(1972), *Biochem. Biophys. Res. Commun. 48,* 1565.
Leary, R. P., Beaudette, N., and Kisliuk, R. L. (1975), *J. Biol. Chem. 250,* 4864.
Leary, R. P., and Gaumont, Y. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 591.
Leary, R. P., and Kisliuk, R. L. (1971), *Prep. Biochem. 1,* 47.
Loeble, R. B., and Dunlap, R. B. (1972), *Biochem. Biophys. Res. Commun. 49,* 1671.
Lorenson, M. Y., Maley, G. F., and Maley, F. (1967), *J. Biol. Chem. 242,* 3332.
Maley, G. F., and Maley, F. (1964), *J. Biol. Chem. 239,* 1168.
Mathews, C. K., and Huennekens, F. M. (1960), *J. Biol. Chem. 235,* 3304.
McHenry, C. S., and Santi, D. V. (1974), *Biochem. Biophys. Res. Commun. 57,* 204.
Mukherjee, K. L., and Heidelberger, C. (1962), *Cancer Res. 22,* 815.
Reyes, P., and Heidelberger, C. (1965), *Mol. Pharmacol. 1,* 14.
Roberts, D., and Loehr, E. V. (1971), *Cancer Res. 31,* 1181.
Santi, D. V., and McHenry, C. S. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1855.
Santi, D. V., McHenry, C. S., and Sommer, H. (1974), *Biochemistry 13,* 471.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Weder, H. G., Schildknecht, J., and Kesselring, P. (1971), *Am. Lab. 3,* 17.
Yoshikawa, M., Kato, T., and Takenishi, T. (1969), *Bull, Chem. Soc. Jpn. 42,* 3505.

The Mechanism of Quinonediimine Acceptor Activity in Photosynthetic Electron Transport†

James A. Guikema and Charles F. Yocum*

ABSTRACT: The rates of electron flow catalyzed by a variety of unsubstituted and C- or N-methylated quinonediimine electron acceptors in a reaction requiring photosystem II in KCN-inhibited chloroplasts vary according to the structure of acceptor used. Quinonediimine, but not quinone, electron acceptor activities are inhibited by a variety of uncouplers. Kinetic analysis of this inhibition shows that it is competitive. Low concentrations of aniline also inhibit the activity of C-methylated quinonediimines, but this appears to be due to a chemical reaction between the acceptor and aniline at low pH inside the chloroplast. Light-induced uptake of a quinonediimine, *p*-phenylenediimine, was shown to occur in a DCMU-sensitive reaction. Methylamine uncoupling inhibits this uptake to the same extent as it inhibits electron flow. Experiments with a lipophobic acceptor, *N,N,N′,N′*-tetramethyl-*p*-phenylenediimine, indicate that it catalyzes electron flow by the same mechanism as other quinonediimines. A model is proposed to account for quinonediimine-catalyzed electron flow.

Saha et al. (1971) have shown that oxidized quinones and oxidized *p*-diaminobenzene compounds (quinonediimines) are effective mediators of electron transport in spinach chloroplasts, and that the efficiency of photophosphorylation obtained with these compounds is approximately half that obtained with electron acceptors such as ferricyanide or methyl viologen. Inhibition of electron transport by treatment of chloroplasts with KCN, which inactivates plastocyanin (Ouitrakul and Izawa, 1973), or by the plastoquinone antagonist DBMIB[1] (Trebst and Reimer, 1973a,b; Izawa et al., 1973), have relatively little effect on quinonediimine photoreduction. These observations implicate the reducing side of photosystem II as the site of electron donation to these compounds. Saha et al. (1971) and Trebst (1974) have proposed models for the electron acceptor activity of quinones and quinonediimines in which the oxidized species of the acceptor molecule, a lipophile, penetrates a membrane (outside to inside), undergoes photoreduction, and then transfers reducing equivalents back to the external medium by a repenetration of the membrane from inside to outside. The penetration of the membrane in these models is ascribed to the lipophilic nature of the acceptor and a shuttle of the acceptor molecules in the light.

