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TWO PATHWAYS OF ELECTRON TRANSFER IN QUINOL-MEDIATED CYCLIC PHOSPHORYLATION IN SPINACH CHLOROPLASTS

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Summary

Low potential quinones are mediators of cyclic phosphorylation in washed spinach thylakoid membranes if they are prereduced to provide the proper redox poise. Cyclic phosphorylation catalyzed by different quinols varies in its sensitivity to the electron transfer inhibitor 2-iodo-6-isopropyl-3-methyl-2',4,4'-trinitrodiphenyl ether (DNPINT), which is thought to inhibit electron flux from the bound plastoquinone (B) to the plastoquinone pool (Trebst, A., Wietoska, H., Draber, W. and Knops, H.J. (1978) Z. Naturforsch. 33c, 919—927). Cyclic phosphorylation catalyzed by uncharged quinols is extremely sensitive to DNPINT, whereas cyclic phosphorylation catalyzed by negatively charged quinols is approximately two orders of magnitude less sensitive. Many quinols have pK_1 values in the physiological range (pH 7—9). Increasing the concentration of the deprotonated quinol either by raising the assay pH, increasing the mediator concentration, or increasing the fractional reduction of the quinone results in a decrease in the sensitivity of cyclic phosphorylation to DNPINT. At very high DNPINT concentrations, cyclic phosphorylation catalyzed by all quinols (and ferredoxin) is inhibited, but not phenazine methosulfate catalyzed cyclic phosphorylation.

These data suggest that the deprotonated form of the quinol can donate electrons directly to the plastoquinone pool, whereas the uncharged quinol most obligately transfer electrons through the bound plastoquinone 'B'. A second site of DNPINT action after the plastoquinone pool is also observed,

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Abbreviations: DNPINT, 2-iodo-6-isopropyl-3-methyl-2',4,4'-trinitrodiphenyl ether, also known as the dinitrophenyl ether of iodonitrothymol; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (dibromothymo-quinone).

which requires much higher DNPINT concentrations for inhibition of phosphorylation.

Introduction

Noncyclic electron transfer in spinach thylakoid membranes is coupled to phosphorylation via the light-induced protonmotive force [1]. Electron flux in two regions of the electon transfer chain results in a transmembrane pH gradient: one region is on the oxidizing side of Photosystem II (i.e., water oxidation) and the other region is associated with electron transfer through the plastoquinone pool [2—4]. Vectorial oxidation and reduction of plastoquinone is thought to be coupled to the shuttling of protons across the thylakoid membrane. The plastoquinone proton shuttle is probably operative in ferredoxin-catalyzed, Photosystem I driven, cyclic phosphorylation in vivo [5].

In vitro, a wide variety of compounds can be used to mediate Photosystem I driven cyclic phosphorylation. They can be grouped in terms of the length of the electron transfer chain that they use. Phenazine methosulfate and diaminodurene appear to require only a very short segment of the electron transfer chain prior to and including Photosystem I. Phosphorylation catalyzed by these mediators is insensitive to the plastoquinone antagonist dibromothymoquinone (DBMIB), which excludes the participation of plastoquinone in the formation of the protonmotive force [6,7]. Cyclic electron transfer catalyzed by naphthoquinones and anthraquinones, on the other hand, is coupled to phosphorylation via the plastoquinone proton shuttle as shown by its sensitivity to dibromothymoquinone [7,8]. The largest segment of the electron transfer chain is used by ferredoxin-catalyzed cyclic phosphorylation. It is inhibited by both antimycin A and dibromothymoquinone, implying a flux of electrons through both cytochrome *b*-563 and plastoquinone [5,9—11].

Trebst et al. [12] have recently characterized the mode of action of the electron transfer inhibitor 2-iodo-6-isopropyl-3-methyl-2',4,4'-trinitrodiphenyl ether (DNPINT). At low concentrations, this compound is thought to inhibit the reduction of the plastoquinone pool, probably at the level of the bound plastoquinone, 'B' [13,14]. This paper extends the characterization of DNPINT and shows how an electrostatic charge on quinol mediators affects electron transfer into the plastoquinone pool.

Materials and Methods

Chloroplasts. Chloroplasts were isolated from spinach leaves as previously described [15] and suspended in 0.3 M sucrose, 20 mM Tricine-KOH (pH 8.0), 10 mM KCl and 5 mM $MgCl_2$. Chlorophyll concentration was determined by the method of Arnon [16].

