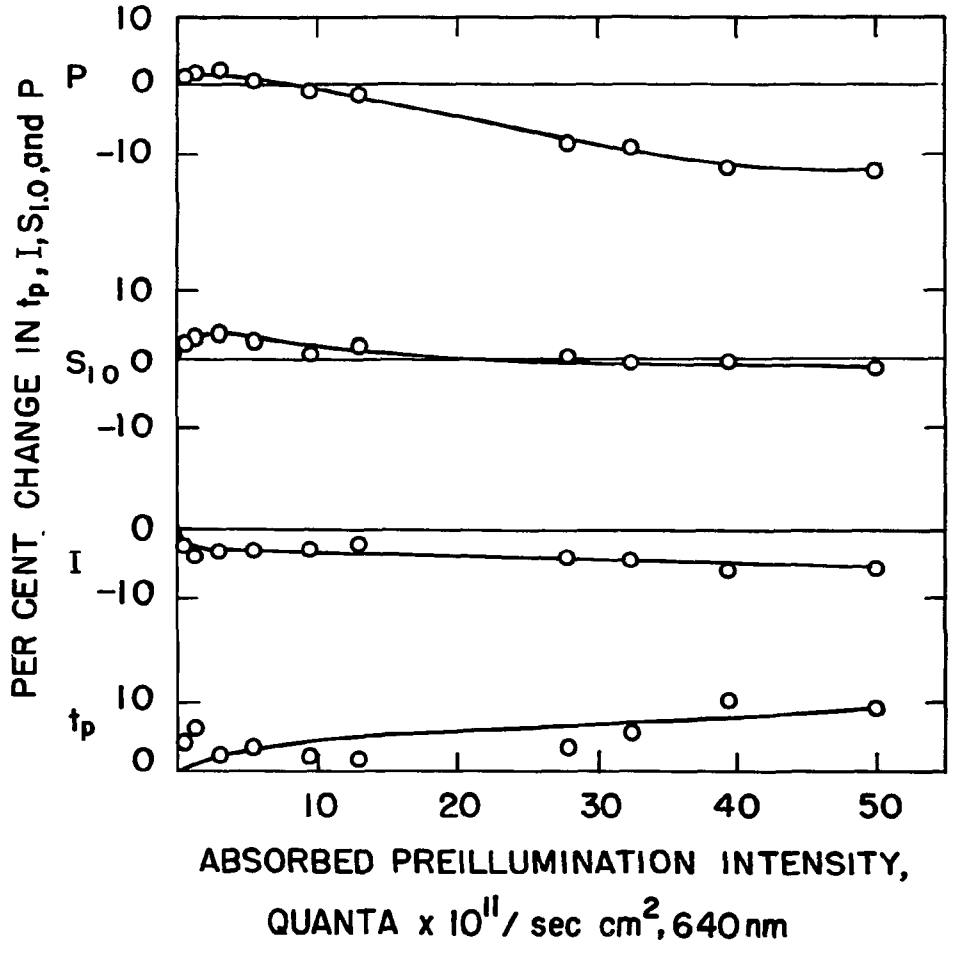


Figure 21. The effect of preillumination at 640 nm in the same culture as in figure 20.

t_p does not decrease at higher intensity as it does in more recent experiments.

A comparison of the old and new results reveals that when the S increase is large, it is accompanied at low intensity by a P increase, while for a small S increase, there is no P increase. Thus, the cause of the S increase, if strong enough, may also raise P.

Clear evidence that there is a correlation between the large S increase and a P increase is shown by another of the earlier experiments portrayed in figure 22. Continuous 705 nm illumination (absorbed intensity 1.2×10^{13} quanta/sec-cm^2, about 10 times the usual preillumination intensity) gradually raised $S_{1.0}$, S_{30}, and P over a 30-minute period. The increase in $S_{1.0}$ was 16%; the increase in P was only 7%. However, interruption of the 705 nm light before the onset of exciting light exposure allowed P to rise even higher. When the interval between the 705 nm light and the exciting light exposure was as much as 33 seconds, the P was 12% higher than it had been before the start of the 705 nm illumination. Obviously, the height of P is controlled by opposing influences: it tends to increase if and when there is a large S increase upon 705 nm illumination but it is depressed below its potential maximum value when there is no dark interval between the 705 nm illumination and the exciting light exposure.

The large S increases due to preillumination 1 cannot be compared easily with results described in the literature, in which absorption in system I decreases fluorescence from system II (7, 8, 19, 38). The

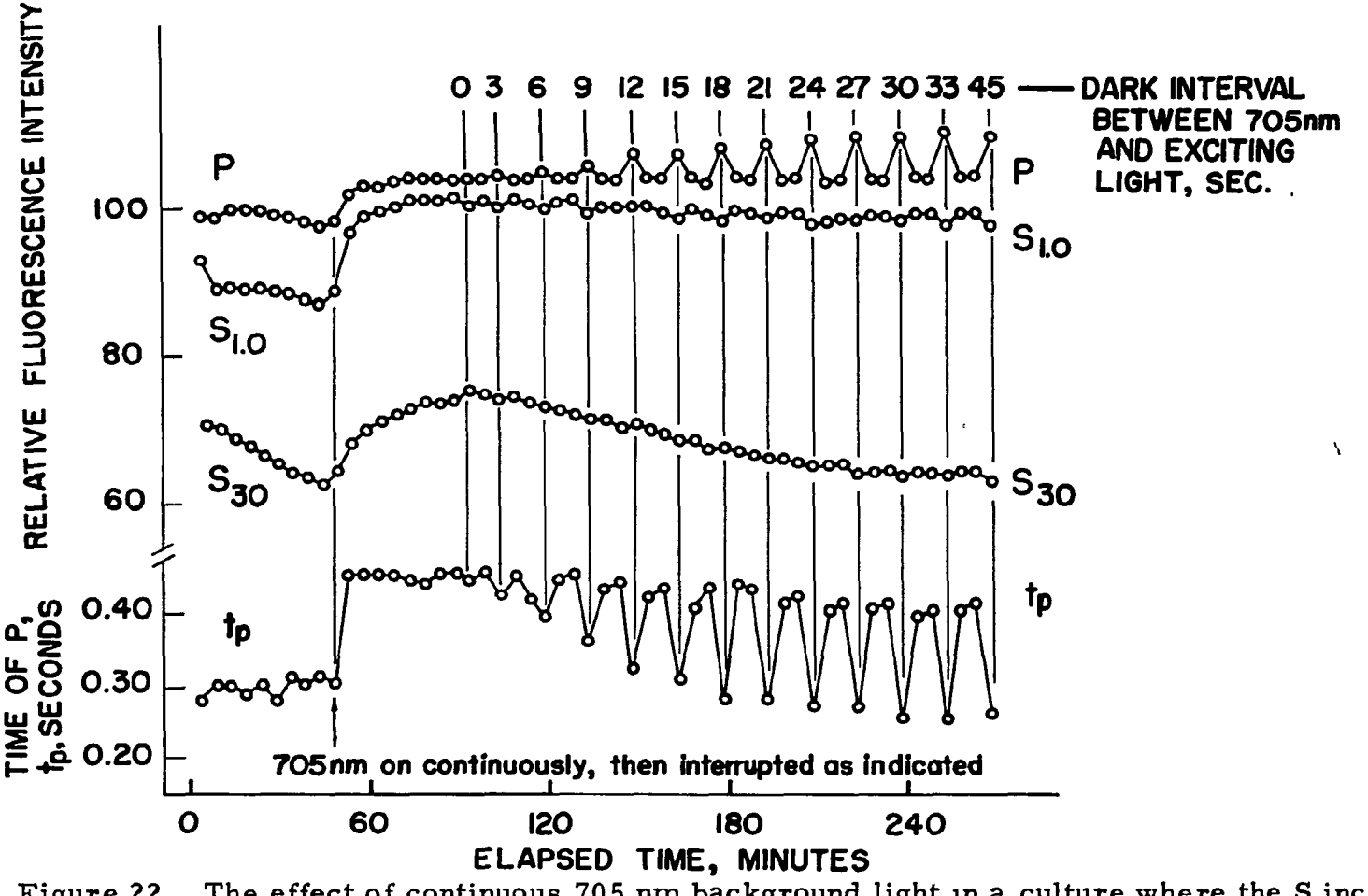


Figure 22. The effect of continuous 705 nm background light in a culture where the S increase is large. P, $S_{1.0}$, and S_{30} are gradually increased over a 30-minute period. But P rises higher, and $S_{1.0}$ and S_{30} decline, if a dark interval is allowed between the 705 nm light and the exciting light. The length of the dark interval allowing the extra rise of P to be half-maximal is 14 seconds. See text for discussion.

reason is that, in the literature, the procedures employed were markedly different from ours. We believe that the results in the literature are obtained under the condition that only the short-term effects of the light absorbed in system I are measured. For example, Govindjee et al. (38) (see Krey and Govindjee (61)), measured the effect of 700 nm light within a few seconds. Duysens and Sweers (19) measured the effects of long-wave light within 1 second to 30 seconds. These short-term effects involve the redox state of Q and other intermediates between the systems. We also believe that the results in the literature are obtained under the condition that the ratio QH/Q is large. The long-wave effect was observed by Duysens and Sweers only if fluorescence was above a base level (i.e., when some Q is reduced). Butler (7) measured effects under the anaerobic condition, when the ratio QH/Q is (nearly) maximal. Our observation in the anaerobic condition (described later in section B of this chapter) is that preillumination 1 decreases P, in agreement with the results of Butler.

Our finding that preillumination 1 increases S (in the aerobic condition) is generally a long-term effect rather than a short-term effect. In figure 22, the S increase was completed only after 30 minutes of 705 nm illumination. As a long-term effect, the S increase most likely has a different basis than changes in the ratio QH/Q, which require just milliseconds to seconds.

Our speculations above were that the S increase involves back reactions or reversal of spatial alterations caused by system II.

Development of the reactions or the reversal might require a long time, which would insure that the S increase would be a long-term effect.

2. Preillumination Quantum Yield Spectra from Single Samples

All the foregoing preillumination results were obtained from different samples because thorough study at each wavelength required 6 to 8 hours, the maximum time cells will display a repeatable transient. Despite consistent results, it is conceivable that differences among the samples might have been the cause of some invalid generalizations.

We were particularly interested in testing the conclusion that decreases of I and P are caused more effectively by longer wavelengths of preillumination. To test this conclusion, we examined single samples for preillumination effects over the entire range of preillumination wavelengths. Because of the time limitation, it was possible to test each wavelength only twice. We used intensities of preillumination below the saturation levels of the preillumination effects. The intensities were determined from the foregoing results.

A relative quantum yield spectrum has been constructed which shows which wavelengths are maximally effective in changing $\bar{I}$, P, and t_p. Figure 23 is a plot of the relative quantum yields as a function of wavelength for the percentage decrease in P, the decrease in I, and the increase in t_p. (S increases are not considered because they were low in these particular experiments).

