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MECHANISM OF TWO PHOTOCHEMICAL REACTIONS IN ALGAE AS STUDIED BY MEANS OF FLUORESCENCE

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1. By means of a new type of apparatus time curves of the fluorescence yield of photosynthetic pigments were studied at various wavelengths of emission and excitation for the red alga *Porphyridium cruentum*, the green alga *Chlorella*, spinach and spinach chloroplasts and a number of other species. The changes in fluorescence yield could be brought about by two actinic beams which did not directly cause a deflection of the recording apparatus. The apparatus was only sensitive to the modulated excitation beam.

2. In all species studied the fluorescence yield of the chlorophyll a_2 belonging to the photochemical pigment system 2 (which is responsible for oxygen evolution and cytochrome reduction), decreased upon illumination with actinic light mainly absorbed by the pigment system 1 (which causes cytochrome oxidation and pyridine nucleotide reduction) and increased upon illumination with light mainly absorbed by system 2. These and other experiments indicate that the decrease is caused by the oxidation of a reactive molecule or " reaction center ". The excitation energy is transferred from the chlorophyll a_2 molecules to these reaction centers and is trapped if the center is in an oxidized state which is designated by Q. Upon excitation of system 2 the reaction center becomes reduced and an unknown precursor of oxygen becomes oxidized. The reduced reaction center, QH, does not trap the excitation energy and thus does not quench the fluorescence of chlorophyll a_2, but is oxidized by a reaction excited by system 1. The inhibitor DCMU prevents the reoxidation of QH.

3. If the algae or spinach leaves are illuminated during more than a few seconds with light mainly absorbed by system 2, the initial rapid increase in chlorophyll a_2 fluorescence is followed by a decrease. This decrease is attributed partly to an increase in the rate of the reoxidation of QH by system 1 and partly to a dark side reaction of QH which converts QH into a compound Q′, which quenches the

Abbreviations: DCMU, 3-(3,4-dichlorophenol)-1,1-dimethylurea; PN, phosphopyridine nucleotide.

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fluorescence and which is slowly converted into Q by a dark reaction. The conversion of QH into Q′ is also inhibited by DCMU.

4. In the red alga *Porphyridium cruentum* and *Porphyra sp.* an increase in the steady state yield of fluorescence occurred not only in the chlorophyll *a* maximum, but also in rather broad bands at about 720 and 730 $m\mu$ respectively, upon switching from blue actinic light to green. In these algae blue light is mainly absorbed by system 1 and green light by system 2. The changes in fluorescence yield in the infrared bands were most pronounced for blue excitation which suggests that these bands are excited by pigment system 1. It is possible that the infrared fluorescing substance is the energy trap for system 1. No changes were observed in phycobilin fluorescence.

Action spectra of chlorophyll *a* fluorescence of various algal species showed that part of the energy of quanta absorbed by carotenoids and phycobilins was transferred to chlorophyll a_1. Since in red and blue-green algae quanta absorbed by the phycobilins were more efficient in exciting chlorophyll *a* than quanta absorbed by chlorophyll *a* itself, it was concluded that part of the chlorophyll is in a non or weakly fluorescent form, and did not, or to a lesser extent, receive energy from the phycobilins (*1, 2*).

Recently, mainly by means of sensitive absorption difference spectrophotometry, experimental evidence was obtained for the red alga *Porphyridium cruentum* that the fluorescent and the weakly-fluorescent chlorophyll *a* were active in photosynthesis in two different photochemical reactions (*3–5*). The action spectra for these photochemical reactions showed that the activity per incident quantum for the reaction driven by the fluorescent chlorophyll *a* was about three to seven times higher in light of 560 $m\mu$ (the maximum of phycoerythrin absorption) than at 680 $m\mu$ (the maximum of chlorophyll absorption). The corresponding activities for the other photochemical reaction were roughly equal at these two wavelengths (*4, 5*). The activity at 430 $m\mu$ is presumably roughly the same as that at 680 $m\mu$ for both reactions. We call the photochemical reaction driven by the weakly fluorescent form of chlorophyll *a* "reaction 1", and the reaction driven by the fluorescent form of chlorophyll *a* "reaction 2". The weakly fluorescent chlorophyll *a* that drives reaction 1 is called "chlorophyll a_1" and the phycoerythrin which transfers excitation energy to chlorophyll a_1 (and thus drives indirectly reaction 1) "phycoerythrin 1". Finally we call the total of pigments 1 "pigment system 1" or "system 1". System 2 is defined in an analogous way.

A variety of experiments can be explained in a simple way by the following scheme:

$$H_2O \rightarrow (\text{system } 2) \rightarrow Q \rightarrow \text{cyt.} \rightarrow P \rightarrow (\text{system } 1) \rightarrow X \rightarrow PN \rightarrow CO_2.$$

The arrows indicate the direction of hydrogen (or electron) transport. PN is phosphopyridine nucleotide; P is the substance the light-driven

oxidation of which is associated with a bleaching at 705 $m\mu$ (KOK), cyt. is an *f* or *c* like cytochrome (*4*, *5*). Q will be discussed in this paper. Photosynthetic phosphorylation (*7*), which may occur between system 2 and 1, provides the adenosine triphosphate which is, in addition to PNH, needed for the reduction of CO_2 by means of the CALVIN cycle. Schemes like this have also been given by other authors (*cf.* *5*).

Evidence for the validity of this scheme was obtained for the red alga *Porphyridium cruentum* (*4*, *5*) and for the blue-green alga *Anacystis nidulans* (*6*, *8*). Other evidence suggests that the scheme is valid for oxygen evolving organisms in general, the phycobilins being replaced by other accessory pigments (*5*). All organisms contain the fluorescent chlorophyll a_2 and the weakly or non-fluorescent chlorophyll a_1.

