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COUPLING BETWEEN ELECTRON TRANSPORT AND ATP FORMATION IN ISOLATED CHLOROPLASTS

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J. Michael Gould

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Norman Good
Major professor

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COUPLING BETWEEN ELECTRON TRANSPORT AND ATP FORMATION IN ISOLATED CHLOROPLASTS

By

J. Michael Gould

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ABSTRACT

COUPLING BETWEEN ELECTRON TRANSPORT AND ATP FORMATION IN ISOLATED CHLOROPLASTS

By

J. Michael Gould

1. The photochemical reduction of lipophilic electron acceptors such as oxidized p-phenylenediamines or p-benzoquionones by chloroplasts is coupled to phosphorylation with an efficiency (P/e_2) approximately one-half that observed when hydrophilic acceptors such as ferricyanide or methylviologen are reduced. The reduction of lipophilic acceptors is largely insensitive to the plastoquinone antagonist dibromothymoquinone (DBMIB) as is the associated phosphorylation. This observation shows that the lipophilic acceptors are reduced by electrons from water at or before the point of involvement of plastoquinone in the electron transport chain. It also shows that there is a site of coupling of electron transport to ATP formation before the involvement of plastoquinone.

2. At concentrations somewhat higher than those required to inhibit electron transport, DBMIB itself behaves as a lipophilic electron acceptor. However, DBMIB seems to be reduced via a pool of electrons (plastoquinone?) whereas other lipophilic acceptors seem to be reduced before the electrons from the photosystem II units are pooled. This

conclusion is based on the fact that DBMIB reduction is exceptionally sensitive to DCMU inhibition at low light intensities whereas the reduction of other lipophilic acceptors is exceptionally sensitive to DCMU at all light intensities.

3. It is possible to insert electrons into the electron transport chain at a point after the site of inhibition by DBMIB by using appropriate exogenous donors such as diaminodurene, diamiotoluene and reduced 2, 6-dichlorophenolindophenol while using methylviologen as the electron acceptor. The reactions depend entirely on photosystem I. The transfer of electrons from these exogenous donors to methylviologen is also associated with phosphorylation, very likely via the coupling site known to be between plastoquinone and cytochrome f.

4. By using lipophilic electron acceptors in the presence of DBMIB one can isolate the functions of photosystem II and the associated phosphorylation reaction (coupling site II). By using exogenous electron donors such as diaminodurene in the presence of DCMU one can isolate the functions of photosystem I and the associated phosphorylation reaction (coupling site I). The efficiency of coupling site II is pH-independent over the range 6-9 (0.3-0.4 ATP molecules per pair of electrons). In contrast, the somewhat higher efficiency of coupling site I is strongly pH-dependent (0.6 at pH 8, $\leq$ 0.1 at pH 6.5). Moreover, the energy transfer inhibitor $HgCl_2$ is a much less effective inhibitor of site II phosphorylation. Electron flow through the less efficient site II is independent of the presence or absence of phosphorylation or of uncouplers, whereas electron flow through coupling site I is greatly increased by phosphorylation or uncouplers.

5. Over a wide pH range the sums of the efficiencies of the two coupling sites measured separately as above are very close to the efficiencies obtained at the same pH's when they are operating simultaneously, as in the overall Hill reaction. This implies that the characteristics of the coupling sites are not altered by the presence of the added inhibitors, donors and acceptors.

6. During the reduction of DBMIB by photosystem II there is an uncoupler-sensitive uptake of protons by the chloroplasts which is reversed when the reduction ceases. About 0.5 protons are reversibly taken up per electron transported over the pH range 6-8.5. The extent of the uptake decreased 40-60% during phosphorylation. This is consistent with the notion that proton gradients are used for phosphorylation as postulated in the chemiosmotic hypothesis. On the other hand, the site-specific inhibitions by Hg^{++} and low pH are difficult to accomodate within the concept of a delocalized trans-membrane gradient as the driving force for phosphorylation. Such site-specificities can be reconciled with a modified version of the chemiosmotic hypothesis. The modified version specifies that protons accumulate inside the membrane, that the resulting strictly local increase in proton activity drives phosphorylation, and that this local proton activity can equilibrate with a trans-membrane gradient. A postulated difference in the hydrophobicity of the microenvironments of the two coupling reactions could then account for the observed site-specificities.

to my wife, Brenda

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CF	coupling factor one
cyt	cytochrome
DAD	diaminodurene
DAT	diaminotoluene
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (dibromothymoquinone)
DCIP	2,6-dichlorophenolindophenol
DCMU	3(3,4-dichlorophenyl)-1, 1-dimethylurea
DMQ	2,5-dimethyl-p-benzoquinone
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
Fecy	ferricyanide; $Fe(CN)_6^{3-}$
H^+/e^-	ratio of the number of protons translocated per electron transported
MV	methylviologen
PC	plastocyanin
PD	p-phenylenediamine
P/e_2	ratio of the number of ATP molecules synthesized per pair of electrons transported
P_i	inorganic phosphate
PSI	photosystem I
PSII	photosystem II

Q	quencher; primary electron acceptor for photosystem II
X	primary electron acceptor for photosystem I

INTRODUCTION

INTRODUCTION

The single most important biological process, upon which all life on earth ultimately depends, is the process of photosynthesis, for it is this process which converts the electromagnetic radiation of sunlight into the chemical bond energy required by all living systems to maintain their entropy gradient with the environment. The mechanisms involved in this energy conversion are incompletely understood, but it is known that at least two distinct processes are involved: i) the use of light quanta to transfer electrons and thereby store energy in oxidized and reduced products; ii) an associated conversion of some of the light energy into the chemical bond energy of ATP. This dissertation deals primarily with studies concerning the nature of the latter process.

A. Electron Transport Pathways

In green plants, the energy of the quanta absorbed by the photosynthetic pigments is ultimately transferred to the chlorophyll embedded in the chloroplast membranes where it is used to move electrons to orbitals which represent higher energy levels. The excited electrons are then transferred to a low potential electron acceptor, and the oxidized chlorophyll which is generated is subsequently reduced by electrons coming from water. There are two such photoreactions occurring in chloroplasts. These photoreactions are arranged in series, and are connected by a sequence of stepwise oxidation-reduction reactions mediated by a variety of electron carriers (Figure 1). One photochemical reaction

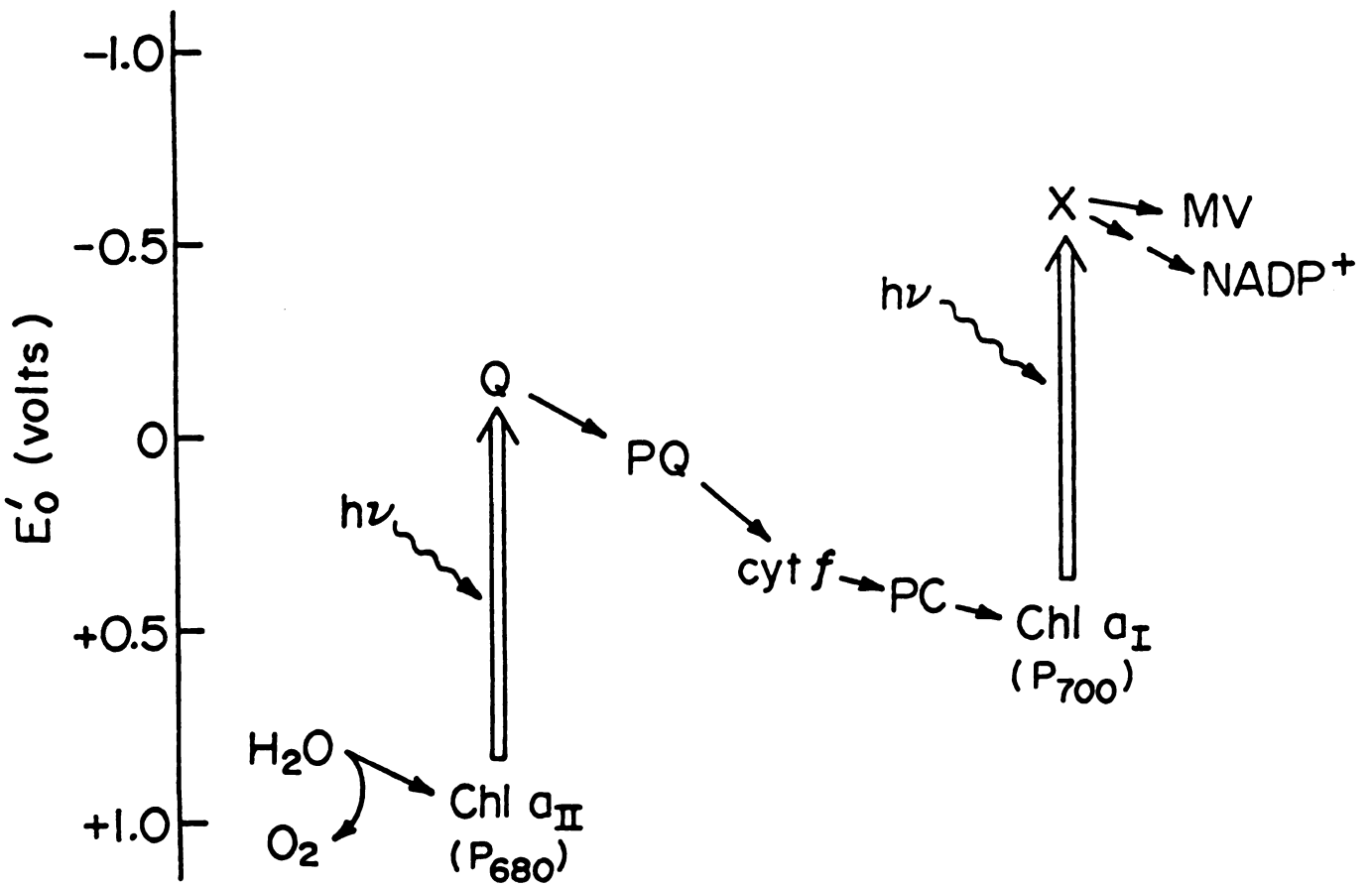

Figure 1. The "Z Scheme" for Non-cyclic Photosynthetic Electron Transport in Isolated Chloroplasts.

(photosystem II) results from the excitation of a special type of chlorophyll a molecule (P_{690}) and causes the sequential accumulation of four electron holes (for four quanta absorbed) in an as yet unidentified primary electron donor (Z), which is subsequently reduced by the four electrons generated by the simultaneous oxidation of two molecules of H_2O to produce molecular oxygen (O_2). The primary acceptor of electrons from the Photosystem II reaction (Q; $E_o' \cong -100$ mV) is also as yet unidentified, although it has been suggested to be a quinone-type compound (Stielh and Witt, 1969). Electrons from Q are rapidly transported to a substituted quinone, plastoquinone ($E_o' \cong 50$ mV), which is present in large excess over the other electron transfer components (excluding chlorophyll). This suggests that plastoquinone may represent an electron pool between the two photochemical reactions. There is strong kinetic evidence to support such a concept (Kok et al., 1969). The oxidation of plastohydroquinone by a c-type cytochrome (cytochrome f, $E_o' = 340$ mV) is believed to be one of the sites along the electron transport chain where the formation of ATP is coupled to the exergonic redox reactions (see below). Reduced cytochrome f transfers electrons to the reaction center chlorophyll of Photosystem I (P_{700}; $E_o' \cong 495$ mV; absorption max. = 700 nm) via a copper containing protein known as plastocyanin ($E_o' \cong 400$ mV). The primary electron acceptor for photosystem I (X; $E_o' < -550$ mV) is also unidentified, although there is some reason to believe that it may be a bound ferredoxin. In intact chloroplasts, the electrons from X are carried via a series of carriers to $NADP^+$ (Figure 1). However, in the chloroplast preparations utilized in this study, the outer envelope and most if not all of the soluble or very weakly bound enzymes are lost,

including the electron carriers from X to $NADP^+$. For this reason it is necessary to introduce into the reaction system an artificial electron acceptor so that ongoing electron transport may be studied. The ability of the chloroplast to catalyze the reduction of artificial oxidants, known as the Hill reaction, has provided an extremely valuable tool for the investigation of electron transport pathways and ATP formation. Examples of the artificial acceptors of electrons from X used in this study are methylviologen and ferricyanide.

The light-driven transport of electrons through the linear transport chain (Figure 1), known as non-cyclic electron transport,is stoichiometrically coupled to the synthesis of ATP (non-cyclic photophosphorylation). It is also possible, by the addition of an appropriate exogenous electron carrier, to observe photophosphorylation which is not coupled to the net flow of electrons from water to some acceptor."Cyclic" photophosphorylation, which is mediated by photosystem I, involves the shuttle of electrons from X (see Figure 1) back to some component of the linear electron transport chain via the exogenous carrier. That is, the exogenous carrier (e.g. pyocyanine, phenazine methosulfate) is reduced by X and subsequently reoxidized by another carrier, probably cytochrome f (see Appendix VII). There is some evidence that a b-type cytochrome (cytochrome b_{563}) may also participate in such cyclic electron flow (Figure 2).

In order to study the number of "sites" of coupling between ATP formation and electron transport it was necessary to operate selected portions of the non-cyclic electron transport chain. In order to do this, one must specifically inhibit the flow of electrons through certain

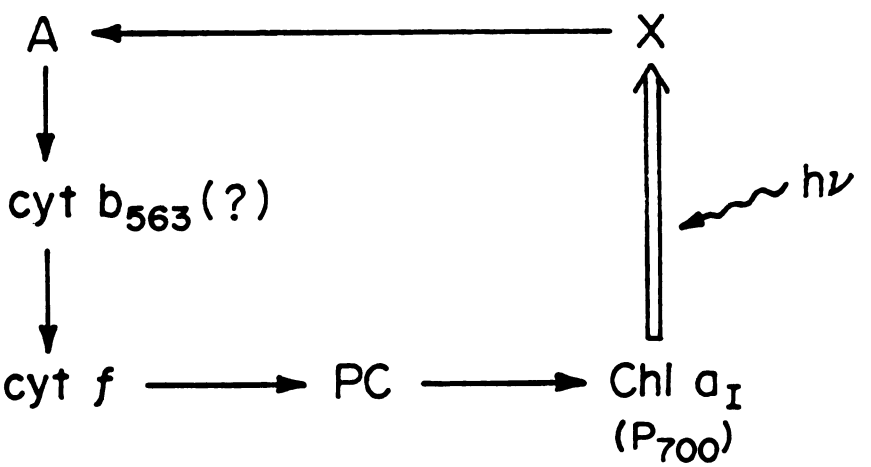

Figure 2. Cyclic Electron Transport Pathway in Chloroplasts. ("A" represents the exogenously added electron transport mediator.)

regions of the chain, and at the same time insert or extract electrons from particular carriers in the chain. The electron transport inhibitors available include compounds or treatments which will block the oxidation of water, the oxidation of Q, the oxidation of plastohydroquinone, the reduction of cytochrome f and the flow of electrons through plastocyanin (Figure 3). When this study was initiated, however, the ability to specifically insert and extract electrons at desired locations was not well established, and one important objective of the work was to define more precisely the regions where exogenous oxidants and reductants interact with the electron transport chain.

Saha et al. (1971) were the first to point out that, of the exogenous electron acceptors available, hydrophilic oxidants (Class I acceptors) such as ferricyanide and methylviologen are reduced almost exclusively by photosystem I, while strong lipophilic oxidants such as p-phenylenediimine and p-benzoquinone (Class III acceptors) are reduced primarily by photosystem II. Inhibitor studies (reviewed in Appendices VII and VIII) have substantiated this conclusion. (Class II acceptors (e.g., indophenols) have been defined as acceptors which uncouple phosphorylation from electron transport).

The site where some reduced compounds donate electrons to the chain has remained unclear for nearly a decade. Compounds such as diaminodurene and reduced dichlorophenolindophenol supply electrons to the transport chain at some point after plastoquinone, so the oxidation of these compounds requires only photosystem I (Izawa et al., 1966). It has also remained uncertain where mediators of cyclic electron transport donate electrons to the transport chain.

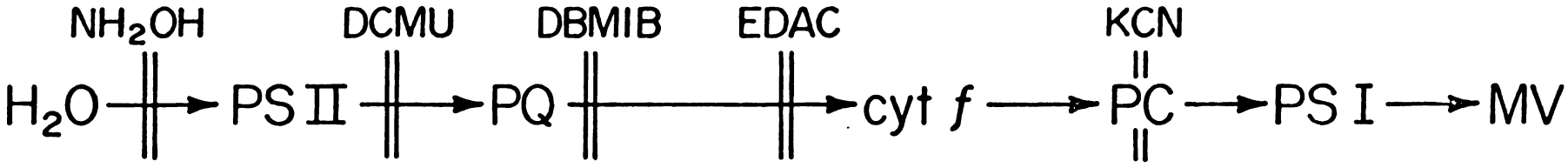

Figure 3. Sites of Action of Various Inhibitors of Non-cyclic Electron Transport in Chloroplasts.

B. Coupling "Sites" and the Hypotheses for the Mechanism of Coupling Between Electron Transport and ATP Formation

A major aspect of the work described in this dissertation deals with determining the stoichiometric relationship between phosphorylation and electron transport in chloroplasts, and in locating the particular electron transfer sequences associated with this coupling phenomenon. It thus becomes important to define exactly what a "coupling site" is. Unfortunately, there is no precise definition available since there is substantial controversy as to the molecular events involved in the mechanism of the coupling process itself. Depending upon the coupling hypothesis, at least three different definitions of a "coupling site" are possible.

1) Chemical coupling. Simply stated, this hypothesis (Slater, 1953) proposes that certain carriers in the electron transport chain interact directly with an as yet unidentified compound to produce a high-energy chemical intermediate. The subsequent hydrolysis of this intermediate by an ATP synthetase is coupled to the condensation of ADP and inorganic phosphate to make ATP. (The enzyme responsible for ATP synthesis in chloroplasts has been identified as a 325,000 MW multisubunit protein known as coupling factor one (CF_1), bound to the surface of the thylakoid membrane.)

According to the formulations of the chemical coupling hypothesis, a coupling "site" represents an electron transfer step which results in the formation of a high-energy chemical intermediate. However, as yet there is no direct evidence for the existence of such an intermediate, although the absence of evidence does not necessarily

constitute evidence for absence. Indeed, it is quite conceivable that a high energy chemical intermediate may be in the form of a charge transfer complex or similar entity which cannot be isolated.

2) Chemiosmotic coupling. This hypothesis, largely based on the ideas of Mitchell (1961, 1966) suggests that the electron carriers are arranged in the membrane in such a manner that the reduction by an electron of a hydrogen carrier near the outer surface of the membrane results in the uptake of a proton, which is subsequently released to the inside of the thylakoid by the oxidation of the hydrogen carrier by an electron carrier near the inner surface of the membrane. This electron transport dependent uptake of protons results in the formation of a pH gradient across the thylakoid. Furthermore, any lag in the movement of charge compensating counterions would result in an electrical potential across the membrane as well. According to this hypothesis, the pH gradient and membrane potential constitute a high-energy state of the membrane which can be discharged by a reversible, proton pumping ATPase (CF_1) to make ATP. By this model, any electron transport step which results in the deposition of protons in the inner phase of the thylakoid can be considered a coupling site.

There is an impressive amount of evidence which lends strong support to the chemiosmotic hypothesis. Chloroplasts do accumulate protons in the light in an electron transport-dependent reaction (Neumann and Jagendorf, 1963). Furthermore a pH gradient produced across the chloroplast membrane, either in the light by electron transport or in the dark by an acid treatment, is capable of driving phosphorylation (Hind and Jagendorf, 1965; Jagendorf and Uribe, 1966). Perhaps the most

convincing evidence comes from the elegant experiments of Racker and Stoeckenius (1974), who employed a simplified system containing bacteriorhodopsin (which catalyzes a light driven proton pump in phospholipid vesicles) and purified mitochondrial hydrophobic proteins and ATPase incorporated into phospholipid vesicles. In the light these vesicles supported substantial rates of phosphorylation even though no electron transport components were present.

3) Conformational Coupling. This hypothesis is less well defined than the other two major hypothesis presented above. It postulates that exergonic electron transport reactions give rise to energetically unstable conformations of membrane proteins. The relaxation of these metastable conformations is coupled to the synthesis of ATP. Thus, according to this model, a coupling site would be any electron transport step giving rise to a conformational change capable of driving phosphorylation.

Actually, there is good evidence that conformational changes in the ATP synthesizing enzyme (CF_1) are involved in energy conservation, although the exact nature of the involvement is still unclear. Energization of chloroplasts induces a conformational change in CF_1 which results in the exposure of 50-100 previously buried tritium exchange sites to the aqueous phase (Ryrie and Jagendorf, 1971, 1972). Similar conditions also expose previously concealed sulfhydryl and lysine residues to attack by N-ethylmaleimide (McCarty et al., 1972) and trinitrobenzenesulfonic acid (Oliver and Jagendorf, 1975), respectively.

In general, regardless of which of these hypotheses one champions, a coupling site is defined as an area in the electron transport

sequence which gives rise to an energy-rich intermediate or state which can be discharged to make ATP. A coupling site is distinct from a phosphorylation site, which is defined as the particular location where the high-energy intermediate or state is enzymatically utilized to make ATP (i.e. the CF_1 molecule).

C. Stoichiometry Between Electron Transport and ATP Formation, and the Location of the Coupling Sites.

The quantitative relationship of electron transport to ATP formation can be expressed as the phosphorylation efficiency, or P/e_2, which is defined as the ratio of ATP molecules synthesized to pairs of electrons passing through the coupling site. Although it is known that at least 1.5 molecules of ATP per 2 electrons are required for the CO_2 fixation reactions, the exact stoichiometry of non-cyclic electron transport has been disputed for some time, with some investigators arguing for a theoretical P/e_2 of only 1.0 (see Avron and Neumann, 1968). However, one of the reasons for the low P/e_2 values observed was the use of suboptimal experimental conditions. With better chloroplast preparations and improved buffers, Winget et al. (1966) were able to obtain P/e_2 ratios consistently and significantly greater than 1.0. This suggested that more than one coupling site may be associated with the non-cyclic electron transport chain. Other indirect evidence supporting this conclusion was supplied by Izawa et al. (1966), and Izawa and Good (1968), who showed that under certain conditions a correction of the electron transport rate for the rate of "basal" electron flow, (i.e. electron flow in the absence of phosphate) gave "corrected" P/e_2 ratios of close to 2.0. A large number of alternate hypothesis (see Avron and Neumann, 1968, for a review) postulating

separate sites for cyclic and non-cyclic phosphorylation, hidden cycles, and even stoichiometries as high as $P/e_2 = 4$ (Lynn, 1967) further confused the issue. However, very little direct evidence for more than one site emerged for nearly a decade.

An important breakthrough in this area came with the work of Saha et al. (1971), who showed that lipophilic strong oxidants such as p-phenylenediimines and p-benzoquinones are reduced by chloroplasts in a reaction coupled to phosphorylation with an efficiency consistently about one-half the efficiency observed when convential hydrophilic Hill oxidants (e.g. methylviologen) are reduced. They concluded that these lipophilic acceptors were being reduced at a point between two coupling sites. Ouitrakul and Izawa (1973) subsequently showed by inactivating plastocyanin with KCN that photosystem I was required for the reduction of hydrophilic oxidants but not for the reduction of lipophilic oxidants. This provided strong circumstantial evidence for the existence of at least two coupling sites associated with the complete non-cyclic chain. However, direct confirmation of this notion requires a knowledge of the exact location of the coupling sites in the electron transport chain.

Evidence for the location of one of the coupling sites comes from studies of the steady-state redox levels of the electron carriers under various conditions. This approach is based on the fact that, in the complete Hill reaction, the rate of electron transport during phosphorylation is considerably higher than the rate in the absence of phosphorylation. When the phosphorylation reaction is uncoupled from electron transport , the highest rates of electron transport are observed. These facts have been taken to mean that the formation of a high-energy

intermediate or an energized state regulates the rate of electron flux through the coupling site by creating a "back-pressure" on the forward phosphorylating reactions. Thus, in the absence of an uncoupler the electron carriers on the photosystem II side of the rate-limiting coupling site should be more reduced, and those on the photosystem I side more oxidized, than when an uncoupler is added (to remove the rate-limitation at the coupling site).

Using this technique, Kok et al. (1969) determined that the rate-limiting step of the Hill reaction came after the electron pool at plastoquinone. Avron and Chance (1966), Larkum and Bonner (1972) and Izawa (1968) similarly showed that the rate-limitation occurred before the reduction of cytochrome f, and Bohme and Cramer (1972) demonstrated directly the existence of a phosphorylation-dependent electron transport reaction between plastoquinone and cytochrome f.

Ouitrakul and Izawa's experiments with KCN cannot completely eliminate the possibility that Class III acceptors are reduced both before and after a single coupling site located between plastoquinone and cytochrome f, since the cyanide inhibition site is at a point in the electron transfer chain after cytochrome f (see Figure 3). One of the objectives of this study was to demonstrate unequivocally the existence of a second coupling site associated with the non-cyclic electron transport chain in chloroplasts. The introduction by Trebst's group (Trebst, et al., 1970) of a new inhibitor (dibromothymoquinone; DBMIB) which blocks electron transport at plastoquinone (i.e. before the known coupling site) made available a new approach to the problem. Indeed, it was found that DBMIB completely inhibited phosphorylation and electron transport

dependent upon the reduction of hydrophilic acceptors, while only slightly affecting the electron transport and phosphorylation associated with the reduction of lipophilic acceptors. This observation provided another, more conclusive demonstration of a second coupling site in chloroplasts. One of the other objectives of this study was to find suitable reactions using defined portions of the electron transport chain so that the individual coupling sites could be functionally separated and investigated without resorting to physical disruption of the chloroplast. In the course of studying these "partial" reactions it was observed that the characteristics of the two coupling sites are substantially different. This was a particularly intriguing development since any theory of the coupling mechanism must accomodate the observed differences.

Finally, the stoichiometric relationship between proton uptake, electron transport and ATP formation associated with the newly isolated coupling site was studied in detail in an attempt to elucidate the role of protons in the energy conservation mechanism.

EXPERIMENTAL METHODS

EXPERIMENTAL METHODS

A. Preparation of Chloroplasts

The chloroplasts employed for the research described in this thesis consisted of intact, naked lamellae. That is, during the isolation procedure the outer chloroplast membrane was stripped away, and enzymatic activities attributable to soluble stroma enzymes were lost. However, the lamellae retained their general morphological appearance, the grana remaining largely intact.

Chloroplasts were isolated from leaves of fresh market spinach (Spinacea oleracea L.) in the cold (4°C). Chilled leaves were washed in cold distilled water before being ground briefly (3-7 seconds) in a Waring Blender. The grinding medium consisted of 0.3 M NaCl, 30 mM N-tris(hydroxymethyl)methylglycine (tricine) -NaOH (pH 7.8), 3mM $MgCl_2$ and 0.5 mM ethylenediaminetetracetic acid (EDTA). After filtering the homogenate through multiple layers of cheesecloth (prerinsed with distilled water), the chloroplasts were sedimented by centrifugation at about 2500 x g for two minutes. The pellet was resuspended in a medium containing 0.2 M sucrose, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.5), 2 mM $MgCl_2$ and 0.05 percent bovine serum albumin. Whole cells and debris were removed by a brief (30-45 second) centrifugation at 2000 x g. Chloroplasts in the resulting supernatant were sedimented at 2000 x g for four minutes, resuspended in a fresh volume of

the same medium, and again sedimented. The final pellet was taken up in a small volume of the suspending medium. In some cases, when the presence of metal chelating agents had to be avoided, the EDTA and bovine serum albumin were omitted from the grinding and suspending media, respectively, while the tricine and HEPES buffers were replaced by 2[N-tris (hydroxymethyl)methyl]aminoethanesulfonic acid (TES) and N-2-hydroxyethylpiperazine-N-3-propanesulfonic acid (HEPPS) buffers respectively.

The concentration of chlorophyll present in the final stock chloroplast suspension was determined spectrophotometrically by the method of Arnon (1949). A 0.1 ml aliquot of the chloroplast suspension was diluted to 10 ml with 80 percent (v/v) acetone-water. After centrifugation at 5000 x g for seven minutes to remove insoluble chloroplast residues, the absorbance (A) of the green supernatant was measured at 663, 652 and 645 nm. The concentration of chlorophyll was determined using the equation

$$100 \left(\frac{[8.02(A_{663}) + 20.2(A_{645})] + [29(A_{652})]}{2} \right) =$$

μgrams chlorophyll/ml stock suspension.

Stock suspensions of chloroplasts were routinely adjusted to 1600-2000 μg chlorophyll/ml and stored in an ice bath. Aliquots of this stock suspension were further diluted with suspending medium before being added to the reaction mixtures.

B. Measurement of Electron Transport

The light-induced flux of electrons through the chloroplast electron transport chain was measured by one of several methods, depending upon the experimental conditions and the nature of the electron acceptor

employed. These methods are described in detail below.

1) Potassium ferricyanide as electron acceptor. When ferricyanide (E_0' = 420 mV) served as the acceptor of electrons from photosystem I, electron transport was generally measured spectrophotometrically. This was accomplished by measuring the decrease in the absorbance of the sample of 420 nm in either a Bausch and Lomb Spectronic 505 spectrophotometer or a spectrophotometer consisting of a Beckman model DU monochrometer equipped with a photomultiplier and associated log conversion electronics. Both spectrophotometers were modified to permit illumination of the sample cuvette (1 x 1 x 4 cm) by an actinic light beam at right angles to the measuring beam. The photomultipliers were protected from stray light by filters which blocked all of the scattered red-orange actinic light (eg. Baird Atomic interference filter; peak transmittance 420 nm, band-width 10nm). The rate of reduction of ferricyanide was determined by continuously recording the decrease in absorbance at 420 nm on a strip chart recorder with a time-base drive. The number of equivalents of ferricyanide reduced was calculated assuming a millimolar extintion coefficient for ferricyanide of 1.06 at 420 nm.

A second method for determining the rate of electron transport when ferricyanide served as the electron acceptor consisted of measuring the rate of oxygen production. Changes in the oxygen concentration of the reaction mixture were determined polarographically using a Clark-type oxygen electrode covered with a teflon membrane (Yellow Springs Instrument Co.), in a thermostatted reaction cuvette in which the reaction mixture was stirred continuously by a small magnetic stirring bar. Changes in the current flow through the electrode (which are directly proportional to

changes in O_2 concentration) were amplified and recorded on a strip chart recorder. The recorder span was calibrated in μequivalents by using a known number of μequivalents of ferricyanide as the electron acceptor and allowing the reaction to continue until all of the ferricyanide had been reduced.

2) Methylviologen as electron acceptor. The reduction of the low potential electron acceptor methylviologen (MV; $E_o' = -446$ mV) by photosystem I was followed polarographically as the rate of change of oxygen concentration in the reaction mixture described in the manner immediately above. However, in the case of methylviologen reduction, the reaction was followed as oxygen uptake rather than as oxygen production, since the reduced methylviologen radical ($MV^{+\cdot}$) is rapidly reoxidized in air. Thus, the reaction proceeds as follows:

$$H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} \tfrac{1}{2} O_2 + 2e^- + 2H^+$$

$$2e^- + 2MV^{++} \longrightarrow 2\ MV^{+\cdot}$$

$$2MV^{+\cdot} + O_2 + 2H^+ \longrightarrow H_2O_2 + 2MV^{++}$$

overall $$H_2O + \tfrac{1}{2}O_2 \longrightarrow H_2O_2$$

Therefore the overall rate of oxygen uptake (due to the reduction and subsequent autoxidation of methylviologen) is equal to the rate of oxygen production (one O_2 consumed equals 4 electrons transported). However, when O_2 evolution by photosystem II is inhibited and an exogenous donor of electrons to photosystem I such as diaminodurene (DAD), diaminotoluene (DAT) or reduced 2, 6-dichlorophenolindophenol ($DCIPH_2$) is employed, the rate of oxygen uptake due to the reoxidation of reduced methylviologen is

twice as great for the same rate of electron transport (one O_2 consumed equals two electrons transported) (Izawa et al., 1966). Thus, for the transport of two electrons, the reaction becomes:

$$AH_2 \xrightarrow[\text{chloroplasts}]{\text{light}} A + 2H^+ + 2e^-$$

$$2e^- + 2MV^{++} \longrightarrow 2MV^{+\cdot}$$

$$2MV^{+\cdot} + O_2 + 2H^+ \longrightarrow 2MV^{++} + H_2O_2$$

overall $$AH_2 + O_2 \longrightarrow H_2O_2 + A$$

where AH_2 represents any one of the exogenous electron donors listed above.

3) Lipophilic (Class III) oxidants as electron acceptors.

Lipophilic oxidized compounds such as p-phenylenediimines or lipophilic p-benzoquinones will serve as electron acceptors, being reduced primarily at a point in the electron transport chain between photosystem II and photosystem I (Saha et al., 1971). These compounds were added to the reaction mixture in the reduced form and subsequently oxidized by the addition of an excess of ferricyanide. Thus, when the Class III acceptor was reduced by chloroplasts in the light, it was immediately reoxidized by the excess ferricyanide present in the reaction mixture. Therefore it was possible to measure electron transport as ferricyanide reduction either spectrophotometrically or as oxygen evolution. (See ferricyanide reduction above.)

When dibromothymoquinone served as the Class III acceptor, it was also possible to measure electron transport as the indirect reduction of ferricyanide as described above or, above pH 8.0, as oxygen uptake

(see methylviologen reduction), since the reduced dibromothymoquinone was rapidly reoxidized by molecular oxygen at pH >8.

C. Actinic Light. Illumination of the reaction mixture was with a 500 W slide projector without the focusing lens. The beam was passed through a one liter round-bottomed flask filled with a dilute (approximately 2%) $CuSO_4$ solution. This functioned both as a condensing lens and as a heat filter. The beam was then passed through a red-orange filter (transmittance > 500 nm) and an I-69 Corning heat filter before impinging on the reaction vessel. Reactions were run in small cuvettes maintained at 18°C by a thermostated circulating water bath. The final reaction mixture volume was generally 2 ml. The light intensity at the surface of the reaction cuvette was 400-700 kergs · cm^{-2} · sec^{-1}.

D. Measurement of ATP Formation

The rate of photophosphorylation was measured dirctly as the incorporation of ^{32}P-labelled orthophosphate into $AT^{32}P$. The reaction mixture contained excess amounts of both radioactive orthophosphate and ADP (5 mM and 0.75 mM respectively). After illumination, a 1.0 ml aliquot of the reaction mixture was pipetted into a 20 x 150 mm test tube and immediately frozen in the dark.

The $AT^{32}P$ present in the frozen sample was determined by a modification of the method of Neilsen and Lehninger (1955). This technique is based on the removal of unreacted ^{32}P-inorganic phosphate from the sample as phosphomolybdic acid. Saha and Good (1970) have shown that virtually all of the nonextractable radioactivity remaining is incorporated into $AT^{32}P$. To the frozen 1.0 ml aliquot of the reaction mixture

was added 10 ml of 1 N perchloric acid saturated with a 1:1 isobutanol-toluene mixture. This resulted in a precipitation of the chloroplast proteins, and prevented any further biological reactions from occurring. In order to improve phase separation, 1.2 ml of acetone was added to the perchloric acid sample mixture. Ammonium molybdate, 1.0 ml of a 10% (w/v) solution was then added . The resulting molybdic acid reacts in the aqueous solution with the excess ^{32}P-labelled orthophosphoric acid to form yellow phosphomolybdic acid. This complex is extremely soluble in organic solvents, and was removed from the perchloric acid phase by adding 7 ml of 1:1 isobutanol-toluene (saturated with 1 N perchloric acid). The phases were mixed briefly using the up and down motion of a glass stirring rod which had been flattened at the end. The mixture was then allowed to stand for about one minute. After this time the phases were mixed for about 60 seconds by the piston action of the flattened stirring rod. After the phases had been allowed to separate, the upper (organic) phase containing the phosphomolybdic acid was carefully removed using suction on a Pasteur pipet connected to a trap. The remaining (aqueous) phase was gravity-filtered through 9 cm Whatman # 4 filter paper pre-wetted with 0.5 ml distilled water. This filtration removed precipitated proteins and remaining traces of the organic phase. The filtered perchloric acid phase was extracted a second time using 0.1 ml ammonium molybdate and 7 ml isobutanol-toluene (saturated with 1 N perchloric acid). After again removing all traces of the organic phase by suction with a pastuer pipet, an aliquot of the final perchloric acid phase was pipetted into a polyethylene scintillation vial (Packard Instrument Co.) containing sufficient distilled water to make a final volume of 15 ml.

The technique used to determine the amount of radioactivity in the perchloric acid extract is based on the method of Gould et al (1972) and utilizes the phenomenon of Cerenkov radiation. Cerenkov radiation is light emitted by some substances when a particle (such as a high energy β-particle) moves through the substance at a velocity greater than the speed of light in that substance. ^{32}P emits β-particles with sufficeint energy (1.71 MeV) to induce Cerenkov radiation in water (minimum energy required, 0.265 MeV). Thus, it was possible to add an aliquot of the perchloric acid extract containing ^{32}P-labelled ATP to distilled water in a scintillation vial and then measure the Cerenkov radiation with the photomultipliers of a liquid scintillation spectrometer. The amount of Cerenkov radiation (measured as counts per minute) is proportional to the amount of ^{32}P over a very wide range.

Experimental samples (in polyethylene vials) were counted at $4^{o}C$ for ten minute intervals in a Packard 3003 Series liquid scintillation spectrometer in the coincidence mode. The relative gain and upper and lower window discriminator settings, which were found to give optimum counting efficiency, were 20% and 40-1000 units respectively. Counting efficiency under these conditions was approximately 25-30%.

In some of the early experiments, and when the extracted perchloric acid phase was highly colored, radioactivity was determined in a Geiger-Meüller immersion tube (20th Century Electronics, Surrey, England) connected to a Nuclear Chicago scaler.

Counts per minute were related to ATP by the following calibration procedure: an aliquot of the stock solution of radioactive phosphate was diluted with 0.1 M Na_2HPO_4 to give a solution containing 0.1 μmole of

the original stock (radioactive) phosphate in each 1.0 ml of the final solution. A 1.0 ml aliquot of this final solution was then diluted to 13.8 ml with 1N perchloric acid and an aliquot of this "standard" was added to a scintillation vial or the immersion tube and counted as described above for the experimental samples.

E. Measurement of Changes in H^+ Concentration (pH Changes)

Light-induced changes in the hydrogen ion concentration in the reaction mixture were detected with a Corning semi-micro ("Tripurpose") combination pH electrode connected to a fast responding Heath/Schlumberger EU 200-30 pH electrometer module equipped with a Heath/Schlumberger EU 200-02 DC offset module to facilitate scale expansion. The output from the electrometer was recorded (using a preamplifier with a gain of 10) on either a Heath/Schlumberger programmable strip chart recorder or on an Esterline Angus strip chart recorder. The response half-time for the pH measuring system was 0.5-1.0 sec. Changes in pH were normally monitored with a scale expansion of 0.1 pH unit full scale (10 inches) on the recorder. The noise level was less than 0.002 pH units.

In routine pH experiments reactions were run in a final volume of 2.0 ml in thermostatted vessels at 18°C with continuous stirring. Prior to illumination the reaction mixture was adjusted to the desired initial pH with small volumes of dilute NaOH or HCl. Actinic illumination was supplied by a 500W slide projector as described earlier. The light intensity was approximately 700 Kergs $\cdot$ cm^{-2} $\cdot$ sec^{-1} (ca. 600-700 nm). At the end of each experiment the pH changes registered on the chart paper were translated into H^+ equivalents by back-titrating the reaction mixture in

the light (Polya and Jagendorf, 1969) with a known amount of 0.001 M HCl.

The overall sensitivity of the pH measuring system was such that 0.5-2.5 mM buffer could be included in the reaction mixture without obscuring the pH change.

F. Inhibitors and Reagents

Plastocyanin was inactivated by treating chloroplasts in the dark with 30 mM KCN (buffered at pH 7.8) at 0°C for 90 minutes as described by Ouitrakul and Izawa (1973). The inhibition was checked by determining the rate of methylviologen reduction as described above.

The inhibitory plastoquinone analog 2.5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (dibromothymoquinone; DBMIB) was prepared by bromination of thymoquinone in water and was recrystallized several times from hot ethanol. This synthesis was kindly performed by Mr. Peter Felker. Stock solutions were prepared by dissolving dibromothymoquinone in ethanol-ethylene glycol (1:1, v/v).

The dihydrochloride salts of diaminodurene, diaminotoluene and p-phenylenediamine were prepared by dissolving the free bases in warm ethanol, treating with acid washed Norit A and adding concentrated HCl. After cooling the solution for a few minutes on ice, the white crystals of the dihydrochloride were collected. Fresh, colorless solutions of these compounds were made up in 0.01 N HCl each day.

2,5-Dimethyl-p-benzoquinone was purified by recrystalization from hot ethanol. This compound appeared to be stable when stored at 0°C in the dark. Ethanolic solutions of the dimethylquinone were yellow in color.

Sodium 2, 6-dichlorophenolindophenol was dissolved in ethanol, filtered, and the concentration determined from the absorbance at 600 nm using a mM extinction coefficient of 21. Ethanolic solutions of 2,6-dichlorophenolindophenol were further diluted with 1 mM Na_2HCO_3.

