

## ON THE REDUCTION OF CHLOROPHYLL- $A_1$ IN THE PRESENCE OF THE PLASTOQUINONE ANTAGONIST DIBROMOTHYMOQUINONE

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### 1. Introduction

In a preceding paper [1] we presented experimental results, which led to the identification of four protolytic reaction sites in the functional membrane of photosynthesis: two sites of proton uptake at the outer side of the membrane, in agreement with prior studies [2], and two sites of proton release into the inner phase. One of these sites had to be attributed to the reduction of the terminal electron acceptor at the outer side of the membrane. Evidence was presented for the coupling of the other sites to the oxidation of water at the inner side of the membrane, to the reduction of plastoquinone at the outer side and its oxidation at the inner side, respectively. This interpretation was corroborated by kinetic studies on proton binding from the outer aqueous phase [3].

In this paper we report on studies on the protolytic reactions at both sides of the functional membrane on excitation with short flashes in the presence of the plastoquinone antagonist dibromothymoquinone (DBMIB)\*. DBMIB is an effective inhibitor of photosynthetic electron transport between photosystem II and photosystem I [4,5]. DBMIB inhibits the oxidation of plastohydroquinone

by photosystem I but not the reduction of plastoquinone by photosystem II [6,7]. For a recent review on the action of DBMIB, see Trebst and Reimer [8]. Although DBMIB blocks the electron transfer from  $PQH_2$  to  $Chl-a_1$ , we find that  $Chl-a_1$  is still reduced during the time interval between subsequent flashes of light. The half-time of reduction is about 1 sec and is independent of the DBMIB conc. (0.5–10  $\mu M$ ). The nature of the 1 sec-electron-donor for LR I in the presence of DBMIB is studied. Our experimental results lead to the conclusion that neither PQ nor DBMIB are the donor for  $Chl-a_1$  in direct reaction. In the presence of DBMIB we observe an anomaly in the protolytic reactions at the inner side of the membrane. While after excitation with a single-turnoverflash two protons per electron are released into the inner phase, if  $PQH_2$  transfer an electron to  $Chl-a_1$ , only one proton per electron is released in the presence of DBMIB. This confirms that one proton released into the inner phase is attributable to the reaction  $1/2 PQH_2 \rightarrow 1/2 PQ + e^- + 1 H^+$ , while the second one is due to the oxidation of water. The deactivation of one protolytic reaction site in the presence of DBMIB leads to the conclusion that there exists an endogenous donor, which slowly reduces  $Chl-a_1$ . This donor does not release a proton on oxidation.

### 2. Experimental

#### 2.1. Chloroplasts

The experiments were carried out with aqueous suspensions of broken chloroplasts. Spinach chloroplasts were prepared as in ref. [9]. Chloroplasts

\* Abbreviations:

BENZ, benzyl viologen; FECY, ferricyanide; DCIP, 2,6-dichlorophenol-indophenol; DBMIB, dibromothymoquinone; PQ, plastoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazide; LR I, light reaction I; LR II, light reaction II,  $Chl-a_1$ , chlorophyll- $a_1$ .

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were suspended in a 20 mm absorption cell containing either of the following standard reaction media: medium A: tricine, 1 mM (pH 8); KCl, 20 mM;  $MgCl_2$ , 2 mM; chlorophyll, 10  $\mu M$ . medium B: cresol red, 30  $\mu M$ ; KCl, 20 mM;  $MgCl_2$ , 2 mM; chlorophyll, 10  $\mu M$ . Deviations from these conditions are indicated in the figures.

## 2.2. Excitation and record of transient absorption changes

Photosynthesis was stimulated by excitation with a 'single turnover flash'. Transient absorption changes were recorded with a rapid kinetic spectrophotometer. The signal to noise ratio was improved by averaging over several transients induced by repetitive flashes. For details and for specific references, please, see our preceding paper [1].

## 2.3. Light reaction activity

The activity of light reaction I in flash light experiments was monitored by the absorption change

at 705 nm, which has been attributed to a redox reaction of  $Chl-a_1$  [10]. It has been shown that both light reactions if active, contribute equal amounts to the electric potential generated in a single turnover flash [2], as monitored by the electrochromic absorption change around 520 nm [11,12]. The activity of both light reactions thus was read out from absorption changes at 520 nm, as previously [1].