In light of 680 $m\mu$ and 430 $m\mu$ system 1 is more active than system 2 in *Porphyridium cruentum*: P and cytochrome, which in the dark are in the reduced state, become oxidized. In light of 560 $m\mu$, system 2 is more active: addition of relatively strong light of 560 $m\mu$ to relatively weak light of 680 $m\mu$ causes reduction of the oxidized cytochrome and of P. We shall call light of a wavelength distribution in which system 1 is more active than system 2 "light 1", and light in which system 2 is more active "light 2". A minor part of light 1 is in general also absorbed by system 2 and *vice versa*.

In *Porphyridium cruentum* and *Porphyra* light of about 680 or of 430 $m\mu$ was light 1, and light of about 560 $m\mu$ was light 2, and in *Anacystis* light of 680 or 430 $m\mu$ was light 1, and light of 620 $m\mu$ was light 2. In the green alga *Chlorella* and in spinach and spinach chloroplasts, light of shorter wavelengths than 680 $m\mu$ was light 2, and light of longer wavelengths than 680 $m\mu$ (we used light of 720 $m\mu$) was light 1. Light 1 causes the intermediates between system 1 and 2 to accumulate in the oxidized state, light 2 in the reduced state.

Basing ourselves on the scheme discussed, we have studied with a specially constructed apparatus the effect of light 1 and 2 on the fluorescence yield of chlorophyll and of other pigments, in the hope that it would be possible to obtain information about the photosynthetic reactions of intermediates which are in contact with fluorescent pigments such as chlorophyll a_2. These reactions may be the primary photosynthetic reactions.

The experimental results give among other things information about the presence and properties of a photosynthetic intermediate Q, which is closely associated with chlorophyll a_2 and may-be is the first substance which is reduced by system 2.

METHODS AND MATERIALS

Growth conditions

The unicellular algae were grown, in continuous light of fluorescent

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lamps, in vertical cylindrical tubes of about 3 *cm* diameter which were constantly bubbled with a mixture of air and about 4% carbon dioxide. The incident intensity given in the following is only approximate.

The red alga *Porphyridium cruentum* was grown in the medium of BRODY and EMERSON (*9*) at 19° at about 3.5 *k*lux, in light of Philips fluorescent lamps TL 32. A three to six days old suspension was concentrated to one of 6 volume per cent of packed wet cells by resuspension in fresh medium after centrifugation for 15 *min* at about $350 \times g$ in a swing-out rotor. Suspensions for other algae were prepared in an analogous way.

The blue-green alga *Anacystis nidulans* was grown three to four days in the slightly modified (*8*) medium C of KRATZ and MYERS (*10*), at 25° in light of 2.2 *k*lux provided by fluorescent lamps TL 34 and of 2.0 *k*lux of tungsten lamps. This alga was centrifuged for 15 *min* at $1400 \times g$. The concentration for measurement was 0.7 per cent by volume.

The green alga *Chlorella ellipsoidea* was grown for three days in the medium described by HASE, MORIMURA and TAMIYA (*11*) at 22° at 2.2 *k*lux provided by TL 34, and centrifuged for 15 *min* at $1400 \times g$; the final concentration was 4 per cent by volume.

Porphyra sp. was collected from the natural habitat, and was obtained from the Zoological Station at den Helder, the Netherlands. It was kept in seawater for a few days. The part of the thallus to be illuminated was stretched between the legs of a u-shaped piece of perspex, which was placed in a Beckmann cuvette of 1 *cm* thickness.

Spinach chloroplasts were prepared from market spinach according to modification of the method of JAGENDORF and AVRON (*12*).

Apparatus

Unless stated otherwise, the suspensions were measured in a cuvette of 1 *mm* thickness. The cuvette with algae, which had an area of about 1×3 *cm*, could be illuminated by means of 500 w 35 *mm* slide projectors. In each of these projectors the slide was replaced by a diaphragm, which was imaged about 1 to 1 upon the cuvette by means of a strong lens, that replaced the objective. Fluorescence from the cuvette was concentrated by means of a lens upon a photomultiplier (RCA 7326).

Interference filters in combination with glass filters were used to isolate relatively narrow wavelength regions. In Table I the filtercombinations are given. The intensity of the beams was adjusted by means of neutral glass filters or by varying the current through the projection lamps. One of the beams, which we call the fluorescence exciting beam, was interrupted with the frequency of the mains (50 *cps*) by means of a disc mounted on a synchronous motor. The modulated fluorescence due to this beam caused a pulsating photomultiplier current. Only the 50 *cps* component of the signal was amplified by an AC amplifier and, after rectification by a phase and frequency sensitive rectifier, recorded by a one second recorder. In

addition to the interrupted exciting beam two non-interrupted beams (continuous beams) could be used. These beams did not cause a deflection of the recorder, except for transient effects and for a small increase in the sensitivity of the photomultiplier. This increase was observed upon admission of a continuous beam of relatively high intensity, and was generally less than a few per cent. Actinic light is the total illumination. When the intensity of the exciting beam was negligible compared to that of the continuous beam, we some times called the latter the actinic beam. The average intensity of all beams could be measured by means of calibrated photocells. These intensities are in general expressed in units of 10^{-11} einstein/(*sec* cm^2); 10^{-11} einstein corresponds roughly to 20 erg. Saturation of photosynthesis occurs at room temperature for most species above several hundred times 10^{-11} einstein/(*sec* cm^2).

