Biochimica et Biophysica Acta, 547 (1979) 282—295

BBA 47698

EFFECTS OF DIBROMOTHYMOQUINONE ON MUNG BEAN MITOCHONDRIAL ELECTRON TRANSFER AND MEMBRANE FLUIDITY

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(Received November 23rd, 1978)

Key words: Dibromothymoquinone; Fluorescence polarization; Electron transfer; Membrane fluidity; (Mung bean mitochondria)

Summary

The effects of the quinone analog dibromothymoquinone on electron transfer in isolated mung bean mitochondria are described. Both the main, cyanide-sensitive and the alternate, cyanide-insensitive pathways are inhibited by dibromothymoquinone but in markedly different fashions. Half-maximal inhibition appeared at 40 μM and 20 μM dibromothymoquinone for the cyanide-sensitive and alternate pathways, respectively. With succinate as the electron donor, dibromothymoquinone inhibited the alternate pathway at a single site; showing a mixed, non-competitive type inhibition. On the succinate, cyanide-sensitive pathway dibromothymoquinone showed two sites of inhibition and neither coincides with the site of inhibition associated with the alternate pathway. With malate as the electron donor, two sites of inhibition by dibromothymoquinone were observed regardless of the pathway measured.

Dibromothymoquinone also inhibited the rate of valinomycin-induced swelling of isolated mung bean mitochondria. Steady-state kinetics showed the inhibition to be non-competitive with respect to valinomycin. Additionally dibromothymoquinone was observed to increase the fluorescence polarization associated with the hydrophobic probe 1,6-diphenylhexatriene. The results indicated that dibromothymoquinone decreased the fluidity of the inner mitochondrial membrane and suggested that the inhibition of mitochondrial electron transfer by dibromothymoquinone may be associated with this decrease in membrane fluidity.

The relationship of the multisite nature of the inhibition of electron transfer

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; 1799, bis-hexafluoroacetonyl-acetone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

by dibromothymoquinone and the possible role of mobile electron carriers such as ubiquinone on the main and alternate respiratory pathways of higher plants is discussed.

Introduction

Plant mitochondria contain a path of electron transfer in addition to the normal cytochrome pathway [1—3]. This 'alternate' pathway is insensitive to classical respiratory inhibitors such as cyanide and antimycin A, but is inhibited selectively by substituted hydroxamic acids [4]. The site of the branchpoint of electrons off the main, cyanide-sensitive chain onto the alternate pathway is not fully known. Bendall and Bonner [5] first localized the branchpoint on the substrate side of the cytochrome *b* pool using the inhibitor antimycin A. Since that time studies by Von Jagow and Bohrer [6] using ubiquinone-depleted mitochondria isolated from *Neurospora*, Storey [7] using stopped-flow kinetic techniques on mung bean mitochondria, and Rich and coworkers [8—10] using EPR spectroscopy and rapid freeze kinetic techniques have each concluded that the branchpoint of the alternate pathway is at the level of a pool of ubiquinone. Rich and Bonner [10] have further suggested that an auto-oxidizable pool of ubiquinone serves the dual role of branchpoint and alternate oxidase. Still unknown is how, if at all, this auto-oxidizable quinone pool differs from the bulk pool of ubiquinone associated with the cyanide-sensitive pathway.

Dibromothymoquinone (2,5-dibromo-6-methyl-3-isopropyl-*p*-benzoquinone, DBMIB) is well established as an inhibitor of photosynthetic electron transport. It has been shown to inhibit electron flow between Photosystems I and II apparently by acting as an antagonist of plastoquinone; preventing the reoxidation of reduced plastoquinone [11,12]. Loschen and Azzi [13] first reported the use of DBMIB to inhibit electron transfer in isolated mitochondria. They showed that DBMIB inhibited both succinate- and malate-linked electron transfer in mitochondria isolated from rat heart. Half-maximal inhibition of electron transfer appeared with 20—25 μM DBMIB which is well above the level of DBMIB (1 μM) required to observe complete inhibition of electron transfer in isolated chloroplasts [12]. Because cytochrome *b*-562 remained oxidized during electron turnover in the presence of DBMIB, Loschen and Azzi [13] concluded that DBMIB was inhibiting electron transfer on the substrate side of cytochrome *b*-562, probably at the level of ubiquinone. Later studies have examined the effects of DBMIB on electron transfer in *Escherichia coli* and have concluded similarly that DBMIB acts to inhibit electron flow at the level of quinone [14—16].

