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

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ABSTRACT

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

By

Rajnikorn Ouitrakul

The purpose of the research reported in this thesis was to study electron transport and photophosphorylation in spinach chloroplasts with different types of electron acceptors.

Three classes of electron acceptors have been identified on the basis of differences in electron transport and phosphorylation. Class I acceptors are ionic and lipid-insoluble and are exemplified by ferricyanide and methylviologen. They resemble the natural acceptor, ferredoxin-$NADP^+$, in that they accept electrons at a slow rate. Phosphorylation increases the electron transport rate. Uncoupling of phosphorylation enhances the rate still more. The phosphorylation efficiency, P/e_2, is 1.2 ±0.1. Class II acceptors, such as 2,6-dichlorophenolindophenol, are acceptor-uncouplers. At low pH where the red, lipid-soluble acid is a major component the electron transport rate is

enhanced. At high pH where the blue, lipid-insoluble anion predominates, the electron transport decreases and is accompanied by low phosphorylation rates. Even at high pH, when the concentration of acceptor increases, the P/e_2 value drops to zero. Class III acceptors are non-ionic and lipid-soluble and are exemplified by quinonediimides. They catalyze a very rapid electron transport which is approximately the same whether in the presence or absence of ADP and phosphate or in the presence of the uncoupler methylamine. Phosphorylation is increased above the phosphorylation mediated by Class I acceptors but the P/e_2 value is half of the value obtained with Class I acceptors. There is, however, no evidence of uncoupling.

The quantum efficiency of electron transport is the same with Class III as with Class I acceptors but the quantum efficiency of phosphorylation with Class III is half that observed with Class I acceptors. In the presence of low concentrations of the electron transport inhibitors diuron, monuron or atrazine, electron transport and the concomitant phosphorylation with oxidized p-phenylenediamine (a Class III acceptor) are inhibited to the same extent at any light intensity. In contrast, the reaction with ferricyanide (a Class I acceptor) is much less inhib ited at high light intensities but at low light intensities the inhibitory effect increases until, at light intensities where the Class I and Class III system are operating at the same

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same rate, the inhibitory effects are equal. The electron transport inhibitor KCN, which probably blocks at plastocyanin inhibits the reduction of Class I acceptors much more strongly than the reduction of Class III acceptors. KCN also inhibits cyclic phosphorylation catalysed by pyocyanin, diaminodurene, reduced 2,6-dichlorophenolindophenol and low concentration of phenazine methosulfate (PMS). Cyclic phosphorylation mediated by high concentration of PMS is only partially inhibited by KCN.

On the basis of the differences in the reaction rate, the P/e_2 value, and the sensitivity to electron transport inhibitors, it was concluded that Class III acceptors accept electrons at or near electron pool A between the two photosystems. There are two phosphorylation sites on the non-cyclic pathway, one site before pool A and one after the pool. The electron transport involving Class III acceptors utilizes only the first phosphorylation site. The second site might be involved in both cyclic and non-cyclic phosphorylation.

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By

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ABBREVIATIONS

A	electron pool A of Kok
ADP	adenosine-5′-diphosphate
ATP	adenosine-5′-triphosphate
Atrazine	2-chloro-4-(2-propylamino)-6-ethylamino-s-triazine
CMU	3-(4-chlorophenyl)-1,1-dimethylurea (monuron)
Chl	chlorophyll
DAD	2,3,5,6-tetramethyl-p-phenylenediamine (diaminodurene)
DAD_{ox}	oxidized form of diaminodurene
DCPIP	2,6-dichlorophenolindophenol
$DCPIPH_2$	reduced 2,6-dichlorophenolindophenol
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron)
Diquat	1,1′-ethylene-2,2′-bipyridylium dibromide
E.T.	electron transport
Fecy	potassium ferricyanide
FMN	riboflavin-5-phosphate
HEPES	N-hydroxymethylpiperazine-N′-ethanesulphonic acid
MA	methylamine
MV	methylviologen
MES	N-morpholino-ethanesulphonic acid
$NADP^+$	nicotinamide adeninedinucleotidephosphate

NADPH	reduced nicotinamide adeninedinucleotidephosphate
PD	p-phenylenediamine
PD_{ox}	oxidized form of p-phenylenediamine or p-benzoquinonediimide
P_i	orthophosphate
PMS	phenazine methosulphate or N-methylphenazonium methosulfate
$PMSH_2$	reduced phenazine methosulfate
PQ-A	plastoquinone A
PQ-C	plastoquinone C
Pyo	pyocyanin
$Pyo\text{-}H_2$	reduced pyocyanin
Q	quencher
QS	quinonesulfonate
Tricine	tris(hydroxymethyl)methylglycine
TAPS	N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
CYT	cytochrome
PC	plastocyanin

INTRODUCTION

The Concept of the Electron Transport System in Chloroplasts

The light reaction in photosynthesis by green plants is a process whereby the plants oxidize water, evolve oxygen and reduce $NADP^+$ with the electrons produced in the process, simultaneously. With this redox reaction there is a concomitant production of ATP. The redox reaction is achieved via the excitation of pigment systems by light. Thus the energies of the absorbed quanta are conserved in the form of ATP and in the reduced compound, NADPH. The ATP and NADPH are then used in light-independent reactions to reduce CO_2 to the level of cell constituents and to form carbohydrates, fat, protein, etc.

The discovery of the light-dependent transfer of electrons from water to artificial electron acceptors in isolated chloroplasts by R. Hill (1939) marked the beginning of biochemical investigations of photosynthesis. This process which has come to be known as the Hill reaction involves the evolution of molecular oxygen which may be considered as an oxidized form of water. In the last decade the concept of two light reactions operating in series to transfer electrons all the way from water to $NADP^+$ has been developed by investigators such as Hill and Bendall (1960), Duysens et al. (1961) and Witt et al. (1961). Hill and Bendall originally proposed as a working model the two-light reaction scheme, now widely known as the "Z" scheme, to describe the electron transport pathway. This scheme was based on the following observations.

1. The cooperativity between two wavelengths of light, known as the Emerson enhancement effect. The photosynthetic efficiency of far red light is increased when this light is supplemented by light of a shorter wavelength and vice versa (Emerson et al., 1957). A similar phenomenon had already been discovered by Blinks (1957). He showed that there was often a transient increase in photosynthetic efficiency following an abrupt change of wavelength. This "chromatic transient" exhibited the same wavelength dependencies ("action spectrum") as the Emerson enhancement phenomenon (Myers and French, 1960).

2. The potential gap of about 0.4 V between cytochrome-f and cytochrome-b_6 in chloroplasts (Davenport and Hill, 1952) suggested that the newly discovered photophosphorylation (Arnon et al., 1954) could be a thermochemical reaction involving the transfer of electrons between these two cytochromes. For this model to function there must be a photochemical reaction reducing cytochrome-b and another photochemical reaction oxidizing cytochrome-f.

The Z scheme has received much attention and has been elaborated further on the basis of more recent discoveries. Duysens, et al. (1960, 1962) have described the photochemical system oxidizing cytochrome-f as "Photosystem I". This oxidation is not sensitive to DCMU. In contrast, "Photosystem II" reduces cytochrome-f and is sensitive to DCMU. These two photosystems can be physically separated by fragmenting the chloroplasts with appropriate detergents and separating the resulting particles into fractions containing large system II particles and small system I particles (Boardman, 1970).

The Z scheme as currently envisioned is presented in Figure I.

