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SITE-SPECIFIC INHIBITION OF PHOTOPHOSPHORYLATION IN ISOLATED SPINACH CHLOROPLASTS BY $HgCl_2$

II. EVIDENCE FOR THREE SITES OF ENERGY CONSERVATION ASSOCIATED WITH NON-CYCLIC ELECTRON TRANSPORT

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SUMMARY

Energy transfer inhibition by $HgCl_2$ has been demonstrated to be selective for certain System I partial reactions. On the basis of different $HgCl_2$ effects on the System I reactions, reduced 2,6-dichlorophenolindophenol → methylviologen, diaminodurene → methylviologen and *N*-phenazine methosulfate cyclic, two sites of energy conservation associated with System I are proposed. Furthermore, these sites are in parallel with each other, in series with the site closely associated with Photosystem II and are shared between non-cyclic and cyclic electron transport.

INTRODUCTION

Recent reports have convincingly demonstrated at least two sites of energy conservation in non-cyclic electron transport. Phosphorylation by Photosystem II reactions has quite different properties from phosphorylation by the second, recognized site, localized [1] between the oxidation of plastoquinone and the reduction of cytochrome *f*. The Photosystem II-dependent energy coupling site exhibits no control over coupled electron transport and has a P/e_2 ratio of about 0.4 [2–5]. Furthermore, this P/e_2 ratio is practically pH independent [4, 5]. On the other hand, the coupling site associated with System I is the rate-limiting step for the Hill reaction, exhibiting control over electron transport and has a pH-dependent P/e_2 of about 0.6–0.7 (pH 8.5) [5]. Besides the physical differences in the properties of the sites, we have recently shown that the two sites respond differently to the energy transfer inhibitor $HgCl_2$ [6].

Abbreviations: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DMQ, 2,5-dimethyl-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, *N*-phenazine methosulfate; DTNB, 5,5′-dithiobis-2-(nitrobenzoic acid); P/e_2: number of ATP molecules formed for every pair of electrons transported; PCMB, *p*-chloromercuribenzoate; FCCP, 4-trifluoromethoxy carbonyl cyanide.

As information accumulates on these two sites of energy conservation, several System I catalyzed phosphorylation reactions remain an enigma. It has been assumed by some that *N*-phenazine methosulfate (PMS) catalyzed cyclic phosphorylation uses a special site not associated with non-cyclic electron transport. This special site could be functioning during the reaction when diaminodurene is the electron donor and methylviologen is the electron acceptor. High rates of electron transport and phosphorylation (which may be equivalent to those of the PMS reaction [7]) are sensitive, however, to the plastocyanine antagonist KCN [5, 8], suggesting that at least some of the components used by this reaction are shared with non-cyclic electron transport.

Since $HgCl_2$ inhibition is selective for the phosphorylation associated with Photosystem I, it was of interest to test the compound using the various System I partial reactions. The results suggest that there are two energy conservation sites associated with System I. Furthermore, these sites are in parallel with each other and are common to non-cyclic and cyclic systems in a specific fashion.

MATERIALS AND METHODS

The buffers employed in this study were specifically selected for their lack of metal binding. During the course of experiments, it has become apparent that various buffers can affect the characteristics of $HgCl_2$ inhibition of isolated chloroplasts. Because Tricine is such a strong metal chelator (nearly 100 times more so than *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)), it was avoided throughout this study. The buffer apparently carries Hg^{2+} through the membrane and results in non-specific inhibition even at low levels of $HgCl_2$. The buffer TES was substituted and even though its pK_a is only 7.5, the pH of the reaction (8.2) was controlled under quite a variety of conditions. In some experiments, *N*-hydroxyethylpiperazine-*N'*-propanesulfonic acid (HEPES), with a pK_a of 8.1 was employed to insure proper maintenance of the pH. Also of importance is the finding that Tris, although it does not bind the metal greatly, seems to relieve $HgCl_2$ inhibition.