None of the electron transfer systems used in the DBMIB studies cited above showed alternate oxidase activity. Recently, however, Drabikowska [17] reported that DBMIB inhibited both the main and alternate pathways in a mutant of *Neurospora* that contains the alternate pathway. When the two pathways were looked at separately, it was found that higher concentrations of DBMIB were required to obtain half-maximal inhibition of the cyanide-sensitive pathway (250 μM) than of the alternate pathway (100 μM). Drabikowska [17] concluded that the results supported the concept that ubiquinone served

as the branchpoint of the alternate pathway and that DBMIB was acting as an antagonist of ubiquinone.

Because DBMIB appears to act as a general quinone antagonist in many different electron transfer systems, it was of interest to examine the effects of DBMIB on the main and alternate pathways of mitochondria isolated from a higher plant (mung bean). Identification of differential effects of DBMIB on the cyanide-sensitive and the alternate pathways should help to clarify the nature of the quinone pool(s) associated with the two electron transfer pathways, and the possible involvement of ubiquinone as the branchpoint of the alternate pathway. Evidence is presented to show that DBMIB inhibits both pathways, but in markedly different fashions. Furthermore, at the concentrations required to observe inhibition of electron transfer, DBMIB is observed to decrease the fluidity of the mitochondrial membrane. The relation of these results to the nature of the DBMIB inhibition of electron transfer and the possible role of ubiquinone on the main and alternate pathways is discussed.

Materials and Methods

Plant material and preparation of mitochondria. Etiolated mung bean hypocotyls (*Phaseolus aureus* Roxb.) were grown for 3—5 days in a dark room maintained at 27°C and approximately 80% relative humidity. Whole mitochondria were isolated either according to the procedure outlined by Bonner [18] or by the method of Moreland and Boots [19]. These methods consistently gave preparations of mitochondria showing good rates of state 3 electron transfer (300+ natoms 0/min per mg protein, succinate; 400—500 natoms 0/min per mg protein, malate) and reasonable values of respiratory control (2.5—2.8, succinate; 4.5—6.0, malate). The level of cyanide-insensitive respiration associated with these mitochondria was 15—20% of the state 3 rate.

Electron transfer measurements. Oxygen uptake rates were measured polarographically using a Clark-type oxygen electrode (Yellow Springs Instruments) in a medium containing either 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 10 mM potassium phosphate (pH 7.2) or 0.15 M KCl, 0.5 mM $MgCl_2$ and 10 mM potassium phosphate (pH 7.2). The electrode was fitted to a 2.0 ml glass reaction cell thermostated at 25°C.

Swelling. Osmotic volume changes were measured at the apparent absorbance decrease at 520 nm. The 2.0 ml reaction mixture contained 0.15 M KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)/NaOH (pH 7.1), and mitochondria (0.4 mg protein/ml). Swelling was initiated by the addition of 0.1 μM valinomycin. To determine inhibition of valinomycin-induced swelling by DBMIB, the compound at concentrations indicated in the text was added 1 min before addition of valinomycin. Swelling rate, expressed as ΔA_{520}/min, was calculated from the initial linear phase of absorbance decrease.

Fluorescence polarization. Mitochondria were labelled with the fluidity probe by incubation at 25°C in: 0.15 M KCl, 10 mM Hepes/NaOH (pH 7.1) and 1 μM 1,6-diphenylhexatriene. After maximum incorporation (maximum fluorescence yield, 15 min), excess probe was removed by centrifugation. Diphenylhexatriene was excited at 365 nm and fluorescence was monitored at 460 nm.

Fluorescence polarization was calculated as described by Shinitzkey and Inbar [20].

Others. Protein was measured by the method of Lowry et al. [21]. DBMIB was synthesized according to a procedure used by Dr. N.E. Good (this procedure was kindly supplied by Dr. C.F. Yocum). bis-Hexafluoroacetylacetone (1799) was a gift of Dr. P.G. Heytler (Du Pont de Nemours, Wilmington, DE). All other reagents were of the highest purity commercially available. Data are averages of at least three separate experiments.