Photosystem I

The energy trapping center of photosystem I is a specially bound or otherwise uniquely situated chlorophyll a molecule which has been called P700 since its oxidation results in a loss of chlorophyll absorbance at about 700 nm. It behaves as a redox reagent, undergoing a one electron oxidation-reduction reaction. The potential seems to be pH-independent and about 0.43 V. P700 can be reversibly oxidized by chemcial reagents such as ferricyanide (Kok, 1965). There are about 400 total chlorophyll molecules for every one of the unique P700 chlorophyll a molecule in

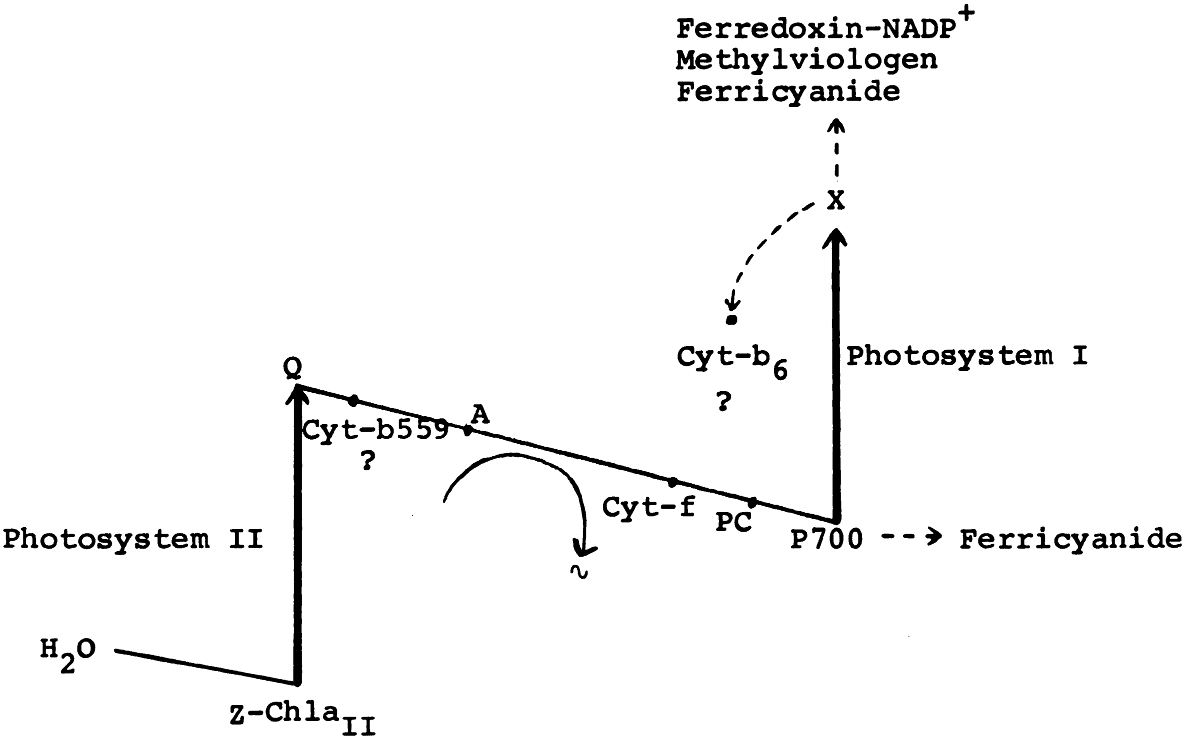


Figure 1. The "Z" scheme for electron transport and phosphorylation in photosynthesis.
Non-cyclic electron transport - the transfer of electrons from water to X.
Cyclic electron transport - the transfer of electron around photosystem I.

chloroplasts and about 200 total chlorophyll molecules for every P700 in the light particles of Boardman (Anderson, *et al.*, 1966).

The primary acceptor of the electrons from excited P700 is still unidentified. This acceptor, X, when reduced is capable of transfering its electrons to such low potential substances as quaternized dipyridyls (e.g., methylviologen or diquat) or to ferredoxin. It has been suggested that the cytochrome c reducing substance of Fugita (Fugita and Murano, 1967; Regitz, *et al.*, 1970) or the related ferredoxin reducing substance of San Pietro (Yocum and San Pietro, 1970) may represent the electron acceptor of photosystem I.

Photosystem II and O_2 Evolution

The reaction center of photosystem II can be considered as a complex of some donor (ZH), another uniquely situated energy trapping chlorophyll a ($chla_{II}$) and an electron acceptor (Q). When a quantum of light is absorbed by photosystem II and transfered to $chla_{II}$, Q is reduced and ZH is oxidized.

The nature of $chla_{II}$ is not yet as clear as the nature of system I counterpart P700 ($chla_{I}$). There have been suggestions that a component of the chlorophyll absorbing at 680 nm is bleached on illumination. The reverse process of this bleaching effect has a very short half-life, about 1/100 the half-life of P700. The bleaching is inhibited by the same concentrations of DCMU which inhibit 0_2 evolution. Furthermore, the 680 nm bleaching occurs even after 0_2 production has been abolished by heating and under this condition DCMU still inhibits the bleaching (Döring, et al., 1969).

Photosystem II differs from photosystem I in that it is responsible for most of the fluorescence observed in vivo. Duysens and Sweer (1963) suggested that the primary electron acceptor of photosystem II, by accepting electrons from $chla_{II}$, quenches the potential fluorescence of this excited molecule. They therefore suggested that the acceptor be called "Q" for "quencher". When Q is fully reduced, as at very high light intensities or when its reoxidation is blocked by inhibitors such as DCMU, the fluorescence yield of absorbed light is maximal.

The "Z" in its oxidized form withdraws hydrogen or electrons from water. The oxidized residue of water is evolved as molecular oxygen. The process of water oxidation is complex, involving both chloride ions and magnese. It has become possible to study this process using the very sensitive technique of Joliot which depends on the modulation of the actinic light with a consequent modulation of the 0_2 production rate and therefore a modulation of a polarograph output. It has been found that the initial step of 0_2 production requires an induction period for Z to withdraw electrons from water. This induction period is not necessary when artificial electron donors such as NH_2OH are oxidized by Z (Cheniae, 1970).

Kinetics of the Transfer of Electrons Between the Two Photosystems

It is generally accepted that the intermediate carriers constitute a pool of measurable size (see review by Hind and Olsen, 1968). The sequence of electron transfer can be represented as follow:

$$H_2O\text{----->}Q\text{--}\overset{\text{DCMU}}{+}\text{----->}A\text{------->}P700\text{----->}X$$

Where A is an electron pool which is considerably larger than either Q or P700.

The existence of the pool designated A is deduced from three main lines of evidence, notably O_2 production and the fluorescence rise curve in the absence of external electron acceptors, and the accumulation of the reducing power in illuminated chloroplasts. In the absence of any external electron acceptors there is a "gush" of O_2 which is much more than can be accounted for by the reduction of Q and P700 (Joliot, 1965). The rise in the fluorescence yield after the onset of illumination is much faster in the presence of DCMU than in its absence. The decay of fluorescence after a brief flash of strong light in the absence of DCMU can be attributed to the transfer of electrons from Q to A. Also from the difference in the time courses of the fluorescence rise (in weak light) in the presence and absence of DCMU, the size of A can be computed if one assumes that the (integrated) number of quanta not emitted in the absence of DCMU represents the number of quanta utilized to fill up A. Finally, chloroplasts which have been illuminated are able to continue to reduce a measurable amount of indophenol dyes in the dark. This amount is again much larger than can be accounted for by Q and P700 (Kok, *et al.*, 1967). The amount of A present seems to be about 10 times higher than Q and P700 (Kok, *et al.*, 1969).

The transfer of electrons from Q to A is potentially much more rapid than the transfer of electrons from A to P700 (Forbush and Kok, 1968; Kok, *et al.*, 1967). I shall return to this matter of the rate of reduction of A by reduced Q in the discussion.

The chemical identity of the "A" pool has not been determined with any degree of certainty. It may be that several substances go to make up the pool. However, there is some reason to suspect that plastoquinone may be involved (see below).

Components of the Electron Transport Chain Which Carry Electrons Between the Two Photosystems

Plastocquinones

Plastoquinones occur as several related molecular species and are quite abundant (Carrier, 1967). The requirement for lipid-soluble plastoquinones in electron transport became evident when chloroplasts were extracted with heptane; such extracted chloroplasts are only capable of the Hill reaction when plastoquinones are added back (Bishop, 1959).

