AN ENERGY-CONSERVATION SITE BETWEEN H_2O AND DBMIB: EVIDENCE FROM msec DELAYED LIGHT AND CHLOROPHYLL FLUORESCENCE STUDIES IN CHLOROPLASTS

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1. Introduction

Trebst et al. [1] reported that DBMIB** by acting as an antagonist of plastoquinone, inhibited electron transport between system II and system I in isolated chloroplasts. Gimmler and Avron [2] studied the effect of DBMIB on electron transport and the fluorescence induction of chlorophyll *a* in *Phorphyridium cruenthum*. They observed, in the presence of DBMIB, a faster increase in fluorescence upon illumination, suggesting that the size of the pool of oxidant available for reoxidation of QH is diminished due to a block in electron transfer caused by the inhibitor. In addition, when DBMIB was added at concentrations higher than 10^{-5} M it caused a quenching of chlorophyll fluorescence independent of the redox state of Q. Very recently, Lozier and Butler [3] discovered that DBMIB can serve as an electron acceptor for system II. In the present work we have monitored the reduction of DBMIB in chloroplasts by following the reversion of chlorophyll *a* fluorescence quenching and in addition we studied the effect of this compound on msec delayed light. These studies indicate that the electron transport span from H_2O to DBMIB includes a site for energy conservation. This has been suggested previously [4].

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** *Abbreviations*: DBMIB, 2,5 dibromo-3-methyl-6 isoprophyl-*p*-benzoquinone; D.L., delayed light; DCMU, 3-(3,4-dichlorophenyl)-1, 1 dimethylurea.

2. Materials and methods

Chloroplasts were isolated from lettuce, *Lactuca savita* var. *romaine* using a procedure described previously [5]. The only difference was that 10 mM sodium ascorbate was present in the grinding medium. The concentration of chlorophyll was measured according to Arnon [6]. Prompt and msec delayed fluorescence were measured using a rotating sector phosphoroscope identical to that described earlier [5]. The exciting light was transmitted through a Balzer Calflex C and a 4 mm Schoot BG 18 filter, giving an intensity of 2.8×10^4 ergs. cm^{-2} sec^{-1} at the cuvette. The delayed light was detected with an EM1 9659 B photomultiplier protected by a 2 mm Schott RG 665 filter. Prompt chlorophyll fluorescence was measured using a second photomultiplier (EM1 9558) placed at right angles to the exciting light beam and shielded from the latter by a filter combination consisting of a Balzer B 40695 and a 6 mm Schott RG 665. The current pulses from the photomultipliers were passed through diode pump circuits with time constants of 100 msec and recorded on a Rikidenki two channel chart recorder.

DBMIB was a gift from Dr. A. Trebst. A stock solution of this compound at a concentration of 1.2 mM was prepared in methanol.

3. Results and discussion

Addition of DBMIB to a suspension of illuminated chloroplasts, caused a series of changes in chlorophyll

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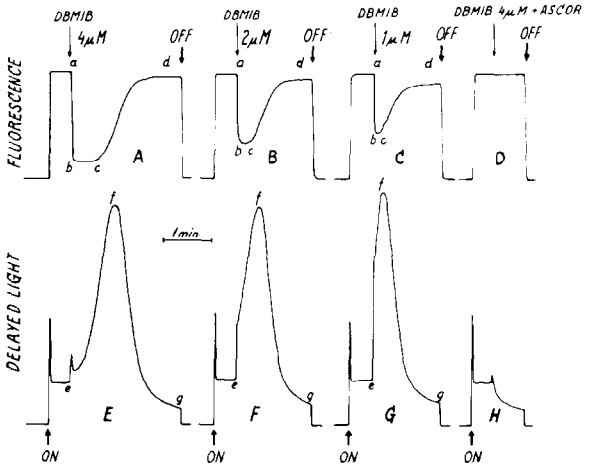

Fig. 1. Effect of various concentrations of DBMIB on prompt fluorescence and msec delayed light. Chloroplasts were suspended in 3 ml of 0.33 M sucrose, 20 mM N-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) at pH 7.0. The chlorophyll concentration was 18.5 μg. DBMIB at the specified concentration was added as indicated. Ascorbate was added at 3 mM.