3(3,4-Dichlorophenyl)-1, 1-dimethylurea (K and K Laboratories) was recrystallized from ethanol. Stock solutions were made in ethanol, with further dilutions being made with 0.01 M NaCl, which helped to prevent absorption of the very dilute inhibitor onto the glass walls of the storage vessel.

Superoxide dismutase was prepared from fresh bovine erythrocytes by the procedure of McCord and Fridovich (1969). The specific activity, assayed as the inhibition of cytochrome c reduction by xanthine oxidase, was > 3000 units per mg. protein.

ADP was purchased from Sigma. ^{32}P-labelled orthophosphate (carrier free) was purchased from International Chemical and Nuclear Corp. (ICN). Buffers were prepared by Dr. N.E. Good.

RESULTS

RESULTS

A. Determination of a Second Coupling Site in Isolated Chloroplasts

The characteristics of the reduction of lipophilic (Class III) electron acceptors can best be understood by assuming that they accept electrons from some intermediate carrier which normally transfers electrons from photosystem II to photosystem I (Saha et al., 1971). Blocking the flow of electrons to photosystem I by inactivating plastocyanin using either KCN (Ouitrakul and Izawa, 1973) or poly-L-lysine (Brand et al., 1971), does not severely inhibit the reduction of Class III acceptors even though the reduction of Class I acceptors is completely blocked (Ort et al., 1973). Furthermore, the reduction of Class III acceptors is coupled to phosphorylation with an efficiency (P/e_2) of approximately one-half that observed when ferricyanide or methylviologen are reduced (Saha et al. 1971). These observations strongly imply that the Class III acceptors intercept electrons before the involvement of plastocyanin but after one of the two sites coupling electron transport to ATP formation.

One of the coupling sites in chloroplasts almost certainly lies between plastoquinone and cytochome f (Avron and Chance, 1966; Kok et al., 1969; Böhme and Cramer, 1972). Thus, if the oxidation of reduced plastoquinone is prevented, this coupling site should become inoperative. An experimental test of this argument became available with the introduction by Trebst et al.(1970) of a new inhibitor, 2, 5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), which was claimed to act as a plastoquinone

antagonist. At very low concentrations DBMIB 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 (Böhme et al., 1971). It also seems to block the transfer of electrons from cytochrome $\underline{b}_{559}$ to cytochrome $\underline{f}$ and it certainly prevents the reduction of cytochrome $\underline{f}$ by electrons from photosystem II (Böhme and Cramer, 1971). These results lend support to the conclusion of Trebst et al. (1970) that DBMIB acts at the level of involvement of plastoquinone in the electron transport chain.

1) The site of DBMIB inhibition and its effects on electron transport and phosphorylation with different electron acceptors. The effects of DBMIB on the reduction of Class I(hydrophilic) and Class III (lipophilic) electron acceptors are very similar to the effects of the plastocyanin antagonists KCN and poly-L-lysine. The flow of electrons to Class I acceptors such as ferricyanide or methylviologen is almost completely sensitive to the inhibitor, while the flow of electrons to Class III acceptors such as oxidized p-phenylenediamine may be largely insensitive (Appendix I, Fig. 1). Regardless of the phosphorylation efficiency (P/e_2) associated with the reduction of the Class III acceptor in the absence of the inhibitor, the P/e_2 ratio always falls to about 0.4 in its presence (Appendix I, Table I).

These results indicate that there is a site of phosphorylation associated with the electron transport pathway before the DBMIB inhibition site. Consequently, precise identification of the inhibition site is a matter of critical concern. Trebst et al. (1970) proposed that DBMIB blocks electron transport at the level of plastoquinone, but their evidence was

somewhat inconclusive. Indeed, the effects of DBMIB described above are identical to the effects of KCN and poly-L-lysine, which almost certainly act at the level of plastocyanin (Izawa et al., 1973; Brand et al., 1972b).

DBMIB differs importantly from KCN and poly-L-lysine in its effects on photosystem I-dependent reactions supported by exogenous electron donors such as diaminodurene. In the presence of DCMU (to block the flow of electrons from photosystem II), the flow of electrons from diaminodurene to methylviologen is strongly inhibited by either KCN or poly-L-lysine. In sharp contrast, however, DBMIB has no appreciable effect on the diaminodurene → methylviologen reaction (Appendix I, Table II). It is clear, therefore, that the site of DBMIB inhibition must come between the point of reduction of Class III acceptors and the site of KCN (or poly-L-lysine) inhibition at plastocyanin. This is entirely consistent with the view that dibromothymoquinone acts as a plastoquinone antagonist.

2) DBMIB as an electron acceptor. Early in the course of these studies it was noted that methylviologen reduction seemed to be more sensitive to DBMIB than was ferricyanide reduction. Furthermore, the phosphorylation efficiency (P/e_2) associated with ferricyanide reduction decreased with increasing concentrations of DBMIB to a plateau near P/e_2 = 0.4. Indeed, at somewhat higher levels of DBMIB than were required for complete inhibition of methylviologen reduction, ferricyanide reduction actually increased to a rate $\geq$ 300 μequivalents · h^{-1} · mg $chlorophyll^{-1}$, about one-half the original (uninhibited) rate (Appendix I, Figs. 2-4). Because the P/e_2 associated with ferricyanide reduction at high DBMIB concentrations (> 5 μM) is similar to the P/e_2 associated with reduction of Class III acceptors in the presence of KCN, poly-L-lysine or low

concentrations of DBMIB (0.5 μM), it seems reasonable to conclude that at higher concentrations DBMIB can act both as an inhibitor of the flow of electrons to photosystem I and as a lipophilic "Class III" acceptor of electrons from photosystem II. The reduced DBMIB is rapidly reoxidized by ferricyanide, but for thermodynamic reasons, not by methylviologen.

The DBMIB reduced by chloroplasts can also react with oxygen to produce H_2O_2 when ferricyanide is not present. However, this reaction requires higher levels of DBMIB (10-20 μM), and the reoxidation of reduced DBMIB by O_2 becomes seriously rate-limiting below pH 8 (Appendix II, Fig. 2). In contrast, the reoxidation of reduced DBMIB by ferricyanide is not rate-limiting under any of the conditions employed in this study since the rate of DBMIB-mediated ferricyanide reduction is constant with time until virtually all of the ferricyanide has been reduced. For these reasons the DBMIB-ferricyanide system was generally employed in the studies described below.

3) <u>The effect of 3(3,4-dichlorophenyl) -1, 1-dimethylurea (DCMU) on DBMIB reduction</u>. DBMIB reduction resembles the reduction of other Class III acceptors in its insensitivity to inhibitors such as KCN and poly-L-lysine (Appendix II, Fig. 1, Table 1), but differs significantly in its sensitivity to DCMU. DBMIB reduction exhibits about the same sensitivity to DCMU as does the reduction of the Class I acceptor ferricyanide. In contrast, other Class III acceptors are extremely sensitive to concentrations of DCMU which have very little effect on ferricyanide reduction (Appendix II, Fig. 4).

DBMIB reduction differs from the reduction of other Class III acceptors in another important characteristic. When the light-intensity is made rate-limiting, DBMIB reduction, like ferricyanide reduction, becomes more sensitive to inhibition by a given concentration of DCMU, while the sensitivity of the reduction of other Class III acceptors is independent of light intensity (Appendix II, Figs, 5,6; Ouitrakul and Izawa, 1973). Again DBMIB behaves more like a Class I acceptor than a Class III acceptor under these circumstances. It should be pointed out that the intensity-dependent and intensity-independent effects of DCMU are not related to the maximum absolute rates of reduction of the electron acceptors in the absence of DCMU and in fact represent an actual difference between the reduction pathways of DBMIB and other Class III acceptors.

It should also be added that the quantum efficiency of DBMIB reduction (DBMIB = 10 μM) is only about 30% of the normal ferricyanide-mediated Hill reaction (Appendix II, Fig.5). Apparently DBMIB has a rather strong inhibitory effect on the photochemical reactions of photosystem II.

4) The site of DBMIB reduction. The fact that KCN and poly-L-lysine have no effect on the rate of electron transport virtually proves that photosystem I is not involved in the reduction of DBMIB. Moreover, DBMIB itself is a potent inhibitor of the transfer of electrons to photosystem I, probably blocking electron transport at the level of plastoquinone (see above). It follows that DBMIB must accept electrons either before or at its own site of inhibition. In these respects DBMIB resembles the reduction of other lipophilic quinones and quinonediimines

(Class III acceptors) which also accept electrons from a carrier close to photosystem II. However, as shown in the preceding section, the reduction of DBMIB more closely resembles the reduction of Class I acceptors in its response to DCMU and light intensity. These differences can be readily explained in terms of the following model.

There are good reasons for believing that the photochemistry and much of the associated thermochemistry of photosystem II takes place in independent structural units (Kok et al., 1970). In other words, it seems that quanta are converted one by one into charge separations within independent structures. Thus the electrons and holes made available by quantum conversions in one structural unit are not directly available for chemical reactions in other units. While the exact nature of the inhibition by DCMU is not clear, it seems likely that DCMU acts by somehow inactivating photosystem II units, one molecule of inhibitor totally suppressing the activity of one unit. A partial inhibition by DCMU probably means that a certain proportion of the photosystem II units have been inactivated.

However, there is no reason to suppose that photosystem I must be confined to the same unit structures as photosystem II. Consequently it is not unreasonable to suppose that the electrons generated by photosystem II may be pooled at some step before the reduction of P_{700}. Indeed one might expect reduced plastoquinone to serve as a common electron pool interconnecting electron transport chains on the basis of its chemical nature and its abundance. In fact, Siggel et al., (1972) and Malkin and Michael (1972) have reached this conclusion from their flash experiments and fluorescence induction studies using chloroplasts poisoned with DCMU.

We are now in a position to understand the effect of light intensity on DCMU inhibition. When light is limiting, the activity of photosystem II is presumably also limiting and the inhibition should be strictly proportional to the number of units inactivated by DCMU. The same will be true, regardless of light intensity, if the electrons are never pooled. However, if the electrons are pooled and the pooled electrons are utilized by a subsequent rate-determining slow step, the situation is quite different. Now a smaller number of functioning photosystem II units can keep the slow reactions draining the electron pool saturated. Thus, as the light intensity increases, fewer and fewer photosystem II units are required and the efficacy of a given concentration of DCMU decreases.

Such considerations suggest that Class III acceptors are reduced before the electrons from photosystem II are pooled (Ouitrakul and Izawa, 1973). Presumably these membrane-permeating strong oxidants react directly with the photosystem II units. Clearly ferricyanide and other Class I acceptors must be reduced after the electrons are pooled since their reduction is less sensitive to DCMU at high light intensities. Although DBMIB is highly lipid-soluble and is a moderately strong oxidant, it does not seem to react directly with photosystem II units but rather with the source of pooled electrons. It seems reasonable to conclude, therefore, that DBMIB, by virtue of its structure, reacts in some specific way at the site of plastoquinone involvement in electron transport, accepting electrons from reduced plastoquinone and at the same time blocking further transport of electrons to cytochrome f.

B. Functional Separation and Characterization of the Two ATP-Generating Coupling Sites.

The conclusion seems inescapable that the non-cyclic electron transport pathway in chloroplasts includes at least two energy conservation sites associated with phosphorylation, one located before the plastoquinone electron pool and a second located after plastoquinone but before cytochome f. It has been shown above that it is possible to operate a "partial" electron transport pathway which includes only photosystem II and utilizes only the energy conservation site before plastoquinone (site II). This is done by using Class III acceptors in conjunction with inhibitors of the flow of electrons to photosystem I (i.e., KCN, poly-L-lysine or DBMIB). Similarly, it is possible to introduce electrons into the transport chain at a point after coupling site II but before coupling site I (See Appendices III,VII and VIII). This is done by using appropriate exogenous electron donors (eg. diaminodurene, diaminotoluene, reduced indophenols) while preventing any contribution of electrons from photosystem II with DCMU. The use of these two types of partial electron transport pathways provides functional separations of the coupling sites so that their characteristics and properties may be studied separately and compared with the characteristics of the overall (Hill) reaction.

1) pH effects. When electrons from water reduce the Class I acceptor methylviologen (MV) through the two coupling sites, both electron flow and phosphorylation show an optimal pH of approximately 8-8.5. The rate of electron transport in the absence of phosphate (basal rate) is much slower but shows a similar pH optimum (Appendix III, Fig. 1). When

diaminodurene (DAD) or reduced 2, 6-dichlorophenylindophenol ($DCIPH_2$) serves as the donor of electrons to photosystem I in the presence of DCMU (coupling site I only), the effect of pH is very similar to that observed in the $H_2O \rightarrow MV$ reaction, with an optimal pH of 8-8.5 and a marked stimulation of the rate of electron transport by phosphorylation (Appendix III, Fig. 1 and Appendix VII, Fig. 4). The reductions of the Class III acceptor 2,5-dimethyl-p-benzoquinone (DMQ) or of DBMIB by electrons from water (coupling site II only) exhibit an entirely different effect of pH, however. The pH optimum is considerably mor acidic (7.3-7.8) and the rate of electron flow is the same in the presence and absence of a complete phosphorylation system (see below).

A study of the effect of pH on efficiency of phosphorylation (P/e_2) associated with each of the electron transport pathways described above revealed some interesting results (Appendix III, Fig. 2, Appendix II, Fig. 3, Appendix VII, Fig. 4). The P/e_2 for the overall reaction ($H_2O \rightarrow MV$) is strongly pH dependent, being optimal at pH 8 to 8.5 and falling sharply at lower pH's to a value of about 0.4 at pH 6.5. In contrast, the P/e_2 associated with the $H_2O \rightarrow DMQ$ and $H_2O \rightarrow DBMIB$ reactions (which utilize only coupling site II) is essentially independent of pH over the range 6.5-9 ($P/e_2 \cong 0.4$). Thus the pH dependent portion of the P/e_2 ratio for the $H_2O \rightarrow MV$ reaction must be attributable to a coupling site located after the site of DMQ and DBMIB reduction. In fact, the P/e_2 ratios for the $DAD \rightarrow MV$ and $DCIPH_2 \rightarrow MV$ reactions are strongly pH-dependent, with an optimum ($P/e_2 \cong 0.6$) at pH 8-8.5. As the pH is lowered the P/e_2 drops sharply to $\leq$ 0.1 at pH 6.5. The pH-dependent nature of the phosphorylation associated with the $H_2O \rightarrow MV$ and DAD (or $DCIPH_2$) $\rightarrow$ MV reactions

suggests that these reactions may involve the same coupling site. Indeed, the fact that the P/e_2 values for the DAD (or $DCIPH_2$) -> MV reaction are lower (by about 0.4) than the P/e_2 values for the H_2O -> MV reaction over the entire pH range tested suggests that a pH independent component of the overall P/e_2 is missing from DAD (or $DCIPH_2$) -> MV reaction. If the P/e_2) values for the two types of partial reactions (eg. H_2O -> DMQ and $DCIPH_2$ -> MV) are added together over the pH range 6-9, the resulting curve is in fact very close to the experimentally obtained curve for the overall reaction H_2O -> MV (Appendix III, Fig. 2). This also suggests that the DAD (or $DCIPH_2$) -> MV reaction is utilizing the coupling site which normally limits the overall Hill reaction (coupling site I). Therefore, the two types of partial reactions described above seem to provide a reliable and convenient assay for the study of the individual coupling sites.

2) <u>The different effects of ADP plus P_i and uncouplers on electron transport associated with each coupling site</u>. The use of the partial reactions described above led to the observation that the two coupling sites differed in several important characteristics. As has already been pointed out, the rate of electron transport in partial reactions which include coupling site I is markedly stimulated by the addition of ADP plus phosphate at pH values higher than 7 (Appendix III, Fig. 1, Table III; Appendix VII, Figs. 2,4), whereas a similar stimulation of electron transport by ADP plus phosphate was not observed in those partial reactions which included only coupling site II (Appendix III, Fig. 1, Table III; Appendix II, Table II; Appendix IV, Figs. 1,2). Apparently the

rate of electron flux through coupling site II is not regulated by the "energized state" of the chloroplast.

Similar results were obtained when uncouplers such as gramicidin D or methylamine were added. The rates of electron flow along transport pathways which included coupling site I only or coupling site I plus coupling site II were increased markedly by uncouplers, while the rates of electron flow along transport pathways which included only coupling site II were unaffected by uncouplers (Appendix II, Table II; Appendix IV, Fig. 3). The effects of ADP plus phosphate and of uncouplers are summarized in Table 1 of Appendix IV.

It is possible to construct a model which explains the differences between the abilities of site I and site II to regulate electron transport. Presumably the stimulation of the rate of electron flow through coupling site I is due to the relaease of "back-pressure" generated against electron flow by the accumulation of the high energy state. Since the potential available between plastoquinone and cytochrome f (coupling site I) is only about 300 mV, such a "back-pressure" could well slow the flow of electrons (see also discussion). However, a very different situation may be encountered at coupling site II. Here the energy conserving electron transport reactions may be essentially irreversible because of the large energy input via photosystem II which seems to drive the reaction. Even though a "back-pressure" due to formation of the high energy state existed, the few hundred millivolts involved would be unlikely to effect a significant reversal of the forward reaction which utilizes 1.8 V.

3) Effects of energy transfer inhibitors. In view of the results presented in the previous section, an attempt was made to determine if the terminal enzymatic steps of ATP formation supported by each coupling site showed the same sensitivities to specific inhibitors of the phosphorylation reaction (energy transfer inhibitors [Good, et al. 1966]). In the overall reaction H_2O –> MV and the partial reaction $DCUPH_2$ –> MV, the energy transfer inhibitor 4´- deoxyphlorizin (Winget et al., 1969) inhibited both ATP formation and that portion of the electron transport dependent upon phosphorylation in a very similar manner (Appendix III, Fig. 3). However, the inhibitor had no effect on the transport of electrons from H_2O –>DAD_{ox} either in the presence or absence of ADP plus P_i, although phosphorylation itself was inhibited. In fact, ATP formation supported by coupling site I and by coupling site II exhibited the same sensitivity to 4' -deoxyphlorizin.

Results similar to those described above were also obtained with rabbit antiserum prepared against chloroplast CF_1 (Gould, 1975b). Again ATP formation supported by the two coupling sites showed equal sensitivity to the inhibitor.

However, very different results were obtained when yet another chloroplast energy transfer inhibitor, $HgCl_2$, was employed. Low concentrations of $HgCl_2$ (approx. 1 atom Hg^{++}/40 molecules chlorophyll) inhibit ATP formation and phosphorylation-dependent electron transport to a plateau of about 50% when the electron transport pathway is from H_2O to MV or ferricyanide (Izawa and Good, 1969; Appendix V, Fig. 1). Neither basal ($-P_i$) nor uncoupled electron transport is affected by these low levels of $HgCl_2$. Electron transport and phosphorylation associated with the

partial reactions $DCIPH_2$ → MV and DAD → MV (coupling site I only) are similarly affected by $HgCl_2$ (Appendix V, Fig. 1; Appendix VII, Fig. 6).

When the electron transport pathway includes only coupling site II, however, entirely different results are obtained. Electron transport and phosphorylation associated with the partial electron transport pathway from water to the Class III acceptors PD_{ox}, DAD_{ox}, DMQ or DBMIB are completely insensitive to the low levels of $HgCl_2$ which inhibit phosphorylation at coupling site I (Appendix V, Table I, Fig. 1). It is unlikely that this remarkable difference in sensitivity to $HgCl_2$ exhibited by the two coupling sites is an artifact arising from fortuitous reaction conditions when Class III acceptors are employed since the chloroplasts were incubated with $HgCl_2$ for 30 seconds before the addition of the acceptor system. Furthermore, UV spectra of PD_{ox}, DAD_{ox}, DMQ and DBMIB remained virtually unchanged in the presence of high concentrations of $HgCl_2$ (33 μ M), which means that there was little or no reaction of these substances with the mercury. Moreover, a similar level of inhibition by a given amount of $HgCl_2$ was obtained at several different concentrations of the Class III acceptor (Gould, 1975b).

C. <u>The Relation of Light-Induced Proton Fluxes to the Electron Transport and ATP Formation Associated with Coupling Site II</u>.

The differences between coupling sites I and II described above, that is, the very different degrees of control of electron transport by phosphorylation and the very different sensitivities to low pH and mercury, prompted us to undertake further investigations into the mechanism by which electron transport may be coupled to phosphorylation at these two sites. The experiments described below deal with a light-induced,

reversible proton uptake (i.e., pH rise in the suspending medium) associated with electron transport through coupling site II, and its relation to the mechanism of energy conservation at that site.

1) Light-induced pH rise associated with DBMIB reduction. As shown earlier, the use of substrate concentrations of DBMIB provides a convenient reaction system for studying the nature of coupling site II since the function of the substrate as an inhibitor effectively blocks further electron transport to photosystem I and thereby isolates site II from site I.

When a weakly buffered reaction mixture containing chloroplasts and 20 μ M DBMIB was illuminated, a dark-reversible rise in the medium pH was observed (Appendix III, Fig. 4; Appendix VI, Figs. 1,2). Above pH 8.1, where the DBMIB is rapidly reoxidized by molecular oxygen, the pH-rise was observed many times over repeated light-dark cycles. Below pH 8, where the reoxidation rate is very slow, the pH shift was maintained only as long as the reduction of DBMIB continued. As the reduction approached completion and electron transport slowed down, there was a gradual reversal of pH rise even in the light and the pH eventually returned to the original level. Subsequent illuminations in the absence of further additions of DBMIB did not restore the pH rise. The uncoupler gramicidin D and the electron transport inhibitor DCMU completely abolished the light-induced pH rise.

2) Demonstration of post-illumination ATP synthesis (X_E) associated with coupling site II. Hind and Jagendorf (1963) discovered that chloroplasts illuminated in the absence of ADP, P_i and Mg^{++} generated a capacity to form ATP when ADP, P_i and Mg^{++} were subsequently added in the

dark. This capacity for post-illumination ATP formation (termed X_E) has been shown to be closely correlated with the uptake of protons by the chloroplasts in the light (Izawa, 1970). Thus, any reaction causing the reversible uptake of protons by chloroplasts should exhibit post-illumination phosphorylation (X_E). Figure 4 shows that the electron transport pathway H_2O $\rightarrow$DMQ, which includes coupling site II only (and the associated proton pump - see above) is capable of generating X_E.

3) Kinetics and stoichiometry of electron transport and proton uptake (H^+/e^-) at site II. The results presented above (Section C, 1 and 2) are most easily explained in terms of a transmembrane H^+ gradient associated with the partial reaction H_2O $\rightarrow$photosystem II $\rightarrow$ Class III acceptor (eg. DBMIB, DMQ). However, any critical evaluation of the relevance of this proton gradient to the coupling mechanism requires a knowledge of the efficiency of the proton uptake (H^+/e^-). Furthermore, since the efficiency of phosphorylation associated with coupling site II is lower than the efficiency of the complete chain where both coupling sites are operating (P/e_2 = 0.3-0.4 versus 1.1-1.2, respectively), one might expect that the efficiency of proton accumulation (H^+/e^-) would be correspondingly lower when only coupling site II is involved.

Because of the technical and/or theoretical problems which have plagued previous attempts to measure H^+/e^- ratios in chloroplasts (see Appendix VI for a discussion of these problems; see also Jagendorf, 1975), a new technique was developed which gave highly reproducible results and which largely avoided the problems alluded to above. This method was based upon the flash-yield tehnique developed by Izawa and Hind (1967) and involved measurement of the pH changes induced by a series of brief

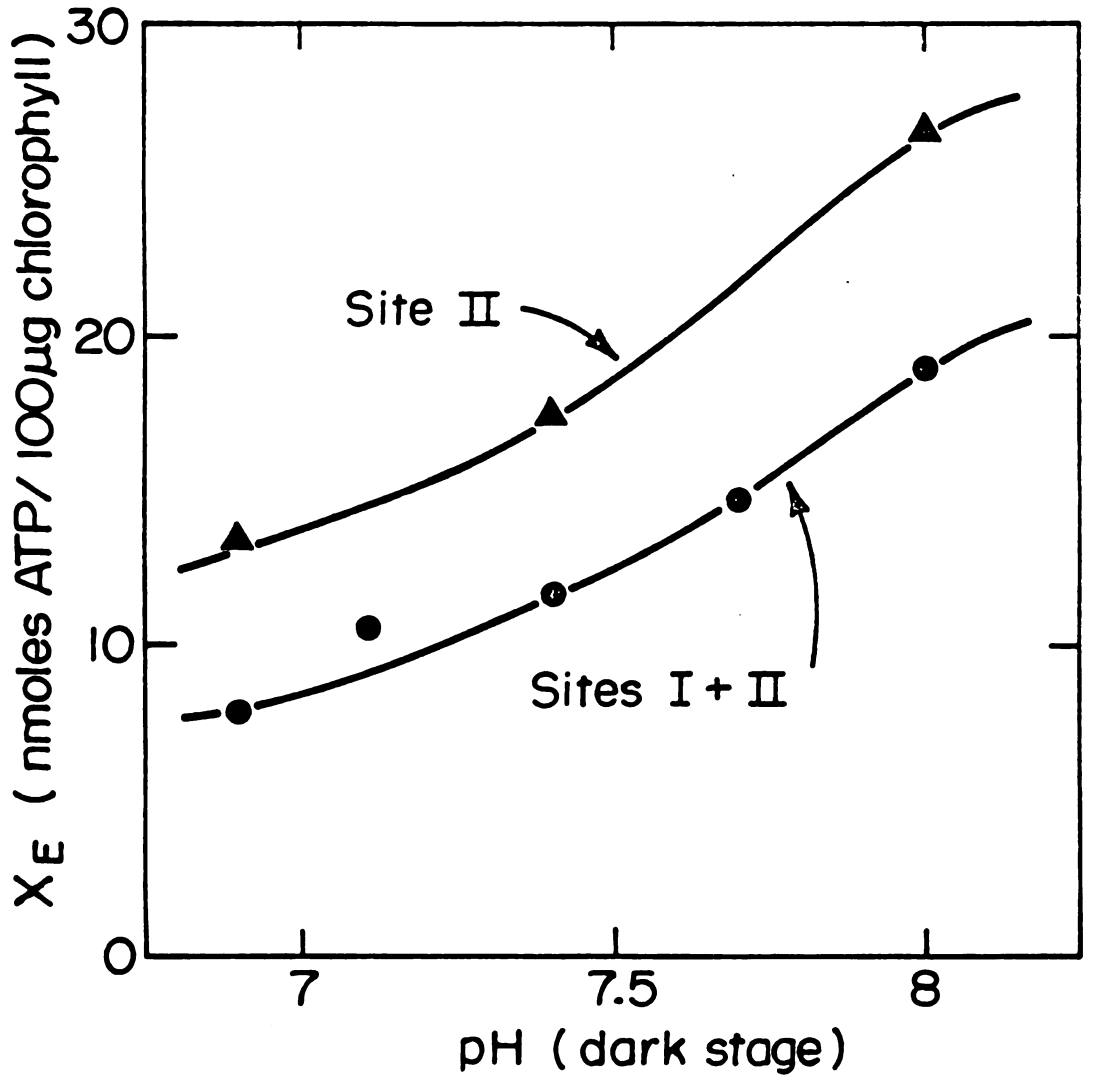

Figure 4. Post-illumination ATP Formation Capacity (X_E) Generated by Electron Transport Through Coupling Sites I and II. (The reaction procedure is given in Appendix III except that the electron transport systems were as follows: Site II, H_2O -> DMQ; Sites I + II, H_2O -> MV.)

illuminations (0.5-3 seconds) with intervening dark periods to allow for the lag due to the relatively slow instrumental response time ($t_{\frac{1}{2}} \tilde{=}$ 0.5 seconds). The sum of the pH changes occurring as the result of each flash plotted against the total illumination time generated a reconstructed time-course of the pH-rise which could be used to calculate the rate of proton uptake. The kinetics of electron transport were measured in a similar manner in the same apparatus by substituting a Clark O_2 electrode ($t_{\frac{1}{2}} \tilde{=}$ 2 seconds) for the pH electrode. Details of the technique are explained more fully in Appendix VI.

The use of the flash-yield technique outlined above made transient differences between initial rates and steady state rates quite obvious. Thus, the initial "pH gush" ($t_{\frac{1}{2}} \leq$ 0.01 seconds) associated with the overall Hill reaction and heretofore attributed to the reduction of the plastoquinone pool (Izawa and Hind, 1967) was easily detected by this method (Appendix VI, Fig. 3). No corresponding initial fast phase in oxygen evolution was detected, however, a fact which throws some doubt on the involvement of the plastoquinone pool in the initial rapid pH change.

No such initial rapid pH changes were associated with DBMIB reduction, the rate of proton uptake (and O_2 evolution) determined for the first flash being essentially the same as the rate determined for a subsequent flash.

Because the rate of proton uptake was practically linear for approximately the first 4 seconds of illumination when DBMIB was the electron acceptor, and for about 3 to 4 seconds after the initial "pH gush" when methylviologen was the electron acceptor, it was possible to determine accurately the initial rate of proton uptake from the reconstructed

time-course obtained by the flash-yield technique. Similarly, the rate of electron transport (as measured by changes in oxygen concentration) was completely linear with illumination time in both systems (Appendix VI, Figs. 3,4). The ratio of the rate of H^+ uptake to the rate of electron transport (both measured by the flash-yield method) was taken to be an accurate reflection of the efficiency of proton accumulation in these systems.

The values determined for the H^+/e^- ratio in the H_2O $\rightarrow$DBMIB reaction were relatively constant (0.35-0.51) over the entire pH range tested (6.2-8.15), averaging about 0.4. This low value is in contrast to H^+/e^- values $\geq$ 1.7 observed for the $H_2O \rightarrow$ MV reaction (Appendix VI, Table I, Fig. 4; see also Izawa and Hind, 1967). Thus the proton pump associated with DBMIB reduction (involving only coupling site II) is distinguished from the proton pump associated with MV reduction in two ways: the reaction involving only site II lacks an initial rapid phase and is less than half as efficient as the combined sites in transporting H^+.

4) Effect of pH on the efficiencies of proton uptake (H^+/e^- and ATP formation (P/e_2) at coupling sites I and II. The remarkable similarity between the effect (or lack of effect) of pH on the H^+/e^- and P/e_2 ratios associated with coupling site II prompted a further investigation into the relationship between proton uptake and ATP formation at coupling site I.

Figure 5 shows that the H^+/e^- values obtained for coupling site I only (using the $Fe(CN)_6^{4-} \rightarrow$ MV reaction, see Izawa and Ort, 1974) were consistently around 1.0 regardless of the pH of the medium. The P/e_2

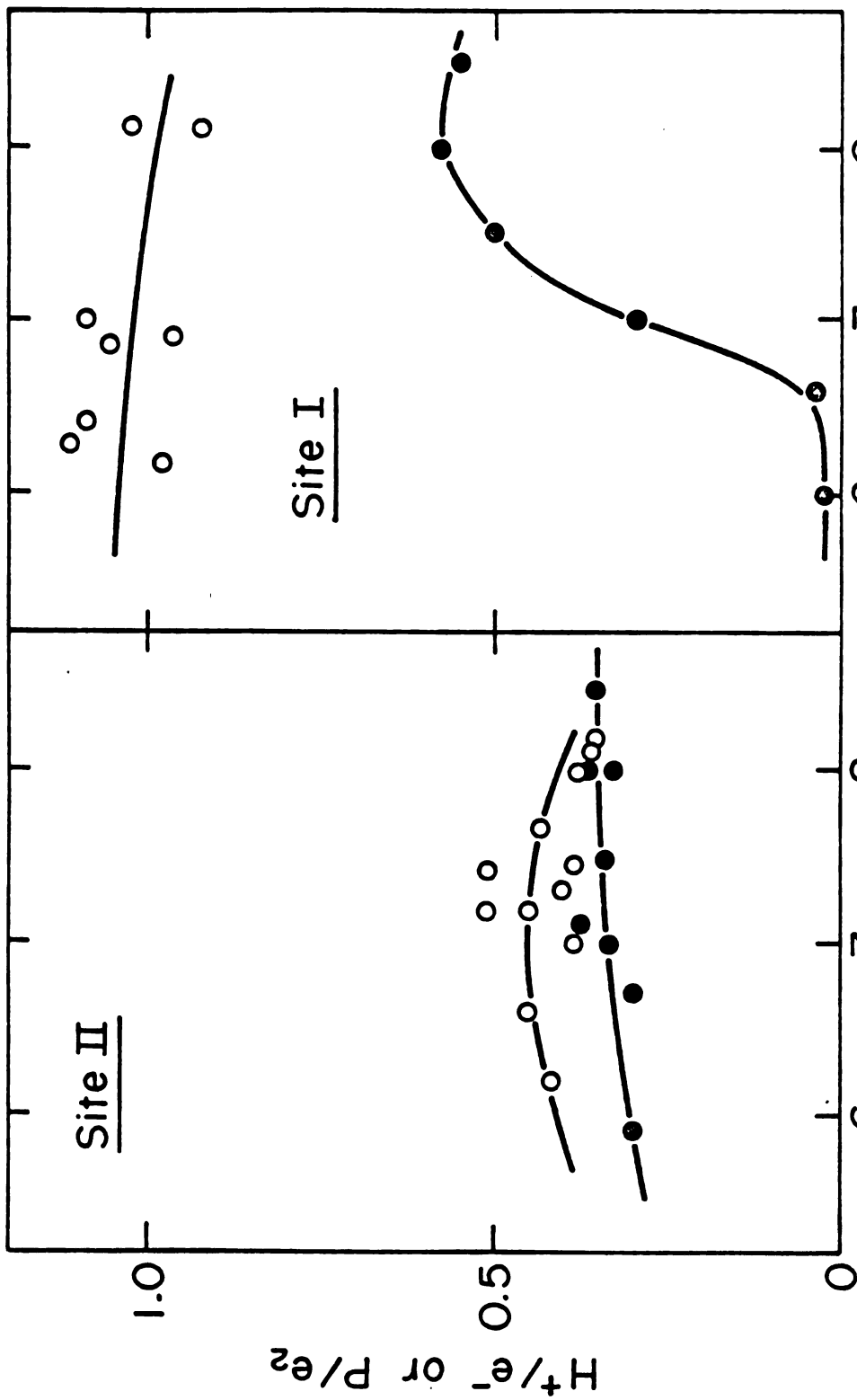

Figure 5. A Comparison of the Efficiencies of Proton Uptake (open circles) and ATP Synthesis (solid circles) at Different pH Values for Coupling Sites I and II. (Reaction conditions are given in Appendices II,VI and VII and in Izawa and Ort, 1974.)

values obtained for the same reaction, however, show the marked pH dependence characteristic of site I phosphorylations (see Results section B1; see also Izawa and Ort, 1974). The reason for the lack of correlation between the efficiencies of proton uptake and phosphorylation at coupling site I and the close correlation between these processes at coupling site II is not at all clear. The implications of this important difference between the two coupling sites will be dealt with more fully in the Discussion (see also Appendix VIII).

5) Effect of phosphorylation and arsenylation on proton uptake. Whether the proton gradient built up by chloroplasts represents an obligatory intermediate of ATP formation, or an energy reservoir on a side-pathway, one might reasonably expect that energy utilization might lower the steady state level of the gradient (Mitchell, 1966). However, both inhibitions and stimulations of proton gradients by phosphorylation have been reported (Dilley and Shavit, 1968; McCarty et al., 1971; Gould and Winget, 1972; Karlish and Avron, 1968). Because ATP formation can increase the rate of electron transport (and proton pumping) drastically, and because ions or ADP alone can greatly increase the extent of proton uptake (Dilley and Shavit, 1968; McCarty et al., 1971), the true effect of ATP formation on the proton gradient could easily be masked. However, use of the photosystem II dependent reduction of DBMIB avoids these complications since the electron transport in this system is not stimulated by phosphorylation (see Results, section B2) nor is proton uptake enhanced by the addition of ADP (Appendix VI, Table II).

The effect of phosphorylation on the proton uptake in the $H_2O \rightarrow$DBMIB reaction was observed directly as: a) the extent of

dark-reversible H^+ uptake superimposed upon the irreversible proton consumption due to ATP formation; b) the extent of the steady-state level of proton uptake in the presence of glucose plus hexokinose (to eliminate the irreversible proton consumption); and c) the extent of the steady-state level of proton uptake in the presence of arsenate instead of phosphate (the unstable, arsenylated ADP hydrolyzes rapidly thereby eliminating the irreversible proton consumption). In each case a consistent lowering of the extent of proton uptake (40-60%) was observed when phosphorylation (or arsenylation) occurred (Appendix VI, Table II, Figs. 5,6).

DISCUSSION

DISCUSSION

A. Functional Separation of the Two Coupling Sites in Chloroplasts

The results presented in this dissertation show clearly that it is possible to divide the chloroplast electron transport chain into two parts through the use of appropriate exogenous electron donors, electron acceptors and electron transport inhibitors. Each part uses only one of the two photosystems and each part uses only one of the two coupling sites. The photosystem II-dependent transport of electron from water to lipophilic oxidants such as p-phenylenediimines and p-benzoquinones (Class III acceptors) is coupled to phosphorylation only at coupling site II when the photosystem I-dependent component of the reduction is inhibited (Results, Section A; see also Appendicies I-IV). Similarly, the photosystem I-dependent transport of electrons from exogenous electron donors such as diaminodurene or reduced dichlorophenolindophenol to hydrophilic acceptors such as methylviologen or $NADP^+$ is coupled to phosphorylation only at coupling site I when the flow of electrons from photosystem II is inhibited (Results, Section B; see also Appendicies I-III, VII).

These conclusions are supported by inhibitor studies. Thus the reduction of Class III acceptors is largely unaffected when the electron transport chain is blocked at plastocyanin (Ouitrakul and Izawa, 1973), cytochrome f (McCarty, 1974), or plastoquinone (Appendicies I-III). On the other hand, the reduction of Class III acceptors is extremely

sensitive to electron transport inhibitors which act close to either the oxidizing side of photosystem II (Ort and Izawa, 1973) or the reducing side of photosystem II (Appendix II; Ouitrakul and Izawa, 1972). The oxidation of exogenous electron donors by photosystem I shows an entirely different pattern of responses to inhibitors; it is severely inhibited when electron transport is blocked at plastocyanin (Appendix I; Ouitrakul and Izawa, 1973) or cytochrome f (McCarty, 1974), but it is completely insensitive to electron transport inhibition at plastoquinone (Appendix I), photosystem II or water oxidation (Appendicies I, III, VII). All of these findings are summarized in the scheme presented in figure 6 (see also Appendix VII).

B. Differences in the Properties of the Two Coupling Sites.

The ability to separate and study the two coupling sites without resorting to disruption of the chloroplast lamellae by physical methods or detergents made possible the discovery of a number of significant differences in the properties of the coupling reactions at sites I and II. These differences between the coupling sites are important in that they must be accomodated by any theory of the mechanism by which electron transport and ATP formation are coupled.

1) Regulation of electron flow by phosphorylation. One of the most obvious differences which can be observed experimentally is the lack of control of the rate of electron flow through site II by phosphorylation. This lack of control can best be considered in the light of how electron flow is probably regulated by phosphorylation at site I.