Plastoquinone can be reduced by light absorbed by system II. The rate of the reduction is greater than the rate of its reoxidation (Witt, *et al*., 1965). This in itself is reminiscent of the "A" electron pool. Moreover, at least two types of plastoquinones, PQ-A and PQ-C are present in amount which correspond to the pool size. The amount of PQ-A required for restoration of the electron transport is about 10 times higher than the amount of PQ-C. Plastoquinone C is more readily extracted than PQ-A (Henninger and Crane, 1966). Since the potential of isolated PQ-C is also lower than the potential of isolated PQ-A (E_0' for PQ-C 55mV, for PQ-A 113 mV; Carrier, 1967) it has been suggested that PQ-C might represent the primary acceptor of photosystem II, Q, and that PQ-A might represent pool A (Hind and Olson, 1968).

Cytochrome-b559

This new autooxidizable b-type cytochrome, also known as cytochrome-b_3, first reported by Lundegårdh (1962), may be located close to system II. (Two potentials have been reported, 20 mV and 370 mV and there may indeed be two different kinds of cytochrome-b559: see Bendall, 1968). This cytochrome is reduced by 650 nm light and oxidized by 713 nm light in CCCP-uncoupled chloroplasts (Hind, 1968). The best evidence for its location on the electron transport chain comes from mutant strains of Chlamydomonas. In a mutant which lacks cytochrome 553, which is equivalent to the cytochrome-f in higher plants, light sensitizes the reduction of cytochrome-b559 but not its oxidation, whereas in a mutant strain lacking cytochrome-b559, light sensitizes the oxidation of cytochrome 553 but not its reduction (Levine, 1969).

Cytochrome-f or cytochrome 555

This is a c-type cytochrome with a rather oxidizing potential, $E_0' = 365$ mV. There is about 1 molecule of cytochrome-f for every 400 chlorophyll molecules (Davenport and Hill, 1952). It was the first cytochrome to be shown to have a role in photosynthesis (Duysens, et al., 1961). A Chlamydomonas mutant which lacks cytochrome 553 cannot reduce $NADP^+$ with $DCPIPH_2$ unless plastocyanin is added. It has been suggested that cytochrome-f and plastocyanin (see below) provide alternative parallel routes of electron transport (Kok, et al., 1964).

The oxidation of cytochrome-f by system I has been reported to occur at -150°C which implies an intimate relationship of cytochrome-f to P700 (Rumberg and Witt, 1964).

However, recent observations have raised the possibility that the observed cytochrome spectral changes at this temperature might really represent the oxidation of cytochrome-b559 by system II (Floyd, *et al*., 1971).

Plastocyanin

Plastocyanin is an intensely blue-colored, copper-containing protein first isolated from *Chlorella* by Katoh (1960). Plastocyanin from spinach chloroplasts has the redox potential, $E_0' = 370$ mV. There seems to be about one plastocyanin molecule for every 600 chlorophyll molecules and therefore about one plastocynin molecule for every cytochrome-f molecule (Katoh, *et al*., 1962). Plastocyanin can be readily dissociated from chloroplasts by heptane treatment, by sonication or by detergent treatment. In contrast the cytochromes are much more tightly bound. Copper can be removed from plastocyanin slowly by copper-chelators such as diethyldithiocarbamate, salicylaldoxime or cyanide (Katoh, 1960). When plastocyanin is added to plastocyanin-depleted chloroplast fragments there is an increased rate of oxidation of cytochrome-f and increased rate of reduction of P700. This suggests the electron transport sequence: donor---> cytochrome-f---> plastocyanin---> P700 (Hind, 1968).

Cytochrome-b_6 or cytochrome 563

Davenport (1952) and Hill (1954) discovered a cytochrome-b_6 component of chloroplasts. It is autooxidizable with a redox potential, $E_0' = 60$ mV. Originally, Hill and Bendall (1960) located the site of action of this cytochrome close to the acceptor of system II, Q. However, Hind and Olson (1966) showed that cytochrome-b_6 is rapidly reduced by system I light. They therefore relocated the site of action on the reducing side of system I and implicated cytochrome -b_6 in "cyclic" electron flow. Rumberg (1966) has also suggested that cytochrome-b_6 is on a side pathway not involving cytochrome-f reduction since cytochrome-f reduction was 10 times faster than cytochrome-b_6 oxidation. According to him cytochrome-b_6 could also be reduced by system II. Thus the location of cytochrome-b_6 in the electron transport pathway is still controversial.

Photophosphorylation and the Theories of the Coupling Mechanisms

That fundamental reaction which is the dehydration of ADP and phosphate coupled to the oxidoreduction steps of electron transport chains has been the subject of investigation for many years. Following is a brief outline of current hypotheses which have been designed to elucidate this problem.

1. "Chemical coupling." This hypothesis was presented by E. C. Slater (1953). The mechanism proposed is analogous to the mechanisms of substrate level phosphorylations. It is postualted that certain electron carriers in the reduced form combine with a "coupling factor" before they can donate electrons to the next carrier. The oxidation of the carrier protein complex changes the complex to a higher energy state. Once the initial high energy intermediate has been formed it can exchange the carrier group with the phosphate available in the medium which then further phosphorylates ADP to ATP. This mechanism would not permit any electron transport unless phosphorylation occurred or an uncoupler was present. Uncoupling of this system would be by means of breakdown of the high energy intermediate, e.g., by hydrolysis of the anhydride bond. Thus the electron transport rate can be increased by elimination of the rate limiting step of ATP formation.

According to this mechanism the phosphorylation sites would be the electron carriers which become high energy states when oxidized.

2. "Chemiosmotic coupling." This hypothesis was proposed by P. Mitchell (1961, 1966). The hypothesis requires an anisotropic membrane surrounding an enclosed space. It is also required that the membrane be fairly impermeable to hydrogen ions. In Mitchell's view of the coupling mechanism there is a vectorial H^+ movement across the membrane during

electron transport. Once the H^+ gradient has been produced it represents a form of potential energy which induces dehydration of ADP and P_i to form ATP via anisotropic ATPase. Thus the "osmotic" energy is transformed into chemical energy. According to this mechanism the "phosphorylation sites" will be those redox steps where proton translocation occurs.

The chemiosmotic hypothesis does not preclude the possibility that some other high energy intermediate can be formed and then ATP formed subsequently as in the chemical hypothesis. The critical difference is that in the chemiosmotic mechanism the first high energy state is the proton gradient across the membrane.

Uncouplers by this scheme are substances that can permeate the membrane to neutralize H^+ or substances that make the membrane permeable to H^+.

3. Coupling by Conformational Changes. This hypothesis was proposed by P. D. Boyer and his associates on the basis of biochemical approach (Mitchell, et al., 1967). They proposed that the high energy compound or state which drives ATP synthesis might be a localized change within the non-aqueous matrix. Morphological evidence from the changes of the mitochondrial inner membrane during respiration has been used to support the hypothesis (Green, 1968). However, no detailed coupling mechanism for the phosphorylation has been described as in the other two hypotheses.

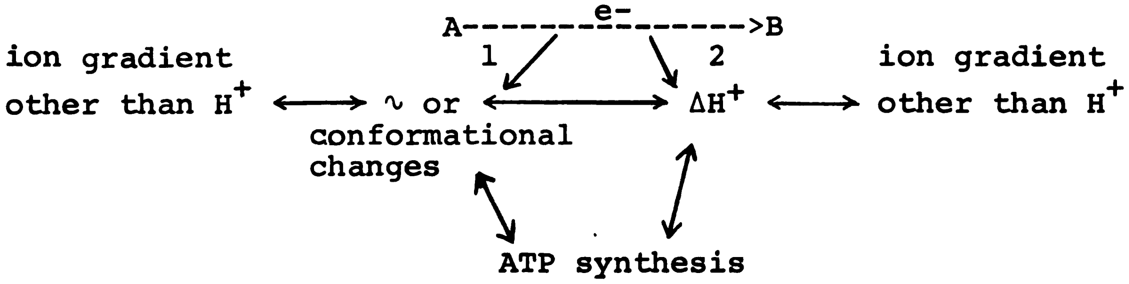
It can be seen that "chemical" coupling and "conformational" coupling are actually very similar in that the oxidoreduction reaction changes the chemical environment in such a way as to form a high energy intermediate, whereas in the "chemiosmotic" mechanism it is the proton gradient across the membrane which constitutes the high energy state.