Cyclic phosphorylation. Reaction mixtures for cyclic phosphorylation contained 20 mM Tricine-KOH (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, 2 mM ADP, 2 mM [^{32}P]phosphate (containing approx. $1 \cdot 10^7$ cpm/ml), 10 μM 3-(3',4'-dichlorophenyl)-1,1-dimethylurea, 10 mM glucose, 0.1 unit/ml hexokinase, 0.25 mg/ml bovine serum albumin and the appropriate mediator. DNPINT

was added as a small volume (3 to 20 μl) of a dimethylsulfoxide solution. In order to prevent autooxidation of the reduced mediators, 1.5 ml of the reaction mixture were added to a 9 ml serum vial, and the vial stoppered and depleted of oxygen as previously described [8]. Chloroplasts were depleted of oxygen separately. A small volume of a 7.5 mM dithionite solution was then added to provide the desired redox poise. Reaction mixtures, containing 10 μg of chlorophyll per 1.5 ml, were placed in a 20°C water bath, illuminated from 10 cm below by a 400 W high-pressure sodium vapor lamp (output 43 000 lumens) for 5 min, and assayed for [γ-^{32}P]ATP as previously described [8]. All additions to stoppered vials were made with gas-tight syringes.

Mediators. Ferredoxin was prepared as described by Petering and Palmer [17] as previously modified [18]. Quinones were recrystallized from alcohol, except for 1,4-naphthoquinone, which was purified by sublimation.

Determination of quinol pK_1. A 350 ml solution of a broad-range buffer (5 mM each of tris(hydroxymethyl)aminomethane, phosphate, carbonate and borate (borate was omitted when titrating 2-hydroxynaphthoquinone)) containing approx. 100 μM of a quinone was deoxygenated by sparging rapidly with nitrogen. The quinone was reduced by adding an excess of dithionite or borohydride, and the pH was titrated by adding small volumes of acid or base while monitoring the pH with a glass pH electrode. Portions of the solution were transferred to an anaerobic cuvette, and the ultraviolet-visible spectra were recorded with an Aminco DW-2 spectrophotometer. The pK_1 was determined from the estimated pH midpoint of the wavelength shift in adsorption peaks [19,20].

Results

Redox poising of quinone mediators in cyclic phosphorylation

In order for an electron transfer mediator to catalyze cyclic phosphorylation efficiently, there must be an optimal balance between the reduced and oxidized forms of the mediator, i.e. the mediator must be redox poised [21]. Many of the quinones used in these experiments are relatively low potential (+100 to −300 mV) and autooxidizable. Dithionite was chosen to prereduce these compounds, and the cyclic phosphorylation assays were performed under anaerobic conditions to preserve the desired redox poise [8].

Fig. 1 shows that the optimal fractional reduction for cylic phosphorylation varies depending upon the mediator employed. Whereas menadione and 1,4-naphthoquinone are most efficient mediators at 60% and 53% reduction, respectively, anthraquinone 2-sulfonate, 2-hydroxynaphthoquinone and phenanthrenequinone show peak rates of ATP synthesis at or near full reduction. Cyclic phosphorylation catalyzed by all these quinones is sensitive to dibromothymoquinone, implying that electron flux passes through the plastoquinone pool [6,8,22], or at least through the plastoquinone oxidation site.

DNPINT inhibition of quinone-mediated cyclic phosphorylation

The dinitrophenyl ether of iodonitrothymol (DNPINT), and inhibitor without intrinsic redox properties which was introduced by Trebst et al. [12], is thought to block electron transfer between 'B' (a bound plastoquinone)