TABLE I

Filter combinations used for isolating the wavelength bands of the incident beams

Only the combination R 2 is used for isolating a fluorescence band. A Calflex filter was used in all combinations except R 1 and R 2. Glass filters and AL filters are from Schott, Mainz, the remaining filters from Balzers, Liechtenstein. B 430 means, filtraflex B 40, wavelength of maximum transmission 430 $m\mu$. BG 38–2 means BG 38 filter of thickness 2 *mm*. All filters were 50×50 *mm*. All numbers indicating wavelengths are in $m\mu$.

filters	B 1	B 2	B 3	G 1
interference	K 1	B 430	AL 475	K 4
glass	BG 38–2 BG 23–2 BG 14–3	BG 38–2 BG 3–1	BG 38–6	BG 38–2 BG 18–1
representative wavelength	420	430	475	550
half width	33	12	20	53

filters	G 2	R 1	R 2	R 3
interference	B 554	AL 718 B 720	B 689	AL 614
glass	BG 38–4	RG 8–2	RG 1–3	OG 2–2
representative wavelength	554	718	689	614
half width	12	20	12	20

RESULTS

Kinetics of fluorescence

The fluorescence time-curves were in general rather complicated and

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varied with species and with experimental conditions. The following two phenomena were found to occur without exception in all actively photosynthesizing organisms studied. These phenomena ware a) a decrease in fluorescence yield at the chlorophyll *a* maximum upon illumination with light 1, and b) an increase in yield upon illumination with light 2. The decrease is only observed if the fluoresence yield is above a base level. This higher level can be obtained by illumination with light 2. The increases and decreases in yield upon switching from light 1 to 2 and *vice versa* occurred rapidly within the indication of 1 *sec* of the recorder at an intensity of 50×10^{-11} einstein/(*sec* cm^2) or higher. In darkness or in very weak light the yield was in general at the base level, that is about the same as in light 1. Fig. 1 illustrates these phenomena for species of three

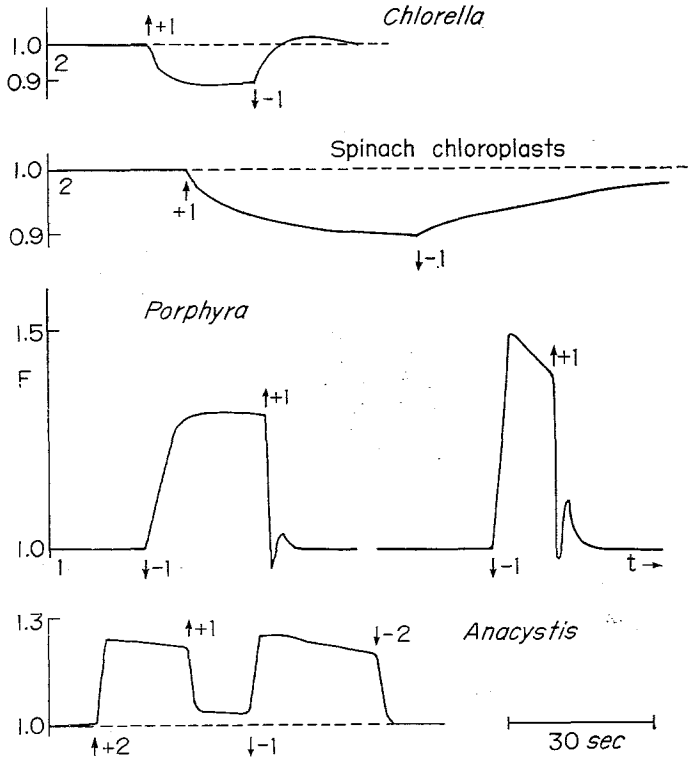

Fig. 1. Increases and decreases in the relative fluorescence yield at 689 $m\mu$ (filter R 2) upon switching from light 1 to 2 and *vice versa*. The moment of switching is indicated by the arrow. The intensities I of the incident beams are expressed in the table below in units of 10^{-11} einstein/(*sec* cm^2). The wavelength of maximum intensity of the incident beam or, if the band is broad, the wavelength which may represent the band in a first approximation are given in $m\mu$. The intensities are given in Table II.

TABLE II

species	exciting beam of light 2			continuous beams					
				light 1			light 2		
	filter	λ	I	filter	λ	I	filter	λ	I
Chlorella	B 3	475	66	R 1	718	40	—	—	—
Spinach chloroplast	B 3	475	5	R 1	718	20	—	—	—
Porphyra									
left curve	G 2	554	0.8	B 1	420	70	—	—	—
right curve	G 2	554	3.9	B 1	420	70	—	—	—
Anacystis	R 3	614	4.9	B 1	420	39	R 3	614	53

different groups of algae and for spinach and spinach chloroplasts.

As shown by comparison of the left and right hand tracing for *Porphyra* the rate of increase upon switching from light 1 to 2 is higher at higher intensities of light 2. The rate of increase upon shutting off light 1 can be considerably enhanced by admitting at this moment strong continuous light 2. If after switching off the light 1 no light at all is given, the fluorescence yield remained low in the dark in most experiments. This was concluded from the observation that after this dark period the fluorescence yield in exciting light 2 started to increase from a low level.

We observed that the effect on the fluoresence time course was the same for continuous light as for light of the same average intensity interrupted with the frequencies 30 and 80 *cps.*, which frequencies were not transmitted by the rectifier. Thus the actinic effect of the fluorescence exciting light, which is interrupted at 50 *cps.*, may be concluded to be the same as that of continuous light of the same wavelength and average intensity.

The fluorescence time curves often showed a pronounced maximum when switching from light 1 to 2. This is illustrated by Fig. 2. The approach from the level O to peak P is often found to occur in two or more phases (see bottom tracing). The fluorescence yield drops relatively slowly (in about 10–20 *sec*) from the peak P to a steady state value S. S is in some experiments not very much above the base level O as is shown by the top tracing of Fig. 1. The latter yield is the minimum yield observable under various conditions. These letters are the same as those used by LAVOREL (*13*) for describing the fluorescence induction curves of *Chlorella* observed when illuminating after a period of darkness with (as we call it) light 2.