## 2.4. Protolytic reactions

pH changes in the outer aqueous phase of the inner chloroplast membrane were measured with the pH indicating dye cresol red at a pH around 8. The absorption changes of cresol red are proportional to changes in the proton concentration in the outer phase of the thylakoids [1]. In the presence of the uncoupler FCCP they indicate the net proton production at both sides of the membrane. The difference between the measurements  $-FCCP$  and  $+FCCP$  represent the proton release into the inner phase. Details are given in our preceding paper [1].

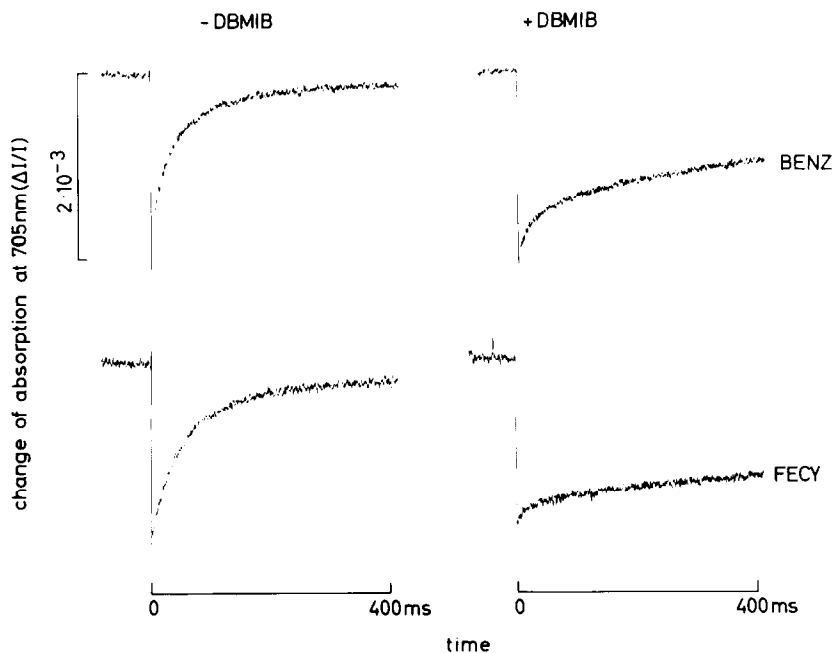


Fig. 1. Absorption changes at 705 nm induced by a single turnover flash at  $t=0$ , in the absence (left) and in the presence (right) of DBMIB (10  $\mu M$ ). Standard reaction medium A, except of electron acceptors (BENZ 60  $\mu M$ , FECY 0.1 mM) and DBMIB. Average over 12 flashes, repetition rate 0.2 Hz.

### 3. Results and discussion

#### 3.1. The activity of the two light reactions in the presence of DBMIB

The activity of the two light reactions as indicated by the extent of the absorption change of Chl- $a_1$  at 705 nm and the electrochromic absorption change at 520 nm, is not affected by DBMIB under excitation with single turnover flashes at a *low repetition frequency* (0.2 Hz). This is evident from a comparison of the left (–DBMIB) with the right traces (+DBMIB) in fig. 1 and fig. 2, respectively. It is independent of whether BENZ or FECY is used as terminal electron acceptor.

However, the half-time of reduction of Chl- $a_1$  (fig. 1) is affected. It increases from 20 msec in the absence of DBMIB to about 1 sec in the presence of DBMIB. In the concentration range of DBMIB covered (0.5–10  $\mu$ M on 10  $\mu$ M Chl) the half-time is independent of the DBMIB concentration. It might be argued that DBMIB, which accepts electrons from LR II, acts as the 1 sec-donor for LR I. The possibility that DBMIB, in analogy to plastoquinone, transfers electrons from LR II to LR I, was investigated in the experiments the results of which are illustrated in fig. 3. There we first forced the system into a steady

state by a series of repetitive flashes the repetition period of which ( $t_d$ ) is indicated at the abscissa, then the steady state extent of the absorption changes at 705 nm and 524 nm was recorded. Since DBMIB is known to be autoxidizable at pH 8 [13], it could act as a steady reductant for Chl- $a_1$  only, if re-reduced either by LR II or by LR I (cycling), respectively. The former can be excluded by the results given in fig. 3. As obvious from the right trace in fig. 3, the absorption change of Chl- $a_1$  at 705 nm in the presence of DBMIB was not influenced by DCMU. The inhibition of LR II by DCMU is demonstrated in the left curve of fig. 3. If DCMU is present only, the dependence of the absorption change at 524 nm on the flash period can be interpreted as follows: there are two components of this absorption change, one of which is half saturated at a repetition period <100 msec, the other one which is half saturated at a repetition period of approx. 1 sec. The former can be attributed to the rapid reduction of DBMIB by LR II, the latter reflects the slow reduction of LR I. Thus DBMIB only via cyclic electron transport around LR I might be involved in the slow reduction of Chl- $a_1$ . As yet, there is no evidence for such a DBMIB mediated cycle in the literature.

