A Second Chlorophyll Reaction in the Electron Chain of Photosynthesis — Registration by the Repetitive Excitation Technique —

G. Döring, H. H. Stiehl, and H. T. Witt
Max-Volmer-Institut, I. Institut für Physikalische Chemie der Technischen Universität Berlin


New absorption changes with a life time of $2 \cdot 10^{-4}$ sec at $\sim 690$ nm probably represent the chlorophyll-a light reaction in photosynthesis which promotes the cleavage of water.

Problems

Light absorbed in the far red limit of the absorption spectrum of green plants shows a poor yield of photosynthesis but is enhanced to much greater efficiency when it is supplemented by shorter wave length light. These two colour effects were a first hint to suppose 20 years ago that the primary reaction of photosynthesis may perhaps be sensitized by two pigments, a long wavelength and a shorter wavelength chlorophyll. — Since then, very different hypotheses have been postulated to explain how a cooperation of two pigments may occur.

The problem remained an open question until those substances which are in action during light excitation could directly be measured by absorption changes. Three techniques have been developed: a static method with continuous light excitation, a high sensitive single flash light method, and a repetitive flash light technique. Using the two colour effects in connection with measurements of the absorption changes, it has been possible to demonstrate directly the existence of two pigment systems coupled in series. Especially by the flash light methods, seven different types of absorption changes and the products and intermediates which cause these changes have been identified. The detailed kinetic analysis of the reactions of these substances between $10^{-6}$ and $10^{-1}$ sec leads to a reaction scheme depicted in fig. 1.

Electrons are transferred from water to NADP® through at least nine intermediates. The flow is generated by the excitation of a chlorophyll-a₁ and by the excitation of an unknown second pigment P₂₇. The promoting chlorophyll-a₁-reaction can be directly followed by absorption changes. The difference spectrum of these changes in the region of the typical chlorophyll absorption bands in the red is depicted in fig. 3. The life time is $2 \cdot 10^{-2}$ sec (22°C). Based on the measurements of action spectra, it was shown that chlorophyll-a₁ can be excited with wave lengths $\lambda < 730$nm, the pigment II, however, only with wave length $\lambda < 700$ nm (see fig. 1). Chlorophyll-a₁-430-703 is, therefore, the proposed long wave length chlorophyll-a. Pigment II must correspond to a shorter wave length chlorophyll-a with an absorption band just beyond 700 nm, probably with a maximum at $\sim 690$ nm.

A detailed description of the characteristics of the chlorophyll-a₁-reaction is given elsewhere. The reaction of the second pigment P₂₇, which is especially active in the cleavage of water has, however, not been discovered. The reasons are as follows.

References

Fig. 1. Reaction scheme of the electron transfer in photosynthesis of green plants. The heavy arrows indicate the direction of the main electron-flow from water to NADP⁺. The thin arrows correspond to the flow of electrons in chloroplasts under special conditions. NADP⁺ = Nicotinamide adenine dinucleotide phosphate, Fd = Ferredoxin, Z and Y = Experimentally detected but chemically unknown intermediates, Chl-α₁ = Chlorophyll-α₁, Cyt-f = Cytochrome-f, Cyt-b = Cytochrome-b, Q = Plastoquinone, Chl-b = Chlorophyll-b, PⅠ = Pigment II = Chlorophyll-α₂-690, ATP = Adenosine triphosphate, ADP = Adenosine diphosphate, P = Phosphate, CMU = N-p-Chlorophenyl-N’-dimethylurea, PMS = N-Methylphenazonium methyl sulfate, Asc = Ascorbate. The figures beside the symbols indicate the maxima of the absorption changes caused by flashing light. Also indicated are the times required for the various electron transfer processes at 20 °C. Details see 1. c. 12.

1. Absorption changes in photosynthesis are only of the order of 1/10 per cent. It depends on the life time of the changes whether they are masked by the random noise or not. As we estimated the reaction time for the electron transfer from water to plastoquinone Q as ~ 10⁻³ sec 12, 15 the reaction time of the second pigment PⅠ promoting this reaction must be of 10⁻⁴ sec or shorter. A life time of, for instance, ~ 10⁻⁴ sec would be ~ 100 times shorter than that of chlorophyll-α₁. This means that the corresponding absorption changes would be blanketed in a ~ 10 times greater noise.