Long wavelengths are clearly more effective than the short wavelengths -- the highest maxima are at 700 nm and 720 nm. At

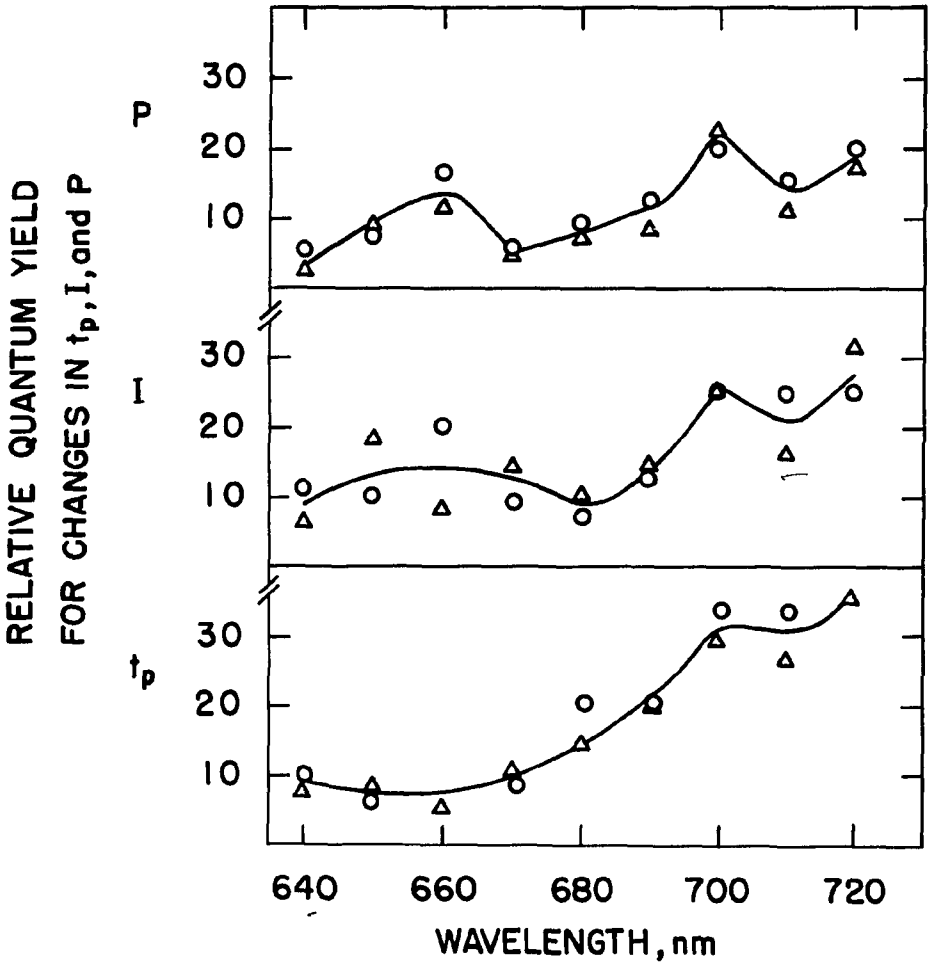


Figure 23. The relative quantum yield for the increase of t_p and the decrease of I and P for preillumination wavelengths from 640 nm to 720 nm. The preillumination intensities were low enough to cause changes of only a few percent, and thus were below the saturation region in figures 12 through 19. Data from two experiments are plotted, one represented by circles, the other by triangles.

670 nm, where both systems have absorption peaks and system II predominates, the quantum yield is much less than at 700 nm and 720 nm. At 650 nm, where system II predominates to a greater extent, the quantum yield is again low. The results support the conclusion that system I is instrumental in decreasing I and P, and increasing t_p.

B. The Anaerobic Case

In anaerobic cells, I is a high spike, D is a deep trough, and P is a high level which remains high without a decline for (at least) several seconds. The separation of I from P is clearer than in aerobic cells. The clearer separation allows an unambiguous determination of the effect of preillumination on I.

If our conclusions thus far are correct, then preillumination 1 should be more effective than preillumination 2 in decreasing the height of I.

700 nm was chosen to represent preillumination 1, and 650 nm represented preillumination 2 (some of the energy of preillumination 2 reaches system I, but the portion is less than half). The effects of these two wavelengths were studied on the same samples, which permitted reliable comparison of absorbed intensities. Changes in I, D, and P from one experiment are shown in figure 24. No changes in t_p and S can be identified because there is no P to S decline; therefore, t_p and S plots are omitted.

As expected, preillumination 1 was more effective than

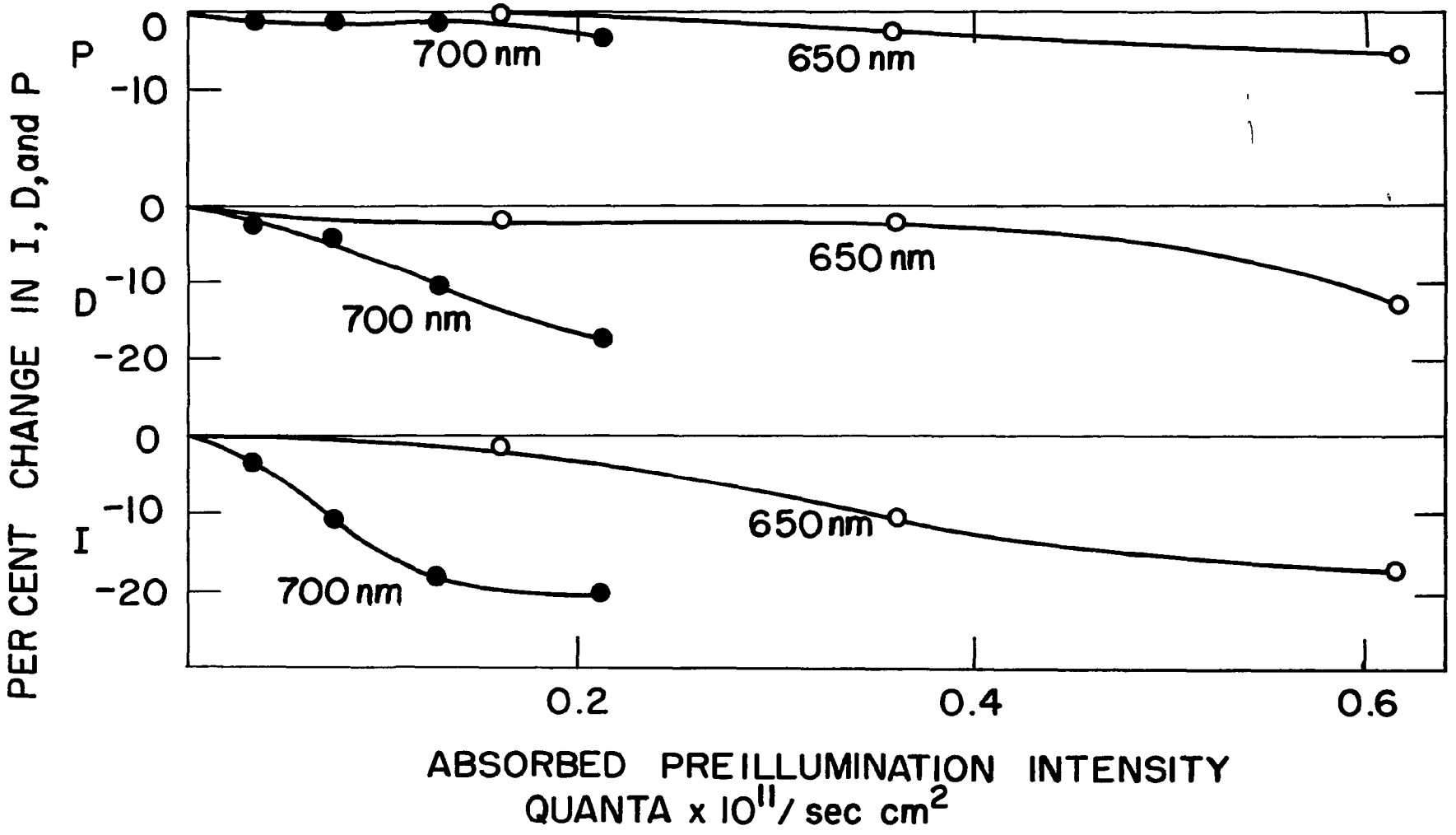


Figure 24. The effect of preillumination in anaerobic Chlorella. Changes in I, D, and P are plotted versus absorbed intensity of preillumination, 700 nm and 650 nm. Plots for t_p and $S_{1.5}$ are omitted; they are not measurable because there is no P - S decline in anaerobic cells.

preillumination 2 in decreasing I, D, and P. Furthermore, preillumination 2 did not increase I. These results confirm our conclusions: system I oxidation of AH lowers I, and system II at low intensities is ineffective in opposing oxidation by system I. We note that P was decreased much less than I and D; the dissimilarity supports our view that if I involves A, P should be explained somewhat differently.

C. Dark Decay of the Preillumination Effect

The preillumination effects seem to involve change of concentrations of electron transport intermediates away from dark-adapted equilibrium values. It is expected that dark reactions will return the concentrations to dark-adapted values, if a dark interval is interposed between the preillumination and exciting light exposure. Thus, we expect a dark interval to cause decay of the preillumination effects.

Study of the dark decay required a modification of the usual experimental procedure. Usually, preillumination completely replaced the four-minute dark period, and so was four minutes in duration. To leave a dark interval after preillumination, yet expose cells to exciting light within four minutes, required that the duration of preillumination be shortened. By experimentation, it was found that the duration of preillumination needed to affect I and P varied with the preillumination intensity. We decided upon a three second exposure of 700 nm and adjusted intensity to obtain a decrease in P of between 10 and 20%.

A three second exposure of preillumination of 700 nm was followed

by different dark intervals. The results from one of several experiments, presented in figure 25, show that the decrease of P decays to one-half its maximum value in a dark interval of 15 seconds.

In the experiment of figure 22 (discussed earlier), involving continuous 705 nm background light rather than a three-second exposure, the half-time of the dark decay was 14 seconds.

The persistence of the effect of preillumination 1 for about 15 seconds is similar to the result of experiments by French (31). Enhancement of oxygen production in the red alga Porphyridium cruentum occurred when a 5-second exposure of red light (system I) preceded a 5-second exposure of green light (system II). The enhancement had a half-life of 18 seconds. When the green light came first, however, there was no enhancement. The enhancement results and the dark decay of the preillumination effect are explainable by the view that A remains oxidized after system I activity for 15 seconds.

It is not upsetting that A fails to remain oxidized any longer, because there are many photosynthetic reductants which are strong enough to reduce A.