Results

Inhibition of electron transfer by DBMIB

Fig. 1 shows the effect of increasing concentrations of DBMIB on electron transfer through both the cyanide-sensitive and the alternate pathways using succinate as the electron donor. The two pathways were separated by running the reactions in the presence of 1.0 mM salicylhydroxamic acid and 0.25 mM KCN, respectively. The addition of increasing concentrations of DBMIB markedly inhibited the rate of oxygen uptake through both pathways. There is, however, a distinct difference to the inhibition patterns observed with the two pathways. The concentration of DBMIB required for half-maximal inhibition of the cyanide-sensitive pathway (40 μM) was approximately twice that required for half-maximal inhibition of the alternate pathway (20 μM). These results were qualitatively similar to those reported by Drabikowska for the inhibition of the two pathways by DBMIB in *Neurospora* [17]. However, Drabikowska

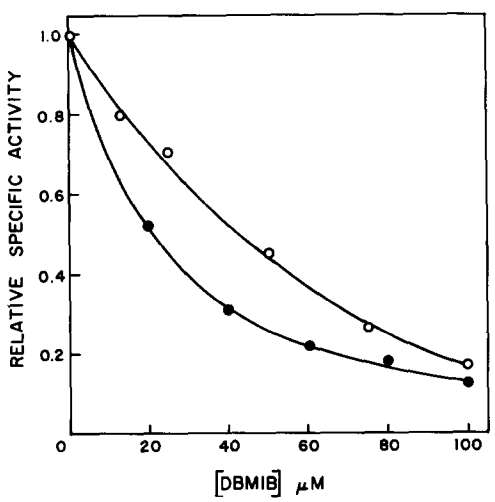


Fig. 1. Effects of DBMIB on cyanide-sensitive and alternate pathway electron transfer in mung bean mitochondria. DBMIB was added as indicated after attainment of a steady-state rate upon the addition of 10 mM succinate. Cyanide-sensitive electron transfer (○) was carried out in the presence of 1.0 mM salicylhydroxamic acid. Alternate pathway electron transfer (●) was carried out in the presence of 0.25 mM KCN. Mung bean mitochondria (0.3—0.5 mg protein/ml) were incubated with ATP (150 μM), 1799 (5 μM), and the appropriate inhibitor for 2 min prior to the addition of substrate. The reaction medium contained 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 10 mM potassium phosphate, pH 7.2. Other conditions are as given in Materials and Methods.

found much higher concentrations of DBMIB were required to obtain half-maximal inhibition of electron transfer. She reported values of 250 μM and 100 μM for inhibition of the cyanide-sensitive and alternate pathways, respectively. Drabikowska also reported a lag associated with inhibition of cyanide-sensitive electron transfer such that no inhibition by DBMIB was observed at concentrations less than 100 μM. No such lag was seen in our experiments and as shown in Fig. 1, the cyanide-sensitive pathway in mung bean mitochondria was inhibited more than 80% in the presence of 100 μM DBMIB. The apparent discrepancy probably resulted from the use of serum albumin (2 mg/ml) in the reaction mixture in the study on *Neurospora* [17]. Bovine serum albumin has been reported to bind DBMIB [22] and was recently used to reverse the inhibition of electron transfer by DBMIB in isolated chloroplasts [23]. Our own studies have shown that 2 mg/ml of bovine serum albumin will substantially protect mung bean mitochondrial electron transfer against inhibition by DBMIB thereby resulting in values that approached those reported by Drabikowska (data not shown). The concentrations of DBMIB required to inhibit cyanide-sensitive electron transfer in mung bean mitochondria are similar to those reported by Loschen and Azzi [13] for the inhibition of electron transfer in rat heart mitochondria.

Several additional points should be made concerning the observed inhibition of electron transfer by DBMIB. Inhibition of either pathway by DBMIB was unaffected by the concentration of mitochondrial protein used in the assay over the range 0.2—0.8 mg/ml. This result coupled with the overall shape of the DBMIB inhibition curves argued against a stoichiometric (i.e. irreversible) binding site for DBMIB on the mitochondrial membrane. In addition, complete inhibition of electron transfer could be achieved on either pathway by the addition of 150—200 μM DBMIB, and no stimulation of oxygen uptake was observed in the presence of added DBMIB up to a concentration of 250 μM. DBMIB was thus unable to serve as an electron acceptor in isolated mung bean mitochondria under the conditions used in this study. The ability of DBMIB to act as an auto-oxidizable electron acceptor has been well characterized in isolated chloroplasts [24,25]. Of those studies where DBMIB has been used to inhibit 'mitochondrial-type' electron transfer, only Poole and Haddock [14] working with *E. coli* membrane particles have reported a stimulation of oxygen uptake at high concentrations of DBMIB. Finally, the electron transfer reactions in this experiment were run in the presence of the uncoupler 1799 (5 μM). This eliminated any interference from the inhibition of energy-linked processes by DBMIB as reported by Sun and Crane [15] and Houghton et al. [16] in *E. coli* membrane particles.