2. In the region of the chlorophyll absorption bands between 650 – 720 nm, plants emit delayed light with half lives between 10⁻⁴ sec and minutes 16. The signal of these emissions is of the order of the absorption changes in photosynthesis and can interfere with them.

3. In the same range between 650 and 720 nm, there exists furthermore a strong chlorophyll fluorescence which causes a signal which, in the optical arrangements of fig. 6, is about 100 times greater than the absorption changes. The time course follows the excitation light flash (half life ~ 10⁻⁵ sec). But through the “tail” of the flash there is still some fluorescence at 10⁻⁴ sec. Therefore, an absorption change of for instance ~ 10⁻⁴ sec in the red region seems to be hopelessly “submerged” in noise, delayed light emission and fluorescence.

However, by the method of repetitive excitation through periodical light flashes the noise/signal-ratio can be decreased by a factor of 100. Furthermore, in view of the extremely high sensitivity of this technique, the fluorescence (and delayed light) can be compensated with high precision. Experimental de-

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13 B. Kok and W. Gott, Plant Physiol. 35, 802 [1960]; B. Kok, Acta bot. neerl. 6, 316 [1957].
tails are given below. The primary results of absorption measurements in the region between 670 to 720 nm with this technique are described in the following paragraphs.

**Results**

To eliminate the fluorescence (and delayed light) which interferes with the changes of absorption firstly the fluorescence (and delayed light) was measured without the absorption changes. This was done by exciting the suspension without measuring light, secondly, the fluorescence (and delayed light) was measured together with the absorption changes. This was done by exciting the suspension with the measuring beam "on". Both signals were subtracted from each other automatically in the averager and there remained on the recorder only the signal indicating the changes of absorption.

The time course of the changes at 690 nm are depicted in fig. 2 a. The kinetic is biphasic. The slow phase has a life time of $2 \cdot 10^{-2}$ sec, the fast one of $2 \cdot 10^{-4}$ sec.

Fig. 2 b indicates to what extent the 100 times greater fluorescence (and delayed light) can be compensated. For this demonstration, two different signals of fluorescence (and delayed light) were subtracted from each other. The remaining uncompensated signal is very small and short. This signal represents the uncertainty which is included in the time course of the absorption changes at 690 nm depicted in fig. 2 a.

The magnitude of the slow phase as a function of the wave length is depicted in fig. 3. The slow phase shows a difference spectrum with a maximum at 703 nm and indicates the well-known reaction of the long-wave-length chlorophyll-$a_1$-430-703.

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**Fig. 2 a.** Absorption changes as a function of time at 690 nm in a suspension of spinach chloroplast fragments. Chlorophyll concentration $0.94 \cdot 10^{-4}$ M/l, activity of $O_2$-production 88 $\mu$Mol(O$_2$/mg(Ch))$ \cdot $ h, temperature 22 °C. Additions: $10^{-4}$ M/l benzylviologene as electron acceptor and $2 \cdot 10^{-3}$ M/l NH$_4$Cl as phosphorylation decoupler and 0.05 M/l tris-buffer, pH = 7.2. Optical path length through the cuvette 1.4 mm. At the time $t=0$ an actinic flash was fired, duration $2 \cdot 10^{-5}$ sec, saturation intensity, wavelength 390—500 nm (colour glasses 4 mm BG 28, 2 mm GG 385, T 8 heat absorption filter). The band width of the measuring light was 7 nm and the intensity 500 ergcm$^{-2}$ sec$^{-1}$. The photomultipliers were protected against actinic light and fluorescence by double interference filters, colour glasses (4 mm RG 1) and a slit of only 2 mm width, which corresponds to the exit slit of the monochromator. 4096 flashes were fired with a frequency of 10 cps. Electrical band width 0.1 cps—13 kcps.

**Fig. 2 b.** Time course of the remaining signal at 690 nm which results from the not completely compensated 100 times greater signal of fluorescence (and delayed light). Details see text.