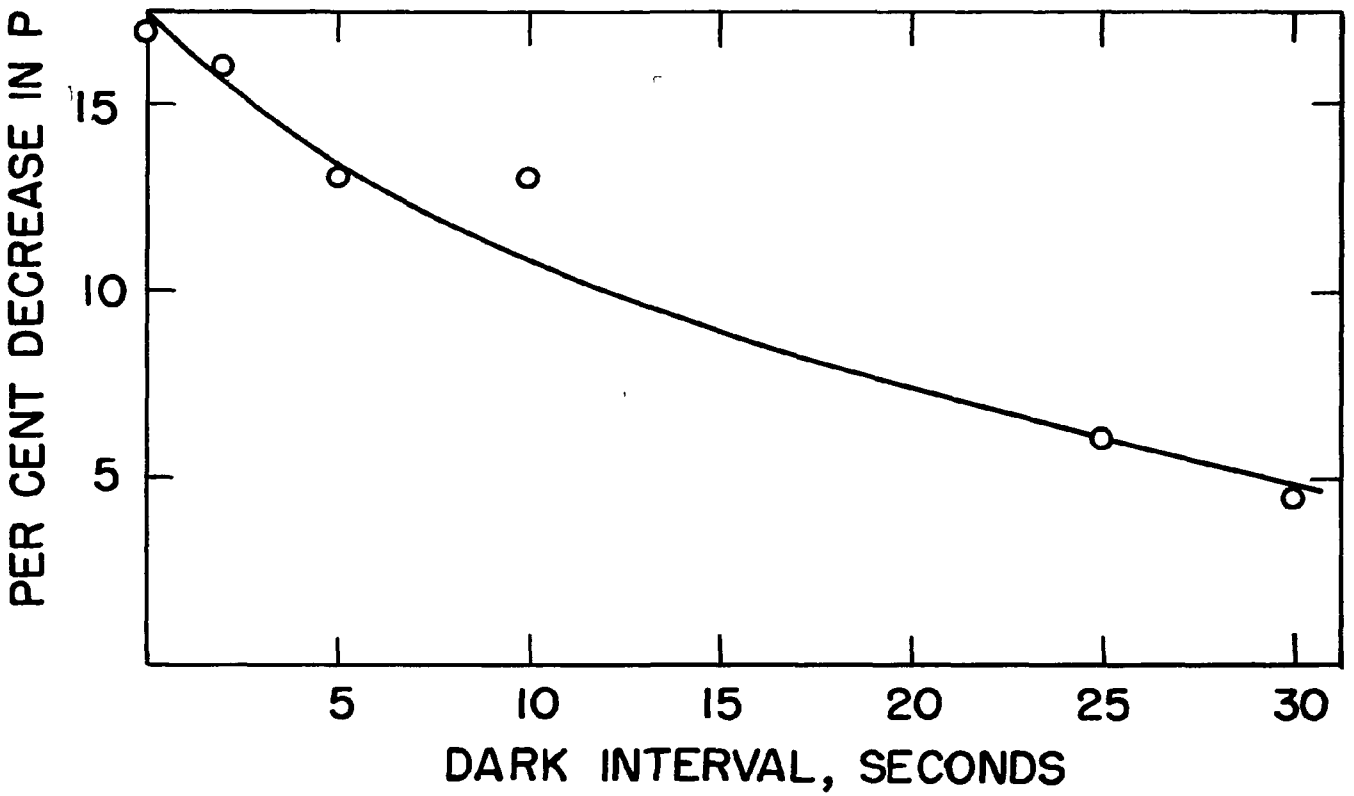


Figure 25. The dark decay of the preillumination effect on P. 700 nm preillumination was followed by different dark periods before the exciting light illumination. The half-time of the decay is 15 seconds. In figure 22, from a different experiment, the half-time was 14 seconds.

VI. ANALYSIS OF THE DIP AND THE PEAK

A. The Light-Activated Dip

The interpretation of the fluorescence transient so far developed is that the points I and D represent development of a system II - system I balance, and that the point P represents reduction of the substance X, with consequent reduction of the substances A and Q. Basic to this view is the finding by Kautsky, Appel, and Amann (50) that the decline from I to D is light-activated. We have presumed that the light-activation is via system I.

This presumption can be tested. If I-D is activated via system I, then addition of system I background light during the transient should hasten D. If, instead, I-D is activated via system II, then system I background light should be ineffective compared to system II light.

D is clearer in anaerobic than in aerobic cells. To take advantage of the clearer D, we used anaerobic cells. 705 nm light was chosen as system I light and 650 nm light as system II light. The full bandwidths were 10 nm. The available 650 nm light was more intense than the 705 nm light, and absorption at 650 nm was greater than at 705 nm. With all available 650 nm light, absorbed intensity (quanta/sec-cm^2) at 650 nm would have been much greater than at 705 nm. To equalize absorbed intensities, the 650 nm intensity was reduced by means of neutral density filters.

Because the absorbed intensities were low, and the time between

I and D normally is so short (75 msec), a normal procedure would have allowed very few quanta to be absorbed before D. The effects would have been small. Therefore, the procedure was modified by using a flashing exciting light instead of the normally continuous exciting light. The flashing light was comprised of 40 msec flashes with intervening dark periods of 40 msec. Because the light and dark periods were equal, the time required for the absorption of the same number of quanta as in continuous light was doubled. This time doubling delayed D and permitted the duration of the continuous background illumination to be longer than normal. With longer duration, more quanta of the background illumination were absorbed, and its effects were then easily noticeable -- the time of D was hastened by about 10%.

Figure 26 shows the fluorescence transient excited by the flashing light. Notice that during each flash there is an increase in fluorescence, and that the level of fluorescence at the end of a flash is higher than the level at the beginning of the next flash. A dark-oxidation of QH is taking place between each flash. Lavorel (66) has studied the kinetics of the dark-oxidation of QH after a 33 msec light flash. In the first 88 msec of dark an oxidation takes place which is inhibited by CMU and o-phenanthroline. A second oxidation phase between 88 msec and 168 msec is not sensitive to these inhibitors. The first phase can be explained as the oxidation of QH by A, and the second phase as an oxidation within the Q-reaction-center complex.

The dark oxidation does not interfere with the light-requiring

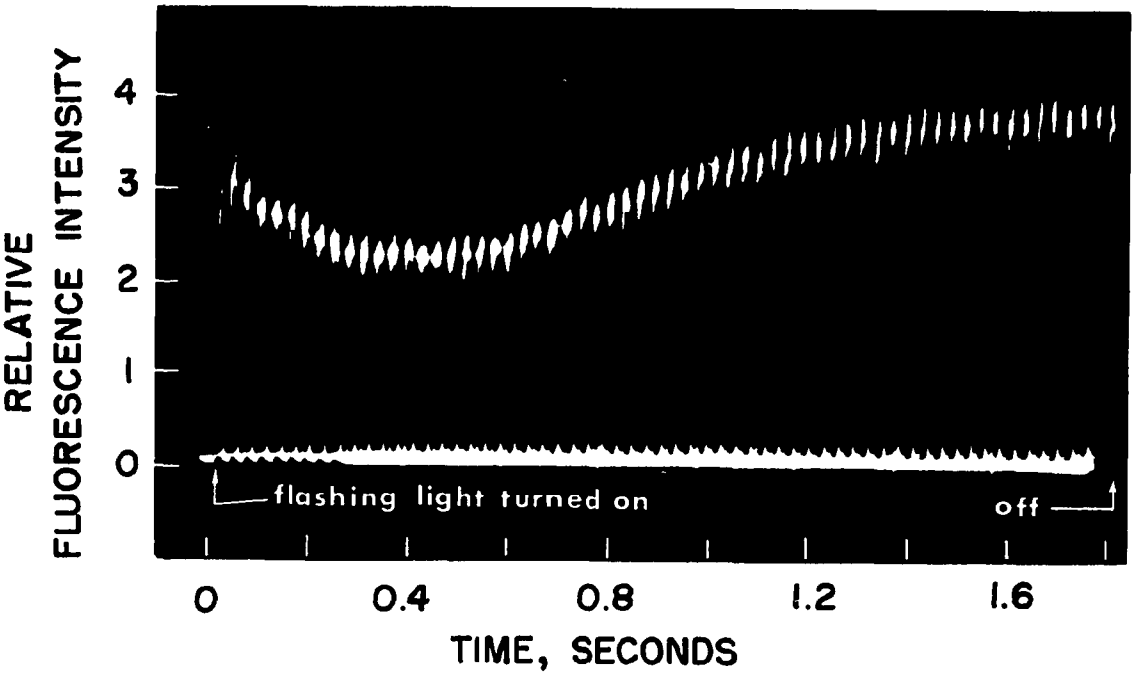


Figure 26. The anaerobic fluorescence transient excited by flashing light (for intensity and wavelength see fig. 1). D is delayed by flashing light, compared to continuous light, because the I - D decline progresses only during the light periods (compare D in fig. 2).

character of I-D discovered by Kautsky et al. (50). Our finding that the time nearly doubles under light and dark flashes of equal length confirms their discovery.

We tested, in several experiments, the effects of 650 nm and 705 nm background light on the transient excited by flashing exciting light. Each experiment involved at least five trials at each wavelength, alternated to eliminate influence of long-term fluorescence changes. For each trial of background light the shutters for the background light and the exciting light were opened simultaneously. The result, from each of several experiments, is that 705 nm light and 650 nm light are almost equally effective in hastening D. The data from one experiment are shown in Table II. The background light effect is expressed as the fractional change in the time of D. The average effect is divided by absorbed intensity to give relative quantum yield. The relative quantum yield for 650 nm is 7.05, and for 705 nm, it is 8.25. According to standard deviations computed for each wavelength (see the table), the two quantum yields are not significantly different.

The result that system I light is not ineffective compared to system II light indicates that the light-activation of D proceeds via system I. The most simple explanation is that system I is oxidizing AH, thereby allowing A to oxidize QH. As QH is oxidized, fluorescence declines from I to D.

We are slightly surprised that the quantum yields for the effects of 650 nm and 705 nm background light are similar. A portion of the

TABLE II

EFFECT ON TIME OF D (t_D)

CAUSED BY 650 nm or 705 nm BACKGROUND LIGHT IN ANAEROBIC CHLORELLA

Exciting Light Exposure	No Background t_D	650 nm - Background t_D	650 nm - Background $\Delta t_D/t_D$	705 nm - Background t_D	705 nm - Background $\Delta t_D/t_D$
1*	0.20 sec.				
2	0.22				
3	0.21				
4	0.20				
5**	0.38				
6	0.38				
7	0.38				
8	0.38				
9	---	0.31	0.184		
10	---	---	---	0.31	0.184
11	0.37	---	---	---	---
12	---	0.33	0.108	---	---
13	---	---	---	0.32	0.135
14	0.36	---	---	---	---
15	---	0.31	0.161	---	---
16	---	---	---	0.30	0.167
17	0.38	---	---	---	---
18	---	0.32	0.158	---	---
19	---	---	---	0.30	0.210
20	0.37	---	---	---	---
21	---	0.34	0.081	---	---
22	---	---	---	0.34	0.081
23	0.37	---	---	---	---
24	---	0.34	0.081	---	---
25	---	---	---	0.33	0.108
	Average $\Delta t_D/t_D$		0.129		0.147
	Standard deviation		0.0407		0.0443
	Absorbed quanta/sec-cm^2		1.83×10^{13}		1.78×10^{13}
	Relative quantum yield		7.05		8.25

*The exciting light was continuous in exposures 1 through 4.
**The exciting light was flashing in exposures 5 through 25.

650 nm light energizes system I, but less than half. On that basis, we expected 650 nm to be somewhat less effective than 705 nm.

The oxidation of AH by system I proceeds despite the ongoing reducing-activity of system II caused by the intense exciting light. We shall consider for a moment the level of QH, which indicates the availability of electrons for reducing A, since QH donates electrons to A. The point O of aerobic cells indicates when Q is oxidized, while the P level of anaerobic cells indicates when most (if not all) Q is reduced. From figure 6 point O (aerobic) has a value of 20, while P (anaerobic) is 108. The difference, or 108 - 20 = 88, is proportional to the total amount of Q. The I hump in the anaerobic condition is 80, or above the point O (aerobic) by 80 - 20 = 60. Therefore, at I (anaerobic), the proportion of QH to total Q is $\frac{60}{88}$ or about two-thirds. Thus, plenty of QH is available to donate electrons to A. Despite the abundance of QH, system I proceeds to oxidize AH, and cause the dip.

Perhaps the abundance of QH at the point I causes significant transfer of excitation energy from system II to system I. Energy absorbed by photosynthetic units of system II is efficiently captured by the reaction centers when Q is oxidized. When Q is reduced, the energy is not captured, and the probabilities of other mechanisms of energy dissipation are increased, such as the probability of fluorescence, and that of energy migration to other reaction centers.
A. Joliot and P. Joliot (45) and Delosme (11) have shown that the S-shape of the O - I rise of the transient excited by intense light can

be explained by energy migration from system II units where Q is reduced to neighboring system II units. The success of this explanation leads to the speculation that transfer from system II to system I will be greater when Q is reduced. If so, then we may understand why the quantum yields for the effects of 650 nm and 705 nm background light are similar: at the point I, where most Q is reduced, a large portion of the 650 nm light absorbed by system II units is being transferred to system I.