Although addition of adenosine diphosphate and inorganic phosphate as substrates for photophosphorylation does not markedly enhance the rate of photoreduction of either quinones or quinonediimines, uncouplers have been shown to affect this pathway of electron flow. Gould and Ort (1973) showed that oxygen evolution in the presence of DBMIB, a reaction which required a quinonediimine acceptor (PDox), was sensitive to the uncoupler methylamine. This effect was attributed to inhibition of photosystem II activity by the uncoupler. Trebst and Reimer (1973a) ob-

† From the Department of Cell and Molecular Biology, The University of Michigan, Ann Arbor, Michigan 48104. *Received March 27, 1975.* This investigation was supported in part by a research grant from the H. H. Rackham School of Graduate Studies of the University of Michigan.

[1] Abbreviations used are: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; PDox, *p*-phenylenediimine; DADox, 2,3,5,6-tetramethyl-*p*-phenylenediimine; DMPDox, *N,N*-dimethyl-*p*-phenylenediimine; DATox, 2,5-diiminotoluene; TMPDox, *N,N,N′,N′*-tetramethyl-*p*-phenylenediimine; DMQ, 2,5-dimethyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; *m*-Cl-CCP, *m*-chlorocarbonylcyanide phenylhydrazone; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

served a similar inhibition by gramicidin when the reaction medium for oxygen evolution was pH 8.0, and these investigators postulated interference with the proton gradient which promotes an optimal amine–imine ratio inside the thylakoid membrane, as the cause of gramicidin inhibition. Cohen et al. (1975) have reported that in the presence of DBMIB or EDAC, electron transport catalyzed by PDox or DADox is inhibited by gramicidin, while that catalyzed by DMQ is not. They suggest that this observation may be due to different sites of quinonediimine and quinone photoreduction.

Since the explanations for the effects of uncouplers on the activity of quinonediimine electron acceptors are at variance with one another, and since electron transfer reactions involving these quinonediimines are largely insensitive to addition of substrates for photophosphorylation, we have undertaken an investigation into the mode of action of these acceptors. Our results indicate that reduced quinonediimines are accumulated by thylakoid membranes in response to lowered internal pH and amine protonation, rather than by imine shuttling. In contrast, quinones appear to act through a passive mechanism.

Materials and Methods

Materials. Tricine, bovine serum albumin, *m*-Cl-CCP, and PD were obtained from Sigma. DCMU was obtained from K and K Laboratories; gramicidin was from Schwarz/Mann; and atebrin was provided by Dr. Helen Gay. The remainder of the electron carriers used in these studies were obtained either from Eastman (TMPD, DMQ, DMPD), Aldrich (DAT), or from Research Inorganics (DAD). Stock solutions of acceptors were prepared from the crystalline dihydrochloride salts.

Preparation of Chloroplasts and KCN-Treated Chloroplasts. Approximately 100 g of deveined spinach leaves was ground in a Waring blender for 15 sec in 250 ml of a medium containing sucrose (0.4 *M*), Tricine (20 m*M*, pH 8.0), NaCl (15 m*M*), and bovine serum albumin (2 mg/ml). The slurry was filtered through 4 layers of cheesecloth and centrifuged at 1000*g* for 1 min to remove debris. The resulting supernatant fluid was centrifuged at 5000*g* (10 min) and the pellets were resuspended in 100 ml of the homogenizing medium and centrifuged again at 5000*g* for 10 min. These pellets were resuspended in sufficient homogenizing medium, supplemented with 10 mg/ml of bovine serum albumin, to give a final concentration of 1–2 mg/ml of chlorophyll, and the preparation was subdivided into aliquots (0.5 ml) which were stored at −70°C until used. Broken chloroplasts prepared and stored by this procedure retained high levels of activity for periods in excess of 4 weeks.

Preparation of KCN-treated chloroplasts was carried out according to the procedure of Ouitrakul and Izawa (1973) except that the KCN concentration was increased to 50 m*M*. Maximal inhibition of electron transport from water to ferricyanide was attained after 75–90 min in KCN, after which the chloroplasts were centrifuged, resuspended, and stored as described above. Chlorophyll was assayed by the method of Arnon (1949). All operations were carried out at 0–4°C.

Assay of Electron Transport Activity. Oxygen evolution by broken chloroplasts was measured polarographically with continuous stirring in a 1.6-ml thermostated (25°C) glass cell. Saturating white light was provided by a microscope lamp or an Oriel light source. The reaction medium contained Tricine (12 m*M*, pH 8.0), NaCl (60 m*M*), ferricyanide (2.4 m*M*), and 12–16 μg of chlorophyll in the presence or absence of a quinone or quinonediimine acceptor (0.24 m*M*). When ionophoric uncouplers were employed for inhibition studies, KCl replaced NaCl in the reaction medium. Additions to reaction mixtures during illumination were made at precise time intervals (1 min) by means of a microliter syringe.