Fig. 2 (top tracing) also shows that, after switching in the steady state from light 2 to 1, illumination during only a few seconds with light 1 is not sufficient to restore the potentiality for showing a rapid pronounced

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increase in fluorescence upon switching back from light 1 to 2. Illumination during a somewhat longer time is necessary. A somewhat longer period of darkness in general has a qualitatively similar effect as illumination with light 1.

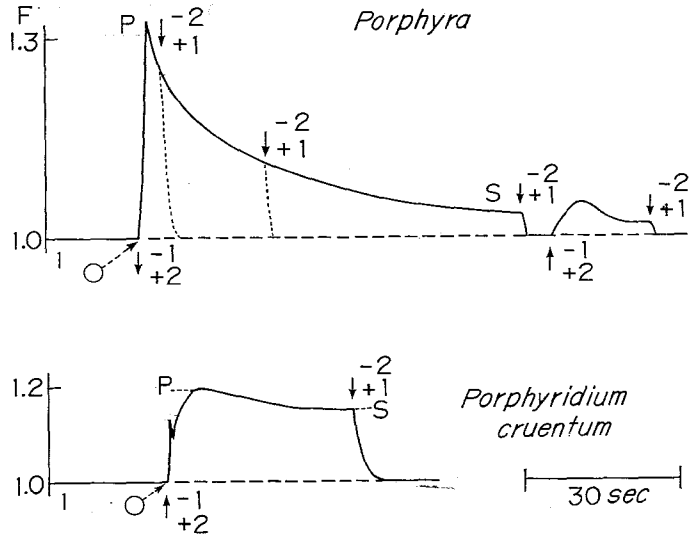

Fig. 2. Examples of time curves of the fluorescence at 689 $m\mu$ for two red algae upon switching from light 1 to light 2 and *vice versa*. The dotted lines were determined in separate experiments. Exciting light is 554 $m\mu$. The continuous beams are isolated by means of the filters B 1 (light 1) and G 1 (light 2); the intensities of these beams were for the top curve 60 and 70×10^{-11} einstein/(*sec* cm^2). The intensities for the bottom curve were about 10 times lower. The precise shapes of the time curves are in general different for different cultures of the same species.

In the presence of the inhibitor DCMU, which inhibits the reduction of cytochrome by system 2 but not the cytochrome oxidation by system 1 (*4, 5*), the fluorescence yield of chlorophyll a_2 in *Porphyridium cruentum* and *Chlorella* is high in the steady state, even at relatively low intensities: this yield was *e. g.* more than doubled at 2×10^{-11} einstein/(*sec* cm^2) of green light in *Porphyridium cruentum*, the species with which the following experiments were performed. We never observed a decrease in the fluorescence upon illumination with light 1, only an increase. Taking into account that the poison is strongly concentrated by the cells (by a factor of more than 20), we found that one DCMU molecule absorbed per about 130 chlorophyll *a* molecules (or per about 60 chlorophyll a_2 molecules), was sufficient to suppress the decrease after the maximum P in *Porphyridium* and *Chlorella* fluorescence. The fluorescence yield of *Porphyridium cruentum* poisoned with DCMU starts from the same base level O as in unpoisoned algae and rapidly increases even at low light intensities. The decrease in

fluorescence yield after darkening is slow. The number of quanta absorbed at 554 $m\mu$ necessary to bring about an appreciable increase in fluorescence was found to be approximately 1/150 of the number of chlorophyll a_2 molecules.

Spectral investigations

In order to facilitate analysis of the complicated fluorescence phenomena we used the following experimental procedure, which will be discussed for the red alga *Porphyridium cruentum*. Blue and green exciting beams of intensity 4.3×10^{-11} einstein/(*sec* cm^2), which were isolated by means of the filters B 2 and G 2, were used. The blue and green continuous beams of intensities 80 and 100×10^{-11} einstein/(*sec* cm^2) were isolated by means of the filters B 1 and G 1. The algae were illuminated alternately during a few minutes with the blue and green continuous beam (light 1 and 2 respectively) and the time course of fluorescence was recorded both in green and in blue exciting light at a number of wavelengths. At both exciting wevelengths fluorescence time curves, in general shape resembling those of Fig. 2, were observed. Since the exciting light is weak compared to the continuous light, the exciting light may be assumed to have only small effects on the fluorescence yield of the fluorescing compounds. If only one fluorescing substance would be the cause of the changes in fluorescence yield, then the time curves for the changes in fluorescence for the two exciting wavelengths would be proportional to each other. As we will discuss now, these time curves were not proportional. It follows that at least two fluorescing substances contributed to the fluorescence changes.

Comparison of the S curves at 689 $m\mu$ (close to the maxima) in Fig. 3 for green and blue excitation shows that the steady state for green excitation was about three times higher than that for blue excitation. However, comparison of the P-S curves shows that the height P-S of the peak above the steady state was seven times higher for green than for blue excitation.

Since the points of the P-S and S curves for either blue or green excitation were obtained from the same time curve at 689 $m\mu$, it is clear that at this wavelength the curves are not proportional. If the peak P-S is mainly caused by one substance, then it can be said that the action spectrum for the excitation of this substance is about 7 times higher in green light than in blue. Fig. 3 also shows that the fluorescence difference spectra for the peaks P-S shows maxima at about 685 $m\mu$, indicating that the fluorescence changes P-S at 689 $m\mu$ are mainly caused by chlorophyll *a*. The value of 7 for the ratio of green to blue indicates that the chlorophyll a_2 (of system 2) is responsible for the P-S changes; if chlorophyll a_1 would be responsible, a value not much different from 1 would have been observed (*cf. 1, 2, 5*).

