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ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION IN CHLOROPLASTS AS A FUNCTION OF THE ELECTRON ACCEPTOR

III. A DIBROMOTHYMOQUINONE-INSENSITIVE PHOSPHORYLATION REACTION ASSOCIATED WITH PHOTOSYSTEM II*

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SUMMARY

Dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) is reputed to be a plastoquinone antagonist which prevents the photoreduction of hydrophilic oxidants such as ferredoxin–$NADP^+$. However, we have found that dibromothymoquinone inhibits only a small part of the photoreduction of lipophilic oxidants such as oxidized *p*-phenylenediamine. Dibromothymoquinone-resistant photoreduction reactions are coupled to phosphorylation, about 0.4 molecules of ATP consistently being formed for every pair of electrons transported. Dibromothymoquinone itself is a lipophilic oxidant which can be photoreduced by chloroplasts, then reoxidized by ferricyanide or oxygen. The electron transport thus catalysed also supports phosphorylation and the P/e_2 ratio is again 0.4. It is concluded that there is a site of phosphorylation before the dibromothymoquinone block and another site of phosphorylation after the block. The former site must be associated with electron transfer reactions near Photosystem II, while the latter site is presumably associated with the transfer of electrons from plastoquinone to cytochrome *f*.

INTRODUCTION

Two quite different arguments lead us to the conclusion that non-cyclic photophosphorylation involves more than one site of energy conservation. Our reasons for believing that there are at least two phosphorylation sites are as follows:

(1) The overall efficiency of photophosphorylation (P/e_2) is considerably higher than one ATP molecule formed for every pair of electrons transported[1]. Furthermore, the P/e_2 ratio approaches 2.0 if one subtracts that part of the electron transport which can occur in the absence of phosphorylation[2].

(2) Lipophilic strong oxidants (Class III acceptors), such as the oxidized form of *p*-phenylenediamine, intercept electrons by reacting with some intermediate

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; P/e_2, ratio of the molecules of ATP formed to the pairs of electrons transported.

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carrier which normally transfers electrons from Photosystem II to Photosystem I (refs 3, 4). This interception of electrons does not abolish phosphorylation but instead decreases the efficiency to about half of the value observed when hydrophilic Class I acceptors are reduced. It therefore seems that the intermediate carrier responsible for the reduction of oxidized *p*-phenylenediamine is situated between two sites of phosphorylation in the electron transport chain.

The work described in this paper was undertaken in an attempt to define the location of the two phosphorylation sites in terms of the sites of action of known electron carriers.

It has long been thought that there must be a rate-determining, phosphorylation-dependent reaction transferring electrons between the two photosystems[5–7]. This rate-determining step presumably lies between plastoquinone and cytochrome *f* since the rate of reduction of cytochrome *f* by Photosystem II and the rate of oxidation of plastoquinone by Photosystem I are accelerated during phosphorylation or when uncouplers are added. However, there is good reason for doubting that this rate-determining phosphorylating process is involved in the reduction of oxidized *p*-phenylenediamine. The reduction of oxidized *p*-phenylenediamine is very fast and independent of phosphorylation[3]. Moreover, the fact that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibition of oxidized *p*-phenylenediamine reduction is independent of light intensity suggests that oxidized *p*-phenylenediamine accepts electrons from a carrier situated close to Photosystem II[4].

We wish now to present evidence which lends further support to the concept of a site of phosphorylation close to Photosystem II and at the same time virtually precludes the participation of a plastoquinone–cytochrome *f* phosphorylating reaction in oxidized *p*-phenylenediamine reduction. The new evidence has been provided by studies of the effects of dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) on electron transport and phosphorylation. This inhibitor was first introduced by Trebst and his associates[8] as a plastoquinone antagonist. At very low concentrations it blocks all of the transfer of electrons from water to Class I acceptors such as ferredoxin–$NADP^+$ or methylviologen and a large part of the transfer of electrons from water to ferricyanide[9]. It also seems to block the transfer of electrons from cytochrome b_{559} to cytochrome *f* and it certainly prevents the reduction of cytochrome *f* by electrons from Photosystem II[10,11]. These observations do indeed suggest that the inhibitor acts at the level of plastoquinone involvement. In any event, it is clear that the inhibitor prevents electron transport at some point after Photosystem II but before cytochrome *f*. Yet dibromothymoquinone does not greatly inhibit either oxidized *p*-phenylenediamine reduction or the associated phosphorylation reaction. It follows that there must be a site of phosphorylation before the site of dibromothymoquinone inhibition and probably therefore before the site of involvement of plastoquinone.