Chloroplasts were isolated from fresh market spinach essentially as described elsewhere [9]. Approx. 50 g of washed deribbed leaves were ground briefly (5 s) in a Waring blendor containing a medium of 0.3 M NaCl, 1.0 mM $MgCl_2$, 1.0 mM EDTA and 0.04 M TES made to pH 7.3 with NaOH. After filtering through several layers of cheesecloth to remove large leaf fragments, the suspension was centrifuged at $4500 \times g$ for 3 min. After suspension of the pellet in a medium of 0.2 M sucrose, 0.5 mM $MgCl_2$ and 0.03 M TES buffer (pH 7.3), unbroken cells and heavy debris were removed by centrifugation at $1500 \times g$ for 75 s. The chloroplasts were removed from the supernatant by centrifugation at $2000 \times g$ for 5 min, resuspended in fresh medium and again sedimented. The final pellet was suspended in a small volume of medium and chlorophyll concentration determined by the spectrophotometric method of Arnon [10].

Crude coupling factor (CF_1) was prepared by modification of the procedure of Lien and Racker [11]. Unwashed chloroplasts, prepared in the usual way, were broken by 10-min suspension in 10 mM NaCl. After centrifugation of the broken chloroplasts at $4500 \times g$ for 10 min, the chloroplasts were suspended to a concentration of 1.5 mg chlorophyll per ml with the NaCl solution. After further dilution

to 100 μg/ml using room temperature EDTA solution (0.75 mM, pH 8.0), the membranes were centrifuged out at 20 000 × *g* for 20 min at room temperature. The supernatant containing the coupling factor was decanted and the protein concentration in the extract was determined by ultraviolet absorption.

The reactions used, with only minor modifications, have been described in other publications [2–8]. Electron transport to $Fe(CN)_6^{3-}$ or Class III acceptors was followed by the loss of absorbance (420 nm) as $Fe(CN)_6^{3-}$ was reduced. The stoichiometric O_2 evolution (when H_2O was the electron donor) or O_2 consumption (during the autooxidation of reduced methylviologen) was measured as previously described [7]. Absorbance changes were followed as previously described using a modified Beckman DU spectrophotometer [9]. O_2 concentration was monitored using a Clark-type oxygen electrode.

Reactions were run in a final volume of 2.0 ml for spectrophotometric measurements and 3.0 ml for the oxygen monitor. Illumination was saturating for the $Fe(CN)_6^{3-}$ reaction and was supplied by a 750-W slide projector.

ATP formation was determined for a 1-ml aliquot of the reaction mixture by extracting unreacted $^{32}P_i$ as phosphomolybdic acid [12]. Radioactivity was measured by the Cerenkov technique of Gould et al. [13]. Correction for quenching was accomplished by the extraction of "dummy" reaction mixtures containing unlabelled phosphate, to which was subsequently added a known amount of ^{32}P. ATPase reactions were measured according to the method of Lien and Racker [11]. Released phosphate was determined by the method of Taussky and Shorr [14].

Diaminodurene (Research Organic/Inorganic Chemical Co.) and *p*-phenylenediamine (Sigma Chemical) were recrystallized as the colorless dihydrochloride salts before use. Water-insoluble compounds were dissolved in ethanol : ethylene glycol (1 : 1, v/v). To avoid solvent damage to the membranes, the stock solutions were prepared with high enough concentrations that solvent concentration in the final reaction mixture never exceeded 1%.

RESULTS

The effect of $HgCl_2$ on the $Fe(CN)_6^{3-}$ Hill reaction is shown by Fig. 1A. These results verify the report of Izawa and Good [15]; $HgCl_2$ inhibits ATP formation by titration to a 50% maximum at approx. 40–50 nmoles/mg chlorophyll. Additional $HgCl_2$ has no effect except much higher concentrations cause a non-specific inhibition. Electron transport coupled to the ATP formation is also inhibited 50%. Basal ($-P_i$) electron transport and electron transport in the presence of 10 mM methylamine are unaffected. Thus the inhibition is characteristic of an energy transfer inhibitor.