Portis et al. (1975) have presented data which support the notion that an energy-dependent conformational change in CF_1 actually

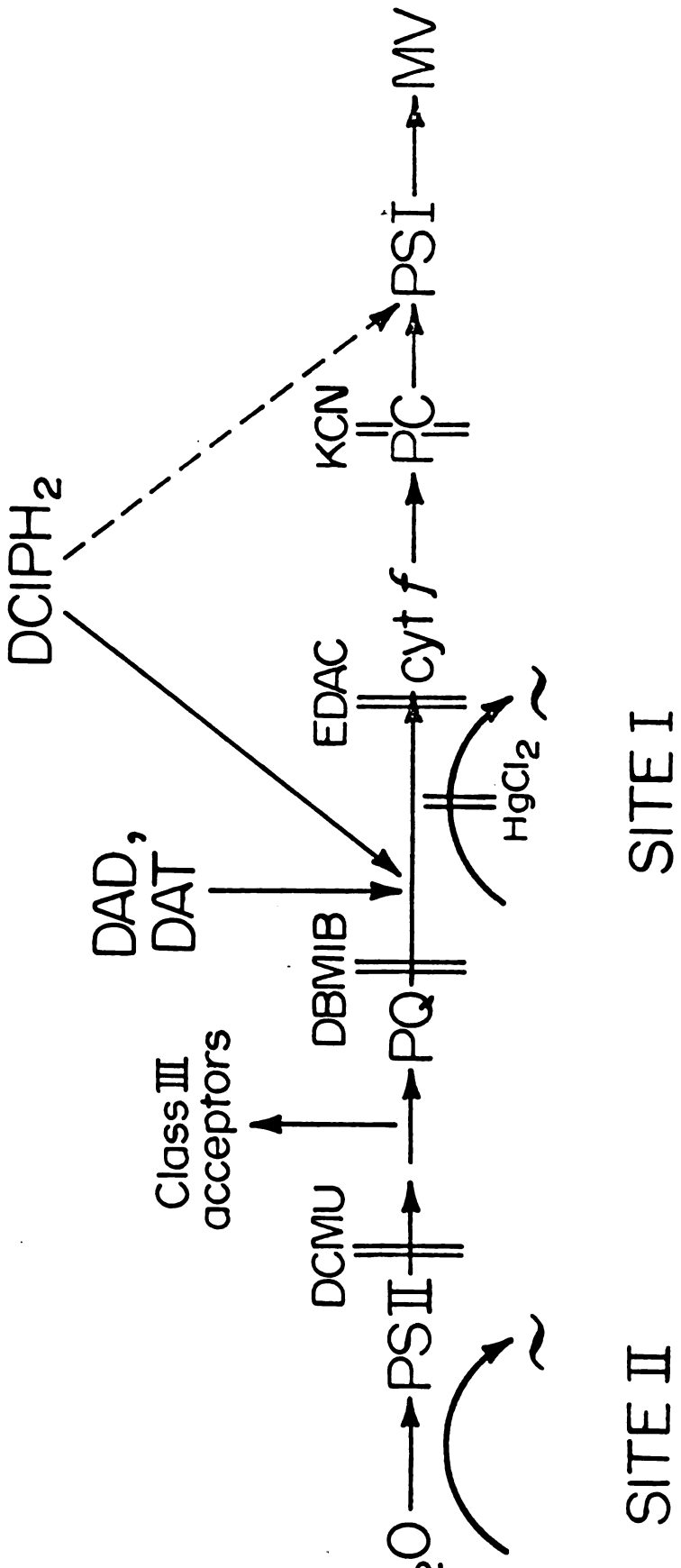

Figure 6. A Scheme for Electron Transport Pathways in Isolated Chloroplasts Showing the Two Sites of Energy Transduction (~) and the Sites of Action of Various Inhibitors.

controls the rate of electron flow through coupling site I. In both the energized conformation and the deenergized conformation, CF_1 is only slightly permeable to protons. However, according to these authors, when the energized conformation is being relaxed during phosphorylation, proton permeability momentarily increases. This effectively lowers the "back pressure" of the internal protons on the proton producing electron transport reactions. However, it is possible that this back pressure represents an almost inconsequential amount of energy in the face of the overwhelming driving energy for the forward electron transport reaction supplied by photosystem II. At coupling site I, where the driving energy is considerably smaller, the back pressure could be considerably more significant, so that the rate of electron flow through this site would be a much stronger function of the proton gradient (see also Appendix VII),

2) $\underline{HgCl_2 \text{ inhibition}}$. Izawa and Good (1969) first showed that mercurials inhibit coupled electron transport and ATP formation in a manner characteristic of energy transfer inhibitors. Energy transfer inhibitors are believed to block phosphorylation by interfering with the terminal enzymatic steps of ATP synthesis, perhaps by binding to the coupling factor (CF_1) or associated membrane proteins. Indeed the amount of $HgCl_2$ required to attain the 50% inhibition plateau at coupling site I in chloroplasts (1 atom Hg^{++}/40-50 chlorophyll molecules; Appendix VII) is in the same order of magnitude as the number of CF_1 molecules associated with the thylakoid (Murakami, 1968).

It has been suggested that $HgCl_2$ and other mercurials inhibit phosphorylation by binding to essential sulfhydryl residues (Izawa and Good, 1969; Bradeen and Winget, 1974) since $HgCl_2$ inhibition is relieved

by cysteine but not by other chelators of Hg^{++}. Further evidence that a sulfhydryl may be involved comes from the unusual effect of $HgCl_2$ on electron transport in the absence of both ADP and P_i. Under these conditions the rate of electron transport is stimulated by $HgCl_2$ to the level of coupled electron flow in the absence of $HgCl_2$(Izawa and Good, 1969). The only other energy transfer inhibitor reported which exhibits this property is N-ethylmaleimide, which reacts with a sulfhydryl residue on the γ-subunit of CF_1 (McCarty et al., 1972; McCarty and Fagan, 1973).

Bradeen et al., (1973) concluded that $HgCl_2$ probably did not inhibit CF_1 directly since the formation of ATP in the dark after an illumination (X_E) was much less sensitive to the inhibitor than steady-state phosphorylation. However, this insensitivity has been observed with other energy transfer inhibitors as well, and probably reflects the introduction of different rate-limiting steps in X_E phosphorylation. In any event, it seems likely that mercurials, like other energy transfer inhibitors, interfere with phosphorylation at a point close to the terminal ATP synthesizing process.

If it is true that mercurials serve as energy transfer inhibitors by reacting with the coupling factor, the very great difference in the sensitivities of site I and site II is of paramount importance; heretofore there has never been any evidence that different coupling sites use different coupling factors, and the chemiosmotic hypothesis specifies a common coupling factor.

Finally, it is interesting to note that the lipophilic mercurial p-hydroxmercuribenzoate is much less site-specific than $HgCl_2$, raising the interesting possibility that the mercury sensitive component may be in

different microenvironments at the different coupling sites. This possibility will be discussed in more detail below.

3) The effects of pH on the efficiences of phosphorylation (P/e_2) and proton uptake (H^+/e^-). It is not at all difficult to understand that the efficiency of proton uptake associated with electron transport through each coupling site is insensitive to the pH of the medium. There is good reason to believe that proton uptake is a consequence of antisotropic arrangement of the electron carriers and hydrogen (i.e. H^+ plus e^-) carriers in the membrane. The reduction by an electron of a hydrogen carrier near the outer side of the membrane results in the uptake of a proton from the medium, and the oxidation of the hydrogen carrier by an electron carrier near the inner side of the membrane results in the loss of a proton to the inside of the thylakoid. In the case of coupling site II, the oxidation of H_2O at the inner membrane interface is almost certainly the source of the internal protons. The ratio of protons translocated to electrons transported can only be affected by pH if i) the apparent hydrogen carrier has a pk_a within the pH range being investigated, loses a proton, and is therefore not really a hydrogen carrier at all, or ii) changing the medium pH drastically alters the arrangement of the membrane so that the release of protons by hydrogen carrier upon oxidation is no longer toward the inside of the thylakoid. The fact that changing the medium pH causes no significant change in the H^+/e^- ratios suggests that neither of these possibilities is occurring over the pH range 6-9 (Appendix VI, VIII).

Even though changing the pH of the medium has no effect on the efficiency of H^+ uptake, changing the pH does affect the efficiency of site

I-dependent phosphorylation dramatically. In contrast, the efficiency of site II phosphorylation is hardly affected. Below pH 7, the efficiency of ATP formation supported by site I has fallen to nearly zero, while the efficiency of ATP formation supported by site II is practically unchanged. What this seems to say is that at low external pH, whether or not ATP is made depends upon exactly where (i.e. at which electron transport reaction) the internal protons are generated. Again, as in the case of Hg^{++} inhibition, low pH seems to be a site-specific inhibition of phosphorylation, which cannot be easily accomodated by the chemiosmotic hypothesis.

C. The Coupling Mechanism and Coupling Site-Specificity

Attempting to explain the data described above within the framework of existing hypotheses of energy conservation is an illuminating exercise since it forces one to define more precisely the molecular mechanism involved. The evidence which has accumulated in support of some sort of chemiosmotic coupling mechanism (as detailed by Mitchell, 1966) makes the chemiosmotic hypothesis attractive. The site-specificities exhibited by Hg^{++} and pH can be fitted into the framework of the chemiosmotic hypothesis as outlined below.

Williams (1969) has suggested that the actual pH gradient involved in energy coupling is confined to a highly localized area of the membrane, and is in equilibrium with a delocalized, transmembrane gradient. Accepting this postulate, one can construct a model for energy coupling which allows site-specificity while at the same time preserving most of the fundamental principles of the chemiosmotic hypothesis. According to the model, the localized pH gradient within the membrane is in the vicinity of a CF_1 molecule. The CF_1 molecule utilizes the protons in the

intramembrane gradient to drive phosphorylation, while any extra protons which are generated by the electron transport reactions are released to the inside of the thylakoid to establish the transmembrane gradient. Thus, protons produced at one coupling site would not be available to support ATP formation at another coupling site except via the relatively slow equilibration of the localized gradients via the transmembrane gradient. The site-specific effects of Hg^{++} and pH can then be understood by postulating that that portion of the energy conserving appratus associated with coupling site II which is sensitive to Hg^{++} and low pH is in a more hydrophobic environment than the corresponding portion at the apparatus associated with coupling site I and therefore is less accessible to mercuric or hydrogen ions.

There is some evidence which supports such a model. For example, it is very likely that the water-splitting reaction, the H^+ generator for site II, is located within a hydrophobic region of the membrane. Furthermore, lipophilic mercurials (e.g. p-hydroxymercuribenzoate) are much less site specific than mercuric chloride (Gould, 1975b). This is consistent with the idea that the inhibition sites are indeed in different microenvironments. However, antiserum against CF_1 shows no site specificity, an observation which indicates that, if there are different coupling factors associated with different coupling sites, at least a part of the CF_1 molecule at each coupling site must be exposed to the aqueous environment. Of course, the observation does not preclude the possibility that some other essential portion of the coupling apparatus besides CF_1 is buried in a hydrophobic environment.

It should be understood that the modification of the chemiosmotic hypothesis proposed above does not necessarily require that the coupling factor itself be directly associated with the coupling sites and in different environments. One can equally well postulate that the suggested local accumulation of hydrogen ions interacts with CF_1 through additional transducers which are unique to each coupling site.

Still other models which could accomodate the apparent site-specificities of Hg^{++} and low pH might be possible, and given enough ingenuity it might be possible to make some of these models conform more closely to the chemiosmotic hypothesis as defined by Mitchell (1966). Final resolution of this matter must await evidence from new and different experiments utilizing new and different approaches.

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APPENDICES

APPENDICES

The following appendices are included as reference material for a large number of experimental data. Appendix VIII represents a summary of much of this work, along with a more complete discussion of the proposed modifications of the chemiosmotic hypothesis. The preponderant portion of the data in the appendices are data obtained in the course of the work described in this dissertation. However, some of the data in some of the appendices represent the work of others. It is impossible to assess the relative contribution of this author to each of these works because of the high degree of interaction and collaboration among this author and his colleagues during the course of these investigations.

APPENDIX I

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

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

S. IZAWA, J. MICHAEL GOULD, DONALD R. ORT, P. FELKER and N. E. GOOD

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Mich. 48823 (U.S.A.)

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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

(*1*) *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	*+dibromo-thymoquinone*	*Control*	*+dibromo-thymoquinone*	*Control*	*+dibromo-thymoquinone*
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

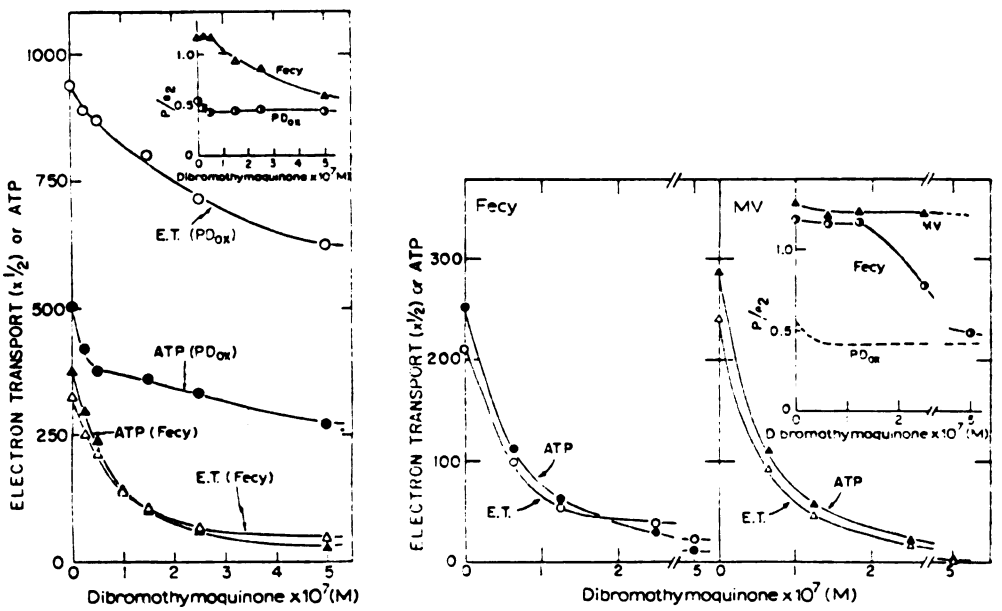

Fig. 1. Effect of dibromothymoquinone on electron transport (E.T.) and phosphorylation (ATP) with ferricyanide (Fecy) or oxidized *p*-phenylenediamine (PD_{ox}) as electron acceptor. Rates are expressed in μequiv or μmoles ATP/h per mg chlorophyll. The 2.0-ml reaction mixture consisted of the following: 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.

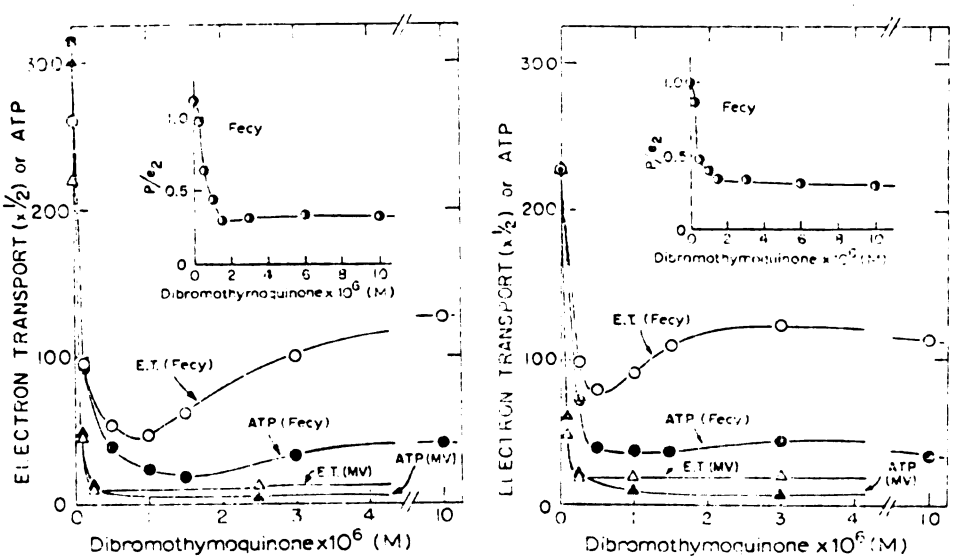

Fig. 3. Effect of higher concentrations of dibromothymoquinone on electron transport and phosphorylation with ferricyanide or methylviologen as acceptors. Reaction conditions, units and abbreviations as in Fig. 2, except that methylviologen was 100 μM. Note the much greater residual electron transport 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 untragmented 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/e₂*
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

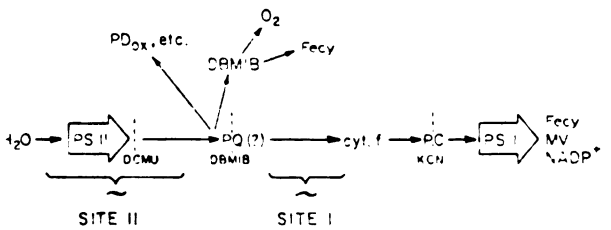

Fig. 5. Simplified scheme of the electron transport pathways, phosphorylation reactions and inhibition sites discussed in this paper. PD_{ox}, oxidized *p*-phenylenediamine; Fecy, ferricyanide; DBMIB, 2,6-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); PQ, plastoquinone; PC, plastocyanin; 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.

ACKNOWLEDGEMENT

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APPENDIX II

PHOTOSYSTEM II ELECTRON TRANSPORT AND PHOSPHORYLATION WITH DIBROMOTHYMOQUINONE AS THE ELECTRON ACCEPTOR

Photosystem-II Electron Transport and Phosphorylation with Dibromothymoquinone as the Electron Acceptor

J. Michael GOULD and Seikichi IZAWA

Department of Botany and Plant Pathology Michigan State University, Michigan

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Dibromothymoquinone has two effects on isolated chloroplasts. At very low concentrations it inhibits the reduction of conventional hydrophilic electron acceptors, probably by acting as a plastoquinone antagonist. At higher concentrations it acts as an electron acceptor, intercepting electrons either before or at the site of its inhibitory activity. Reduced dibromothymoquinone can be readily reoxidized by excess ferricyanide in the reaction mixture or by molecular oxygen. The transfer of electrons to this substance from water is coupled to phosphorylation. The pH optima for this reduction and associated phosphorylation are both at 7.3, considerably lower than the pH optimum of 8.4 observed with the normal Hill reaction. The ratio of molecules of ATP formed to pairs of electrons transported is relatively constant (0.3—0.4) between pH 6 and 9. The rate of reduction is independent of the presence or absence of ADP and phosphate or uncouplers.

The reduction of dibromothymoquinone resembles the reduction of other lipid-soluble oxidants such as oxidized *p*-phenylenediamines and 2,5-dimethylquinone in several respects. Both reactions are insensitive to the plastocyanin inhibitors KCN and polylysine, but are sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea. They support phosphorylation with a similar efficiency. However, dibromothymoquinone reduction differs from the reduction of other lipophilic oxidants in that its sensitivity to dichlorophenyl-dimethylurea decreases with increasing light intensities. This implies that it may be reduced *via* a pool (plastoquinone?) which is a common electron acceptor for independent photosystem II units whereas oxidized *p*-phenylenediamines and 2,5-dimethylquinone are reduced directly by these independent units.

The transport of electrons from photosystem II to photosystem I can be inhibited in several ways. Treatment of chloroplasts with KCN [1] or poly-L-lysine [2] seems to block the flow of electrons from cytochrome *f* to P_{700} by inactivating plastocyanin [3,4]. In contrast, dibromothymoquinone probably acts as a plastoquinone antagonist [5] and therefore blocks electron transport at an entirely different site. These inhibitors abolish the transport of electrons from water to conventional hydrophilic electron acceptors such as methylviologen or ferredoxin-$NADP^+$ but they do not prevent the reduction of lipophilic acceptors such as the oxidized forms of *p*-phenylenediamine and diaminodurene.

Saha *et al.* [6] were the first to point out that these lipophilic "class III" oxidants could be reduced at exceptionally high rates by illuminated chloroplasts and that this rapid electron transport was coupled to phosphorylation. However, they also showed that the overall efficiency of phosphorylation, as measured by the ratio of molecules of ATP formed to the pairs of electrons transported ($P/e_2 \approx 0.5$) is much lower than the efficiency with conventional hydrophilic "class I" acceptors ($P/e_2 \approx 1.2$). These findings led Saha *et al.* to postulate that class III acceptors might intercept electrons by reacting with an intermediate carrier situated between two sites of phosphorylation. The experiments with inhibitors alluded to above have strongly supported this concept. Ouitrakul and Izawa [1] have shown that the reduction of class III acceptors is largely insensitive to KCN treatment and Ort *et al.* [7] have shown that the same is true for polylysine treatment. Thus we may conclude that plastocyanin, which is required for the reduction of class I acceptors, is not required for the reduction of class III acceptors. Similarly we have shown that the plastoquinone antagonist dibromothymoquinone does not inhibit the reduction of class III acceptors

Abbreviation. P/e_2, ratio of the molecules of ATP formed to the pairs of electrons transported.

Trivial names. Dibromothymoquinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; dimethylbenzoquinone, 2,5-dimethyl-*p*-benzoquinione; dichlorophenyl-dimethylurea, 3(3,4-dichlorophenyl)-1,1-dimethylurea; P_{700}, primary electron donor to photosystem I.

[8]. Consequently it seems likely that class III acceptors intercept electrons before the site of involvement of plastoquinone in the electron transport chain. Furthermore, since the reactions insensitive to dibromothymoquinone, KCN and polylysine continue to support phosphorylation even in the presence of these inhibitors, we concluded that there must be a site of phosphorylation associated with the photosystem II-driven transfer of electrons from water to plastoquinone [8].

In the course of our investigations we noted that, at concentrations somewhat higher than are required for inhibition of the reduction of class I acceptors, dibromothymoquinone itself acts as an electron acceptor [8,9]. Furthermore, the photoreduction of dibromothymoquinone was coupled to phosphorylation with an efficiency characteristic of the reduction of class III acceptors. This paper deals with a more detailed investigation of the role of dibromothymoquinone as an electron acceptor. The study has shown that this lipophilic oxidant has unique properties which distinguish it from other class III acceptors.

MATERIALS AND METHODS

The procedures employed were similar to those described in previous papers [6,8]. Washed chloroplasts were isolated from fresh market spinach (*Spinacia oleracea* L.) by differential centrifugation as detailed elsewhere [6]. Electron transport with ferricyanide as the electron acceptor was measured spectrophotometrically by continuously recording the decrease in absorbance of the reaction mixture at 420 nm. Electron transport with oxidised diaminodurene, dimethylbenzoquinone or dibromothymoquinone as the electron acceptor was followed as the reduction of excess ferricyanide since the reduced acceptors were immediately reoxidized by ferricyanide present in the reaction mixture [6]. Oxygen uptake (Mehler reaction) was measured with a Clark-type membrane-covered oxygen electrode. No H_2O_2 trap was necessary since the chloroplast preparations were free of catalase activity. Saturating intensities of orange actinic light (> 600 nm) were supplied by a 500-watt slide projector and the appropriate colored filters. For some experiments the intensity of the actinic beam was varied with a series of calibrated neutral-density screen filters. Absolute light intensity was measured with a YSI radiometer shielded from infrared by a Corning heat filter (C.S. 1—69). All reactions were carried out in thermostatted cuvettes at 19 °C.

ATP formation was measured as the residual radioactivity remaining in the reaction mixture after extraction of the unreacted [^{32}P]orthophosphate as phosphomolybdic acid [10].

Cyanide-treated chloroplasts were prepared as described by Ouitrakul and Izawa [1] by incubating chloroplasts at 0 °C for 60 min in a 30 mM KCN solution buffered at pH 7.8. Control chloroplasts for these experiments were incubated in a similar manner substituting KOH for KCN. Dibromothymoquinone was prepared as described earlier [8] and dissolved in ethanol—ethylene glycol (1 : 1, v/v). 3(3,4-Dichlorophenyl)-1,1-dimethylurea was dissolved in ethanol and further diluted with 0.01 M NaCl. Stock solutions were prepared in such a way that the final concentration of organic solvent in the reaction mixture never exceeded 1.5%. Details of the treatment of chloroplasts with poly-L-lysine (M_n 194000) are described elsewhere [7].

RESULTS

Dibromothymoquinone-Catalyzed Reduction of Oxygen (Mehler reaction)

In the absence of added electron acceptor, illuminated chloroplasts consume oxygen slowly (Fig. 1). This phenomenon, which represents the reduction of small amounts of endogenous acceptors, their reoxidation by molecular oxygen, and the formation of H_2O_2 is known as the Mehler reaction [11]. There are two reasons for believing that the Mehler reaction catalyzed by endogenous acceptors involves photosystem I. The electron transport is coupled to phosphorylation with an efficiency characteristic of the Hill reaction with class I acceptors ($P/e_2 \approx 1.2$) and treatments of chloroplasts with KCN or polylysine, which block the flow of electrons at plastocyanin [1—4] abolish the reaction. However, when dibromothymoquinone (15 μM) is added to the reaction mixture the rate of oxygen uptake is greatly increased and now neither KCN nor polylysine inhibits. The efficiency of phosphorylation also falls to the level characteristic of the reduction of class III acceptors (Table 1). It seems clear therefore that this dibromothymoquinone-catalyzed Mehler reaction involves only photosystem II.

Since dibromothymoquinone is itself a powerful inhibitor of the transport of electrons to photosystem I, it totally suppresses the endogenous Mehler reaction at low concentrations (Fig. 2). As the concentration of dibromothymoquinone is increased, the inhibitor begins to act as an electron acceptor, catalyzing the dibromothymoquinone-KCN-polylysine-insensitive, photosystem II Mehler reaction. With the inhibition of the endogenous reaction the phosphorylation efficiency (P/e_2) promptly falls to a constant level of 0.3—0.4. It should be noted that this efficiency is quite independent of the rate of electron transport, depending rather on the electron transport pathway.

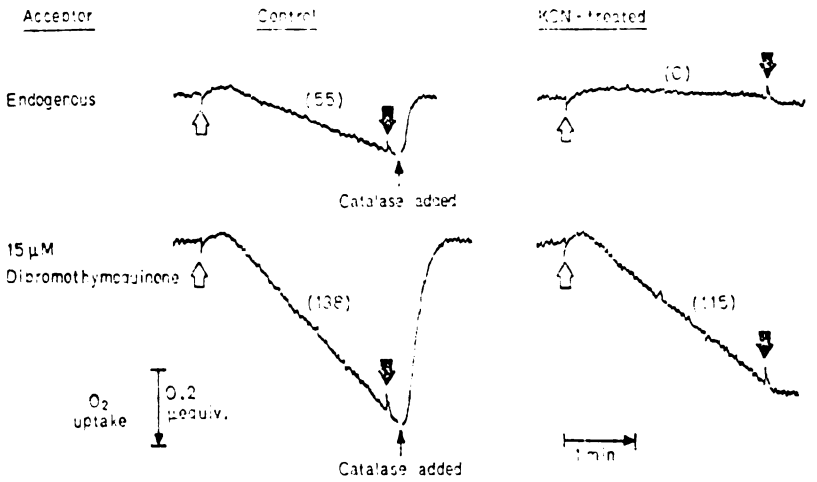

Fig. 1. *The effect of KCN on endogenous and dibromothymoquinone-catalyzed oxygen uptake (Mehler reaction) in illuminated chloroplasts.* The reaction mixture (2 ml) contained 0.1 M sucrose, 50 mM tricine buffer pH 8.1, 2 mM $MgCl_2$, 0.5 mM ADP, 5 mM P_i, and chloroplasts containing 40 μg chlorophyll. When added, a small amount of catalase was injected into the sample chamber with a microliter syringe. Numbers in parentheses are μequiv. × h^{-1} × mg chlorophyll^{-1}

Other Characteristics of the Electron Transport and Phosphorylation Associated with Dibromothymoquinone Reduction

The unusual dual action of dibromothymoquinone, inhibiting the transport of electrons to photosystem I while at the same time serving as an electron acceptor, makes its reduction a convenient reaction for the study of photosystem II. However, the reoxidation by oxygen becomes seriously rate-limiting below pH 8.0 or when the concentration of the inhibitor-acceptor is lowered (Fig. 2). This difficulty can be overcome by the addition of ferricyanide since it has already been shown that ferricyanide rapidly reoxidizes reduced dibromothymoquinone [8]. Indeed we have shown that this chemical reoxidation by ferricyanide is not rate-limiting under any of the conditions employed in this study; the rate of dibromothymoquinone reduction, measured as the consequent reduction of excess ferricyanide, is constant with time until virtually all of the ferricyanide has been reduced. Since the dibromothymoquinone-catalyzed reduction of oxygen and of ferricyanide both support a phosphorylation reaction having the same efficiency and the same sensitivity to photosystem I inhibitors (Table 1), it seems probable that the two reactions are biologically equivalent. For these reasons, and because less dibromothymoquinone is required to catalyze ferricyanide reduction, we used the dibromothymoquinone-ferricyanide system in the experiments described below.

The pH optimum for photosystem II electron transport and phosphorylation (Fig. 3) seems to be considerably lower than the optimum observed for the Hill reaction with class I acceptors (7.3 *vs* 8.4). This lower pH optimum observed in the presence of

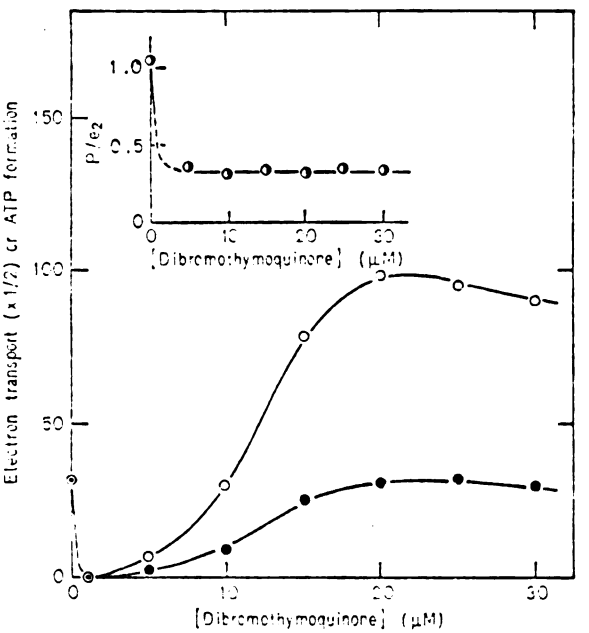

Fig. 2. *Effect of dibromothymoquinone on electron transport and phosphorylation in the absence of added electron acceptor.* Reaction conditions were as in Fig. 1. Electron transport (○) was measured as oxygen uptake as described in Methods. (●) ATP formation. (◑) P/e_2. Units are as in Table 1. Note that low concentrations of dibromothymoquinone abolish the endogenous Mehler reaction ($P/e_2 = 1.1$). Higher concentrations catalyze a photosystem II Mehler reaction which supports phosphorylation with a P/e_2 of about 0.4

dibromothymoquinone cannot be an effect of pH on the phosphorylation mechanism since the same optimum is seen in the presence or absence of ADP

Table 1. *Effect of photosystem-I inhibitors on electron transport and photophosphorylation with various electron acceptors*
The reaction mixture (2 ml) contained 0.1 M sucrose, 50 mM tricine-NaOH pH 8.1, 2 mM $MgCl_2$, 0.5 mM ADP, 5 mM $NaH_2{}^{32}PO_4$, chloroplasts containing 40 μg chlorophyll and the indicated acceptor system. In the dibromothymoquinone to ferricyanide (DBMIB → FeCy) system the reaction was buffered at pH 7.5 with 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate. The acceptor systems used were: FeCy, 0.4 mM ferricyanide; PD_{ox}, 0.5 mM *p*-phenylenediamine plus 1.5 mM ferricyanide; DBMIB → O_2, 15 μM dibromothymoquinone; DBMIB → FeCy, 10 μM dibromothymoquinone plus 0.4 mM ferricyanide. KCN-treated chloroplasts were prepared as described in Methods. Poly-L-lysine-treated chloroplasts were prepared as detailed by Ort *et al.* [7]. Rates of electron transport (E.T.) and phosphorylation (ATP) are given as μequiv. or μmol ATP × h^{-1} × mg chlorophyll^{-1}. Note that the reduction of the class III acceptor PD_{ox} has a cyanide and polylysine-sensitive component. Since dibromothymoquinone blocks the transfer of electrons to photosystem I, its reduction lacks this component. Note also that the residual photosystem II electron transport supports phosphorylation with an efficiency (P/e_2) of about 0.3—0.4. The lower efficiency after polylysine treatment of the chloroplasts is probably due to the well-known uncoupling effect of this substance [18]

Expt No.	Condition	Electron acceptor system											
		FeCy			PD_{ox} (→ FeCy)			DBMIB (→ O_2)			DBMIB (→ FeCy)		
		E. T.	ATP	(P/e_2)	E. T.	ATP	(P/e_2)	E. T.	ATP	(P/e_2)	E. T.	ATP	(P/e_2)
I	control	401	253	(1.26)	1070	310	(0.58)	150	20	(0.27)	262	50	(0.38)
	KCN	35	5	(—)	605	108	(0.36)	146	17	(0.23)	237	43	(0.36)
II	control	560	347	(1.24)	1390	429	(0.61)	110	20	(0.36)	275	43	(0.31)
	polylysine	20	0	(—)	980	218	(0.26)	116	19	(0.33)	285	36	(0.25)

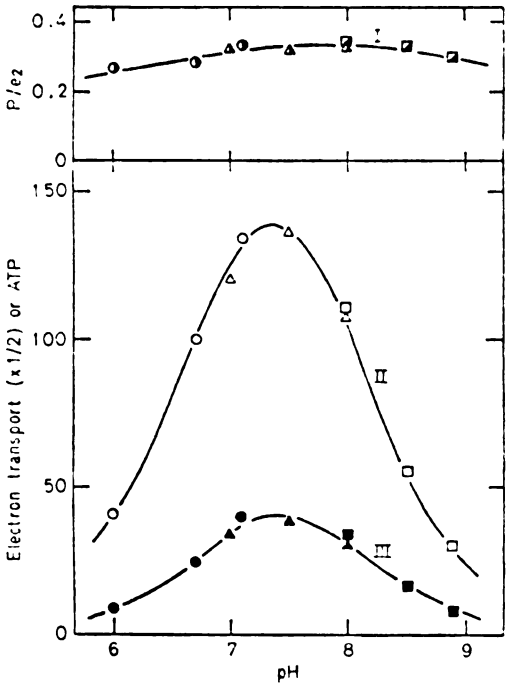

Fig. 3. *Effect of pH on dibromothymoquinone reduction and associated phosphorylation.* The basic reaction mixture used is described in Table 1. 10 μM dibromothymoquinone plus 0.4 mM ferricyanide was the acceptor system. The buffers (50 mM) employed were: (●, ○, ◑) 2-(*N*-morpholino)ethanesulfonate, (▲, △, ◭) *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate, and (■, □, ◪) tricine. Units for electron transport (II) and phosphorylation (III) are μequiv or μmol ATP × h^{-1} × mg chlorophyll^{-1}. Note that the efficiency, P/e_2 (I), of the phosphorylation is almost independent of pH from 6 to 9

and phosphate: indeed the rate of electron transport during the reduction of dibromothymoquinone (Table 2) or other class III acceptors [6] is quite

Table 2. *The lack of effect of phosphate, ADP or uncouplers on dibromothymoquinone reduction*
The basic reaction mixture (pH 7.5) was as in Table 1. The acceptor system used was 10 μM dibromothymoquinone plus 0.4 mM ferricyanide. Additions were made in small volumes of distilled water (0.05 ml) to the final concentrations indicated below: ADP, 0.5 mM; P_i, 5 mM; methylamine hydrochloride, 10 mM; gramicidin D, 4 μg/ml

Experiment No.	Additions	Electron transport rate
		μequiv. × h^{-1} × mg chlorophyll^{-1}
I	None	251
	ADP	243
	ADP + P_i	250
	ADP + P_i + methylamine	253
II	None	204
	ADP + P_i	205
	ADP + P_i + gramicidin D	221

independent of phosphorylation. It should also be noted that the phosphorylation efficiency with dibromothymoquinone as the electron acceptor is almost independent of pH over a wide range. This is in striking contrast to the effect of pH on the phosphorylation efficiency when class I acceptors are reduced (optimum pH = 8.5—9.0) [12].

Effect of Dichlorophenyl-dimethylurea on Dibromothymoquinone Photoreduction

Although dibromothymoquinone reduction is not affected by electron transport inhibitors acting close to photosystem I (Table 1), it is sensitive to inhibitors such as 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (Gould and Bradeen, unpublished observations) and

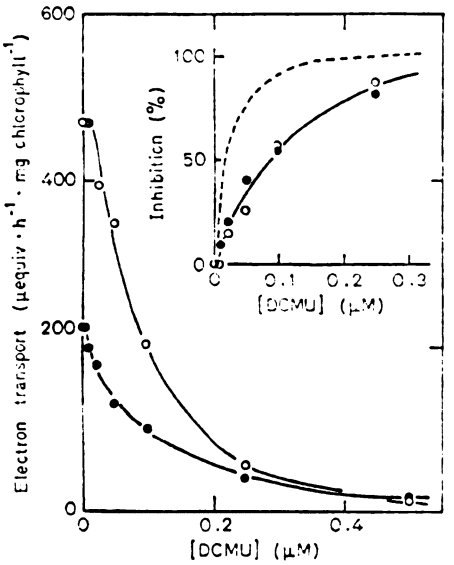

Fig.4. ***Inhibition by dichlorophenyl-dimethylurea of electron transport with various acceptors.*** The basic reaction mixture is described in Table 1. When ferricyanide (0.4 mM) was used as the electron acceptor (O) the reaction das buffered with 50 mM tricine-NaOH pH 8.1. When dibromothymoquinone (10 μM plus 0.4 mM ferricyanide) was the acceptor system (●), the reaction was buffered with 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate pH 7.5. (----) Reduction of oxidised diaminodurene from a separate experiment [1]. Note that at the high light intensities employed the photosystem II reduction of dibromothymoquinone and the reduction of ferricyanide have the same sensitivity to dichlorophenyl-dimethylurea (DCMU) while the reduction of oxidised diaminodurene is much more sensitive

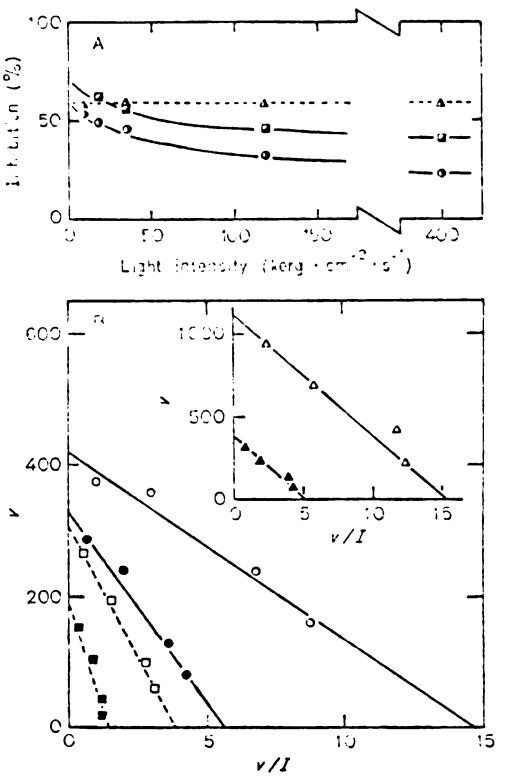

Fig.5. *The effect of light intensity on dichlorophenyl-dimethylurea inhibition of electron transport supported by various acceptors.* Reaction conditions were as described in Fig.4. (△, ▲, ◭) 0.5 mM diaminodurene dihydrochloride plus 1.5 mM ferricyanide as electron acceptor, (○, ●, ◑) ferricyanide reduction and (□, ■, ◪) dibromothymoquinone reduction. Solid symbols, 0.075 μM dichlorophenyl-dimethylurea added. (B) v represents the rate of electron transport in μequiv. $\times h^{-1} \times$ mg chlorophyll^{-1} and I represents the light intensity in kerg $\times$ cm$^{-2} \times$ s^{-1}. The intercept on the v-axis represents the rate of electron transport extrapolated to infinitely high light intensity (V) while the intercept on the v/I-axis gives the quantum efficiency extrapolated to zero light intensity. Note that the primary effect of dichlorophenyl-dimethylurea is to lower the quantum efficiency, presumably by blocking independent photosystem II chains (see Discussion). Dibromothymoquinone also has a pronounced secondary effect on the quantum efficiency. Note also from (A) however, that the inhibition by dichlorophenyl-dimethylurea decreases with increasing light intensity when ferricyanide or dibromothymoquinone are reduced but not when oxidised diaminodurene is reduced

dichlorophenyl-dimethyl urea which block the electron flow close to photosystem II. Fig.4 shows the effect of the latter substance on the rate of dibromothymoquinone reduction. This exhibits about the same sensitivity to dichlorophenyl-dimethylurea inhibition as does the reduction of the class I acceptor ferricyanide. Dibromothymoquinone differs from class III acceptors in this respect, since the reduction of the oxidation products of *p*-phenylenediamine and diaminodurene is extremely sensitive to concentrations of dichlorophenyl-dimethylurea which have very little effect on ferricyanide reduction [1].

The effect of light intensity on dichlorophenyl-dimethylurea inhibition of electron transport with various electron acceptors is shown in Fig.5. As the light intensity becomes rate-limiting, dibromothymoquinone reduction and ferricyanide reduction become more sensitive to this inhibition, while the high dichlorophenyl-dimethylurea sensitivity of the reduction of oxidised diaminodurene remains unchanged. Again dibromothymoquinone behaves more like a class I acceptor than a class III acceptor under these conditions.

