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The effects of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone on isolated pea chloroplasts

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The effects of a quinone analogue, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), on the photochemical activities of isolated pea chloroplasts have been investigated. DBMIB completely inhibits pseudo-cyclic flow with methyl viologen (MV) and partially inhibits non-cyclic flow with ferricyanide (FeCN) in both coupled and uncoupled systems. It is also shown that, under some circumstances, DBMIB acts like an uncoupler in that it stimulates electron flow and inhibits cyclic photophosphorylation.

Trebst et al. (*1*) first reported the inhibitory effects of the quinone analogue, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) on electron flow in spinach chloroplasts. They showed that it strongly inhibited the Hill reaction, as well as coupled and uncoupled flow with either anthroquinone-2-sulphonate or methyl viologen (MV) as electron acceptors. On the other hand, DBMIB only partially inhibited the same reactions with ferricyanide (FeCN) as electron acceptor. Bohme et al. (*2*) extended these observations and showed that exogenous plastoquinone could reverse the inhibitions caused by DBMIB and that electron flow became insensitive to DBMIB when diaminodurene or 2,6-dichlorophenol indophenol (DCPIP) plus ascorbate replaced water as the electron donor. These workers concluded that DBMIB acted as an antagonist to plastoquinone.

We have studied the effects of DBMIB on electron flow in pea chloroplasts, and present results that cannot be explained solely in terms of DBMIB being a specific inhibitor of plastoquinone-mediated reactions. In particular, evidence is presented to suggest that DBMIB has some uncoupling properties.

Materials and methods

Freshly harvested pea leaves (*Pisum sativum* variety Green Feast) were prechilled and homogenised for about 7 sec in a Waring Blendor in a medium containing 0.4 M sucrose, 1 mM ethylene-glycol-bis-(β-amino-ethyl ether)-*N,N'*-tetraacetic acid,

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, dichlorophenyl-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; FeCN, ferricyanide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; MV, methyl viologen; PMS, phenazine methosulphate.

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5 mM $MgCl_2$, 40 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) buffer, pH 7.3 and 5 mg/ml bovine serum albumin. The slurry was filtered through a layer of 'miracloth' and centrifuged in a Sorvall RC2 refrigerated centrifuge. The centrifuge was accelerated to 6000 rpm, maintained for 30 sec and quickly brought to rest again. The chloroplasts were resuspended in 0.4 M sucrose containing 0.5 mM $MgCl_2$, 30 mM *N*-tris(hydroxymethyl)methylglycine (Tricine) buffer pH 7.5 and 5 mg/ml bovine serum albumin. All operations were carried out at about 3°C and the chloroplast suspension was stored in ice.

Changes in oxygen concentration were measured at 25°C with a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, England), illuminated by a Rank Aldis slide projector with a 150 W quartz iodine lamp and a Wratten No. 29 red filter, giving an intensity of about 2×10^5 ergs·cm^{-2}·sec^{-1} at the reaction vessel. Chlorophyll was determined according to the method of Arnon (*3*). ADP concentration was determined enzymically (*4*). The oxygen concentration of air-saturated medium (100%) at 25°C was taken as 250 μM.

Cyclic photophosphorylation was measured by following pH changes according to the method of Chance and Nishimura (*5*) using a Philips combined pH electrode (model CA 14/02) connected to a Beckman research pH meter and a Beckman potentiometric recorder. The pH changes measured were calibrated by adding known amounts of standard acid at the end of each experiment.

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Results

The effects of DBMIB on Hill reaction rates with MV and FeCN as electron acceptors are shown in Fig. 1. The Hill reaction with MV is completely inhibited by 1 μM DBMIB whereas the Hill reaction with FeCN is only partially inhibited. Phosphorylation (ADP plus Pi) and uncoupling stimulate the Hill reaction rate, and do not alter the effect of DBMIB except that the percentage inhibition of FeCN reduction becomes greater (Fig. 1). The residual FeCN reduction, in the presence of 1 μM DBMIB is completely inhibited by DCMU (4×10^{-6} M) in pea chloroplasts. The above results are in complete agreement with those published by Trebst et al. (*1*), for isolated spinach chloroplasts.

It has been reported that higher concentrations of DBMIB have no further effect (*1*) on, or cause further inhibition (*2*) of, FeCN reduction in spinach chloroplasts. However, in pea chloroplasts higher concentrations of DBMIB lead to some recovery of the FeCN reduction previously inhibited by 1 μM DBMIB (Table 1). A similar recovery of electron flow is not as apparent in the MV system although higher concentrations of DBMIB do show a slight stimulation in the rate of oxygen uptake (Table 1).