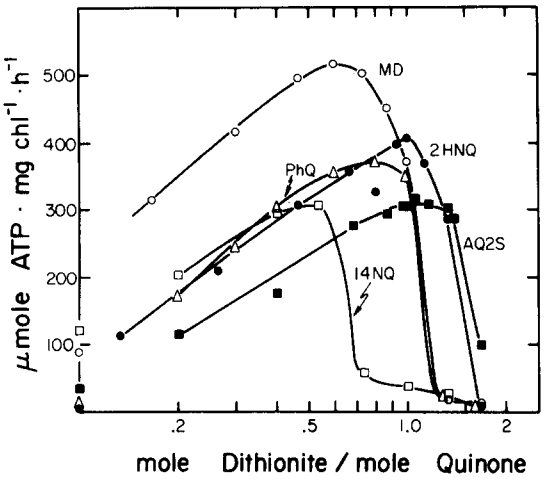

Fig. 1. The influence of the reduction level of quinone mediators on the rate of cyclic phosphorylation. Cyclic phosphorylation was assayed with 150 μM of the indicated quinone, except in the case of phenanthrenequinone (100 μM). A small volume of a dithionite solution was added to reaction mixture to provide the indicated ratios of dithionite to quinone. Symbols: MD (○), menadione; 2 HNQ (●), 2-hydroxynaphthoquinone; PhQ (△), phenanthrenequinone; AQ2S (■), anthraquinone 2-sulfonate; 14NQ (□), 1,4-naphthoquinone.

and the plastoquinone pool. Fig. 2 shows the effect of DNPINT on cyclic phosphorylation catalyzed by various quinone mediators under conditions of optimal redox poise for each quinone. Cyclic phosphorylation catalyzed by menadione and 1,4-naphthoquinone, at pH 8.0, is extremely sensitive to inhibition by DNPINT. The half inhibition (I_{50}) of phosphorylation with menadione and 1,4-naphthoquinone occurs at 54 and 80 nM DNPINT, respectively. In contrast, cyclic phosphorylation catalyzed by either 2-hydroxynaphtho-

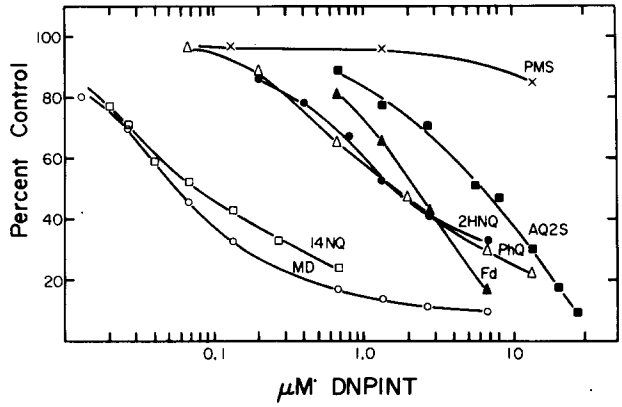

Fig. 2. Inhibition of cyclic phosphorylation by DNPINT. Rates of cyclic phosphorylation were assayed and are represented as a percent of the control rate (without added inhibitor). Mediators were present at a concentration of 150 μM, except in the case of phenanthrenequinone (100 μM) and ferredoxin (50 μM). In all cases the mediators were reduced to the optimal redox poise. Control rates (in μmol ATP/mg Chl per h) were: phenazine methosulfate (PMS (X)), 1000; anthraquinone 2-sulfonate (AQ2S (■)), 358; ferredoxin (Fd (▲)), 162; phenanthrenequinone (PhQ (△)), 371; 2-hydroxynaphthoquinone (2 HNQ (●)), 351; 1,4-naphthoquinone (14NQ (□)), 230; menadione; menadione (MD (○)), 533.

quinone, phenanthrenequinone or anthraquinone 2-sulfonate is much less sensitive to DNPINT and requires from 20- to 100-fold more DNPINT for 50% inhibition. The I_{50} values for 2-hydroxynaphthoquinone, phenanthrenequinone and anthraquinone 2-sulfonate are 1.6, 1.6, and 7.0 μM, respectively. Fig. 2 also shows that cyclic phosphorylation mediated by ferredoxin is relatively insensitive to DNPINT. Half inhibition occurs at approx. 2.6 μM. Cyclic phosphorylation mediated by phenazine methosulfate (poised with ascorbate) is completely insensitive to DNPINT, indicating that DNPINT is not acting as an uncoupler or energy transfer inhibitor.