Because the cyanide-sensitive and alternate pathways showed different sensitivities toward inhibition by DBMIB, we attempted to quantify the nature of the two inhibitions. The data were analyzed with the method of Dixon, in which the reciprocal of the specific activity was plotted versus inhibitor concentration [27]. A Dixon plot of the DBMIB inhibition of the alternate pathway with succinate at two concentrations as the electron donor is shown in Fig. 2. DBMIB inhibited the alternate pathway in a simple hyperbolic fashion suggesting a single site of action. The intersection of the two lines in the second quadrant showed the inhibition to be of a mixed non-competitive type (Line-

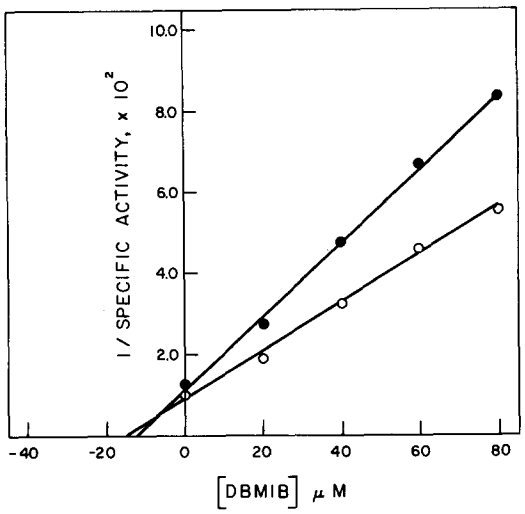


Fig. 2. Inhibition of alternate pathway electron transfer by DBMIB in mung bean mitochondria, Dixon plot. Mung bean mitochondria (0.45 mg protein/ml) were incubated with 0.25 mM KCN prior to the addition of substrate. The reaction was initiated by the addition of either 2.0 mM (●) or 10 mM (○) succinate. All other experimental conditions were as given in Fig. 1.

weaver-Burk double-reciprocal plots confirmed this), with DBMIB showing a higher affinity for the free enzyme (K_{EI}) than for the enzyme-substrate complex K_{ESI} (see Eqn. 1).

$$\begin{array}{ccc} & E + S \rightleftharpoons ES \rightarrow E + P & \\ K_{EI} \downarrow & \quad K_{ESI} \downarrow & \\ EI & \quad ESI & \end{array} \qquad (1)$$

The two inhibition constants obtained from Fig. 2 were 7 μM (K_{EI}) and 15 μM (K_{ESI}). The simplest interpretation of the two K_i values in mitochondrial terms was that K_{EI} represented binding to oxidized mitochondria while K_{ESI} was associated with mitochondria in some reduced state. K_{EI} was found to vary from 7 to 15 μM (average = 12 μM) whereas K_{ESI} was always equal to approximately $2K_{EI}$ (Table I).

Inhibition of succinate-supported, cyanide-sensitive electron transfer by DBMIB gave markedly non-linear Dixon plots (Fig. 3). The curves became increasingly parabolic with decreasing succinate concentrations. Trivial explanations for non-linear Dixon plots involve the irreversible binding of DBMIB at its site of action or the interaction of a significant fraction of the DBMIB with a non-inhibitory site either on the mitochondria or with some component of the reaction mixture [26]. These explanations were eliminated because, as noted previously, the degree of inhibition by DBMIB was insensitive to the level of mitochondrial protein. In addition, inhibition of the cyanide-sensitive pathway by DBMIB was not influenced by varying the concentration of salicylhydroxamic acid between 0.5 and 5.0 mM. The non-linear Dixon plots were therefore not the result of an interaction between DBMIB and the salicyl-

TABLE I

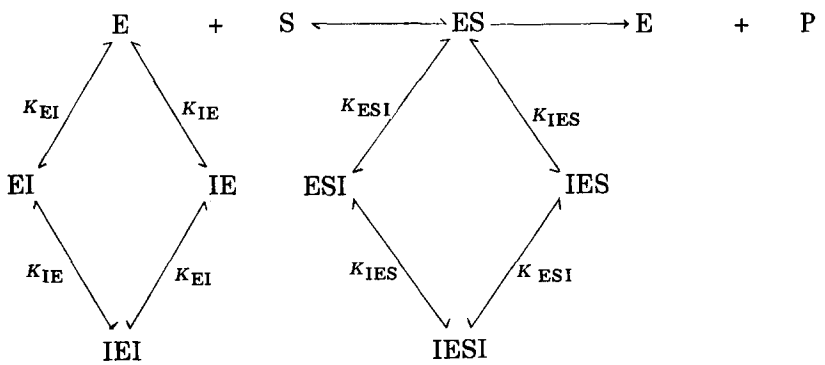
INHIBITION CONSTANTS FOR THE INHIBITION OF CYANIDE-SENSITIVE AND ALTERNATE PATHWAY ELECTRON TRANSFER BY DBMIB IN MUNG BEAN MITOCHONDRIA

