

Light-Induced Valence Change of a Manganese-Containing Chlorophyll Derivative

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K_3 (Mn-(III)-chlorin e_6)acetate, a water-soluble chlorophyll derivative with manganese as central atom was prepared. The influence of reducing agents, light, and bicarbonate on the oxidation states of manganese in the chlorin complex was investigated. In alkaline solutions the central Mn^{3+} was reducible to Mn^{2+} . The illumination of the partially reduced complex first accelerated the reduction (light reduction: $Mn^{3+} \rightarrow Mn^{2+}$), which was then followed by a strong reoxidation (light oxidation: $Mn^{2+} \rightarrow Mn^{3+}$) continuing after switching off the light. This redox effect of light was obtained only in presence of hydroxylamine as reducing agent. Illumination time, intensity, and frequency had an influence on this effect. In the light/dark change the process was reversible. In regard to the requirement of manganese for the photosynthetic oxygen evolution the observed valence change of manganese in the chlorin complex was compared to the supposed reaction mechanism on the donor side of photosystem II.

Introduction

It is known, that manganese is involved in photosynthesis. McHargue [1] proved, that the synthesis of chlorophyll depends on manganese. Pirson [2] showed the influence of manganese on photosynthesis of *Chlorella*. Gerretsen [3] proposed a participation of the redox system Mn^{2+}/Mn^{3+} in the evolution of the photosynthetic oxygen. A direct light dependent oxidation of Mn^{2+} to Mn^{3+} in chloroplast suspensions was observed by Kenten and Mann [4]. Cheniae and Martin [5] pointed to the existence of two manganese pools and their requirement for maximum quantum efficiency of oxygen evolution. Both pools are different in function; but both are located on the oxidizing side of system II. Keller and Bachofen [6] supposed bound manganese in the protein fraction of the chloroplasts. Indeed, metal protein complexes were isolated in the last years (Lagoutte and Duranton [7]; Holdsworth and Arshad [8]).

Kok *et al.* [9] proposed a model for the oxygen evolution, in which a photochemically activated intermediate accumulates four oxidation equivalents before it reacts with water and evolves oxygen. As manganese can exist in different oxidation states, it might play a central role in charge accumulation. According to Wydrzynski *et al.* [10] only one third to

one fourth of the loosely bound manganese is present in dark-adapted chloroplasts as Mn^{2+} . The remainder is present in a higher oxidation state, probably as Mn^{3+} . Renger [11] suggested a mechanism, in which the cooperation of two double-oxidized functional groups in the secondary donor system Mn is assumed to be the essential step for the oxygen evolution. Electron paramagnetic resonance measurements performed by Blankenship and Sauer [12] led to a model in which 60% of the total manganese of the chloroplasts are loosely bound, whereas the remaining 40% are tightly bound in the thylakoid membrane. Wydrzynski and Sauer [13] showed that after a series of flashes of light the Mn(II) released by heat treatment oscillates over periods of four flashes. They chose a binuclear manganese complex as a model to interpret the data in terms of the present four-step mechanism for oxygen evolution. Manganese is successively oxidized in the first two steps, followed by a partial reduction in the third step.

These facts make it conceivable that manganese is complexed with protein, which is bound to the chloroplast membrane. At present there exists no detailed information about the molecular environment of manganese. It is therefore not surprising, that already several years ago synthetic manganese complexes were thought to evolve molecular oxygen from water. Thus a number of metal-chelate complexes with biologically important ligands was pre-

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pared. One of the most common structures in cells, especially in photosynthetic units, is the porphyrin ring. Therefore several manganese porphyrin complexes were investigated (Loach and Calvin [14]); (Boucher and Garber [15]). Glikman and Zabroda [16] as well as Vierke and Müller [17] obtained contradictory results with regard to the participation of Mn-(III)-chlorins in the evolution of H_2O_2 and perhaps O_2 .

Up to now it has not been possible to detect a manganese porphyrin complex in plants. It was therefore only possible to study an artificial preparation. Especially suitable seemed the manganese chlorin complex, first synthesized by Glikman and Zabroda [16], because the porphyrin ligand of this water-soluble complex can be gained from plant material.