Correlation between the quinol pK_1 and the sensitivity of quinol mediated cyclic phosphorylation to DNPINT

The physical basis for the difference in sensitivity to DNPINT of the various quinol mediators in cyclic phosphorylation was studied. No correlation could be found between the apparent redox midpoint potential of the quinone/quinol and the sensitivity to DNPINT inhibition. However, an examination of the pK_1 values of the quinols (the negative logarithm of the dissociation constant for the first proton) revealed that all of the quinones used in this study have a pK_1 in the pH range 7.0—9.3 (Table I). Table I shows that anthraquinol 2-sulfonate, phenanthrenequinol and 2-hydroxynaphthoquinol (mediators with a low sensitivity to DNPINT) have pK_1 values at or below pH 8.0 (the pH at which phosphorylation was assayed in Figs. 1 and 2), whereas 1,4-naphthoquinol and menadiol (mediators very sensitive to DNPINT) have pK_1 values substantially above pH 8.0. The pK_1 values of the quinols correlate well with both the sensitivity of phosphorylation to DNPINT and the optimum redox poise for the mediator (Table I). Apparently, mediators assayed at a pH above their pK_1 are insensitive to DNPINT inhibition and are most efficient at or near full reduction, whereas mediators assayed at a pH below their pK_1 are sensitive to DNPINT and function optimally at partial reduction.

This observation suggests that the mediator sensitivity can be altered by merely changing the pH of the assay medium. Decreasing the pH should

TABLE I

EFFECT OF THE pK_1 OF THE QUINOL HYDROXYL GROUP ON CYCLIC PHOSPHORYLATION ACTIVITY

The pK_1 was determined by spectrophotometric absorption peak shifts. Other parameters are described in Figs. 1 and 2. Midpoint potentials are from Ref. 7.

Mediator	E'_o (mV)	pK_1 (reduced form)	Cyclic phosphorylation at pH 8.0	
			Optimal fractional reduction (%)	DNPINT concn. required for 50% inhibition
Anthraquinone 2-sulfonate	—225 mV	8.0	100	6.8 μM
Phenanthrenequinone	+28 mV	7.0	80	1.7 μM
2-Hydroxynaphthoquinone	—152 mV	8.0	100	1.6 μM
1,4-Naphtoquinone	+50 mV	9.2	53	80 nM
Menadione	—10 mV	9.3	60	54 nM

increase the DNPINT sensitivity of DNPINT-insensitive quinol mediators, whereas increasing the pH should decrease the DNPINT sensitivity of DNPINT-sensitive quinol mediators. Fig. 3 shows that this prediction is confirmed for 150 μM 2-hydroxynaphthoquinone. As the pH is raised, the DNPINT concentration required for 50% inhibition of phosphorylation (I_{50}) increases. Fig. 4 is a replot of data similar to Fig. 3 in which I_{50} values for cyclic phosphorylation mediated by anthraquinone 2-sulfonate, menadione and 2-hydroxy naphthoquinone are plotted as a function of pH. In all these cases, increasing the pH lowers the DNPINT sensitivity of phosphorylation. Apparently, only electron transfer catalyzed by the dihydroquinol, and not the monohydroquinol anion, is very sensitive to inhibition by DNPINT.

Fig. 4 also shows the effect of increasing the fractional reduction of the mediator on the sensitivity of cyclic phosphorylation to DNPINT. Cyclic phosphorylation catalyzed by both menadiol and 2-hydroxynaphthoquinol becomes more insensitive to DNPINT as the fractional reduction of the mediator is increased from 60% to 100%. This results in a 2- to 6-fold increase in the DNPINT concentration needed for 50% inhibition (depending upon the pH and the mediator employed). In contrast, the DNPINT sensitivity of phosphorylation catalyzed by anthraquinol 2-sulfonate, which contains a negatively-charged sulfonate group, is unchanged when changing the fractional reduction.

Since increasing the amount of reduced mediator decreases the sensitivity of phosphorylation to DNPINT, the effect of increasing the total quinol concentration by increasing the total mediator concentration (holding both the fractional reduction and the pH constant) was determined. These results (Table II) show that increasing the concentration of menadione (at 60% reduction) increases the amount of DNPINT required for 50% inhibition of cyclic phosphorylation. Again, increasing the quinol concentration causes the DNPINT sensitivity to decrease. In contrast, the sensitivity of quinol-mediated cyclic phosphorylation to dibromothymoquinone is largely independent of the pH and fractional reduction, as shown in Table III. It should be noted that anthra-