Measurement of p-Phenylenediamine Uptake. Accumulation of *p*-phenylenediamine by chloroplasts was assayed at 25°C using a microcentrifuge technique similar to that described by Rottenberg et al. (1971). The reaction mixture was identical with that described for oxygen evolution assays except that the ferricyanide concentration was increased to 6.0 m*M* and higher concentrations of chloroplast material (500–650 μg of chlorophyll/1.6 ml) were used. Aliquots (0.4 ml) of the reaction mixture were distributed among four 0.5-ml polypropylene microcentrifuge tubes. The loaded tubes were placed in a Coleman microcentrifuge and illuminated from the top with red light (>600 nm, 5×10^5 ergs per cm^2 per sec) which was passed through a 4-cm water filter.

After 1 min of illumination centrifugation was begun, and after 45 sec illumination and centrifugation were terminated simultaneously. Aliquots (0.2 ml) of supernatant fluid were immediately withdrawn from each of the four tubes and deproteinized by addition of 0.02 ml of 30% trichloroacetic acid and centrifugation (3000*g*) at 4°C for 5 min. The concentration of *p*-phenylenediamine in the supernatant fluid from this step was assayed by a modification of a colorimetric procedure described by Snell and Snell (1967). Aliquots (0.05–0.10 ml) of the supernatant fluid were added with mixing to a solution containing Mes buffer (50 μmol) (pH 6.0), ferricyanide (2 μmol), and sufficient water to give a final volume of 1.0 ml. The color reaction was initiated by addition of 0.02 ml of 0.1 *M* aniline in ethanol, and the blue color which formed was assayed spectrophotometrically 3–4 min later at 670 nm, using a blank which contained all components except aniline. The concentration of *p*-phenylenediamine was determined using a standard curve prepared by subjecting the reaction mixture minus chloroplasts to the complete procedure described above. Linear correspondence between concentration and absorbance was obtained between 0 and 40 nmol of *p*-phenylenediamine/ml; 25 nmol of *p*-phenylenediamine/ml produced an absorbance of 0.40 at 670 nm.

Uptake was also assayed in chloroplast pellets. The same procedure was used except that the supernatant fluid was carefully removed from the tubes, and the pellets were resuspended in 0.8 ml of reaction mixture containing 0.05% sodium deoxycholate by flushing the tubes with this solution. Complete removal of the pellets could not be obtained under any condition tried, and when experiments were done to assay *p*-phenylenediamine concentrations in both the pellets and supernatants, total recovery varied between 85 and 90%. Since the supernatant assay was more reliable and less laborious, it was used for the uptake measurements, in combination with occasional pellet assays to check recovery.

Spectrophotometric Assays. All spectrophotometric assays were performed in a Zeiss PMQ-3 single-beam spectrophotometer using 1-cm quartz cuvettes having a volume of 1.5 ml.

Results

Effect of Acceptor Structure on Electron Transport Rate and Susceptibility to Inhibition by Uncoupling. Incubation

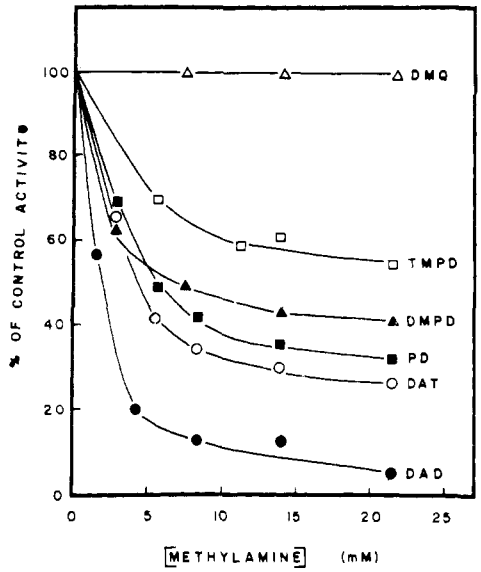

FIGURE 1: Effect of methylamine concentration on the rate of oxygen evolution catalyzed by quinone and quinonediimine electron acceptors in KCN-treated chloroplasts. The 100% activity levels (0 m*M* methylamine) are those given in Table I. Conditions are described in Materials and Methods.