The purpose of the present investigation was to prepare and to characterize this compound in more detail, to measure its photoreactions and to investigate a possible influence on the change of the manganese oxidation state by bicarbonate. This is to provide insight into the supposed reaction mechanism on the donor side of photosystem II.

Materials and Methods

$\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{acetate}$ was prepared from chlorophyll *a* isolated from the blue-green alga *Anacystis nidulans*, which was cultivated on a large scale (Jüttner *et al.* [18]). Extraction of chlorophyll *a* was performed by methanol. The pigment was purified of yellow pigments by shaking a methanol extract with petroleum benzine (boiling range: 40–60 °C); 100 ml 2.5 M NaCl solution and 180 ml petroleum benzine were added to 400 ml methanol extract. Further purification was achieved following a precipitation method of Iriyama *et al.* [19]. The preparations of chlorophyllin and chlorin e_6 were performed in accordance with Oster *et al.* [20, 21]. The insertion of manganese was executed according to a modified method of Ashkinazi *et al.* [22]: 4 g Mn-(II)-acetate were added to a filtered solution of 100 mg chlorin e_6 in 80 ml glacial acetic acid; the mixture, through which oxygen was continuously passed, was heated (60 °C in the dark) for 6 h on a reflux condenser. The product was extracted with chloroform. To obtain the water-soluble salt $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{acetate}$ the ion-exchange resin Dowex 50 Wx 8 (Serva, Heidelberg) was used.

The resulting complex is hygroscopic; it was therefore impossible to obtain a defined complex concentration by weighing. For this reason the extinction at 357 nm (absorption maximum for the Mn^{3+} -complex) was adjusted to 0.8 (corresponding to approximately 5×10^{-5} M $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{acetate}$ in 0.1 M glycine/NaOH buffer pH 12.8) to guarantee the same conditions in all experiments. In order to follow redox reactions spectroscopically, 2.5 ml of the complex solution were filled into a 1 cm quartz cuvette, which was sealed by a special rubber (Suba Seal Turnover Type Vaccine Closures, W. Freeman, England). The cuvette was degassed with pure nitrogen for three minutes by an injection needle under nitrogen atmosphere in a special self-made box similar to a glove bag. All preparations were performed in the dark. A crossed beam allowed to follow the kinetics in a Cary 14 UV Recording Spectrophotometer (Varian) during actinic light (sharp cut filters GG 475 and RG 695, Jenaer Glaswerk Schott, Mainz). A filter (BG 12, Jenaer Glaswerk Schott, Mainz) protected the photomultiplier against actinic light, which might disturb the measurement. The reducing agent was injected through the rubber, and the cuvette was shaken three times. The first recording of the extinction took place 15 s after injection. A Barr and Stroud Fibre Optics Light Source (Type LS 2) was employed for illumination. Light was conducted through a light guide into the cell interior.

Reductive titration was recorded by a Perkin-Elmer 356 Two Wavelength Double Beam Spectrophotometer. Elemental analysis was performed in an Elemental Analyzer Model 1104 (Carlo Erba) at a temperature of 1020 °C. The voltamograms were recorded on a Multipolarograph Model 471 (AMEL, Mailand, Italy) with the attached polarography stand Model 460; working electrode: dropping mercury electrode, 38 s capillary, Hg-pressure 48 cm; reference electrode: saturated calomel electrode; counter electrode: platinum ring electrode; voltage sweep: 200 mV/s; delay time: 6 s; degassing: 10 min with pure nitrogen. All experiments were performed at room temperature.

Results

The UV-spectrum of $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{acetate}$ in H_2O shows absorption maxima at 370, 418, 464, 549, and 665 nm. The complex was also char-

acterized by elemental analysis. Found: C, 52.06; H, 5.01; N, 6.42%. Calculated for $C_{36}H_{36}N_4O_9K_3Mn$: C, 51.42; H, 4.28; N, 6.66%.