That DBMIB under continuous light conditions

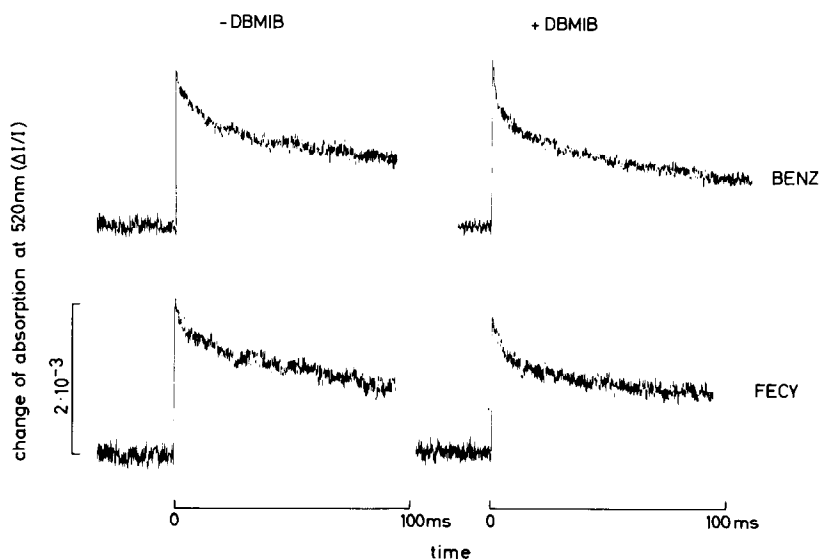


Fig. 2. Absorption changes at 520 nm induced by a single-turnover-flash at  $t=0$ , in the absence (left) and in the presence (right) of DBMIB (10  $\mu$ M). Reaction medium and repetition rate, as given in fig. 1. Average over 30 flashes.

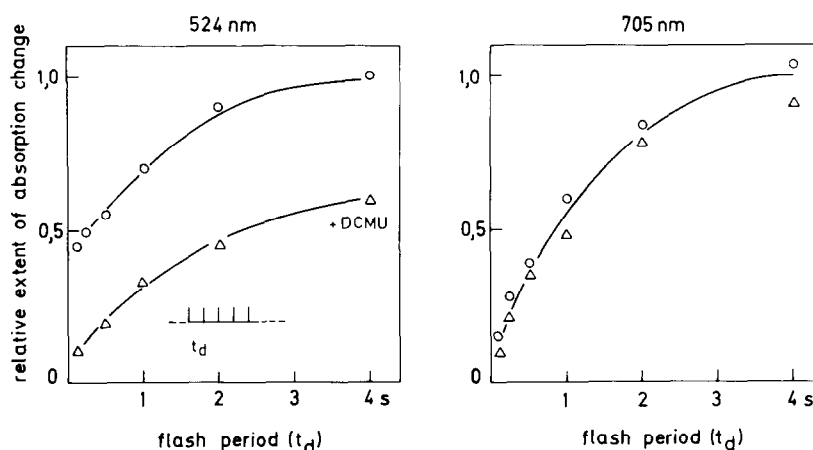


Fig. 3: Dependence of the absorption changes at 524 nm (left) and 705 nm (right) on the flash period ( $t_d$ ). Standard reaction medium A, electron acceptor: BENZ (60  $\mu$ M). ○ = in the presence of DBMIB (10  $\mu$ M) △ = in the presence of DBMIB (10  $\mu$ M) + DCMU (3  $\mu$ M). Each signal was averaged over 100 flashes.

acts as electron acceptor for LR II is known from studies of Gould and Izawa [13,14]. In our flash light experiments in the presence of DBMIB LR II remains even active if several hundred flashes with a repetition period of  $t_d = 40$  msec was applied. We

conclude that DBMIB acts as a large acceptor pool, the electron transfer from LR II (perhaps via PQ) to DBMIB being very rapid (half-time of reduction  $\leq 40$  msec), otherwise the activity of LR II would be limited by the size of the plastoquinone pool,