Further experiments were performed to insure that $HgCl_2$ is indeed an energy transfer inhibitor. These tests were deemed necessary since there have been a number of recent reports on electron transport inhibition and electron acceptance by $HgCl_2$ [16, 17]. To insure that uncoupled electron transport is indeed insensitive to $HgCl_2$, and that the results with the amine were not due to some peculiar interactions between the compounds, a variety of uncoupled reactions were subjected to high levels of $HgCl_2$. Table I shows that electron transport stimulation by the ADP-independent uncouplers methylamine, gramicidin, 4-trifluoromethoxycarbonylcyanide (FCCP), and atebrin is insensitive to mercury. The ADP-dependent arsenate-uncoupled electron

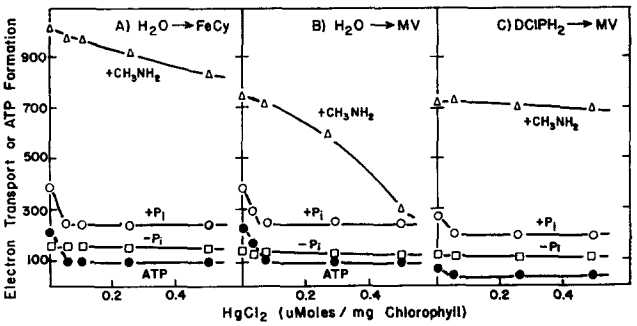

Fig. 1. Effect of $HgCl_2$ on partial reactions utilizing the rate-limiting phosphorylation site. Reaction mixtures all contained: 50 mM TES–NaOH buffer (pH 8.2); 0.1 M sucrose; 2 mM $MgCl_2$, 1 mM ADP and, if added, 5.0 mM phosphate and 20 mM methylamine · 2 HCl. Donor/acceptor additions were: (A), 0.4 mM $K_3Fe(CN)_6$; (B), 50 μM methylviologen; (C), 0.4 mM DCIP, 50 μM methylviologen, 1.0 μM DCMU and 5.0 mM ascorbate. In this and all figures, open symbols represent electron transport and solid symbols represent ATP formation rates. Symbol shape designates reaction conditions: uncoupled with methylamine (△–△) phosphorylating conditions (○–○) and basal conditions ($-P_i$) (□–□). Note that the three reactions have similar sensitivity to the energy transfer inhibitor. Rates are expressed as μmoles or μequiv/h per mg chlorophyll.

transport, however, is sensitive to $HgCl_2$. Since arsenate, by mimicking phosphate, utilizes the phosphorylation mechanism and thus stimulates electron transport, mercury affects this reaction as it does the phosphorylating reaction.

A further test that $HgCl_2$ acts on the coupling mechanism is its effect on chloroplasts stripped of coupling factor, i.e. EDTA-extracted chloroplasts. Fig. 2 shows that $HgCl_2$ has no inhibitory effect on electron transport catalyzed by EDTA-treated chloroplasts. The other curves in the figure demonstrate that the preparative procedures (salt treatment and osmotic shock during extraction) are not responsible for the relief of inhibition. This evidence argues strongly for the involvement of $HgCl_2$ at the level of the phosphorylating mechanism. The results presented in this figure also demonstrate that the degree of organelle integrity is not critical to the response

TABLE I

EFFECT OF $HgCl_2$ ON UNCOUPLED ELECTRON TRANSPORT

Uncoupler	Concn (mM)	Increase of electron transport* over basal	
		$-HgCl_2$	$+HgCl_2$
Methylamine	20.0	617	560
Gramicidin	0.01	625	664
Atebrin	0.06	191	189
FCCP	0.0013	329	289
Arsenate**	5.0	372	196

* Increase in rates (in μmoles/h per mg chlorophyll) over basal ($-P_i$) electron transport, which was 161 in this experiment. $HgCl_2$ was present as 0.5 μmoles/mg chlorophyll.

