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Properties of the Photoactive Chlorophyll-a_{II} in Photosynthesis

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1. The complete difference spectrum of the reaction of the photoactive chlorophyll-a_{II} is presented.
2. The reaction of excited chlorophyll-a_{II} is of the type of a sensitizer. It is not engaged directly in the electron transfers. This is in contrast to the photoactive chlorophyll-a_I which is an electron donor in its excited state.
3. The chlorophyll-a_{II}-reaction can be separated from the overall reaction by heating chloroplasts 5 min at 50 °C.
4. Chlorophyll-a_{II} is the reaction center of the well-known poison DCMU.
5. Properties of chlorophyll-a_{II} are depicted in Tab. 1. They are compared with those of chlorophyll-a_I and the O_2-evolution system.

The electron transport chain of photosynthesis is driven by the light reactions of chlorophyll-a_I and chlorophyll-a_{II}. Chlorophyll-a_{II} has been detected by absorption changes with a half-life time of $2 \cdot 10^{-4}$ sec at 22 °C [1]. We found two characteristic absorption changes at 435 nm and 682 nm [2]. Therefore the pigment was called chlorophyll-a_{II}-435-682. It is located within the electron transport chain between plastoquinone and water [1] (see fig. 1).

In fig. 1 the substance X-320 is that molecule out of the pool of ~8 PQ-molecules which is complexed with Chl-a_{II} and which is converted into a semiquinone [3].

In the following we have extended the measurements on the properties of Chl-a_{II}.

Material and Methods

Whole chloroplasts of spinach were prepared as described in l. c. [4]. Suspensions in which the reaction system II is enriched were prepared according to BOARDMAN [5]. The spectroscopic measurements were performed by the repetitive flash technique described in l. c. [1]. The oxygen yield per flash was measured with the Clark-electrode [6]. The double-flash experiments were performed by combining the techniques in l. c. [7] and l. c. [8]. Further details are described in the legend of the figures.

Results

a) Fig. 2 (left) shows the typical time course of the absorption changes at 690 nm with a superposition of chlorophyll-a_I and chlorophyll-a_{II} changes. In fig. 2 (right) the relative changes are plotted in a log scale. The fast component ($\tau_{1/2} = 0.24$ ms) belongs to Chl-a_{II}, the slow component ($\tau_{1/2} = 16$ ms) to Chl-a_I [1].

The absorption changes of chlorophyll-a_{II} have been measured in the whole visible region between 420 and 725 nm in suspensions in which the reaction system II is enriched. The results are depicted in fig. 3. They represent the complete dif-

[1] G. DÖRING, H. H. STIEHL, and H. T. WITT, Z. Naturforschg. **22 b**, 639 [1967].
[2] G. DÖRING, J. L. BAILEY, W. KREUTZ, and H. T. WITT, Naturwissenschaften **55**, 220 [1968].
[3] H. H. STIEHL and H. T. WITT, Z. Naturforschg. **23 b**, 220 [1968]; H. H. STIEHL and H. T. WITT, Z. Naturforschg., in press.
[4] G. D. WINGET, S. IZAWA, and N. E. GOOD, B. B. Res. Commun. **21**, 438 [1965].
[5] J. M. ANDERSON and N. K. BOARDMAN, Biochim. biophysica Acta [Amsterdam] **112**, 403 [1966].
[6] L. C. CLARK, Trans. Amer. Soc. Artificial Internal Organs **2**, 41 [1956].
[7] J. VATER, G. RENGER, and H. T. WITT, reported in: H. T. WITT, in: Fast Reactions and Primary Processes in Chemical Kinetics (Nobel Symposium V), ed. S. CLAESSON, p. 81, Almqvist & Wiksell, Stockholm 1967; Intersience Publ. New York, London, Sydney.
[8] H. E. BUCHWALD and H. RÜPPEL, Nature [London] **220**, 57 [1968].

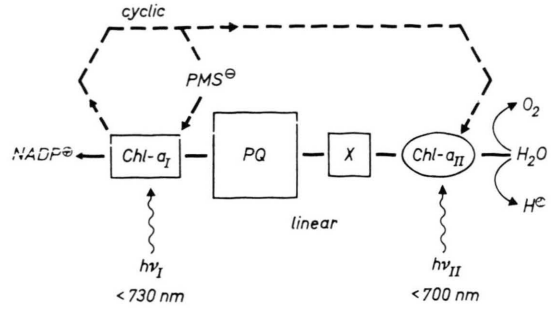

Fig. 1. Simplified electron transfer system in photosynthesis. $NADP^⊕$ = nicotinamide adenine dinucleotide phosphate, Chl-a_I = chlorophyll-a_I, PQ = plastoquinone pool, X = plastosemiquinone, Chl-a_{II} = chlorophyll-a_{II}, $PMS^⊖$ = reduced N-methylphenazonium-methylsulfate.