We will assume (there is no evidence to the contrary) that oxidation of AH continues uninterrupted as long as oxidized X is available. (X is a hypothetical electron acceptor for system I. For a discussion of X, see the review by Avron, ref. 2.) Oxidation of AH and QH by system I is in progress between I and D, according to our experiments, and is of a high enough rate to overcome reduction of Q and A by system II. We believe therefore that fluorescence rises from D to P because the system I reaction slows down, due to a limitation in the amount of oxidized X.

B. The Peak

The view that P results from a limitation in the amount of oxidized X can be tested. One method is to observe the effect of a short system I exposure during the phase D - P. What will be the effect on P? Let us assume the opposite of our claim. We shall assume P does not involve X, instead, that it involves only a temporary

maximum in AH and QH. Then, the effect of the system I exposure should be increased oxidation of AH, and a reduction and/or delay in P.

The experiment involved a 150 msec flash produced by a shutter. The flash was timed so that it started after D, but terminated more than 100 msec before P usually occurs. 700 nm light was chosen to excite system I.

Many trials in several experiments showed that the 700 nm flash hastens P. Figure 27 shows superimposed drawings of one transient excited normally, and the succeeding transient modified by the flash. The effect of the flash seems small, but in fact t_p was reduced by 7%. Larger effects were observed for 697, 695, and 693 nm, for which absorption is greater. These wavelengths also preferentially excite system I (9). The results indicate by contradiction that P is not simply due to a maximun in AH. Since the short flash causes extra reduction of X, and hastens P, P must be due to reduction of X, with consequent reduction of Q and A.

Another test of the view that P involves X was designed around an exogenous electron acceptor. If most X is normally reduced at P, then P should be absent if most X can be kept oxidized. This is accomplished by adding an exogenous electron acceptor which can remove electrons from X. For the test to be conclusive, the acceptor must not be able to remove electrons from intermediates of systems I and II other than X. Since the other intermediates have redox potentials more positive than -0.2 v., while X has a potential more negative

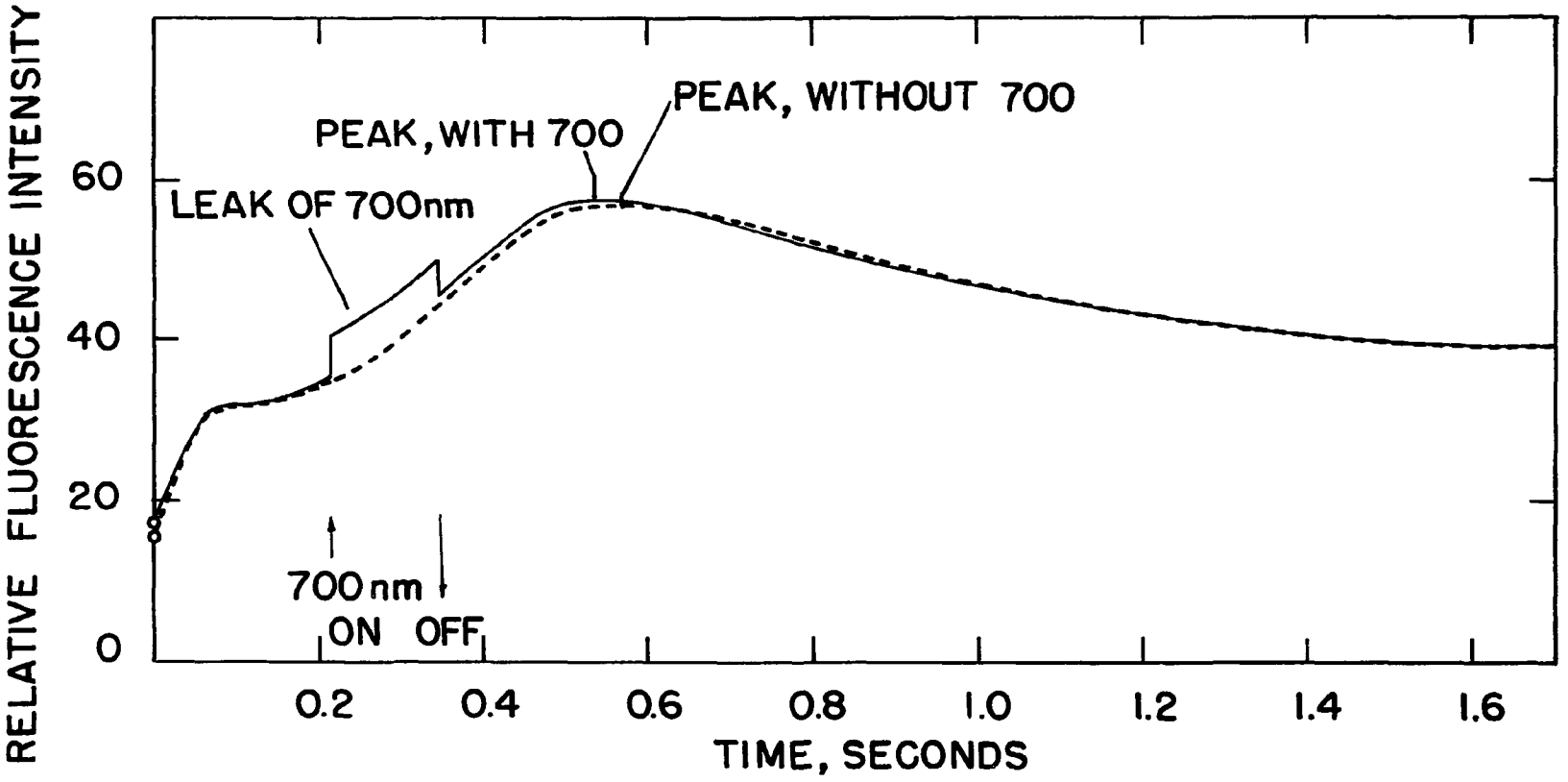


Figure 27. The effect on P of a 700 nm (system I) flash after I. The flash was 150 msec in duration, and had an absorbed intensity of 2.5×10^{13} quanta/sec-cm^2. Transient without flash, dotted line, followed by transient with flash, solid line. Some of the flash intensity leaked into the detection system as shown. The flash caused an increase in P, and caused t_p to decrease by 7%. (In this experiment, the exciting light intensity was reduced slightly to allow more time between I and P.)

than -0.4 v (59), an acceptor with a potential near -0.4 v. will be suitable. Several investigators have used the dye methyl viologen (paraquat dichloride, i.e., 1,1'-dimethyl-4,4'-dipyridilium dichloride) which has a potential E_o' = -0.445 v. In chloroplasts, methyl viologen has been shown to undergo a 1-electron photoreduction (58, 59). It facilitates cyclic photophosphorylation (1, 42). A concentration of 10^{-4} moles/liter has a saturating effect on the absorption change of P_{700}, the trap for system I (59). The potential of methyl viologen, and its effects on chloroplasts, prove that it interacts only with system I. It apparently has not been shown to penetrate intact cells, but because of its small size it is expected to penetrate.

Methyl viologen at 10^{-4} moles/liter caused the progressive disappearance of P in 15 minutes (penetration is thus demonstrated). As P disappeared, t_p decreased. A mild depression developed between P and $S_{1.5}$. There was no effect on the phase O - I. Figure 28 shows the transient after treatment.

Anaerobic cells treated with methyl viologen displayed a normal anaerobic transient. Admission of air led to display of the methyl viologen-treated aerobic transient, but return to anaerobiosis led to display once more of a normal anaerobic transient. These events are explained by the fact that methyl viologen after photoreduction donates its electron (perhaps via intermediates) to oxygen. When no oxygen is present, photoreduction is unopposed and all of the methyl viologen accumulates in the reduced form. Then it can no longer accept electrons

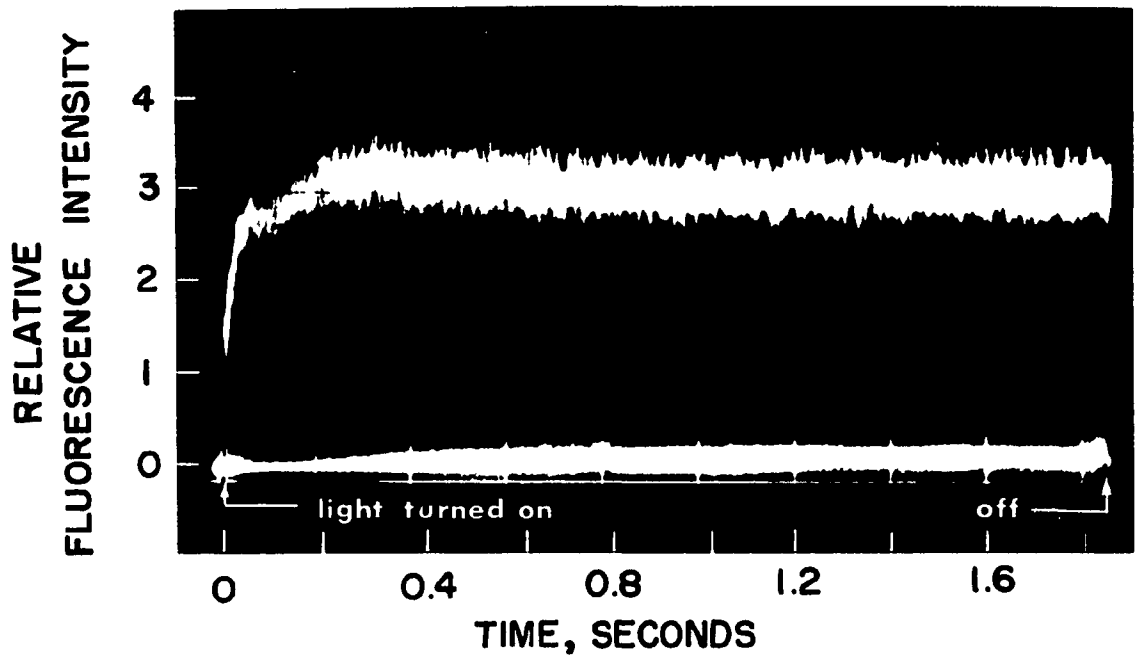


Figure 28. The fluorescence transient after addition of methyl viologen. Concentration after addition, 10^{-4} moles/liter. Methyl viologen ($E_o' = -0.445$) accepts electrons from system I. It largely eliminates P but has little effect on the O - I rise.

from system I.

The results confirm the belief that reduction of X and then of A and Q causes the peak P.

VII. MATHEMATICAL ANALYSIS OF THE TRANSIENT

The aim of mathematical analysis is to compare the time course of in vivo fluorescence with fluorescence predicted by supposed mechanisms of photosynthesis. If a mechanism is found whose predicted fluorescence matches the in vivo fluorescence, then we have circumstantial evidence for believing that this mechanism is homomorphic with the actual mechanism. We emphasize that mathematical analysis can provide only circumstantial evidence. The quality of the evidence increases as the ratio of fact to assumption increases in the supposed mechanism. Ultimately, our willingness to accept mathematical evidence depends on imperfectly-defined subjective judgment.

Sections A, B, and C of this chapter constitute a survey of recent mathematical analyses of the transient. The emphasis is on axioms and relations between the analyses. Section D is an investigation of models which may explain the dip, D.

A. Basic Assumptions

We shall assume that the following statements are true:

(i) The fluorescence at O of the aerobic transient represents a fluorescence which is constant with time and independent of the photosynthetic reactions. This assumption is an approximation of the real situation and was discussed in chapter III.

(ii) The excess of fluorescence above O is emitted by system II. This assumption is supported by the evidence reviewed in chapter I.

We require next a statement of the exact nature of the system II photoreaction. It is not known whether it is a photooxidation of the chlorophyll molecule in the reaction center complex, a photoreduction, or an electron transfer from a donor to Q mediated physically (rather than chemically) by the chlorophyll. Each possibility has its complexities which require definition before reaction rate equations can be written. We have found for these possibilities that the rate equations are too complex to solve with information now available.