** Note that arsenate stimulation of electron transport is most affected by $HgCl_2$.

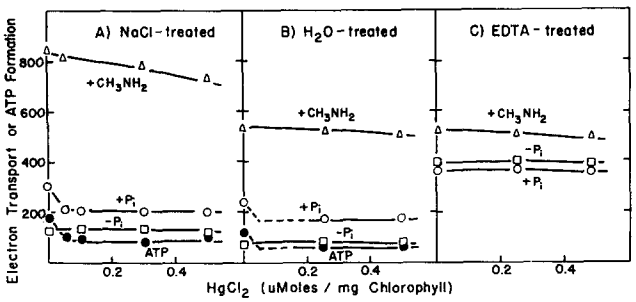

Fig. 2. Effect of $HgCl_2$ on broken, osmotically shocked and EDTA-extracted chloroplasts. (A) Chloroplasts were broken by suspension in 10 mM NaCl and washed in TES suspension medium. (B) Chloroplasts were treated just as during the EDTA extraction except that distilled water replaced the EDTA solution. Despite this harsh treatment, notice that the 50% inhibition plateau results. (C) Chloroplasts were extracted of coupling factor as described in Materials and Methods. The resulting chloroplasts were incapable of $^{32}P_i$ incorporation. Note that electron transport by these membranes is insensitive to $HgCl_2$. Reaction conditions as in Fig. 1A.

to $HgCl_2$. Even when the membranes are osmotically shocked (broken), the 50% $HgCl_2$ inhibition plateau results. Furthermore, this inhibition level is seen under a variety of chemical conditions (see Fig. 1) and with day to day changes in the quality of the chloroplast preparation, suggesting that the maximum effect is not fortuitous.

Fig. 1B shows the effect of $HgCl_2$ on pseudocyclic electron transport to methylviologen. Basal ($-P_i$) electron transport is unaffected; phosphorylating electron transport is inhibited to the 50% maximum in similar manner to the $Fe(CN)_6^{3-}$ reaction. The methylamine-uncoupled electron transport rates, however, are quite markedly affected by fairly low concentrations of $HgCl_2$. The non-specific inhibition previously seen at higher concentrations of $HgCl_2$ is also observed at much lower mercury levels when methylviologen is the electron acceptor. These latter results suggest that there is a site of $HgCl_2$ inhibition (electron carrier) which is omitted when $Fe(CN)_6^{3-}$ accepts electrons but which is involved in the water to methylviologen reaction. Upon addition of $HgCl_2$, this mercury-sensitive oxidation–reduction step soon becomes the rate determining step.

Reduced DCIP has recently been shown to donate electrons in a reaction which is not sensitive to plastoquinone antagonists [5]. Thus it appears to involve only System I components. The effect of $HgCl_2$ on electron transport and phosphorylation during this reaction is shown by Fig. 1C. Neither uncoupled nor basal electron transport is affected by $HgCl_2$, but phosphorylation and coupled electron transport are inhibited to the 50% plateau. Sensitivity is similar to that of the Hill reaction. Notice that the secondary electron transport effect of $HgCl_2$ seen with the H_2O–methylviologen system (Fig. 1B) is absent here, perhaps due to the presence of ascorbate.

Several compounds have been found to accept electrons in System II catalyzed reactions (Class III acceptors). The effect of $HgCl_2$ on the partial reactions to these compounds was tested and the results shown in Fig. 3. Electron transport and phosphorylation of these partial reactions are insensitive to $HgCl_2$. Transport to the diimides is virtually unaffected, while that to 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) is inhibited significantly at higher mercury levels. Since this partial reaction is known to be 'loosely coupled' (electron transport is not dependent

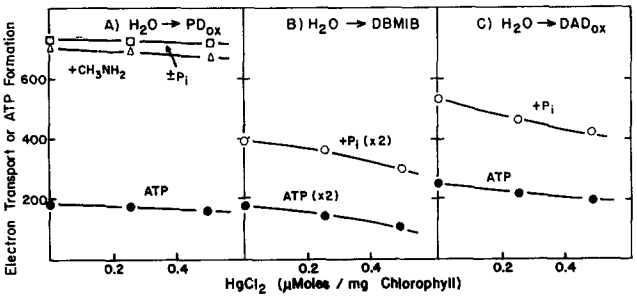

Fig. 3. Effect of $HgCl_2$ on Photosystem II partial reactions to Class III acceptors. Reaction mixtures were the same as in Fig. 1 with the donor/acceptor conditions: (A), 0.5 mM phenylenediamine, 0.5 μM DBMIB and 1.4 mM $Fe(CN)_6^{3-}$; (B), 10.0 μM DBMIB and 0.4 mM $Fe(CN)_6^{3-}$; and (C) 0.5 mM diaminodurene, 0.5 μM DBMIB and 1.4 mM $Fe(CN)_6^{3-}$. The chloroplasts and $HgCl_2$ were combined for approx. 30 s before the addition of the donors, acceptors and inhibitors to avoid binding with these substances. Note that the reactions are insensitive to the energy transfer inhibitor.

on phosphorylation), an energy transfer inhibitor should inhibit ATP formation but not electron transport. It is most probable, then, that $HgCl_2$ is affecting the efficiency of DBMIB electron acceptance, which is already the rate limiting step of the reaction. A fourth Class III acceptor reaction to dimethylquinone shows similar insensitivity to $HgCl_2$ [6].