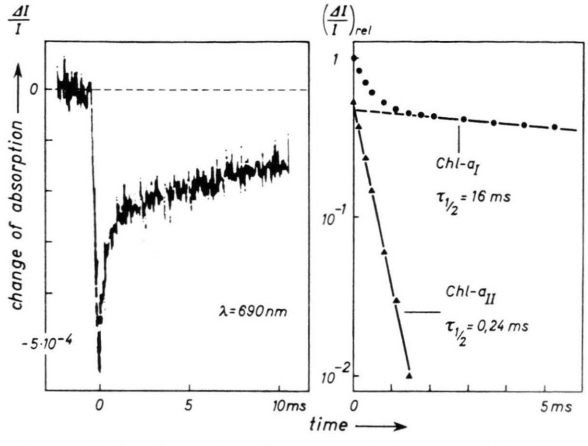

Fig. 2. *Left:* Absorption changes at 690 nm in whole chloroplasts as function of time. At t=0 a flash was fired. *Right:* Relative absorption changes as function of time plotted in a log scale. The fast phase (0,24 ms) is caused by Chl-a_{II}, the slow phase (16 ms) by Chl-a_I. Chlorophyll content $5\cdot10^{-5}$ M, activity of the O_2-production 88 moles O_2/mole Chl.h. Tris-buffer pH 7,2 0,05 M. Phosphorylation uncoupler: NH_4Cl $2\cdot10^{-3}$ M, electron acceptor: benzylviologene 10^{-4} M. Optical length through the cuvette 1,2 mm. Excitation 385—550 nm (4 mm BG 28, 2 mm GG 385, T8) duration $2\cdot10^{-5}$ sec, repetitive flash technique, frequency 10 cps, saturating intensity. 4096 flashes were fired. Measuring beam grating monochromator, optical bandwidth 7 nm. Temperature 22 °C. Electrical bandwidth 0,1 cps—13 kcps.

ference spectrum of the reaction of chlorophyll-a_{II}. Besides the two bands at 435 and 682 nm we found a smaller band at 640 nm. The difference spectrum in fig. 3 is caused by one and the same substance. This follows from the same characteristic kinetics at all wavelengths in dependence of different parameters (poisons, temperature, etc.).

b) The temperature sensitivity of the chlorophyll-a_{II}-reaction has been investigated in whole chloroplasts of spinach.

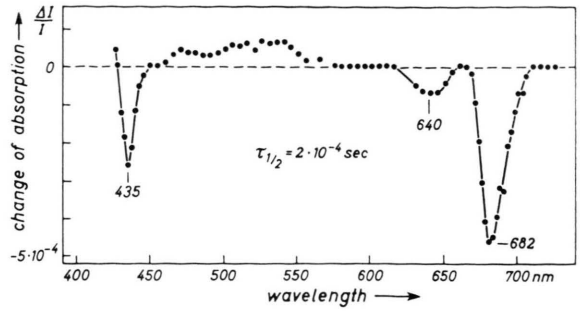

Fig. 3. Absorption changes with a life time of $2\cdot10^{-4}$ sec as a function of the wavelength in spinach chloroplasts. It was used the 10.000 *g* precipitate of digitonin-treated chloroplasts, preparation see l. c. [5]. Chlorophyll content $5\cdot10^{-5}$ M, activity of O_2-production 103 moles O_2/mole Chl.h. Tris-buffer pH 7,2 0,05 M. Phosphorylation uncoupler: NH_4Cl $2\cdot10^{-3}$ M, electron acceptor: $K_3Fe(CN)_6$ $5\cdot10^{-4}$ M. Optical path length through the cuvette 1,2 mm. Excitation: 610—710 nm (1 mm RG 610+T8), 380—500 nm (2 mm BG 28+T8), duration $2\cdot10^{-5}$ sec. Repetitive flash technique, frequency 10 cps, saturating intensity. For each measuring point 4.096 flashes were fired. Measuring beam: grating monochromator, optical band width 5 nm. Temperature 22 °C. Electrical band width 0,1 cps—13 kps.