Many investigators simplify matters by assuming that the primary reaction is the photoreduction of Q. The immediate source of the electron used in the reduction is not specified -- it could be chlorophyll or a donor formed from water. The reaction rate equations which have been written on this basis presume in effect that the electron comes from a donor molecule whose concentration is constant. In accord with practice, we write that

(iii) the primary reaction of system II is the photoreduction of Q.

The last statement needed concerns the relation between Q and fluorescence. With this relation we can write an equation of fluorescence as a function of time. The problem is that different relations have been proposed in the literature, and the physical basis which ties these relations together is not always evident. For the purpose of

clarification, we discuss briefly the physics of excited molecules, and their use in finding a relation between Q and fluorescence.

B. Rate Laws and Equations for Fluorescence

Once a photon has excited a molecule to an electronic excitation state, the excitation energy has many possible fates: fluorescence, change to chemical energy, change to heat energy, or transfer to a second molecule. The change to heat energy may proceed by internal conversion. There are several ways for transfer to occur: by radiative transfer, by diffusion-controlled non-radiative transfer, by long-range dipole-dipole resonance interaction, by exciton migration, and by electron and hole migration (96).

Three mathematical axioms apply to excited molecules. One is conservation of energy. Another is the concept of rate equations for the possible fates of excitation energy. The third is the definition of quantum yield for any of the possible fates.

The usual application of these axioms to problems of photochemistry is straightforward. The rate equations can be written first. The rate equation for fluorescence depends on the fact that the rate of fluorescence is proportional to the concentration of excited molecules. Let F be the rate of fluorescence, k_F the proportionality constant, and $[C^*]$ the concentration of excited molecules.

Then

$$F = k_F [C^*] . \qquad (1)$$

The rate equation for change to heat is simplest when the change occurs by internal conversion. Then the rate is proportional to $[C^*]$. Let H be the rate of change, and k_H the proportionality constant.

$$H = k_H [C^*] . \tag{2}$$

The rate of change to chemical energy, which we shall call P, is dependent on $[C^*]$. We assume that it depends also on the concentration of another molecule, which we shall call B.

Then

$$P = k_P [C^*][B] . \tag{3}$$

We shall omit for the moment the possibility of energy transfer.

Let I be the rate of absorption of quanta. An application of the conservation of energy gives

$$\frac{d}{dt} [C^*] = I - F - H - P = I - (k_F + k_H + k_P [B]) [C^*] . \tag{4}$$

The term $\frac{d}{dt}[C^*]$ accounts for the possibility that the concentration of C* will vary in response to changing rates of excitation and de-excitation. Such a term is especially important in Q-switched lasers, where the Q-switching produces wide variations in the concentration of excited atoms and molecules. Over long enough times, the variations of $\frac{d}{dt}[C^*]$ average to zero, and equation (4) may be written as

$$I = (k_F + k_H + k_P [B]) [C^*] . \tag{4a}$$

If we are interested in fluorescence, the equations (1) and (4a) may be

used to obtain

$$F = I - H - P = I - k_H[C^*] - k_P[C^*][B]. \qquad (5)$$

An equally valid expression for F can be obtained from a quantum yield expression. The quantum yield of fluorescence, $\frac{F}{I}$, designated by ϕ_F, is

$$\phi_F \equiv \frac{F}{I} = \frac{F}{F + H + P}$$

$$= \frac{k_F[C^*]}{k_F[C^*] + k_H[C^*] + k_P[B][C^*]}$$

$$= \frac{k_F}{k_F + k_H + k_P[B]} \quad . \qquad (6)$$

Therefore,

$$F = I\ \phi_F = \frac{Ik_F}{k_F + k_H + k_P[B]} \quad . \qquad (7)$$

Notice that we have <u>three</u> valid equations for F, equations (1), (5), and (7).

C. <u>Application of the Equations to Photosynthetic Units of System II</u>

Equations (1), (5), and (7) cannot be applied straightaway to analysis of the <u>in vivo</u> fluorescence transient, by the simple identification of B with Q, because the equations presume that all molecules behave

identically, while in the photosynthetic unit there is a distinction between the behavior of bulk molecules and the energy trapping molecule at the reaction center. For the bulk molecules, energy transfer is dominant, but for the trap, the reduction of Q is dominant.

Murata, Nishimura, and Takamiya (78) have proposed equations for the fluorescence transient which do not distinguish between the bulk and trap molecules.

One method of accounting for the bulk-vs.-trap distinction is to apply equation (7) to the trap, and apply to the bulk a modified equation (7) which incorporates the rate of energy transfer rather than the rate of photochemistry. It is necessary to use different I values in each case because of the energy transfer from bulk to trap. This method is cumbersome and will not be considered further.

Another method, used by Duysens (14), is to modify equation (5). It is given that the number of quanta absorbed is equal to the number trapped, plus the number fluoresced or changed to heat energy. The number trapped is equal to the number absorbed multiplied by a probability of trapping. Let the probability be K.

Then

$$I = KI + F_2 + H . \qquad (8)$$

The subscript 2 of F_2 reminds us that we are considering only fluorescence from photosynthetic units of system II. Duysens assumes that H is proportional to F. This assumption is also made by Murata et al. (78) and Delsome (11). Then

$$I = KI + c_1 F_2 \qquad (8a)$$

where the constant c_1 allows for the proportionality.

We consider this assumption to be a weak point in analyses of the transient. A simple calculation shows why. Under optimum conditions, utilization of light energy for photosynthesis is very efficient. This conclusion is based on the series scheme and its theoretical requirement of 8 quanta per oxygen molecule evolved, and the experimental value of Emerson and Chalmers (21) of 8 under optimal conditions. We may safely assume that the quantum efficiency of each photoreaction under optimal conditions is probably above 90%. Fluorescence wastes 3%; however, in system II, the fluorescence yield is greater (18) and may be as high as 6%. Therefore, in system II roughly 4% of the quanta may be lost to heat. When cells are treated with DCMU, the quantum efficiency of the system II photoreaction drops nearly to zero; an upper limit for the efficiency of the photoreaction in this case is 10%. Fluorescence approximately triples (8). Now, if we presume that the only alternative left to the quanta is a loss to heat, the percentage of quanta lost to heat must be 72%. Thus the percentage of quanta lost to heat apparently rises nine times as much as the percentage lost to fluorescence, whereas the assumption is that the rises are equal.

A way out of this difficulty is to presume that quanta have another alternative when the system II photoreaction is inhibited by DCMU (or saturated by high light intensity). Perhaps the quanta are trapped despite the DCMU inhibition, but used for some other reaction

than photoreduction of Q.

We will consider this matter to be unsettled, but we will proceed with our survey as if equation (8a) is acceptable.

The probability K depends on the concentration of Q; if we know this dependence, we can substitute it into equation (8a) and obtain the relation we desire between F_2 and Q.

Duysens (14) gives a short derivation of the K - Q dependence, but we prefer the more rigorous derivation by Delosme (11). Delosme assumes that energy absorbed by a bulk molecule may be transferred to another bulk molecule or to the trap, or may be fluoresced, while energy absorbed by the trap or transferred to the trap will be used for chemistry. By an infinite series, Delosme determines the probability that energy is ultimately trapped. The probability is·

$$K = 1 - \frac{(1 - a)(1 - T)}{1 - a\ (1 - T)} = \frac{T}{1 - a\,(1-T)} \qquad (9)$$

where $\underline{a}$ is the probability of each transfer and $\underline{T}$ the fraction of chlorophyll molecules which are traps. Since the ratio of traps to Q is unity, T equals the ratio of total Q to total chlorophyll. Delosme used this ratio (which we call Q_o) in place of T. Replacement of T by Q_o, and substitution of the expression for K into equation (8a) gives

$$I = I \left\{ 1 - \frac{(1 - a)(1-Q_o)}{1 - a\,(1 - Q_o)} \right\} + c_1 F_2. \qquad (10)$$

Rearranging, we get

$$F_2 = \frac{1}{c_1} \; I \left\{ \frac{(1 - a)(1-Q_o)}{1 - a(1 - Q_o)} \right\}. \qquad (10a)$$

We will find it convenient to use, instead of equation (10a), the following, from equation (8a):

$$F_2 = \frac{1}{c_1} \; I(1 - K). \qquad (10b)$$

As it stands, equation (10a) is correct only when all Q is oxidized, ready for photoreduction. F_2 of equation (10a) contributes to the fluorescence at O, which presumably includes in addition a contribution from system I.

In a photosynthetic unit where Q is already reduced, photoreduction cannot occur, and energy arriving at the trap cannot be used. The energy is then subject to further transfer and is more likely to be fluoresced. T in this case must be, not Q_o, but zero, if no transfer occurs between units.

Delosme (11) thought to account for this situation by replacing Q_o in equation (10a) with $\frac{Q}{Q_o}$, the fraction of Q_o in the oxidized form. The resulting equation was used to draw theoretical fluorescence transients. Since the theoretical curves did not match experimental curves for appropriate values of $\underline{a}$ and Q_o, equation (10a) was subjected to further modification. The replacement of Q_o by $\frac{Q}{Q_o}$ is incorrect, however, because it assumes that each quantum of excitation energy has free access to all oxidized Q, contrary to the original assumption that each quantum has access only to the oxidized Q of the photosynthetic

unit where it is absorbed. The correct procedure is to consider each photosynthetic unit in turn, applying equation (10a) with the value of T appropriate to the unit in question. When Q is oxidized, $T = Q_o$. Thus, from equation (10a),

$$F_o = \frac{1}{c_1} I \left\{ \frac{(1 - a)(1 - Q_o)}{1 - a(1 - Q_o)} \right\} \tag{11}$$

where the subscript o stands for "oxidized". We write an equivalent form using equation (10b) and K_o (standing for K in the oxidized units):

$$F_o = \frac{1}{c_1} I (1 - K_o). \tag{11a}$$

Where Q is reduced, T = 0, therefore K = 0, and

$$F_r = \frac{1}{c_1} I \tag{12}$$

where r stands for "reduced". K = 0 signifies that the energy has a probability of zero of being trapped.

The units contributing F_o are a fraction $\frac{Q}{Q_o}$ of the total, and the units contributing F_r are a fraction $\frac{QH}{Q_o}$ where $Q + QH = Q_o$. Let $[Q_o]$ be the concentration of total Q, $[Q]$ the concentration of oxidized Q, and $[QH]$ the concentration of reduced Q. The ratios $\frac{Q}{Q_o}$ and $\frac{QH}{Q_o}$ may be replaced by $\frac{[Q]}{[Q_o]}$ and $\frac{[QH]}{[Q_o]}$. Then, total fluorescence from all units of system II, F_2, is

$$F_2 = \frac{[Q]}{[Q_o]} F_o + \frac{[QH]}{[Q_o]} F_r = F_o + (F_r - F_o) \frac{[QH]}{[Q_o]}. \tag{13}$$

We may use equations (11a) and (12) to substitute for $(F_r - F_o)$:

$$F_2 = F_o + \frac{K_o I}{c_1} \frac{[QH]}{[Q_o]} . \qquad (13a)$$

Equation (13) is isomorphic with the basic equation used by Malkin and Kok (72) in an analysis of the fluorescence transient in isolated chloroplasts. In their equation, however, F_o and F_r do not have the specific meanings given by equations (11a) and (12).