Several other partial reactions have been tested. Diaminodurene will donate electrons in a System I catalyzed reaction [7]. The effect of $HgCl_2$ on electron transport and phosphorylation by this system is seen in Fig. 4A. These kinetics do not correspond to any of the previous systems. The low levels of $HgCl_2$ seem to inhibit phosphorylation but not electron transport. At approx. 0.2 μmoles/mg chlorophyll, electron transport begins to be affected by the mercury. Total inhibition is seen at approx. 1.0 μmole/mg chlorophyll.

Fig. 4B shows that PMS-catalyzed cyclic phosphorylation, when the catalyst

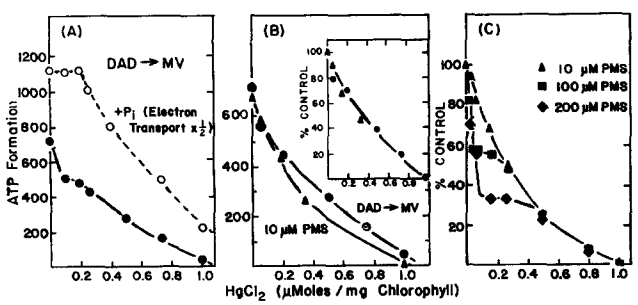

Fig. 4. Effect of $HgCl_2$ on Photosystem I catalyzed partial reactions. Donor/acceptor/inhibitor additions to the standard reaction mixture included: (A), 2.0 mM diaminodurene, 50.0 μM methylviologen and 1.0 μM DCMU; (B) and (C), 1.0 μM DCMU plus appropriate PMS. $HgCl_2$ inhibits diaminodurene methylviologen and 10 μM PMS reactions but probably only as an electron transport inhibitor (A). The reactions are similarly sensitive as shown by the insert (B). Phosphorylation catalyzed by PMS becomes more sensitive to low levels of $HgCl_2$ as the cofactor concentration is increased.

is present in low (10 μM) concentrations, is inhibited by mercury with sensitivity paralleling that of the diaminodurene–methylviologen reaction. The similar results suggest that the two reactions may be utilizing the same rate-limiting $HgCl_2$-sensitive step, but that this step is not the same $HgCl_2$-sensitive step seen during inhibition of the $Fe(CN)_6^{3-}$ Hill reaction.

Ouitrakul and Izawa [8] have reported that high concentrations of PMS (up to 200 μM) result in high rates of phosphorylation but that as the PMS concentration is increased, the reaction becomes increasingly insensitive to KCN treatment. It was of interest to see if any difference in $HgCl_2$ sensitivity would result from raising the PMS concentration. Fig. 4C shows that quite different results were obtained at high PMS concentrations. As PMS concentration is increased, a greater proportion of the phosphorylation becomes sensitive to low levels of $HgCl_2$. At 200 μM PMS, Ouitrakul and Izawa [8] found that KCN inhibited activity only about 30%. At these high co-factor concentrations, $HgCl_2$ (at low levels) inhibits phosphorylation to a level of about 30% of the control. These results suggest that high levels of PMS catalyze a reaction using the same $HgCl_2$-sensitive site that the $Fe(CN)_6^{3-}$ reaction uses, but that low PMS reactions omit this step.