High concentrations of DBMIB also stimulate electron flow under other circumstances. Fig. 2 shows that the state 4 rate (*6*) and the ATP-inhibited Hill reaction

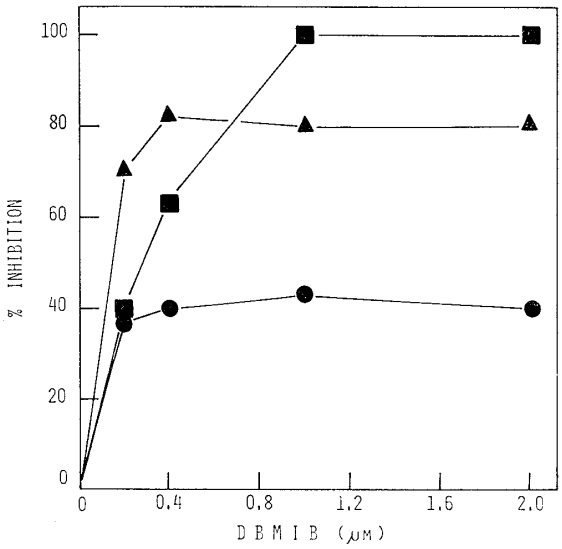

Fig. 1. *DBMIB inhibition of photosynthetic electron flow.* The reaction medium (2.8 ml) contained 0.33 M sorbitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 12 mM K_2HPO_4-KH_2PO_4 buffer pH 7.5, 40 mM HEPES buffer pH 7.5 and 110 μg chlorophyll. Electron acceptors were either 64 μM MV with 0.93 mM azide or 1.5 mM FeCN. -●-, Hill reaction with FeCN. -■-, Hill reaction with MV. -▲-, Coupled reduction of FeC_N (1.0 μmole ADP added).

rate (*13*) of ferricyanide reduction can also be stimulated by DBMIB. Such a response is typical of uncoupling agents. It is difficult to test the uncoupling properties of a compound in a system where the compound is a potent inhibitor of electron flow. Photo-oxidation of reduced DCPIP by photosystem I is not inhibited by DBMIB (*2*), and electron flow in the reduced DCPIP-MV couple can be stimulated by uncouplers (*7*, *8*). The strong stimulatory effect of CCCP and of nigericin plus K^+ on oxygen uptake in the reduced DCPIP-MV couple is shown in Table 2, together with the effect of DBMIB. It can be seen that DBMIB has the same effect as the other uncouplers. However, this result should not be explained simply on an uncoupling basis because under the same conditions electron flow is not stimulated by phosphorylation (ADP+Pi).

If DBMIB is an effective uncoupler it should also inhibit cyclic photophosphorylation. As can be seen from Fig. 3 DBMIB does cause some reduction in the rate of ATP formation in a PMS-catalysed system. It is not possible to

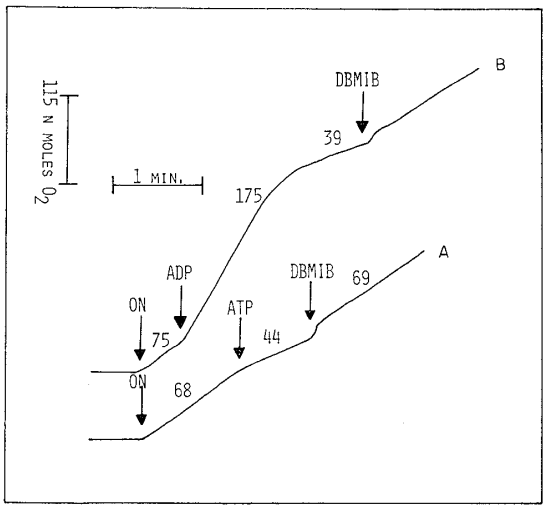

Fig. 2.(A) *DBMIB stimulation of ATP-inhibited Hill reaction with FeCN.* The reaction mixture was the same as for Fig. 1. Final concentrations of chlorophyll, ATP and DBMIB were 65 μg, 1 μmole and 4.3 μM respectively. Rates are expressed as μmoles O_2/mg chlorophyll·hr.
Fig. 2.(B) *DBMIB stimulation of ADP-limited oxygen evolution with FeCN.* Conditions as for Fig. 1. Chlorophyll and ADP added were 65 μg and 0.5 μmole. DBIMB added to a final concentration of 4.3 μM. Rates are expressed as μmoles O_2/mg chlorophyll·hr.

Table 1 *The effect of DBMIB on nigericin-uncoupled electron flow with FeCN and MV in pea chloroplasts*

DBMIB addition	Change in oxygen (μmoles/mg chlorophyll·hr)	
	FeCN (O_2 evolved)	MV (O_2 consumed)
(Control)	368	320
First (1 μM)	44	0
Second (4.6 μM)	77	6

The reaction medium (2.8 ml) contained 0.33 M sorbitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM KH_2PO_4-K_2HPO_4 buffer pH 7.6, 40 mM HEPES, 0.46 μM nigericin, 55 μg chlorophyll and either 64 μM MV with 0.93 mM azide or 1.5 mM FeCN. The first addition of DBMIB gave a final concentration of 1 μM and the second 4.6 μM.