MATERIALS AND METHODS

The procedures employed in this study were similar to those employed in the earlier papers of the series[3,4]. Chloroplasts were isolated from commercial spinach (*Spinacia oleracea* L.) as already described[3]. Cyanide-treated chloroplasts were prepared by incubating chloroplasts at 0 °C for 90 min in a 30 mM KCN solution

buffered at pH 7.8 as described in the previous paper[4]. Control chloroplasts were suspended for the same time in a similar medium containing KOH instead of KCN.

The inhibitor dibromothymoquinone was prepared by bromination of thymoquinone in water and was recrystallized several times from alcohol. Stock solutions were prepared by dissolving dibromothymoquinone in ethanol–ethylene glycol (1:1, v/v). The concentration of the stock was such that the organic solvent in the reaction mixture never exceeded 1%.

The reduction of ferricyanide was measured as the decrease in absorbance of the reaction mixture at 420 nm. In experiments with oxidized *p*-phenylenediamine, recrystallized colorless *p*-phenylenediamine dihydrochloride was added to the buffered reaction mixture, then oxidized immediately before the reaction with excess ferricyanide. Electron transport was measured as reduction of the excess ferricyanide since the oxidized *p*-phenylenediamine reduced during the reaction is immediately reoxidized by ferricyanide. Reactions involving other aromatic diamines and quinones were measured in the same indirect way. The reduction of methylviologen was measured as oxygen uptake since reduced methylviologen reacts rapidly with oxygen to form H_2O_2[12]. For these measurements a Clark-type, membrane-covered electrode was used. Phosphorylation was measured by a modification of the method of Avron[13] as the residual radioactivity after extraction of the ^{32}P-labeled orthophosphate from the reaction mixture as phosphomolybdic acid. In all experiments the temperature was 19 °C.

RESULTS

(*I*) *The sensitivity of electron transport and phosphorylation to dibromothymoquinone with different electron acceptors*

As we have reported elsewhere[3], lipophilic oxidants tend to increase the rate of electron transport in illuminated chloroplasts, decrease the dependence of electron transport on phosphorylation and reduce the efficiency of phosphorylation (P/e_2) toward one-half. The extent to which acceptors are able to intercept electrons between two phosphorylation sites can be roughly judged by the increase in the rate of electron transport and the decline in the P/e_2 ratios. (These criteria only apply, of course, if the P/e_2 ratios fall to a plateau rather than to zero and it can be shown that the acceptor is not an uncoupler.) Among the lipophilic acceptors listed in Table I, oxidized *p*-phenylenediamine is most nearly a typical Class III acceptor while 2,5-dimethyl-*p*-benzoquinone is the least typical. Ferricyanide ion is not lipophilic at all and, in our chloroplasts, seems to intercept very few of the electrons generated by Photosystem II. As can be seen in Table I, the transport of electrons to lipophilic acceptors has a large component which is resistant to dibromothymoquinone. This component is largest with the best Class III acceptor, oxidized *p*-phenylenediamine and smallest with the worst Class III acceptor, 2,6-dimethyl-*p*-benzoquinone. Clearly, that part of the electron transport which results from the interception of electrons between the phosphorylation sites is largely insensitive to the inhibitor. This is even more obvious when one notes the effect of dibromothymoquinone on the P/e_2 ratios. Regardless of the ratio in the absence of the inhibitor, that is regardless of what proportion of the electrons are intercepted between the phosphorylation sites, the P/e_2 ratio always falls to about 0.4 in the presence of the inhibitor. This is true even

TABLE I

THE EFFECT OF DIBROMOTHYMOQUINONE ON ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION IN CHLOROPLASTS WITH DIFFERENT ELECTRON ACCEPTORS