The inhibition constants were determined from Dixon plots of the reciprocal of the rate of electron transfer against DBMIB concentration. For multisite inhibitions, best fits to Eqn. 3 were obtained as described in the text. Experimental conditions were as described in Figs. 2 and 3 for succinate reactions. Malate-stimulated electron transfer was carried out under the conditions of Figs. 2 and 3 except that succinate was replaced with 10 mM and 25 mM malate as the electron donor. All constants are expressed as dissociation constants and represent the average of at least three separate determinations.

Pathway	Inhibition constant (μM)			
	K_{EI}	K_{ESI}	K_{IE}	K_{IES}
Succinate				
Alternate	12	24	—	—
Cyanide-sensitive	54	141	25	73
Malate				
Alternate	18	36	30	80
Cyanide-sensitive	33	87	23	70

hydroxamic acid (1.0 mM) that was used in these assays to block the alternate pathway.

The parabolic nature of the curves obtained at low succinate concentrations suggested that the inhibition was proportional to the square of the inhibitor concentration. This would be expected if DBMIB had two sites of inhibition on the cyanide-sensitive pathway. The kinetic scheme depicting such an interaction is shown in Eqn. 2 and the resulting rate expression for two non-interacting sites of inhibition is given as Eqn. 3.



(2)

$$\frac{1}{v} = \frac{K_m}{V[S]}\left[1 + \frac{[I]}{K'_1} + \frac{[I]^2}{K'_1K'_2}\right] + \frac{1}{V}\left[1 + \frac{[I]}{K'_3} + \frac{[I]^2}{K'_3K'_4}\right] \quad (3)$$

Where

$$K'_1 = \frac{K_{EI}K_{IE}}{K_{EI} + K_{IE}} \qquad K'_3 = \frac{K_{ESI}K_{IES}}{K_{ESI} + K_{IES}}$$

$$K'_2 = K_{EI} + K_{IE} \qquad K'_4 = K_{ESI} + K_{IES}$$

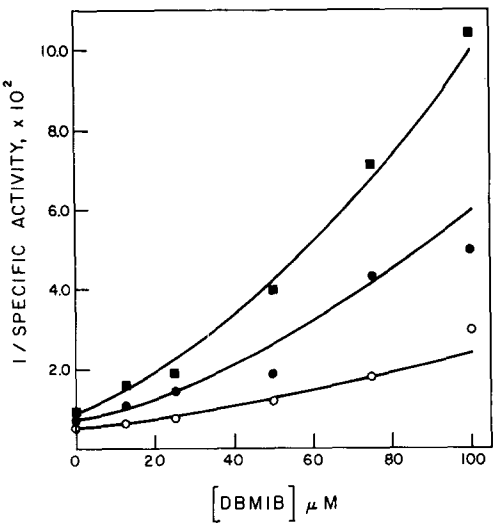


Fig. 3. Inhibition of cyanide-sensitive electron transfer by DBMIB in mung bean mitochondria, Dixon plot. Mung bean mitochondria were incubated with 1.0 mM salicylhydroxamic acid prior to the addition of substrate. The reaction was initiated by the addition of either 0.5 mM (■), 1.0 mM (●), or 10 mM (○) succinate. Other experimental conditions were as given in Fig. 1.

In Eqn. 3, v, V, K_m, [S], and [I] represent initial velocity, maximal velocity, Michaelis constant, substrate concentration, and inhibitor concentration, respectively. K_m and V were calculated from Lineweaver-Burk plots of the rates obtained in the absence of DBMIB. The solid lines shown in Fig. 3 represent theoretical lines obtained from the solution of Eqn. 3 using values for K_{EI} and K_{IE} of 20 μM and 50 μM and for K_{ESI} and K_{IES} of 70 μM and 150 μM. This kind of solution cannot distinguish inhibition constants corresponding to the K_{EI}—K_{ESI} pair from those corresponding to K_{IE}—K_{IES}. The correspondence between the calculated lines and the observed data was quite good and supported the concept of two sites of inhibition by DBMIB on the cyanide-sensitive electron transfer pathway. The inhibition constants did not vary appreciably with different preparations of mitochondria as shown by the averages obtained with several preparations (Table I).