The reduction potential for the transfer Mn^{3+} to Mn^{2+} in the complex was determined by D. C. Peak Voltammetry in 0.1 N KOH as -0.330 V versus saturated calomel electrode (Fig. 1). In 0.1 N KNO_3 the reduction potential of the complex shifted by 60 mV for each pH unit between pH 10 and pH 13 (Fig. 2). The higher the pH the more negative the potential.

The reduction of $K_3(Mn(III)\text{-chlorin } e_6)\text{acetate}$ by hydroxylamine in 0.1 M glycine/NaOH buffer pH 12.8 is illustrated by Fig. 3. The absorption maxima at 357, 461, 658, and shoulders at 563 and 602 nm decrease, whereas maxima increase at 416, 538, 572, and 624 nm. After addition of hydroxylamine four isosbestic points at 374, 446, 522, and 642 nm can be observed. But the absorption spectrum of the complex before reduction does not pass these isosbestic points.

A comparison of various reducing agents (ascorbic acid, hydroxylamine, sodium sulfide) shows, that at equimolar concentrations hydroxylamine is the most efficient one. Reduction with hydroxylamine yields a maximum at 418 nm, with ascorbic acid and sodium sulfide at 424 nm. A pH effect is excluded.

The light effect depends upon the hydroxylamine concentration and reaches a maximum at 6×10^{-4} M $NH_2OH \cdot HCl$.

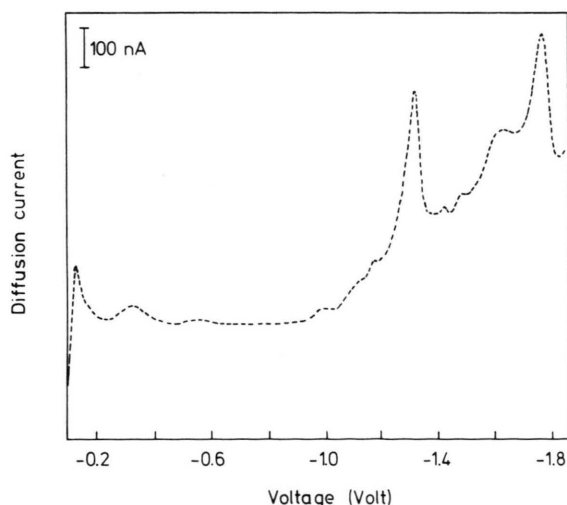


Fig. 1. D. C. peak voltammogram of $K_3(Mn(III)\text{-chlorin } e_6)\text{acetate}$ ($\sim 4.4 \times 10^{-4}$ M) in 0.1 N KOH; voltage range: -0.1 to -1.85 V.

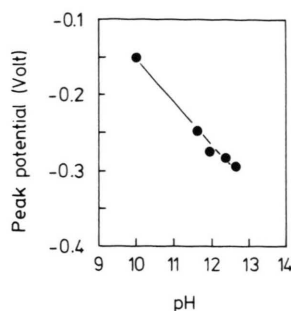


Fig. 2. Reduction potentials of Mn^{3+}/Mn^{2+} in $K_3(Mn(III)\text{-chlorin } e_6)\text{acetate}$ in dependence of the pH value; electrolyte: 0.1 N KNO_3 .