Evidence supporting the chemiosmotic hypothesis has been obtained mostly by Jagendorf's group. It has been shown that light-induced electron transport can induce a pH rise in the chloroplast bathing medium. Associated with this pH change is a "high energy state" which has been designated "X_E". This X_E state can be used to phosphorylate ADP subsequently in the dark.

A similar and perhaps identical X_E state can also be created by bathing the chloroplasts in a permeant weak acid. Upon transfering the chloroplasts to an alkaline medium containing ADP + P_i, ATP is formed. However, there is some evidence that the X_E state may represent a side pool of energy storage and not a high energy intermediate directly resulting from oxidoreduction reactions. In other words, it is not certain that the X_E state is a necessary intermediate of phosphorylation (Jagendorf and Uribe, 1967). There is also abundant evidence that electron transport can bring about the transport of ions other than the hydrogen ions such as K^+. In the presence of valinomycin, a K^+ gradient can also drive ATP synthesis in mitochondria (Cockrell, et al., 1967).

Recently Packer (1970) showed in his spectrophotometric and electron micrographs studies that configurational and conformational changes do occur in mitochondrial membranes. He concluded that changes in configuration can affect the energy coupling but they may not be a prerequisite for the energy coupling per se.

Taken all together the possibilities can be represented as in the diagram below.



Where ∿ or "squiggle" signifies any unidentified high energy substance or state. It has not yet been elucidated whether 1 or 2 is the primary mechanism coupled to oxidoreduction reactions.

The Number of Phosphorylation Sites in the Electron Transport Chain of Chloroplasts

By assuming that the phosphorylation coupled to a redox reaction involves the simultaneous transfer of two electrons (by analogy with substrate level phosphorylation reactions and for that matter by analogy with the more common of the reactions of organic chemistry), the ratio of

ATP molecules formed to pairs of electrons transferred is taken to indicate the number of sites of phosphorylation. Early observations on the stoichiometry (P/e_2) suggested that this ratio was 1.0 and therefore it was taken as granted that the non-cyclic electron transport pathway involved one phosphorylation site. However, electron transport in chloroplasts can occur in the absence of phosphorylation. The question therefore arises whether this non-phosphorylating electron transport continues under phosphorylation conditions concurrently with the phosphorylating electron transport. There is a good deal of evidence which suggests that the non-phosphorylating electron transport often does continue during phosphorylation; when phosphorylation is varied in a number of ways, the amount of ATP formed is proportional to the extra electron transport which occurs during phosphorylation (Good, 1960; Izawa and Good, 1968). Moreover, under these conditions two ATP molecules are formed for every extra pair of electrons transported. Therefore it seems probable that the P/e_2 ratio of the phosphorylating electron transport is 2.0. Hence it is not unreasonable to suspect the existence of two phosphorylation sites.

The idea of two phosphorylation sites on the non-cyclic pathway had already been put forward by Krogmann, _et al_., (1959). They observed that the P/e_2 can be slightly above 1.0 with ferricyanide as electron acceptor and they also raised the question of how to compute the non-phosphorylating electron transport which might occur during phosphorylation. When Winget, et al. (1965) replaced tris-HCl buffer with a wide range of other buffers they found that the overall P/e_2 ratio was consistently above 1.0 and sometimes reached 1.3. This high value has been repeatedly confirmed in this laboratory. Furthermore, Saha and Good (1970) showed that the $^{32}P_i$ incorporated, which had been used to estimate ATP synthesis was indeed virtually all in the form of ATP. Still higher values of the P/e_2 ratio have been reported (Hall, et al., 1971; Lynn and Brown, 1967). However, these reports are based on less direct measurements and need to be confirmed.

The Location of Phosphorylation Site(s)

Non-cyclin Phosphorylation.

The original scheme of Hill and Bendall (1960) located the phosphorylation site between system I and system II. Avron and Chance (1967) found that illumination with either 650 nm or 730 nm light resulted in the oxidation of cytochrome-f, which indicates that a rate limiting step is on the reducing side of cytochrome-f. The addition of $ADP+P_i$ or of uncouplers accelerated the rate limiting step and thereby caused a decrease in cytochrome-f oxidation. They concluded, therefore, that the phosphorylation site is before cytochrome-f. The work on *Chlamydomonas* also showed that a mutant strain which lacked P700 could perform noncyclic but not cyclic phosphorylation (Givan and Levine, 1967). Their interpretation is that cyclic phosphorylation requires system I but that ferricyanide can accept electrons from cytochrome-f or plastocyanin. Therefore phosphorylation associated with ferricyanide reduction requires only system II and the electron transport system between Q and cytochrome-f.

Recently Böhme and Trebst (1969) found that when ascorbate was used as an artificial electron donor, the overall stoichiometry of ATP formation to pairs of electron transported was 0.5:1. When water was the electron donor the stoichiometry was 1:1. They concluded that the half efficiency of ATP formation with ascorbate as donor indicated the involvement of only one of two phosphorylation sites. They suggested therefore that there might be a phosphorylation site at the water oxidizing steps and that ascorbate bypasses the site when it replaces water as the electron donor.

Another possible location of the phosphorylation sites has been put forward by Kok, et al. (1967). They suggested that the photoacts, that is the primary charge separations themselves, might generate the high energy states. According to this picture the high energy states can be explained either in terms of a proton gradient or a conformational change. They were led to this concept by their findings of a rather high redox potential for Q. They concluded that there was not enough energy available for the oxidation of reduced Q by P700 to support a phosphorylation reaction. This concept is also supported by the measurements of the steady state concentrations of open trapping centers Q and P700 at different wavelengths of light (Joliot, et al., 1968).

However, Cramer and Butler (1969) reported new values for the redox potential of Q in isolated spinach chloroplasts. On the basis of measurements of the fluorescence yield at pH 7.0 and various redox levels they concluded that Q had two mid-point potentials,-35 mV and -270 mV. Both showed pH dependence. Presumably, therefore, the reduction of Q involves a two-step reduction mechanism. Even if we take the value of -35 mV, the potential gap between Q and cytochrome-f is 395 mV which is almost enough for synthesis of 2 ATP molecules per each pair of electron transferred. Therefore, phosphorylation coupled to a purely thermochemical process involving only electron carriers between the two photosystems is possible. Amesz, et al, (1970) basing their opinion on *in vivo* measurements of redox levels of Q and cytochrome-f during illumination in *Porphyridium* have come to the same conclusion.

Cyclic Phosphorylation

The problem of whether the cyclic electron flow involves the same phosphorylation site as the non-cyclic flow is still unsolved. Hill and Walker (1969) observed that in Swiss chard and sugar beet chloroplasts, with pyocyanin as acceptor, there seemed to be two pH optima; at 7.0 and 7.7.

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When a combination of FMN and Vitamin K_3 was the acceptor, only a pH optimum at 7.7 was observed. They suggested the possibility of two phosphorylation sites with the cyclic system utilizing both sites. A similar suggestion has also been made by Duane, et al., (1965) for phosphorylation in Anabaena lamellar fragments. In this system PMS-mediated phosphorylation had two pH optima; 6.8 and 7.6. FMN and Vitamin K_3-mediated phosphorylations were best at pH 7.6 but also showed a lower peak at pH 6.8.

Laber and Black (1969) reported that n-heptane treated chloroplasts lost about 90% of non-cyclic phosphorylation while electron transport was not affected. At the same time cyclic phosphorylation was inhibited by only 50%. They concluded that n-heptane selectively uncoupled the non-cyclic site. According to this model the cyclic electron flow involves two sites, one shared by the non-cyclic pathway. Therefore the phosphorylation was inhibited by 50%.