For such information we heated the chloroplasts 5 min at higher temperatures and measured the absorption changes always at 22 °C. The magnitude of the chlorophyll-a_{II}-absorption change at 690 nm in dependence of the heating temperature is depicted in fig. 4. The characteristic temperature of desactivation is observed for

Chlorophyll-a_{II} at 55 °C. (50% of maximal value)

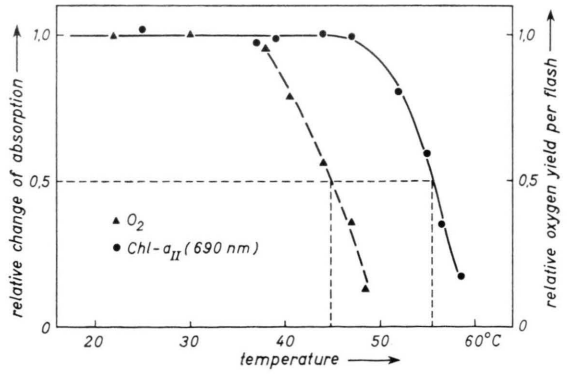

Fig. 4. Relative changes of absorption of Chl-a_{II} at 690 nm and relative oxygen yield per flash as a function of the heating temperature in spinach chloroplasts. The chloroplasts had been exposed to the marked temperatures for 5 min before the measurement. Chlorophyll content: $5\cdot10^{-5}$ M, activity of O_2-production (before heating): 152 moles O_2/mole Chl.h. Tris-buffer pH 7,2 0,05 M. Phosphorylation uncoupler: NH_4Cl $2\cdot10^{-3}$ M, electron acceptor: benzylviologene 10^{-4} M (resp. $K_3Fe(CN)_6$ for the O_2-measurements). All other details see fig. 3, except: frequency of the flashes 10 cps for Chl-a_{II} and 8 cps for O_2.

Corresponding measurements have been made simultaneously for the O_2-production per flash during the cleavage of H_2O (see fig. 1). According to fig. 4 the characteristic temperature of desactivation is observed for

O_2-production at 44 °C. (50% of maximal value)

The oxygen yield per flash is practically zero at 50 °C, whereas the absorption change of chlorophyll-a_{II} at 50 °C is influenced nearly not at all (see fig. 4 and also fig. 5).

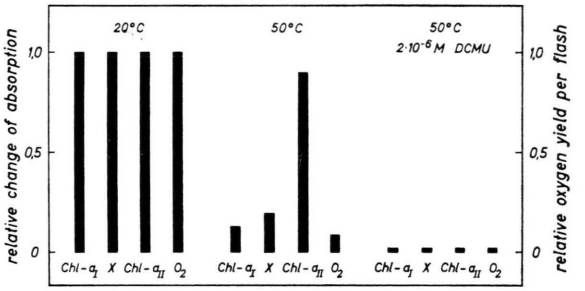

Fig. 5. Relative change of absorption of Chl-a_I, X and Chl-a_{II}, and relative oxygen yield per flash in spinach chloroplasts. *Left:* normal conditions, *center:* after 5 min heating to 50 °C, *right:* after 5 min heating to 50 °C and addition of $2 \cdot 10^{-6}$ M DCMU. Chl-a_I was measured by absorption changes at 703 nm, X at 335 nm and Chl-a_{II} at 690 nm. All other details see fig. 4.

The difference of the results in l. c. [9] in which a temperature of desactivation for the O_2-production was reported at 35 °C is caused by different preparations of the chloroplasts. In l. c. [9] we prepared the suspensions without dimethylsulfoxide, which has a stabilizing effect against temperature desactivation.

c) It is of interest to know which other members of the electron transport chain are blocked when the reaction of O_2-production is totally desactivated at 50 °C. We checked this question by measuring the absorption changes of chlorophyll-a_I at 703 nm and X-320 at 335 nm. The results are depicted in fig. 5. With the desactivation of the O_2-production the reaction of chlorophyll-a_I and X-320 are also strongly reduced.

To avoid misconceptions it should be mentioned that for the chlorophyll-a_I-reaction itself [separated from the overall reaction by adition of the artificial electron donor $PMS^{\ominus}$ (see fig. 1)] the temperature of desactivation is 65 °C [10, 11] (see also table 1).