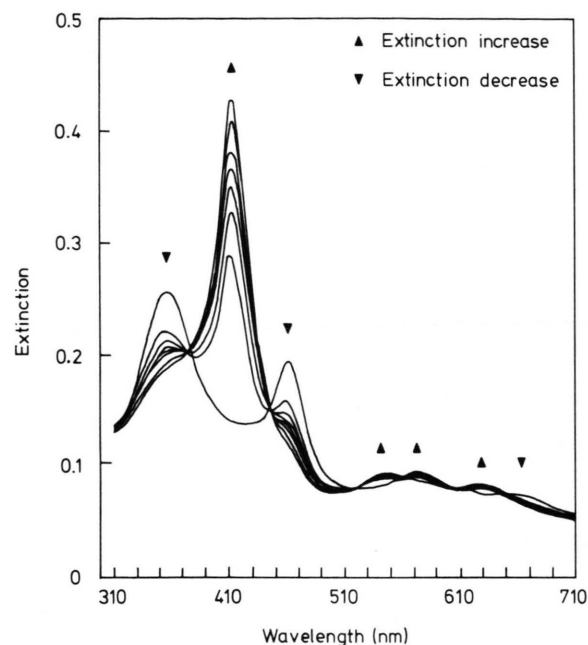


Fig. 3. Reduction of $K_3(Mn(III)\text{-chlorin } e_6)\text{acetate}$ ($\sim 1.75 \times 10^{-5}$ M) in 0.1 M glycine/NaOH buffer pH 12.8; reducing agent: 3×10^{-4} M hydroxylamine; the spectra were recorded in an interval of several minutes.

After 5 min of dark reduction with hydroxylamine the samples were illuminated for one minute (Fig. 4). The true light effect is presented by the light-minus-dark curve. Immediately after beginning of the illumination the difference (light-minus-dark) grows; this corresponds to an intensified complex reduction, *i. e.* the ratio Mn^{3+} to Mn^{2+} shifts to the $Mn(II)$ -complex. During the last 30 s of light the curve decreases, and it decreases even more in the following dark period. The decrease in the dark period is slower than the increase at the beginning of the light

period. At the end of the dark period the starting level is almost reached. A new illumination shows again rapid ascent, *i. e.* an increased rate of reduction. The decrease of the curve starts with the beginning of the dark period. A comparison between the light periods illustrates, the reduction in the first light period is much stronger than in the second one. This may perhaps be explained by a dark period, which is too short after the first light period, because at the starting point of the second light period the curve did not reach the zero line. The second dark period in comparison to the first one indicates a slower decrease. Illumination causes a reinforcement of reduction, *i. e.* a light reduction occurs. After the light period the rate of reduction in the dark moves towards the dark control.

Light periods from 10 to 120 s indicate only small differences (Fig. 5). The increase of the rate of reduction after starting the irradiation remains constant in the first 10 s. 20 and 30 s of illumination enhance the reduction. After 60 s of light the reduction maximum is reached. Even doubling of the light time (120 s) has no bigger effect. In the following dark period the curve decreases. With samples illuminated for 10 and 20 s this happens more slowly than with samples illuminated for 30, 60, and 120 s.

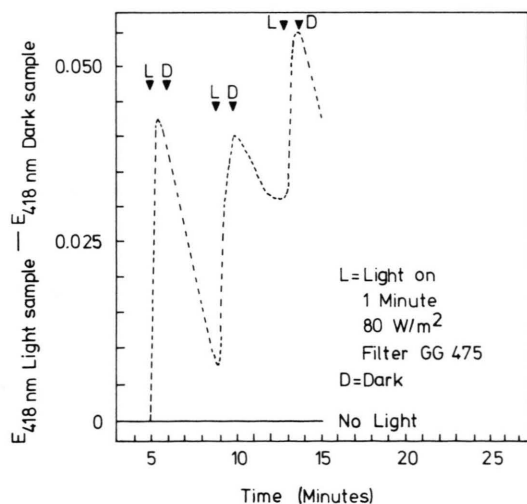


Fig. 4. Reversibility of the light reduction. Kinetics: 1) 5 min of reduction in the dark after the addition of hydroxylamine (6×10^{-4} M); 2) 1 min of illumination; 3) 3 min of dark period; 4) another minute of illumination; 5) 3 min of dark period etc; measured at 418 nm in 0.1 M glycine/NaOH buffer pH 12.8; $K_s(\text{Mn(III)-chlorin } e_a)\text{acetate}$: $\sim 3 \times 10^{-5}$ M.