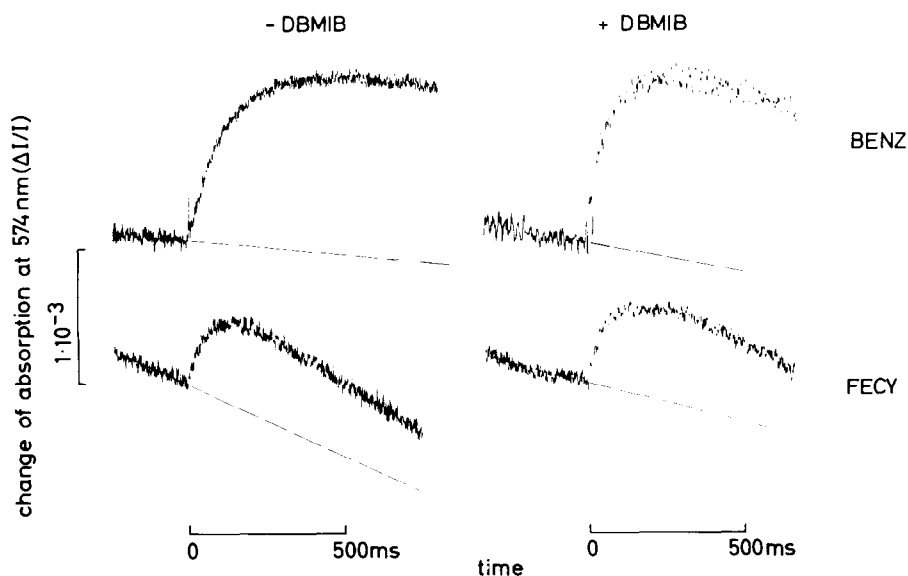


Fig. 4. Absorption changes of cresol red at 574 nm induced by a single turnover flash at  $t=0$ . left: in the absence of DBMIB; right: in the presence of DBMIB (10  $\mu$ M). Standard reaction medium B, electron acceptors: BENZ (60  $\mu$ M), FECY (0.1 mM). Average over 10 flashes, repetition rate 0.2 Hz.

which amounts approx.  $10 e^-/LR II$  [15].

Our results, that the activity of LR I in the presence of DBMIB is independent of the redox state of DBMIB (see above) in the covered conc. range ( $0.5\text{--}10\text{ }\mu\text{M}$ ), led us to suggest that reduced DBMIB is not the direct donor for  $\text{Chl-}a_1$ . More conclusive evidence comes from measurements of the protolytic reactions.

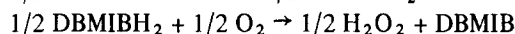
### 3.2. Protolytic reactions at both sides of the membrane in the presence of DBMIB

The proton uptake of the acceptor sites of LR II and LR I at the outer side of the membrane was not affected by DBMIB if both light reactions are active, as shown in fig. 4.

The rise of absorption at 574 nm indicates alkalinisation. No signal was obtained in the presence of buffer, as it has been checked in the presence of tricine, 1 mM, pH=8. In our preceding paper [1] we have shown, that with FECY ( $0.1\text{ mM}$ ) only one proton is taken up from the outer phase (at the reducing site of LR II). Due to its low  $pK$ -value of 4.3, FECY doesn't bind a proton, if reduced at the reducing site of LR I. From the experiments depicted in fig. 4 we conclude, that the rapid electron

transfer from PQ to DBMIB at the acceptor site of LR II, leads to the binding of one proton per electron, too.

Our conclusion is in agreement with results of Gould and Izawa [13], who measured a reversible proton uptake under continuous light conditions, if DBMIB acts as electron acceptor for LR II. They further demonstrated that this proton uptake is independent, whether reduced DBMIB is reoxidized by molecular oxygen (pH 8.1) or not (pH 7.4). That indicates, that DBMIB itself binds a proton on reduction. The reoxidation by oxygen doesn't change the protolytic reaction.