[9] B. RUMBERG, P. SCHMIDT-MENDE, B. SKERRA, J. VATER, J. WEIKARD, and H. T. WITT, Z. Naturforschg. **20 b**, 1086 [1965].

[10] B. RUMBERG and H. T. WITT, Z. Naturforschg. **19 b**, 693 [1964].

[11] B. RUMBERG, Z. Naturforschg. **19 b**, 707 [1964].

d) It is long known that DCMU poisons the O_2-production [12]. Firstly, we repeated such measurements for the oxygen production in dependence of the DCMU concentration (see fig. 6 top). The characteristic concentration of the desactivation is for

O_2-production at $\sim 10^{-7}$ M. (50% of maximal value)

Secondly, corresponding measurements were done after heating the chloroplasts for 5 min at 50 °C. At these conditions the O_2-production is nearly zero at all DCMU-concentrations (see fig. 6 top).

Thirdly, corresponding measurements were carried out for the reaction of chlorophyll-a_{II}. According to fig. 6 bottom the characteristic concentra-

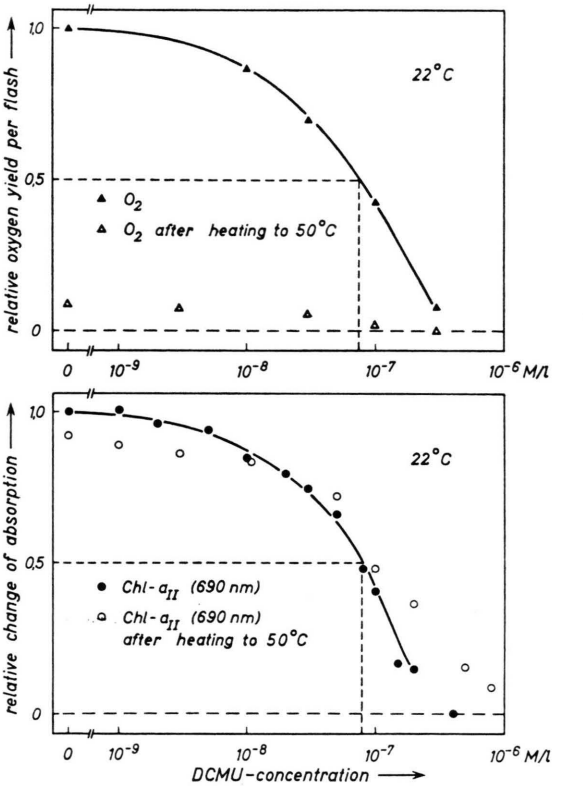

Fig. 6. *Top.* Relative oxygen yield per flash, *bottom*, relative change of absorption of Chl-a_{II} at 690 nm as a function of the DCMU-concentration in spinach chloroplasts before and after heat treatment. The heated chloroplasts had been exposed to 50 °C for 5 min before the measurements. Frequency of the flashes 10 cps. For further details see Fig. 3 and Fig. 4.

[12] N. I. BISHOP, Biochim. biophysica Acta [Amsterdam] **27**, 205 [1958]; T. NAKOMOTO, D. W. KROGMANN, and B. VENNESLAND, J. biol. Chemistry **234**, 2783 [1959]; A. T. JAGENDORF and M. MARGULIES, Biochem. Biophysics **90**, 184 [1960].

Properties	Chlorophyll-a_I	Chlorophyll-a_{II}	H_2O/O_2
Type of Reaction	Electron Donor	Sensitizer	Cleavage of Water
Characteristic Change of Absorption	430, 655, 680—703 nm Band Splitting	435, 640, 682 nm No band splitting	——
Half-Life Time (20 °C)	$2 \cdot 10^{-2}$ sec *	$2 \cdot 10^{-4}$ sec	$6 \cdot 10^{-4}$ sec
Redox Potential	+ 0,46 V (Chl-a_I/Chl-a_I)	——	+ 0,81 V (pH 7)
Range of Excitation	$\lambda < 730$ nm	$\lambda < 700$ nm	——
Range of pH	3—11	?	5—9
Temperature of Inactivation	65 °C	55 °C	44 °C
Sensitive to Aging	no	$t_{1/2} \approx 95$ h (~ 0 °C)	$t_{1/2} \approx 36$ h (~ 0 °C)
Sensitive to DCMU	no	$c_{1/2} \approx 10^{-7}$ M/l	——

* When the electron donor is in the oxidized state.