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The spectra of the steady state changes S in Fig. 3 indicate that these spectra are also mainly caused by chlorophyll *a*. The lower ratio of 3 for the S spectra at 689 $m\mu$ may be explained by the assumption that in ad-

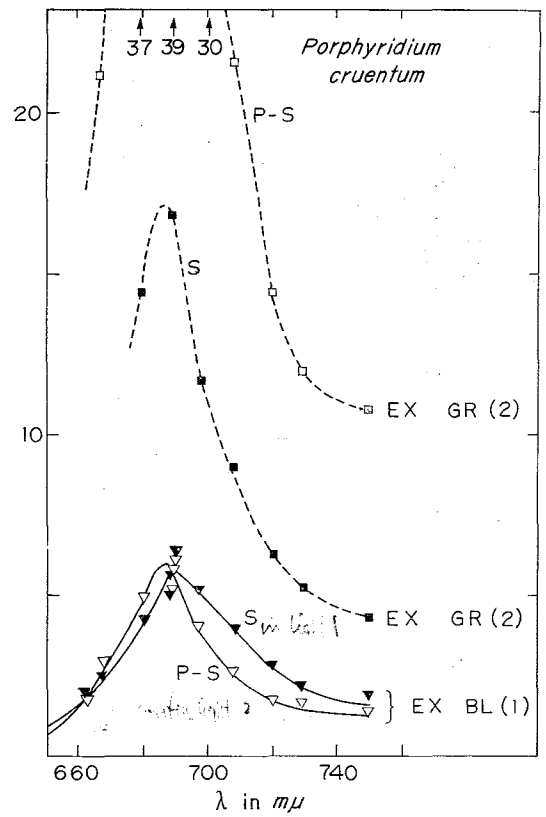

Fig. 3. Difference spectra for fluorescence. The points of the two curves for blue excitation (EXBL) were determined from the fluorescence time curve at the wavelength on the abscissa. This fluorescence time curve was obtained by switching from light 1 to light 2 as described in the text. The ordinate value of a point on the P–S curve is the height P–S of the peak above the steady state in light 2, and that of a point on the S curve is the height S of the steady state level above the base level 0 (in light 1). The two curves for green excitation were obtained in an analogous self explanatory way. The incident intensities for blue and green excitation in einstein/(*sec* cm^2) were the same. The intensity distribution for the fluorescence spectra was obtained with a calibrated tungsten lamp. The units used were einstein/(*sec* cm^2) per unit of wavelength interval. The letters P and S may designate not only points on the curves, but also the heights of these points above the base level 0.

dition to a change in chlorophyll a_2 fluorescence, change in chlorophyll a_1 fluorescence appreciably contributes to the steady state. A comparison of the S with the P-S spectrum for blue excitation indicate that a fluorescence

band is present in the S spectrum between 710 and 720 $m\mu$ and that the chlorophyll maximum is slightly shifted to longer wavelengths. In *Porphyra* a more pronounced band with a maximum at 730 $m\mu$ was found in the S spectrum for blue excitation, corresponding to the fluorescence maximum at 730 $m\mu$ observed earlier (*1*). These experiments indicate that in addition to the chlorophyll a_2, chlorophyll a_1 and presumably a pigment with a fluorescence band at 715 or 730 $m\mu$ show changes in fluorescence. The fluorescence of the two last mentioned pigments presumably are excited by light absorbed by system 1. The steady state fluorescence yield of these pigments decreases in blue (light 1) and increases in green light (light 2). Another perhaps less plausible interpretation is that the band at 715 $m\mu$ is caused by a second band of chlorophyll a_1.

After eliminating artifacts caused by changes in photomultiplier sensitivity, we were not able to detect changes in fluorescence attributable by phycoerythrin and phycocyanin when switching from light 1 to 2 and *vice versa*. Such changes, if they exist, were much smaller than those in chlorophyll *a*. FRENCH and YOUNG (*14*) found that changes in the fluorescence yield of chlorophyll a_1, which they observed in "old" cultures of *Porphyridium cruentum*, were not accompanied by changes in phycobilin fluorescence.

DISCUSSION

By various authors, amongst which KAUTSKY, FRANK and WASSINK and coworkers, numerous investigations have been made concerning the time course of the red fluorescence of photosynthetic organisms, mainly of *Chlorella* and higher plants. For a review of the earlier work we may refer to RABINOWITCH's monograph on photosynthesis (*15*). In various hypotheses it was implicitly assumed that only one photochemical pigment system was present and that the fluorescence changes were caused by chlorophyll *a*. The first precisely measured fluorescence spectra of *Chlorella* for exciting light of 480 and 430 $m\mu$ confirmed the assumption that mainly chlorophyll *a* was responsible for the red fluorescence (*2*). Our results indicate that at least two chlorophyll *a* types belonging to two different pigment systems, and perhaps other pigments contribute to the red fluorescence changes. Since in the earlier investigations in general (what we called) light 2 was used for excitation (and as actinic light) the main part of the fluorescence changes studied in these investigations was probably caused by chlorophyll a_2. The lack of agreement in the theoretical interpretations between the various earlier authors may at least partly have been caused by the incorrect assumptions mentioned. Only KAUTSKY had suggested in some of his papers the occurrence of two light reactions (but sensetised by one pigment system). These were presumably not generally accepted because they were only supported by indirect evidence from the kinetics

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of fluorescence. A recent paper of KAUTSKY *et al.* (*16*) contains a scheme, which is formally similar to ours.

The first direct experimental suggestion of a different effect on fluorescence of two light beams of different colors was obtained by GOVINDJEE *et al.* (*17*). These authors concluded from experiments with *Chlorella* that the total fluorescence caused by a far-red and red beam was smaller than the sum of the fluorescence intensities in each beam, and also that the fluorescence yield at both wavelengths was independent of intensity. No explanation was given of the phenomena. The first conclusion is consistent with our results. However, we find that the steady state fluorescence yield increases with increasing intensity of light 2. Such an increase is in our experience necessary for obtaining a subsequent decrease upon illumination with light 1.