The 2.0-ml reaction mixture consisted of the following: 0.1 M sucrose, 50 mM Tricine buffer (pH 8.2), 2 mM $MgCl_2$, 1 mM ADP, 5 mM $^{32}P_i$, chloroplasts containing 30 μg chlorophyll, and the indicated acceptor system. These acceptor systems were: 0.5 mM potassium ferricyanide (Fecy); 0.5 mM *p*-phenylenediamine *plus* 1.5 mM ferricyanide (PD_{ox}); 0.5 mM diaminodurene *plus* 1.5 mM ferricyanide (DAD_{ox}); 0.5 mM 2,5-dimethyl-*p*-benzoquinone *plus* 0.5 mM ferricyanide (DMQ); 0.5 mM 2,5-diaminotoluene *plus* 1.5 mM ferricyanide (DAT_{ox}). When used dibromothymoquinone was 0.5 μM. Rates are expressed in μequiv or μmoles ATP/h per mg chlorophyll.

Electron acceptor	*Rate of electron transport*		*Rate of ATP formation*		P/e_2	
	Control	*+dibromothymoquinone*	*Control*	*+dibromothymoquinone*	*Control*	*+dibromothymoquinone*
Fecy	430	58	228	13	1.06	0.45
PD_{ox}	1260	695	292	149	0.46	0.43
DAD_{ox}	735	383	244	56	0.66	0.39
DMQ	902	294	325	52	0.72	0.36
DAT_{ox}	791	396	280	95	0.71	0.48

for the tiny residue of electron transport with ferricyanide as acceptor. We have also found that, regardless of the acceptor, the dibromothymoquinone-resistant component of the electron transport is always independent of the presence or absence of ADP and phosphate.

Further effects of dibromothymoquinone are illustrated in Figs 1–4. Again, the transport of electrons from water to oxidized *p*-phenylenediamine has two components: one large, insensitive to dibromothymoquinone and supporting phosphorylation with a P/e_2 ratio of about 0.4; the other smaller, sensitive to dibromothymoquinone with a computed P/e_2 about 1.0 (Fig. 1). In contrast, the transport of electrons to ferricyanide is mostly sensitive to the inhibitor while the transport to methylviologen is almost all sensitive (Fig. 2). Once again the small residue of dibromothymoquinone-insensitive ferricyanide reduction supports phosphorylation with a P/e_2 ratio of 0.4–0.5.

This residual dibromothymoquinone-resistant ferricyanide reduction deserves attention since we have here the unusual situation of an inhibitor seeming to catalyze the reaction it inhibits; increasing concentrations of dibromothymoquinone actually increase the rate of ferricyanide reduction (Figs 3 and 4). The inhibitor, in addition to blocking electron transport, is itself a lipophilic oxidant which accepts electrons before or at its own site of inhibition. Apparently the reduced dibromothymoquinone is quickly reoxidized by ferricyanide and thus the inhibited ferricyanide reduction is in part restored. However, this dibromothymoquinone-mediated ferricyanide reduction is quite different from the usual ferricyanide Hill reaction, having instead many of the characteristics of oxidized *p*-phenylenediamine reduction; the rate is independent of the presence or absence of ADP and phosphate or of uncouplers such as methylamine, and the efficiency of phosphorylation (P/e_2) is only 0.3–0.4. Although