Table I: Stimulation of Oxygen Evolution in KCN-Treated Chloroplasts by Quinone and Quinonediimine Acceptors.[a]

Acceptor	Rate[b]	Acceptor	Rate[b]
None	30	DMPDox	340
PDox	465	TMPDox	205
DATox	455	DMQ	200
DADox	390		

[a] Conditions as given in Materials and Methods. [b] Micromoles of oxygen evolved per hour per milligram of chlorophyll.

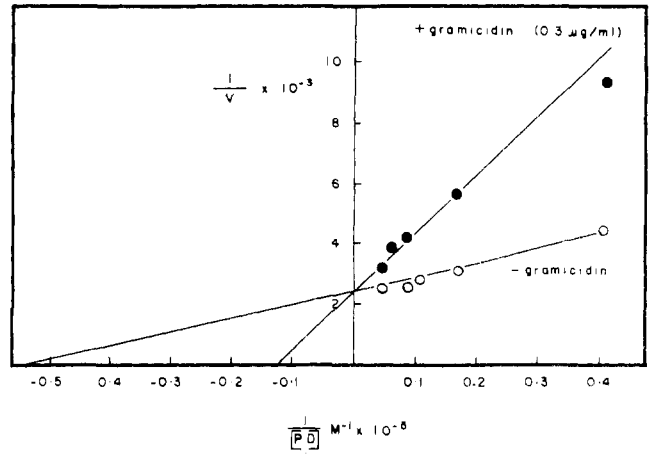

FIGURE 2: Double reciprocal plots of PDox-catalyzed oxygen evolution by KCN-treated chloroplasts in the presence and absence of gramicidin. Conditions are described in Materials and Methods.

Table II: Effect of Gramicidin on Oxygen Evolution Catalyzed by Quinones and Quinonediimines in KCN-Treated Chloroplasts.[a]

Acceptor	Control[b]	+ 3 μg/ml of Gramicidin	% Control Rate
None	30	59	197
DMQ	170	170	100
TMPDox	200	145	73
DMPDox	313	195	62
PDox	340	215	63
DATox	405	238	59
DADox	325	98	30

[a] Conditions as given in Materials and Methods. [b] Micromoles of oxygen evolved per hour per milligram of chlorophyll.

of chloroplasts with KCN produces two effects. High rates of electron flow require a quinone or quinonediimine acceptor in addition to ferricyanide, while the complication of bypass reactions through photosystem I observed in DBMIB-inhibited chloroplasts (Trebst and Reimer, 1973a,b) is minimized owing to inactivation of plastocyanin (Ouitrakul and Izawa, 1973). Table I shows that identical concentrations (0.24 m*M*) of quinonediimine or quinone acceptors produce differing rates of electron flow in KCN-inhibited chloroplasts. Since electron flow is saturated at this concentration for each acceptor shown, the variation in rate represents the effect of acceptor structure on activity. The highest rates are produced by PDox, DATox, and DADox. The activity of DMPDox is somewhat lower, while the cation radical TMPDox and the quinone DMQ catalyze the lowest rates observed in this experiment. The reason for this variation is not clear. With the exception of TMPDox, all of these acceptors are lipophilic (Saha et al. 1971), exist as uncharged species under our assay conditions, and possess redox potentials (Hauska et al., 1974) which facilitate their reduction by photosystem II.

When methylamine, an uncoupler, was added in increasing amounts to reaction mixtures containing KCN-inhibited chloroplasts plus a quinone or quinonediimine acceptor the results shown in Figure 1 were obtained. At high concentrations of methylamine all quinonediimine-mediated electron transport rates are decreased to levels which depend on the acceptor used. Note, however, that the DMQ rate is unaffected by methylamine. These findings are in agreement with those of Cohen et al. (1975), but differ from the results of Trebst and Reimer (1973a) who reported that uncoupling of DBMIB-inhibited chloroplasts produced a lowered rate of electron transport which was independent of quinonediimine acceptor structure. In order to see whether this disagreement was due to the uncoupler used, gramicidin uncoupling was also examined and the results are presented in Table II. Again, DMQ-mediated electron transport is insensitive to uncoupling while the quinonediimine rates vary in the presence of gramicidin, depending on the structure of the acceptor. We have also tested nigericin and *m*-Cl-CCP and have obtained results similar to those shown in Figure 1 and Table II. Our data show that uncoupler-induced inhibition is a general property of quinonediimine-catalyzed electron transport in KCN-inhibited chloroplasts.