The absorption changes of cresol red in the presence of FCCP, which indicate the net proton production at both sides of the membrane [1], are depicted in fig. 5. From a calculation of the proton release in the inner phase, according to:

$$\Delta\text{H}_i^+ = (\Delta\text{H}_o^+ + \Delta\text{H}_i^+) - \Delta\text{H}_o^+$$

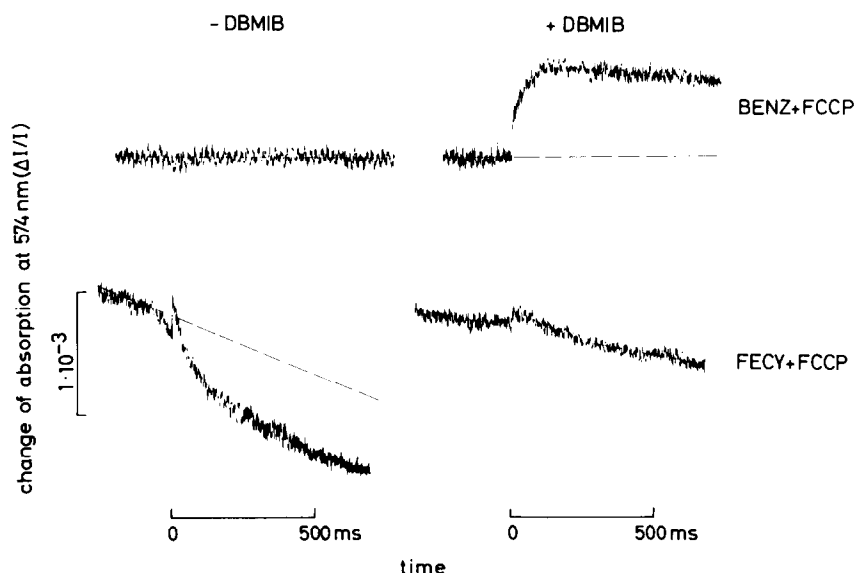


Fig. 5. Absorption changes of cresol red at 574 nm in the presence of FCCP ( $1\text{ }\mu\text{M}$ ), induced by a single turnover flash of light at  $t=0$ . left: in the absence of DBMIB. right: in the presence of DBMIB ( $10\text{ }\mu\text{M}$ ). Reaction medium and measuring conditions as given in fig. 4. The net acidification in the lower left trace and the net alkalinisation in the upper right trace correspond to about  $1\text{ H}^+/e^-$ .

Table 1  
Summary of the experimental results from figs. 1–5

	$\Delta\phi$ (524 nm)	LR I (705 nm)	LR II (calculated)	$\Delta H_o^+/ \Delta e^-$ (574 nm)	$(\Delta H_o^+ + \Delta H_i^+) / \Delta e^-$ (574 nm)(+FCCP)	$\Delta H_i^+ / \Delta e^-$ (calculated)
BENZ	1	1	1	-2	0	2
BENZ + DBMIB	1	1	1	-2	-1	1
FECY	1	1	1	-1	+1	2
FECY + DBMIB	1	1	1	-1	0	1

$\Delta\phi$ , the electric potential generated (relative units); LR I and LR II, activity of the indicated light reactions (relative units);  $\Delta H_o^+ / e^-$ , proton uptake from the outer phase per electron;  $\Delta H_i^+ / e^-$ , proton release from into the inner phase per electron.

wherein  $\Delta H_i^+$  is the proton release into the inner phase per single turnover flash,  $\Delta H_o^+$  is the proton uptake from the outer phase (–FCCP) per single turnover flash, and  $(\Delta H_o^+ + \Delta H_i^+)$  is the net proton production (+FCCP) per single turnover flash, it is obvious from fig. 5, that in agreement with our earlier results [1] two protons are released without DBMIB, but only one proton is released if DBMIB is present. The results from fig. 1–5 are summarized in table 1. The deactivation of one site of proton release into the inner phase in the presence of DBMIB shed doubt on the interpretation, that reduced DBMIB acts as slow donor for LR I (otherwise the reaction  $1/2 \text{ DBMIBH}_2 + \text{Chl-}\alpha_1 \rightarrow 1/2 \text{ DBMIB} + \text{Chl-}\alpha_1 + \text{H}^+$  should lead to the release of 1  $\text{H}^+$  within

1 sec). This is corroborated by the results depicted in fig. 6. There comparison is made between the kinetics of the  $\text{Chl-}\alpha_1$  reduction by the as yet unidentified donor and the kinetics of the net proton production at both sides of the membrane (+FCCP). If DBMIB is substituted for PQ as proton binding electron carrier between the two light reactions (with different kinetics, of course) then one would expect that it binds a proton rapidly on reduction and release it upon oxidation by  $\text{Chl-}\alpha_1$  within 1 sec. However, it is obvious from fig. 6 that there is no proton release with a time-constant of 1 sec detectable. This excludes that DBMIB acts as electron donor for  $\text{Chl-}\alpha_1$ . We conclude, that there exists an unidentified endogenous electron donor for LR I,