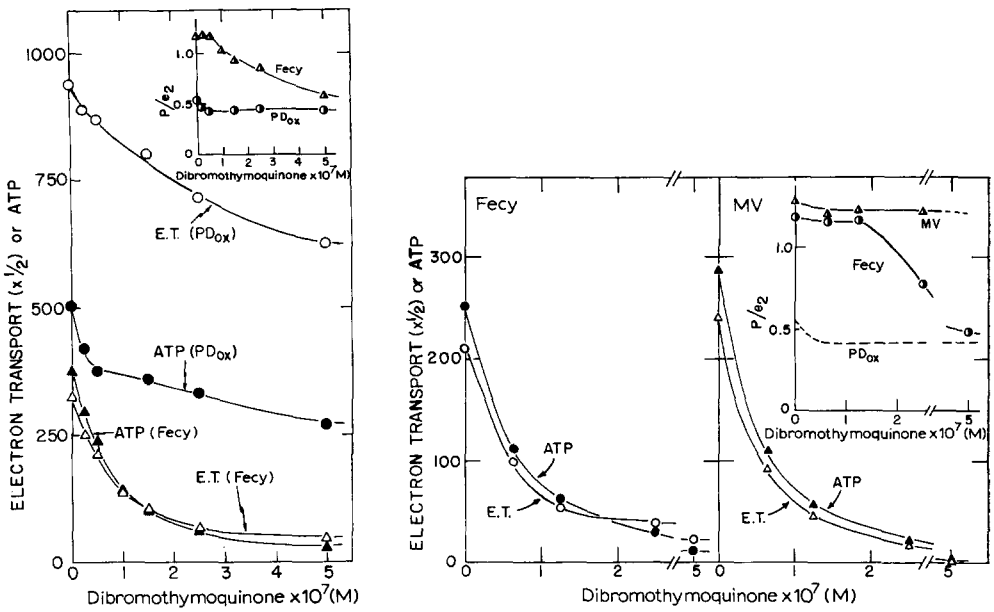

Fig. 1. Effect of dibromothymoquinone on electron transport (E.T.) and phosphorylation (ATP) with ferricyanide (Fecy) or oxidized *p*-phenylenediamine (PD_{ox}) as electron acceptor. Rates are expressed in μequiv or μmoles ATP/h per mg chlorophyll. The 2.0-ml reaction mixture consisted of the following: sucrose, 0.1 M; Tricine–NaOH buffer (pH 8.2), 50 mM; $MgCl_2$, 2 mM; ADP, 1 mM; $^{32}P_i$, 10 mM; chloroplasts containing 40 μg (ferricyanide) or 30 μg (PD_{ox}) chlorophyll; and either 0.5 mM potassium ferricyanide or a combination of 1.5 mM ferricyanide and 0.5 mM *p*-phenylenediamine dihydrochloride. Note the great sensitivity of ferricyanide reduction to the inhibitor, the relative insensitivity of oxidized *p*-phenylenediamine reduction and the high rate of ATP formation associated with the resistant oxidized *p*-phenylenediamine reduction.

Fig. 2. Effect of dibromothymoquinone on electron transport and phosphorylation with ferricyanide and methylviologen (MV) as electron acceptors. Reaction conditions, units and abbreviations as in Fig. 1 except that $^{32}P_i$ was 5 mM, potassium ferricyanide was 0.4 mM and methylviologen was 50 μM. Electron transport was measured as oxygen production with ferricyanide and as oxygen consumption with methylviologen. The dotted curve for oxidized *p*-phenylenediamine (PD_{ox}) in the inset figure is taken from Fig. 1 for comparison. Note from the inset figure that the small amount of residual ferricyanide reduction supports phosphorylation with the efficiency characteristic of the oxidized *p*-phenylenediamine-reducing system. Presumably ferricyanide can intercept electrons, either directly or indirectly, between two sites of phosphorylation as can oxidized *p*-phenylenediamine whereas methylviologen cannot.

this catalysis of ferricyanide reduction is most conspicuous at high dibromothymoquinone concentrations, there is no reason to doubt that it is already taking place at the point of apparent maximum inhibition of ferricyanide reduction and even there constitutes a large fraction of the residual reaction. This is clearly indicated by the fact that the P/e_2 ratio associated with ferricyanide reduction declines to 0.4–0.5 as the dibromothymoquinone inhibition approaches its maximum (Figs 1 and 2). No such decline is observed when the low potential acceptor methylviologen is being reduced; for thermodynamic reasons reduced dibromothymoquinone cannot donate electrons to methylviologen and therefore the inhibitor cannot catalyze methylviologen reduction.