Table 2 *Effect of DBMIB, CCCP and nigericin plus K^+ ions on electron transport from $DCPIPH_2$ to MV*

Additions	Electron transport (μmoles O_2/mg chll·hr)
Control	32
DBMIB 2.3 μM	52
DBMIB 4.6 μM	97
DBMIB 9.2 μM	203
CCCP 9.2 μM	203
Nigericin 0.46 μM	224

Reaction mixture (2.8 ml) contained 0.33 M sorbitol; 5 mM $MgCl_2$; 1 mM $MnCl_2$; 40 mM HEPES buffer, pH 7.5; 35 μM DCPIP, 4.63 mM ascorbate; 64 μM MV; 0.97 mM azide, 4.2 μM DCMU and 40 μg chlorophyll. 21 mM KCl was also added with nigericin.

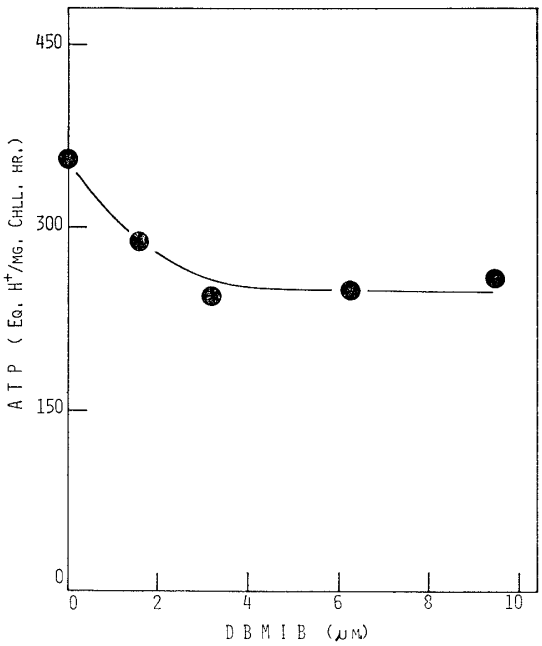

Fig. 3. *The effect of DBMIB on cyclic photophosphorylation in pea chloroplasts.* The reaction mixture contained 0.4 M sucrose, 5 mM $MgCl_2$, 12 mM K_2HPO_4-KH_2PO_4 buffer pH 8.0, 15 μM PMS, 3 mM ascorbate, 1.0 μmole ADP, 3 μM DCMU and 84 μg chlorophyll, in a volume of 2.8 ml.

determine whether this is due to an inhibition of the rate of electron flow or to an uncoupling of photophosphorylation. Nevertheless, the results are in contrast to those of Bohme et al. (*2*) who observed no effect of DBMIB on PMS-catalysed cyclic photophosphorylation in spinach chloroplasts.

Discussion

Some of the above results confirm the reports (*1*, *2*) that DBMIB can be a potent inhibitor of electron flow in isolated chloroplasts. Its site of action is between the two photosystems and more specifically at or about plastoquinone (*1*, *2*). The complete inhibition of MV photoreduction from water by DBMIB is understandable. But the incomplete inhibition of FeCN photoreduction and the apparent uncoupling properties of DBMIB require further interpretation.

Sites of FeCN reduction

The incomplete inhibition of FeCN reduction by DBMIB (1 μM) shows that there are at least two sites of FeCN reduction. If the incomplete inhibition of FeCN reduction was due to reducing equivalents bypassing the site of DBMIB inhibition (i.e. incomplete inhibition by DBMIB) the complete inhibition of MV-mediated electron flow would not have been observed.

One of these sites of FeCN reduction is located near photosystem I and is coupled to phosphorylation and sensitive to both DBMIB and DCMU. The other site of FeCN reduction must occur after the site of DCMU inhibition and prior to the site of DBMIB inhibition. These sites are presented diagrammatically in Fig. 4.

In agreement with Trebst et al. (*1*) we have found that electron flow from water to FeCN, in the presence of DBMIB, is not affected by phosphorylating conditions, energy-transfer inhibitors or by known uncouplers. Trebst et al. (*1*) have suggested that the DBMIB-insensitive reduction is non-phosphorylating. However, their interpretation need not be correct and there may be a coupling site between water and the DBMIB-insensitive site of FeCN reduction. It is conceivable that the rate of reaction between FeCN and this site of reduction is too slow to detect any rate response (or even phosphorylation) indicative of coupling. Clarification of this point may be difficult because procedures which tend to expose this site for FeCN reduction (e.g. sonication (*2*)) also tend to uncouple (*6*).