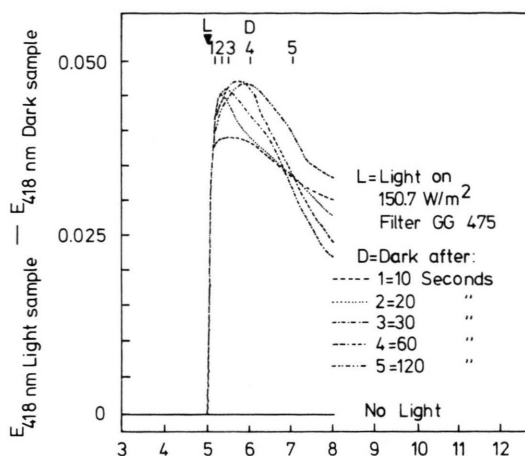


Fig. 5. Light effect in dependence of the light period length. Kinetics: 1) 5 min of reduction in the dark after the addition of hydroxylamine (6×10^{-4} M); 2) 10, 20, 30, 60, and 120 s of illumination; 3) dark period; measured at 418 nm in 0.1 M glycine/NaOH buffer pH 12.8; $K_s(\text{Mn(III)-chlorin } e_a)\text{acetate}$: $\sim 3 \times 10^{-5}$ M.

The time of illumination determines the size of reduction till a certain limit.

Reduction was investigated at four different light intensities (Fig. 6). Within the first 30 s of light reduction there exists an identical increase. The strongest reduction is reached at a light intensity of 69.5 W/m^2 , the lowest at 150.7 W/m^2 , between the values of 34.3 , and 124.3 W/m^2 . The relation between illumination intensity and size of reduction is not clear. The curves for 69.5 , 124.3 , and 150.7 W/m^2 are similar, they offer a decrease after 10 to 20 s in the light period. The 34.3 W/m^2 curve shows this after the beginning of the dark period. The three curves for $\geq 69.5 \text{ W/m}^2$ do not decrease after the dark period, but in contrast to the 34.3 W/m^2 curve they increase again in order to diminish after 30 (124.3 and 150.7 W/m^2) respectively after 60 s (69.5 W/m^2).

These three curves prove: the higher the light intensity, the stronger the decrease of the light reduction. It is interesting, that the 150.5 W/m^2 sample falls short of the starting level, *i. e.* light causes an oxidation in the last 10 s of the light period; so the rate descends below the value of the reduction rate of the dark control.

The influence of the light quality (using the sharp cut filters, red: RG 695, yellow: GG 475; light in-

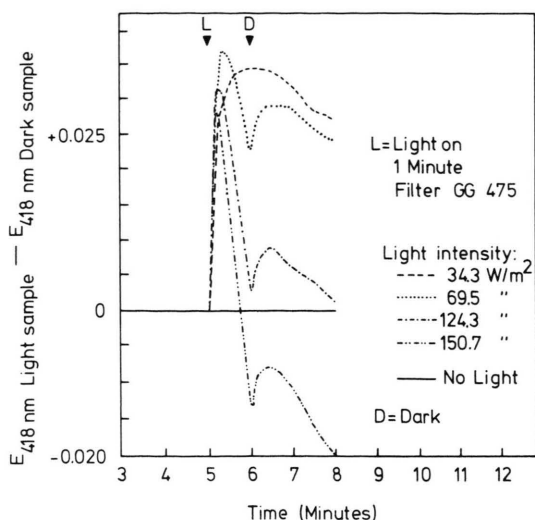


Fig. 6. Light effect in dependence of the intensity of illumination. Kinetics: 1) 5 min of reduction in the dark after the addition of hydroxylamine (6×10^{-4} M); 2) 1 min of illumination with different intensities of light; 3) dark period; measured at 418 nm in 0.1 M glycine/NaOH buffer pH 12.8; $K_3(\text{Mn(III)-chlorin } e_6)\text{acetate}$: $\sim 3 \times 10^{-5}$ M.