Although the time curves of fluorescence varied strongly for various cultures even of the same species, the rapid increase and decrease in chlorophyll a_2 fluorescence upon changing from light 1 to 2 and *vice versa* occurred without exception in all organisms studied. This strongly suggests that these fluorescence changes reflect important photosynthetic reactions. The antagonistic effects of light 1 and 2 indicate that the hypothetical substance responsible for the fluorescence changes participates in the reactions between the pigment systems 1 and 2. According to our scheme this substance then must be oxidized by system 1. The decrease in chlorophyll a_2 fluorescence in light 1 then indicates that the substance in the oxidized form quenches the chlorophyll a_2 fluorescence. By an analogous reasoning it is concluded that it does not quench in the reduced form. This conclusion is consistent with the finding that the fluorescence yield strongly increases upon addition of the reducing agent hydrosulfite (sodium dithionite) to spinach chloroplasts.

We call the fluorescence quenching substance Q and the reduced form, which does not quench the chlorophyll a_2 fluorescence, QH. Then excitation of system 2 reduces Q to QH and excitation of system 1 reoxidizes QH. This "explains" the increases and decreases in chorophyll a_2 fluorescence upon illumination with light 2 and 1 respectively. Since in *Porphyridium cruentum* the absorption of only one quantum per one hundred chlorophyll a_2 molecules appears sufficient to reduce Q, the concentration of Q is only roughly 1/150 of chlorophyll a_2 if this reduction is a one quantum process and $1/(n\times100)$ if the reduction requires n quanta. Since Q quenches the chlorophyll a_2 fluorescence, Q is presumably closely associated with chlorophyll a_2.

In the presence of DCMU even upon illumination with weak light the chlorophyll a_2 fluorescence yield rapidly increases and remains high in the steady state, and system 1 is not able to decrease the fluorescence. This indicates that DCMU inhibits the reoxidation of QH by system 1. DCMU

in a small concentration (*18*) inhibits photosynthesis even at low light intensities (*19*).

The observations of the preceding paragraph can be understood, if it is assumed that light energy absorbed by chlorophyll a_2 or phycoerythrin 2 is transferred by induced resonance to a trapping center Q, present in a small concentration. If energy trapping centers are made inactive by photochemical reduction, the fluorescence yield of chlorophyll a_2 increases. The experimental results to be discussed can be explained by the assumption that a certain pigment molecule of system 2 can transfer its excitation energy to one, and only one, reaction center Q. This reaction center Q together with all the pigment molecules which are able to transfer Q, is a special model of a so called photosynthetic unit. The pigment system 2 thus consists of a number of such units, between which no energy transfer occurs. When more precise results will become available, modification of this simple model may become necessary. A rough theoretical estimation showed that transfer from chlorophyll *a* molecules to a "reaction center" is possible with good efficiency by induced resonance, if the ratio of the number of reaction centers to the number of chlorophyll molecules is not much less than 1/200 (*2*). This calculation was based, amongst other things on a reestimate of the fluorescence yield of chlorophyll *a in vivo*, which was assumed to be of the order of one per cent. This value of the yield was more recently confirmed by precise measurements (*20*). By means of a semi-experimental method the number of transfers between chlorophyll *a* molecules *in vivo* was estimated to be about 300 (*21*).

Since DCMU inhibits the oxidation of QH, but not the oxidation of cytochrome or P by system 1, and since this poison inhibits the reduction of cytochrome and P (*5*) but not the reduction of Q, DCMU probably inhibits a reaction between Q and cytochrome (see scheme in the introduction). Since photoreduction is not inhibited by DCMU (*19*), oxidized cytochrome or another component between Q and system 1 may react with the hydrogen. This is consistent with the observation that points of the action spectra for photoreduction of *Porphyridium cruentum* and *Ankistrodesmus* show that these spectra resemble more the action spectrum of system 1 than that of system 2 (AMESZ, VAN DONGEN: unpublished observations in this laboratory). In order to explain why DCMU inhibition of photosynthesis occurs also at low light intensities, and why DCMU appears to act on intermediates in the scheme except Q as if it puts out of action a part or the whole of system 2 (*4, 5*), we postulate that a DCMU molecule is absorbed close to the trapping center and inhibits the reoxidation of QH but not the photoreduction of Q. Thus one DCMU molecule puts one photosynthetic unit out of action.

KAUTSKY *et al.* (*16*) found that phenylurethane had a similar effect on the fluorescence as that which we reported for DCMU. DCMU. methylurethane and also hydroxylamine at not too high concentrations were found to inhibit

the reduction of cytochrome (*4*, *5*, *8*). Thus all these poisons may interfere with the reoxidation of QH by an intermediate oxidized by system 1. KAUTSKY *et al.* (*16*) concluded that the rate of increase in fluorescence in the presence of phenylurethane is proportional to a hypothetical substance, which is similar to Q in our scheme. The authors estimated from the time course of fluorescence of *Chlorella* that the ratio of the total amount of Q to total chlorophyll *a* was 1/400 or less. These observations are a support for the model of a photosynthetic unit discussed and were interpreted as such by KAUTSKY *et al.* Since in *Chlorella* perhaps a little less than half of the chlorophyll *a* belongs to system 1 (*cf.* *5*), the ratio Q/chlorophyll a_2 is somewhat less than 1/200.