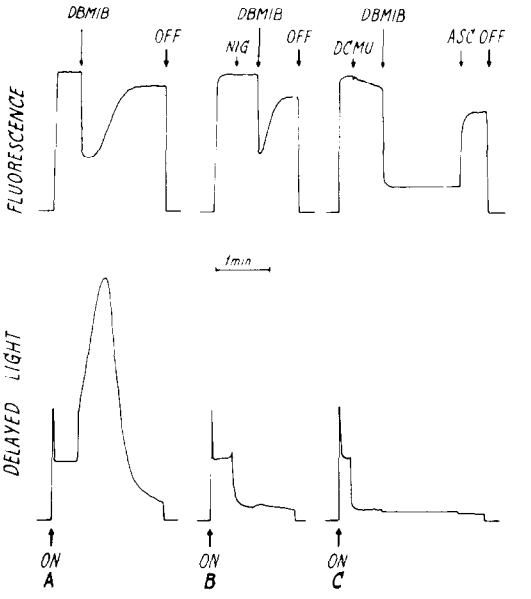

Fig. 2. Effect of nigericin and DCMU on prompt fluorescence and msec delayed light in the presence of DBMIB. DBMIB was added at 2 μM, nigericin at 0.1 μM, DCMU at 10 μM and sodium ascorbate at 3 mM. Other conditions as in fig. 1.

a fluorescence and msec delayed light which is described in fig. 1. Initially, prompt fluorescence is quenched as indicated by '*ab*'. The extent of '*ab*' is determined by the amount of DBMIB added (compare A, B and C in fig. 1). In another experiment, in which we have followed the fluorescence changes by using an oscilloscope we have found (data not shown), that DBMIB quenches both the 'live' and 'dead' fluorescence, where 'dead' fluorescence is taken as the initial fluorescence level after a long dark period often referred to as the f_o level [7]. the DBMIB quenching of chlorophyll *a* fluorescence is relieved in the light after a lag period '*bc*', the length of which is a function of DBMIB concentration (fig. 1). The rise of fluorescence after the lag is seen in '*cd*'. In accordance with the proposal of Lozier and Butler [3], we suggest that the relief of chlorophyll *a* fluorescence quenching with time seen in fig. 1 is the result of the photoreduction of DBMIB. The cycle of quenching and relief can be repeated by adding another sample of oxidized DBMIB (data not shown).

When DBMIB is added in the presence of DCMU (fig. 2C top part) chlorophyll a fluorescence is quenched with no subsequent relief. This would be expected since in the presence of DCMU no photoreduction of DBMIB can take place. Ascorbate reduces DBMIB chemically and thus when both compounds are added together, there is no quenching (fig. 1D); Addition of ascorbate to a chloroplast suspension in which fluorescence was quenched by DBMIB, causes a restoration of fluorescence, even in the presence of DCMU (fig. 2C, top).

The addition of DBMIB to intact cells of *Chlamydomonas reinhardi* caused a quenching of chlorophyll *a* fluorescence and inhibition of O_2 evolution. However, contrary to chloroplasts, the quenching of fluorescence was not relieved; neither was there a stimulation of msec D.L. (see below for the effect of DBMIB on msec D.L. of chloroplasts). Apparently, in intact cells of *Chlamydomonas reinhardi* DBMIB can act as an electron blocking agent but not as an electron acceptor.

It is known that the addition of electron acceptors to chloroplasts stimulates msec delayed light emission considerably by increasing the proton motive force across the thylakoid membrane [8]. As known from

Table 1
Effect of ADP and nigericin on the rate of photoreduction of DBMIB and maximum extent of msec delayed light.