The nature of uncoupler inhibition of quinonediimine acceptor activity in KCN-inhibited chloroplasts was further explored by kinetic analyses. In these experiments, the concentration of acceptor added during assay was varied in the presence or absence of an uncoupler and the results were fitted to double-reciprocal Lineweaver–Burk plots. Representative results of such an experiment are shown in Figure 2, where PDox was the acceptor and gramicidin was the uncoupler. The inhibition observed with gramicidin is clearly competitive; the K_m for PDox is 2×10^{-5} *M*, while in the presence of gramicidin it is calculated to be 8.6×10^{-5} *M*. Similar results were obtained in the presence of 6.0 m*M*

Table III: Effect of Aniline on PDox-Catalyzed Oxygen Evolution by Untreated and KCN-Treated Chloroplast.[a]

Chloroplast Preparation	Addition	Rate[b]
Untreated		175
Untreated	PDox	425
Untreated	Aniline (0.6 m*M*)	185
Untreated	PDox + aniline (0.6 m*M*)	300
KCN-treated		30
KCN-treated	PDox	490
KCN-treated	Aniline (0.6 m*M*)	32
KCN-treated	PDox + aniline (0.6 m*M*)	310

[a] Conditions are given in Materials and Methods. [b] Micromoles of oxygen evolved per hour per milligram of chlorophyll.

Table IV: Accumulation of PDox by Illuminated Chloroplasts.[a]

Preparation	Addition	Uptake[b]	$[PD]_{in}/[PD]_{out}$[c]
Untreated chloroplasts		0.240	23.6
	DCMU (6 μM)	0.020	1.6
	Methylamine (10 m*M*)	0.080	8.0
KCN-treated chloroplasts		0.140	12.0
	DCMU (6 μM)	0.004	0.4
	Methylamine (10 m*M*)	0.050	4.8

[a] Conditions are described in Materials and Methods. [b] Light *minus* dark uptake, micromoles per milligram of chlorophyll. [c] Calculated assuming a constant external PD_{ox} concentration of 0.25 m*M* and an internal chloroplast volume of 40 μl/mg of chlorophyll.

methylamine, which produced a shift of the PDox K_m to 5 $\times 10^{-4}$ *M*. We have repeated these experiments with all of the quinonediimine acceptors listed in Table II and a variety of uncouplers (methylamine, gramicidin, nigericin, *m*-Cl-CCP). In all experiments, competitive inhibition was obtained. Since the uncouplers we have examined are presumed to act by decreasing the proton concentration within the thylakoid membrane, the competitive inhibition by uncoupling of quinonediimine electron transfer activity shows that a proton gradient is required for optimal rates of activity.

Correlation of PDox Uptake with Acceptor Activity. Previous work has established that uncouplers are competitive inhibitors of amine uptake (Crofts, 1968) and that aromatic amines such as aniline accumulate in chloroplasts in response to a light-induced proton gradient (Portis and McCarty, 1973). Since we have observed competitive inhibition with *p*-diaminobenzene compounds, it was of interest to see whether internal accumulation was occurring during electron transport. Experiments were done in which aniline was included with PDox in the assay medium. The concentration of aniline (0.6 m*M*) which was used does not uncouple electron flow, but nevertheless an inhibition of electron transport was seen with chloroplasts and KCN-inhibited chloroplasts, as shown in Table III. Similar results were obtained with DATox and DADox. This inhibition was thought to be due to a competition between the acceptor species and aniline for internal protons, but a kinetic analysis of the phenomenon produced the unexpected result that the inhibition was uncompetitive rather than competitive. One explanation for this result was that aniline reacts chemically with the acceptor. A chemical reaction between PDox and aniline was in fact observed to occur in the pH range between 4.0 and 6.0. The product of this reaction, a Wurster's blue derivative (Lu Valle et al., 1948), appears to be formed under oxidizing or reducing conditions, but the blue color is seen only in the presence of an oxidant, such as ferricyanide.