Now we discuss the part OP of the flurescence time course in the absence of poisons. As stated in the section on results, the fluorescence does not in general increase monotonously to the steady state but, after a rapid increase, a second slower increase or a dip may occur in the curve, such as shown in the bottom curve of Fig. 3. KAUTSKY *et al.* (*16*) studied the dip in the OP curve and concluded that it was caused by a delayed light reaction. The *ad hoc* scheme of photosynthesis proposed to explain the results was partly similar to our hypothesis. Using the language of our scheme, KAUTSKY's suggestion was that system 1 produces, after a short delay, during the first seconds of illumination an oxidized substance which rapidly oxidizes QH. After a short time the light-driven oxidation of this substance stops (because of exhaustion of an intermediate), and the fluorescence rises to P. The drop occurring after P was left unexplained.

It was noticed a long time ago that increases and decreases in fluorescence may occur simultaneously with decreases and increases in the rate of carbon dioxide consumption (see the review by FRANCK and GAFFRON, *22*), but a precise quantitative relationship had not been established. DELOSME *et al.* (*23*), using JOLIOT's rapid apparatus for measurement of rate of oxygen evolution, presented experimental data which showed that upon illumination of *Chlorella* with constant light a precise inverse linear relation occurred between the rate of oxygen evolution and the intensity of fluorescence during the first part of the time curves.

From our model of a unit such an inverse linear relationship is readily derived. A formally similar derivation was given by DELOSME *et al.* If it is assumed that the reduction of Q is accompanied (with negligible time delay) by the production of oxygen, then the rate of oxygen production at a constant light intensity is proportional to the rate of reduction of Q which is in turn proportional to the concentration of Q, [Q]. According to our model the fluorescence intensity of chlorophyll a_2 is equal to $F = a[Q] + b[QH]$, in which a and b are constants and [QH] is the concentration of QH. The term a[Q] is the fluorescence of the units containing Q, and the term b[QH] of the units containing QH. Since we assume that [Q]+[QH] remains constant, it follows that the fluorescence F is a linear

function of [Q], and since the fluorescence increases when [Q] decreases, F must be an inverse linear function of [Q], and thus of the rate of oxygen evolution. It should be remarked that F is the fluorescence of chlorophyll a_2, but the inverse linear relationship remains valid if a constant fluorescence would be present in addition to that of chlorophyll a_2. As we have seen in the section on results, changes in the fluorescence of other substances also occur in the region of chlorophyll a_2 fluorescence, but these changes are presumably relatively small compared to those of chlorophyll a_2 for excitation by light 2 in the first seconds of illumination. We will see that after prolonged illumination [Q]+[QH] probably will not remain constant so that the inverse linear relationship neither will remain valid. Although we do not offer an explanation for the oxygen "burst" and carbon dioxide "gulp" accompanying the dip in the part OP of the fluorescence time course, the fact that these phenomena can be correlated with the hypotheses proposed is a support for our scheme.

The explanation for the kinetics of the part of PS of the fluorescence time curves requires additional hypotheses. LAVOREL (*13*) made a quantitative study of this part of the time curve under different conditions for *Chlorella*. The time curve was obtained by illumination with light 2 after a period of darkness. LAVOREL attributed the last decreasing part of the P-S curve to the conversion of the fluorescent chlorophyll into a a non-fluorescent and a non-photoactive form by a dark reaction with a rate depending on the concentration of the fluorescent form and on the temperature. He also proposed that the non-photoactive form was converted by a second dark reaction into the photo-active form. This explains part of the decrease in PS and also the requirement of a dark period to get a large increase OP. To explain the part PS we will use this part of his conclusion, but not his further suggestion that these reactions are in the main sequence of the reactions of photosynthesis.

We will give evidence that LAVOREL's dark reactions are side reactions. In the main hydrogen transport chain QH is reoxidized by system 1 as already stated. This reaction, which we have demonstrated to take place, was not considered by LAVOREL. It probably causes part of the decrease in the PS curve. If this reaction is assumed to be the only one, we can estimate the level S from the ratio of the steady state activities of the photochemical systems 1 and 2. This will be done for *Porphyridium cruentum*. At 560 $m\mu$ system 1 may be estimated to be roughly 30 per cent less active than system 2 (*5*). The rate of the reduction of Q by system 2 has to be lowered with about 30% to equal the rate of the reoxidation by system 1. This will happen if 30% of the total number of reaction centers Q is converted into the form QH. The level S which is proportional to [QH] then would be about 30% of the maximum attainable level which (*e. g.* in the presence of DCMU) is about twice the level O. However, the steady state level S was in general lower than 30% of the

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maximum attainable level (see *e. g.* top curve of Fig. 3). The low level S can be explained if it is assumed that QH can also be converted by a dark reaction into a fluorescence quenching non-photoactive form Q′. The reaction sequence may be simply written as QH→Q′→Q. Then the concentration of QH and thus the fluoresence will drop to a lower level, determined among other things by the rate of the dark reactions of QH to Q′. The sum of [Q′] and [QH] then remains 30%. From the right part of the curve given in Fig. 3 it can be concluded that upon switching in the steady state from light 2 to light 1, QH is oxidized within 1 *sec* and that during the subsequent 6 *sec* illumination with light 1 little Q′ is converted into Q, as indicated by the absence of a high peak upon switching from light 1 to light 2. We conclude that the dark reaction converting Q′ to Q is much too slow to be in the reaction sequence from system 1 to 2. Q′ traps the excitation energy of the unit which otherwise might cause photo-bleaching of the chlorophyll a_2 or other harmful effects. The main part of the "excess" QH, which might be harmful to the cells, is removed by conversion into Q′. The fact that DCMU not only inhibits the reoxidation of QH by system 1, but also the decrease in fluorescence occurring during prolonged illumination, indicates that DCMU also inhibits the dark conversion of QH to Q′.