	10 mM KCl		50 mM KCl	
	Rate of DBMIB photoreduction	Max. extent of msec delayed light	Rate of DBMIB photoreduction	Max. extent of msec delayed light
Control	3.6	39	6.7	38
+ 0.2 mM ADP			8.6	25
+ 0.2 μM Nigericin	5.6	2	9.3	2

The chloroplasts were suspended in 3 ml of 0.33 M sucrose, 20 mM *N*-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), 3 mM NaP_i, 1.4 mM $MgCl_2$ and either 10 or 50 mM KCl. The pH of the reaction mixture was adjusted to 7.6. The amount of chloroplasts added was equivalent to 32 μg chlorophyll. DBMIB at 4 μm was added to the suspension before illumination. The slopes of the fluorescence rise were used as indices for electron transport. Both the rate of photoreduction and the extent of msec D.L. are presented in relative units.

previous works [8,9], and shown in fig. 1E, even in the absence of an exogenous electron acceptor a signal of msec D.L. is developed, presumably due partly to endogenous electron flow. Upon addition of DBMIB, there is a marked increase in the signal of D.L. (fig. 1 '*ef*') and a subsequent decrease (fig. 1 '*fg*'). We suggest that the increase in D.L. is a result of electron flow from H_2O to DBMIB, and the subsequent decrease in the signal is due to the exhaustion of the oxidant; no such rapid decrease in the signal of msec D.L. in the light is observed in the presence of other electron acceptors [8,9]. As expected, the signal of msec D.L. can be restored by the addition of another sample of oxidized DBMIB (data not shown). The level of D.L. (fig. 1 '*g*') eventually drops below the steady-state level obtained in the absence of an exogenous electron acceptor (fig. 1 '*e*') indicating that reduced DBMIB inhibits the non-cyclic endogenous electron flow. This can be seen directly by adding reduced DBMIB (fig. 1H). Since the effects of DBMIB on msec D.L. and prompt chlorophyll *a* fluorescence can be repeated by a further addition of oxidized DBMIB it is clear that reduced DBMIB does not inhibit the process in which the oxidized form is reduced.

Addition of DCMU caused a strong inhibition of the signal of msec D.L. obtained in the absence of an electron acceptor (fig. 2C). The signal remained inhibited after the addition of DBMIB since the latter can't be photoreduced in the presence of DCMU. Ascorbate which reduces DBMIB chemically (irrespective of the presence of DCMU) relieves the quenching of chlorophyll *a* fluorescence (fig. 2C top), but as expected, does not relieve the inhibition of msec D.L. (fig. 2C bottom).

Thus, the stimulation of D.L. by DBMIB is presumably the result of the formation of a high energy state supported by electron flow from H_2O to DBMIB. Consequently, the addition of the uncoupler nigericin causes an almost total inhibition of msec D.L. (fig. 2B and table 1). The addition of phosphorylating reagents also causes an inhibition of D.L. (table 1). Inhibition of msec D.L. by nigericin (or by other uncouplers) and by phosphorylating reagents was shown previously in other electron transport systems [8,9].

As shown above, oxidized DBMIB quenches markely chlorophyll *a* fluorescence however, during illumination when DBMIB is photoreduced, the quenching is relieved and the original level of chlorophyll *a* fluorescence is restored. It is reasonable to assume that the *rate* of fluorescence rise would depend on the rate of photoreduction of DBMIB. The fact that the rate of the fluorescence rise is stimulated by the addition of nigericin or phosphorylating reagents (table 1), supports again the notion that an energy transduction site exists between H_2O and DBMIB.

4. Conclusions

As shown in previous works and supported in the present publication DBMIB both in its oxidized and

reduced forms inhibits electron flow between the two photosystems. However, oxidized DBMIB can serve as an electron acceptor from photosystem II prior to the site of inhibition.

Addition of oxidized DBMIB causes a quenching of chlorophyll *a* fluorescence. However, upon illumination when DBMIB is reduced, chlorophyll quenching is abolished and fluorescence restored. This process can be followed kinetically.

Addition of DBMIB caused a marked increase in msec D.L. Both nigericin and phosphorylating reagents which are known to dissipate the high energy intermediate inhibited the signal of msec D.L. while stimulating electron transport. It is concluded that a site of energy transduction is located in the span $H_2O \rightarrow$ DBMIB.

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