**Fig. 3.** Absorption changes with a life time of $2 \cdot 10^{-2}$ sec (see fig. 2 a) as a function of wave length in chloroplast fragments of spinach. The difference spectrum with a life time of $2 \cdot 10^{-2}$ sec is the well-known one which is caused by the reaction of chlorophyll-$a_1$-703-430. A new absorption change with a life time of $2 \cdot 10^{-4}$ sec (see fig. 2 a) has its maximum at $\sim 690$ nm. The difference spectrum is not precisely known and therefore only marked by lines. Optical path length through the cuvette 20 mm. Between 3000 and 12000 flashes were fired for each measuring point. For further details see fig. 2 a.

The fast phase is new and had not been previously observed. This phase has a maximum at shorter wave length, namely at $\sim 690$ nm. Above and below this wave length the magnitude of the fast phase is smaller. The fast phase must, therefore, be caused by a reaction of a shorter-wave-length chlorophyll-$a$. We call the new compound chlorophyll-$a_{II}$-690.
precise difference spectrum of this substance is as yet not known, because a third type of absorption change at $\lambda \approx 690$ nm with a half life between $2 \cdot 10^{-4}$ and $2 \cdot 10^{-2}$ sec complicates the analysis.

The magnitude of the slow and fast phases of the absorption changes, as a function of the light intensity, is shown in fig. 4. The intensity curve of the slow phase (caused by chlorophyll-a$_{11}$-430-703) shows a linear increase at low intensities and is intensity independent due to saturation at higher intensities. This curve corresponds in its absolute values to the light intensity curve of photosynthesis. The fast phase (caused by chlorophyll-a$_{11}$-690) shows a similar behaviour, which indicates that it must be functionally involved in the electron transport chain of photosynthesis.

If we assume for the extinction coefficient of Chl-a$_{11}$ that of chlorophyll-a in vitro ($\varepsilon = 10^5$ l/M·cm) and for Chl-a$_{11}$' $\varepsilon \approx 0$ or $\varepsilon = 7.5 \cdot 10^4$ l/m·cm respect, in relation to the bulk of chlorophyll the following ratio was estimated: chlorophyll-a$_{11}$-690 / total chlorophyll $\geq 1:400$ or 1:1000 respect. In relation to Chl-a$_{11}$ this means $\frac{\text{Chl-a$_{11}$}}{\text{Chl-a$_{11}$'}} \approx \frac{1}{4}$ or $\approx 1$ respect.

Because of the uncertainty in signal height after flash illumination (fig. 2 a) this estimation was taken only from the amplitude of absorption change 100 $\mu$sec after the flash. Measurements with higher time resolution will give a greater value. Such investigations are in preparation.

The question arises as to where the new chlorophyll-a$_{11}$-690 is functionally located in the electron chain of fig. 1. Four arguments support the assumption that chlorophyll-a$_{11}$-690 may be identical with the as yet unknown pigment II.

1. The fact that the reaction time of chlorophyll-a$_{11}$-690 is shorter than $10^{-3}$ sec ($\tau_{11} = 2 \cdot 10^{-4}$ sec) is just what one would expect for the reaction time of pigment II (see problems).

2. The maxima of the changes at 690 nm and 703 nm resp. indicate that chlorophyll-a$_{11}$-690 absorbs at shorter wavelengths than chlorophyll-a$_{11}$-430-703. This is a property which was already predicted for pigment II.

3. The electron flow from water to Q promoted by the reaction of the pigment II can be totally blocked by DCMU$^{17}$ (see fig. 1). Upon the addition of DCMU, the electron-flow and all absorption changes disappear (compare figs. 5 a and 5 b).

Under these circumstances, artificial electron donors such as ascorbate (with PMS as electron coupler), can supply chlorophyll-a$_{11}$-430-703 with electrons at positions indicated in fig. 1$^{12}$. Under these conditions, chlorophyll-a$_{11}$-430-703 should be reactivated, but not pigment II. Fig. 5 c shows the absorption changes of chloroplasts with the addition of DCMU + ascorbate (+ PMS). The slow phase indicating the reaction of chlorophyll-a$_{11}$-430-703 reappears in full, the fast phase, however, not. This result corresponds also to the assumption that the chlorophyll-a$_{11}$-690 is identical with pigment II.