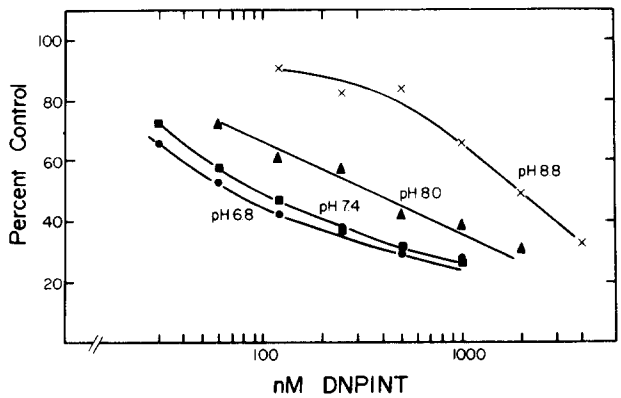

Fig. 3. Effect of pH on DNPINT inhibition of 2-hydroxynaphthoquinone-mediated cyclic phosphorylation. The concentration of 2-hydroxynaphthoquinone was 150 μM and was reduced 60% by dithionite. Control rates (μmol ATP/mg Chl per h) were: pH 6.8 (•), 113; pH 7.4 (■), 235; pH 8.0 (▲) 286; pH 8.8 (X), 277.

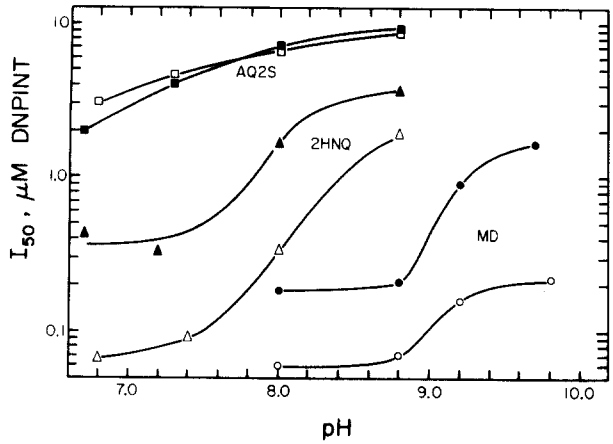

Fig. 4. Effect of pH on DNPINT sensitivity of cyclic phosphorylation. Menadione (MD: circles), 2-hydroxynaphthoquinone (2 HNQ: triangles), and anthraquinone 2-sulfonate (AQ2S: squares) were present at a concentration of 150 μM. Dithionite was added to reduce either 60% (open symbols) or 100% (dark symbols) of the indicated mediator. The DNPINT concentration required for 50% inhibition of phosphorylation (I_{50}) at the given pH was determined by interpolating a DNPINT titration curve similar to the curves shown in Fig. 3. Reaction mixtures with 2-hydroxynaphthoquinone or anthraquinone 2-sulfonate contained in addition 20 mM morpholinopropane sulfonate and reaction mixtures with menadione contained 20 mM glycine. All control phosphorylation rates were greater than 100 μmol ATP/mg Chl per h except for: 2-hydroxynaphtoquinone (100% reduced) at pH 6.7, 63; anthraquinone 2-sulfonate (100% reduced) at pH 6.7, 64; anthraquinone 2-sulfonate (60% reduced) at pH 6.8, 51.

TABLE II

EFFECT OF MEDIATOR CONCENTRATION OF DNPINT SENSITIVITY OF CYCLIC PHOSPHORYLATION

Cyclic phosphorylation was assayed at pH 8.1. In all cases, dithionite was added to reduce 60% of the menadione present.

Menadione concn. (μM)	Rate of ATP synthesis (μmol ATP/mg Chl/h)	DNPINT concn. required for 50% inhibition of phosphorylation (nM)
50	281	25
100	372	49
150	457	54
300	372	150

TABLE III

EFFECT OF CHANGES IN pH OR FRACTIONAL REDUCTION ON DIBROMOTHYMOQUINONE SENSITIVITY OF CYCLIC PHOSPHORYLATION

Cyclic phosphorylation was assayed with serum albumin omitted from the reaction mixture. Reaction mixtures with anthraquinone 2-sulfonate (AQ2S) contained additionally 20 mM morpholinopropane sulfonate and reaction mixtures with 2-hydroxynaphthoquinone (2HNQ) contained 20 mM glycine.