The trend with the inhibition constants observed for the alternate pathway (i.e. $K_{EI} < K_{ESI}$) was also observed with both sites on the cyanide-sensitive pathway, although the difference was closer to three-fold if the pairings shown in Table I were used. However, neither of the two sites found for DBMIB inhibition of the cyanide-sensitive pathway corresponded to the single site of inhibition associated with the alternate pathway.

Studies similar to those outlined above were also carried out with malate as the electron donor. However, with malate both the cyanide-sensitive and alternate pathways gave parabolic Dixon plots, indicating that electron flow from NADH (internal) was inhibited by DBMIB at two sites regardless of the pathway measured. The inhibition constants obtained from best fits of Eqn. 3 to the observed data appear in Table I. .These results showed that a 25—75 μM (K_{EI}—K_{ESI}) DBMIB inhibition site was associated with both cyanide-sensitive

and alternate electron flow from malate. The second site on the malate alternate pathway (18—36 μM) was more sensitive to DBMIB than the second site on the cyanide-sensitive pathway (33—87 μM). In addition, the second site of inhibition by DBMIB on the malate alternate pathway corresponded closely to the single site of inhibition observed with the succinate alternate pathway (12—24 μM). Finally, the second site of inhibition on the malate cyanide-sensitive pathway (33—87 μM) was more sensitive to DBMIB than the second site on the succinate cyanide-sensitive pathway (54—141 μM).

The above results were markedly reproducible given their complexity. Because DBMIB is considered to act as a quinone antagonist in the chloroplast electron transfer chain, it is tempting to speculate that multiple DBMIB inhibition sites on the mung bean mitochondrial electron transfer chain are associated with multiple sites of action of ubiquinone. Multiple quinone pools have been postulated to occur in *E. coli* [28] and speculation concerning multiple sites of action of ubiquinone has been made with regard to mitochondria from higher organisms [29—31]. Whereas ubiquinone may have multiple sites of action on the mitochondrial electron transfer chain, the results as tabulated in Table I defy interpretation in terms of a linear scheme of electron flow. Furthermore, added ubiquinone-10 was unable to reverse the inhibition of electron transfer by DBMIB on either the cyanide-sensitive or the alternate pathway (data not shown). Interpretation of the above results will be deferred until further data outlining a possible mechanism of inhibition of electron transfer by DBMIB have been presented.

Effect of DBMIB on membrane fluidity

The inhibition of electron transport by DBMIB at multiple sites described above is not consistent with a site-specific mode of action of DBMIB; e.g. inhibition at the level of quinone, as with chloroplast electron transport [12]. Alternatively, DBMIB may cause a general membrane perturbation that is manifested as multiple sites of inhibition.

The effect of DBMIB on membrane fluidity was therefore determined. An indicator of membrane fluidity is the rate of valinomycin-induced swelling of mitochondria suspended in isotonic KCl. The inner membrane of mung bean mitochondria is normally permeable to Cl^- but impermeable to K^+ [32]. When the inner membrane is made permeable to K^+ by the ionophore valinomycin [33], the mitochondria swell (apparent absorbance decrease) because of net uptake of K^+ and Cl^- (Fig. 4, 0 μM curve). Low concentrations of DBMIB inhibited the rate of valinomycin-induced swelling (Fig. 4). The results suggested that DBMIB reduced membrane fluidity because valinomycin is a mobile carrier that requires a fluid membrane for transport of K^+ [33,34].

The concentration dependence of DBMIB inhibition of valinomycin-induced swelling is shown in Fig. 5. Maximal inhibition of swelling rate (approximately 85%) required about 50 μM DBMIB. Half-maximal inhibition was attained at 4 μM DBMIB.

The effect of DBMIB on fluorescence polarization of 1,6-diphenylhexatriene, which is a direct measure of membrane fluidity [20], is also presented in Fig. 5. As shown, concentrations of DBMIB that inhibited valinomycin-induced swelling significantly increased the fluorescence polarization of the

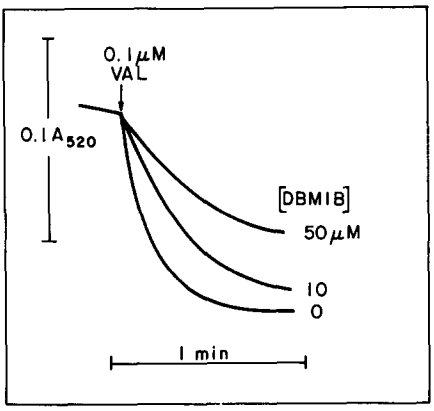


Fig. 4. Inhibition of valinomycin-induced swelling of mung bean mitochondria by DBMIB. For details, see Materials and Methods.