Equations (11), (12), and (13) when taken together imply that units are separate, in other words, that no energy transfer can take place between units. If this is true, then equation (13) enables us to develop an equation expressing F_2 as a function of time. The time dependence of F_2 results from the time dependence of $[QH]$. This dependence can be derived from the rate of trapping, KI; the method of derivation is based on that used by Delosme (11). To find the rate of trapping we use equations (8a), (11a), (12), and (13). From (8a),

$$KI = I - c_1 F_2 .$$

Substituting for F_2 from equation (13a), we get

$$KI = I - c_1 F_o - K_o I \frac{[QH]}{[Q_o]} .$$

Substituting for F_o from equation (11a), we get

$$KI = I - I(1 - K_o) - K_o I \frac{[QH]}{[Q_o]}$$

Rearranging, we get

$$KI = K_o I \left(1 - \frac{[QH]}{[Q_o]}\right). \tag{14}$$

Since energy which is trapped is used for photoreduction of Q, the rate of trapping is equal to the rate of reduction of Q. Therefore,

$$KI = - \frac{d}{dt}[Q]$$

where the minus sign appears because $[Q]$ decreases as reduction takes place. From

$$[Q] + [QH] = [Q_o], \quad - \frac{d}{dt}[Q] = + \frac{d}{dt}[QH], \text{ and } KI = + \frac{d}{dt}[QH].$$

Substituting for KI from equation (14),

$$\frac{d}{dt}[QH] = K_o I \left(1 - \frac{[QH]}{[Q_o]}\right). \tag{15}$$

We assume that there is no oxidation of QH. The solution for $[QH]$ is

$$[QH] = [Q_o]\left(1 - e^{-K_o I t} \frac{1}{[Q_o]}\right) \tag{16}$$

with the boundary condition that $[QH] = 0$ when $t = 0$. Substituting equation (16) in equation (13a), we get

$$F_2 = F_o + \frac{K_o I}{c_1} \left(1 - e^{-K_o I t} \frac{1}{[Q_o]}\right). \tag{17}$$

According to this equation, fluorescence will rise exponentially to a steady state. The assumptions used in derivation of equation (17) are

that units are separate, reduction of Q proceeds unopposed, and the rate of change to heat energy is proportional to fluorescence.

Morin (75) and Delosme (11) recorded the O - I rise in Chlorella in high intensity light, when reduction of Q is rapid enough to proceed unopposed by dark oxidation via A. The results were that the O - I rise is not exponential, which conflicts with the prediction of equation (17). A. Joliot and P. Joliot (45) and Delosme (11) have suggested that energy reaching a trap where Q is reduced may be transferred to another trap. Assuming this to be true, Delosme (11) obtained an equation (ref. 11, equation (8)) analogous to equation (13a). When written with our symbols, his equation is:

$$F_2 = F_o + \frac{K_o I}{c_1} \quad \frac{(1 - p)\ \frac{[QH]}{[Q_o]}}{1 - p\ \frac{[QH]}{[Q_o]}} \qquad (18)$$

where $\underline{p}$ is the probability of transfer from one trap to another.

As before, $[QH]$ was determined from the rate of trapping. The equation by Delosme (ref. 11, equation (9)) in our terminology is (except for one trivial constant associated with I which we have assumed equal to unity):

$$K_o I\, t = p\,[QH] + (p - 1)\,[Q_o]\left(\ln \frac{[Q]}{[Q_o]}\right). \qquad (19)$$

Equations (18) and (19) give an S-shaped rise of fluorescence which matches the experimental O - I rise observed in intense light. The

match is strong circumstantial evidence that the traps of photosynthetic units of system II are not isolated from each other, i.e., that energy may travel from the trap of one unit to the trap of another unit.

A property of two basic equations in this section is that fluorescence monotonically follows $[QH]$, increasing when $[QH]$ increases, and decreasing when $[QH]$ decreases. In equation (13), this property is explicit. In equation (18), it can be seen by dividing both numerator and denominator of the right-hand term by $[QH]$; then, $[QH]$ will appear only once, as the inverse, $\frac{1}{[QH]}$, in the denominator. The monotonic relation is then obvious.

We have reviewed in this section the development of equations for fluorescence emitted by units of system II. We emphasize points which are unsettled in the development to date: whether the loss of quanta to heat is proportional to fluorescence, and whether quanta may be trapped even when the photoreduction of Q cannot occur. The equations of this section apply only when there is no oxidation of QH.

D. Mathematics of the Dip

In this section we extend the mathematical analysis to a later phase of the transient, D, where oxidation of QH must be considered. We obtain differential equations with terms representing oxidation of QH for two types of systems. These two types are named "nonlinear" and "linear", because of the mathematical properties of their associated equations. We do not in general obtain explicit expressions for

fluorescence as a function of time; rather, we examine equations in [QH] in the light of the fact that fluorescence monotonically follows [QH].

1. Nonlinear Systems

We assume in this part that Q has only two forms, oxidized and reduced, rather than three, as in the Joliot (46) or the Duysens and Sweers (19) models.

The experiments described in chapter V show that by the end of the O - I phase, at moderate exciting light intensity, the rise of [QH] is being opposed by the interaction of QH with A. The dip after O - I, according to chapter VI, represents a decrease in [QH]. Thus, in a discussion of the dip, we must withdraw the assumption (at equation 15) that there is no oxidation of QH. The rate equation for [QH] must be re-written to include the oxidation of QH by A. We assume that the rate of oxidation is $k_a [A][QH]$. Then

$$\frac{d}{dt}[QH] = K_o I \left(1 - \frac{[QH]}{[Q_o]}\right) - k_a [A][QH]. \qquad (20)$$

This equation is insoluble because [A] is a function of time which is unknown. The concentration of A depends not only on its reaction with QH but also on the reaction of AH with the succeeding electron transport intermediate. We resort to a simplifying assumption in order to get a solution. We assume that [A] is constant. Once we have the solution, we shall see what happens to it as [A] varies. The solution when [A] is constant is:

$$[QH] = \frac{[Q_o]\, I\, K_o}{K_o I + k_a [A][Q_o]} \left(1 - e^{-\left\{K_o I \frac{1}{[Q_o]} + k_a [A]\right\} t}\right) \qquad (21)$$

with the condition that $[QH] = 0$ when $t = 0$. According to this equation, $[QH]$ will rise exponentially to a final, maximum value. This value is determined by the rate constants of the opposing reactions, $\frac{K_o I}{[Q_o]}$ for the production of QH, and $k_a [A]$ for the oxidation of QH.

The concentration of A is not constant; it decreases as A is reduced by QH. Any decrease in $[A]$, by equation (21), causes the rate of approach of $[QH]$ to its final value to slow down, and causes the final value to increase. If $[A]$ decreases to zero, then the final value of $[QH]$ is $[Q_o]$. The important feature of equation (21) is that if $[A]$ only decreases, $[QH]$ cannot ever decrease and permit a dip. Since the dip is an experimental fact, it must not be true that $[A]$ only decreases. (Only if Q has more than two forms can this conclusion be avoided. The situation when Q has more than two forms is discussed below in part 2 of this section.)

According to equation (21), $[QH]$ will decrease if $[A]$ increases. An increase in $[A]$ may occur in the series scheme of photosynthesis by two mechanisms: one is oxidation of AH by dark reactions with substances at a more positive redox potential. In chapter V, we have presented evidence that such dark oxidation does not completely oxidize AH even after 30 minutes of dark. This mechanism, therefore, is slow and/or ineffective. The other mechanism of oxidation of AH is

the action of system I. We conclude that the dip must involve an increase in $[A]$. The increase is most likely due to oxidation of AH by system I. (The conclusion about the dip is based here on the mathematical analysis. The same conclusion was reached in chapter VI on the basis of experimental evidence.)

With this conclusion we can write a rate equation for $[A]$. We assume that system I oxidizes AH by a first order reaction, whose rate is $k[AH]$. Then,

$$\frac{d}{dt}[A] = -k_a[A][QH] + k[AH] = -k_a[A][QH] + k([A_o] - [A].) \quad (22)$$

where $[A_o] = [A] + [AH]$. Equations (20 and (22) must be solved simultaneously. Unfortunately, the equations are nonlinear,* and cannot be solved easily. The analytical methods and analogue computer methods used with such equations require knowledge of specific values for the constants (see ref. 10), if the explicit behaviors of $[A] = f(t)$ and $[QH] = g(t)$ are to be determined. In general, such equations lead to a variety of behaviors, only sometimes periodic and/or stable. An interesting question is whether the initial oxidation of AH by system I greatly lags reduction of A by system II, and whether a lag is the main factor in shaping the dip. We do not answer this question for the following reason. It has been shown for some systems, whose equations are more simple than equations (20) and (22), that the

*Technically speaking, it is the differential equation obtained by dividing either of equations (20) and (22) by the other which is nonlinear.

dependent variables cause cyclical variations in each other, and that there is hysteresis in the relationship between them. A good example is Volterra's problem of the growth of two conflicting biological populations (for the solution of Volterra's problem see ref. 10, pp. 102-109). Because hysteresis naturally arises in some systems, presumption of a special initial lag to explain the dip is unwarranted without a thorough study of equations (20) and (22).

2. Linear Systems

The difficulty of getting explicit solutions to the non-linear equations led to a search for suitable linear equations. We analyzed various systems of chemical reactions whose rate equations are linear.

i) A Linear System with Three Components

A simple reaction system which is well-known is the sequence

$$X_1 \longrightarrow X_2 \longrightarrow X_3 \longrightarrow \cdots \longrightarrow X_n. \qquad (23)$$

The solutions for n (arbitrary) consecutive reactants were first obtained by Rakowski (85). The solution of the rate equation for $[X_2]$ is the most interesting to us, because it shows that $[X_2]$ increases from zero to a maximum and then subsides back to zero. The rise and fall are grossly similar to the O - I - D phase of the fluorescence transient.

To make the similarity greater, $[X_2]$ must fall not to zero but to some level above zero. System (23) can be modified to produce this behavior in $[X_2]$. The modified system is

$$X_1 \longrightarrow X_2 \rightleftharpoons X_3 \,. \tag{24}$$

Another variant of system (23) is

$$X_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} X_3. \tag{24a}$$

The rate equations for these systems may be solved without difficulty. We obtained the solutions, and then discovered that the same system (24a) had already been discussed several times in the chemical literature. A thorough analysis of system (24a) was published by Lowry and John (70). Our solution for $[X_2]$ is

$$[X_2] = X_{2m}\left\{ - \frac{m_2(k_3-m_1)}{k_3(m_2-m_1)} e^{-m_1 t} + \frac{m_1(k_3-m_2)}{k_3(m_2-m_1)} e^{-m_2 t} + 1 \right\}$$

where

$$X_{2m} = \frac{k_1 k_3}{k_2 k_4 + k_1 k_3 + k_1 k_4}, \qquad m_{1,2} = \frac{1}{2}(p \mp q),$$

$$p = k_1 + k_2 + k_3 + k_4, \text{ and}$$

$$q = (p^2 - 4\{ k_2 k_4 + k_1 k_3 + k_1 k_4 \})^{1/2}. \tag{25}$$

The solution for $[X_2]$ has the following properties. The steady-state value is not zero. The transient in $[X_2]$ preceding the

steady-state depends on the ratio of k_1 to k_3. If $k_1 > k_3$ (and k_2 is small) then $[X_2]$ rises from zero to a temporary maximum which is higher than the steady-state value.

The behavior of $[X_2]$ when $k_1 > k_3$ is similar to the O - I- D phase of the fluorescence transient. We may consider, then, whether the elements of the system (24a) can be identified with elements of the mechanism of system II. Specifically, X_2 must be identified with QH.

ii) The Joliot Model

During our consideration of system (24a), a review of Kok and Cheniae (57) presented a novel arrangement of a portion of the Joliot (46) model, the portion for the mechanism of system II. It was suddenly evident to us that this portion of the Joliot model is homomorphic with system (24a) by a relabeling of X_1, X_2, and X_3, as Q_1H, QH, and Q.