Table II shows the effect of $HgCl_2$ on ATPase reactions of chloroplasts and coupling factor. In the table we can see that trypsin-treated chloroplasts, which will catalyze a Ca^{2+}-dependent, light independent ATPase activity, are unaffected by $HgCl_2$ at the low levels dealt with here. The lack of effect is not due to competing sulfhydryls, since trypsin and inhibitor were washed out and, to our surprise, no new DTNB-detectable sulfhydryls are exposed by trypsin treatment. Coupling factor (crude CF_1) ATPase is similarly insensitive to low levels of $HgCl_2$. It is possible that the EDTA present during the reaction masked inhibition, but this is unlikely considering that EDTA will not relieve $HgCl_2$ inhibition of the $Fe(CN)_6^{3-}$ reaction and that the ATPase reaction was run for 10 min, plenty of time for equilibrium to have been reached.

We have previously demonstrated that $HgCl_2$ does not inhibit any step involved in the utilization of the chloroplast high energy state (X_E). After X_E was formed in the absence of $HgCl_2$, the addition of $HgCl_2$ did not inhibit the ATP

TABLE II

EFFECT OF $HgCl_2$ ON Ca^{2+}-DEPENDENT, LIGHT-INDEPENDENT ATPase ACTIVITY OF COUPLING FACTOR AND WHOLE CHLOROPLASTS

Both chloroplasts and coupling factor were trypsin-activated as described in Materials and Methods. The chloroplasts were washed free of trypsin and inhibitor before being used for the experiment. Rates for both coupling factor and chloroplasts are expressed in μmoles/min per mg chlorophyll. Back calculation was made to correlate the amount of coupling factor with the source chloroplasts. In this case 660 μg protein resulted from extraction of chloroplasts containing 1.0 mg chlorophyll.

Component	Rate of ATP hydrolysis		
	$-HgCl_2$	$+HgCl_2$	
		0.25 μmoles/mg chlorophyll	0.50 μmoles/mg chlorophyll
Coupling factor	3.6	3.2	2.9
Chloroplasts	4.3	4.2	4.18

formed when ADP and phosphate were added. It is unlikely then that $HgCl_2$ inhibits a step terminal to the high energy state of the chloroplasts. This data localizes the site of $HgCl_2$ action close to the redox reactions (electron transport) as must be the case for site specificity.

DISCUSSION

Energy transfer inhibition by $HgCl_2$

$HgCl_2$ (and other mercuric compounds such as *p*-chloromercuribenzoate (PCMB), *p*-chloromercurisulfonate and mercuric acetate) have been shown to inhibit energy transfer during the Hill reaction to the electron acceptor, $Fe(CN)_6^{3-}$ [15].

The Hill reaction, with $Fe(CN)_6^{3-}$ (Fig. 1A) or methylviologen (Fig. 1B) as electron acceptor, is inhibited by $HgCl_2$ in proportion to the amount added up to levels equivalent to 40 nmoles $HgCl_2$/mg chlorophyll. At this level of $HgCl_2$, coupled electron transport and phosphorylation is inhibited to 50% of the control activity. At levels less than approx. 1.0 μmole/mg chlorophyll, more $HgCl_2$ has no additional effect. The $HgCl_2$ titration is complete when 1 molecule of $HgCl_2$ is bound for every 40 chlorophyll molecules. This value corresponds with the number of sulfhydryl groups detectable by DTNB [18].

$HgCl_2$ inhibition of photophosphorylation is reversed by the addition of cysteine but is unaffected by EDTA [15]. This reversal by sulfhydryl compounds and the strong affinity of mercury for reduced and oxidized sulfhydryl groups suggest that a sulfhydryl residue is involved in the phosphorylation mechanism of at least one site. Sulfhydryl groups have been implicated in active site involvement in a variety of phosphorylation mechanisms including phosphorylation reactions [19–22] and chemical "model systems" [23–25].

The data presented in this study indicates that fast-reacting sulfhydryl groups, easily accessible to $HgCl_2$, are not associated with the terminal steps of phosphorylation. This is in agreement with other studies which indicate few active site sulfhydryls in transphosphorylation reactions [26], but is contrary to those other interpretations based on inhibition of coupling factor ATPase by PCMB [27]. We have found that $HgCl_2$ inhibits ATPase only at high levels; the organic mercurials were not tested, but they could react more readily with the protein to produce a conformational change. Neither the ATPase activity (Table III) nor utilization of the high energy intermediate X_E is inhibited by $HgCl_2$ at the low levels dealt with here. The results suggest that $HgCl_2$ acts on a site of the phosphorylation mechanism prior to the formation of $X \sim Y$. These findings are consistent with the current concept of a pooled high energy intermediate and shared coupling factor (CF_1) (i.e. site specificity would be impossible with inhibition of a common transphosphorylation enzyme). The results, however, suggest that EDTA extraction removes more than just the terminal enzyme and that each site possesses its own set of 'early' enzymes to form $X \sim Y$.