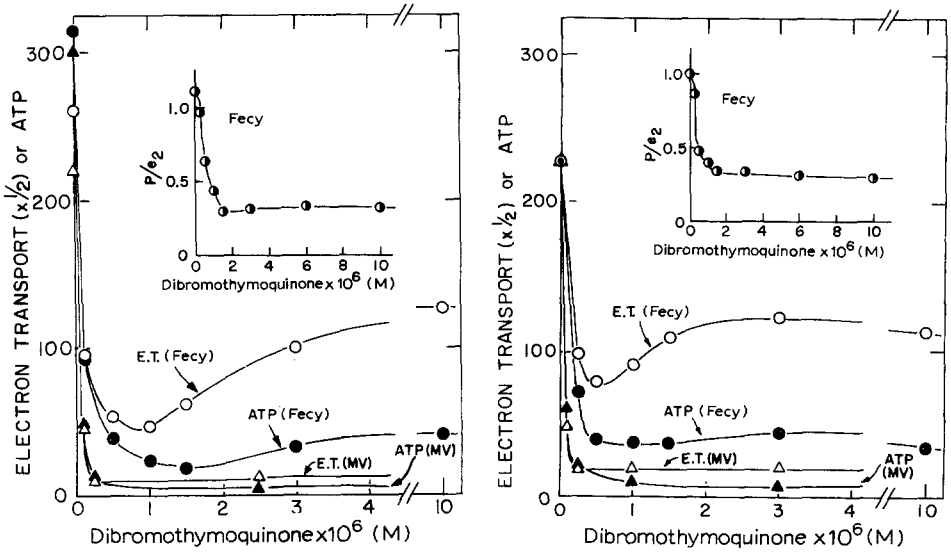

Fig. 3. Effect of higher concentrations of dibromothymoquinone on electron transport and phosphorylation with ferricyanide or methylviologen as acceptors. Reaction conditions, units and abbreviations as in Fig. 2, except that methylviologen was 100 μM. Note the much greater residual electron transport with ferricyanide and the increasing rate of ferricyanide reduction with increasing dibromothymoquinone concentration. Note also from the inset figure that the inhibitor-insensitive electron transport again supports phosphorylation with the efficiency characteristic of the oxidized *p*-phenylenediamine-reducing system. Apparently the inhibitory lipid soluble quinone dibromothymoquinone is reduced by chloroplasts, in a reaction which does not include the site of dibromothymoquinone inhibition and is then rapidly reoxidized by the excess ferricyanide.

Fig. 4. Effects of dibromothymoquinone on digitonin-treated chloroplasts. Conditions, units and abbreviations as in previous figures. Chloroplasts were treated with 0.05% digitonin in buffer (pH 7.4) at 0 °C for 15 min, spun down at 4000 × *g* for 10 min and washed twice in buffer. The chloroplast material subjected to this mild treatment consisted in the main part of unfragmented lamellae or large fragments. Rates of electron transport and phosphorylation were only slightly lowered by the treatment. Note, however, that the treatment lowered the concentration of dibromothymoquinone required to catalyze ferricyanide reduction (see also Fig. 3), thereby producing the illusion that ferricyanide reduction is in large part insensitive to the inhibitor.

The dibromothymoquinone reduced by chloroplasts can react with oxygen to produce H_2O_2 when ferricyanide is not present. This dibromothymoquinone-insensitive Mehler reaction becomes substantial when the concentration of inhibitor is above 10 μM. As will be described in another paper, this reaction also supports phosphorylation with a P/e_2 ratio of 0.4. Thus the reaction provides a mechanism for "pseudocyclic" photophosphorylation which probably involves only Photosystem II.

A similar reaction — the reduction of dibromothymoquinone by illuminated chloroplasts and its subsequent reoxidation by oxygen in the dark— has been noted by Lozier and Butler[14].

All of the dibromothymoquinone-resistant reactions we have tested, electron transport and phosphorylation alike, are inhibited by DCMU which inactivates Photosystem II and are largely insensitive to KCN which inactivates plastocyanin.