Tab. 1. Properties of chlorophyll-a_I, chlorophyll-a_{II} and of the H_2O/O_2 system.

tion of desactivation is the same as for the O_2-production, namely

Chlorophyll-a_{II} at $\sim 10^{-7}$ M.
(50% for maximal value)

Fourthly, the measurements were performed for chlorophyll-a_{II} after heating the chloroplasts for 5 min at 50 °C after which all electron transfers are blocked (see b and c). According to fig. 6 bottom the chlorophyll-a_{II}-reaction has nearly not changed in comparison with the behavior of chlorophyll-a_{II} before heating. It shows the same dependence on the DCMU concentration with a characteristic concentration of desactivation

Chlorophyll-a_{II} at $\sim 10^{-7}$ M.
(50% of maximal value)

This indicates that DCMU acts directly on chlorophyll-a_{II} (see discussion).

The DCMU dependency depends on the chlorophyll content. In the experiments reported above this content was $5 \cdot 10^{-5}$ M (s. also fig. 6).

e) From measurements of the O_2-production and of the absorption change of X-320 in double flashes it follows that the rate limiting time of the electron transport from water to X-320 is $6 \cdot 10^{-4}$ sec (characteristic time distance of the second flash for 50% of maximal value) [13]. In case that the chlorophyll-a_{II}-reaction is an electron carrier between H_2O and X-320, this reaction should also decrease to 50% in its absorption change in a second flash when this flash has a time distance of $t_d = 6 \cdot 10^{-4}$ sec from the first one. Fig. 7 shows, however, that even with $t_d = 2{,}5 \cdot 10^{-4}$ sec the absorption change in the second flash is decreased not at all. This means that chlorophyll-a_{II} is not engaged directly in the electron transfer between H_2O and X-320.

It is to be mentioned that the double-flash experiment was possible only by using the high frequency flash technique [7] and by using the high frequency modulated measuring light technique [8] be-

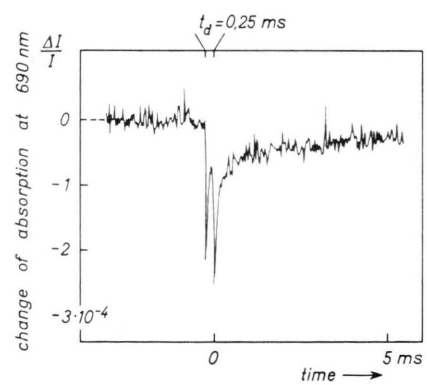

Fig. 7. The kinetics of the absorption changes of Chl-a_{II} at 690 nm in a double-flash experiment in spinach chloroplasts. Chlorophyll content: $2 \cdot 10^{-6}$ M, activity of O_2-production 138 moles O_2/mole Chl.h. Tris-buffer pH 7,2 0,05 M. Phosphorylation uncoupler: NH_4Cl $2 \cdot 10^{-3}$ M, electron acceptor: benzylviologene 10^{-4} M. Optical path length through the cuvette 50 mm. Excitation: 380—550 nm (2 mm BG 28+T8), duration $2 \cdot 10^{-5}$ sec, darktime t_d between the two flashes of a group $2{,}5 \cdot 10^{-4}$ sec. Repetitive flash technique, frequency of the groups 5 cps, saturating intensity. 16.384 double-flashes were fired; after every 4.096 double-flashes the sample has been changed. Measuring beam: interference filter 690 nm, optical band width 5 nm. Temperature 22 °C. Electrical band width 3 cps—120 cps.

[13] J. VATER, G. RENGER, H. H. STIEHL, and H. T. WITT, Progress in Photosynthesis Res. **Vol. 3**, Biochem. of Photosynthesis.

[14] K. SEIFERT, unpublished measurement.

cause of the extremely high fluorescence in the red region.

JOLIOT [15] found after addition of DCMU in the dark that only the first flash is able to cause an electron transfer from which it was concluded that DCMU acts possible to an excited state in vivo.

BERTSCH et al. [16] concluded from delayed light emission that DCMU blocks the electron flow in light reaction II. ZWEIG et al. [17] had assumed from oxygen evolution and fluorescence that DCMU may inhibit the energy migration to light reaction II. WESSELS et al. [18] had supposed that the phenyl ureas might associate the active cyclopentanone ring of chlorophyll through hydrogen bonds. Such association would prevent the transfer of excitation energy from chlorophyll to an acceptor.

In respect to the results with DCMU in d) the following should be mentioned.