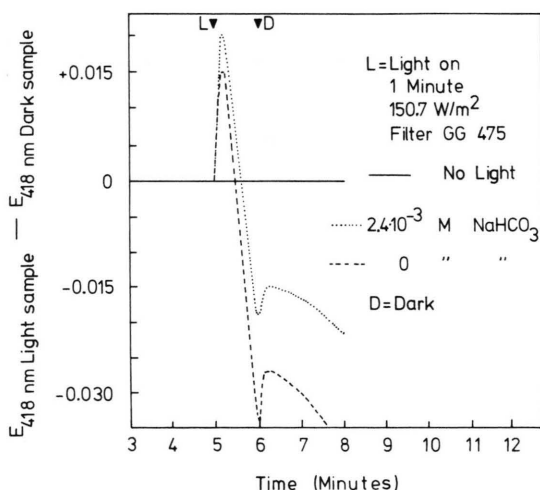


Fig. 7. Light effect and influence of NaHCO_3 . Kinetics: 1) 5 min of reduction in the dark after the addition of hydroxylamine (6×10^{-4} M); 2) 1 min of illumination; 3) dark period; measured at 418 nm in 0.1 M glycine/NaOH buffer pH 12.8; $K_3(\text{Mn(III)-chlorin } e_6)\text{acetate}$: $\sim 3 \times 10^{-5}$ M.

tensity: 48 W/m^2) was examined. Red light shows no effect. Light reduction occurs only at the shorter wavelengths; in this case the reduction strongly increases, and then decreases slowly in the dark period.

The influence of bicarbonate was investigated. NaHCO_3 was added to the sample before injecting

hydroxylamine. It does not affect the reduction in the dark before the light period. After switching on the light the reduction is higher in the sample containing bicarbonate (Fig. 7). After one minute of illumination the light oxidation is stronger in the bicarbonate-free sample than in the HCO_3^- -containing one. In the dark period the back reaction is stronger in the bicarbonate-free sample.

If the differences are significant, light has at first a larger reductive, then a weaker oxidative influence on the bicarbonate-containing sample than on the bicarbonate-free control, *i.e.* NaHCO_3 causes a stronger reduction.

When hydroxylamine was replaced by ascorbic acid and sodium sulfide, a reduction in the dark happened, too. After 5 min of reduction light was switched on for one minute (107 W/m^2). However, opposed to the experiments with hydroxylamine, this irradiation did not increase the reduction. Even in the following dark period there existed no difference to the dark control.

Discussion

Within photosystem II a primary photooxidation of chlorophyll a is suggested. It is supposed, that the chlorophyll radical accepts the missing electron from a complex which is able to reach different oxidation states. It is possible to attribute manganese a participation in this complex.

Brown and Harriman [23] proved, that excited chlorophyll can withdraw electrons from manganese complexes. For Mn(IV)-hematoporphyrin IX at pH 7 Loach and Calvin [24, 25] supposed a very strong oxidation power, which was thought to be able to evolve oxygen from water. But in the presence of this manganese porphyrin they detected neither H_2O_2 nor O_2 formation. Tabushi and Kojo [26] investigated the reduction of Mn(IV)- to Mn(III)-hematoporphyrin in acid solution. Among the reaction products they found water-soluble peroxide.

In this paper $K_3(\text{Mn(III)-chlorin } e_6)\text{acetate}$ was investigated as model substance. The spectrum of this complex in H_2O resembled that of Glikman and Zabroda [16], but the absorption bands were shifted by 5 nm to the long wavelength region. The elemental analysis data were in good agreement with the theoretical ones, taking into account the bad burning of such complexes (Strell and Urumow [27]).

The measured redox potential for the $\text{Mn}^{3+}/\text{Mn}^{2+}$ transfer is in accord with the given potentials for different Mn-(III)-porphyrin complexes as by Boucher [28].

Hydroxylamine reduces $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{-acetate}$ in alkaline solutions; in 0.1 M glycine/NaOH buffer pH 12.8 a change of the oxidation state in favor of Mn^{2+} is observed. This transfer is definitely expressed by an increase of the extinction at 418 nm and a simultaneous decrease at 357 and 461 nm. The time course of this reaction in the dark shows four isosbestic points. But the maxima present a slight shift. A pH effect is excluded here, this effect may have its origin in a slow association of the complex with hydroxylamine, respectively one of its decomposition products. Yet Davis and Montalvo [29] proved, that N-containing ligand molecules could coordinate with Mn-(III)- and Mn-(II)-hematoporphyrin IX. In photosystem II hydroxylamine can replace the natural electron donor (Bennoun and Joliet [30]).