Mediator (150 μM)	Assay pH	Fractional reduction (%)	DBMIB concn. required for 50% inhibition (nM)
AQ2S	7.0	100	150
AQ2S	8.0	100	92
2HNQ	8.0	60	75
2HNQ	8.0	100	110
2HNQ	9.0	100	180

quinone 2-sulfonate-mediated cyclic phosphorylation is much more sensitive to DBMIB (I_{50} = 92 nM) than to DNPINT (I_{50} = 6.8 μM) at pH 8.0.

Discussion

When quinol-mediated cyclic phosphorylation is assayed at pH 8.0, two classes of quinols are apparent: The first class, containing menadiol and 1,4-naphthoquinol, is both highly sensitive to DNPINT and is optimally poised at partial reduction (Figs. 1 and 2). The second class, consisting of anthraquinol 2-sulfonate, phenanthrenequinol and 2-hydroxynaphthoquinol, is both less sensitive to DNPINT, and optimally poised at or near full reduction. Cyclic phosphorylation catalyzed by both classes is sensitive to dibromothymoquinone and insensitive to antimycin A [8]. These two classes do not correlate well with lipophilic/hydrophilic groupings nor with midpoint potential (Table I). However, the pK_1 values of the quinol hydroxyl of these compounds do correlate with these same classes. Cyclic phosphorylation catalyzed by quinols that are appreciably deprotonated (in the quinoxide form) at the pH of the assay is relatively insensitive to DNPINT. Additionally, the DNPINT sensitivity of cyclic phosphorylation can be changed by varying the pH of the assay, which simultaneously various the concentration of quinoxide present, as seen in Figs. 3 and 4. The range of assay pH is limited; appreciable phosphorylation rates can only be observed between pH 6.7 and 9.8. For both menadiol- and 2-hydroxynaphthoquinol-mediated cyclic phosphorylation, inflection points in Fig. 4 correlate well with the pK_1 of the quinols obtained spectrophotometrically, being shifted in the thylakoid system to slightly lower pH values.

Menadiol- and 1,4-naphthoquinol-mediated cyclic phosphorylation at lower pH values is extremely sensitive to DNPINT, suggesting that electrons donated by these quinols must obligately pass through the bound plastoquinone 'B'. This is unusual. A priori, one would expect menadiol and 1,4-naphthoquinol to be able to transfer electrons to the plastoquinone pool also. Apparently, there is a path of electron transfer available to the quinoxide (QH^-) form that is not available to the uncharged quinol (QH_2). In the case of menadiol-catalyzed cyclic phosphorylation, when the total concentration of quinoxide is increased either by (i) raising the pH or increasing the fractional reduction

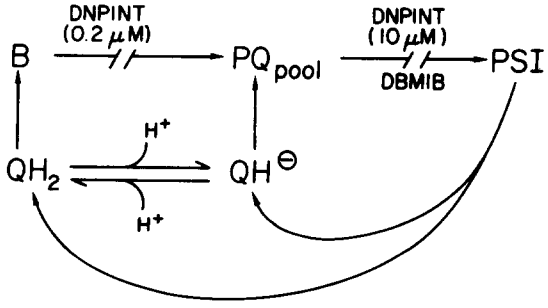

Fig. 5. Model for cyclic electron transfer mediated by quinols. Symbols: B, bound plastoquinone; PQ pool, plastoquinone pool; PSI, Photosystem I; QH_2 and QH^-, neutral and ionized forms of the quinol, respectively; DBMIB, dibromothymoquinone.

(Fig. 4) or (ii) raising the total mediator concentration (Table II), a decrease in the sensitivity to DNPINT is seen. Apparently, a negative charge on the quinol anion confers the ability for electron transfer to be less sensitive to DNPINT. Anthraquinone 2-sulfonate, which is always negatively charged in the physiological pH range, is relatively insensitive to DNPINT both above and below its pK_1 (Fig. 3). These data are summarized in the model shown in Fig. 5.

If the DNPINT concentration is increased to the μmolar range, cyclic phosphorylation catalyzed by all quinols is inhibited (Fig. 2). Apparently, there is an additional, less sensitive site for DNPINT action. This site is presumably after the plastoquinone pool, but before the oxidation site of phenazine methosulfate. It is possible that this is the same site of electron transfer susceptible to inhibition by DBMIB.

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