Only the system II portion of the model by Joliot will be considered in terms of system (24a). We avoid the rest of the model because it includes nonlinear interactions. Even the inclusion that [A] varies with time introduces nonlinearity which is unmanageable at the present time. The equations which can be written for the model in its entirety can only be solved by numerical integration, or portrayed by an analogue computer. Both methods require the assumption of values for the numerous rate constants, total concentrations, and initial concentrations. Until nearly all of these values seems well-established experimentally, it is not useful to mathematically test the model by

comparing its predicted fluorescence curves with the experimental transient.

The condition $k_1 > k_3$ which is necessary for the maximum in $[X_2]$ may be achieved in the Joliot model by a high light intensity. Thus, if system (24a) is homomorphic with the mechanism of system II, then a higher light intensity should make the hump I and the subsequent dip more noticeable. We find that under normal conditions the prediction cannot be tested. We have observed that the higher light intensity causes a higher P and a shorter t_p, and the rise to P eliminates the dip. However, Delosme (11) found that in extremely high light intensity (about 10 to 100 times our highest intensity), a clear I - D phase appears as intensity increases. Delosme did not have an interpretation for this finding. We suggest that it could be explained by system (24a).

If P can be eliminated, then the O - I - D phase can be observed free from the rise to P. It then will be possible to test the prediction that higher light intensity accentuates I and D. Our success in eliminating P with methyl viologen (chapter VI) shows that elimination of P is possible, but we have not yet experimented with high light intensity in the presence of methyl viologen.

We have a reservation about identifying X_1, X_2, and X_3 as Q_1H, QH, and Q in order to explain the dip. The reservation is based on the simultaneous measurements by Joliot (47) of rate of oxygen evolution and fluorescence during the fluorescence phases O, I, and P. The measurements show that preilluminated Chlorella show the fluorescence phases O, I, and P, but do not show an activation phase in oxygen

evolution. The lack of an activation phase indicates that the preillumination has converted all $Q_i H$ to the active forms QH and Q. Since the usual fluorescence phases appear when $Q_i H$ is apparently not present, it seems unlikely that I and D are due to the interaction of $Q_i H$ with QH and Q.

This reservation applies only to the identification of system (24a) with the Joliot model. It is still possible that a form Q' such as suggested by Duysens and Sweers (19) may participate in D according to system (24a).

We remarked in part 1 of this section that if Q had only two forms, then the dip required that [A] increase after the point I. However, if Q has three forms connected as in system (24a), then [A] need not increase for the dip to occur. For any value of [A] allowing $k_1 > k_3$, the dip will occur.

iii) The Malkin Model

A different linear system has recently been proposed by Malkin (71). Its novelty lies in the assumption of a chemically first-order interaction between QH and A. This assumption allows Q and A to be treated mathematically as a single entity, Q, A. The sequence of reactions is:

$$Q, A \xrightarrow{r} QH,\ A \xrightarrow{k} Q,\ AH \xrightarrow{r} QH,\ AH \qquad (26)$$

The steps with the rate constant $\underline{r}$ apply to the photochemical reduction of Q, and the step with the rate constant $\underline{k}$ applies to the oxidation of QH by A. Malkin has solved the differential equations of this system.

With the use of the solutions and equation (13) above, he obtained a successful match between the predicted and the experimental fluorescence transient for isolated spinach chloroplasts. The transient shows a hump similar to I, but does not show a dip.

We are interested in whether this system allows a dip. Analysis shows that it does not. The proof follows.

The solutions by Malkin employ the labels n_1 for the species $[Q, A]$, n_2 for $[QH, A]$, n_3 for $[Q, AH]$, and n_4 for $[QH, AH]$. The sum of all the species is a constant, n_o. Under the conditions that at $t = 0$, only n_1 is present, and that $k \neq r$, the solutions are:

$$n_1 = n_o e^{-rt}$$

$$n_2 = \frac{n_o r}{k-r} (e^{-rt} - e^{-kt})$$

$$n_3 = \frac{n_o kr}{k-r} t e^{-rt} - \frac{n_o kr}{(k-r)^2} (e^{-rt} - e^{-kt})$$

$$n_4 = n_o - (n_1 + n_2 + n_3). \qquad (27)$$

The case $k = r$ is a singularity for which equations (27) are not valid. This case will receive special attention later.

Malkin employed equation (13) in his analysis. We have pointed out that this equation may be incorrect concerning the detailed relation between $[QH]$ and fluorescence, because it assumes that system II units are separate, an assumption made unlikely by observations of Morin (75) and Delosme (11). However, we know that fluorescence monotonically follows $[QH]$, which is the crucial fact to our discussion

of the dip. Equation (13) incorporates this monotonic feature and is therefore adequate. We replace $\frac{F_r - F_o}{[Q_o]}$ in equation (13) by $\underline{b}$ and obtain

$$F_2 = F_o + b\,[QH]. \tag{28}$$

$[QH]$ is equivalent to $[QH, A] + [QH, AH] = n_2 + n_4$ in Malkin's model. We substitute for $[QH]$ in equation (28) to obtain:

$$F_2 = F_o + b\,(n_2 + n_4). \tag{29}$$

The dip requires that the two points I and D of the transient have zero slope. Between I and D, the slope of F_2 must be negative. In addition, there will be, by definition of the steady-state, a zero slope when $t \to \infty$. At points of zero slope,

$$\frac{d}{dt}\,(F_2) = 0. \tag{30}$$

From equation (29):

$$\frac{d}{dt}(F_2) = b\,\frac{d}{dt}(n_2+n_4) = b\,\frac{d}{dt}\,(n_o - n_1 - n_3)$$

$$= b\,\frac{d}{dt}\left[n_o - n_o e^{-rt} - \frac{n_o kr}{(k-r)}\,t\,e^{-rt} + \frac{n_o kr}{(k-r)^2}\,(e^{-rt} - e^{-kt})\right]$$

$$= b\left[r n_o e^{-rt} + \frac{n_o kr^2}{(k-r)}\,t\,e^{-rt} - \frac{n_o kr}{(k-r)}\,e^{-rt} - \frac{n_o kr^2}{(k-r)^2}\,e^{-rt} + \frac{n_o k^2 r}{(k-r)^2}\,e^{-kt}\right]. \tag{31}$$

We factor $\frac{n_o r e^{-rt}}{(k-r)^2}$ and obtain:

$$\frac{d}{dt}(F_2) = b \frac{n_o r e^{-rt}}{(k-r)^2} \left[(k-r)^2 + krt(k-r) - k(k-r) - kr + k^2 e^{-(k-r)t} \right]$$

$$= b \frac{n_o r e^{-rt}}{(k-r)^2} \left[-2rk + r^2 + krt(k-r) + k^2 e^{-(k-r)t} \right]. \tag{32}$$

The exponential term outside the parentheses in equation (32) insures that $\frac{d}{dt}(F_2) \longrightarrow 0$ as $t \longrightarrow \infty$, fulfilling the requirement that F_2 is constant in the steady-state. Let R stand for the terms inside the parentheses:

$$R = -2rk + r^2 + krt(k-r) + k^2 e^{-(k-r)t}. \tag{33}$$

If $\frac{d}{dt}(F_2)$ is to be negative for any finite t (in order to allow a dip), then R must be negative at some finite t, because the terms other than R are positive for finite t. We investigate R by examining its derivatives with respect to time:

$$\frac{d}{dt}(R) = kr(k-r) - k^2(k-r)e^{-(k-r)t} \tag{34}$$

$$\frac{d^2}{dt^2}(R) = k^2(k-r)^2 e^{-(k-r)t}. \tag{35}$$

$\frac{d^2}{dt^2}(R)$ is positive for finite t. Therefore, the condition $\frac{dR}{dt} = 0$, if satisfied for any finite t, will indicate a minimum in R.

$\frac{d^2}{dt^2}$ (R) changes exponentially, as well as being positive for finite t. Therefore, the function $\frac{dR}{dt}$ is monotonically increasing for finite t.

$\frac{dR}{dt}$ is negative at t = 0, since

$$\frac{dR}{dt}_{t=0} = -k(k-r)^2. \tag{36}$$

However, at large t, $\frac{dR}{dt}$ is positive, since

Case I

$$k > r: \quad \lim_{t \to \infty} \frac{dR}{dt} = kr(k-r), \tag{37}$$

and

Case II

$$k < r: \quad \lim_{t \to \infty} \frac{dR}{dt} = \infty. \tag{37a}$$

Since $\frac{dR}{dt}$ is negative at t = 0 but positive at large t, and is changing monotonically, it is zero at one intermediate value of t (call it t_m).

At t_m the function R is a minimum. Is the minimum of R negative, allowing a dip? The minimum in R occurs when $\frac{dR}{dt} = 0$. From $\frac{dR}{dt} = 0$, we get

$$t_m = \frac{1}{k-r} \ln\left(\frac{k}{r}\right). \tag{38}$$

R at t_m is

$$R_{t_m} = -2kr + r^2 + kr \ln\left(\frac{k}{r}\right) + k^2 e^{-\ln\left(\frac{k}{r}\right)}$$

$$= r^2 + kr \ln\left(\frac{k}{r}\right) - kr$$

$$= r^2 - kr\left[1 - \ln\left(\frac{k}{r}\right)\right]. \qquad (39)$$

We investigate whether R_{t_m} is negative for any values of r and k. The two cases, $k > r$ and $k < r$, are examined separately.

Case I

$k > r$:

We rearrange R to get

$$R_{t_m} = r^2 + kr\left[\ln\left(\frac{k}{r}\right) - 1\right]. \qquad (40)$$

If $k \geqslant er$, where e is the base of Napierian logarithms,

$$\ln\left(\frac{k}{r}\right) - 1 \geqslant 0, \text{ and } R_{t_m} \geqslant r^2 \text{ and is positive.} \qquad (41)$$

The rest of the domain of k is $r < k < er$. The upper value as we have just seen gives positive $R_{t_m} = r^2$. The limit of R_{t_m} for the lower value is approached monotonically from the positive value r^2 and is

$$\lim_{k \to r} R_{tm} = O^+. \qquad (42)$$

Therefore for $k > r$, R_{t_m} is never negative, $\frac{d}{dt}(F_2)$ is never

negative, and F_2 cannot show a dip.

Case II

$k < r$:

We rearrange R_{t_m} by changing $\ln\left(\frac{k}{r}\right)$ to $\ln\left(\frac{r}{k}\right)$:

$$R_{t_m} = r^2 - kr\left[1 + \ln\left(\frac{r}{k}\right)\right]. \tag{43}$$

For R_{t_m} to be negative, we must have

$$r^2 < kr\left[1 + \ln\left(\frac{r}{k}\right)\right].$$

Or, by dividing through by kr,

$$\frac{r}{k} < 1 + \ln\left(\frac{r}{k}\right). \tag{44}$$

The lower limiting value of $\frac{r}{k}$ is arbitrarily close to 1. For $\frac{r}{k} = 1$, $\ln\left(\frac{r}{k}\right) = 0$, and the inequality cannot be satisfied. For $\frac{r}{k} > 1$ and increasing, the left-hand side of the inequality increases faster than the right-hand side, because by differentiating each side with respect to $\frac{r}{k}$, we have that

$$\frac{d}{d\left(\frac{r}{k}\right)}\left[\frac{r}{k}\right] = 1 > \frac{d}{d\left(\frac{r}{k}\right)}\left[1 + \ln\left(\frac{r}{k}\right)\right] = \frac{1}{\frac{r}{k}}. \tag{45}$$

Since the inequality (44) is not satisfied for $\frac{r}{k} = 1$, and its left-hand side increases faster than the right, as $\frac{r}{k}$ increases beyond 1, the inequality is never satisfied. Therefore, for $k < r$, R_{t_m} is never

negative, $\frac{d}{dt}(F_2)$ is never negative, and F_2 cannot show a dip.