It is possible to explain without further assumptions the rapid transient increase in rate of oxygen evolution observed when switching from light 1 to light 2 and the transient decrease when switching from light 2 to light 1 (*24*, *25*). The intensities at both wavelengths were adjusted in such a way that the steady states were about the same. The explanation of the rapid phases of these so called chromatic transients is as follows. In light 1 all reaction centers are in the state Q. Upon switching to light 2, the initial rate of photosynthesis, which is proportional to [Q], is high, but it decreases after a short time since some of the Q is converted into Q′. Immediately after switching back to light 1, system 2 limits the rate of oxygen evolution. This rate, which is initially relatively low due to the presence of Q′, increases since Q′ is converted into Q. This conversion takes place because the concentration of QH, from which Q′ is formed, is low in light 1. Similar but in general smaller effects may be expected if one switches from light 2 of one wavelength to light 2 of another wavelength at which system 1 and 2 absorb in different proportions. Such effects have been observed.

Concerning the measurement of photosynthesis in flashing light, we restrict ourselves to giving a few remarks about some fundamental observations. EMERSON and ARNOLD (*26*) discovered that in a succession of flashes of a duration of about 10^{-5} *sec* the yield of oxygen per flash at saturating intensity, and for sufficiently long dark periods between the flashes, was 1 oxygen molecule per about 2,000 chlorophyll molecules. Assuming that the quantum requirement per oxygen atom is 10, this means

that one photosynthetically active quantum is absorbed per about 2,000/10 =200 chlorophyll molecles, or per about 100 chlorophyll a_2 molecules. This suggests that during one flash the total amount of reducible Q can be reduced only once, and that thus the reoxidation of QH to Q takes longer than 10^{-3} *sec*. TAMIYA *et al.* (*27*) discovered that in flashes of the duration of the order of 10^{-3} *sec* the maximum yield per flash became much higher. This indicates that the reoxidation of QH occurs in less than 10^{-3} *sec*, perhaps in about 10^{-4} *sec*, so that Q can be reduced and reoxidized several times during 10^{-3} *sec*. KOK (*28*) confirmed these experiments with a precise technique both for long and short flashes. Since pigment system 1 too may behave analogously to system 2 in flashing light, the yield in the longer flashes may be expected to be a complicated function of the duration, intensity and color of the flashes, the duration of the dark periods and the state of the cells.

For purple bacteria an increase in the yield of bacteriochlorophyll flourescence was found to be quantitatively correlated with a bleaching in the absorption of a pigment P, which had a major absorption maximum at 880 $m\mu$, about coinciding with the maximum of bacteriochlorophyll (*29*). The functional relationship found and the rate of bleaching per absorbed quantum were interpreted as follows: P is a non-fluorescent pigment which traps the energy from the bacteriochlorophyll with an efficiency proportional to the concentration of unbleached P and with a quantum requirement of less than 3 (and possibly of 1) per bleached molecule P. The ratio P/bacteriochlorophyll is roughly 1/50. These observations suggest that in purple bacteria photosynthetic units based on the energy transfer model occur with P as a trapping center.

Spectral investigations

The constancy of fluorescence of the phycobilins in red and blue-green algae is consistent with the hypothesis that these pigments transfer their energy to the bulk of the chlorophyll *a*. Such a transfer to chlorophyll a_2 is demonstrated by the strong chlorophyll a_2 fluorescence upon excitation of the phycobilins (*1*, *2*, *14*). Because of the presence of the chlorophyll a_2 fluorescence, it was not possible yet to measure the action spectrum for the weak chlorophyll a_1 fluorescence and to demonstrate directly energy transfer from phycobilin 1 to chlorophyll a_1. However, the absence of fluorescence changes in the phycobilins and reasons of analogy argue against the hypothesis of a direct photochemical reaction of the phycobilins in system 1.

As remarked in the section on results, comparison of the fluorescence difference spectra indicated that changes in fluorescence occur with a maximum around 720 $m\mu$ in *Porphyridium cruentum* and around 730 $m\mu$ in a *Porphyra sp.* The fact that these changes appear more pronounced in blue than in green exciting light suggests that the fluorescence of these

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substances is excited by system 1. It was earlier suggested that energy was transferred from the weakly fluorescent chloroyhyll a_1 to a substance showing a fluorescence peak at 730 $m\mu$ in *Porphyra* (*1*). The changes in fluorescence then may be ascribed to an oxidative quenching of the fluorecence at 720 or 730 $m\mu$ upon exciting system 1 and a reductive restoration of this fluorescence by exciting system 2. It is possible that excitation of system 1 bleaches a fluorescing substance and thus eliminates its fluoresconce, and that illumination with light 2 restores the substance. If the latter interpretation is correct, the fluorescing component may be the one designated as P 700 by KOK (*6*), but evidence for this from parallel studies of the kinetics of the absorption changes of P 700 and the fluorescence at 720 $m\mu$ or 730 $m\mu$, is not yet available. In different groups of algae, including green and red algae, and also in concentrated solutions of chlorophyll BRODY (*30*) observed at a temperature of −160°, but not at room temperature, a strong fluorescence at 720 $m\mu$ which he attributed to a chlorophyll dimer. There is some evidence (*31–33*) that at room temperature fluorescence at 720 $m\mu$ also occurs in higher plants and also in green algae, which would suggest that the infrared fluorescence earlier observed in a few red and blue-green algae (*2*) is present in all chlorophyll *a* containing organisms. Studies by others on the kinetics of this fluorescence have not yet been reported. Although different lines of evidence suggest that the infrared fluorescing substances may participate in photosynthesis, and various hypotheses have been proposed, the kind of action of these subtances has not yet been established. In our opinion the relation between the fluorescence at room temperature in algae and the low temperature fluorescence in algae and in concentrated chlorophyll solutions is still uncertain.

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