Some evidence for the existence of an entirely different cyclic phosphorylation site has been obtained. Lee, et al., (1969) reported that in Anabaena lamellar fragments PMS-cyclic phosphorylation required a protein component which was not required for electron transport from reduced indophenols to $NADP^+$. The latter process required plastocyanin while PMS-cyclic did not require plastocyanin. One interpretation of these observations is that the cyclic and non-cyclic electron transport systems are entirely different.

Work with a Chlamydomonas mutant showed that PMS can mediate cyclic phosphorylation in a cytochrome-f deficient mutant but not in a plastocyanin deficient mutant (Gorman and Levine, 1965). In a mutant lacking P700, cyclic phosphorylation is abolished (Given and Levine, 1967).

A separate cyclic phosphorylation site was also proposed by Huaska, et al., (1969). In digitonin system I particles, cyclic phosphorylation with DAD did not require added plastocyanin but involved endogenous plastocyanin which is located in the membrane at a point which is inaccessible to the outside medium. Cyclic electron flow can be stimulated by exogenous plastocyanin without increasing phosphorylation.

Characteristics of Phosphorylation when Oxidized p-Phenylenediamine (PD_{ox}) is the Electron Acceptor

The oxidized form of p-phenylenediamine (PD_{ox}) is probably largely p-benzoquinonediimide. Trebst (1964) found that in the presence of a high concentration of ferricyanide (about 6 X 10^{-4}M), PD_{ox} at about 10^{-4}M can have a slight uncoupling effect. More recently Saha (1970) has shown that, in isolated spinach chloroplasts, PD_{ox} can support high rates of electron transport and phosphorylation. The closely related oxidized diaminodurene (DAD_{ox}) behaves in a very similar manner.

The characteristics of PD_{ox} reduction can be summarized as follows.

1. High rates of electron transport whether or not ADP and P_i are present. These rates are approximately equal to the maximum uncoupled rate of ferricyanide reduction in the presence of MA (metylamine-hydrochloride).

2. Phosphorylation is increased, exceeding the best rates observed when ferricyanide is reduced. The rate of phosphorylation at pH 7.0 with PD_{ox} as acceptor is several times higher than when ferricyanide is the acceptor.

3. Electron transport and phosphorylation with PD_{ox} as acceptor are more sensitive to DCMU than the reactions with ferricyanide.

4. Phosphorylation with PD_{ox} has half quantum efficiency of phosphorylation with ferricyanide.

5. The K_m for phosphate is the same for PD_{ox} or ferricyanide-mediated phosphorylation but different from PMS-mediated phosphorylation.

On the basis of these results and evidence presented by Böhme and Trebst (1969), Saha (1970) postulated that there are two phosphorylation sites, with one site probably at the water oxidizing step. According to his postulation PD_{ox} accepts electrons at some site before the phosphorylation step between system I and system II. Thus PD_{ox} phosphorylation involves only one of the two sites of ph

it

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phosphorylation. This site is "loosely coupled," therefore, it can support high electron transport rates whether or not ADP and P_i or an uncoupler are present.

The Purpose of the Present Work

I attempted to show that electron acceptors such as quinonediimide and quinone with lipophilic properties can accept electrons between the two photosystems, i.e., they are the "photosystem II acceptors". Electron acceptors are classified into three classes on the basis of the characteristics of electron transport, phosphorylation and P/e_2 ratio. When the electrons are accepted at different sites on the electron transport chain, it is possible to investigate the different kinetics of the different partial reactions. Studies on electron transport limiting factors, e.g., low light intensities or electron transport inhibitors such as DCMU and KCN helped to locate the accepting sites. The conditions for KCN to be an effective inhibitor of electron transport in photosynthesis have been established. These observations have some relevance to the question of the nature of the noncyclic and cyclic phosphorylation sites.

MATERIALS AND METHODS

Preparation of Chloroplasts

Leaves of spinach (Spinacea oleracea L.) obtained from a local market were washed after removing the petioles and most of the midribs. The washed leaves were ground for about 5 seconds in a Waring blender in a medium containing 0.3 M NaCl, 0.002 M $MgCl_2$, and 0.04 M HEPES-NaOH or Tricine-NaOH (pH 7.6). The homogenate was squeezed through 8 layers of cheesecloth and then centrifuged for about 4 minutes in a Sorvall Angle Centrifuge Model XL at about 2000 x g. The pellet was resuspended in a washing medium containing 0.2 M sucrose, 0.002 M $MgCl_2$, and 0.01 M HEPES-NaOH (pH 7.4), and centrifuged at 2000 x g for about 25 seconds to sediment cell debris and intact cells. The supernatant solution was recentrifuged at the same speed for 4 minutes. The pellet was then taken up in a small volume of the same washing medium. Chlorophyll was estimated by the method of Arnon (1949). Chloroplasts were diluted in the same medium to give a standard concentration of the chloroplast stock suspension. For each transfer of chloroplasts during washing the supernatant solution was carefully drained off to remove possible traces of the cytoplasmic and vacuolar contaminants. All operations were carried out at as near 0°C possible.

Preparation of the Electron Donor and Electron Acceptor Solutions

Compound sparingly soluble or unstable in water, e.g., 2,5-dimethyl-p-benzoquinone (DMQ) and N-methylphenazonium methosulfate (PMS), were dissolved in a mixture of methanol and ethyleneglycol (1:1.V/V) to give a concentration of 1-2 x 10^{-2} M and then diluted further with water to the required concentration before use. DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, was dissolved in ethanol and ethylene glycol (1:1,V/V) and diluted with 0.1 M NaCl. The final concentration of organic solvent in the reaction mixture never exceeded 5%. Stable water soluble compounds such as potassium ferricyanide, methylviologen (MV) and pyocyanin were dissolved in water. The chloride or sulfate salts of p-phenylenediamine (PD) and 2,3,5,6-tetramethyl-p-phenylenediamine (DAD) were either dissolved in water or in 0.01 N HCl at a maximum concentration of 1.2 x 10^{-2}M.

PD_{ox} and DAD_{ox} (oxidized forms of PD and DAD, respectively) were prepared by adding the calculated amount of ferricyanide (2 equivalents) plus 0.4 μmole/ml excess to the reaction mixture immediately before adding the chloroplasts. In the experiments which measured PD_{ox} reduction in the absence of ferricyanide, PD was mixed with exactly the required 2 equivalents of ferricyanide. After illumination the amount of PD formed by reduction of the PD_{ox} was determined by titration with ferricyanide.

Sodium 2,6-dichlorophenolindolphenol (DCPIP) was prepared in methanol-ethylene glycol solution at a concentration about 10^{-2} M. Further dilution was made with water. The actual concentration of the compound was measured at 600 nm using the molar extinction coefficient, 21 x 10^3.

Preparation of the $Na_2H^{32}PO_4$ Solution

Stock solutions of Na_2HPO_4 (0.11 M and 0.1 M) were prepared and purified by treatment with acid-washed charcoal (Norit) at about 0°C for about an hour. The solution was then filtered through Whatman #1 filter paper.

About 2 mc of carrier-free $H_3{}^{32}PO_4$ were incubated in 1.0 ml 0.2 N HCl (or occasionally H_2SO_4) overnight at 85°C. After heating, the Norit-treated 0.11 M Na_2HPO_4 solution was added until the phosphate concentration was 0.1 M. This was stored in the cold as a highly radioactive stock solution. At intervals it was further diluted by the addition of the Norit-treated 0.1 M solution of Na_2HPO_4.