Bohme et al. (*2*) regard this DBMIB-insensitive FeCN reduction as being due to a chemical reaction between endogenous reduced plastoquinone and FeCN. If this interpretation is correct it seems surprising that exogenous plastoquinone

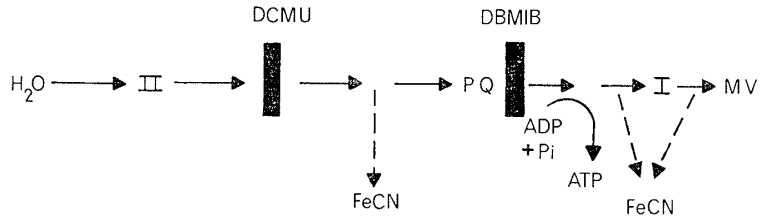

Fig. 4. *A schematic representation of the sites of reaction of inhibitors and electron acceptors.*

should recover both electron flow and photophosphorylation with FeCN. Exogenous plastoquinone would tend to short-circuit the electron flow without including a coupling site.

The precise location of the site of FeCN reduction which is sensitive to DBMIB, has not been resolved. The arguments are discussed by Bohme et al. (*2*) who conclude that it occurs after photosystem I. The only definite conclusion that can be made is that it occurs after plastoquinone. However, if the DBMIB-insensitive site of FeCN reduction (from water) is not coupled to phosphorylation (as current evidence suggests) then two coupling sites (*13*) must be located between the site of DBMIB inhibition and the second site of FeCN reduction. The energy drop between plastoquinone and photosystem I does not seem large enough to support two coupling sites. In which case it would be necessary to use photosystem I energy to reduce FeCN in the absence of DBMIB.

DBMIB as an uncoupler

The stimulation of ADP-limited oxygen evolution in a FeCN system (Fig. 2) with higher concentrations of DBMIB suggests an uncoupling property. However, these higher concentrations also stimulate a DBMIB-inhibited system (Table 1). This stimulation must be independent of uncoupling because DBMIB is a strong inhibitor of an uncoupled system (Fig. 1). There are three possible explanations:

(a) DBMIB tends to expose the first (DBMIB-insensitive) site of FeCN reduction.
(b) Excess DBMIB carries reducing equivalents around its own site of inhibition within the electron transfer chain.
(c) DBMIB is reduced by the chain and then chemically reduces FeCN.

The first possibility is considered unlikely. The second has merit, but, if it were the only explanation, the pseudo-cyclic MV system should show the same recovery as the non-cyclic FeCN system. Therefore, the chemical reaction between FeCN and reduced DBMIB is the most likely explanation.

Light-induced transfer of reducing equivalents from reduced DCPIP to MV is usually stimulated by uncoupling agents (Table 2, and *7*, *8*). DBMIB is as effective as known uncouplers in stimulating this type of electron flow which strongly suggests it has properties in common with other uncouplers. Phosphorylating conditions do not induce a similar increase in the rate of electron flow but ATP formation has been reported (*9*, *10*). Weak uncoupling of photophosphorylation by DBMIB may explain why phosphorylation lags behind electron flow in the plastoquinone recovery experiment of Bohme et al. (*2*).

The inhibition of the rate of PMS-catalysed cyclic photophosphorylation by DBMIB (Fig. 3) differs from the result reported by Bohme et al. who showed that DBMIB had no effect on PMS-catalysed cyclic phosphorylation. No likely explanation for this difference is readily available, however, it should be noted that Bohme et al. (*2*) subjected their chloroplast preparation to an osmotic shock.

Inhibition of PMS-catalysed cyclic photophosphorylation could be due to an uncoupling property of DBMIB. However, it could also be due to an inhibition of that part of the cyclic flow which proceeds through plastoquinone. The latter explanation conforms with the model of cyclic flow proposed by Bohme and Cramer (*11*). However, if the sole action of DBMIB is the inhibition of plastoquinone-mediated electron flow, it becomes difficult to explain the stimulation by DBMIB,

of electron flow between reduced DCPIP and MV.

Another quinone analogue 2,3-dimethyl-5-hydroxy-6-phytyl-β-benzoquinone has been used as an inhibitor of electron flow in isolated lettuce chloroplasts (*12*). This compound also inhibits PMS-catalysed cyclic photophosphorylation but does not stimulate electron flow in the reduced DCPIP-MV couple. In fact, it inhibited the uncoupler-stimulated electron flow of this system. Such conflicting results emphasise the caution needed in interpreting data obtained with these inhibitors.

Although differences in quality of chloroplast preparation or in the conditions of assay may explain some of the dissimilar results obtained with DBMIB, it is clear that DBMIB is not merely a plastoquinone antagonist.

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