The only remaining case is $k = r$. For this case, denominator terms in equations (27) vanish, the equations are no longer valid, and new solutions must be obtained. They are:

$$n_1 = n_o e^{-rt}$$

$$n_2 = r n_o t e^{-rt}$$

$$n_3 = \frac{r^2 n_o t^2}{2} e^{-rt}$$

$$n_4 = n_o - (n_1 + n_2 + n_3). \qquad (46)$$

As before, we find $\frac{d}{dt}(F_2)$:

$$\frac{d}{dt}(F_2) = b n_o r e^{-rt}\left[1 + \frac{r^2 t^2}{2} - rt\right]. \qquad (47)$$

The factor e^{-rt} insures that $\frac{d}{dt}(F_2) \to 0$ as $t \to \infty$. For finite time, $\frac{d}{dt}(F_2)$ can be zero only if the term inside the brackets becomes zero for some value of t. By a procedure analogous to the analysis of R of equations (32) and (33), we have determined that the minimum value of the term inside the brackets is + 1/2, at the time $t = 1/r$. Thus, the term is never zero. The same conclusion is reached by directly solving the term for t, assuming that the term equals zero. Then,

$$1 + \frac{r^2 t^2}{2} - rt = 0. \qquad (48)$$

By the quadratic formula,

$$t = \frac{1}{r} \pm \frac{i}{r} \quad . \tag{49}$$

The permitted solutions for t are complex. Therefore, no real finite time allows $\frac{d}{dt}(F_2)$ to vanish. Consequently, for $k = r$, F_2 cannot show a dip.

Thus, we have shown that for all cases: $k > r$, $k < r$, and $k = r$, F_2 cannot show a dip.

The consequence of this conclusion is that the Malkin scheme cannot be used to explain the dip in the fluorescence transient of Chlorella pyrenoidosa. Two important qualifications must be added. First, the Malkin scheme was proposed to explain the transient in isolated chloroplasts, not in intact algae. The scheme may still correctly explain the isolated chloroplast transient. Second, we cannot conclude that the Malkin scheme is completely inapplicable to the algal transient -- our only consideration was whether the scheme could explain a particular feature of the algal transient, the dip. It is conceivable that the scheme is applicable, that is has nothing to do with the dip, and that the dip is explainable as a lagging production of A by system I.

E. Summary

Fluorescence above the point O is emitted by system II from those photosynthetic units where Q is reduced. In applying rate equations

and quantum yield expressions to photosynthetic units, a distinction must be made between bulk and trap molecules. The promising approaches reported so far make this distinction via expressions of trapping probability. The trapping probability depends on the redox state of Q; the probability is near unity in those units where Q is oxidized; the probability does not equal zero in a unit where Q is reduced, because of energy transfer to other units where Q might be in the oxidized state. Fluorescence monotonically follows $[QH]$.

Analysis of the dip of the fluorescence transient, for the case where Q has only two forms, suggests that the dip must involve a sudden increase in the concentration of A. The increase is probably due to oxidation of AH by system I. The equations which are written on this basis are nonlinear and cannot be solved easily. A thorough study of the equations might be fruitful.

A search was made for linear equations which might explain the dip. The search produced a reaction scheme where Q has three forms connected by chemically first-order reactions. Analysis of the Malkin model showed that it cannot explain the dip.

The variety of models allowed by the experimental data must be reduced before it will be possible to use mathematics to obtain values of rate constants and reactant concentrations. The present utility of mathematics lies in determining whether proposed models can explain particular features of the transient.

VIII. GENERAL REMARKS

A. Comparison of the Algal and Isolated Chloroplast Fluorescence Transients

Murata et al. (78) and Malkin and Kok (72) have analyzed the isolated chloroplast transient for concentrations of Q and A, and the rate constant for the QH/A reaction, while the algal transient has been analyzed by Delosme (11) for energy transfer probabilities. It is interesting that the isolated chloroplast and algal transients have been analyzed for different information. Moreover, the shapes of the transients are different: The isolated chloroplast transient has a two-phase monotonic rise to a steady-state, while the algal transient has a four-phase non-monotonic rise. One may wonder whether the mechanisms underlying the two transients are also different. We do not think so. First, Kautsky and Hirsch (53), Virgin (90), and Rosenberg et al. (86) have observed algal-like transients in leaves of higher plants. Second, the procedure of isolating chloroplasts from higher plants is known to cause oxygen production rates to be much lower than in intact leaves, and to cause rapid decay of these rates. Only recently have rates been obtained which are a large fraction of in vivo rates. Jensen and Bassham (43) recently announced that they have been able to obtain initial rates that are 60% of rates of intact leaves. Third, one effect of isolation is the loss of endogenous electron acceptors for system I. Malkin and Kok (72) confirmed this finding by fluorescence study. They reported that upon addition to isolated chloroplasts of $NADP^+$ plus ferredoxin, which are

electron acceptors for system I, the maximum fluorescence decreases. After a time proportional to the amount added, fluorescence rises back to its maximum. These observations suggest that the transient of chloroplasts is a modification of the natural transient shown by leaves and algae, and that the modification is caused by the isolation procedure.

B. Intermediates Reduced at P

We emphasized in the introduction that most investigators have until now thought that P of the algal transient is caused by the reduction of A and Q. The only exceptions are Kautsky et al. (50), who thought that P involved reduction of another intermediate as well as A and Q. Our conclusion is that X is also reduced at P.

This view of P does not invalidate conclusions by Malkin and Kok (72) concerning the amount of reduction of oxidants during the course of the isolated chloroplast transient. But we believe that the reason for the reduction is not the one they suggested. They suggested that system II for most wavelengths of illumination is more active than system I, and is therefore able to reduce Q and A despite oxidation by system I. We believe instead that the reduction proceeds because of the lack of electron acceptors for system I brought about by the isolation procedure. Malkin and Kok, themselves, present evidence for this view by reporting that addition of $NADP^+$ plus ferredoxin lowers the maximum fluorescence.

C. Unsolved Problems

i. Cause of the Anaerobic Effect; the Role of Oxygen

The effect of oxygen removal remains unexplained. Because partial effects can be achieved within four minutes, the effects are probably due to low oxygen concentration at the site of photosynthesis.

P involves reduction of X, we presume that the P-S decline involves oxidation of X. Because the P-S decline is retarded by partial anaerobiosis, we speculate that oxygen assists, either directly or indirectly, the oxidation of intermediates between system I and the carbon cycle.

O-I-D involves reduction of Q, and oxidation of QH by A. The effect of complete anaerobiosis in raising I suggests that very small amounts of oxygen are normally consumed in oxidizing QH.

ii. Numbers of Fluorescence Quenchers and Intermediates

Delosme (11) believes there is, in addition to Q, a second fluorescence quencher, R, which is converted to a non-quenching form during the I-P phase. His argument is: If only Q were operative, high intensity light would reduce all Q to QH before any oxidation of QH occurred, and fluorescence would rise smoothly from O to P without the hump I. However, I does not disappear as intensity increases. We suggest that the variation of intensity be tried under an anaerobic condition, because then I is a spike nearly as high as P, and the rise to I is smooth, suggesting that only one quencher is operative. At the moment, the possibility that P involves a second quencher is unappealing,

but it may be reconciled with our view of P.

Malkin and Kok (72) decided from the two phases of the isolated chloroplast transient that two components are undergoing reduction, while Murata et al. (78) decided that there are three components, because 3.2×10^{-7} moles/liter DCMU produced a three-phased transient. The DCMU studies should be repeated. If there are three components, Q, A_1, and A_2, perhaps Malkin and Kok measured the concentrations of A_1 and A_2 rather than Q and A_1. According to the results of Murata et al., $[A_1] + [A_2] = 7[Q]$. Being of smaller concentration, Q might escape detection under some circumstances.

The question whether or not Q has three forms is raised by the Joliot (46) model. Joliot proposed a third (inactive) form of Q to account for the lack of oxygen production by a saturating light flash after a dark period. This proposal may be obviated by the assumption that oxygen production requires interaction of two oxidized Z molecules (where reduced Z is the system II electron donor).

iii. Differences between our Chlorella and that of Delosme

An interesting difference exists between the transient of our Chlorella pyrenoidosa and the Chlorella of Delosme (11) (no species reported). At the saturating intensities (10^4 ergs/sec -cm^2 incident) used in our experiments, I appears at 50 msec and P at 500 msec. Delosme exposes his cells to such high intensity that I appears at between 0.5 to 5.0 msec, yet P appears later than 1000 msec. We do not know the reason for these differences. Any comparison of reports

from us and Delosme will be questionable until the reason for the differences is discovered.

IX. SUMMARY

The fluorescence transient of aerobic, dark-adapted Chlorella pyrenoidosa, excited by intense light, has a base level (O), and four sequent phases in the first second· a hump (I), dip (D), peak (P), and a decline to a steady level (S). In anaerobic cells, I is a spike, and there is no decline from P. We investigated the relation between the transient and the series mechanism of photosynthesis, where light absorbed in pigment system II transports electrons from water to system I, and absorption in system I transports them to a carbon cycle intermediate. The technique uses a phototube and oscillography. Experiments involved modification of aerobic and anaerobic transients via preillumination, background light, change in exciting light intensity, and addition of methyl viologen. Results fit the current hypothesis that fluorescence monotonically follows reduction of Q, the electron acceptor for system II. The analysis includes a survey of mathematical approaches and models.

D of anaerobic cells is hastened by addition of system I light during the phase O - I - D. In aerobic cells, a 100 msec flash of system I light during D - P hastens P. Methyl viologen, an exogenous acceptor for system I electrons, eliminates P. Hence, O - I - D represents balancing between reduction of Q by system II, and oxidation by system I via A, an intrasystem intermediate D - P represents depletion of a post-system I intermediate and the resulting rise in concentration of reduced Q.

The effect of preillumination (replacing darkness) depends on wavelength and intensity (we used 1 to 750 ergs/sec-cm^2 incident). In aerobic cells, preillumination 1 (preillumination of system I) is more effective than preillumination 2 in lowering and delaying I; in anaerobic cells, preillumination 1 is more effective in removal of I. These results confirm a system II-system I balancing at O - I - D.

Preillumination 1 lowers and delays I and P, and raises S. Preillumination 2 lowers I and S. It lowers P, but delays P only at low intensity; at higher intensity, it hastens P. The probable explanation is that preillumination 2 is absorbed by both systems; at weak intensity, portion (2) reaching system II is wasted by a back reaction, at high intensity, portion (2) prevents dark restoration of the transient, hastening P and lowering S.

The effect on P of system I preillumination persists for 15 seconds, suggesting that oxidized A is protected from strong reductants.

Fluorescence intensity versus light intensity curves for O, I, P and S show that as light intensity increases, fluorescence yield at P increases three-fold. The ratio of P of anaerobic cells to O of aerobic cells is six. These high ratios restrict the Franck-Rosenberg model of photosynthesis, where the permitted fluorescence increase is two-fold.

Mathematical analysis of D shows that if Q has only two forms, oxidized and reduced, D must involve a sudden increase in concentration of A. The increase is probably due to system I oxidation of AH.

Equations written on this basis are nonlinear and cannot be solved yet because reaction rate constants and reactant concentrations are not accurately known. Our search for linear equations which might explain D produced a reaction scheme where Q has three forms connected by chemically first-order reactions. Analysis of the Malkin model for the spinach chloroplast transient shows that it cannot explain D of Chlorella.

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