Aniline inhibition of PDox activity by a chemical reaction requiring lowered pH showed that both amine species were probably accumulating inside chloroplasts during the electron transport assay, but the extent of accumulation could not be determined under these conditions. The colored reaction product produced by aniline and PDox was therefore used as a means of measuring PDox accumulation as described in Materials and Methods. The results of a number of experiments are summarized in Table IV, which shows that PDox uptake occurs in both chloroplasts and KCN-inhibited chloroplasts. Uptake varied from 100 to 280 nmol/mg of chlorophyll depending on whether KCN-inhibited or untreated chloroplasts were used for assay. The reason for this variance is not clear but KCN-inhibition lowers the rate of photosystem II electron flow and this may in turn limit the extent of accumulation. The sensitivity of PDox uptake to DCMU and methylamine shows that electron transport and proton conservation within the membrane are necessary for optimal accumulation. Furthermore, the extent to which uptake by KCN-inhibited chloroplasts is inhibited by methylamine (60%) corresponds closely to the extent of inhibition of electron transport (62%) observed with the same concentration of uncoupler (Figure 1), showing that optimal accumulation is related to optimal rates of electron transport. Similar measurements of uptake were attempted with other acceptors, but were unsuccessful owing either to failure to obtain a suitable color reaction (DATox, DADox) or to rapid decomposition in the assay medium (TMPDox, DMPDox), probably by demethylation (Lu Valle et al., 1948).

Analysis of TMPDox-Mediated Electron Flow. The cation radical TMPDox represents a unique exception among quinonediimine acceptors because it is an effective catalyst in spite of its lipophobic nature (Saha et al., 1971). Our results show that TMPDox is similar to other quinonediimines in that its catalytic activity is impaired by uncoupling. If the mechanism of TMPDox activity is similar to that of PDox, then it could only accumulate in the membrane after photoreduction. Saha et al. (1971) have in fact proposed a similar mechanism, but our observations of uncoupler inhibition of TMPDox activity suggest certain alternate explanations. Under our conditions of assay (pH 8.0) where highest rates of electron flow are obtained with quinonediimines, TMPDox can be shown to decay. This decomposition can readily be observed spectrophotometrically at either 562 or 610 nm. It is therefore possible that a decomposition product is the catalytic species rather than TMPDox. An experiment was done to test this hypothesis in which TMPDox was incubated in the light in a complete reaction mixture without chloroplasts for 5 or 10 min, after which KCN-inhibited chloroplasts were added and electron transport activity was measured. The results showed that control activity (107 μmol of oxygen evolved per hr per mg of chlorophyll) at zero time was higher than the activities after 5 min (94 μmol per hr per mg of chlorophyll) or 10 min (79 μmol per hr per mg of chlorophyll) of incubation. The concentrations of TMPDox were estimated spectrophotometrically at 562 nm after these incubation times, and were

found to be 0.21 mM (5 min) and 0.19 mM (10 min). These concentrations of fresh TMPDox were then added to assay mixtures, and the rates of electron flow were assayed and found to be 92 and 80 μmol of oxygen evolved per hr per mg of chlorophyll, respectively. Since these rates are in reasonable agreement with those obtained in the decomposition experiments, it appears unlikely that it is a decomposition product, rather than TMPDox, which is responsible for electron transport activity under our conditions of assay.

It has been demonstrated by Kraayenhof et al. (1972) that charged amines such as atebrin undergo energy-dependent binding to chloroplast membranes. It was therefore of interest to see whether atebrin would inhibit the uncoupler sensitive activity catalyzed by TMPDox, a result which might be expected if TMPDox were binding to the chloroplast membrane. Concentrations of atebrin (38 μM) which produced a weak uncoupling (10–15%) of ferricyanide reduction in uninhibited chloroplasts produced no effect on the photoreduction activity seen with PDox in KCN-inhibited chloroplasts. Similar experiments in assays where TMPDox was the catalyst produced rates in the presence of atebrin (122 μmol of oxygen evolved per hr per mg of chlorophyll) which were somewhat higher than in corresponding control experiments (94 μmol per hr per mg of chlorophyll). These results suggest that if membrane binding is occurring as part of the mechanism of TMPDox electron transport activity, the binding is either inhibitory or must be occurring at sites on the membrane which are insensitive to atebrin binding.