We have checked if DCMU has any influence on the photochemistry of chlorophyll reactions in vitro. Chlorophyll soluted in "oxygen free" ($< 10^{-7}$ mole/l) butanol is not influenced in the kinetics of its singulet as well as its triplet state by the addition of DCMU up to 10^{-2} M [14].

Discussion

1. In l. c. [10] evidence was given how it is possible to separate the reaction of chlorophyll-a_I from the overall reaction. From the results above it is now known how to separate also the reaction of chlorophyll-a_{II} from the overall reaction. It can be simply done by heat-treating the chloroplasts for 5 min at 50 °C.

2. The fact that DCMU has the same influence on the chlorophyll-a_{II}-reaction before and after heat-treating (see fig. 6) and the fact that heat-treating blocks all electron transfer events (see fig. 5) indicates clearly that in vivo the action center of DCMU is chlorophyll-a_{II}.

3. DCMU has without heat-treating the same influence on the chlorophyll-a_{II}-reaction as on the O_2-production (see fig. 6). This indicates together with the conclusion in 2 that chlorophyll-a_{II} is the active pigment in the cleavage of H_2O.

This has already been shown in other ways in l. c. [1].

4. The fact that heat-treating stops O_2-production indicates that no linear electron transfer from H_2O to $NADP^{\odot}$ takes place. The fact that under this condition also the reactions of the members of the electron chain, as chlorophyll-a_I and X-320 are blocked indicates that also no cyclic electron transfer is in action (see fig. 1).

When without any electron movements in linear and cyclic ways nevertheless chlorophyll-a_{II} is in action, it can be concluded that the reaction of chlorophyll-a_{II} is probably not of the type of a light induced redox reaction, but of the type of a sensitizer *. The same conclusion follows from the double flash experiment in fig. 7 during intact electron transfers. If chlorophyll-a_{II} donates or accepts electrons in its excited state, chlorophyll-a_{II} should be able to act in a following second flash not before the electron transfer time from H_2O to X-320 ($6 \cdot 10^{-4}$ sec). This is, however, not the case.

In contrast to this behaviour of chlorophyll-a_{II} it has clearly been shown that the reaction of chlorophyll-a_I is a light induced redox reaction [19, 10, 11]. In its excited state chlorophyll-a_I donates an electron to an acceptor.

5. The properties of chlorophyll-a_I have been investigated in detail in l. c. [10, 11, 19, 20]. The properties of chlorophyll-a_{II} have been investigated as yet only in combination with the H_2O/O_2-system and this only indirectly. This was done by the analysis of the positive absorption change of chlorophyll-a_I [21]. These positive changes indicate the reduction of chlorophyll-a_I [21] or the production of electrons in the chlorophyll-a_{II}-H_2O/O_2-system. By the difference spectrum of chlorophyll-a_{II} its properties can now be measured directly and (by heating to 50 °C) separately from the properties of the H_2O/O_2-system. The results so far obtained here and in l. c. [1, 2] are summarized in Tab. 1. Additionally, the properties of chlorophyll-a_I [19, 10, 11, 20] and those of the oxygen evolution system [9, 13] are depicted in Tab. 1.

The most remarkable fact is that the two photoactive chlorophylls are obviously engaged in two completely different types of reactions. It is unknown in which way the excited chlorophyll-a_{II} sentisizes the electron transfer from H_2O to X-320.

[15] P. JOLIOT, private information.

[16] W. F. BERTSCH, J. B. DAVIDSON, and J. R. AZZI, Photosynthetic mechanism of green plants, National Academy of Sciences—National Research Council, Washington D. C., p. 701 [1963].

[17] J. S. C. WESSELS and R. VAN DER VEEN, Biochim. biophysica Acta [Amsterdam] **66**, 196 [1963].

[18] B. KOK, Biochim. biophysica Acta [Amsterdam] **48**, 527 [1961].

[19] G. DÖRING, J. L. BAILEY, W. KREUTZ, J. WEIKARD, and H. T. WITT, Naturwissenschaften **55**, 219 [1968].

* To assume aster heat-treating an electron back reaction between Chl-a_{II} and an unknown intermediate is also unlikely because the kinetics of Chl-a_{II} does not change.

[21] H. T. WITT, in: Fast Reactions and Primary Processes in Chemical Kinetics (Nobel Symposium V), ed. S. CLAESSON, p. 261, Almqvist & Wiksell, Stockholm 1967; Interscience Publ., New York, London, Sydney.