These differences in sensitive to dichlorophenyl-dimethylurea and the effects of light intensity on the inhibition could be explained in two ways: either the high rates involved in reduction of oxidised diaminodurene make the dichlorophenyl-dimethylurea site more critically rate-determining or oxidised diaminodurene intercepts electrons close to the site of dichlorophenyl-dimethylurea inhibition (see Discussion). The experiment illustrated in Fig.6 all but eliminates the first possibility. The photoreduction

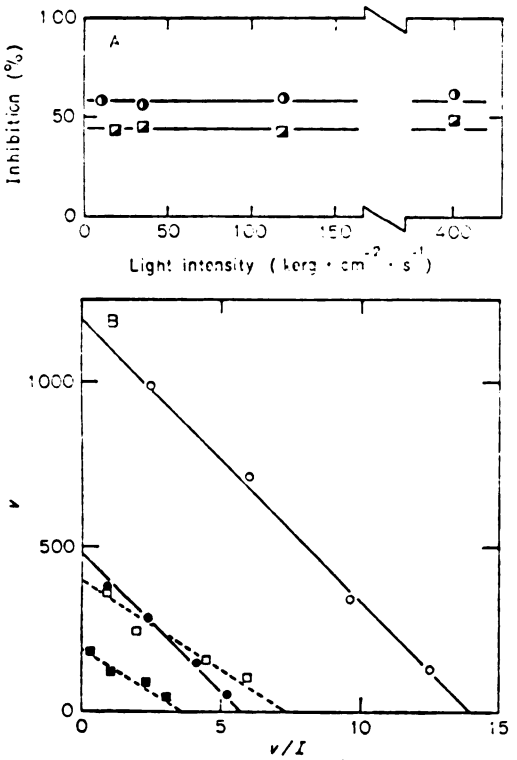

Fig. 6. *Effect of light intensity on dichlorophenyl-dimethylurea inhibition of dimethylbenzoquinone reduction.* The basic reaction mixture (pH 8.1) is described in Table 1. Dimethylbenzoquinone (0.5 mM) plus 0.4 mM ferricyanide was the acceptor system. (○, ◑) No additions; (●, ■) 0.075 μM dichlorophenyl-dimethylurea added; (□, ◪, ■) 0.5 μM dibromothymoquinone added, sufficient to block electron flow to photosystem I but not high enough to accept electrons at a significant rate [8]. Note that the dichlorophenyl-dimethylurea inhibition of dimethylbenzoquinone reduction is independent of light intensity both in the presence and absence of dibromothymoquinone. Note also from (B) that the latter has a secondary effect on the quantum efficiency of this reduction

of 2,5-dimethylbenzoquinone has two components. One component is sensitive to KCN, polylysine and low concentrations of dibromothymoquinone [8]. This component clearly involves both photosystems and is in every way analogous to the reduction of class I acceptors. The other component is insensitive to these three inhibitors and supports phosphorylation with the efficiency characteristic of the reduction of class III acceptors. Clearly dimethylbenzoquinone intercepts some electrons in the same manner as oxidised *p*-phenylenediamine and diaminodurene, but it does so slowly. Thus the residual transport of electrons to dimethylbenzoquinone in the presence of low concentrations of dibromothymoquinone is approximately equal to the rate observed when dibromothymoguinone itself is used as an electron acceptor. Nevertheless dimethylbenzoquinone reduction remains more sensitive to dichlorophenyl-dimethylurea than is dibromothymoquinone reduction and this sensitivity does not vary with light intensity. It should be added here that the quantum efficiency of dibromothymoquinone reduction (*i.e.* dibromothymoquinone-mediated ferricyanide reduction) is only 30% of the normal ferricyanide Hill reaction (Fig. 5B). Apparently dibromothymoquinone has a rather strong secondary effect on the quantum efficiency of photosystem II (see also Fig. 6B). This is also reflected in the fact that at very low light intensities dibromothymoquinone reduction becomes even more sensitive to dichlorophenyl-dimethylurea than does the reduction of oxidised diaminodurene (Fig. 5A).

DISCUSSION

Dibromothymoquinone as an Electron Acceptor

The fact that KCN and polylysine treatments of chloroplasts have no effect on the rate of electron transport (Table 1) virtually proves that photosystem I is not involved in the reduction of dibromothymoquinone since these treatments block the transfer of electrons to P_{700} [3, 4]. Moreover dibromothymoquinone is itself a potent inhibitor, blocking electron transport on the photosystem II side of cytochrome *f* [14]. It follows that dibromothymoquinone must accept electrons either before or at its own site of inhibition. In some respects dibromothymoquinone reduction resembles the reduction of other lipophilic quinones and quinonediimides (class III acceptors) which also accept electrons from a carrier close to photosystem II [1, 6, 8]. However, the reduction of dibromothymoquinone is unlike the reduction of class III acceptors in other respects. For instance, the concentration of dibromothymoquinone which is optimal for electron transport is 50 times lower than the optimal concentration of oxidised diaminodurene or *p*-phenylenediamine. This observation could be interpreted as implying some degree of specificity but it could also be interpreted in terms of a partition coefficient which favors accumulation of dibromothymoquinone in the membranes.

A much more important difference which distinguishes dibromothymoquinone from class III acceptors is the nature of the inhibition of its photoreduction by dichlorophenyl-dimethylurea. The effect of this compound on dibromothymoquinone reduction is similar to the effect on the reduction of ferricyanide (class I) and is quite different from the effect on the reduction of oxidized *p*-phenylenediamine and diaminodurene (class III). The much greater sensitivity of chloroplasts to low concentrations of dichlorophenyl-dimethylurea with class III acceptors (Fig. 4, inset) can readily be explained in terms of the model presented in Fig. 7. This model also explains the fact that dichlorophenyl-dimethylurea inhibition of elec-

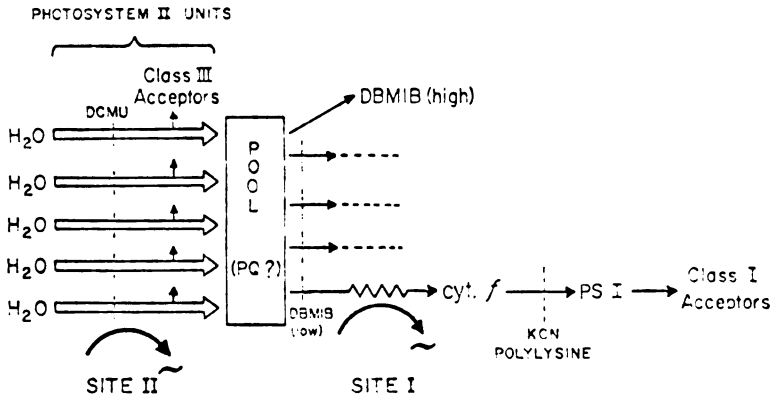

Fig. 7. *A simplified model of electron transport in chloroplasts.* The open arrows represent functional structures each of which contains pigments and a reaction center of photosystem II, a single oxygen-producing site, and a site sensitive to dichlorophenyl-dimethylurea (DCMU). The straight arrows represent electron transfer reactions and curved arrows represent energy conservation reactions (phosphorylation sites). The zig-zagged line at site I indicates the primary rate-limiting step of electron transport to class I acceptors. For further explanation, see the text. DBMIB = dibromothymoquinone

tron transport is independent of light intensity when class III acceptors are reduced but becomes intensity-dependent when class I acceptors are reduced (Fig. 5).

There are good reasons for believing that the photochemistry and much of the associated thermochemistry of photosystem II takes place in independent structural units [15]. In other words, it seems that quanta are converted one by one into charge separations within independent structures. Thus the electrons and holes made available by quantum conversions in one structural unit are not directly available for chemical reactions in other units. We have represented these water-oxidizing, electron-producing photosystem II units in Fig. 7 by the open arrows. While the exact nature of dichlorophenyl-dimethylurea's inhibition is not clear, it seems likely that it acts by somehow inactivating photosystem II units, one molecule of inhibitor totally suppressing the activity of one unit. A partial inhibition by dichlorophenyl-dimethylurea probably means that a certain proportion of the photosystem II units have been inactivated.

However, there is no reason to suppose that photosystem I must be confined to the same unit structures as photosystem II. Consequently it is not unreasonable to suppose that the electrons generated by photosystem II may be pooled at some step before the reduction of P_{700}. Indeed one might expect reduced plastoquinone to serve as a common electron pool interconnecting electron transport chains on the basis of its chemical nature and its abundance. In fact, Siggel *et al.* [16] and Malkin and Michaeli [13] have reached this conclusion from their flash experiments and fluorescence induction studies using chloroplasts poisoned with dichlorophenyl-dimethylurea.

We are now in a position to understand the effect of light intensity on dichlorophenyl-dimethylurea inhibition. When light is limiting, the activity of photosystem II is presumably also limiting and the inhibition will be strictly proportional to the number of units inactivated by dichlorophenyl-dimethylurea. The same will be true, regardless of light intensity, if the electrons are never pooled. However, if the electrons are pooled and the pooled electrons are utilized by a subsequent rate-determining slow step, the situation is quite different. Now a smaller number of functioning photosystem II units can keep the slow reactions draining the electron pool saturated. Thus, as the light intensity increases, fewer and fewer photosystem II units are required and the efficacy of a given concentration of dichlorophenyl-dimethylurea decreases.

These considerations suggest to us that class III acceptors are reduced before the electrons from photosystem II are pooled [1]. Presumably these membrane-permeating strong oxidants react directly with the photosystem II units. Clearly ferricyanide and other class I acceptors must be reduced after the electrons are pooled since their reduction is less sensitive to dichlorophenyl-dimethylurea at high light intensities. Although dibromothymoquinone is highly lipid-soluble and is a moderately strong oxidant, it does not seem to react directly with photosystem II units but rather with the source of pooled electrons. We are tempted to postulate that dibromothymoquinone, by virtue of its structure, reacts in some specific way at the site of plastoquinone involvement in electron transport, accepting electrons from reduced plastoquinone and at the same time blocking further transport of electrons to cytochrome *f*.

It is not immediately clear how the model presented in Fig. 7 can be reconciled with the experiments of Izawa and Good [19], which suggest that the number of dichlorophenyl-dimethylurea inhibition sites is

smaller than the number of O_2-producing units (1 O_2 producing unit per 500 chlorophylls [15]). We are now inclined to question the interpretations offered by Izawa and Good for their observations.

Photosystem II Phosphorylation with Dibromothymoquinone as Electron Acceptor

The data presented in this paper strongly support our contention that there is a phosphorylation reaction associated with the transfer of electrons from water to plastoquinone [8]. Moreover it is very probable that the energy conservation occurs before the electrons produced by individual photosystem II units are pooled. If this is so, each of the units must be equipped with an appropriate energy-conserving mechanism (site II in Fig.7). Perhaps the photosystem II units are so oriented that the photoactivation or subsequent electron transport occurs across the thylakoid membrane and the resulting membrane potential or ion gradient drives phosphorylation, as Witt [17] has suggested. On the other hand the as-yet unassigned cytochrome b_{559} may be associated with photosystem II units and may be involved in some chemical mechanism of energy conservation.

In any event, photosystem II phosphorylation is very different from the better-known phosphorylation associated with the reduction of class I acceptors, both in efficiency and in pH dependence. The lower efficiency with which photosystem II electron transport is coupled to phosphorylation may simply result from the fact that only one of two (or more) in a sequence of phosphorylation sites is being used [6]. On the other hand the complete lack of dependence of electron transport on phosphorylation may reflect a fundamentally inefficient coupling mechanism which in turn may reflect a basic difference between the oxidation-reduction reactions performed at site II and site I. Indeed, the insensitivity to pH of the phosphorylation efficiency at site II (Fig.3) is in striking contrast to the pH dependence of the P/e_2 attributable to site I [12].

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J. M. Gould and S. Izawa, Department of Botany and Plant Pathology, 166 Plant Biology Building, Michigan State University, East Lansing, Michigan 48823, U.S.A.

APPENDIX III

STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION I. SEPARATION OF SITE I AND SITE II BY PARTIAL REACTIONS OF THE CHLOROPLAST ELECTRON TRANSPORT CHAIN

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STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION

I. SEPARATION OF SITE I AND SITE II BY PARTIAL REACTIONS OF THE CHLOROPLAST ELECTRON TRANSPORT CHAIN

J. MICHAEL GOULD and S. IZAWA

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Mich. 48823 (U.S.A.)

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SUMMARY

1. The transport of electrons from H_2O to lipophilic oxidants such as oxidized *p*-phenylenediamines and 2,5-dimethylquinone, when observed in the presence of the plastoquinone antagonist dibromothymoquinone, has a pH optimum of approximately 7.5 and is independent of the presence or absence of ADP and phosphate. Nevertheless the electron transport supports phosphorylation with an efficiency (P/e_2) of 0.3–0.4 and this efficiency is practically pH independent. A reversible proton uptake is associated with the electron transport. The energy coupling site responsible for the phosphorylation, which must be before plastoquinone, we have designated Site II, while the well-known rate-determining coupling site after plastoquinone and before cytochrome *f* is referred to as Site I.

2. The transport of electrons from reduced 2,6-dichlorophenolindophenol (DCIP) to methylviologen, when observed in the presence of the Photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, is remarkably similar in most respects to the overall Hill reaction (*e.g.* $H_2O \rightarrow$ methylviologen). The rate of electron flow is markedly stimulated by ADP and phosphate. Electron transport and phosphorylation have the same pH optimum of about 8.5. The P/e_2 ratio is also strongly pH dependent, showing a similar pH optimum of 8.0–8.5. However, the absolute value of the P/e_2 ratio observed for the partial reaction reduced DCIP→methylviologen is lower than the P/e_2 ratio observed for the overall reaction $H_2O \rightarrow$ methylviologen at all pH values. The maximum P/e_2 value observed for the reduced DCIP→methylviologen raction is 0.5–0.6 at pH 8.0–8.5 while the maximum value for the $H_2O \rightarrow$ methylviologen reaction under the same conditions is about 1.1.

3. When the P/e_2 ratios for the two partial reactions ($H_2O \rightarrow$ dimethylquinone and reduced DCIP→methylviologen) are added together at all pH values from 6 to 9, the resulting curve is very close to the P/e_2–pH profile experimentally obtained for

Abbreviations: P/e_2, the ratio of the molecules of ATP formed per pairs of electrons transported; 4'-deoxyphlorizin, 4,6'-dihydroxy-2'-glucosidodihydrochalcone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxyethyl)methylglycine; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

the overall Hill reaction $H_2O \rightarrow$methylviologen. It seems probable, therefore, that the transport of electrons from reduced DCIP to methylviologen utilizes only the rate-determining coupling Site I while the overall transport of electrons from H_2O to methylviologen utilizes both Site I and Site II.

INTRODUCTION

The existence of two sites of energy coupling associated with noncyclic electron transport in isolated chloroplasts has been postulated for some time based on various lines of indirect evidence[1-7]. A new approach to this problem has recently been made possible with the use of the lipophilic "Class III" electron acceptors (such as oxidized *p*-phenylenediamines)[8]. In previous papers we have shown that the transport of electrons from water to these Class III acceptors is insensitive to the plastocyanin inhibitors KCN[9] and poly-L-lysine[10], and also to the plastoquinone antagonist dibromothymoquinone[11]. These inhibitors strongly inhibit the reduction of conventional (Class I[8]) acceptors such as ferricyanide or methylviologen, which require participation of both Photosystem II and Photosystem I. Furthermore, Class III acceptors are reduced at a point before the electrons from the independent Photosystem II units are pooled[9,12]. From these observations we have concluded that the reduction of Class III acceptors takes place predominately before plastoquinone[12]. Moreover, since the reduction of Class III acceptors is firmly coupled to phosphorylation even in the presence of these inhibitors, we have concluded further that there is a coupling site before plastoquinone (Site II) in addition to the coupling site believed to be located after plastoquinone and before cytochrome *f*[13] (Site I).

The phosphorylation reaction associated with Site II is characterized by (i) a pH optimum between 7 and 8, (ii) a low coupling efficiency ($P/e_2 = 0.3$–0.4) which is practically pH independent, and (iii) the lack of effect of ADP and phosphate or uncouplers on the rate of electron transport. The characteristics of conventional noncyclic photophosphorylation, which utilizes both Site II and Site I, are quite different. The pH optimum for both electron transport and ATP formation is 8.5 or above. The phosphorylation efficiency is also a strong function of pH, showing a similar optimum (where the P/e_2 is 1.0–1.1 in average chloroplast preparations). Furthermore, the rate of electron transport responds sharply to phosphorylating or uncoupling conditions. One may therefore reasonably deduce that these prominent features of conventional noncyclic photophosphorylation originate almost entirely from the coupling reaction at Site I. The existence of a rather inconspicuous coupling reaction at Site II must be largely masked, except for its contribution to the overall efficiency of phosphorylation.

In order to verify these deductions, however, it is essential to find a partial reaction of the electron transport chain which includes only coupling Site I. Such a reaction should very much resemble the complete noncyclic reactions except for its efficiency of phosphorylation (P/e_2), which should be approximately 0.6–0.7, instead of slightly above 1.0.

Larkum and Bonner[14] and Izawa[15], on the basis of spectral evidence, and Neumann *et al.*[16] on the basis of uncoupler studies, have postulated that reduced 2,6-dichlorophenolindophenol (DCIP) in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) donates electrons to the electron transport chain on the Pho-

tosystem II side of the coupling site preceeding cytochrome *f*. The studies reported in this paper have provided strong evidence that the electron flow from reduced DCIP to methylviologen indeed constitutes a partial reaction which includes coupling Site I but not coupling Site II. In addition, we report here and in a subsequent paper[17] on further characterization of the coupling reaction at Site II including the demonstration of a "proton pump" driven by a partial reaction which involves only Site II.

MATERIALS AND METHODS

Chloroplasts were isolated in the cold (4 °C) by a technique similar to that used in previous studies[8,11,12]. Leaves of fresh market spinach (*Spinacia oleracea* L.) were washed in cold distilled water and ground briefly (3–7 s) in a Waring blendor containing a medium consisting of 0.3 M NaCl, 30 mM *N*-tris(hydroxyethyl)methylglycine (Tricine)–NaOH (pH 7.8), 3 mM $MgCl_2$ and 0.5 mM EDTA. After filtering the homogenate through multiple layers of cheesecloth, the chloroplasts were sedimented at 2500 × *g* for 2 min. The pellet was resuspended in a medium containing 0.2 M sucrose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaOH (pH 7.5), 2 mM $MgCl_2$ and 0.05% bovine serum albumin. After a brief centrifugation to remove whole cells and debris (45 s at 2000 × *g*) the chloroplasts were sedimented at 2000 × *g* for 4 min, resuspended in a fresh volume of the same medium, and again sedimented. The final pellet was taken up in a small volume of the suspension media.

The reduction of 2,5-dimethyl-*p*-benzoquinone, oxidized *p*-phenylenediamines and high concentrations of dibromothymoquinone was measured spectrophotometrically as described earlier[8] as the decrease in absorbance of the reaction mixture at 420 nm due to the reduction of excess ferricyanide. Methylviologen reduction (either water or artificial reductants as electron donor) was measured as oxygen uptake resulting from the reoxidation of reduced methylviogen[18]. A Clark-type membrane-covered oxygen electrode was used for assay. No catalase inhibitor was needed since our chloroplast preparations were free of catalase ativity.

Reactions were run in a final volume of 2.0 ml in thermostatted cuvettes at 19 °C. Actinic light (>600 nm) was supplied by a 500-W slide projector and the appropriate filters.

ATP formation was determined for a 1-ml aliquot of the reaction mixture by extracting unreacted ^{32}P-labeled orthophosphate as phosphomolybdic acid into butanol–toluene (1:1, v/v) as detailed by Saha and Good[19]. Radioactivity in the final aqueous phase was measured by the Cerenkov technique of Gould *et al.* [20].

KCN-treated chloroplasts were prepared by incubating chloroplasts at 0 °C in a 30 mM KCN solution buffered at pH 7.8 as described by Ouitrakul and Izawa[9].

Stock solutions of 2,5-dimethyl-*p*-benzoquinone and dibromothymoquinone were made in ethanol–ethylene glycol (1:1, v/v). DCIP was dissolved in ethanol and diluted with glass distilled water. DCMU was dissolved in ethanol and further diluted with 0.01 M NaCl. At all times the concentration of organic solvent in the final reaction mixture was 1% or less.

RESULTS

The effect of pH on the rate of electron transport and phosphorylation using three different electron donor–acceptor systems is shown in Fig. 1. When electrons

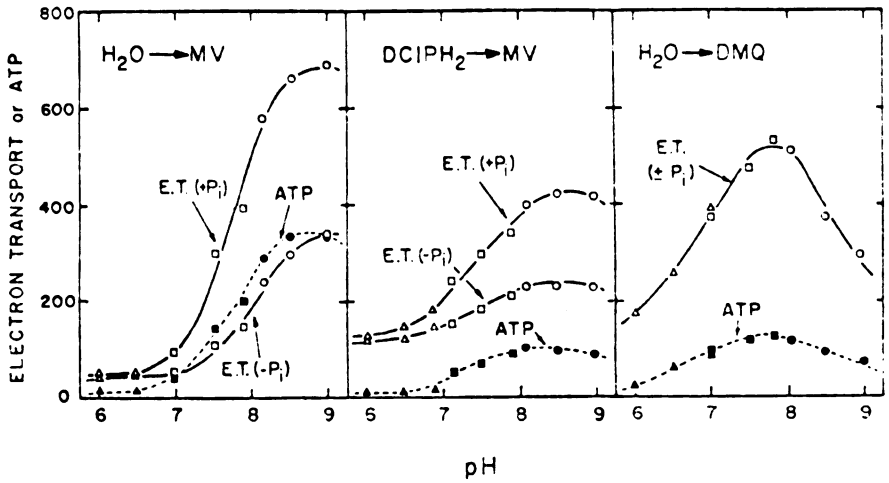

Fig. 1. Effect of pH on the rates of electron transport and phosphorylation associated with various electron donor–acceptor systems. The reaction mixture (2 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM buffer, 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$ (when added), chloroplasts containing 40 μg chlorophyll, and the indicated electron donor or acceptor system. These systems were: methylviologen, 50 μM; 2,5-dimethyl-*p*-benzoquinone, 0.5 mM *plus* 0.4 mM ferricyanide; reduced DCIP ($DCIPH_2$), 0.4 mM *plus* 2.5 mM ascorbate, 1 μM DCMU and 50 μM methylviologen. The buffers employed were 2-(*N*-morpholino)ethanesulfonic acid–NaOH (triangles), HEPES–NaOH (squares) and Tricine–NaOH (circles). Open symbols are for electron transport (E.T.) and solid symbols are for phosphorylation (ATP). When 2,5-dimethyl-*p*-benzoquinone was the electron acceptor 0.5 μM dibromothymoquinone was added to the reaction mixture to block the Class I component of 2,5-dimethyl-*p*-benzoquinone reduction. Rates are in μequiv or μmoles ATP/h per mg chlorophyll. Note the similarity between H_2O→methylviologen and reduced DCIP→methylviologen but not H_2O→2,5-dimethyl-*p*-benzoquinone. Abbreviations: MV, methylviologen; $DCIPH_2$, reduced DCIP; DMQ, 2,5-dimethyl-*p*-benzoquinone.

from water reduce the Class I acceptor methylviologen through the two sites of energy coupling, both electron flow and phosphorylation show a pH optimum around pH 8.5. The rate of electron transport in the absence of phosphate (basal rate) is much slower but shows a similar pH optimum. This is in good agreement with previous reports for ferricyanide reduction and the associated phosphorylation[1]. When reduced DCIP serves as electron donor and methylviologen as acceptor (in the presence of DCMU) the effect of pH is very similar to that observed for the H_2O→methylviologen reaction. Again the optimal pH for electron transport and phosphorylation is about 8.5 and a marked stimulation of electron transport by the concomitant phosphorylation is observed. Clearly the two reaction systems H_2O→methylviologen and reduced DCIP →methylviologen are governed by the same rate-limiting phosphorylation reaction. However, when the Class III acceptor 2,5-dimethyl-*p*-benzoquinone is reduced *via* Photosystem II by electrons from water, a different effect of pH is evident. 2,5-Dimethyl-*p*-benzoquinone reduction and the associated phosphorylation (which involves only coupling Site II) exhibit a considerably more acidic pH optimum than is observed for the H_2O→methylviologen system or the reduced DCIP→methylviologen system.

The effect of pH on the phosphorylation efficiency (P/e_2) of each of the three types of electron donor–acceptor systems mentioned above produced some striking results (Fig. 2). The P/e_2 ratio for the H_2O→methylviologen system is strongly pH

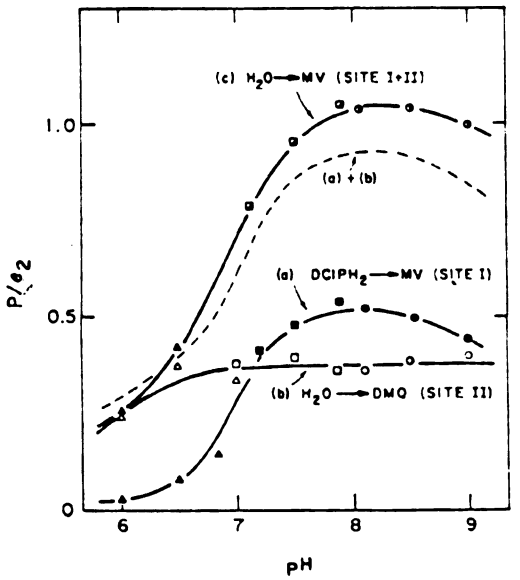

Fig. 2. Effect of pH on the phosphorylation efficiency (P/e_2) of three different electron donor–acceptor systems. P/e_2 values for each system were computed from the data presented in Fig. 1. Note that if the P/e_2 values for H_2O→2,5-dimethyl-*p*-benzoquinone (Photosystem II only) are added to the values for reduced DCIP→methylviologen (Photosystem I only), the sum (dashed curve) is close to the P/e_2 values obtained for H_2O→methylviologen (Photosystem II *plus* Photosystem I). Note also that the P/e_2 ratio associated with 2,5-dimethyl-*p*-benzoquinone reduction is practically constant over a wide pH range, whereas the P/e_2 ratios for H_2O→methylviologen and reduced DCIP→methylviologen are strongly pH dependent. Abbreviations: see Fig. 1.

dependent, showing a pH optimum of about 8 to 8.5. In contrast, the P/e_2 ratio for the H_2O→2,5-dimethyl-*p*-benzoquinone system, which utilizes only coupling Site II is essentially pH independent from pH 6.5 to 9. Thus the pH-dependent portion of the P/e_2 ratio for the H_2O→methylviologen system must be located after the site of 2,5-dimethyl-*p*-benzoquinone reduction. The effect of pH on the P/e_2 ratio associated with the reduced DCIP→methylviologen reaction once again resembles very closely the effect observed for the H_2O→methylviologen system. This similarity again suggests that the coupling site associated with the reduced DCIP→methylviologen system may be the same rate-limiting coupling site (Site I) associated with the H_2O→methylviologen reaction. However, the P/e_2 values observed for the reduced DCIP→methylviologen system are markedly lower than those for the H_2O→methylviologen system over the entire range of pH values tested, as though a pH-independent component is absent in the reduced DCIP system. Indeed, when the P/e_2 values for the two partial reactions (H_2O→2,5-dimethyl-*p*-benzoquinone and reduced DCIP→methylviologen) are added together, the resulting curve (Fig. 2, dashed line) is in fact very close to the experimentally obtained curve for the overall reaction H_2O→methylviologen. This implies very strongly that the partial reaction reduced DCIP→methylviologen is indeed utilizing only the coupling site that normally limits the rate of electron transport to Class I acceptors (*i.e.* Site I).

The curve derived by adding the P/e_2 values for the two partial reactions is

slightly lower than the observed curve for $H_2O \rightarrow$methylviologen, reflecting the fact that the P/e_2 ratios observed for the reduced DCIP→methylviologen reaction (maximum 0.55) are slightly lower than the values one would expect (0.6–0.7) from the difference in P/e_2 between the complete system $H_2O \rightarrow$methylviologen and the partial system $H_2O \rightarrow$2,5-dimethyl-*p*-benzoquinone. This discrepancy could be explained in two ways. If reduced DCIP had a secondary, uncoupling effect the P/e_2 values for the reduced DCIP→methylviologen reaction would be lowered. This seems unlikely, however, since it has already been shown that the reaction exhibits a constant P/e_2 over a wide range of reduced DCIP concentrations[21]. To further eliminate this possibility the effect of reduced DCIP on the post-illumination phosphorylation ("X_E") was examined. If reduced DCIP had an uncoupling action, then its presence in the dark (phosphorylation) stage of the experiment should decrease the yield of X_E. The high sensitivity of this method in detecting an uncoupling effect has been previously demonstrated by Hind and Jagendorf[22]. Table I shows that neither reduced DCIP nor 2,5-dimethyl-*p*-benzoquinone decreases the X_E yield to any significant extent, thus practically eliminating the possibility of these compounds having a significant uncoupling effect. These results are also important in that they ensure that the low efficiencies of phosphorylation observed for systems involving 2,5-dimethyl-*p*-benzoquinone ($P/e_2 = 0.3$–0.4) or reduced DCIP ($P/e_2 = 0.5$–0.6) are not due to an uncoupling effect of these compounds.

TABLE I

EFFECTS OF REDUCED DCIP *PLUS* ASCORBATE AND 2,5-DIMETHYL-*p*-BENZOQUINONE ON POST-ILLUMINATION ATP FORMATION (X_E)

Reduced DCIP (0.13 mM *plus* 0.8 mM ascorbate), 2,5-dimethyl-*p*-benzoquinone (0.17 mM) or methylamine (3.3 mM) were present only in the dark phosphorylation stage of the experiment. Chloroplasts containing 100 μg chlorophyll were illuminated for 20 s in a continuously stirred reaction mixture (2 ml) containing 0.1 M sucrose, 50 mM NaCl, 2 mM $MgCl_2$, 10 mM 2-(*N*-morpholino)ethanesulfonic acid–NaOH buffer (pH 6.0) and 5 μM pyocyanine. Immediately after shutting off the light 1 ml of a strongly buffered ADP–phosphate mixture (0.1 M Tricine–NaOH buffer (pH 8.2), 2 mM ADP, 10 mM $Na_2H^{32}PO_4$) containing the additions was quickly injected into the suspension to initiate ATP formation. After 20 s the dark phosphorylation was terminated by addition of 0.5 ml 1 M $HClO_4$. All reactions were run in a thermostated water bath at 19 °C. Note that both reduced DCIP (*plus* ascorbate) and 2,5-dimethyl-*p*-benzoquinone did not inhibit the yield of X_E, whereas the known uncoupler methylamine did.

Addition (dark stage)	*Expt*	*ATP formed (nmoles/100 μg chlorophyll)*	*Effect*
None	a	7.4	—
	b	7.7	
Reduced DCIP	a	9.2	Slight stimulation
	b	8.4	
2,5-Dimethyl-*p*-benzoquinone	a	6.9	None
	b	7.5	
Methylamine	a	2.6	Inhibition
	b	2.4	

TABLE II

EFFECT OF KCN TREATMENT ON ELECTRON TRANSPORT AND PHOSPHORYLATION ASSOCIATED WITH THE PHOTOSYSTEM I-DEPENDENT REACTION REDUCED DCIP→METHYLVIOLOGEN

Reaction conditions are as described in Fig. 1. KCN treatment of chloroplasts is described in Methods. The rates of electron transport (E.T.) and phosphorylation (ATP) are given in μequiv or μmoles ATP/h per mg chlorophyll. Electron transport from H_2O to methylviologen in the KCN-treated chloroplasts used here was completely inhibited.

Reaction pH	*Control chloroplasts*		*KCN-treated chloroplasts*	
	E.T.	*ATP*	*E.T.*	*ATP*
6.0	127	4	90	2
7.0	240	49	123	5
8.0	397	102	154	4
9.0	420	93	148	1

Alternatively, some of the electrons from reduced DCIP may be donated to Photosystem I *via* a secondary, nonphosphorylating pathway. In fact, there is already strong evidence for this possibility. Ouitrakul and Izawa[9] have shown that the phosphorylation associated with the reaction reduced DCIP→methylviologen is abolished by KCN treatment but the electron transport itself is only partially inhibited[9]. Recently, Izawa *et al.*[23] have shown by EPR studies that reduced DCIP can donate electrons directly to P_{700}, by-passing a KCN block at plastocyanin. They suggested that this portion of the donor reaction is not coupled to phosphorylation. Table II shows the effect of KCN treatment on the electron transport from reduced DCIP to methylviologen and the associated phosphorylation. Undoubtedly the reduced DCIP→methylviologen reaction contains a minor component which is KCN resistant and not coupled to phosphorylation. (It should be noted here that KCN treatment itself has no appreciable uncoupling or inhibitory effect on phosphorylation[9].) It therefore seems reasonable to conclude that the "true" P/e_2 values for this partial reaction are slightly higher than those shown in Fig. 2 (curve a). Thus the true sum of the P/e_2 values obtained for the partial reactions reduced DCIP→methylviologen (involving Site I only) *plus* H_2O→2,5-dimethyl-*p*-benzoquinone (involving Site II only) must indeed be very close to the values obtained for the overall reaction H_2O→methylviologen (Site II *plus* Site I).

The conclusion that the complete noncyclic electron transport H_2O→methylviologen and the partial electron transport reduced DCIP→methylviologen are governed by the same energy coupling reaction (Site I), is further strengthened by the experiments of Fig. 3 in which the effects of the energy transfer inhibitor 4'-deoxyphlorizin[31] on electron transport and phosphorylation were examined. In both systems ATP formation and that portion of the electron transport which is dependent upon the presence of ADP and phosphate are inhibited in a very similar manner. However, the phlorizin derivative has no effect on electron transport from H_2O to oxidized diaminodurene (a Class III acceptor[8]) either in the presence or absence of phosphate, although phosphorylation is inhibited. These observations are directly in line with the concept that the coupling site near Photosystem II (Site II) has no control over

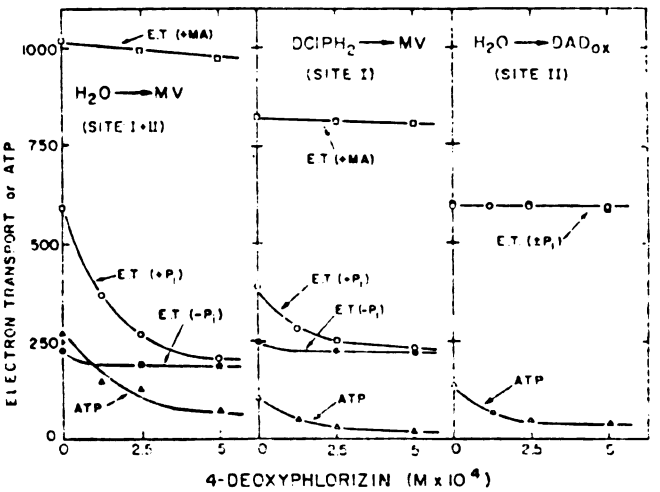

Fig. 3. Effect of the energy transfer inhibitor 4′-deoxyphlorizin on electron transport and phosphorylation associated with different electron donor–acceptor systems. Reaction conditions are essentially as described in Fig. 1. The buffer used was 50 mM Tricine–NaOH (pH 8.0). Methylamine (MA) was 10 mM when added. The chlorophyll concentration was 20 μg/ml. When oxidized diaminodurene (DAD_{ox}) was the electron acceptor the chlorophyll was 10 μg/ml and 0.5 μM dibromothymoquinone was added to block the Class I component of oxidized diaminodurene reduction. Rates of electron transport (E.T.) and phosphorylation (ATP) are in μequiv or μmoles ATP/h per mg chlorophyll. Note that for H_2O → methylviologen and reduced DCIP → methylviologen both ATP formation and that portion of the electron transport dependent upon phosphorylation are inhibited by 4′-deoxyphlorizin. However, when the Class III acceptor oxidized diaminodurene is being reduced, electron transport is not affected by the absence of phosphate or the presence of 4′-deoxyphlorizin, although phosphorylation is inhibited by the latter. Also note that ATP formation associated with all three electron donor–acceptor systems exhibits about the same sensitivity to 4′-deoxyphlorizin. Abbreviations: see Fig. 1.

electron transport while the site between plastoquinone and cytochrome *f* (Site I) does.

Relevant to these findings is the question of why the reduction of oxidized *p*-phenylenediamines (typical Class III acceptors) did not seem to be stimulated by ADP and phosphate at all[8], despite the fact that some portions of these acceptors are reduced *via* the complete electron transport pathway (as are Class I acceptors), utilizing both coupling sites (Site II and Site I)[9]. We have reinvestigated this problem and found that the concomitant phosphorylation does stimulate electron flow quite consistently (Table III). The stimulation may seem quite small, but this is simply because the very fast reduction of these compounds by Photosystem II. The absolute stimulation is in fact approximately equivalent to the stimulation observed when electrons flow from water to methylviologen. However, when this "Class I component" of the reduction of Class III acceptors is eliminated by the addition of low concentrations of dibromothymoquinone (or by KCN treatment[9]), the electron transport, now mediated only by Photosystem II and utilizing only coupling Site II, becomes completely independent of phosphorylating conditions. The genuine Photosystem II electron transport from H_2O to dibromothymoquinone (high concentration) is also not influenced by phosphorylation (Table III) or by uncouplers[12].

TABLE III

EFFECT OF PHOSPHORYLATING CONDITIONS ON ELECTRON TRANSPORT AS A FUNCTION OF THE ELECTRON ACCEPTOR

The 2.0-ml reaction mixture contained 0.1 M sucrose, 2 mM $MgCl_2$, 1 mM ADP, 5 mM $Na_2H^{32}PO_4$ (if added), 50 mM Tricine-NaOH (pH 8.1), chloroplasts, and the indicated acceptor. These acceptors were: methylviologen, 50 μM; oxidized *p*-phenylenediamine, 0.5 mM *plus* 1.5 mM ferricyanide; dibromothymoquinone, 4.5 μM *plus* 0.4 mM ferricyanide. The concentration of chlorophyll was 20 μg/ml when methylviologen or dibromothymoquinone acted as the electron acceptor and 15 μg/ml when oxidized *p*-phenylenediamine was the electron acceptor. Rates are given in μequiv or μmoles ATP/h per mg chloropnyll. Note that the reduction of oxidized *p*-phenylenediamine contains a phosphate-sensitive Class I component. If this component is abolished with a low concentration of dibromothymoquinone (which blocks electron transport to cytochrome *f*[30]) the effect of phosphate on oxidized *p*-phenylenediamine reduction is eliminated, even though a substantial rate of phosphorylation remains. Similarly, the Photosystem II-driven reduction of high concentrations of dibromothymoquinone, which accepts electrons from plastoquinone[12], is firmly coupled to ATP formation even though the electron transport shows no effect by phosphate.

Electron transport system	*Acceptor class**	*Coupling site involved***	*Electron transport rate*				
			$-P_i$	$+P_i$	*ΔE.T.*	*ATP*	P/e_2
$H_2O \rightarrow$ methylviologen	I	Site II + Site I	190	458	(268)	276	1.20
$H_2O \rightarrow$ ox. *p*-phenylenediamine	III (+I)	Site II (+ Site I)	1370	1560	(190)	405	0.52
$H_2O \rightarrow$ ox. *p*-phenylenediamine (+ $5 \cdot 10^{-7}$ M dibromothymoquinone)	III	Site II	860	860	(0)	185	0.43
$H_2O \rightarrow$ dibromothymoquinone	III	Site II	198	200	(2)	34	0.34

* See ref. 8 and Introduction.

** Proposed sites of phosphorylation (see Fig. 5 and refs 9, 11, 12).

Fig. 4 shows a light induced pH rise ("proton uptake") associated with coupling Site II when 20 μM dibromothymoquinone was the electron acceptor. It can be seen that at pH 8.1, where reduced dibromothymoquinone is rapidly reoxidized by molecular oxygen[12], repeated periods of illumination induced the familiar reversible pH rise in the reaction medium. If DCMU is added (abolishing electron transport) the pH rise is also abolished. Similary, at pH 7.4 a brief illumination induces the pH rise. However, at this pH reduced dibromothymoquinone is not reoxidized by molecular oxygen. Thus, repeated illuminations do not induce a pH rise once all of the dibromothymoquinone is reduced and electron transport cannot proceed. This is comfirmed by the fact that addition of oxidized dibromothymoquinone restores the pH rise. The Photosystem II-dependent pH rise is also abolished by conventional uncouplers such as methylamine and gramicidin.

DISCUSSION

In previous papers[9,11,12] we have amply documented evidence that there is a site of energy coupling (Site II) near Photosystem II or, more specifically, before plastoquinone, in addition to the well-recognized coupling site (Site I) which lies between plastoquinone and cytochrome *f* and governs the rate of noncyclic electron

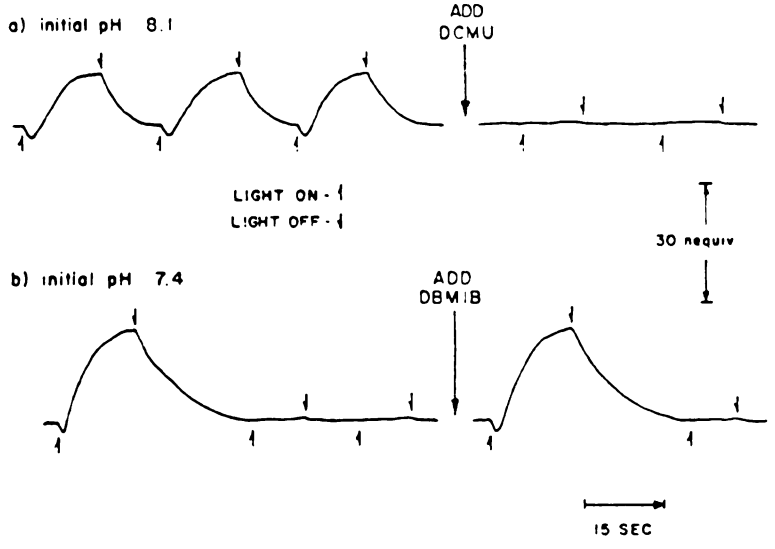

Fig. 4. Light-induced pH rise in the medium ("proton uptake") associated with the reduction of dibromothymoquinone by Photosystem II. The continuously stirred sample was illuminated in a thermostatted cuvette (19 °C) with strong white light. The change of pH of the medium was recorded using a Sargent miniature combination electrode and a Corning expanded scale pH electrometer. The scale was calibrated by adding in the light a known amount of HCl. The 2.0-ml sample contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM NaCl, 20 μM dibromothymoquinone, 0.5 mM Tricine–NaOH buffer at the indicated pH and chloroplasts containing 140 μg chlorophyll. When DCMU was added (Expt a) the final concentration was 2.1 μM. In Expt b 40 nmoles dibromothymoquinone (DBMIB) were added to restore the initial pH rise.

flow[13,24]. The main body of evidence comprises the fact a unique type of phosphorylation is observed when the lipophilic Class III oxidants (*e.g.* 2,5-dimethyl-*p*-benzoquinone, oxidized *p*-phenylenediamines, *etc.*) are reduced *via* Photosystem II alone. To observe these genuine Photosystem II reactions, the simultaneous reduction of the acceptors through Photosystem I needs to be eliminated (a) by blocking plastoquinone with dibromothymoquinone[25,26] or (b) by inactivating plastocyanin with KCN[9,10,23] or (c) by using dibromothymoquinone itself as a Class III acceptor (at high concentrations)[12]. The phosphorylation efficiency (P/e_2) of these Photosystem II reactions is always between 0.3 and 0.4, regardless of the acceptor used[9,11,12] and therefore regardless of the electron transport rate, which ranges from about 50 μequiv/h per mg chlorophyll when dibromothymoquinone is used as the acceptor to about 1500 when oxidized *p*-phenylenediamine is the electron acceptor. (This makes it extremely unlikely that the phosphorylation associated with the reduction of Class III acceptors is due to a Photosystem II-catalyzed cyclic electron flow.) The efficiency of phosphorylation supported by Site II is practically independent of pH over a wide range (ref. 12; see also Fig. 2, this paper). The electron transport is not stimulated by ADP and phosphate (ref. 12; see also Table III, this paper). When the electron transport chain is not blocked at plastoquinone or at plastocyanin, that is, when Class III acceptors are being reduced in part by Photosystem II alone and in part *via* both Photosystem II and Photosystem I, then the characteristics of the associated phosphorylation reactions become intermediate between those outlined above and those observed for

standard noncyclic photophosphorylation reactions such as with ferricyanide or methylviologen (Class I acceptors). There seems no doubt that we have succeeded, by the combined use of Class III acceptors and the inhibitors which block electron transport at or after plastoquinone, in disclosing and functionally "isolating" a coupling site (Site II) located before plastoquinone.