The observations described above concerning the effect of dibromothymoquinone or ferricyanide are somewhat at variance with the observations of Böhme *et al.*[9]. They found that nearly half of the transport of electrons to ferricyanide in "intact" chloroplasts was resistant to dibromothymoquinone, while in our chloroplasts the resistant portion never exceeded 20% and was sometimes much less. Presumably this discrepancy resulted from some difference in the state of the chloroplast membranes. As shown in Fig. 4 (compare with Fig. 3), the ferricyanide reduction became markedly less sensitive to dibromothymoquinone when the membranes were slightly modified by treating the chloroplasts with a low concentration of digitonin. A more serious discrepancy, however, is to be found in the fact that the dibromothymoquinone-resistant ferricyanide reduction in our chloroplasts remains firmly coupled to phosphorylation. A constant P/e_2 ratio of 0.3–0.4 is observed over a wide range of conditions, even after the digitonin treatment. In contrast, the data of Böhme *et al.* show a continual decline in phosphorylation efficiency with increasing dibromothymoquinone until a P/e_2 ratio of zero is reached (see Fig. 2 of ref. 9). Böhme *et al.* concluded from their observations that the dibromothymoquinone-insensitive portion of ferricyanide reduction is not coupled to phosphorylation whereas we are forced to conclude from our observations that it is coupled. The cause of the discrepancy is as yet unknown. We agree with Böhme *et al.* that the reduction of ferricyanide by sonicated chloroplasts is quite resistant to dibromothymoquinone, but here again we noted that considerable phosphorylation was associated with the dibromothymoquinone-insensitive electron transport. In a sonicated chloroplast preparation the P/e_2 ratio was 0.2 with inhibitor and 0.6 without inhibitor. Presumably further disruption would have still further increased the resistance to the inhibitor while abolishing phosphorylation in dibromothymoquinone-treated and control alike.

(*2*) *Evidence bearing on the site of dibromothymoquinone inhibition* (*Table II*)

Our observation that there is a site of phosphorylation on the electron transport pathway before the dibromothymoquinone inhibition site makes precise identification of the inhibition site a matter of critical concern. There is already evidence that dibromothymoquinone interferes with electron transport at the level of plastoquinone[9–11] but this evidence is not absolutely conclusive. Moreover, the most striking features of dibromothymoquinone inhibition described here (the strong inhibition of electron transport with hydrophilic acceptors, the weak inhibition with lipophilic acceptors, and the lowering of the P/e_2 ratio to 0.4) are also characteristic of KCN inhibition[4]. Yet KCN almost certainly inhibits because it reacts with plastocyanin. Therefore, it seemed important to us to prove that the site of dibromothymoquinone inhibition is different from and precedes the site of KCN inhibition.

Table II shows that the effects of the two inhibitors are indeed quite distinct. DCMU-insensitive, Photosystem I-dependent reactions such as the transport of electrons from diaminodurene to methylviologen and the diaminodurene-mediated cyclic phosphorylation system are inhibited by KCN but, as Böhme *et al.*[9] have already implied, are not inhibited by dibromothymoquinone. The transfer of electrons from water to Class I acceptors such as methylviologen is inhibited by both KCN and dibromothymoquinone. In contrast, the DCMU-sensitive transfer of electrons from water to oxidized *p*-phenylenediamine is inhibited by neither. Thus we must

TABLE II

INHIBITION OF VARIOUS REACTIONS IN CHLOROPLASTS BY DIBROMOTHYMOQUINONE AND KCN

Reaction conditions and concentrations of reactants were as in Table I and the figures unless otherwise specified. Units are as in Table I. PD_{ox} represents products of the oxidation of *p*-phenylenediamine. When KCN was used, the chloroplasts were pretreated as described in Materials and Methods. When dibromothymoquinone was used, it was 0.5 μM. Density of chloroplast suspended in the 2.0 ml reaction mixture was: water→methylviologen, 40 μg chlorophyll; water→PD_{ox}, 30 μg chlorophyll; diaminodurene→methylviologen and diaminodurene (cyclic), 10 μg chlorophyll. In the diaminodurene→methylviologen system ascorbate (1 mM), DCMU (1 μM), diaminodurene (0.5 mM) and methylviologen (0.1 mM) were added and the pH was lowered to 7.7 to eliminate much of the non-biological ascorbate oxidation. The diaminodurene (cyclic) phosphorylation system was similar except that methylviologen and ascorbate were omitted and 0.1 mM ferricyanide was added to establish an appropriate diaminodurene/oxidized diaminodurene ratio.