Site specificity of $HgCl_2$ inhibition

On the basis of inhibition of partial reactions by DCMU, KCN and DBMIB [2–8], it seems apparent that the site associated with Photosystem II is in series with the rate determining site after plastoquinone. Inhibition of one of two phosphoryla-

TABLE III

SUMMARY OF INHIBITION AND REACTION CHARACTERISTICS OF THE PARTIAL REACTIONS OF PHOTOSYNTHETIC ELECTRON TRANSPORT AND PHOSPHORYLATION

	1	2	3	4	5	6
Donor	H_2O	H_2O	Reduced DCIP	Diamino-durene	PMS^*_{low}	PMS^*_{high}
Acceptor	$Fe(CN)_6^{3-}$ Methylviologen	Class III	Methyl-viologen	Methyl-viologen	PMS^*_{low}	PMS^*_{high}
Reaction rates**	Moderate	High	Moderate	High	High	High
Coupling***	+	−	+	−	...	...
Electron transport inhibition						
DCMU	+	+	−	−	−	−
DBMIB	+	−†	−	−	−	−
KCN	+	−†	+	+	+	30 % (−)†††
Energy transfer inhibition						
$HgCl_2$††	50 %	0 %	50 %	0 %	0 %	70 % (100 %)†††

* PMS concentrations were: low; 10 μM and high; 200 μM.

** Relative reaction rates of electron transport and/or phosphorylation under phosphorylating conditions.

*** Electron transport regulated by availability of ADP and phosphate or stimulated by uncouplers.

† DBMIB and KCN inhibit only the System I component of Class III reactions. System II component considered here is unaffected.

†† The maximal inhibition by low levels of $HgCl_2$.

††† The numbers in parentheses are valid if the complementary relationship between KCN and $HgCl_2$ inhibition is considered to indicate separate electron transport pathways and separate sites.

tion sites in series would give one of the following results: (1) Inhibition of the first 'loosely coupled' site would result in inhibition of ATP formation at this site but would not affect electron transport, which is not limited by this phosphorylation mechanism. (2) Inhibition of the second, tightly coupled site would inhibit electron transport to basal ($-P_i$); and phosphorylation to that which could be supported by the first site under the low electron transport. In neither case would we see 50% inhibition of both ATP formation and coupled electron transport. The 50% maximum inhibition, as demonstrated by Fig. 1, is a phenomenon of phosphorylation at the rate-determining site; apparently the insensitivity to $HgCl_2$ of System II phosphorylation is not involved in determining the extent of inhibition (also see [6]).

If a third, as yet unproposed, site of phosphorylation were functioning in parallel with the rate-limiting site only, the data could be explained. To achieve a 50% inhibition of activity, however, one-half of the coupled electron transport and phosphorylation would be provided by each of the two parallel sites. In such a proposed scheme, complete inhibition of one of the sites would yield the observed results. Precisely one half of the electrons from plastoquinone are donated to each site. This requirement for equal distribution of electrons to these sites is not as demanding as it first appears. Since the phosphorylation sites are the rate-limiting steps of electron transport (both exert control), the build-up of pools of reduced carriers on the Photosystem II side of the sites, would provide a continuous, ample supply of electrons. Thus, complete inhibition of one site would result in 50% inhibition of electron transport and associated phosphorylation.

The existence of this third site is suggested by the reaction and inhibition characteristics of Photosystem I partial reactions (Table III). These data suggest three different types of reactions catalyzed by System I: (1) reduced DCIP → methylviologen which is tightly coupled with moderate rates of phosphorylation and electron transport and which is inhibited to the 50% maximum by $HgCl_2$; (2) diaminodurene → methylviologen (and 10 μM PMS) which is loosely coupled, with high phosphorylation and electron transport rates and which is insensitive to $HgCl_2$; and (3) 200 μM PMS which is probably totally sensitive to $HgCl_2$.