Discussion

The results presented here confirm previous observations (Gould and Ort, 1973; Trebst and Reimer, 1973a; Cohen et al., 1975) that catalysis of photosystem II electron transport by quinonediimines is sensitive to uncoupling. We have further shown that this inhibition is competitive, implying that the proton gradient in chloroplasts is necessary for optimal rates of electron flow with these compounds, and our experiments demonstrating uptake of PDox by chloroplasts and the inhibition of this process by uncoupling show that uptake and catalysis are related phenomena. Our attempts to show that catalysis of electron flow by TMPDox requires a decomposition product or energized binding to the chloroplast membrane were unsuccessful, implying that the catalytic mechanism for lipophobic quinonediimines is the same as for uncharged species.

These findings cannot be satisfactorily explained by the previous hypotheses that lipophilicity alone (Saha et al., 1971) or in concert with an imine/amine shuttle (Trebst and Reimer, 1973a) constitute the mechanism for catalysis of electron flow by quinonediimines. While lipophilicity is certainly necessary as a basic requirement for activity, it does not explain inhibition of catalysis by uncouplers; the catalytic activity of TMPDox argues against imine penetration as a significant factor in activity, as do the uptake experiments reported here. Membrane penetration by a lipophobe (TMPDox) is difficult to envision, and the protonation of PDox (pK_1 = 4; Clark, 1961), which must occur to facilitate uptake, is not favored by the internal pH presumed to prevail under our experimental conditions (Portis and McCarty, 1973).

Because of these difficulties we would propose an alternate hypothesis to account for our results. This mechanism requires a minimum of the three steps: (1) photoreduction and protonation of acceptor on the external surface of the thylakoid membrane with generation of lowered internal pH by concomitant photooxidation of water; (2) penetration into the membrane by the reduced amine and protonation to form the amine cation; (3) repenetration by free amine base, after internal deprotonation, into the external medium. If this proposal is correct, several criteria must be met. The primary acceptor for photosystem II must reside on the external surface of the thylakoid membrane. The extensive experiments of Arntzen et al. (1974a,b) have shown that this is so. Penetration by reduced amine requires that this species be lipophilic. The reduced species of DADox and TMPDox are well-known photosystem I donors (Trebst, 1972), and the reduced forms of DATox and PDox have also been shown to donate to photosystem I (Ort and Izawa, 1974). Since the sites of donation of these compounds (plastocyanin, cytochrome f) reside inside the thylakoid membrane (Hauska et al., 1971; Trebst, 1974), these reduced species must be lipophilic. Protonation inside the membrane requires that the amine pK_1 value favor protonation at a pH near 5.0 (Portis and McCarty, 1973). The pK_1 of reduced PDox is 6.08 (Perrin, 1965). The pK_1 values of DADox and DATox are probably somewhat lower owing to C-methylation, while the N-methylation of aromatic amines produces higher pK_1 values (Sober, 1970).

While the topology of the chloroplast membrane and the chemical properties of quinonediimines are in accord with our proposal, there are three factors which are of concern. Our proposal envisions photoreduction and reoxidation of acceptors to occur on the same side of the chloroplast membrane. Accumulation of amine inside the membrane therefore adds to the mechanism what appears to be an unnecessary complication. We cannot explain this complication, but it is possible that accumulation facilitates electron flow by actively withdrawing lipophilic reducing equivalents from the donor site. This accumulation is presumed to involve protonation of the amine species with protons arising from water oxidation, and this implies that reducing equivalents are accumulated near the site of water oxidation. Even if this were to occur, the reductants are not likely to interfere with water oxidation since increasing concentrations of quinonediimines stimulate rather than inhibit oxygen evolution, and the stoichiometries of photophosphorylation obtained with quinonediimine acceptors (Saha et al., 1971) are consistent with noncyclic, rather than cyclic, electron flow.

The last, but perhaps most important, consequence of our proposed model concerns the possibility that internal protonation of p-phenylenediamines will lead to uncoupling of photophosphorylation. Several considerations argue against this, however. The low pK_2 values for p-phenylenediamines (3.29 in the case of PD; Sober, 1970) indicate that one proton, out of two generated by water oxidation during photoreduction of the quinonediimine to the amine species, is required for formation of the amine cation inside the membrane. It is unlikely that this reversible protonation step constitutes uncoupling, however, since it is known that other low-pK_a amines which are taken up by a mechanism similar to that which we propose do not uncouple appreciably, even at concentrations approaching a 100-fold excess over the concentrations of the p-phenylenediamines used in our assays (Portis and McCarty, 1973; Nelson et al., 1971). Rather, these compounds act as internal buffers which stimulate the extent of proton uptake by chloroplasts, and it is possible that p-phenylenediamines might produce a similar effect on proton uptake.