In this paper we have presented strong evidence which shows that the Photosystem I-dependent transport of electrons from reduced DCIP to methylviologen involves only Site I (Figs 1–3). The noninvolvement of Site II in this reaction is also consistent with the fact that the reaction is totally insensitive to dibromothymoquinone[25]. The lack of dibromothymoquinone inhibition indicates that plastoquinone, and therefore the coupling site before plastoquinone (Site II), does not participate in the reduced DCIP→methylviologen partial reaction.

Photosystem I-dependent reactions with reduced DCIP as electron donor have often been regarded as complex, involving a cryptic cyclic phosphorylation which could make phosphorylation appear completely unrelated to the observed rates of electron flow[32]. However, at least under the conditions employed in this study, the relation of phosphorylation to observed electron flow seems quite rigid, judging by the effect of ADP and phosphate, the uncoupler methylamine, and the energy transfer inhibitor 4′-deoxyphlorizin. Trebst and Pistorius[27] have presented brief data which led them to the same conclusion. Neumann *et al.*[16] have postulated, based on their uncoupler studies, that the reduced DCIP→methylviologen reaction and the H_2O→methylviologen reaction share the same rate-limiting phosphorylation site (our Site I). Shavit and Shoshan[28] have pointed out that high concentrations of ATP (where ATP acts as an energy transfer inhibitor) affect the reduced DCIP→$NADP^+$ system and the H_2O→$NADP^+$ system in a very similar manner. However, a detailed comparison of the phosphorylation reactions associated with the reduced DCIP system and the complete noncyclic system, such as we have presented here, has not been previously reported.

The failure of earlier investigators to detect significant phosphorylation associated with the photooxidation of reduced DCIP by Photosystem I may have been due largely to the nature of the chloroplast material used. Swollen, broken, or otherwise "leaky" chloroplast preparations give a high rate of electron transport (reduced DCIP→methylviologen) with very little, if any, phosphorylation. This electron transport is mostly KCN insensitive (unpublished data of J. M. Gould) and therefore probably represents increased access of reduced DCIP directly to P_{700} (ref. 23). Larkum and Bonner[14] have found that the reduced DCIP-induced cytochrome *f* response is also greatly diminished in broken chloroplasts.

The main conclusions we have drawn from this study are summarized in the scheme presented in Fig. 5. When Class I acceptors (*e.g.* methylviologen) are being reduced by electrons from water or reduced DCIP, the rate of electron flow is limited by the energy coupling reaction between plastoquinone and cytochrome *f* (Site I). The electron flux through Site I responds strongly to the addition of ADP and phosphate, uncouplers, or energy transfer inhibitors. The efficiency of phosphorylation (P/e_2) at this site is also pH dependent, having a maximum at pH 8.0–8.5 (observed maximum P/e_2, 0.5–0.6; predicted, 0.6–0.7). As mentioned above, Site II exerts no apparent control over electron flow. The efficiency of phosphorylation at Site II ($P/e_2 = 0.3$–0.4) is essentially pH insensitive. One possible explanation for this pH

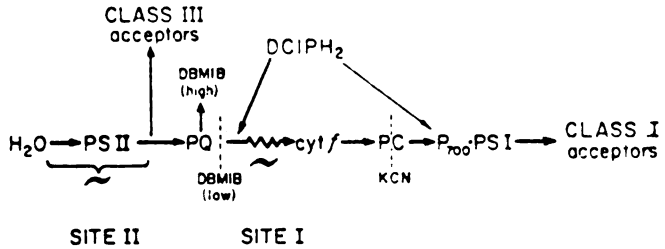

Fig. 5. A scheme for electron transport pathways in isolated chloroplasts showing the two sites of energy coupling (~). PS II, Photosystem II; DBMIB, dibromothymoquinone; PQ, plastoquinone; cyt *f*, cytochrome *f*; PC, plastocyanin; P_{700}, primary donor to Photosystem I (PS I). The zig-zagged line at Site I represents the primary rate-limiting step of electron transport to Class I acceptors. Class I acceptors include methylviologen, ferricyanide, flavins and ferredoxin-$NADP^+$. Class III acceptors include oxidized *p*-phenylenediamine, oxidized diaminodurene, 2,5-dimethyl-*p*-benzoquinone, *etc.* Since Class III acceptors can also act to some extent as Class I acceptors, genuine Photosystem II reactions are observed only when plastoquinone or plastocyanin is blocked (by dibromothymoquinone or KCN, respectively). Thus, in the presence of these inhibitors, the partial reaction $H_2O \rightarrow$Class III acceptor includes coupling Site II but not Site I. Similarly, the partial reaction reduced DCIP→Class I acceptor includes coupling Site I but not Site II, whereas the overall reaction $H_2O \rightarrow$Class I acceptor includes both Site II and Site I. Note also that the reduced DCIP→Class I acceptor contains both a KCN-sensitive and KCN-insensitive component.

insensitivity could be that coupling Site II is buried in a hydrophobic region of the membrane and therefore does not "see" the medium pH. This is consistent with the idea put forth by Böhme and Trebst[6] and Yamashita and Butler[7] that there is a coupling site on the water oxidizing side of Photosystem II, perhaps associated with the water-splitting reaction itself. It has been suggested that the protons lost during the oxidation of water are released to the inside of the thylakoid[29].

$$H_2O \rightarrow 2e^- + \tfrac{1}{2}O_2 + 2H^+_{(inside)}$$

The pH rise observed in the external medium when dibromothymoquinone (high concentration) is the electron acceptor (Fig. 4) would therefore represent the loss of protons from the medium for the reduction of the lipophilic dibromothymoquinone

$$2e^- + \text{dibromothymoquinone} + 2H^+_{(outside)} \rightarrow \text{reduced dibromothymoquinone}$$

in the thylakoid membrane. It is therefore probable that a transmembrane proton gradient is associated with the pathway $H_2O \rightarrow$Photosystem II→dibromothymoquinone as well as with reduced DCIP→Photosystem I→methylviologen[21].

Finally, it should be mentioned that the relationship between Site I (see Fig.5) and the two coupling sites postulated by Neumann *et al.*[16] to be associated with Photosystem I is still unclear. Preliminary experiments have indicated that ATP formation coupled to the Photosystem I-dependent electron flow from diaminodurene to methylviologen may not utilize the coupling site which limits the H_2O (or reduced DCIP) →methylviologen reaction (unpublished data of J. M. Gould). Experiments are in progress to determine if further subdivision of Site I into two sites may be necessary.

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APPENDIX IV

STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION III. THE DIFFERENT EFFECTS OF METHYLAMINE AND ADP PLUS PHOSPHATE ON ELECTRON TRANSPORT THROUGH COUPLING SITES I AND II IN ISOLATED CHLOROPLASTS

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STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION

III. THE DIFFERENT EFFECTS OF METHYLAMINE AND ADP *PLUS* PHOSPHATE ON ELECTRON TRANSPORT THROUGH COUPLING SITES I AND II IN ISOLATED CHLOROPLASTS

J. MICHAEL GOULD and DONALD R. ORT

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Mich. 48824 (U.S.A.)

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SUMMARY

1. The reduction of lipophilic (Class III) oxidants such as oxidized *p*-phenylenediamine consists of two components. One component requires both Photosystem II and Photosystem I and includes both sites of energy coupling associated with noncyclic electron transport. The second component requires only Photosystem II and includes only the site of energy coupling located before plastoquinone (Site II). When oxidized *p*-phenylenediamine is being reduced by both pathways, the overall rate of electron transport is stimulated by the addition of ADP *plus* phosphate or the uncoupler methylamine. However, if the Photosystem I component of oxidized *p*-phenylenediamine reduction is eliminated by a low concentration of the plastoquinone-antagonist dibromothymoquinone, the stimulation of electron transport by ADP *plus* phosphate or methylamine is also abolished, although the remaining Photosystem II-dependent electron transport remains firmly coupled to phosphorylation (*via* coupling Site II). These results indicate that coupling Site II, unlike the well-known rate-limiting coupling site between plastoquinone and cytochrome *f* (Site I), does not exert any control over the rate of associated electron transport.

2. When substituted *p*-benzoquinones (*e.g.* 2,5-dimethyl-*p*-benzoquinone) or quinonediimides (*e.g.* *p*-phenylenediimine) are used as Class III acceptors in conjunction with dibromothymoquinone, a small but significant stimulation of electron transport by ADP *plus* phosphate is observed. However, it can be shown that this stimulation does not arise from coupling Site II but rather is due to a low rate of electron flux through coupling Site I even in the presence of dibromothymoquinone. Apparently the *p*-benzoquinones can catalyze an electron "bypass" around the dibromothymoquinone-induced block at plastoquinone, possibly by substituting partially for the natural electron carrier. If this bypass electron flow is blocked at plastocyanin by KCN treatment, the stimulation of electron transport by ADP *plus* phosphate is eliminated, although a high rate of phosphorylation (from Site II only) remains.

Abbreviations: P/e_2 ratio, the ratio of the number of molecules of ATP formed to the number of pairs of electrons transported; dimethylquinone (DMQ), 2,5-dimethyl-*p*-benzoquinone.

3. These results provide strong evidence that a profound difference exists between the two sites of energy coupling associated with non-cyclic electron transport in isolated chloroplasts. That is, the rate of electron flow through coupling Site I, which is the rate-determining step in the Hill reaction, is strictly regulated by phosphorylating conditions, whereas the rate of electron flux through coupling Site II is independent of phosphorylating conditions.

4. A model is presented which accounts for the lack of control over electron transport exhibited by coupling Site II. It is postulated that Site II is coupled to an essentially irreversible electron transport step, so that conditions which affect the phosphorylation reaction would have no effect on the rate of electron transport through the coupling site. Two essentially irreversible reactions, closely associated with Photosystem II —the water-splitting reaction and the System II photoact itself— are discussed as possible locations for coupling Site II.

INTRODUCTION

Saha *et al.*[1] were the first to point out that lipophilic strong oxidants such as oxidized *p*-phenylenediamines could intercept electrons from the chloroplast electron transport chain primarily at a point between the two photosystems. Subsequent work has shown that the preponderant portion of the electron transport to these "Class III" oxidants is insensitive to the plastocyanin inhibitors KCN (ref. 2) and poly(L)-lysine (ref. 3) and the plastoquinone antagonist dibromothymoquinone[4,5]. Thus, when electron flow to Photosystem I is blocked by one of these inhibitors, Class III acceptors are reduced by an electron pathway which includes only Photosystem II. Recently our laboratory[4,5] and Trebst's laboratory[6] have shown that there is a site of energy conservation closely associated with Photosystem II-driven photoreduction of Class III acceptors.

Evidence is now accumulating that this newly discovered coupling site close to Photosystem II differs in several fundamental aspects from the well-known coupling site located after plastoquinone and before cytochrome *f*[7,8]. When the two coupling sites are functionally isolated by partial reactions of the electron transport chain[9], the coupling site between plastoquinone and cytochrome *f* (Site I) exhibits a pH-dependent phosphorylation efficiency (P/e_2 ratio) (optimal $P/e_2 = 0.6$ at pH 8.0–8.5) whereas the coupling site located before plastoquinone (Site II) is less efficient, with a pH-independent P/e_2 ratio of 0.3–0.4. In addition, we have noted[9,10] that coupling Site II apparently exerts no control over the rate of coupled electron transport. That is, the rate of electron flux through coupling Site II is not stimulated by the presence of uncouplers or ADP *plus* phosphate (P_i). Conversely, Site I exerts tight control over the associated electron flow, responding sharply to the presence of phosphorylating or uncoupling conditions. From these results we have concluded that coupling Site I alone constitutes the rate-determining step in the reduction of conventional Hill oxidants such as ferricyanide or methylviologen[9].

However, Trebst and Reimer[6] have reported data which indicates that electron transport through coupling Site II is regulated by phosphorylating conditions. They reported that the reduction of substituted *p*-benzoquinones in the presence of dibromothymoquinone was stimulated by the addition of ADP *plus* P_i or amine

uncouplers. In an effort to resolve the apparent discrepancy between their data and our own, we have re-examined this problem in considerable detail. In this paper we report conclusive evidence that substituted *p*-benzoquinones such as 2,5-dimethylquinone not only accept electrons at a point before the site of dibromothymoquinone inhibition[5] (*i.e.* before plastoquinone[11]) but also catalyze a "bypass" around the dibromothynoquinone block, allowing electrons to pass through coupling Site I to Photosystem I. When KCN[2] is used to block the bypass electron flow by inactivating plastocyanin[12], no stimulation of electron transport by ADP *plus* P_i is observed, indicating that Site II in fact does not exert control over coupled electron transport. This important difference in the properties of coupling Sites I and II may reflect a fundamental difference in the mode of energy transduction at the two sites.

EXPERIMENTAL METHODS

The techniques employed in this study were similar to those described in previous papers. Chloroplasts were isolated from fresh market spinach (*Spinacia oleracea* L.) as described earlier[9]. The photoreductions of 2,5-dimethylquinone and oxidized *p*-phenylenediamine were measured spectrophotometrically as the decrease in absorbance of the reaction mixture at 420 nm due to the reduction of excess ferricyanide[1]. Reactions (in a final volume of 2.0 ml) were run in thermostated cuvettes at 19 °C. Actinic light (>600 nm; 400 kergs·s^{-1}·cm^{-2}) was supplied by a 500-W slide projector and the appropiate colored glass filters.

ATP formation was determined for an aliquot of the reaction mixture as the residual radioactivity in the aqueous phase after extracting unreacted orthophosphate as phosphomolybdic acid into a butanol–toluene mixture (1:1, v/v) as described by Saha and Good[13]. Radioactivity in the final aqueous phase was determined using the Cerenkov technique of Gould *et al.*[14].

KCN-treated chloroplasts were prepared by incubating chloroplasts in 30 mM KCN (buffered at pH 7.8) at 0 °C for 90 min as described by Ouitrakul and Izawa[2].

Stock solutions of 2,5-dimethylquinone and dibromothymoquinone were prepared in ethanol–ethylene glycol (1:1, v/v) and diluted so that the final concentration of organic solvent in the reaction mixture never exceeded 2%.

RESULTS

We noted previously that dibromothymoquinone, which blocks electron flow at plastoquinone, strongly inhibits electron transport and ATP formation when ferricyanide is the electron acceptor, but only partially inhibits electron transport and phosphorylation when the lipophilic Class III oxidant *p*-phenylenediimine (oxidized *p*-phenylenediamine) serves as the electron acceptor[5]. Since the reduction of Class III acceptors is known to contain two components, one solely dependent on Photosystem II and one requiring both Photosystem II and Photosystem I (ref. 2), it was concluded that dibromothymoquinone was blocking the Photosystem I component of oxidized *p*-phenylenediamine reduction. This conclusion was confirmed by the observation that the reduction of Class III acceptors in the presence of dibromothymoquinone is completely insensitive to plastocyanin inhibition by KCN[10].

Fig. 1 shows the effect of dibromothymoquinone on the reduction of ferri-

cyanide and oxidized *p*-phenylenediamine in the presence and absence of a complete phosphorylating system. As the dibromothymoquinone concentration approaches $5 \cdot 10^{-7}$ M the rate of electron transport to oxidized *p*-phenylenediamine in the

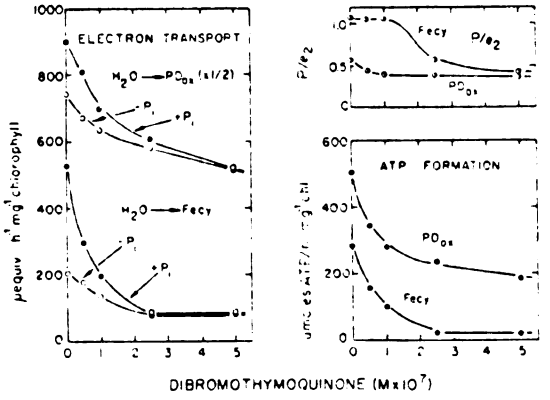

Fig. 1. Effect of the plastoquinone-antagonist dibromothymoquinone on electron transport and ATP formation associated with the photoreduction of ferricyanide and oxidized *p*-phenylenediamine by isolated chloroplasts. The reaction mixture contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM Tricine–NaOH buffer (pH 8.0), 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$ (when added), chloroplasts equivalent to 15 ug chlorophyll, and the indicated acceptor system. The acceptor systems were: Fecy, 0.4 mM potassium ferricyanide; PD_{ox}, 0.5 mM *p*-phenylenediamine *plus* 1.5 mM potassium ferricyanide. Note that as the Photosystem I component of PD_{ox} reduction is eliminated, the stimulation of electron transport by P_i is also eliminated, even though a high rate of ATP formation remains. Also note that at dibromothymoquinone concentrations $\geq 2.5 \cdot 10^{-7}$ M, where dibromothymoquinone itself functions as a Class III acceptor[10], the stimulation of electron transport by P_i is not observed, although phosphorylation, with the characteristic Site II P/e_2 ratio of 0.3–0.4, does occur.

phosphorylating ($+P_i$) system falls to the level of the nonphosphorylating ($-P_i$) system. Nevertheless, this dibromothymoquinone-insensitive electron transport remains firmly coupled to ATP formation, even though the stimulation of electron transport by ADP *plus* P_i is no longer observed. Similar results can be seen when ferricyanide acts as the electron acceptor. This is because dibromothymoquinone, at concentrations greater than $2.5 \cdot 10^{-7}$ M, not only blocks at plastoquinone but also functions as a Class III electron acceptor[10], the reduced dibromothymoquinone being rapidly reoxidized by the excess ferricyanide present in the reaction mixture. In both systems the P/e_2 ratio falls to around 0.4, the characteristic efficiency of coupling Site II[2,4,5,9,10]. Since both systems (in the presence of $5 \cdot 10^{-7}$ M dibromothymoquinone) are insensitive to plastocyanin inhibition by KCN, we can conclude that only coupling Site II is involved.

While the data in Fig. 1 clearly show that coupling Site II exerts no control over Photosystem II electron transport, Trebst and Reimer[6], using substituted *p*-benzoquinones as Class III acceptors, have shown that electron transport to these compounds is stimulated by ADP *plus* P_i and by amine uncoupling, even in the presence of dibromothymoquinone concentrations which completely block electron transport to Photosystem I. From this data they concluded that coupling Site II does exert control over the rate of electron transport. To resolve this apparent discrepancy

with our own results, we performed similar experiments using 2,5-dimethyl-*p*-benzoquinone as the Class III acceptor.

Fig. 2A shows that in the mixed system (*i.e.* dimethylquinone reduction by both Photosystem II alone and Photosystem II *plus* Photosystem I), considerable stimulation of electron transport by the complete phosphorylation system ($+P_i$) is observed. These data confirm the earlier results of Saha *et al.*[1]. If $5 \cdot 10^{-7}$ M dibromothymoquinone is added (Fig. 2B), however, the rate of electron transport is inhibited, indicating that the Photosystem I component of dimethylquinone reduction[2,5] has been largely eliminated. When no dimethylquinone is present in the reaction mixture (dimethylquinone = 0, Fig. 2B), the residual rate of electron transport, which is due to the Class III-type reduction of ferricyanide *via* dibromothymoquinone[10], shows no stimulation by ADP *plus* P_i. Nevertheless, when dimethylquinone is added, a small but significant stimulation of electron transport by ADP *plus* P_i is observed. This confirms the findings of Trebst and Reimer[11] that control

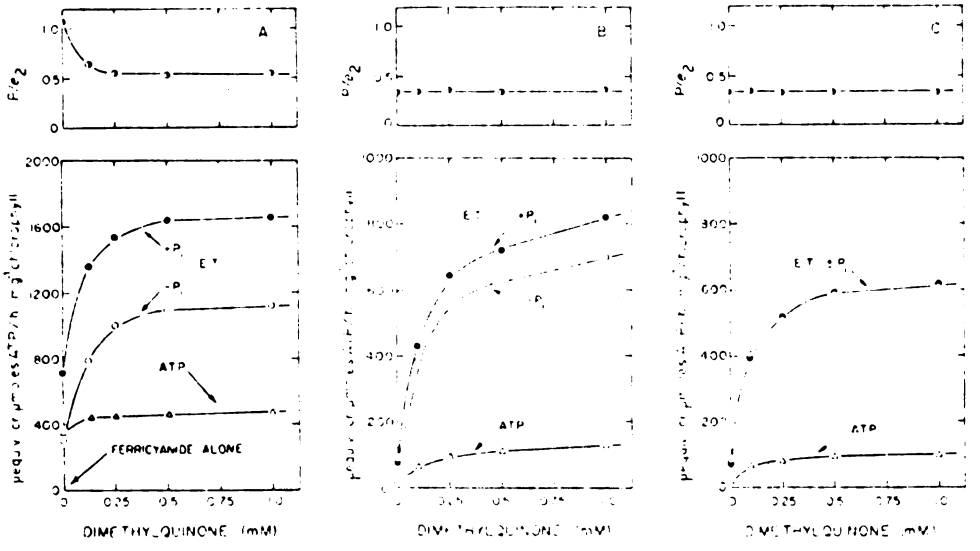

Fig. 2. Effect of the electron transport inhibitors dibromothymoquinone and KCN on electron transport (E.T.) and phosphorylation (ATP) when dimethylquinone is the electron acceptor. The basic reaction mixture is as described in Fig. 1 when ferricyanide was the electron acceptor. Specific conditions are as follows: (A) No inhibitor added. Note that when no dimethylquinone is present (*i.e.* ferricyanide is being reduced *via* the normal Hill reaction) there is a large stimulation of electron transport by the complete phosphorylating system ($+P_i$). When dimethylquinone is added, a large increase in the rates of electron transport is observed, although the absolute amount of stimulation by P_i remains about the same. (B) Dibromothymoquinone ($5 \cdot 10^{-7}$ M) was added. Here rates of electron transport are lower since dibromothymoquinone blocks the Photosystem I component of dimethylquinone reduction. Note that in the absence of dimethylquinone (*i.e.* when dibromothymoquinone serves as the electron acceptor) no stimulation by ADP *plus* P_i is observed. When increasing concentrations of dimethylquinone are added, however, a small but significant stimulation of electron transport by the complete ($+P_i$) system is observed. (C) Chloroplasts blocked at plastocyanin by KCN treatment (see Methods); $5 \cdot 10^{-7}$ M dibromothymoquinone added. Note that the stimulation of electron transport by the complete phosphorylating system (see B) is abolished by plastocyanin inhibition, indicating that electrons are bypassing the dibromothymoquinone-induced block. Nevertheless, a substantial rate of ATP formation remains even when this bypass is blocked by KCN.

is present in this system, even in the presence of a dibromothymoquinone block.

However, by using the plastocyanin inhibitor KCN it is possible to show that this control of electron transport by phosphorylating conditions is not due to coupling Site II. If electron flow is completely blocked at plastoquinone by dibromothymoquinone, then inactivation of plastocyanin by KCN should have no effect on dimethylquinone reduction. However, as Fig. 2C shows, treatment of chloroplasts with KCN completely eliminates the stimulation of electron transport by ADP *plus* P_i seen in Fig. 2B. Nevertheless, the remaining electron transport is coupled to ATP formation with an efficiency of 0.3–0.4. Thus, it can be concluded that in the presence of dibromothymoquinone *plus* dimethylquinone, there is a small amount of electron leakage around the dibromothymoquinone block which allows a slow rate of electron flux through coupling Site I. Since Site I exerts tight control over electron transport[9], this would account for the stimulation of electron flow by ADP *plus* P_i which is observed in this system. Indeed, when this electron leakage is blocked at plastocyanin, the remaining pure Photosystem II reaction, utilizing only coupling Site II, is not stimulated by ADP *plus* P_i. It should be pointed out that we have not observed this leakage phenomenon when oxidized *p*-phenylenediamine (Fig. 1)

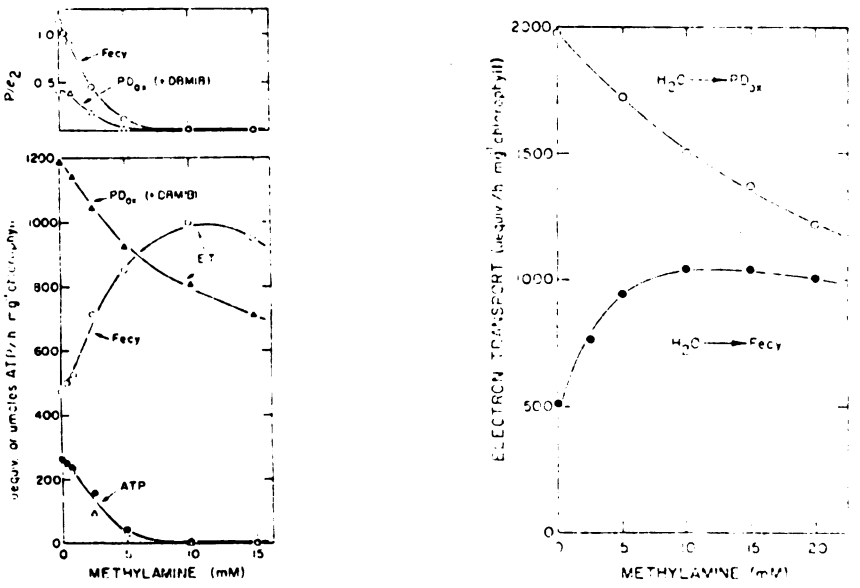

Fig. 3. Effect of the uncoupler methylamine hydrochloride on electron transport (E.T.) and ATP formation when ferricyanide (Fecy) and oxidized *p*-phenylenediamine (PD_{ox}) serve as electron acceptors. Reaction conditions are as in Fig. 1. When PD_{ox} served as the electron acceptor, $5 \cdot 10^{-7}$ M dibromothymoquinone (DBMIB) was added to block the Photosystem I component of PD_{ox} reduction. Note that the ferricyanide system shows a big stimulation of electron transport as the rate limitation at coupling Site I is relieved, while electron transport in the PD_{ox} system is inhibited as Site II becomes uncoupled.

Fig. 4. Effect of methylamine on the rate of electron transport when ferricyanide (Fecy) and oxidized *p*-phenylenediamine (PD_{ox}) are the electron acceptors. Reaction conditions are as in Fig. 3 except that dibromothymoquinone was omitted from the PD_{ox} system to eliminate a secondary effect of the inhibitor on the quantum efficiency of Photosystem II (ref. 10).

or other, substituted quinonediimides are used as Class III acceptors, suggesting that the *p*-benzoquinones may be able to substitute partially for the natural electron carrier plastoquinone.

The effect of the uncoupler methylamine on electron transport and phosphorylation associated with the reduction of ferricyanide and oxidized *p*-phenylenediamine (in the presence of $5 \cdot 10^{-7}$ M dibromothymoquinone) is shown in Fig. 3. As is already widely known, methylamine stimulates the rate of electron transport when ferricyanide is the electron acceptor by uncoupling electron flow from a rate-limiting energy conservation reaction[15]. Since we have shown previously that coupling Site I is the rate-determining step for the Hill reaction[9], we can conclude that methylamine's preponderant effect on electron transport is by releasing the rate-limitation at Site I. Methylamine has an entirely different effect on a Photosystem II partial reaction (utilizing only Site II), however. Instead of stimulating the rate of electron transport as Site II becomes uncoupled, methylamine inhibits electron flow. Since this inhibition increases with increasing concentrations of methylamine, even after ATP formation has been completely abolished, it seems likely that the inhibition is actually due to a secondary effect of the amine on Photosystem II. Indeed, high concentrations of methylamine have been shown to inhibit the water-splitting reaction[16].

In this experiment (Fig. 3) the rate of electron transport in the presence of $\geq$5 mM methylamine is somewhat lower when oxidized *p*-phenylenediamine is the electron acceptor than when ferricyanide is the electron acceptor. This is due to the dibromothymoquinone present in the *p*-phenylenediamine system, since dibromothymoquinone has been shown to have a secondary effect on the quantum efficiency of Photosystem II[10]. In the absence of dibromothymoquinone (Fig. 4) similar results are obtained for the effect of methylamine on ferricyanide and oxidized *p*-phenylenediamine reduction. It is clear that, as the rate limitation at coupling Site I is released, the rate of ferricyanide reduction increases until the secondary inhibition of Photosystem II by methylamine becomes the rate-limiting factor.

DISCUSSION

There is an impressive amount of evidence accumulating which indicates that the mechanisms of energy conservation at Sites I and II are not identical. When these sites are isolated by partial reactions of the electron transport chain[9], it can be demonstrated that they differ in their coupling efficiencies (P/e_2 ratios) and in the effect of pH on these efficiencies. Site II exhibits a characteristic P/e_2 ratio of 0.3–0.4 which is practically pH-independent from pH 6 to 9, whereas Site I, which is the rate-limiting step for the Hill reaction, exhibits a strongly pH-dependent P/e_2 ratio having an optimal value of about 0.6 at pH 8.0–8.5. Furthermore, it has recently been shown that $HgCl_2$, which is an energy transfer inhibitor in chloroplasts[17], can preferentially inhibit the coupling reaction at Site I without affecting ATP formation supported by Site II[18]. In this paper we have reconfirmed that, unlike Site I, coupling Site II does not exert control over the rate of associated electron flux. That is, the rate of electron transport through coupling Site II is independent of the presence of ADP *plus* P_i or uncouplers. Nevertheless, under phosphorylating conditions ATP formation supported by Site II can occur at very high rates.

Furthermore, we have presented data which allows a reinterpretation of the findings of Trebst and Reimer[6], who concluded from experiments with dibromothymoquinone and 2,6-dimethyl-*p*-benzoquinone that coupling Site II does exert control over electron transport. Apparently certain *p*-benzoquinones, in the presence of dibromothymoquinone, can catalyze an electron bypass around the dibromothymoquinone-induced plastoquinone block, perhaps by substituting for plastoquinone. This does not seem unlikely in view of the structural similarities between these *p*-benzoquinones and plastoquinone. When KCN blocks this bypass, however, no control over electron transport by Site II is observed. Indeed, Trebst and Reimer[6] noted that when they used higher concentrations of dibromothymoquinone in the presence of ferricyanide (so that dibromothymoquinone itself functioned as a Class III acceptor) no effect of uncouplers on electron transport was observed. This observation is in agreement with our own results obtained in a more extensive study of dibromothymoquinone as an electron acceptor[10]. It is also important to note that oxidized *p*-phenylenediamines, when used as Class III acceptors, do not catalyze this bypass reaction around the dibromothymoquinone block.

It is possible to construct a model which explains the observed differences between Site II and Site I in their ability to regulate electron transport. In many respects coupling Site I resembles sites associated with the mitochondrial respiratory chain[9]. By analogy with mitochondria[19], the electron transport between adjacent electron carriers (A and B) at Site I can be viewed as an equilibrium system. Thus, in the light, when Photosystem I is rapidly draining electrons from B, the reaction (A→B) would be pulled to the right. However, as the high energy state (~) generated in the coupled energy conservation reaction begins to accumulate, a back pressure is created against the flow of electrons from A to B by reversal of the energy conservation steps. When a complete phosphorylating system (*i.e.* ADP *plus* P_i) is present, however, the pool of high energy intermediate is much smaller since it is continually being utilized to drive ATP formation. Thus the back pressure exerted by this pool is diminished and electron transport from A to B is stimulated. Similarly, in the presence of uncouplers, the high energy state is rapidly dissipated and no significant back pressure is present, allowing very high rates of electron transport.

Coupling Site II does not exhibit the tight control over electron transport seen at Site I. This can be readily explained, however, if the oxidation-reduction reaction which gives rise to the energy conservation step at Site II is essentially an irreversible step. That is, the nature of the forward reaction (A→B) is such that the reverse reaction is thermodynamically prevented. In this case, the accumulation of the high energy intermediate or state (~) would still exert a back pressure on the forward reaction, but this back pressure would have no effect on the rate of electron transport from A to B. This would account for the fact that conditions which drain or dissipate the high energy intermediate pool do not effect the rate of electron flow at Site II.

Several observations lead us to believe that this model does indeed provide a reasonable explanation for the differences between Site II and Site I discussed in this paper. We have shown previously that coupling Site II occurs at a point in the electron transport chain before the electrons from the independent Photosystem II units are pooled[10]. This indicates that coupling Site II is located prior to plastoquinone in the electron transport chain. The insensitivity of the phosphorylation

associated with the reduction of Class III acceptors to the plastoquinone antagonist dibromothymoquinone lends strong support to this argument. Indeed, the existence of a Photosystem II-driven "proton pump" before plastoquinone has been demonstrated[9].

There are at least two reactions in this portion of the electron transport chain which could be considered essentially irreversible. One of these is the System II photoact itself. It has been suggested that a photochemical quantum conversion results in the formation of a reduced acceptor Q^- and an oxidized donor Z^+ on opposite sides of the thylakoid membrane (e.g. ref. 20). According to this model the resulting electrical field or membrane potential could serve as an energy reservoir to drive ATP formation as elaborated by Mitchell[21].

A second essentially irreversible reaction closely associated with Photosystem II electron transport is the water-splitting reaction. The existence of a coupling site on the water-oxidizing side of Photosystem II has already been considered by several authors[9,22–24]. It has been suggested that the protons from water oxidation are released to the inside of the thylakoid membrane, generating a transmembrane H^+ gradient which is capable of driving ATP formation[25].

Experiments are currently in progress in an attempt to further define the exact location of coupling Site II in chloroplasts.

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APPENDIX V

SITE-SPECIFIC INHIBITION OF PHOTOPHOSPHORYLATION IN ISOLATED SPINACH CHLOROPLASTS BY MERCURIC CHLORIDE

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Site-specific Inhibition of Photophosphorylation in Isolated Spinach Chloroplasts by Mercuric Chloride[1]

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DAVID A. BRADEEN AND G. DOUGLAS WINGET
Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221
J. MICHAEL GOULD AND DONALD R. ORT
Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Photophosphorylation associated with noncyclic electron transport in isolated spinach (*Spinacia oleracea*) chloroplasts is inhibited to approximately 50% by low concentrations of $HgCl_2$ (less than 1 μmole Hg^{2+}/mg chlorophyll) when the electron transport pathway includes both sites of energy coupling. Reactions involving only a part of the electron transport system can give a functional isolation of at least two sites coupled to phosphorylation. Only one of these sites, located between the oxidation of plastoquinone and the reduction of cytochrome *f*, is sensitive to mercuric chloride. The energy conservation site located before plastoquinone and close to photosystem II is unaffected by $HgCl_2$ concentrations up to 10-fold those required to inhibit phosphorylation by the coupling site after plastoquinone. This site-specific inhibition may reflect a mechanistic difference in the mode of energy coupling at the two coupling sites or a variable accessibility of $HgCl_2$ to these sites.

Concentrations of $HgCl_2$, which inhibit steady state phosphorylation, do not inhibit dark phosphorylation after illumination (X_2), suggesting that $HgCl_2$ affects a step in the coupling mechanism prior to the terminal step of ATP formation.

Recent data from several laboratories indicate that there are at least two sites of energy coupling associated with noncyclic electron transport in isolated chloroplasts (5–7, 11, 17–20). By utilizing various electron donor-acceptor systems in conjunction with several new inhibitors of electron transport, these energy-coupling sites can be functionally isolated and characterized. One site closely associated with photosystem II (5), differs in several respects from a second, well recognized site coupled to electron transport from plastoquinone to cytochrome *f*. (3). The photosystem II-dependent energy coupling site exhibits no control over coupled electron transport and has a P/e_2[2] ratio of about 0.4 (5–7, 17, 18). Furthermore, this P/e_2 ratio is practically pH independent (5, 6). On the other hand, the coupling site between plastoquinone and cytochrome *f*, which is the rate-limiting step for the Hill reaction, exhibits control over electron transport and has a pH-dependent P/e_2 ratio of about 0.6 (pH 8.0–8.5) (6). Because of the apparent differences in the characteristics of the two coupling sites, it was of interest to study the effect of specific inhibitors of the phosphorylation reaction (energy transfer inhibitors) on each coupling site. In this communication, we report that the energy transfer inhibitor $HgCl_2$ (11) specifically inhibits ATP formation supported by the coupling site between plastoquinone and cytochrome *f* while not affecting ATP formation supported by the coupling site close to photosystem II.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) chloroplasts were prepared as described previously (21), except TES-NaOH buffer replaced Tricine in the isolation media, since Tricine strongly binds Hg. Reactions were run in a thermostatted vessel at 19 C using strong light (>400 kerg/cm²·sec). In all cases, the chloroplasts were incubated with $HgCl_2$ for 30 sec before the addition of donors, acceptors, or inhibitors.

Electron transport using oxidized *p*-phenylenediamines, substituted *p*-benzoquinones, or ferricyanide as the electron acceptor was followed spectrophotometrically, as described elsewhere (19). MV reduction was measured as oxygen uptake (10) with a Clark-type membrane-covered electrode. ATP formation was assayed using a modified procedure of Avron (2). Radioactivity was measured using the Cerenkov technique of Gould *et al.* (4).

RESULTS AND DISCUSSION

Figure 1 shows the effect of low concentrations of $HgCl_2$ on electron transport and phosphorylation using three different electron donor-acceptor systems. As previously reported (11), the over-all Hill reaction with ferricyanide (Fig. 1A) or MV (Table I) as the electron acceptor is inhibited in direct proportion to the added $HgCl_2$ up to approximately 35 to 40 nmoles of $HgCl_2$/mg Chl. Although very high concentrations of $HgCl_2$ (greater than 1 μmole/mg Chl) result in nonspecific electron

[1] J.M.G. and D.R.O. were supported by Grants GB 22657 and GB 37959X from the National Science Foundation.

[2] Abbreviations: P/e_2 ratio: the ratio of the number of molecules of ATP formed to the number of pairs of electrons transported; $DCIPH_2$: reduced 2,6-dichlorophenolindophenol; HEPPS: hydroxyethylpiperazinepropanesulfonic acid; FeCy: potassium ferricyanide; DBMIB: dibromothymoquinone; DMQ: 2,5-dimethyl-*p*-benzoquinone; PD_{ox}: oxidized *p*-phenylenediamine; DAD_{ox}: oxidized diaminodurene; MV: methylviologen.

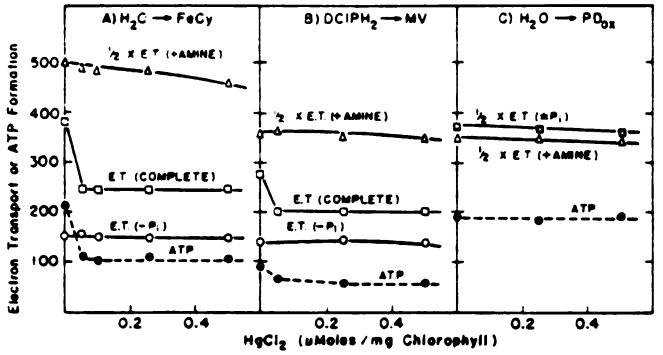

FIG. 1. Effect of $HgCl_2$ on electron transport (E.T.) and phosphorylation associated with various electron transport pathways. The reaction mixture (2 ml) contained 50 mM HEPPS-NaOH buffer (pH 8.2), 2 mM $MgCl_2$, 0.1 M sucrose, 1 mM ADP, 5 mM $Na_2H_{32}PO_4$, chloroplasts containing 40 μg of chlorophyll, and the indicated donor-acceptor system. These systems were: A, 0.4 mM ferricyanide; B, 0.4 mM $DCIPH_2$, 2.5 mM L-ascorbate and 50 μM MV; C, 0.5 mM *p*-phenylenediamine (PD) plus 1.4 mM ferricyanide. When added, methylamine was 10 mM. In the $DCIPH_2 \rightarrow MV$ system (B), 1 μM DCMU was added to block electron transport from photosystem II. When PD_{ox} was the electron acceptor (C), 0.5 μM DBMIB was added to block the photosystem I component of PD_{ox} reduction (1). Note that only the $H_2O \rightarrow PD_{ox}$ system, which does not utilize the rate-determining coupling site after plastoquinone, is insensitive to inhibition by $HgCl_2$. Rates of electron transport and ATP formation are given in μmoles/hr·mg chlorophyll.