In reaction Type (2), we consider the partial reactions diaminodurene → methylviologen and 10 μM PMS (low PMS) to be equivalent. From the characteristics of the reactions (cf. Columns 4 and 5 of Table III) it seems likely that the reactions utilize the same components and phosphorylation site associated with Photosystem I. Since the measurable properties (reaction rates and inhibition characteristics) of these two reactions are identical, it may be assumed that other (immeasurable) properties are similar. It is likely, then, that PMS-catalyzed phosphorylation shows the same loosely coupled nature as the diaminodurene → methylviologen reaction. We would suggest, however, that interaction of donors and chloroplasts results in the loose coupling of physiologically tightly coupled sites.

It is unlikely that three different sites of phosphorylation are located in association with Photosystem I. Furthermore, the different responses of these sites suggest that the different systems are utilizing: (a) a $HgCl_2$-insensitive site (diaminodurene and low PMS), (b) a $HgCl_2$-sensitive site (high PMS) and (c) both sites operating in parallel (Fig. 5).

KCN (by inactivation of plastocyanin [8]) inhibits electron transport of the Hill reaction, diaminodurene → methylviologen and low PMS but not that catalyzed

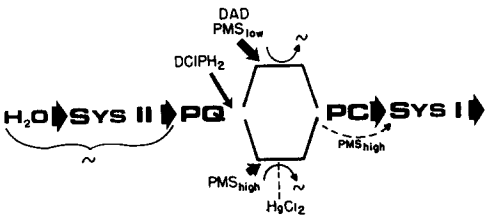

Fig. 5. A scheme of electron transport and phosphorylation sites in parallel. On the basis of $HgCl_2$ inhibition data, three sites of phosphorylation are proposed: (1) a System II phosphorylation site which is insensitive to $HgCl_2$ and in series with each of the other two, (2) a System I phosphorylation site which is insensitive to $HgCl_2$, and (3) a second System I phosphorylation site in parallel with the second, which is similar to the second except that it is totally sensitive to $HgCl_2$.

by high levels of PMS [8]. These results make it difficult to postulate a role for plastocyanin in the electron transport scheme as we have outlined it. As noted by these authors, however, the fact that high concentrations of PMS can react with P-700 [30, 31] does not necessarily explain the fact that high concentrations of PMS also relieve KCN inhibition. If electron donation is directly to P-700, the possible sites of phosphorylation are few. Ouitrakul and Izawa [8] suggested that phosphorylation in the presence of high concentrations of PMS does not differ substantially from the other System I reactions (diaminodurene–methylviologen, low PMS) and that high concentrations of the donor provide a bypass around plastocyanin. The latter suggestion is supported by the known formation of a PMS–P-700 complex [32, 33], which may accept electrons without the involvement of plastocyanin. Thus it is quite possible that the high PMS reaction utilizes the same phosphorylation site as the Hill reaction, but that plastocyanin is bypassed during the reaction. This explanation is far from satisfactory but awaits a more thorough understanding of the role of plastocyanin in electron transport.

Our proposal of parallel sites is certainly not a simple or obvious interpretation of the $HgCl_2$ inhibition data. It is possible that peculiar properties of the electron donors and acceptors used have produced misleading results. Moreover, the data could be explained if we consider the accessibility of whole electron transport systems so arranged in the membrane that some react with mercury and others do not. The proposal is not substantiated, however, by evidence for precise orientation of electron carriers in the membrane [34, 35]. Thus it is difficult to imagine that one-half of the electron transport chains are inaccessible to mercury (for example, on the inner side of the membrane).

Nor can we offer a satisfactory explanation of the selective sensitivity of sites to $HgCl_2$. The phenomenon may be due to site-specific requirements of substrate or cofactors. Mercurials are known to inhibit phosphate transport in mitochondria [36, 37] and chloroplasts [38] and ATPase-dependent ion movements in mitochondria [39–41]. The necessity for the transport of these ions, however, is a property of each site and in no way alters our proposed scheme of phosphorylation.

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