Electron Transport and Phosphorylation Reactions

A reaction medium containing 0.1 M sucrose, 3 x 10^{-3} M $MgCl_2$, 10^{-3} M ADP, 5 x 10^{-3} M $Na_2H^{32}PO_4$ and 4 x 10^{-2} M buffer was used. The buffers were selected to buffer well at the required pH, MES-NaOH for pH 6.0-6.5; HEPES-NaOH for pH 7.0-7.5; Tricine-NaOH for pH 8.0-8.2; TAPS-NaOH for pH 8.5-9.0. Unless otherwise specified, the following condition was used routinely; reaction medium pH 8.2; electron acceptors, 4 x 10^{-4}M ferricyanide or 10^{-4}M MV or 6 x 10^{-4}M PD_{ox} (or DAD_{ox} or DMQ) or 6 x 10^{-5}M DCPIP; 5 x 10^{-3}M MA if used; chloroplasts equivalent to 40 µg chlorophyll; total volume 2 ml in a 1 cm cuvette; temperature 19°C.

Electron transport was usually measured as the rate of reduction of ferricyanide by following the absorbance change at 420 nm. These absorbance changes were followed using a Bausch and Lomb Spectronic 505 modified for illumination of the sample by actinic light or by using a similarly modified Beckman DU quartz monochromator combined with a Ledland Log Converter and a recorder. The actinic light was from a 500 W slide projector. The beam was passed through a 1 liter round-bottomed flask containing dilute copper sulfate solution which acted as a combined heat filter and a condensing lens. Broad-band red light (600-700 nm) was used routinely. The light intensity was about 700 Kergs/cm^2/sec. In experiments which measured the effect of light intensity, 650 nm light (obtained with an interference filter) or broad-band orange light (560-700 nm) was used. Light intensity was varied by passing the beam through neutral filters. Light intensity was measured by a wavelength-independent Radiometer (Yellow Spring Instrument-Kettering, Model 65).

In the later part of the research when MV was used as electron acceptor, the rate of electron transport was measured as O_2 uptake (Izawa, et al., 1967) with a Clark-type O_2 electrode. In these experiments the volume of the total reaction mixture was 1.8 ml.

Measurement of Organic Phosphate

Organic-$^{32}P_i$ was estimated by a method adapted from Avron (1960) and Nielson and Lehninger (1955). This method measures the organic phosphate formed as the radioactivity remaining after extraction of all of the residual orthophosphate as phosphomolybdic acid.

1.0 ml of the reaction mixture was collected after the experiment and mixed with 10 ml of 10% perchloric acid, 1.2 ml of acetone, 1.0 ml of 10% ammomium molybdate, and about 7.0 ml of butanol-benzene mixture (1:1, V/V saturated with 10% parchloric acid). The two-phase mixture was stirred throughly with up and down movement of a glass rod flattened at the end, for about 1 min. After the layers had separated, the upper organic phase was sucked off carefully through a pasteur pipette connected to a vacuum line with two traps. The aqueous solution was then filtered through Whatman #4 filter paper presoaked with 0.5 ml water. Denatured chloroplasts and any minute droplets of organic phase which contained high radioactivity of inorganic phosphate were removed by this procedure. The clear filtrate was mixed once more with 0.1 ml of 10% ammonium molybdate and about 7.0 ml of butanol-benzene mixture, stirred and the organic phase removed as before but without filtration. The aqueous layer containing the organic-$^{32}P_i$ was poured into a Geiger-Müller immersion tube (20th Century Electronic, England) and the radioactivity was measured with a Nuclear-Chicago counter. The activity of the sample was compared with the standard $Na_2H^{32}PO_4$ (0.1 μmole) in identical volume. Counts were taken for 3-5 min., depending on the amount of radioactivity.

RESULTS

Electron Transport and Phosphorylation with "Class I" Electron Acceptors

No important differences have been observed in the electron transport and phosphorylation reactions when ferredoxin-NADP$^+$, FMN, methylviologen or ferricyanide served as chloroplasts electron acceptors. All are reduced at low rates in the absence of phosphorylation and at considerably higher rates when ADP and P_i are present. Uncouplers enhance the rate above those observed during phosphorylation. Figure 2 illustrates the characteristics of electron transport and phosphorylation with ferricyanide as acceptor. It can be seen that electron transport in the presence of ADP and P_i was enhanced about two-fold. When electron transport was uncoupled with methylamine (MA), the electron transport was also increased by a factor of about four.

When ferricyanide concentration was increased the rate of electron transport and phosphorylation was slightly decreased. This effect of increasing ferricyanide concentrations may have been in part due to inhibition by ferricyanide but is more likely an expression of the aging of the chloroplasts since the measurements with low ferricyanide concentrations were made before those

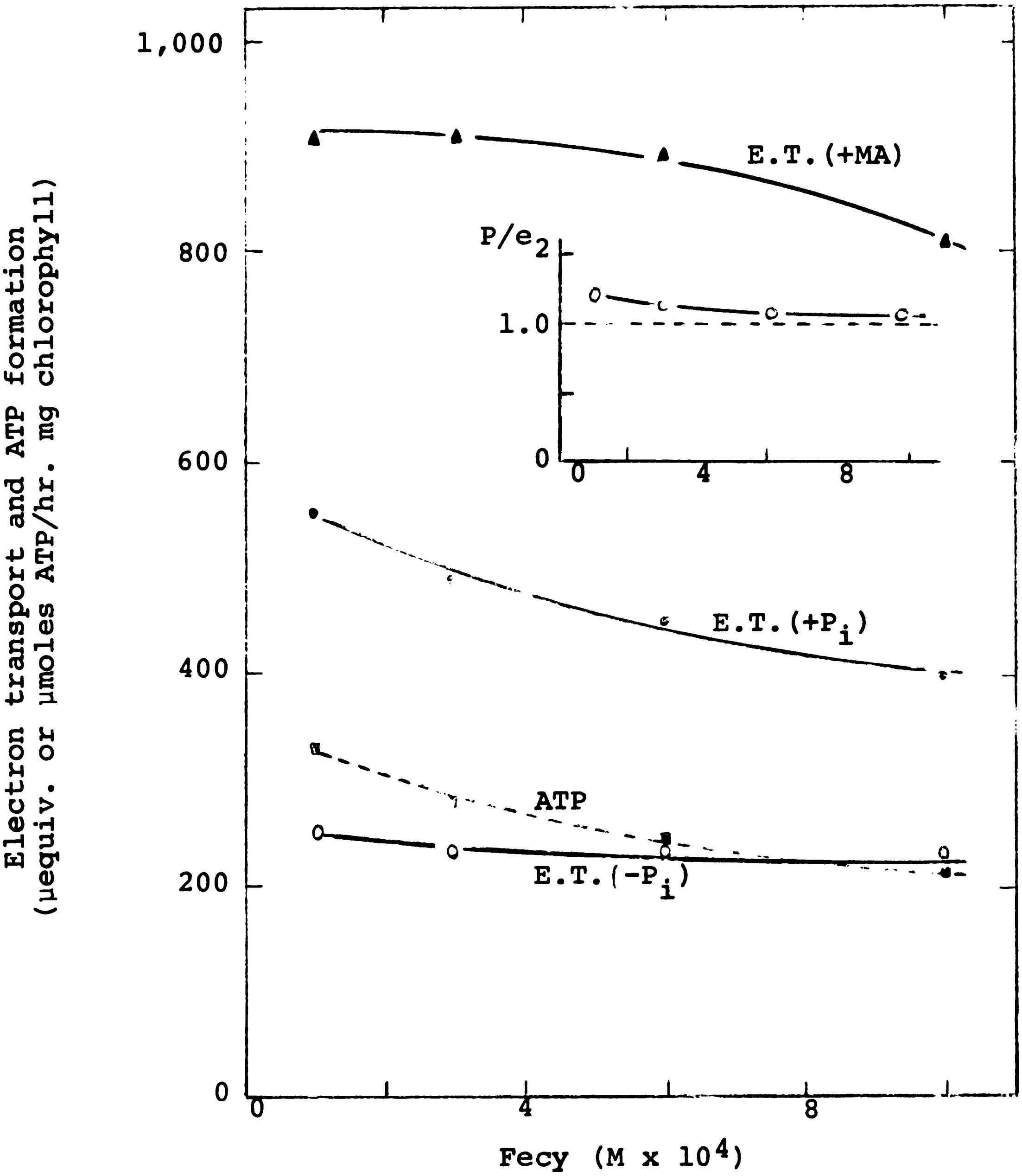


Figure 2. Electron transport, ATP formation and their ratio, P/e$_2$, with potassium ferricyanide as the electron acceptor. Reaction conditions as described in Materials and Methods.