4. Far red actinic light between $\lambda = 700 - 730$ nm excites only chlorophyll-a$_{11}$ and not pigment II. Therefore, with permanent far red background light "on",

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17 N. I. Bishop, Biochim. biophysica Acta [Amsterdam] 27, 205 [1958].
Fig. 5. Absorption change as a function of time at 690 nm in a suspension of spinach chloroplast fragments. Chlorophyll concentration $4.7 \times 10^{-5} \text{M}$. 8192 flashes were fired with a frequency of 10 cps. a) Additions as in fig. 2 a, b) additions as in fig. 2 a plus $2 \times 10^{-6} \text{M/l}$ DCMU, c) additions as in fig. 2 a plus $2 \times 10^{-6} \text{M/l}$ DCMU plus $10^{-5} \text{M/l}$ PMS and $10^{-3} \text{M/l}$ ascorbate. For further details see fig. 2 a.

Chlorophyll-a$_1$ is partially permanently oxidized. Therefore, the magnitude of the absorption changes of chlorophyll-a$_1$ caused by flashing light, should depend on the intensity of far red background light, but not the changes of pigment II. This effect has been proved on the absorption changes at 690 nm. The slower phase ($2 \times 10^{-2} \text{sec}$) which is caused by the reaction of chlorophyll-a$_1$ decreases with increasing far red light intensities. However, the magnitude of the fast phase ($2 \times 10^{-4} \text{sec}$) is not influenced by the background light.

More problems and results will be discussed in a later publication.

The periodical flash photometry apparatus

The technique of repetitive chemical excitation was developed since 1960. Since the signal/noise-ratio can be increased by the square root of the number of the measurements, a sensitivity more than 100 times greater than that of the sensitive single excitation ($0.1\%$ in $10^{-5} \text{sec}$) has been demonstrated by excitation with periodical light flashes complicated photochemical reactions could be analysed with excellent results. By excitation with periodical microwave impulses (causing periodical temperature jumps) also non-photochemical reactions have been investigated.

Details of the first type of periodical flash photometry using an electrical integrator were published in l.c. The time resolution is limited only by the flash duration ($10^{-6} \text{sec}$). During each excitation of the signal

![Diagram of the periodical flash photometer](image)

Fig. 6. Schematic block diagram of a second type of the periodical flash photometer. (For first type see l.c.) For details see text.


one amplitude is evaluated. A second type used since 1964\textsuperscript{18} and supplied with a commercial magnetic aver-
gager is depicted in fig. 6. In this case during each exci-
tation 1024 different amplitudes are registered. But this restricts the time resolution to $3 \times 10^{-5}$ sec.

Cuvette C contains the photoactive system and is located inbetween two monochromatic measuring beams $M_1$ and $M_2$. The measuring light is detected by two photomultipliers. The system can be excited firstly by periodical flashes $F_{11}$ of $10^{-6} - 10^{-5}$ sec duration with frequencies between 0 and 1000 cps, secondly by flashes $F_{12}$ of $10^{-2} - 1$ sec duration with frequencies between 0 and 10 cps, and thirdly simultaneously by steady light.

The light intensities can be measured by the cali-
brated thermopile TP. Colour filters, and interference filters F are additionally located inbetween the beams. All light sources are stabilized by transistors (not depicted). Absorption changes of the system produced by the periodical flashes cause periodical changes in the intensities of $M_1$ and $M_2$ which are recorded by the multipliers as changes in potential. By this means, ab-
sorption changes at two different wave lengths or fluorescence and absorption changes can be measured simultaneously.

Through low and high pass filters the potential changes are dc-amplified in $A_1$ and $A_2$. Out of the time course of each amplified potential change an averager (Enhancetron 800, Nuclear Data) picks up $s = 1024$ different samples which can be observed on the oscillo-
graph CRO\textsuperscript{1}. These 1024 samples are reproduced $n$-times by $n$-excitations through $n$-flashes, e.g. $n = 10,000$. All these data are accumulated and summarized in the averager. In this way the information increases linearly with $n$. The noise increases linearly with $\sqrt{n}$. Therefore the output of the averager gives a signal/ noise-ratio, which is increased by $\sqrt{n}$. The output signal can be observed directly on the oscillograph CRO\textsuperscript{2} or can be written on the recorder. A master generator triggers the Enhancetron and oscilloscopes. To registrate the zero-line before the signal, the flashing lights are fired with a delay. A detailed description of this apparatus will be published else-
where\textsuperscript{21}.