probe, which indicated a substantial decrease in membrane fluidity [20]. Some quenching of fluorescence was observed upon the addition of DBMIB. This might affect the observed polarization if it decreased the fluorescence lifetime. However, a plot of the polarization ($1/p$—$1/3$) versus total fluorescence ($I_{\parallel} + 2I_{\perp}$) indicated that the observed polarization did not originate from a decreased fluorescence lifetime due to quenching.

Further support for the hypothesis that DBMIB reduced membrane fluidity is provided by the results presented in Fig. 6, which showed that DBMIB reduced the maximum rate of swelling without affecting the apparent K_m for valinomycin. This result eliminates the possibility that DBMIB inhibited

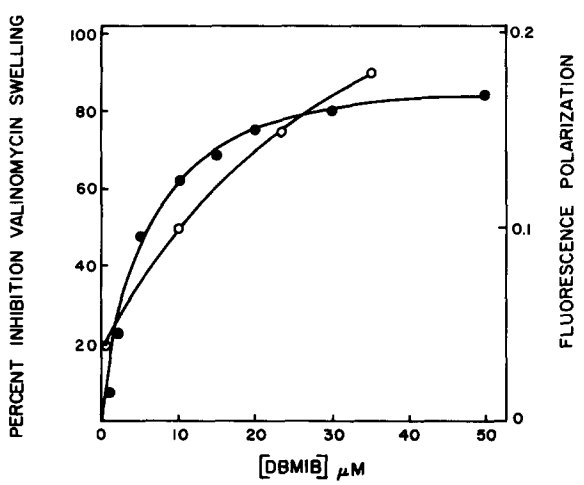


Fig. 5. Effect of DBMIB concentration on the rate of valinomycin-induced swelling (●) and fluorescence polarization of 1,6-diphenylhexatriene (○) with mung bean mitochondria. For details, see Materials and Methods.

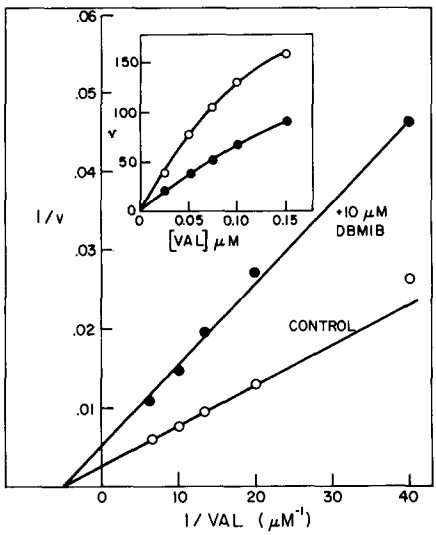


Fig. 6. Valinomycin dependence of mitochondrial swelling rate in the presence and absence of 10 μM DBMIB. The insert is a standard plot of swelling rate as a function of valinomycin concentration.

swelling by reducing the partitioning the valinomycin-K^+ complex into the membrane (which would produce competitive inhibition).

Discussion

The results presented in this paper show a strong correlation between the concentrations of DBMIB required to inhibit electron transfer and bring about a decrease in the fluidity of the inner mitochondrial membrane. Given this marked correlation, the inhibition of electron transfer by DBMIB in isolated mung bean mitochondria may be attributable to a decrease in membrane fluidity and not to the specific interaction of DBMIB with ubiquinone. The possibility that DBMIB specifically interacts with ubiquinone and that the multisite nature of the DBMIB inhibition can be attributed to multiple ubiquinone pools was ruled out for several reasons. First, the concentration of DBMIB required to specifically inhibit plastoquinol oxidation in isolated chloroplasts is roughly stoichiometric with the levels of plastoquinone in the chloroplast membrane [12]. In contrast, complete inhibition of mitochondrial electron transfer required the addition of DBMIB at a concentration almost two orders of magnitude greater than that of the ubiquinone present in the mitochondrial membrane. Second, there was no tendency for added DBMIB to undergo reduction (and subsequent auto-oxidation) in mung bean mitochondria suggesting that DBMIB is unable to interact directly with the ubiquinol pool as it does with isolated chloroplasts [24,25]. Third, the addition of exogenous ubiquinone-10 was unable to reverse the inhibition of mitochondrial electron transfer by DBMIB. This is again in contrast to isolated chloroplasts where added plastoquinone can completely reverse the inhibition of photosynthetic electron transfer by DBMIB [12]. Given differences between the

inhibition of mitochondrial and chloroplast electron transfer by DBMIB, the multisite nature of the DBMIB inhibition, and the marked correlation between the concentrations of DBMIB required to inhibit electron transfer and decrease membrane fluidity in plant mitochondria, it seemed more reasonable to attribute the inhibition of mitochondrial electron transfer by DBMIB to the more general affects associated with a decrease in membrane fluidity.