Table I. *Effect of $HgCl_2$ on Photophosphorylation in Spinach Chloroplasts with Various Electron Acceptors*

The reaction mixture (3 ml) contained 20 mM HEPPS-NaOH buffer (pH 8.2), 50 mM sucrose, 1 mM $MgCl_2$, 1 mM ADP, 5 mM $Na_2H^{32}PO_4$, chloroplasts containing 60 μg of Chl, and the indicated electron acceptor system. These systems were: 1: 50 μM MV; 2: 10 μM DBMIB plus 0.4 mM ferricyanide; 3: 0.5 mM DMQ plus 0.4 mM ferricyanide; 4: 0.5 mM DAD plus 1.4 mM ferricyanide. A low concentration of dibromothymoquinone (0.5 μM) was added to reactions 3 and 4 to inhibit the photosystem I component of DAD_{ox} and DMQ reduction (2). Rates are given in μmoles ATP/hr·mg Chl. Note that only the $H_2O \rightarrow MV$ system is sensitive to $HgCl_2$.

Experiment	Electron Acceptor	Phosphorylation Rate		
		Micromoles $HgCl_2$ added/mg Chl		
		None	0.05	0.25
No.		*μmoles ATP/hr·mg Chl*		
1	MV	200	102	105
2	DBMIB	47	46	42
3	DMQ	71	65	64
4	DAD_{ox}	217	200	195

transport inhibition (11, 13), the low levels of $HgCl_2$ used here (less than 1 μmole/mg Chl) inhibit ATP formation (and that portion of the electron transport dependent upon phosphorylation) to a plateau of approximately 50%. Contrary to the results of Miles *et al.* (16), however, we find the same degree of inhibition by $HgCl_2$ when electron transport is measured spectrophotometrically or as oxygen evolution. Neither basal (−Pi) or uncoupled (+methylamine) electron transport is significantly affected by $HgCl_2$, indicating that $HgCl_2$ does act as an energy-transfer inhibitor rather than an electron-transport inhibitor at these concentrations (11). Chloroplasts which have been uncoupled by EDTA treatment, which removes CF_1 (1), are also insensitive to $HgCl_2$.

$DCIPH_2$ (in the presence of DCMU and ascorbate) donates electrons at a point before the rate-limiting coupling site on the electron transport chain (*i.e.* before cytochrome *f*) (6, 9, 15). It has recently been shown that the photosystem I-dependent partial reaction $DCIPH_2 \rightarrow MV$ includes only the rate-limiting coupling site after plastoquinone and not the coupling site before plastoquinone (6). Figure 1B shows that $HgCl_2$ affects the partial reaction $DCIPH_2 \rightarrow MV$ and the over-all reaction $H_2O \rightarrow FeCy$ similarly, indicating that both pathways include the $HgCl_2$-sensitive site. Moreover, since this coupling site constitutes the primary rate-limiting step for both electron transport reactions, a similar 50% sensitivity to $HgCl_2$ should be observed for both systems (compare Fig. 1, A and B).

However, when electrons from water reduce lipophilic acceptors (class III acceptors [19]), such as oxidized *p*-phenylenediamine (PD_{ox}), a different effect of HgCl is observed. These lipophilic oxidants (in the presence of the plastocyanin inhibitors KCN or poly-L-lysine (17) or the plastoquinone antagonist dibromothymoquinone (12, 20)) accept electrons at a point in the electron transport chain before plastoquinone (5). Thus the partial reaction $H_2O \rightarrow PD_{ox}$ includes the photosystem II-dependent phosphorylation site, but not the rate-determining site after plastoquinone. As Figure 1C shows, electron transport and phosphorylation associated with the partial reaction $H_2O \rightarrow PD_{ox}$ is completely insensitive to $HgCl_2$ inhibition. Several other class III acceptors were also tested for $HgCl_2$ inhibition with similar results (Table I).

The absence of inhibition by $HgCl_2$ with class III electron acceptors is probably not a result of fortuitous reaction conditions that mask the inhibition. In all cases, chloroplasts were incubated with $HgCl_2$ for 30 sec before the addition of the acceptor system. Since SH-compounds can reverse $HgCl_2$ inhibition (11), it seems likely that Hg^{2+} is reacting with a membrane sulfhydryl group. Thus it is unlikely that binding between Hg^{2+}

Table II. *Effect of $HgCl_2$ on Postillumination ATP Formation (X_E)*

$HgCl_2$, triphenyltin chloride, or methylamine (at the final concentrations indicated) were present in the dark, phosphorylation stage only. Chloroplasts containing 100 mg of Chl were illuminated with white light (>400 kergs/cm²·sec) for 20 sec in a continuously stirred reaction mixture (2 ml) containing 0.1 M sucrose, 50 mM NaCl, 2 mM $MgCl_2$, 10 mM MES-NaOH buffer (pH 6.0), and 5 μM pyocyanine. Immediately after shutting off the light, 0.25 ml of a stock solution containing the test compound was added to the reaction mixture with a syringe. After 5 sec 0.75 ml of a strongly buffered ADP phosphate mixture (0.15 M HEPPS-NaOH buffer (pH 8.0), 3 mM ADP, 15 mM $Na_2H^{32}PO_4$) was quickly injected to initiate ATP formation. After 20 sec the dark phosphorylation was terminated by addition of 0.5 ml of 1 N perchloric acid. All reactions were run in a thermostatted cuvette at 19 C. Note that $HgCl_2$ did not significantly affect the yield of X_E at concentrations which strongly inhibit steady state phosphorylation. The tributyltin analog triphenyltin, which is a potent energy transfer inhibitor in chloroplasts (unpublished observations of J.M.G.), does abolish X_E, however, as does the uncoupler methylamine.

Addition (dark stage)	ATP Formed	Inhibition
	nmoles/mg Chl	*%*
None	64	
$HgCl_2$ (20 nmoles/mg Chl)	58	9.5
$HgCl_2$ (200 nmoles/mg Chl)	52	18
Triphenyltin chloride (10 μM)	8.8	87
Methylamine hydrochloride (5 mM)	12	81

and the quinonediimides or substituted benzoquinones used here as class III acceptors would be strong enough to reverse the inhibition. Furthermore, UV spectra of DMQ, DAD_{ox}, PD_{ox}, and DBMIB remained virtually unchanged in the presence of high concentrations (33 μM) of $HgCl_2$, again suggesting that little or no binding is occurring. This evidence suggests that $HgCl_2$ is not interacting chemically with the class III acceptors used here and supports our contention that the energy-coupling site close to photosystem II is insensitive to $HgCl_2$.

Table II presents evidence that $HgCl_2$ acts on an early step of energy conservation rather than a terminal reaction of ATP formation. Concentrations of $HgCl_2$, which strongly inhibit steady state phosphorylation (*e.g.* $H_2O \rightarrow MV$), were demonstrated to have virtually no effect on the chloroplast's ability to synthesize ATP in the dark after brief illumination (X_F) (8). However, methylamine, an uncoupler, and triphenyltin chloride, an energy transfer inhibitor which acts on a terminal reaction of ATP formation, decrease the yield of X_F significantly (8; unpublished observations of J.M.G.). The idea that $HgCl_2$ affects an early stage of energy transfer is also supported by the fact that the trypsin activated ATPase activity of CF_1 and whole chloroplasts is not affected by the low levels of $HgCl_2$ used here (data not shown).

The results presented herein lend strong support to the argument that the two known sites of energy coupling associated with noncyclic electron flow in chloroplasts may exhibit mechanistic differences in their mode of energy transfer (5, 6). It has previously been shown that these coupling sites differ in their response to pH (5, 6), uncouplers, ADP + P_i (7), and in phosphorylation efficiency (P/e_2 ratio) (6). In addition, we have now demonstrated that the energy-transfer inhibitor $HgCl_2$ is specific for the rate-determining coupling site after plastoquinone and before cytochrome *f*. This selectivity may be due to a variable accessibility of $HgCl_2$ to the coupling sites, or, alternatively, to a basic difference in the mechanism of the early steps of energy conservation at the two sites.

A discussion of why the $HgCl_2$ sensitive coupling site is only partially sensitive (50%) to the inhibitor is beyond the scope of this report. In a subsequent publication, a more detailed study of $HgCl_2$ inhibition of chloroplast reactions will be presented, with emphasis on the nature of $HgCl_2$ inhibition and the significance of the 50% inhibition plateau.

Acknowledgments—The authors wish to thank S. Izawa for many valuable suggestions.

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APPENDIX VI

STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION IV. THE RELATION OF PROTON FLUXES TO THE ELECTRON TRANSPORT AND ATP FORMATION ASSOCIATED WITH PHOTOSYSTEM II

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STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION

IV. THE RELATION OF PROTON FLUXES TO THE ELECTRON TRANSPORT AND ATP FORMATION ASSOCIATED WITH PHOTOSYSTEM II

J. MICHAEL GOULD and S. IZAWA

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Mich. 48824 (U.S.A.)

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SUMMARY

1. By using dibromothymoquinone as the electron acceptor, it is possible to isolate functionally that segment of the chloroplast electron transport chain which includes only Photosystem II and only one of the two energy conservation sites coupled to the complete chain (Coupling Site II, observed P/e_2 ~ 0.3–0.4). A light-dependent, reversible proton translocation reaction is associated with the electron transport pathway: $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone. We have studied the characteristics of this proton uptake reaction and its relationship to the electron transport and ATP formation associated with Coupling Site II.

2. The initial phase of H^+ uptake, analyzed by a flash-yield technique, exhibits linear kinetics (0–3 s) with no sign of transient phenomena such as the very rapid initial uptake ("pH gush") encountered in the overall Hill reaction with methylviologen. Thus the initial rate of H^+ uptake obtained by the flash-yield method is in good agreement with the initial rate estimated from a pH change tracing obtained under continuous illumination.

3. Dibromothymoquinone reduction, observed as O_2 evolution by a similar flash-yield technique, is also linear for at least the first 5 s, the rate of O_2 evolution agreeing well with the steady-state rate observed under continuous illumination.

4. Such measurements of the initial rates of O_2 evolution and H^+ uptake yield an H^+/e^- ratio close to 0.5 for the Photosystem II partial reaction regardless of pH from 6 to 8. (Parallel experiments for the methylviologen Hill reaction yield an H^+/e^- ratio of 1.7 at pH 7.6.)

5. When dibromothymoquinone is being reduced, concurrent phosphorylation (or arsenylation) markedly lowers the extent of H^+ uptake (by 40–60%). These data, unlike earlier data obtained using the overall Hill reaction, lend themselves to

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Abbreviations: P/e_2, ratio of the number of molecules of ATP formed to the number of pairs of electrons transported; H^+/e^-, ratio of the number of hydrogen ions transported to the number of electrons transported; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone).

an unequivocal interpretation since phosphorylation does not alter the rate of electron transport in the Photosystem II partial reaction. ADP, P_i and hexokinase, when added individually, have no effect on proton uptake in this system.

6. The involvement of a proton uptake reaction with an H^+/e^- ratio of 0.5 in the Photosystem II partial reaction $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone strongly suggests that at least 50% of the protons produced by the oxidation of water are released to the inside of the thylakoid, thereby leading to an internal acidification. It is pointed out that the observed efficiencies for ATP formation (P/e_2) and proton uptake (H^+/e^-) associated with Coupling Site II can be most easily explained by the chemiosmotic hypothesis of energy coupling.

INTRODUCTION

The electron transport pathway in isolated chloroplasts can be divided into several segments by the use of appropriate electron donor–acceptor combinations in conjunction with specific inhibitors of certain intermediate electron carriers [1]. When this is done it becomes clear that there are two sites of energy conservation in the phosphorylation-coupled transport of electrons from water to Photosystem I [1–5]. These two coupling sites differ markedly in their characteristics.

Coupling Site I refers to the energy conservation site associated with the transfer of electrons from plastoquinone to cytochrome *f* [6, 7]. This site provides the rate-determining step for the overall Hill reaction [1]. The rate of electron flux through Coupling Site I is greatly increased by ADP plus phosphate under phosphorylating conditions and still more by the presence of phosphorylation uncouplers. The efficiency of phosphorylation (P/e_2) at Site I is dependent on the pH of the medium, with an optimum at pH 8–8.5 where the observed P/e_2 is 0.6–0.7 (ref. 1). ATP formation supported by Coupling Site I is inhibited by the chloroplast energy transfer inhibitor $HgCl_2$ (ref. 8).

Coupling Site II is associated with the transfer of electrons from water to plastoquinone or with the transfer of electrons from water to exogenous lipophilic "Class III" oxidants which intercept electrons before they reach plastoquinone [5, 9]. Coupling Site II differs from Site I in a number of important respects. The rate of electron transport through Site II is independent of phosphorylation and uncoupling [1, 9, 10]. The efficiency of phosphorylation is practically independent of pH (6–9), and the observed P/e_2 ratio is characteristically 0.3–0.4 [1–3, 5, 9, 10]. Moreover, ATP formation supported by Coupling Site II is insensitive to $HgCl_2$ (ref. 8).

These differences suggest that the modes of energy transduction at Sites I and II are somehow different. Therefore we have been prompted to undertake a further investigation into the mechanism by which electron transport may be coupled to phosphorylation at these two sites. This paper deals primarily with further characterizations of the light-induced proton uptake (pH rise) associated with the transfer of electrons from water to dibromothymoquinone via Photosystem II [1]. In previous papers [3, 9, 13] we established that this plastoquinone antagonist [11], which at low concentrations (0.5 μM) blocks the transport of electrons from reduced plastoquinone to cytochrome *f* [12], can act at higher concentrations (25 μM) as an acceptor of electrons from Photosystem II, probably via plastoquinone [9]. In

addition, we have shown that the phosphorylation reaction associated with dibromothymoquinone reduction exhibits all of the characteristics of Coupling Site II outlined above [9].

The use of substrate concentrations of dibromothymoquinone provides a convenient reaction system for studying the nature of Coupling Site II, since the function of the substrate as an inhibitor effectively blocks further electron transport (to Photosystem I) and thereby isolates Site II from Site I. Furthermore, we have recently presented evidence that a reversible "proton pump" is associated with dibromothymoquinone reduction [1]. Since electron transport in this system is unaffected by uncouplers or by phosphorylation, it has been possible to observe directly the effects of ATP formation on the "proton pump" without a complication encountered by previous investigators [14, 15]. Heretofore it has been impossible to study the effect of phosphorylation on proton uptake without increasing the electron transport rate, thereby inadvertently modifying the associated proton uptake. Therefore it has previously not been possible to study the proton pump and phosphorylation as opposing processes in an unambiguous way.

The results presented in this paper show that there is a quantitative relationship between the electron transport-dependent accumulation of hydrogen ions and ATP formation at Coupling Site II. The results also suggest that Coupling Site II in chloroplasts may be identified with the water-oxidation reaction, which seems to release protons to the inside of the chloroplast lamellar membrane.

MATERIALS AND METHODS

Chloroplasts (intact, naked lamellae) were isolated in the cold from leaves of fresh market spinach (*Spinacia oleracea* L.) as described in a previous paper in this series [1]. For some experiments the HEPES–NaOH buffer in the suspension medium was replaced with either phosphate or arsenate and the bovine serum albumin was omitted. The concentration of chloroplasts in the final stock suspension was adjusted so that the amount of buffer carried over into the reaction mixture gave a final concentration of 0.5 or 1.0 mM.

Changes in hydrogen ion concentration in the reaction mixture were detected with a Corning semi-micro ("Tri-purpose") combination pH electrode connected to a fast responding Heath/Schlumberger EU200-30 pH electrometer module equipped with a Heath/Schlumberger EU200-02 DC offset module to facilitate scale expansion. The output from the electrometer was recorded on an Esterline-Angus strip chart recorder. The response half-time for the pH measuring system was in the order of 0.5–1 s. Changes in pH were normally monitored with a scale expansion of 0.1 pH unit full scale (10 inches) on the recorder. The noise level at this amplification was less than 0.002 pH unit.

In routine pH experiments, reactions were run in a final volume of 2.0 ml in thermostated vessels at 18 °C with continuous stirring. Prior to illumination the reaction mixture was adjusted to the desired initial pH with small volumes of dilute NaOH or HCl. Actinic illumination was supplied by a 500-W slide projector. The beam was passed through a 500-ml round-bottomed flask containing a dilute $CuSO_4$ solution (which served both as an infrared filter and as a condensing lens) and through an orange glass filter (transmission $\geq$ 600 nm) plus a Corning 1-69 heat filter. The

light intensity was approximately 700 kergs $\cdot s^{-1} \cdot cm^{-2}$ (600–700 nm). At the end of each experiment the pH changes registered on the chart paper were translated into H^+ equivalents by titrating the reaction mixture in the light with a known amount of 0.001 M HCl.

Electron transport was measured as oxygen evolution (dibromothymoquinone as acceptor) or oxygen uptake (methylviologen as electron acceptor) [16] using a Clark-type membrane-covered oxygen electrode. For these experiments a 3.0-ml reaction mixture was used. When both electron transport and proton uptake were determined, the pH rise was measured in an identical reaction mixture in the same apparatus, substituting the pH electrode for the oxygen electrode.

RESULTS

Light-induced pH rise associated with dibromothymoquinone reduction

We have previously presented evidence that the electron transport pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone, which includes Coupling Site II but not Coupling Site I, is associated with a light-dependent, reversible proton uptake [1]. These results have been confirmed and extended (Fig. 1).

Fig. 1 depicts the general pattern of the light-induced pH rise in the medium. Above pH 8.1, where reduced dibromothymoquinone is rapidly reoxidized by molecular oxygen [9] (and therefore no exhaustion of the electron acceptor occurs), the pH rise can be observed many times using repeated light cycles (trace A). Below pH 8, where the reoxidation rate is very slow, the pH shift can be maintained only as long as the reduction of dibromothymoquinone continues. As the reduction approaches completion and the electron transport slows down, a gradual reversal of the pH change is observed even in the light, and eventually the pH returns to the original level (trace B). Subsequent illuminations do not restore the pH rise. If the light is turned off before the complete exhaustion of the electron acceptor, a second light cycle does induce a small pH rise, the extent of which depends upon the amount of oxidized dibromothymoquinone remaining. The uncoupler gramicidin D (4 μg/ml) completely abolishes the light-induced pH response (data not shown).

If ferricyanide is added to the reaction mixture so that the photoreduced dibromothymoquinone is continually reoxidized by the excess ferricyanide, a reversible pH rise superimposed on an irreversible pH drop is observed (Fig. 1 trace C). This is repeatable even below pH 8 since the reoxidation of reduced dibromothymoquinone by ferricyanide is very rapid at any pH above 6. The irreversible pH drop is due to the protons produced by the oxidation of water according to the equation:

$$1/2\ H_2O + Fe^{3+} \rightarrow 1/4\ O_2 + Fe^{2+} + H^+.$$

Thus the rate of proton production can also be used as a means for determining the rate of electron transport. As trace D shows, both the transient pH rise and the lag in the light-induced pH drop due to the superimposed proton uptake are eliminated by the uncoupler gramicidin, although the rate of electron transport (measured as proton production) is scarcely affected. This confirms our previous observations that the rate of electron transport associated with dibromothymoquinone reduction is not increased by uncouplers [9].

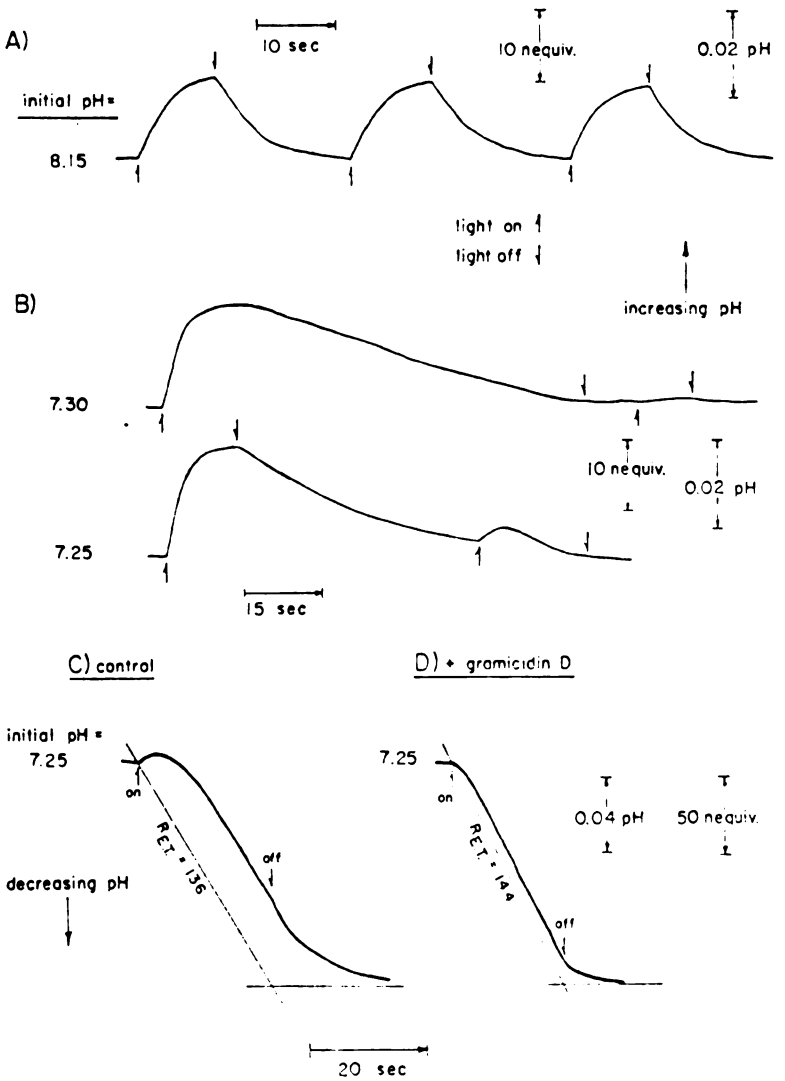

Fig. 1. Light-induced pH changes associated with the partial electron transport pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone in isolated chloroplasts. The 2.0-ml reaction mixture contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM NaCl, 0.5 mM HEPES-NaOH buffer, 20 μM dibromothymoquinone and chloroplasts containing 100 μg chlorophyll. In the experiments presented in traces C and D, 0.5 mM potassium ferricyanide was added to the reaction to continually reoxidize the photoreduced dibromothymoquinone. Note that the rate of electron transport (R_{e^-}; in nequiv · h^{-1} · mg chlorophyll^{-1}) is not increased by the uncoupler gramicidin D. For further explanation see the text.

Stoichiometry between electron transport and proton uptake (H^+/e^-)

The data presented in Fig. 1 are most easily interpreted in terms of a transmembrane H^+ gradient associated with the Photosystem II partial reaction $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone. However, the significance of this proton gradient and its relation to the coupling mechanism cannot be evaluated critically

until the efficiency of proton uptake (H^+/e^-) is determined. Moreover, determination of H^+/e^- in this case is of special interest, since the dibromothymoquinone partial reaction includes only one (Site II) of the two coupling sites associated with the complete Hill reaction [3, 9]. Thus the observed efficiency of ATP formation (P/e_2) supported by this partial electron transport pathway is lower than the efficiency of the complete chain where both coupling sites are operating ($P/e_2 = 0.3$–0.4 versus 1.1–1.2, respectively) [1]. Therefore one might reasonably expect the efficiency of proton uptake associated with Site II to be correspondingly low. However, the determination of an H^+/e^- ratio involves a variety of difficulties, and, in fact, no truly unequivocal method of measuring it has as yet been developed. For a more complete discussion of these difficulties, see review articles by Jagendorf [17], Walker and Crofts [18] and Schwartz [19]. In the experiments outlined below we have observed the initial kinetics of proton uptake and electron transport by two different methods in an attempt to circumvent these difficulties and to accurately measure the H^+/e^- in the dibromothymoquinone partial reaction.

The response time ($t_{\frac{1}{2}} \leq 1$ s) of the pH assay system used in these experiments (see Methods) was considerably faster than the apparent kinetics of the pH rise ($t_{\frac{1}{2}} \simeq 4$–5 s) and therefore it seemed possible that the relatively linear initial phase

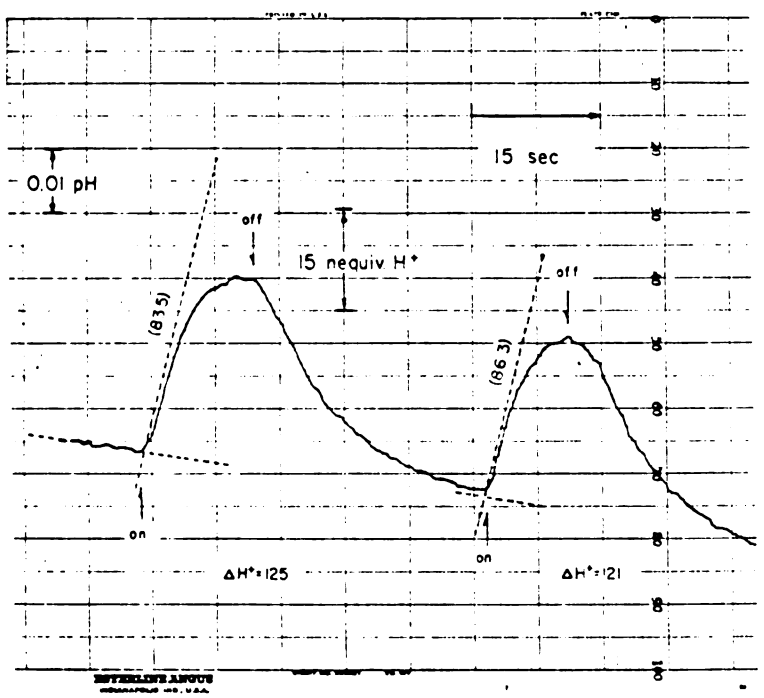

Fig. 2. Recorder tracing of the apparent kinetics of the reversible pH rise associated with Photosystem II electron transport from water to dibromothymoquinone. The reaction mixture was as in Fig. 1, trace A, except the HEPES buffer was replaced by 2.25 mM Na_2HPO_4 and contained chloroplasts equivalent to 145 μg chlorophyll. The initial pH was 8.16. Numbers in parentheses are the apparent initial rates of proton uptake in μequiv $H^+ \cdot h^{-1} \cdot$ mg chlorophyll^{-1}. The steady-state extent of the pH rise (ΔH^+; in nequiv · mg chlorophyll^{-1}) was determined by titrating the reaction mixture in the light with known volumes of 0.001 M HCl (not shown).

of the pH tracings (Fig. 2) might provide a reasonably accurate estimate of the initial rate of proton uptake. This assumption would be invalid, of course, if the pH rise involved any transient kinetics faster than the instrument response time, such as the initial "pH gush" ($t_{\frac{1}{2}} \simeq 0.10$ s) described earlier by Izawa and Hind [20]. In order to detect the possible involvement of such transient kinetics, we have examined pH changes induced by a series of brief illuminations (flash duration 1–3 s) with intervening dark periods to allow for the lag due to instrument response time. That is, a sufficient dark period was introduced between successive flashes to allow the instrument to record the entire pH change which had been completed during the flash. This dark period was not long enough to allow any significant decay of the pH change, however. Somewhat surprisingly, we could detect no evidence for any significant "gush" phenomena at all associated with dibromothymoquinone reduction (Fig. 3, trace b), the rate of proton uptake determined for the first flash being essentially the same as the rate determined for a subsequent flash. Plot c of Fig. 3 represents the early phase of proton uptake reconstructed by summing the flash yields and illumination times of trace b. A very good agreement was found between the initial slope of the linear phase of the reconstructed time-course curve (< 4 s; trace c) and the initial slope of the continuous recorder tracing (trace a). This is not surprising since, as trace b shows, there was no significant overshoot of the pH response after a flash except for the small amount attributable to the instrumental time lag. Nor was there a rapid enough dark decay to obscure the flash yield. In other words, each flash yield determination did seem to accurately represent the extent of the pH rise which had been essentially completed during the flash. Thus the initial slope of a pH rise curve obtained under continuous light must in fact be a fairly reliable measure of the actual initial rate of proton influx.

The rate of electron transport in the same time range (0–5 s after light on) was measured as O_2 evolution using a duplicate reaction mixture in the same apparatus (Fig. 3, traces d, f). Flash experiments were even more important in this case, because of the very slow response time ($t_{\frac{1}{2}} \simeq 2$ s) of the membrane-covered oxygen electrode used. The flash experiments again detected no sign of transient phenomena (Fig. 3, trace e) and again the reconstructed slope agreed rather precisely with the steady-state slope determined under continuous light (traces d, f). (Transient kinetics such as those discovered by Joliot et al. [21] are far beyond the resolution of our instrument.) The ratio H^+/e^- determined from the data presented in this set of experiments (Fig. 3) was 0.51.

The use of intermittent brief periods of illumination described above makes transient differences between initial rates and steady state rates quite obvious when these differences do exist. Thus the initial "pH gush" known to be associated with the reduction of methylviologen by chloroplasts [20] is clearly revealed by the flash technique (Fig. 4b) even though the pH changes recorded during continuous illumination conceal the transient (Fig. 4a). However, if the tracing for the continuous illumination is corrected for the instrument response time according to the method of Izawa and Hind [20], there is good agreement between the kinetics of the pH rise as determined by the flash yield technique and the corrected kinetics for continuous illumination. The "pH gush" amounted to one mole of H^+ for each 20–30 moles chlorophyll in this experiment. However, no corresponding initial fast phase in oxygen production could be detected (Fig. 4b), indicating that the "pH-gush" observed under some

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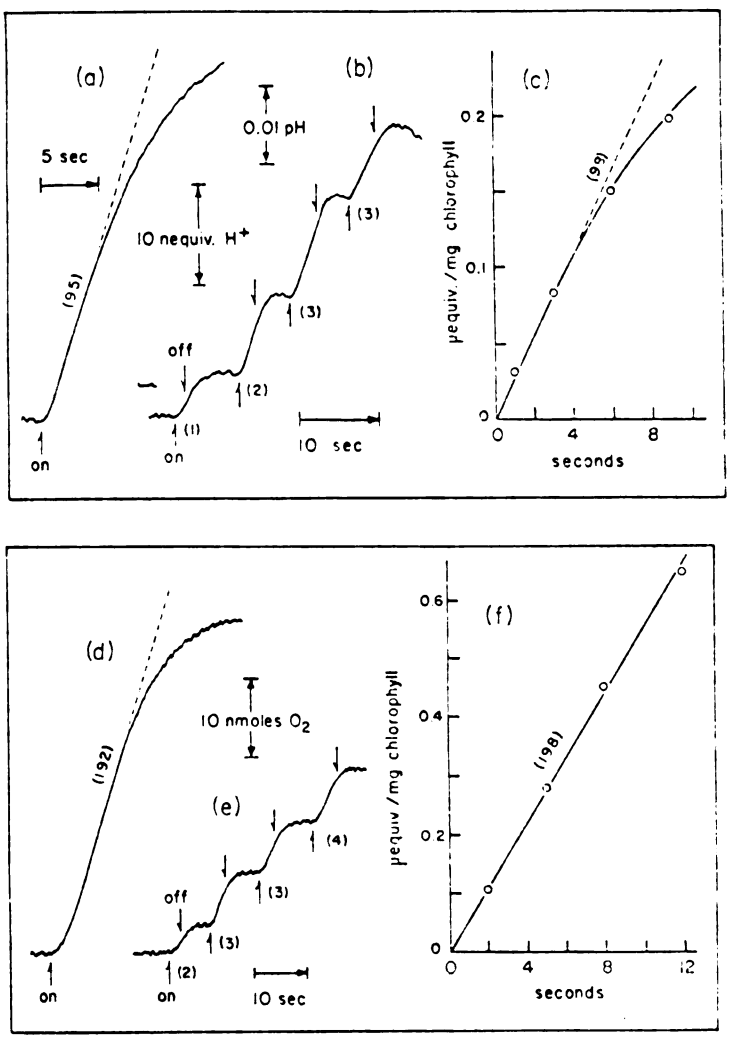

Fig. 3. Initial kinetics of the light induced pH rise and electron transport measured under both flash and continuous illumination with dibromothymoquinone as the electron acceptor. The reaction mixture (3.0 ml) was as in Fig. 1, trace a, except that the concentration of dibromothymoquinone was 50 μM and the reaction contained chloroplasts equivalent to 150 μg chlorophyll. The initial pH was 7.4. Trace a: recorder tracing of the apparent initial kinetics of the pH rise observed under continuous illumination. The rate of H^+ uptake in μequiv $H^+ \cdot h^{-1} \cdot$ mg chlorophyll^{-1} was determined from the essentially linear initial portion of the tracing and is shown in parentheses. Trace b: recorder tracing of the pH rise induced by flash illumination. Numbers in parentheses represent the flash duration (in s). Trace c: reconstructed time course of the initial portion of the pH rise from flash yield determinations (trace b). Note that the rate of proton uptake measured by the flash technique agrees very well with the rate determined under continuous illumination (trace a). Trace d: recorder tracing of the kinetics of O_2 evolution under continuous illumination. The rate of electron transport (in μequiv $\cdot h^{-1}$ $\cdot$ mg chlorophyll^{-1}) is shown in parentheses. Trace e: recorder tracing of O_2 evolution measured by the flash yield technique. Numbers in parentheses represent flash duration (in seconds). Trace f: reconstructed time-course for O_2 evolution determined from the flash experiment (trace e). Note that the rate determined by this technique agrees very well with the steady-state rate determined under continuous illumination (trace d).

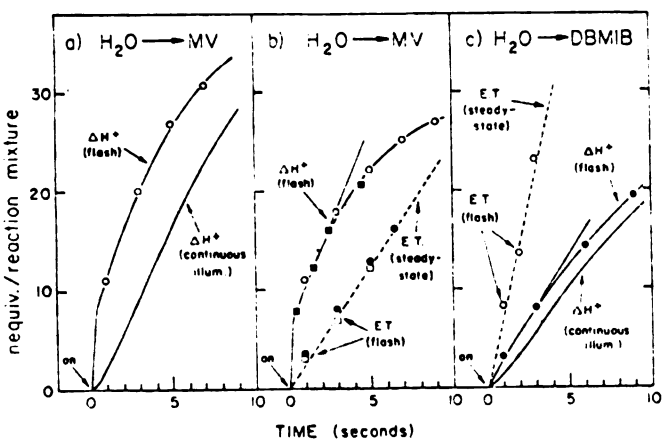

Fig. 4. Comparison between the initial rates of proton uptake and electron transport for the complete electron transport chain ($H_2O \rightarrow$ methylviologen) and the Photosystem II partial reaction $H_2O \rightarrow$ dibromothymoquinone. Reaction conditions were as in Fig. 3 with the following exceptions. In the experiments presented above in a and b, dibromothymoquinone (DBMIB) was omitted and 100 μM methylviologen (MV) was added. When dibromothymoquinone was the electron acceptor, electron transport (E.T.) was observed as O_2 evolution. When methylviologen was the acceptor, electron transport was followed as O_2 uptake [16]. The chlorophyll concentration was 50 μg/ml. (a) Comparison between the observed pH change (ΔH^+) under continuous illumination and by a flash yield technique. Note that the large "pH gush", which is clearly evident in the kinetics obtained by the flash technique, is masked in the continuous tracing due to the instrumental response lag. Nevertheless the slope of the relatively linear portion of the pH rise immediately following the "gush" is in good agreement with the slope obtained with continuous illumination. The initial pH for this experiment was 6.8. (b) Comparison between the kinetics of H^+ uptake (ΔH^+) and electron transport (E.T.) when methylviologen is the electron acceptor. The data shown is from two identical experiments done at pH 7.6. Note that the rate of electron transport determined under continuous illumination (dashed line) agrees very well with the electron transport rate determined by the flash technique (points), and that there is apparently no initial burst of electron transport corresponding to the initial "pH gush". The H^+/e^- ratio determined for this experiment (see text) was 1.7. (c) An experiment similar to b above except the electron transport pathway included only Photosystem II and only Coupling Site II. Dibromothymoquinone served as the electron acceptor. Note that the initial "pH gush" is absent in this system. The H^+/e^- ratio calculated for this experiment (pH 7.2) was 0.45 (See also Fig. 3).

conditions cannot be simply explained in terms of an initial rapid rate of electron transport associated with plastoquinone reduction. The ratio H^+/e^- observed during methylviologen reduction (calculated from the relatively linear portion of the early phase of the pH rise after the initial gush) was 1.7. Note again (Fig. 4c) that there was no significant initial rapid phase of proton uptake when dibromothymoquinone was being reduced and that the ratio H^+/e^- was much lower, about 0.5. Thus the proton pump associated with dibromothymoquinone reduction (involving only Coupling Site II) is distinguished from the proton pump associated with methylviologen reduction (involving both Sites I and II) in two ways: the reaction involving only Site II lacks an intitial rapid phase and is less than half as efficient as the combined sites in transporting H^+ across the lamellar membranes.

Table I summarizes the results of three independent series of experiments performed with different chloroplast preparations. While the ratio H^+/e^- for the dibromothymoquinone reducing system is relatively constant (0.35–0.5) over a wide

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TABLE I

STOICHIOMETRY BETWEEN PROTON UPTAKE AND ELECTRON TRANSPORT (H^+/e^-) WITH DIBROMOTHYMOQUINONE OR METHYLVIOLOGEN AS ELECTRON ACCEPTOR

Reactions were run as described in Figs 3 and 4. The basic reaction mixture was as in Fig. 5 except in Expt 1, where the phosphate buffer was replaced by 0.5 mM HEPES–NaOH. The chlorophyll concentration was 100 μg/2ml. Note that when dibromothymoquinone served as the electron acceptor so that only Coupling Site II was being utilized, the ratio H^+/e^- was relatively constant (0.35–0.51) over a wide pH range. When methylviologen served as the electron acceptor (utilizing Coupling Sites I and II), the H^+/e^- ratio was much higher.

Expt No.	Electron acceptor	Initial pH	Initial rate (μequiv · h^{-1} · mg chlorophyll^{-1})		H^+/e^-
			H^+ uptake	Electron transport	
1	Dibromothymo-quinone	6.22	93**	218**	0.42
		6.60	102	225	0.45
		7.00	89	234	0.38
		7.48	81	212	0.38
2*	Dibromothymo-quinone	7.20	100**	198**	0.51
		7.60	63	146	0.43
		8.00	48	125	0.38
3*	Dibromothymo-quinone	7.30	69**	172**	0.40
		8.13	60**	168**	0.36
		8.15	53	153	0.35
	Methylviologen	7.40	103**	59**	1.74
		7.75	108**	63**	1.71

* Phosphate buffer (2.5 mM).

** Measured by flash yield determinations. Other values are from continuous tracings.

range of pH values (6.2–8.2), there is some tendency for the lower H^+/e^- values to be associated with the higher pH values. Several considerations lead us to believe that the true efficiency of proton translocation in this system is even more pH-independent than actually observed, however, and that the true value for the ratio H^+/e^- is most likely about 0.5, the highest value actually encountered at the lower pH regimes (pH 6–7). At higher pH's, especially above pH 8, the outward diffusion of protons in the light is probably much faster and therefore competes more efficiently with proton accumulation (uptake). This was apparent in the experiments, since the dark decay process observed after turning off the light was approximately twice as fast at pH 8 as at pH 7 (decay half-time = 7 s at pH 8; 15 s at pH 7). It was also apparent in the flash experiments that the initial linear phase of the pH rise at pH 8 was somewhat shorter than at pH 7.3 (see Fig. 3, trace c).

Effect of phosphorylation and arsenylation on proton uptake

Both the chemiosmotic and the chemical coupling hypotheses predict that concomitant phosphorylation or arsenylation should decrease the steady-state extent of the proton uptake [22]. Several workers have indeed observed a decrease in the pH rise by concomitant phosphorylation or arsenylation [15, 23], but the opposite effect — a stimulation of proton uptake by arsenylation — has also been reported [14]. As has been pointed out by Dilley and Shavit [15], the acceleration of electron transport in ordinary noncyclic electron transport systems by phospho-

rylation (or in some cases by the effect of ions in the phosphorylation medium, e.g. Mg^{2+}), and resultant increases in the rate of proton flux could easily mask the true effect of phosphorylation on proton gradients. Moreover, the addition of ADP (or ATP) itself has also been shown to cause marked increases in proton uptake [23]. However, by using the Photosystem II-dependent dibromothymoquinone reduction one avoids these complications since the electron transport in this system is not stimulated by phosphorylation [9], nor is proton uptake enhanced by the addition of ADP (cf. Table II). This reaction therefore provides a convenient system in which to test the effect of phosphorylation on proton uptake.

TABLE II

EFFECT OF PHOSPHORYLATION AND ARSENYLATION ON THE EXTENT OF THE LIGHT-INDUCED PROTON UPTAKE ASSOCIATED WITH THE ELECTRON TRANSPORT PATHWAY $H_2O \rightarrow$ PHOTOSYSTEM II $\rightarrow$ DIBROMOTHYMOQUINONE

Reactions were run as in Figs 5 and 6. Final concentrations of the additions were: P_i, 2.5 mM; ADP, 75 μM; hexokinase, 1 μg/2ml (plus 10 mM glucose); $HAsO_4^{2-}$, 2.5 mM. The extent of the proton uptake was determined by titration in the light as described in Methods. Note that the presence of a complete phosphorylation or arsenylation system significantly lowers the steady-state extent of the proton uptake, although the individual phosphorylating agents have no effect by themselves (Expt 1).