System	*Condition*	*Electron transport*	*ATP formation*	P/e_2
Water→methylviologen	Control	646	395	1.13
	+dibromothymoquinone	44	6	—
	KCN treated	0	0	—
Water→PD_{ox}	Control	1720	386	0.45
	+dibromothymoquinone	1300	257	0.40
	KCN treated	1200	198	0.33
Diaminodurene→methylviologen	Control	4180	733	0.35
	+dibromothymoquinone	4580	705	0.31
	KCN treated	440	33	(0.15)
Diaminodurene (cyclic)	Control	—	702	—
	+dibromothymoquinone	—	605	—
	KCN treated	—	12	—

conclude that the site of dibromothymoquinone inhibition falls between the DCMU inhibition site and the KCN inhibition site. This is consistent with the view that dibromothymoquinone acts as a plastoquinone antagonist.

DISCUSSION

In the first paper of this series[3] we noted that lipophilic strong oxidants (*e.g.* oxidized *p*-phenylenediamine) can be reduced very rapidly by illuminated chloroplasts whether or not phosphorylation occurs. Nevertheless, in the presence of ADP and phosphate, the high rate of electron transport is associated with a great deal of phosphorylation. We have called such oxidants Class III electron acceptors. Conventional hydrophilic oxidants such as methylviologen, ferredoxin–$NADP^+$ and ferricyanide we have called Class I acceptors. Since the reduction of Class III acceptors supports only half as much phosphorylation as the reduction of an equivalent amount of Class I acceptor, we suggested that lipophilic oxidants have access to

and accept electrons from some electron carrier which lies between two sites of phosphorylation. Furthermore, we suggested that the second phosphorylation site, the one not employed in the reduction of Class III acceptors, is responsible for limiting the rate of the Hill reaction. Hence the high rate of electron transport in the presence of Class III acceptors.

In the second paper[4] we showed that KCN treatment of chloroplasts prevents the reduction of Class I acceptors but not the reduction of Class III acceptors. This virtually proves that Class III acceptors do react directly with some intermediate carrier in the electron transport chain, a carrier operating before the KCN block. Moreover we postulated that this intermediate carrier is close to Photosystem II on the basis of the kinetics of DCMU inhibition of the reduction of Class III acceptors. This in turn implies the existence of a phosphorylation site closely associated with Photosystem II, since the reduction of Class III acceptors *via* the KCN-insensitive shortened pathway is still coupled with a P/e_2 ratio of 0.3–0.4.

In the present paper, we have shown that dibromothymoquinone also inhibits the reduction of Class I acceptors without severely inhibiting the reduction of Class III acceptors. Regardless of the Class III acceptor used (oxidized *p*-phenylenediamine, oxidized diaminodurene, oxidized diaminotoluene or 2,6-dimethyl-*p*-benzoquinone) and therefore, regardless of the rate of electron transport and the P/e_2 ratio in the absence of the inhibitor, the dibromothymoquinone-resistant portion of electron transport is coupled to phosphorylation with a P/e_2 ratio of 0.35–0.45. A very similar P/e_2 ratio (0.3–0.4) is associated with the residual ferricyanide reduction in the presence of low concentrations of dibromothymoquinone. It is quite clear that all these reactions involve only Photosystem II and that segment of the electron transport chain which ends in the dibromothymoquinone block. We must therefore conclude that there is a site of phosphorylation associated with Photosystem II and located before the dibromothymoquinone inhibition site. If dibromothymoquinone indeed blocks electron transport at the site of plastoquinone involvement, as the evidence suggests, there must be a site of phosphorylation both before and after plastoquinone. Thus the rate-limiting phosphorylation reaction presumed to occur between plastoquinone and cytochrome f[6,7] may be equated to the slow step postulated in our first paper[3].