The relationship between membrane fluidity and the inhibition of electron transfer may be associated with the decreased movement of some 'mobile' electron carrier(s) within the mitochondrial membrane in a manner not unlike the inhibition of valinomycin-induced swelling. A 'site' of inhibition would then represent a step in the electron transfer chain that required a fluid membrane. In this regard, ubiquinone (or a ubiquinone-protein complex [29]) has been postulated to act as a mobile electron carrier across the inner mitochondrial membrane [30,35]. The inhibition of electron transfer by DBMIB might, therefore, still be at the level of ubiquinone, but its mechanism of action would be indirect, i.e. the result of decreased membrane fluidity. Other mobile electron carriers may be inhibited similarly by DBMIB.

Given the rather general nature of the inhibition of electron transfer resulting from a decrease in membrane fluidity, it would not be surprising if other lipophilic inhibitors of mitochondrial electron transfer acted by decreasing membrane fluidity. This has been postulated by Moreland and Huber [32] to be the case with a number of herbicides. Previous studies concerned with the inhibition of mitochondrial electron transfer by DBMIB have found that half-maximal inhibition required at least 10—40 μM DBMIB [13—17], which is within the range of concentrations required to substantially decrease membrane fluidity (Fig. 5). It is probable that the inhibition of electron transfer observed in these studies resulted from a DBMIB-induced decrease in membrane fluidity. In chloroplasts, the DBMIB inhibition of electron transfer is probably more specific as complete inhibition is observed with submicromolar levels of DBMIB [12]; somewhat below the concentrations required to affect membrane fluidity in mung bean mitochondria. Recent reports of a second site of action of DBMIB on the chloroplast electron transfer chain showing inhibition with 10 μM DBMIB [23,36] may indicate that the fluidity of the chloroplast membrane is also affected at high concentrations of DBMIB.

It is tempting to try to correlate the multisite inhibition of electron transfer by DBMIB with sites of action of mobile electron carriers. Using the results outlined in Table I, there appears to be a site in Complex I, a site associated with the alternate pathway, and one or two sites in Complex III depending upon whether malate or succinate is the electron donor. Relating this to the role of ubiquinone, Mitchell has recently presented a model (the protonmotive Q cycle) that outlines two sites in Complex III where ubiquinone shuttles across the inner mitochondrial membrane and two additional sites where ubiquinone movement (parallel to the membrane plane) might also be necessary [30,35]. The involvement of this Q cycle in plant mitochondrial electron transfer and in particular its role in the branching of electrons from the cyanide-sensitive pathway onto the alternate pathway has been postulated by Rich and Moore [8] and more recently by Rich and Bonner [10]. The results given in this paper generally support the suggestion that the branchpoint of the

alternate pathway is located in Complex III prior to the antimycin-sensitive site. The observations that the alternate pathway is inhibited by DBMIB would also indicate that a mobile electron carrier is required for the alternate pathway to operate. This might be expected if the Q cycle is involved in the operation of the alternate pathway [35] and might also be necessary if the alternate pathway is associated with the C-side of the inner mitochondrial membrane as has been suggested [4].

Conclusions

1. DBMIB inhibited both the cyanide-sensitive and the alternate pathways of electron transfer in isolated mung bean mitochondria with either succinate or malate as the electron donor.

2. The inhibition patterns derived from steady-state kinetic analyses supported the suggestion that the branchpoint of the alternate pathway is located in Complex III.

3. DBMIB was observed to decrease the fluidity of the inner mitochondrial membrane as evidenced by: (a) inhibition of the rate of valinomycin-induced mitochondrial swelling; (b) an increased fluoresence polarization of the fluorescent probe diphenylhexatriene.

4. The inhibition of mitochondrial electron transfer by DBMIB may result from a DBMIB-induced decrease in the fluidity of the inner mitochondrial membrane. This nonspecific mechanism of action manifests itself as multiple sites of inhibition of DBMIB on the mitochondrial electron transfer chain.

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