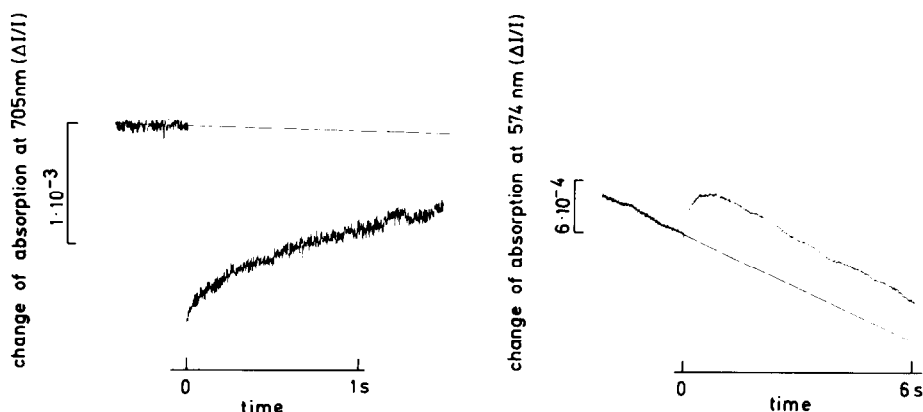


Fig. 6: Absorption changes at 705 nm and of cresol red at 574 nm in the presence of DBMIB (10  $\mu\text{M}$ ) + FCCP (1  $\mu\text{M}$ ), induced by a single turnover flash of light at  $t=0$ . left: reaction medium A + BENZ (60  $\mu\text{M}$ ). Average over 3 flashes, repetition rate 0.1 Hz, right: reaction medium B + BENZ (60  $\mu\text{M}$ ). Average over 6 flashes, repetition rate 0.1 Hz.

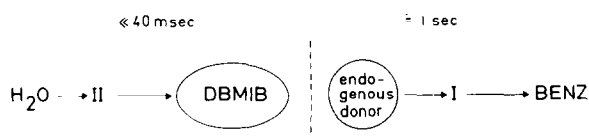


Fig. 7: Kinetic scheme for the reactions between the light reactions and DBMIB and the endogenous electron donor, respectively.

which is non-proton binding. This donor reduces  $Chl-a_1$  with a half-time of 1 sec (see fig. 7).

The deactivation of one proton-release site in the inner phase, if PQ is replaced by an unknown endogenous donor for LR I, confirms our previous conclusion that the oxidation of plastoquinone is stoichiometrically coupled to the release of one proton.

## References

- [1] Junge, W. and Ausländer, W. (1974) *Biochim. Biophys. Acta* 333, 59–70.
- [2] Schliephake, W., Junge, W. and Witt, H. T. (1968) *Z. Naturforsch.* 23b, 1561–1578.
- [3] Ausländer, W. and Junge, W. (1974) *Biochim. Biophys. Acta*, in press.
- [4] Trebst, A., Harth, E. and Draber, W. (1970) *Z. Naturforsch.* 25b, 1157–1159.
- [5] Böhme, H., Reimer, S. and Trebst, A. (1971) *Z. Naturforsch.* 26b, 341–351.
- [6] Böhme, H. and Cramer, W. A. (1972) *Biochemistry* 11, 1155–1160.
- [7] Lozier, R. H. and Butler, W. L. (1972) *FEBS Letters* 26, 161–164.
- [8] Trebst, A. and Reimer, S. (1973) *Biochim. Biophys. Acta* 305, 129–139.
- [9] Siggel, U., Renger, G., Stiehl, H. H. and Rumberg, B. (1972) *Biochim. Biophys. Acta* 256, 328–335.
- [10] Rumberg, B. (1964) *Z. Naturforsch.* 19b, 707–716.
- [11] Junge, W. and Witt, H. T. (1968) *Z. Naturforsch.* 23b, 244–254.
- [12] Emrich, H. M., Junge, W. and Witt, H. T. (1969) *Z. Naturforsch.* 24b, 1144–1146.
- [13] Gould, J. M. and Izawa, S. (1973) *Biochim. Biophys. Acta* 314, 211–223.
- [14] Gould, J. M. and Izawa, S. (1973) *Eur. J. Biochem.* 37, 185–192.
- [15] Stiehl, H. H. and Witt, H. T. (1969) *Z. Naturforsch.* 24b, 1588.