The extreme complexity of quinonediimine acceptor activity precludes assignment of a rate controlling function to any one step in our model. It is attractive to assume that protonation/deprotonation reactions limit the rate by virtue of the equilibrium between free amine and the cation radical form inside the membrane, but the possible effects of the lipophilic membrane environment on p*K*'s probably add complications which cannot be readily identified or quantitated at the present time. It is possible, however, that uptake and protonation are responsible, at least in part, for the differences in rate between quinonediimines and DMQ. The former substances are subject to the effects of differential concentration gradients across the membrane, while DMQ, lacking an ionic species which accumulates, is not.

An obvious criticism of our model must be noted. We have measured the uptake of only one acceptor, PDox, for the reasons noted in Results, and therefore we have no evidence that accumulation is occurring with any of the other quinonediimine acceptors used in these studies. We would point out, however, that all quinonediimines used in our experiments are sensitive to inhibition by uncouplers, and it is therefore likely that uptake is also occurring with these other acceptors.

Acknowledgments

We gratefully acknowledge the many helpful suggestions of Drs. N. E. Good, Donald R. Ort, and H. Ikuma.

References

Arnon, D. I. (1949), *Plant Physiol. 24,* 1.

Arntzen, C. J., Armond, P. A., Zettinger, C. S., Vernotte, C., and Briantais, J. M. (1974a), *Biochim. Biophys. Acta 347,* 329.

Arntzen, C. J., Vernotte, C., Briantais, J. M., and Armond, P. (1974b), *Biochim. Biophys. Acta 368,* 39.

Clark, W. M. (1961), Oxidation-Reduction Potentials of Organic Systems, Baltimore, Md., Williams and Wilkins, pp 396–400.

Cohen, W. S., Cohn, D. E., and Bertsch, W. (1975), *FEBS Lett. 49,* 350.

Crofts, A. (1968), in Regulatory Functions of Biological Membranes, Jarnefelt, J., Ed., New York, N.Y., Elsevier, pp 252–255.

Gould, J. M., and Ort, D. R. (1973), *Biochim. Biophys. Acta 325,* 157.

Hauska, G., McCarty, R. E., Berzborn, R. J., and Racker, E. (1971), *J. Biol. Chem. 246,* 3524.

Hauska, G., Reimer, S., and Trebst, A. (1974), *Biochim. Biophys. Acta 357,* 1.

Izawa, S., Gould, J. M., Ort, D. R. Felker, P., and Good, N. E. (1973), *Biochim. Biophys. Acta 305,* 119.

Kraayenhof, R., Izawa, S., and Chance, B. (1972), *Plant Physiol. 50,* 713.

Lu Valle, J. E., Glass, D. B., and Weissberger, A. (1948), *J. Am. Chem. Soc. 70,* 2223.

Nelson, N., Nelson, H., Naim, Y., and Neumann, J. (1971), *Arch. Biochem. Biophys. 145,* 263–267.

Ort, D. R., and Izawa, S. (1974), *Plant Physiol. 53,* 370.

Ouitrakul, R., and Izawa, S. (1973), *Biochim. Biophys. Acta 305,* 105.

Perrin, D. D. (1965), Dissociation Constants of Organic Bases in Aqueous Solution, London, Butterworths, pp 58–96.

Portis, A., and McCarty, R. E. (1973), *Arch. Biochem. Biophys. 156,* 621.

Rottenberg, H., Grunwald, T., and Avron, M. (1971), *FEBS Lett. 13,* 41.

Saha, S., Outrakul, R., Izawa, S., and Good, N. E. (1971), *J. Biol. Chem. 246,* 3204.

Snell, F. T., and Snell, C. T. (1967), Colorimetric Methods of Analysis, Princeton, N.J., Van Nostrand, p 380.

Sober, H. A., Ed. (1970), Handbook of Biochemistry, Selected Data for Molecular Biology, 2nd ed, Cleveland, Ohio, The Chemical Rubber Co., p J-208.

Trebst, A. (1972), *Methods Enzymol. 24,* 146–165.

Trebst, A. (1974), *Annu. Rev. Plant Physiol. 25,* 423.

Trebst, A., and Reimer, S. (1973a), *Biochim. Biophys. Acta 325,* 546.

Trebst, A., and Reimer, S. (1973b), *Z. Naturforsch. C 28,* 710.