On the basis of cross-over point determinations, Böhme and Cramer[7] concluded that only one phosphorylation site in the electron transport chain exerted a control over the rate of electron transport. However, our observations are in no way inconsistent with their conclusion. The transport of electrons to Class III acceptors proceeds at high rates whether or not phosphorylation occurs and it is axiomatic that cross-over data cannot yield information on sites of phosphorylation unless the electron transport through the site is phosphorylation dependent.

We have presented the bare bones of our conclusions in Fig. 5. No doubt alternative interpretations of the data could be devised but none has occurred to us. The precise location of Site II, the site close to Photosystem II which we have proposed in this paper, remains a matter for conjecture. Neumann *et al.*[15] have provided a model of non-cyclic photophosphorylation in which two sites of phosphorylation are assumed to be involved in Photosystem I reactions. We find it difficult to reconcile their model with our data unless Site I in Fig. 5 is further divided into two sites. It is, however, possible that there is another site close to Photosystem I which

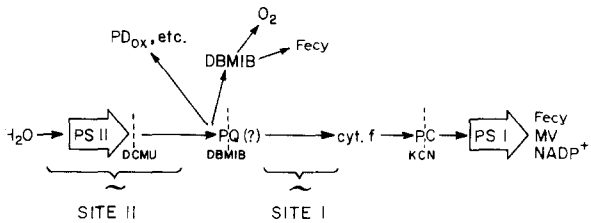

Fig. 5. Simplified scheme of the electron transport pathways, phosphorylation reactions and inhibition sites discussed in this paper. PD_{ox}, oxidized *p*-phenylenediamine; Fecy, ferricyanide; DBMIB, 2,6-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); PQ, plastoquinone; PC, plastocyanin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MV, methylviologen; PS I and PS II, Photosystems I and II, respectively. It should be noted that dibromothymoquinone and KCN both block reduction of the hydrophilic electron acceptors but not the reduction of the lipophilic acceptors. Moreover, the residual electron transport with either inhibitor present supports phosphorylation with an efficiency (P/e_2 ratio) of 0.4.

is responsible for some DCMU-insensitive cyclic photophosphorylation reactions, but this possibility is outside the scope of our present investigation.

POSTSCRIPT

After we had completed the manuscript of this paper we received a communication from Dr Achim Trebst describing similar experiments conducted in his laboratory which have led him also to conclude that there is an energy conservation step associated with Photosystem II reactions.

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REFERENCES

1 Winget, G. D., Izawa, S. and Good, N. E. (1965) *Biochem. Biophys. Res. Commun.* 21, 438–443
2 Izawa, S. and Good, N. E. (1968) *Biochim. Biophys. Acta* 162, 380–391
3 Saha, S., Ouitrakul, R., Izawa, S. and Good, N. E. (1971) *J. Biol. Chem.* 246, 3204–3209
4 Ouitrakul, R. and Izawa, S. (1973) *Biochim. Biophys. Acta* 305, 105–118
5 Avron, M. and Chance, B. (1966) *Brookhaven Symp. Biol.* 19, 149–160
6 Kok, B., Joliot, P. and McGloin, M. P. (1969) in *Progress in Photosynthesis Research*, pp. 1042–1056, International Union of Biological Sciences, Tübingen
7 Böhme, H. and Cramer, W. A. (1972) *Biochemistry* 11, 1155–1160
8 Trebst, A., Harth, E. and Draber, W. (1970) *Z. Naturforsch.* 25b, 1157–1159
9 Böhme, H., Reiner, S. and Trebst, A. (1971) *Z. Naturforsch.* 26b, 341–352
10 Böhme, H. and Cramer, W. A. (1971) *FEBS Lett.* 15, 349–351
11 Knaff, D. B. (1972) *FEBS Lett.* 23, 142–144
12 Good, N. E. and Hill, R. (1955) *Arch. Biochem. Biophys.* 57, 355–366
13 Avron, M. (1960) *Biochim. Biophys. Acta* 40, 257–272
14 Lozier, R. H. and Butler, W. L. (1972) *FEBS Lett.* 26, 161–164
15 Neumann, J., Arntzen, C. J. and Dilley, R. A. (1971) *Biochemistry* 10, 866–873