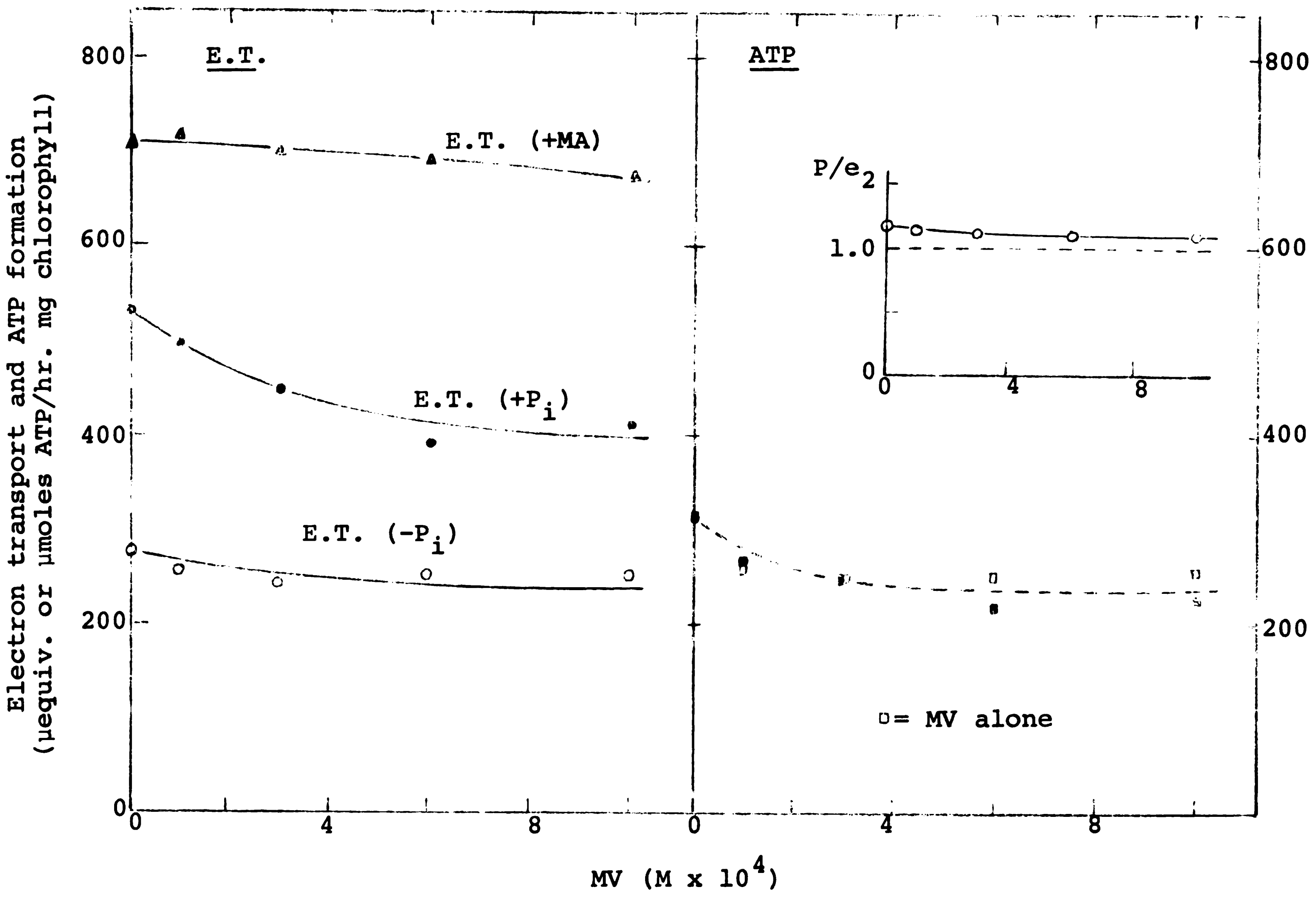


Figure 3. Electron transport, ATP formation and their ratio, P/e_2, with methylviologen as the electron acceptor.

measurements with high ferricyanide concentrations. The phosphorylation efficiency, P/e_2, also declined slightly and possibly for the same reasons (Figure 2).

When methylviologen was used as acceptor, the reaction was run in the presence of 4×10^{-4} M ferricyanide for electron transport assay. At zero concentration of MV, the rate is of course the rate observed with 4×10^{-4} M ferricyanide alone. In other experiments the reactions were also run in the presence of MV alone and the same phosphorylation rates were observed, (Figure 3).

Acceptors of this type, e.g., ferricyanide, methylviologen, etc., are highly polar substances and have low lipid solubility. In these characteristics they resemble the natural acceptor ferredoxin-$NADP^+$. We have designated such substances as Class I acceptors.

Electron Transport and Phosphorylation with a "Class II" Electron Acceptor

The reduction of dichlorophenolindophenol (DCPIP) was analyzed by Kok, *et al.* (1967). In their view, the dye can be reduced rapidly by photosystem I acceptor and with a slower rate from the intermediate "A" pool or by Q, the quencher, or by P700. It was also early recognized that the rate of the indophenol reduction was higher than the rate of ferricyanide reduction but with much less ATP formation (Krogmann and Jagendorf, 1959). We reinvestigated this uncoupling property of DCPIP as expressed by its effects on ferricyanide reduction and associated phosphorylation. Figure 4 shows that with DCPIP present, the basal (non-phosphorylating electron transport) is faster than with ferricyanide alone. At this high pH (8.2) the increase in electron transport rate due to DCPIP does not equal the increase due to ADP + P_i or to uncoupler methylamine. With increasing concentrations of DCPIP, the phosphorylation and amine-uncoupled electron transport are inhibited. However ATP formation is inhibited much more severely so that the P/e_2 ratio falls to zero.

A part of the apparent inhibition of electron transport by high concentrations of DCPIP may actually be due to the strong light absorption by this dye and therefore merely represent a light limitation.

At acidic pH values, with ferricyanide and DCPIP, the electron transport rate is very high--comparable to the highest uncoupled rate with methylamine, and phosphorylation was completely inhibited. With increasing pH the electron transport rate with DCPIP dropped until at values above pH 7.5 the rate was about the same as the rate with ferricyanide alone. Above pH 7.5 some phosphorylation can be detected but still at a much lower rate than with ferricyanide alone. The P/e_2 value increases as the phosphorylation rate increases but less than half the value for the ferricyanide alone (Figure 5).