Expt No.	Initial pH	Additions	H^+ extent neauiv/mg chlorophyll	(%)
1	8.14	None	42	(100)
		ADP	42	(100)
		Hexokinase	40	(98)
		ADP, P_i, hexokinase	26	(62)
2	8.15	P_i	56	(100)
		ADP, P_i, hexokinase	22	(40)
3	8.22	P_i	70	(100)
		ADP, P_i, hexokinase	39	(56)
4	8.27	$HAsO_4^{2-}$	55	(100)
		$HAsO_4^{2-}$, ADP	44	(80)
5	8.25	$HAsO_4^{2-}$	73	(100)
		$HAsO_4^{2-}$, ADP	59	(81)

In Fig. 5, trace A demonstrates the reproducibility of the pH rise in a phosphate-containing suspension over repeated light cycles. This reproducibility greatly facilitated the experiments since the effect of an additive (e.g. ADP) could be examined without preparing a new reaction mixture. Trace B shows that in the absence of hexokinase, the addition of ADP initiates ATP formation which can be followed as the irreversible consumption of protons according to the equation:

$$ADP^{3-} + HPO_4^{2-} + H^+ \rightarrow ATP^{4-} + H_2O \qquad (pH\ 8)$$

Clearly the extent of the reversible proton uptake (seen as a proton efflux after turning off the light) is smaller under phosphorylating conditions (+ ADP) than under non-phosphorylating conditions (− ADP). This is demonstrated in a different way in trace C, where the hexokinase + glucose system is used to consume the ATP

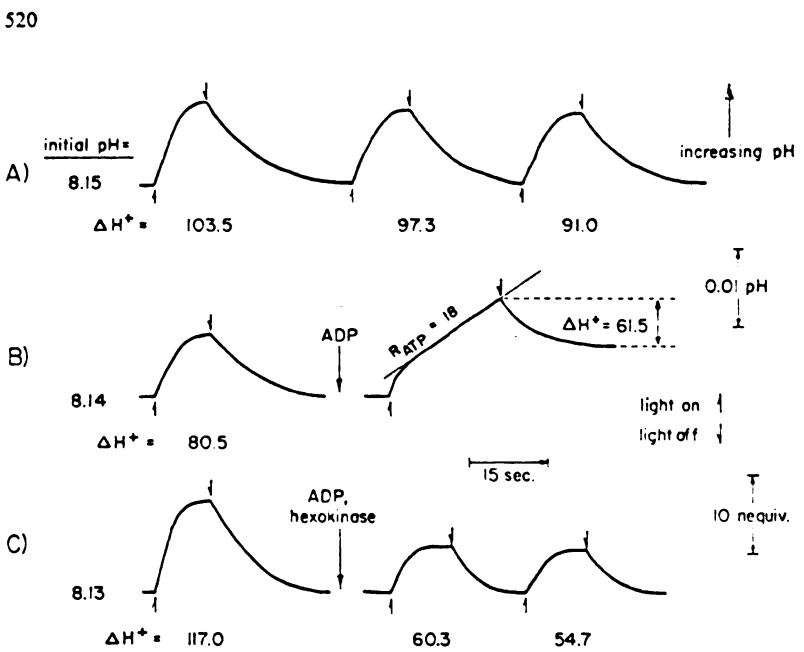

Fig. 5. Effect of phosphorylation on the extent of the light-induced proton uptake associated with electron transport from water to dibromothymoquinone. Reaction conditions are as in Fig. 1, trace A, except that the HEPES buffer was replaced by 2.5 mM Na_2HPO_4. The extent of the proton uptake (ΔH^+) was determined as described in Methods. Trace A illustrates the repeatability of the pH rise over repeated light–dark cycles. Trace B shows that the addition of ADP (final concentration 75 μM) causes an irreversible proton consumption in the light (due to ATP formation) and results in a decrease in the extent of the reversible proton uptake (seen as a dark efflux after turning off the light). This irreversible proton consumption was eliminated in trace C by the addition of hexokinase (1 μg/2ml) and glucose (10 mM) with the ADP (75 μM). Again a significant decrease in the extent of the proton uptake is observed. (The additives themselves had only a negligible effect on the buffering capacity of the reaction mixture.)

as it is produced and thus eliminate the irreversible proton consumption due to ATP formation. Hexokinase alone has no effect on the extent of the pH rise (cf. Table II).

If arsenate replaces phosphate (with or without hexokinase) a situation similar to the ADP+P_i+hexokinase system should be observed, since the unstable arsenylated ADP does not accumulate. Fig. 6 shows that indeed this is the case. A marked lowering in the extent of proton uptake is observed upon addition of ADP to an arsenate containing medium (in this case without hexokinase).

Table II summarizes the effect of various additions on the extent of the proton uptake associated with dibromothymoquinone reduction. It can be seen that in this system ADP, hexokinase or phosphate alone have no effect on the extent of proton uptake. The lack of effect by ADP in this system is in contrast with the observation of McCarty et al. [23]. Using the methylviologen system, they too noted that hexokinase had no effect on proton uptake, but they did observe a large stimulation by low concentrations of ADP. We have confirmed their observation using methylviologen as electron acceptor, although in our chloroplast preparations the maximum stimulation obtained was only 30–40%. The complete lack of ADP effect on proton uptake in the partial reaction involving only Coupling Site II suggests that the stimu-

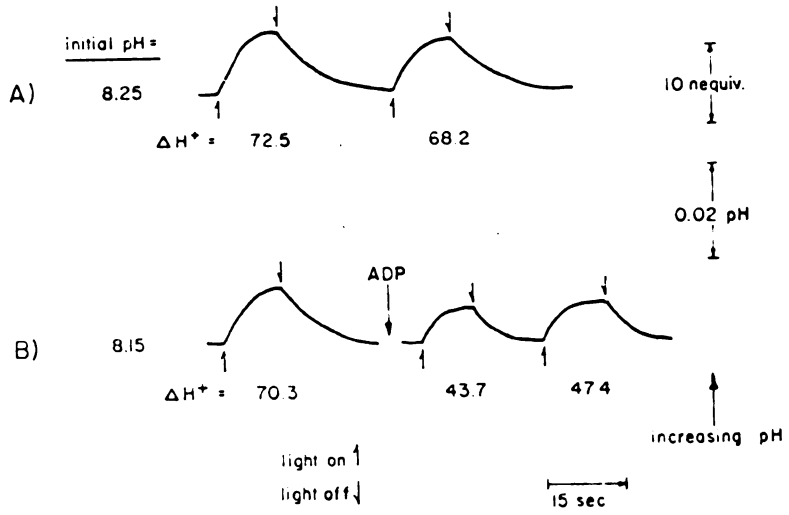

Fig. 6. Effect of arsenylation on the extent of the light induced proton uptake associated with electron transport to dibromothymoquinone. Reaction conditions were the same as in Fig. 5. except the Na_2HPO_4 buffer was replaced by 2.5 mM Na_2HAsO_4 and no hexokinase plus glucose was added. Note that the extent of the H^+ uptake was significantly smaller when ADP was added to the reaction mixture. The arsenylation of ADP does not result in an irreversible proton consumption, however, since the arsenylated nucleotide hydrolyzes rapidly.

latory effect of ADP may be expressed only when the electron transport system includes Coupling Site I.

DISCUSSION

The characteristics of the proton pump associated with the electron pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone, revealed in this study, may be summarized as follows: (i) approximately one proton is taken up for every pair of electrons transported (or H^+/e^- ratio $\approx$ 0.5; observed range, 0.4-0.5), (ii) the efficiency of proton uptake is essentially pH-independent (between 6.2 and 8.2), and (iii) the kinetics of proton uptake, as observed by flash-yield determinations, show no sign of burst phenomena (such as the "pH-gush" which is clearly seen in the methylviologen reducing system). It should be stressed here that the light-induced pH rise in the medium (proton uptake) described in this paper has no direct bearing on the chemistry of the oxidation or reduction of dibromothymoquinone per se, as demonstrated by the fact that gramicidin abolishes the reversible pH rise but has no effect on dibromothymoquinone reduction [1, 9] (see also Fig. 1, traces C and D).

In other words, the reduction of dibromothymoquinone consumes the same number of H^+ as are generated in the oxidation of water and therefore any pH changes observed in the medium must represent the formation of proton gradients.

Mechanism of proton translocation associated with Photosystem II electron transport

The mechanism of the Photosystem II "proton pump" described above and its efficiency ($H^+/e^- \approx 0.5$) can be explained most easily if we assume that the mem-

brane-bound electron transport enzymes are arranged within the membrane in such a manner as to favor the vectoral movement of protons directly involved in electron transport. Such an anisotropy in the electron transport chain could result in a proton uptake with the observed stoichiometry (H^+/e^- ratio) according to two models:

$$H_2O \rightarrow 1/2\ O_2 + 2e^- + 2H^+_{(inside)}$$
$$DBMIB + 2e^- + H^+_{(inside)} + H^+_{(outside)} \rightarrow DBMIBH_2$$
$$H_2O + DBMIB + H^+_{(outside)} \rightarrow 1/2\ O_2 + DBMIBH_2 + H^+_{(inside)}$$

or

$$H_2O \rightarrow 1/2\ O_2 + 2e^- + H^+_{(inside)} + H^+_{(outside)}$$
$$DBMIB + 2e^- + 2H^+_{(outside)} \rightarrow DBMIBH_2$$
$$H_2O + DBMIB + H^+_{(outside)} \rightarrow 1/2\ O_2 + DBMIBH_2 + H^+_{(inside)}$$

where DBMIB and $DBMIBH_2$ represent the oxidized and reduced forms of dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) respectively. The stoichiometric formation of the fully reduced dibromothymoquinone below pH 8 has in fact been confirmed (see Fig. 3). It should be noted concerning the above models that, in theory, any intermediate situation between these two extremes is possible. One thing is clearly indicated by these formulations, however. That is, in any situation, a large portion (50–100%) of the protons produced in the oxidation of water must be released to the inside of the thylakoid, and a similar and complementary portion of the protons needed for the hydrogenation of dibromothymoquinone must come from the outside (i.e. 50–100%, inversely related to the percentage of protons from water released inside).

The exact point at which the protons eventually used in the reduction of dibromothymoquinone enter into the electron transport chain is not known. However, there is kinetic evidence suggesting that dibromothymoquinone is primarily reduced via the plastoquinone pool [9], in line with the fact that its inhibitory site for electron transport is on the Photosystem I side of plastoquinone [24]. It seems reasonable to assume, therefore, that the protons which eventually are used in the reduction of dibromothymoquinone actually enter the electron transport chain at the level of one of the plastoquinones, or even at the level of the primary electron acceptor for Photosystem II, which may also be a quinone-type substance [25].

However, we hasten to point out that it is still premature to assign the H^+/e^- ratio of 0.5, observed for this "isolated" Photosystem II reaction, to the proton translocation efficiency intrinsic to the electron transport pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ plastoquinone since, for example, we have no clear idea of how the membrane permeating electron/hydrogen carrier dibromothymoquinone might affect the anisotropy of that region of the membrane surrounding the plastoquinones. Some indication that the intrinsic value might be higher than 0.5 has been offered by Reinwald et al. [26], who showed a kinetic correspondence between the initial fast proton uptake ("pH gush") and plastoquinone reduction. They calculated the $\Delta H^+/\frac{1}{2}\Delta$ plastoquinone ratio ($= H^+/e^-$) to be exactly 1. On the other hand their interpretation of the "pH gush" may be questioned. As we have shown in Fig. 3e and f the "gush" as observed in this laboratory is not associated with an increased rate of elec-

tron transport. Furthermore there are also reports describing anomalously high $H^+/h\nu$ ratios (e.g. 5) [27, 28] at the onset of illumination. This too suggests a different mechanism of pH change rather than an increased rate of electron transport. Nevertheless, it seems quite possible that in the normal, complete electron transport system the efficiency of proton translocation (H^+/e^-) attributable to the $H_2O \rightarrow$ Photosystem II $\rightarrow$ plastoquinone portion of the chain may be somewhat higher than the values approaching 0.5 observed when this system is isolated from the complete chain.

The relation between the accumulation of H^+ and the mechanism of phosphorylation at Coupling Site II

As shown in Table I, the efficiency of proton uptake (H^+/e^-) associated with dibromothymoquinone reduction (i.e. associated with Coupling Site II) is 0.4–0.5 over a wide pH range (6.2–8.2). A striking parallel is found in the efficiency of phosphorylation, $P/e_2 = 0.3$–0.4 (ref. 9) which is also essentially pH-independent over the same pH range. Furthermore, we have demonstrated unequivocally that concurrent phosphorylation markedly depresses the steady-state extent of proton uptake in this system (see Fig. 5 and Table II). These observations tempt us to postulate a coupling mechanism for Site II which involves the obligatory participation of H^+. Indeed, the H^+/e^- ratio is so close to the P/e_2 ratio that one is inclined to give considerable credence to the "chemiosmotic" coupling hypothesis of Mitchell [29].

The chemiosmotic hypothesis, as applied to chloroplast photophosphorylation, postulates that the efflux of 2 protons through the membrane-bound ATP synthesizing enzyme produces 1 ATP ($H^+/ATP = 2$). Various attempts have been made in recent years to determine this H^+/ATP ratio experimentally. Junge et al. [30] obtained a value of 3 from their studies of flash-induced proton uptake and 515 nm absorbance changes. The same value was also obtained by Schröder et al. [31] who studied the kinetics of the dark proton efflux after the steady state. (A prototype of this latter experiment by Schwartz [32] gave a value of 2). Using somewhat more direct measurements based on post-illumination phosphorylation (X_E) experiments Izawa [33] found a value of 2.4. Thus, the experimentally determined H^+/ATP ratios range between 2 and 3, which should be regarded as in good agreement with the hypothetical value of 2.

In the Photosystem II reaction dealt with here, an approximation of the H^+/ATP ratio may be made from the H^+/e^- and P/e_2 values by assuming (a) that the H^+/e^- ratio determined from initial rates applies to the steady state, and (b) that all protons translocated are available for ATP formation. If we take the Site II ratio of H^+/e^- as 0.5 (see Results) and the Site II P/e_2 as approximately 0.35 (see refs 3, 9), it follows that the requirement for protons in ATP formation (H^+/ATP) is approximately 2.9. While this is in good agreement with Mitchell's hypothetical value of 2 (ref. 29), it most likely represents an overestimation of the true H^+/ATP ratio since there is probably a non-coupled, unspecific outward diffusion of protons competing with the ATP synthesizing pathway. Based on the data presented in refs 31 and 33, a rough estimation of the amount of this unspecific proton efflux may be made. Summarizing those published data, it can be reasonably concluded that the non-phosphorylating efflux constitutes 20–40% of the total proton efflux (pH 8). The H^+/ATP ratios corrected for this component would now fall very near 2.

These considerations strongly implicate proton gradient formation as the mechanism of energy conservation at Coupling Site II. In addition, it seems likely that Site II can be identified as the water-oxidation reaction: the protons lost from water being discharged to the inside of the thylakoid. If this is so, the insensitivity of the electron transport rate to uncouplers of phosphorylation is easily understood [10]; the electron transport results from an essentially irreversible photochemical reaction which is, as a consequence of its irreversibility, insensitive to concentrations of its proton product.

ACKNOWLEDGEMENTS

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APPENDIX VII

THE PHOSPHORYLATION SITE ASSOCIATED WITH THE OXIDATION OF EXOGENOUS DONORS OF ELECTRONS TO PHOTOSYSTEM I

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THE PHOSPHORYLATION SITE ASSOCIATED WITH THE OXIDATION OF EXOGENOUS DONORS OF ELECTRONS TO PHOTOSYSTEM I

J. MICHAEL GOULD*

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Mich. 48824 (U.S.A.)

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SUMMARY

1. The Photosystem I-mediated transfer of electrons from diaminodurene, diaminotoluene and reduced 2,6-dichlorophenolindophenol to methylviologen is optimal at pH 8–8.5, where phosphorylation is also maximal. In the presence of superoxide dismutase, the efficiency of phosphorylation rises from $\leqslant 0.1$ at pH 6.5 to 0.6–0.7 at pH 8–8.5, regardless of the exogenous electron donor used.

2. The apparent K_m (at pH 8.1) for diaminodurene is $6 \cdot 10^{-4}$ M and for diaminotoluene is $1.2 \cdot 10^{-3}$ M. The concentrations of diaminodurene and diaminotoluene required to saturate the electron transport processes are > 2 mM and > 5 mM, respectively. At these higher electron donor concentrations the rates of electron transport are markedly increased by phosphorylation (1.5-fold) or by uncoupling conditions (2-fold).

3. Kinetic analysis of the transfer of electrons from reduced 2,6-dichlorophenolindophenol ($DCIPH_2$) to methylviologen indicates that two reactions with very different apparent K_m values for $DCIPH_2$ are involved. The rates of electron flux through both pathways are increased by phosphorylation or uncoupling conditions although only one of the pathways is coupled to ATP formation. No similar complications are observed when diaminodurene or diaminotoluene serves as the electron donor.

4. In the diaminodurene → methylviologen reaction, ATP formation and that part of the electron transport dependent upon ATP formation are partially inhibited by the energy transfer inhibitor $HgCl_2$. This partial inhibition of ATP formation rises to about 50 % at less than 1 atom of mercury per 20 molecules of chlorophyll, then does not further increase until very much higher levels of mercury are added.

Abbreviations: $DCIPH_2$, reduced 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; P/e_2, ratio of the number of molecules of ATP formed to the number of pairs of electrons transported; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea. DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; HEPPS, *N*-2-hydroxyethylpiperazine-*N*-propanesulfonic acid; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

* Present address: Section of Biochemistry, Molecular and Cell Biology, Wing Hall, Cornell University, Ithaca, N.Y. 14853, U.S.A.

5. It is suggested that exogenous electron donors such as diaminodurene, diaminotoluene and $DCIPH_2$ can substitute for an endogenous electron carrier in donating electrons to cytochrome *f* via the mercury-sensitive coupling site (Site I) located on the main electron-transporting chain. If this is so, there would seem to be no reason for postulating yet another coupling site on a side branch of the electron transport chain in order to account for cyclic photophosphorylation.

INTRODUCTION

The transport of electrons from water to conventional Hill reaction oxidants such as ferricyanide or methylviologen is coupled to ATP formation at two sites [1–4]. By the use of appropriate combinations of exogenous electron donors, electron acceptors and electron transport inhibitors it is possible to divide the complete electron transport process into two partial reactions each of which involves one of the two coupling sites [5]. This approach makes possible the study of segments of the electron transport chain and therefore the study of the functionally isolated coupling sites without physical disruption of the chloroplast lamellae by detergents or mechanical treatments. Through the use of these partial reactions it has been discovered that the two coupling sites in chloroplasts behave differently in many respects [5, 13, 42].

Activities of the recently discovered coupling site [1–4] believed to be associated with water oxidation (see ref. 6), Coupling Site II, can best be separated from activities of the well-known coupling site between plastoquinone and cytochrome *f* [7, 8], Coupling Site I, by using lipophilic oxidants as acceptors of the electrons from Photosystem II. These "Class III" acceptors (e.g. oxidized *p*-phenylenediamine) intercept electrons primarily at a point between the photosystems [9] (presumably before the plastoquinone pool [10]). Thus when a Photosystem-I component of the reduction of these acceptors is eliminated by inactivating plastocyanin with KCN [11] or poly-L-lysine [12], or by blocking electron transport at plastoquinone with 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) [1–3], the resulting pure Photosystem II reaction includes only Coupling Site II. Studies of Coupling Site II isolated in this manner have revealed the following characteristics: (a) Site II does not control the rate of electron transport since the rate is independent of phosphorylating or uncoupling conditions (refs 9, 10 and 13; but see refs 3 and 14); (b) Site II exhibits a characteristic phosphorylation efficiency (P/e_2) of 0.3–0.4 [2–5, 10, 11] which is independent of pH over the range 6–9 [5, 10]; (c) a light-driven reversible H^+-uptake reaction is associated with the partial reaction [5]. The efficiency of this proton uptake (H^+/e) is 0.4–0.5 over the pH range 6–8.5 [15]; finally (d) phosphorylation supported by Coupling Site II is not inhibited by the chloroplast energy transfer inhibitor $HgCl_2$ [16, 17].

Similarly, it is possible to isolate Coupling Site I from Site II by inhibiting electron flow from Photosystem II with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) while using an appropriate exogenous source of electrons for Photosystem I. In an earlier paper it was reported that the Photosystem I-dependent transport of electrons from reduced 2,6-dichlorophenolindophenol ($DCIPH_2$) to methylviologen utilizes Coupling Site I but not Coupling Site II [5]. Site I is probably a primary rate-

determining step for the overall Hill reaction ($H_2O \rightarrow$ methylviologen) [7]. In fact, many of the characteristics of the $DCIPH_2 \rightarrow$ methylviologen and the water $\rightarrow$ methylviologen reactions are similar. For instance, the rate of electron flux from $DCIPH_2$ to methylviologen is markedly stimulated by phosphorylation or by uncoupling conditions [5, 18–20] as is the overall Hill reaction. ATP formation supported by the transfer of electrons from $DCIPH_2$ to methylviologen is partially inhibited by $HgCl_2$, as is phosphorylation in the overall Hill reaction [16, 17]. Moreover, the efficiency of phosphorylation (P/e_2) is strongly pH dependent in the $DCIPH_2 \rightarrow$ methylviologen reaction [5] as is the efficiency of phosphorylation in the overall Hill reaction.

There are, however, some characteristics of the $DCIPH_2 \rightarrow$ methylviologen reaction which detract from its usefulness as a system for studying isolated Coupling Site I. For example, $DCIPH_2$ donates electrons to the transport chain in at least two places. Most of the electrons from $DCIPH_2$ are donated on the Photosystem II side of cytochrome *f* [21, 22]. This electron transport pathway is coupled to phosphorylation (via Site I) and is sensitive to inhibition by KCN [5]. A second, smaller portion of the electrons from $DCIPH_2$ are donated to P_{700} via a KCN-insensitive pathway which is not coupled to phosphorylation [23, 24]. Furthermore, the proportion of the electron transport which is KCN-insensitive is a function of the physical state of the chloroplasts, being much greater in damaged or "leaky" preparations [5].

In an effort to avoid some of the difficulties encountered with $DCIPH_2$ as electron donor, we have used other compounds which have long been known to be donors of electrons to Photosystem I (for a more complete discussion of Photosystem I reactions, see reviews by Trebst, contained in references 39 and 41). Several investigators have reported that diaminodurene is an extremely efficient donor of electrons to Photosystem I, supporting very high rates of ATP formation [18, 25, 26], but the properties of the coupling site responsible for this phosphorylation have not previously been characterized, nor has the coupling site been definitely located. Trebst and coworkers have recently postulated that lipophilic exogenously added donors of electrons catalyze phosphorylation via an "artificial" coupling site formed when protons are released to the inner phase of the thylakoid upon oxidation of the donor by plastocyanin or P_{700} [39, 40]. In this paper we arrive at a slightly different conclusion: that the electron-transport pathway from diaminodurene (or from the chemically related compound diaminotoluene) to methylviologen very likely includes the coupling site located just before cytochrome *f* (Site I). If so, the high rates of electron transport and ATP formation associated with these partial reactions, as well as the absence of secondary electron pathways, make these systems superior to the $DCIPH_2 \rightarrow$ methylviologen reaction for the isolation and study of Coupling Site I in chloroplasts.

MATERIALS AND METHODS

Chloroplasts were prepared from leaves of fresh market spinach (*Spinacia oleracea* L.) as described earlier [5]. However, for experiments with $HgCl_2$ it was necessary to avoid the use of tricine buffer since this compound strongly complexes Hg^{2+}. Chloroplasts for use in these experiments were isolated in a similar manner, substituting *N*-2-hydroxyethylpiperazine-*N*'-propanesulfonic acid (HEPPS)/NaOH

for the tricine or *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) where appropriate. In addition, EDTA was omitted from the grinding medium and bovine serum albumin was omitted from the suspension medium when $HgCl_2$ was used.

Electron transport was measured as the oxygen uptake resulting from the aerobic reoxidation of reduced methylviologen [18]. A membrane-covered Clark-type oxygen electrode was used. Reactions (2.0 ml final volume) were run in thermostatted vessels at 19 °C. Orange actinic illumination (> 600 nm; > 500 kerg $\cdot$ cm$^{-2} \cdot$ s^{-1}) was supplied by a 500-W tungsten projector lamp. The beam was passed through a 1-l round-bottom flask, containing a dilute $CuSO_4$ solution, which served both as a condensing lens and as a heat filter. ATP formation was determined as the $^{32}P_i$ incorporation into ATP as described elsewhere [27]. Radioactivity was measured as Cerenkov radiation by the technique of Gould et al. [28].

Diaminodurene (Research Organic/Inorganic Chemical Corp.) and 2,5-diaminotoluene (Aldrich) were dissolved in 0.1 M HCl, treated with Norit A, and recrystallized as the dihydrochlorides from concentrated HCl. Sodium 2,6-dichlorophenolindophenol (Matheson) was dissolved in ethanol and filtered. Fresh stock solutions of diaminodurene, diaminotoluene and DCIP were made before each experiment. Diaminodurene and diaminotoluene were dissolved in 0.01 M HCl. Ethanolic solutions of DCIP were further diluted with 1 mM Na_2HCO_3 so that the final concentration of ethanol in the reaction mixture did not exceed 0.5 %. The concentration of the DCIP solution was determined from the absorbance at 600 nm using an extinction coefficient (ε) of $2.1 \cdot 10^4$.

Superoxide dismutase was prepared from fresh bovine erythrocytes by the procedure of McCord and Fridovich [29]. The specific activity, assayed as the inhibition of cytochrome *c* reduction with xanthine oxidase, was > 3000 units mg protein.

RESULTS

Diaminodurene → methylviologen and diaminotoluene → methylviologen

Earlier studies of the Photosystem I-catalyzed transport of electrons from diaminodurene to methylviologen indicated that very high rates of electron transport can occur. However, a tight coupling between electron transport and phosphorylation was not apparent in these studies: the rate of electron transport being only slightly increased by phosphorylation or uncoupling conditions [18, 25]. Furthermore, the efficiency of phosphorylation (P/e_2) was found to be rather low, usually about 0.3 [18].

In this paper we have re-examined the diaminodurene → methylviologen reaction in an effort to better characterize the relationship between electron transport and phosphorylation. Recently several workers have shown that the photooxidations of a number of exogenous electron donors can be associated with misleadingly high rates of oxygen uptake when methylviologen serves as the electron acceptor [30–33]. This is because significant (and variable) portions of the superoxide radicals ($\cdot O_2^-$) generated by the aerobic reoxidation of the methylviologen radical react directly with the exogenous electron donor (AH_2) and are reduced to peroxide ($2 \cdot O_2^- + AH_2 \rightarrow 2\, HO_2^- + A$). This leads to an exagerated rate of O_2 uptake since some of the $\cdot O_2^-$, which normally dismutates to regenerate 1/2 of the consumed O_2 ($2 \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), becomes trapped as additional H_2O_2; to the extent that superoxide

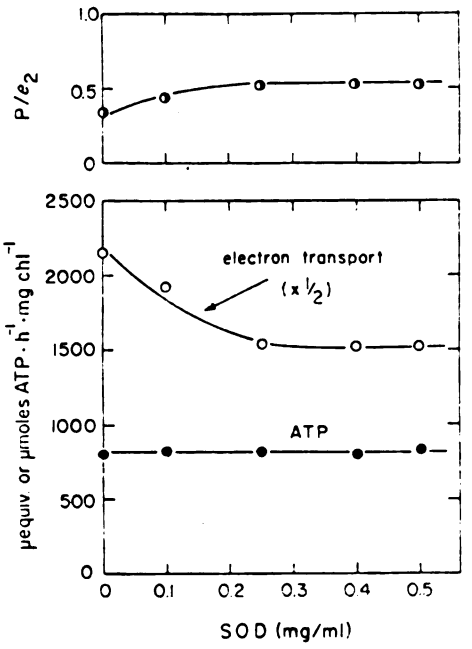

Fig. 1. Effect of bovine erythrocyte superoxide dismutase on the electron transport and phosphorylation associated with the diaminodurene → methylviologen reaction. The 2.0-ml reaction mixture consisted of: 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM tricine NaOH (pH 8.1), 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$, 2.5 μM DCMU, 2.5 mM ascorbate, 2.5 mM diaminodurene, 100 μM methylviologen, chloroplasts containing 5 μg chlorophyll and the indicated amount of superoxide dismutase (SOD). Note that although the oxygen uptake is lowered approximately 30 % by SOD, the rate of ATP formation is unaffected and therefore there is an increase in the apparent P/e_2 ratio from 0.3 to 0.5. For explanation see the text and ref. 30.

reacts with the donor, the transport of a single electron results in the uptake of a whole molecule of oxygen. However, if the dismutation of $\cdot O_2^-$ is greatly enhanced with superoxide dismutase, the reaction with the electron donor is eliminated and the rate of O_2 uptake becomes a reliable measure of the actual rate of electron transport, the uptake of a molecule of oxygen representing the transport of exactly two electrons ($O_2 = e_2$). This is, of course, only true if the chloroplast preparation is free of catalase activity (which was the case with the chloroplasts used in this study).

Fig. 1 shows the effect of bovine erythrocyte superoxide dismutase on the Photosystem I reaction diaminodurene → methylviologen. As increasing amounts of the enzyme compete more effectively for $\cdot O_2^-$, the rate of O_2 uptake is lowered by about 30 %, beyond which further addition of the enzyme has little effect. However, as might be expected, the rate of phosphorylation and therefore presumably the actual rate of electron transport is independent of superoxide dismutase. Because of this decrease in O_2 uptake the apparent P/e_2 (P/O_2) ratio increases from 0.3 to about 0.5. These data confirm the observation of Ort and Izawa [30], and indicate that accurate determinations of electron transport rates in the diaminodurene → methylviologen reaction can only be obtained in the presence of a suitable amount of superoxide

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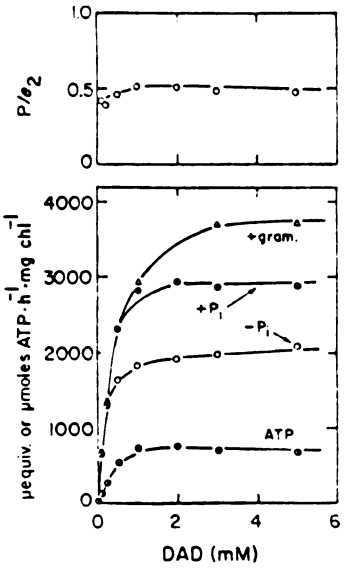

Fig. 2. Effect of diaminodurene (DAD) concentration on the rate of electron transport and phosphorylation in the diaminodurene → methylviologen reaction. The reaction mixture was as described in Fig. 1, except that the diaminodurene concentration was varied as indicated. The concentration of superoxide dismutase was 0.4 mg/ml. When added, gramicidin was 4 μg/ml.

dismutase. Very similar effects of superoxide dismutase were also observed when 5 mM diaminotoluene or 2,6-dichlorophenolindophenol served as the electron donor.

Since electron transport from diaminodurene (or diaminotoluene) to methylviologen exhibits many of the characteristics of the complete Hill reaction (H_2O → methylviologen), both reactions probably share the same rate-limiting step (i.e. Coupling Site I). At high diaminodurene concentrations (> 2 mM), the electron transport from diaminodurene to methylviologen is considerably accelerated by phosphorylation or to an even greater extent by uncoupling (Fig. 2). The failure of some earlier investigators to detect these large stimulations is perhaps due in part to the low concentrations of diaminodurene generally employed in the study of this reaction: typically < 0.5 mM. At these lower concentrations the donation of electrons by diaminodurene can be rate-limiting, and the effects of phosphorylation or uncoupling on electron transport may be largely concealed. At the higher diaminodurene concentrations used here, however, the diaminodurene → methylviologen reaction resembles very much both the $DCIPH_2$ → methylviologen and the H_2O → methylviologen reaction in its response to phosphorylating or uncoupling conditions, even though all rates are much higher in the diaminodurene system.

The kinetics of the diaminodurene → methylviologen reaction are shown by a double-reciprocal plot in Fig. 3. The rate-limitation imposed by the energy coupling mechanism is clearly revealed in this plot as departures from linearity. The data also indicate that this rate limitation can only be completely relaxed in the presence of an uncoupler.

The reaction diaminotoluene → methylviologen exhibits very similar characteristics to the diaminodurene → methylviologen reaction, except (a) electron trans-

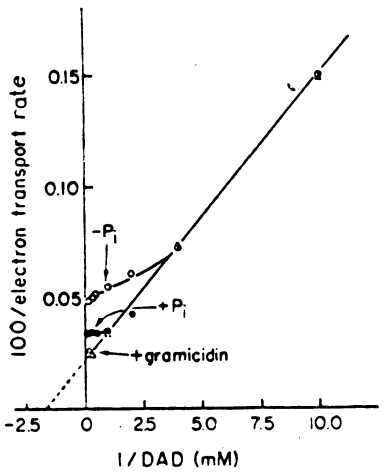

Fig. 3. Double-reciprocal plot showing the effect of diaminodurene concentration on electron transport in the diaminodurene → methylviologen reaction. The data are replotted from Fig. 2. (Electron transport rates are in μequiv · h^{-1} per mg chlorophyll.) Note that, at very low diaminodurene concentrations, electron transport in the presence or absence of phosphate (P_i) or the uncoupler gramicidin is limited by the same rate-determining step. As the concentration of diaminodurene is increased, different rate-limitations become evident unless the reaction is uncoupled by gramicidin.

port supported by diaminotoluene saturates at slightly higher concentrations ($>$ 5 mM), and (b) the absolute rates of electron transport and phosphorylation are about 30 % lower under optimal conditions with diaminotoluene as the electron donor. Some of the characteristics of Photosystem I reactions catalyzed by diaminodurene, diaminotoluene and $DCIPH_2$ are summarized in Table I.

TABLE I

DIAMINODURENE, DIAMINOTOLUENE AND REDUCED 2,6-DICHLOROPHENOL-INDOPHENOL AS DONORS OF ELECTRONS TO PHOTOSYSTEM I

Reactions were run as described in Fig. 2 when diaminodurene and diaminotoluene were the electron donors, or as in Fig. 5 when $DCIPH_2$ served as the electron donor.

Electron donor	Apparent K_m (mM)	Approximate concentration required to saturate rates (mM)	Approximate V (μequiv · h^{-1} per mg chlorophyll)*
Diaminodurene	0.6	3	4000
Diaminotoluene	1.2	5	3000
$DCIPH_2$	0.03** | 0.5** |	>0.6	>1000

* Determined in the presence of 4 μg/ml gramicidin D. The rates are those obtained at the saturating or nearly saturating light intensities used in this study (see Methods).

** High- and low-affinity components determined as described in ref. 34. See Fig. 5 and text for further explanation.

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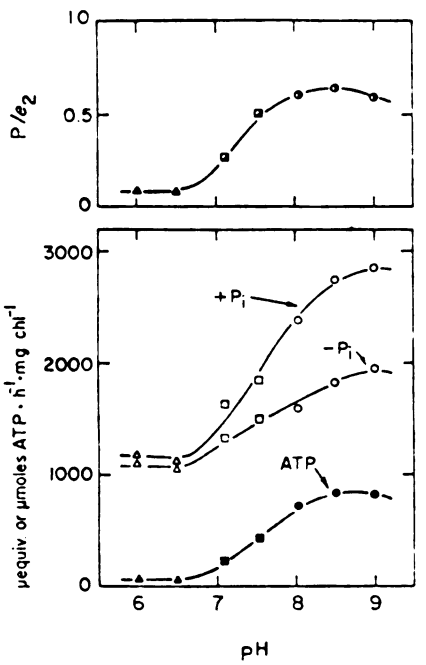

Fig. 4. Effect of pH on the rate of electron transport and phosphorylation associated with the diaminodurene → methylviologen reaction. The reaction mixture was as described in Fig. 1, except that the concentration of diaminodurene was 3 mM. The concentration of superoxide dismutase was 0.37 mg/ml. The buffers (50 mM) employed were 2-(*N*-morpholino)-ethanesulfonic acid/NaOH (triangles), HEPES/NaOH (squares) and tricine/NaOH (circles). Note that the efficiency of phosphorylation (P/e_2) is strongly pH-dependent, being optimal at pH 8–9, and that a large stimulation of electron transport by phosphorylation is also seen at the higher pH values. These characteristics resemble very closely the characteristics of Coupling Site I in chloroplasts (see Introduction and ref. 5).

Effects of pH on the diaminotoluene → methylviologen reaction are shown in Fig. 4. The rates of both electron transport and ATP formation are optimal at pH 8.5 or higher, with little or no phosphorylation occuring below pH 7. The P/e_2 ratio, therefore, shows the strong pH-dependence characteristic of Coupling Site I [5]. Indeed, the effects of pH on the H_2O → methylviologen, $DCIPH_2$ → methylviologen and diaminodurene → methylviologen reactions are strikingly similar (compare Fig. 4 and ref. 5, Fig. 1).

$DCIPH_2$ → methylviologen

The widely used Photosystem I reaction transporting electrons from reduced $DCIPH_2$ to methylviologen probably utilizes the coupling site after plastoquinone and before cytochrome *f* (i.e. Site 1) [21, 22]. However, it has been shown that the donation of electrons to Photosystem I by $DCIPH_2$ has two components [5, 23, 24]. One component is coupled to phosphorylation (presumably via Site 1) and is sensitive to inactivation of plastocyanin by KCN treatment. The other component is not coupled to phosphorylation and is insensitive to KCN (ref. 5, see below).

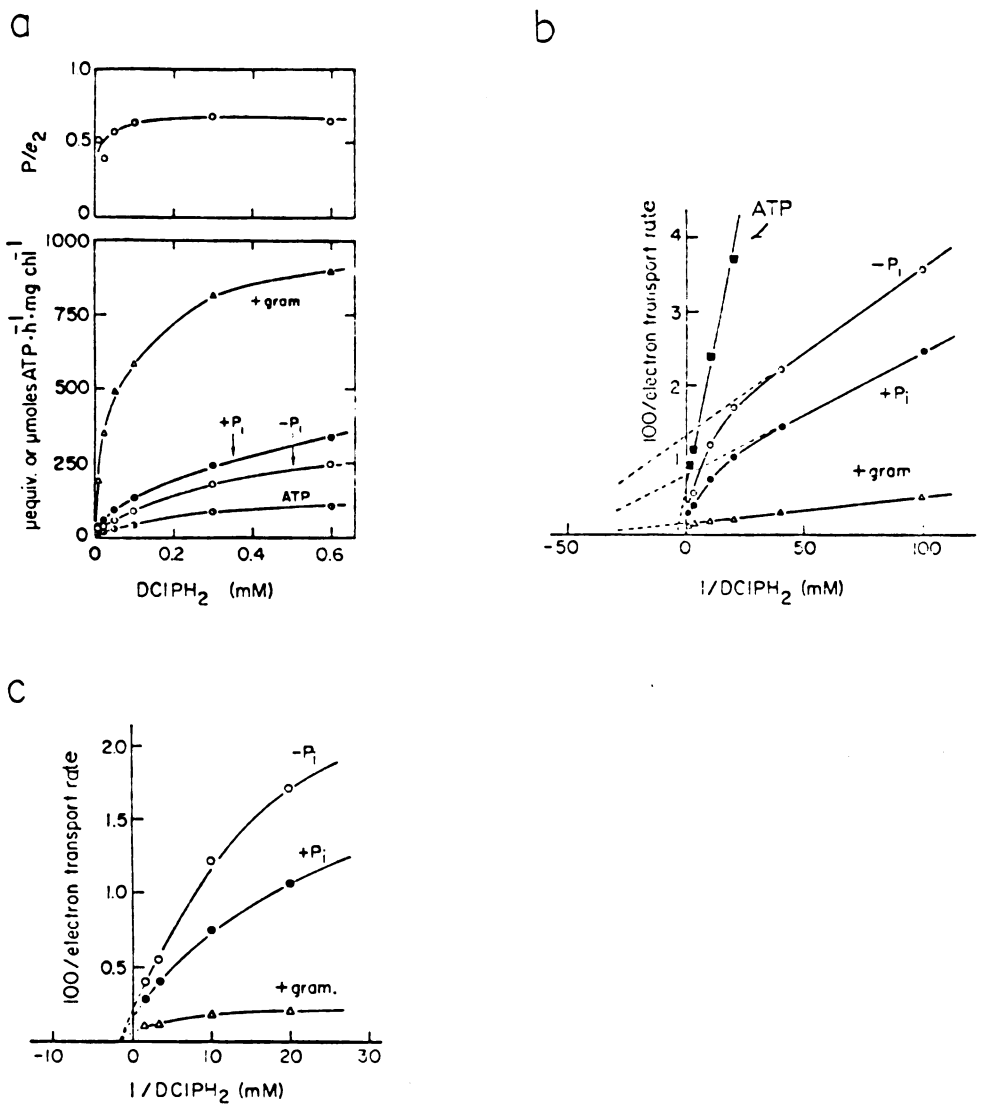

Fig. 5. a. Effect of $DCIPH_2$ concentration on electron transport and phosphorylation in the $DCIPH_2$ → methylviologen reaction. The reaction mixture (2.0 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM tricine/NaOH (pH 8.1), 0.75 mM ADP, 2.5 μM DCMU, 100 μM methylviologen, 2.5 mM ascorbate, 250 μg superoxide dismutase, chloroplasts containing 40 μg chlorophyll, and the indicated amount of $DCIPH_2$. When added, $Na_2H^{32}PO_4$ (P_i) was 5 mM; gramicidin was 4 μg/ml. Note that even at the lowest levels of $DCIPH_2$ tested, where the rates of coupled electron flow are very low, a large stimulation of electron transport by gramicidin can be observed. b. Double-reciprocal plot of the data presented in a. Electron transport (circles, triangles) is in μequiv · h^{-1} per mg chlorophyll; ATP formation (squares) is in μmol ATP · h^{-1} per mg chlorophyll. Note the biphasic nature of the plots, indicating that two different reactions with different affinities for $DCIPH_2$ are competing for $DCIPH_2$ as a substrate [34]. Note also that the rate of electron transport from $DCIPH_2$ to methylviologen is increased by phosphorylation and uncoupling conditions in both reactions. c. Reploi of some of the data presented in b showing the component of the $DCIPH_2$ → methylviologen reaction with the higher apparent K_m.