The course of the reduction of the illuminated $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{-acetate}$ is complex. In the presence of hydroxylamine light accelerated the reduction rate considerably. Without addition of this reductant there is no change of the manganese oxidation state; therefore the aromatic ring system is not able to function as donor. Very soon the photoreduction slows down. The reoxidation starts already during the illumination, but it does not reach the rate of photoreduction. Apparently Mn^{2+} acts here once more as an electron donor. A premature switch off of the light signifies the scale of oxidation more distinctly. As soon as the light is switched on, the photoreduction starts without any delay. Hence $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{-acetate}$ is a redox system, whose oxidation state depends on light.

The intensity dependency of the light effect is interesting. Above a comparatively low intensity (34.3 W/m^2) the reaction rate reaches a maximum. At higher intensities the reoxidation begins already during the light time. Thus light provokes a reduction of the central Mn^{3+} . If one component of the system is consumed, the arising Mn^{2+} for its part is effective as electron donor. At 150 W/m^2 the reoxidation takes a completely different course; after eight minutes there exists more Mn^{3+} -complex compared to the start after five minutes and the dark control. Quantitatively the light effects can vary at different charges (compare Figs 5, 6, and 7).

Since Franck [31] has detected, that chloroplast suspensions show a higher activity in the presence of CO_2 , there were several thorough investigations of the CO_2 , respectively the bicarbonate effect in photosystem II (Warburg and Krippahl [32–34]; Stenler and Radmer [35]). In spite of all endeavours we are still insufficiently informed about the action site of CO_2 . Therefore influences of bicarbonate in model reactions deserve particular interest.

The addition of NaHCO_3 to $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{-acetate}$ accelerates the reduction of Mn^{3+} ; it inhibits, however, the photooxidation of the central atom. In the presence of HCO_3^- , respectively CO_3^{2-} anions a lower number of Mn^{3+} -ions is formed. It is not clear, whether this is caused by a delayed electron flow or whether the arising Mn^{3+} -ions are reduced very rapidly by bicarbonate anions.

When $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{-acetate}$ was converted into the divalent form by hydroxylamine treatment, the complex was photooxidizable. So far there exists a similarity with the behaviour of the natural manganese complex of the chloroplasts. We may assume that part of bound manganese atoms is found in the divalent state (Siderer *et al.* [36]; Wydrzynski *et al.* [10]). After adaptation of the cells to the dark, trivalent and divalent manganese might exist side by side; thus the synthesized complex is comparable with the natural manganese complex.

An essential difference between the photoreactions of the artificial manganese porphyrin and there of the manganese complex in the chloroplast is, that the primary light reaction of photosystem II consists in an oxidation of chlorophyll molecules. Apparently the electron transition between the manganese complex and the oxidized chlorophyll is a secondary dark reaction. Quite contrary the photons, in case of the artificial manganese porphyrin, are absorbed by the respective complex itself. It would be too speculative to discuss, whether synthetic manganese complexes may be used as models for the charge accumulation in the so-called S-states of the chloroplasts. Especially with regard to a simulation of photosynthetic reactions in models, it would be of great value to synthesize a complex, which is able to split water in light. Theoretically this should be possible with manganese compounds. But $\text{K}_3(\text{Mn}(\text{III})\text{-chlorin } e_6)\text{-acetate}$ is not able to oxidize water, because its potential is too negative. To split water there should be a redox system whose potential should lie far below the potential of $+0.8 \text{ V}$ of the

oxygen electrode. In fact it is unlikely, that the bound manganese of the thylakoids is part of a porphyrin. Its binding to a protein is more probable. But the redox potential of such a complex may lie at a considerably different voltage.

Moreover it is not sure, that the natural manganese complex of the thylakoid membranes exists in an aqueous medium. Therefore in further investiga-

tions it is recommendable to use aprotic solvents and especially to look for complexes, whose redox potentials lie in a strongly positive voltage region.

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