The two-component nature of this reaction, also noted by Arntzen et al. [24], is illustrated in the double reciprocal plots in Figs 5b and 5c. The biphasic nature of the plots is characteristic of a system in which two reactions compete for the same substrate [34], $DCIPH_2$ in this case. It is important to note, however, that both reactions appear to be dependent on the coupling state of the chloroplast, although only the reaction with the lower affinity for $DCIPH_2$ seems to support ATP formation (Fig. 5b); even at the lowest concentrations of $DCIPH_2$, where the high affinity, non-phosphorylating reaction predominates, significant stimulations of electron transport by uncouplers and by ADP+P_i are observed. Perhaps the access of the reduced indophenol to its electron donation sites within the lipid membrane is limited to some extent by the high energy state (proton gradient?) associated with the membrane.

$HgCl_2$ inhibition

$HgCl_2$ has been shown to act as a unique energy transfer inhibitor in chloroplasts at extremely low levels [35]. Concentrations of 50 nmol Hg^{2+} per mg chlorophyll or less inhibit ATP formation in the Hill reaction to a 50 % inhibition plateau. It has recently been shown that $HgCl_2$ inhibits phosphorylation associated with $H_2O \rightarrow$ methylviologen (Coupling Sites II and I) and $DCIPH_2 \rightarrow$ methylviologen (Site I only)

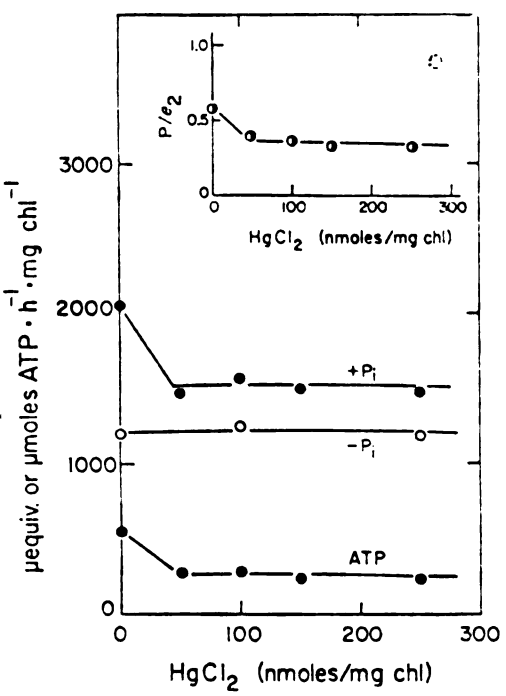

Fig. 6. Effect of the energy transfer inhibitor $HgCl_2$ on electron transport and phosphorylation associated with the diaminodurene → methylviologen reaction. The reaction mixture was as in Fig. 1, except the buffer employed was 50 mM HEPPS/NaOH (pH 8.0). The concentration of superoxide d smutase was 0.37 mg/ml. Chloroplasts were incubated with the indicated amount of $HgCl_2$ for 20 s in the dark before addition of the remainder of the reaction mixture. Note that ATP formation and that portion of the electron transport dependent upon phosphorylation are inhibited to a plateau of about 50 % by low concentrations of $HgCl_2$.

but not phosphorylation associated with $H_2O \rightarrow$ Class III acceptors (Site II only) [16]. The implication is that $HgCl_2$ acts as a specific inhibitor of Coupling Site I. (The reason for the peculiar 50 % inhibition plateau is not at all understood.) Fig. 6 shows that the diaminodurene → methylviologen reaction also includes the Hg-sensitive coupling site.

The effects of $HgCl_2$ on the diaminodurene → methylviologen reaction presented here are somewhat at variance with those reported by Bradeen and Winget [17]. They found that phosphorylation-coupled electron transport from diaminodurene to methylviologen was insensitive to low concentrations of $HgCl_2$ (250 nmol/mg chlorophyll) although phosphorylation itself was inhibited about 30 % by 50–250 nmol $HgCl_2$ per mg chlorophyll. (Higher concentrations of mercury result in electron transport inhibition due to plastocyanin inactivation [36].) However, it should be pointed out that a small inhibition of a very fast reaction can often be difficult to detect when the electron transport is measured with the slow-responding, Clark-type oxygen electrode.

DISCUSSION

There are a variety of compelling reasons for believing that the primary pathway of electron transport from $DCIPH_2$ to methylviologen includes the coupling site located between plastoquinone and cytochrome *f*, which we have called Site I. Izawa [21] and Larkum and Bonner [22], on the basis of spectral evidence, and Neumann et al. [19], on the basis of uncoupler studies, have suggested that $DCIPH_2$ donates electrons to the transport chain at a point before a phosphorylation-dependent rate-limiting reaction on the Photosystem II side of cytochrome *f*. It has also been shown that the phosphorylation-coupled $DCIPH_2 \rightarrow$ methylviologen reaction resembles the coupled overall $H_2O \rightarrow$ methylviologen reaction in its response to pH, to $ADP+P_i$, to uncouplers, to $HgCl_2$, to other energy transfer inhibitors and to KCN [5]. It seems highly probable therefore that both reactions share the same rate-determining coupling site. This conclusion is further strengthened by the fact that the sums of the phosphorylation efficiencies of the two partial reactions ($H_2O \rightarrow$ Photosystem II → Class III acceptors, and $DCIPH_2 \rightarrow$ Photosystem I → methylviologen are very close to the efficiencies observed for the overall $H_2O \rightarrow$ methylviologen reaction over the wide pH range 6–9 (see refs 5 and 42).

Unfortunately the $DCIPH_2 \rightarrow$ methylviologen reaction is not altogether satisfactory for the study of Coupling Site I. As we have seen, the reaction is complex. The electron transport has a non-phosphorylating component which utilizes a different pathway and the magnitude of this component varies with the state of the chloroplast membrane (see Fig. 5 and refs. 5, 22–24).

The results presented in this paper seem to indicate that the photooxidations of diaminodurene or diaminotoluene are partial reactions of the electron transport chain which also involve Coupling Site I. Again the effects of pH, of $ADP+P_i$, of uncouplers, of $HgCl_2$ and of KCN are similar in the diaminodurene (or diaminotoluene) → methylviologen, in the $DCIPH_2 \rightarrow$ methylviologen and in the overall $H_2O \rightarrow$ methylviologen reactions. Therefore, the criteria of Site I involvement applied to the $DCIPH_2$ reaction may also be applied to the diaminodurene (or diaminotoluene) → methylviologen reactions. This conclusion is strongly supported by the recent finding

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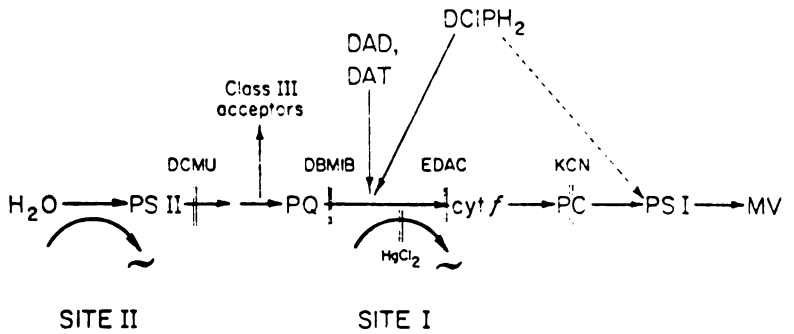

Fig. 7. A scheme for electron transport pathways in isolated chloroplasts showing the two sites of energy transduction (~). PS II, Photosystem II; PS I, Photosystem I; PQ, plastoquinone; cyt *f*, cytochrome *f*; PC, plastocyanin; DAD, diaminodurene; DAT, diaminotoluene; MV, methylviologen; Class III acceptors include lipophilic strong oxidants such as oxidized *p*-phenylenediamines or *p*-benzoquinones. Note that in this scheme diaminodurene, diaminotoluene and $DCIPH_2$ donate electrons directly to the main electron transport chain at a point before the rate-determining Coupling Site I, which also limits the rate of electron transport from H_2O to methylviologen. Note also the two component pathways of the $DCIPH_2$ → methylviologen reaction, one KCN-sensitive and one KCN-insensitive [5]. For further explanation see refs 2–6, 8–11, 16, 23 and 37.

of McCarty [37] that the electron transport inhibitor 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), which apparently blocks electron flow from plastoquinone to cytochrome *f* at some point after the DBMIB inhibition site, inhibits the transport of electrons from diaminodurene to methylviologen. This suggests that, while the chemiosmotic model for Photosystem I phosphorylation may be basically correct, the actual acceptor of electrons from diaminodurene is not plastocyanin (as suggested by Trebst [39, 40]) but cytochrome *f*, as is clearly the case with the coupled $DCIPH_2$ → methylviologen reaction [21, 22]. This is consistent also with the fact that no energy control over electron transport can be detected between cytochrome *f* and $NADP^+$ [7, 8].

A scheme summarizing these observations and conclusions is presented in Fig. 7.

One important implication of the results summarized above concerns the probable location of the coupling site associated with Photosystem I cyclic phosphorylation reactions such as those catalyzed by pyocyanine, diaminodurene/oxidized diaminodurene, or $DCIPH_2$/DCIP. It has long been postulated that cyclic photophosphorylation is associated with a separate coupling site not on the main electron transport chain (for a review, see ref. 38). In light of the evidence presented here it seems more likely that the reduced form of the cycling cofactor (i.e. $DCIPH_2$, diaminodurene or reduced pyocyanine) donates electrons directly to the main electron transport chain at a point before Coupling Site I (i.e. before cytochrome *f*, but see ref. 40) while the oxidized form of the cycling cofactor (DCIP, oxidized diaminodurene or pyocyanine) accepts electrons from Photosystem I. Trebst and co-workers have recently presented a similar view [4, 39, 40]. Such schemes eliminate altogether the need to postulate a special "cyclic" coupling site associated with a separate electron-transport side chain. Instead these schemes have cyclic phosphorylation resulting from the proton translocating properties of the exogenously added lipophilic electron

donor: i.e. the donor is reduced (consuming protons) on the outside and oxidized (releasing protons) on the inside of the thylakoid, thereby generating a transmembrane proton gradient which may drive phosphorylation. The scheme presented in Fig. 7 is more specific in that cytochrome *f* is suggested to be the acceptor of electrons from the exogenous (artificial) electron donor (e.g. diaminodurene) as well as the endogenous (natural) electron donor plastoquinone. Studies with electron transport inhibitors also lend support to the scheme. Ouitrakul and Izawa [11] found that KCN inactivation of plastocyanin inhibited cyclic phosphorylation catalyzed by $DCIPH_2$/DCIP, diaminodurene/oxidized diaminodurene, pyocyanine and low concentrations of *N*-methylphenazonium methosulfate (PMS)*, showing that cyclic electron transport shares at least this carrier with non-cyclic electron transport. Furthermore, McCarty [37] has shown that pyocyanine-mediated cyclic phosphorylation is inhibited by EDAC. Thus it seems clear that cytochrome *f* (and therefore probably Coupling Site I) is involved in the cyclic reaction [37].

If we are to equate the site of cyclic photophosphorylation and other Photosystem I-mediated phosphorylation reactions with the rate-limiting Coupling Site I of the non-cyclic electron transport system, a problem of relative rates arises, since the Photosystem I photophosphorylation can be several times faster than the non-cyclic photophosphorylation associated with the Hill reaction. This apparent conflict, however, may be easily explained in terms of the differences in the concentrations and reactivities of the different electron donors (e.g. diaminodurene vs. natural electron donor) at the common site of oxidation, where energy conservation takes place. Electron transport rates of all of these reactions can be under comparable degrees of energy control, regardless of the electron flux, since the steady-state level of the high-energy intermediate or state (proton gradient?), and therefore the back pressure it imposes on electron transport through the coupling site [13], may well increase or decrease in approximate proportion to the level of electron flux. The result would be a similar degree of dependence of electron flow on phosphorylation and on uncoupling regardless of the absolute level of electron flux.

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* Cyclic phosphorylation reactions catalyzed by PMS are complex, the properties apparently varying with the PMS concentration. See refs 11, 17 and 23.

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APPENDIX VIII

ELECTRON TRANSPORT REACTIONS, ENERGY CONSERVATION REACTIONS AND PHOSPHORYLATION IN CHLOROPLASTS

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ELECTRON TRANSPORT REACTIONS, ENERGY CONSERVATION REACTIONS AND PHOSPHORYLATION IN CHLOROPLASTS.

S. Izawa, D. R. Ort, J. M. Gould, and N. E. Good

Department of Botany and Plant Pathology, Michigan State University
East Lansing, Michigan 48824 (U. S. A.)

I. Location of the Sites of Phosphorylation

This part of the paper deals with the use of a variety of electron donors, electron acceptors, and specific electron transport inhibitors to isolate quite different oxidation-reduction reactions responsible for ATP formation in chloroplasts. Figure 1 summarizes the partial reactions thus investigated.

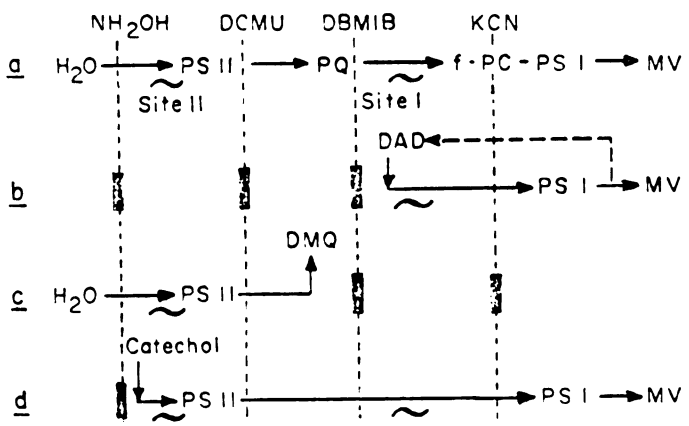

Figure 1. Chloroplast reactions currently available for the study of photosynthetic electron transport and phosphorylation

Figure 1a. The scheme shows the overall electron transport system, the probable positions of the transport blocks utilized, and the apparent location of the two sites of energy conservation. Let us first review the nature of the inhibitions indicated by the

Abbreviations used: DAD, diaminodurene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCIPH2, reduced form of 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ, 2,5-dimethyl-p-benzoquinone; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

vertical bars across the transport chain. Hydroxylamine has long been known as an inhibitor of photosynthesis and the Hill reaction. More recently it has been recognized that this amine inactivates the water-oxidizing system (1-3) as do other amines including Tris (4). The great advantage of hydroxylamine-treatment is that it can be carried out in such a manner as to totally and specifically abolish water oxidation without adversely affecting the ability of the chloroplasts to phosphorylate ADP (5). Hydroxylamine-treated chloroplasts are highly active in the Photosystem II-dependent oxidation of those exogenous electron donors which can substitute for water. DCMU is the standard specific inhibitor of Photosystem II reactions. Dibromoisopropylbenzoquinone (DBMIB), introduced by Trebst and his associates (6), is a plastoquinone antagonist which seems to block the reoxidation of reduced plastoquinone (7,8) and thus prevent the transfer of electrons from Photosystem II to Photosystem I. Transfer of electrons between the two photosystems is also blocked by prior treatment of the chloroplasts with KCN (9). In this case the inhibition results from inactivation of plastocyanin (9,10) and some of the consequences of the block are quite different from the consequences of DBMIB inhibition (see below). The partial reactions of the electron transport system made available by the use of these inhibitors are shown in Figure 1 (b,c and d).

Figure 1b. A number of exogenous electron donors such as Diaminodurene (DAD) and reduced indophenol ($DCIPH_2$) can be oxidized by Photosystem I while $NADP^+$ or methylviologen (MV^+) is being reduced. These familiar reactions are insensitive to hydroxylamine, DCMU and DBMIB (7). In the past the electron transport has been difficult to measure accurately for two reasons. Superoxide formed by the aerobic oxidation of reduced methylviologen can spontaneously oxidize the electron donor with the consequence that the electron transport may be overestimated (11-14). Furthermore the tendency of the oxidized form of the donor to accept electrons, superceding the acceptor added, can result in a hidden cyclic electron flow with the consequence that the electron transport may be underestimated. This latter problem is particularly acute when the relatively inefficient $NADP^+$-ferredoxin acceptor system is used (15-17). Thus, at the present time the only reliable procedure for measuring the Photosystem I oxidations of exogenous electron donors involves the use of an efficient acceptor such as methylviologen under aerobic conditions, the use of ascorbate to prevent the accumulation of the oxidized form of the

donor, and the use of large amounts of superoxide dismutase (18). When these precautions are taken the efficiency of the associated phosphorylation reaction can be measured with some precision. The ratio of ATP molecules formed to electron pairs transferred (P/e_2) measured in this manner is consistently about 0.6 regardless of the electron donor used (13,18 but see 19). We have called the site responsible for the phosphorylation "Site I" for historical reasons since this seems to be the site best known to contain the rate of reduction of cytochrome f (20-24). As already implied, there are good reasons for believing that this is also the site involved in the so-called "cyclic phosphorylation" mediated by electron carriers such as DAD or pyocyanine. In any event, the reactions, cyclic or non-cyclic, are inhibited if plastocyanin is inactivated by KCN (9) or poly-L-lysine (23).

Figure 1c. Lipophilic strong oxidants such as dimethylquinone (DMQ), oxidized p-phenylendiamine (PD_{ox}) or oxidized N,N,N',N'-tetramethyl-p-phenylendiamine (DAD_{ox}) can intercept electrons between the two photosystems (24). Thus the electron transport is totally independent of Photosystem I if DBMIB is present (25-27) or the chloroplasts have been treated with KCN (9) (see figure 2). The Photosystem II - dependent electron transport, which can be very rapid, is then coupled to phosphorylation with an efficiency (P/e_2) of about 0.4 (9,25-28). We have called the site of phosphorylation involved "Site II", both because it was the second identified and because it is associated with Photosystem II.

Figure 1d. As we have already indicated, hydroxylamine-treated chloroplasts can no longer obtain electrons from water but they can oxidize a number of exogenous substances by reactions which depend on Photosystem II and are therefore inhibited by DCMU. Thus electrons can be transported from ascorbate, benzidine (9), catechol (18), ferrocyanide, iodide, etc. (29), to methylviologen in the absence but not in the presence of DCMU. Again the electron transport is coupled to phosphorylation - but the efficiency of phosphorylation depends on the nature of the electron donor (29). With some donors both Site I and Site II are operative but with other donors only Site I seems to be operative. This observation will be considered in more depth in the next section of the paper since it may be relevant to an understanding of the mechanism of phosphorylation at Site II.

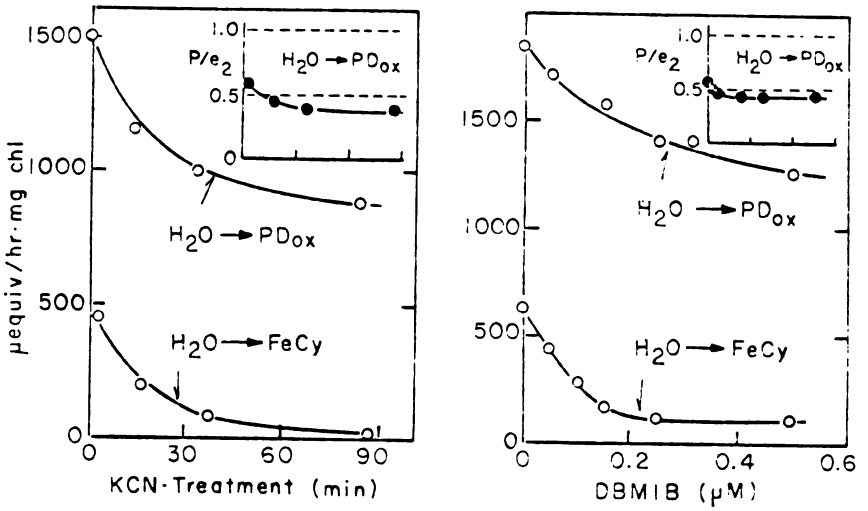

Figure 2. Reduction of a lipophilic strong oxidant with electrons from water. The reduction of oxidized p-phenylenediamine (PD_{ox}) has two components, a large component which depends only on Photosystem II and is therefore insensitive to KCN-treatments or DBMIB and a smaller component which is eliminated by those inhibitors. Note that the large Photosystem II component of the electron transport supports phosphorylation with the same efficiency (P/e_2) of about 0.4 regardless of which inhibitor is used to eliminate the Photosystem I contribution. Ferricyanide reduction, which involves both photosystems in these chloroplasts, is shown for comparison.

II. Characterization of the Sites of Phosphorylation

(a) Proton production and phosphorylation at Site II.

A great deal of evidence has accumulated which interrelates electron transport, the formation of hydrogen ion gradients, and ATP formation in chloroplasts. Much of this evidence supports the "chemiosmotic" explanation of phosphorylation proposed by Mitchell (30) and, indeed, some predictions of Mitchell's theory have been confirmed by experiments so beautifully that the theory has gained considerable popularity. Figure 3 illustrates the presumed workings of the chemiosmotic mechanism in chloroplasts when they are oxidizing water (upper) or exogenous donors (lower) (31; for a review see 32,

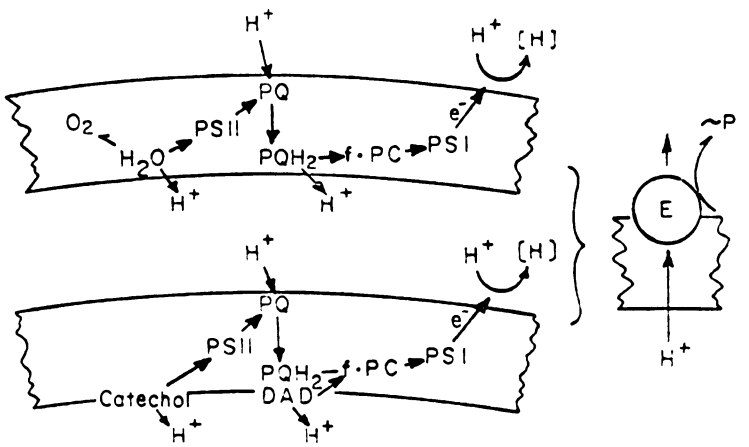

Figure 3. A conventional chemiosmotic interpretation of phosphorylation in chloroplasts. The upper figure shows a segment of a thylakoid membrane oxidizing water. The lower segment shows a segment oxidizing exogenous electron donors. The essential features of this model are: a) Photosystem I oxidations and Photosystem II oxidations both result in the accumulation of protons inside the thylakoid. b) These protons are extruded through the coupling factor in such a manner as to generate ATP.

According to the model hydrogen donors are oxidized on or near the inside of the membrane and as a result hydrogen ions are extruded into the inner space of the thylakoid. This is believed to happen when water is oxidized by Photosystem II (33-35) and again when some intermediate hydrogen carrier such as plastoquinone is oxidized by Photosystem I via cytochrome f (34). The inner protons then somehow generate ATP while diffusing out of the thylakoid via the coupling factor (30). The two proton-producing reactions thus constitute the two "sites" of phosphorylation described above.

The concept of internal proton production as an essential intermediate step in ATP formation has received very strong support from experiments with chloroplasts. As Hauska et al. (36) have pointed out, the fact that the oxidation of DAD by Photosystem I supports phosphorylation while the oxidation of TMPD does not may be a consequence of the fact that DAD oxidation results in hydrogen ion production whereas the removal of an electron from TMPD produces only the free radical. We have now shown that a similar correlation between proton release and ATP formation is observed when exogenous

electron donors are oxidized by Photosystem II. Figure 4 compares the oxidation of catechol by hydroxylamine-treated chloroplasts with the oxidation of ferrocyanide by the same chloroplasts. When catechol is oxidized the efficiency of phosphorylation is almost

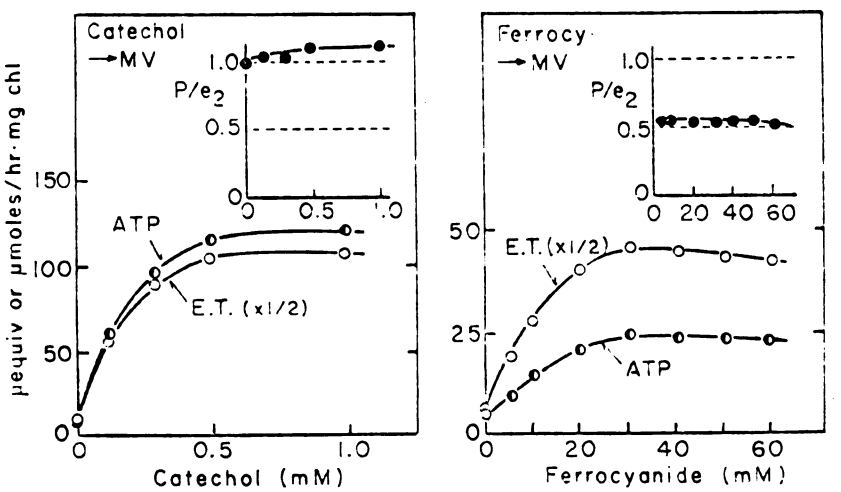

Figure 4. The oxidation of catechol and ferrocyanide by hydroxylamine-treated chloroplasts with methylviologen as electron acceptor. These reactions are inhibited by DCMU and therefore require Photosystem II. Note that the oxidation of catechol supports phosphorylation with an efficiency which indicates both Site I and Site II whereas the oxidation of ferrocyanide supports phosphorylation with the efficiency which is characteristic of the operation of Site I alone.

the same as when water is oxidized and therefore both Site I and Site II must be operative (13). On the other hand, when ferrocyanide is oxidized the efficiency of phosphorylation is lowered to about half although ferrocyanide at the concentrations used has no uncoupling effect (29). Indeed the phosphorylation associated with the transport of electrons from ferrocyanide to methylviologen has all of the properties of Site I phosphorylation and none of the properties of Site II phosphorylation (29; see also figure 6 below). Therefore it is reasonable to suppose that Site II is inoperative when ferrocyanide (pure electron donor) substitutes for water or for catechol (hydrogen donors). Incidentally, the fact that this reaction requires very high concentrations of ferrocyanide (29) tends to confirm the chemiosmotic notion that Photosystem II oxidations occur inside of the thylakoid membrane.

A similar correlation between proton production and the operation of Site II has been observed when other exogenous donors are oxidized by Photosystem II. On the basis of the efficiencies of phosphorylation, it would seem that oxidations of proton-producers such as hydroquinones and benzidine invariably result in energy conservation at Site II. In contrast, oxidations of stable metal complexes or of halide ions do not directly involve proton production and oxidations of such substances apparently do not support phosphorylation at Site II.

Table 1. Phosphorylation Efficiency as a Function of the Donor of Electrons to Photosystem II.

PS II donor	Conc. (mM)	E.T.*	P/e
(H_2O)	-	[illegible]	[illegible]
Catechol	0.5	[illegible]	[illegible]
p-Hydroquinone	0.5	[illegible]	[illegible]
p-Aminophenol	0.5	[illegible]	[illegible]
Benzidine	0.5	[illegible]	[illegible]
Dihydroxybiphenyl	1.0	[illegible]	[illegible]
--	20	200	0.45
Ferrocyanide	10	121	0.56
Fe(dipyridyl)$_3$**	1.5	[illegible]	[illegible]
Mn(oxine)$_2$**	0.5	145	0.91
Mn(dipyridyl)$_2$**	0.6	[illegible]	[illegible]

*Electron transport (equiv/hr.mg chl)
**Secondary reactions associated with the oxidation of these metal complexes may result in large pH changes.

(b) Phosphorylation as a function of proton gradients and pH

As we have seen, an excellent case can be made for the involvement of protons in chloroplasts phosphorylation reactions. However the case for the involvement of trans-membrane proton concentration or activity gradients is not nearly as persuasive. In fact the simple version of the chemiosmotic mechanism depicted in Figure 1 seems to be in conflict with a number of observations. Two major areas of conflict are illustrated by Figure 5. This figure shows the time-course of phosphorylation at Site I and at Site II, both at pH 6.5 and pH 8.0.

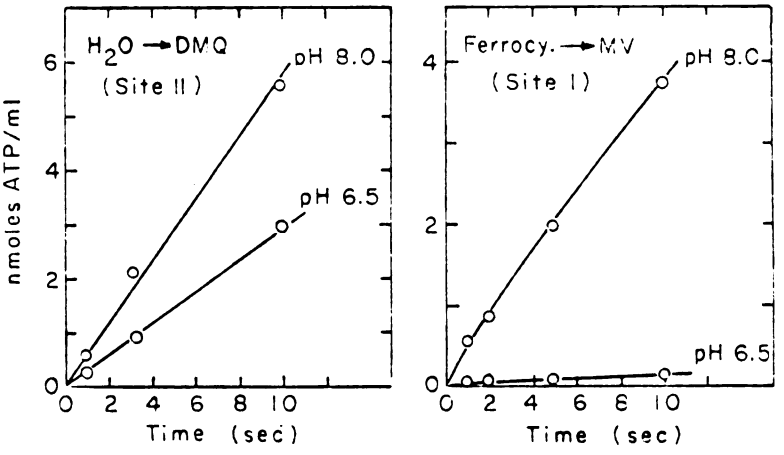

Figure 5. Phosphorylation as a function of the time of illumination.

Clearly phosphorylation is linear with time and therefore must have reached full efficiency within a small fraction of a second. Actually, there is reason to believe that phosphorylation starts very much sooner than this. The flash experiments of Boeck and Witt (37) suggest that phosphorylation occurs long before there is any appreciable difference between the concentrations of protons inside and outside the thylakoid. Therefore, if the escape of protons from the inner aqueous phase is to drive phosphorylation, the protons must be under the impetus of a very large, electron-transport-induced membrane potential. But the internal oxidation of ferrocyanide should produce the same charge separation and the same membrane potential as the oxidation of water or catechol and the oxidation of TMPD should produce the same membrane potential as the oxidation of DAD. Why then is there no phosphorylation by Site I or Site II when TMPD and ferrocyanide are oxidized if the requirement is a membrane potential rather than proton accumulation?

The second problem raised by figure 5 is equally puzzling from the chemiosmotic point of view. At pH 8 phosphorylation proceeds at a good rate whether driven by events at Site II or by events at SiteI. At pH 6.5 or lower phosphorylation driven by Site II still goes on apace but phosphorylation driven by Site I stops. The precipitous drop in Site I phosphorylation at pH 6.5 is not primarily due to a decrease in electron transport. It is mostly due to a decrease in

the efficiency with which electron transport supports phosphorylation. As figure 6 shows, the P/e_2 at Site I is very sensitive to changes in pH whereas the P/e_2 ratio at Site II is quite indifferent.

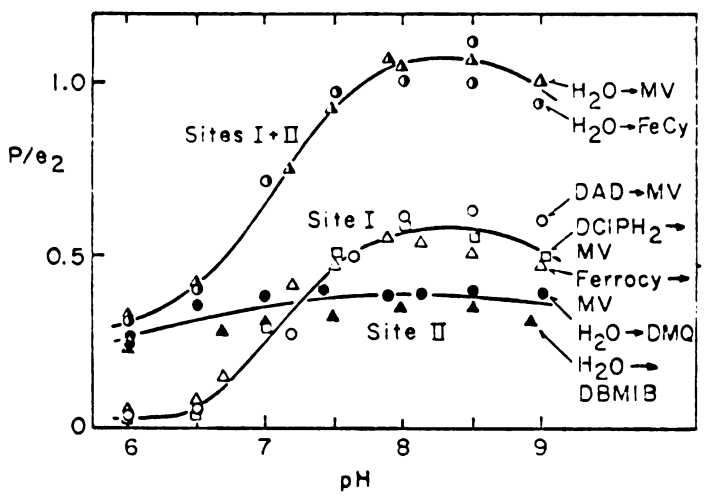

Figure 6. Photophosphorylation efficiency as a function of the external pH.

The sensitivity of Site I phosphorylation to low pH seems to be independent of the chemical nature of the electron donor used: ferrocyanide (anion of a strong acid); $DCIPH_2$ (anion of a weak acid); DAD (a weak base). This makes it extremely improbable that an identical uncoupling effect (due to the donors themselves) is responsible for the low pH inhibition. It is also important to point out that these pH dependence curves are not likely to be the trivial consequences of any other abnormal aspects of the reaction conditions employed. The sum of the curves for Site I and Site II phosphorylation efficiencies, regardless of the electron acceptors and electron donors used, is very close to the curve for the efficiency of the normal Hill reaction which uses both Site I and Site II (see also 38). Consequently it seems safe to conclude that the pH profiles for Site I and Site II, as measured by partial reactions and depicted in figure 6, approximate the true pH dependencies of those individual sites when they are operating in concert in the overall Hill reaction.

The conclusion that Site II is responsible for all of the phosphorylation below pH 6.5 is very difficult to reconcile with the chemiosmotic model. Proton uptake associated with Photosystem I re-

actions is very active, with a probable efficiency (H^+/e^- ratio) of about 1.0 regardless of pH. (See figure 7). Indeed the efficiency of proton accumulation seems independent of pH when the electron transport is through either site. How then can it be that below pH 6.5 the protons can be used for phosphorylation only if they are accumulated through the action of Site II? The implications of this

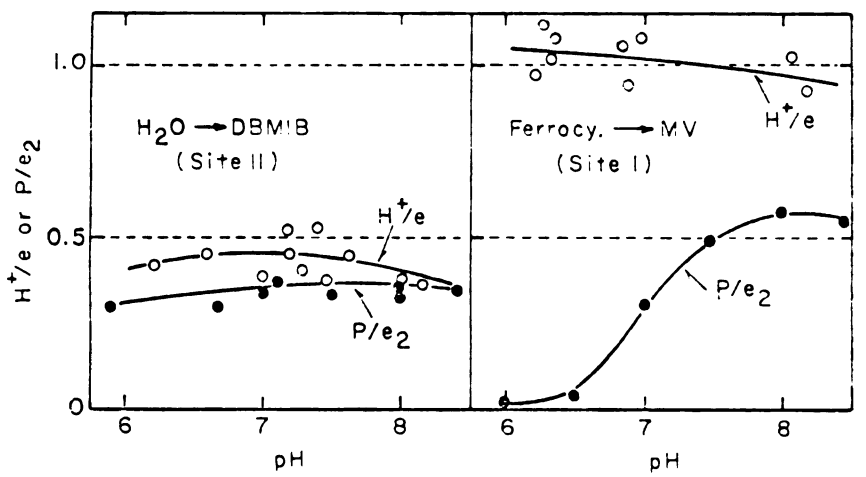

Figure 7. A comparison of the efficiencies of proton uptake and ATP synthesis at different pH's.

question are very important. If we accept the contention that accumulated protons are used to drive phosphorylation we are faced with a chemiosmotic conundrum: Utilization of the accumulated protons for phosphorylation seems to depend on how the protons are accumulated. Clearly this cannot be if both Site I and Site II deposit the protons destined to drive phosphorylation in a common pool, the inner space in the thylakoid.

III. A Proposed Refinement of the Chemiosmotic Theory

Considerations of the chemiosmotic theory of phosphorylation are usually predicated on this assumption that the gradients involved in the conservation of energy result from different concentrations of ions inside and outside the thylakoid. According to the model, there is only one energized state (the inside-outside gradient) and one ATP-synthesizing enzyme (the coupling factor) common to all phosphorylation sites. Furthermore, these "phosphorylation sites" must be thought of as the redox reactions which generate the critical trans-membrane proton gradient.

The concept of a single energized state in the form of a trans-

membrane hydrogen ion activity gradient is in accord with a great deal of evidence. When energization of the chloroplasts is separated in time from ATP synthesis, as in preilumination experiments (39) or in "acid-bath" experiments (40), the energized state almost certainly *is* an inside-outside gradient. There is also little doubt that the space inside the thylakoid becomes acid while electron transport is going on (41,42) and little doubt that concurrent ATP synthesis decreases (but does not abolish) the internal acidification (35, 43-45). Finally, as we have seen, it seems that proton production is required for phosphorylation. Such facts must be accomodated by any theory of phosphorylation and the chemiosmotic theory accomodates them admirably.

However, when it pictures the driving force of steady-rate phosphorylation strictly in terms of an inside-outside proton activity gradient, the chemiosmotic theory has corollaries which are not so obligingly consistent with observations. If the energized state of the chloroplast responsible for ATP synthesis consisted of a trans-membrane gradient, or indeed of any property of the membrane as a whole, site specificity with regard to the utilization of the conserved energy would be impossible. Thus there could be no site-specific inhibitions of photophosphorylation other than inhibitions of the redox reactions responsible for the gradient formation. Nevertheless, there are reasons for believing that the forbidden site specificities do exist. As we have shown above, low pH seems to produce an inhibition which is specific for Site I and this inhibition seems to have nothing to do with the formation of the common proton pool within the thylakoid; at low pH the proton gradient is formed but cannot be used for Site I phosphorylation. Similarly, very low concentrations of mercury partially inhibit Site I phosphorylation without inhibiting Site II phosphorylation at all (46). Again the inhibition seems to affect phosphorylation without affecting the earlier steps in the energy conservation.

For these reasons we are inclined to question the chemiosmotic theory as it is illustrated in figure 3. It may be that this simple model should be replaced by a modification which is basically similar to one already considered by Williams (47). Our version of this modified model is illustrated in figure 8. It differs from the original only in that the critical proton activity gradients are within the hydrophobic membrane rather than between the inner and outer aqueous phases. Being strictly local, a steep gradient could

be formed almost instantaneously and the time-factor-proton-capacity problem alluded to in the discussion of figure 5 need not arise. Furthermore if Site I and Site II reside in different regions of the thylakoid – and the fact that Photosystem I can be physically separated from Photosystem II (48) implies that they do – each local gradient would presumably have to be utilized by a local coupling factor. In this connection it is of interest to note that the number of molecules of coupling factor in the chloroplast is of the same order of magnitude as the number of electron transport "chains" (48,50). These differently situated coupling factors could respond quite differently to pH or to mercury. Perhaps some critical region of Site II and its coupling factor is less accessible than the equivalent region of Site I to external hydrogen ions or added mercury.

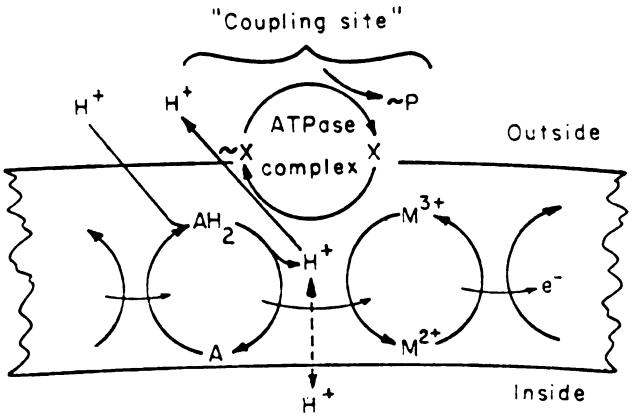

Figure 8. A modified model of the chemiosmotic mechanism which allows for site specificity in the utilization of the energized state for phosphorylation. In this model protons are produced and used within a hydrophobic region of the thylakoid membrane. During steady-state phosphorylation protons migrate to the outside through an ATP-synthesizing complex, the coupling factor, without having been in the inner aqueous phase of thylakoid. However protons can diffuse to the inner space from the local hydrophobic site of proton production-utilization and therefore the site may be in approximate equilibrium with the inner space. According to the model, each proton-producing oxidation center is of necessity associated with its own ATP-synthesizing coupling factor. Thus the coupling factors associated with Site I and Site II oxidation centers are differently situated and may respond in different ways to external conditions.

The fact that trans-membrane gradients are formed during electron transport (51) and the fact these gradients are used for ATP synthesis in the dark (39) can be accomodated by such a "micro-

chemiosmotic" model if we retain the concept of an anisotropically organized membrane. The localized site of proton production and utilization need only be in communication with the inner space in order to permit massive net accumulations of protons. Presumably, such communication would also permit the utilization of inner space protons for phosphorylation as in "acid bath" or preillumination experiments. Thus the acidification of the inner space of the thylakoid, which has been taken to be an essential intermediate step in phosphorylation, may in fact represent a side-reaction which is only indirectly related to steady-state phosphorylation.

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