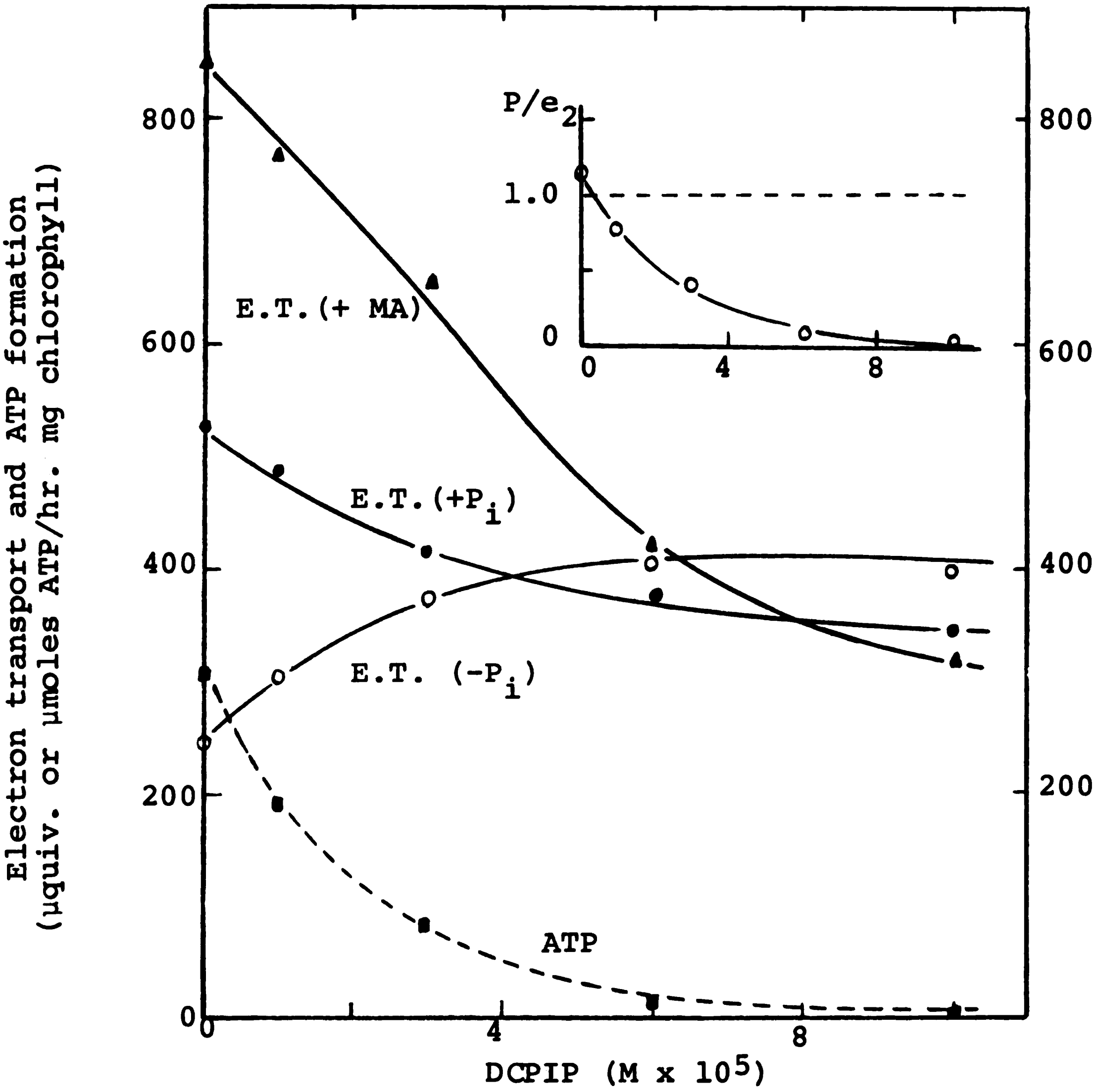


Figure 4. Electron transport, ATP formation and their ratio, P/e_2, as affected by the presence of dichlorophenolindophenol. The reaction was run in the presence of 4 x 10^{-4} M ferricyanide in order to assay the electron transport. In order to maximize the intensity of actinic light the chlorophyll concentration for the DCPIP system was reduced to 3 μg/ml and diluted ferricyanide solution was used as a light filter in the round bottom flask, replacing the $CuSO_4$ solution. The heating effect was controlled by using a glass heat filter instead of $CuSO_4$ solution.

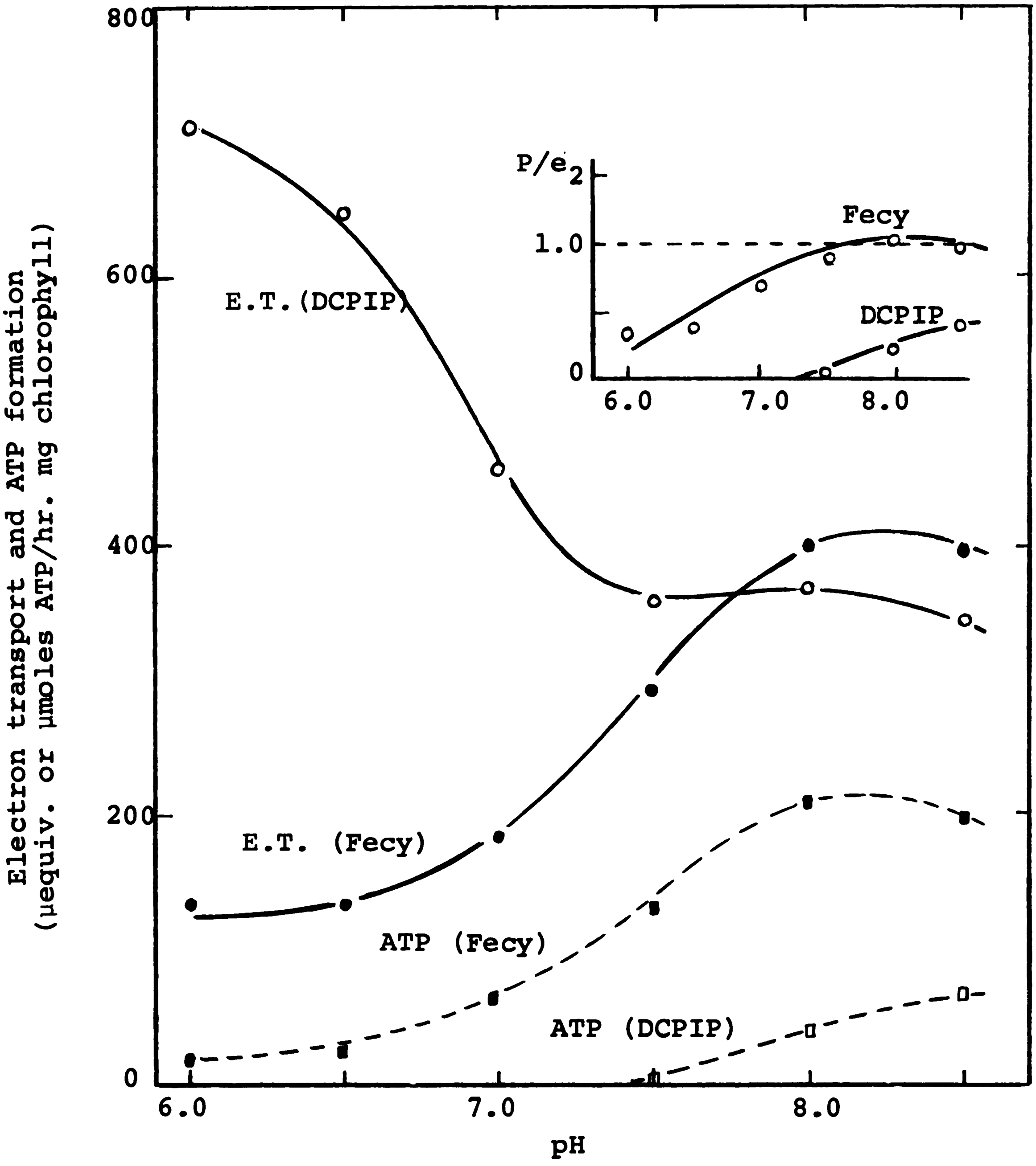


Figure 5. The effect of pH on electron transport and ATP formation when DCPIP is present. The conditions were similar to those of Figure 4. Chlorophyll concentration in the DCPIP-containing system was 6 μg/ml; DCPIP concentration, 6 x 10^{-5} M.

DCPIP-type of acceptors was designated as Class II. These acceptors exhibited very clear uncoupling properties at acidic pH values but much less uncoupling at higher pH values. The uncoupling effect of DCPIP is correlated with the presence of the red, lipid-soluble acid form of the dye which occurs as a major component below pH 6.5. At higher pH values the major component is the blue lipid-insoluble anionic form. It can be imagined that in this form the dye accepts electrons with measurable phosphorylation while the lipid-soluble indophenolic acid behaves as an uncoupler. The uncoupling effect could be a function of red indophenolic acid molecules as is the case of other membrane soluble weak acids, e.g., fatty acids (McCarty and Jagendorf, 1965), phenols (Siow and Unrau, 1968) and carbonylcyanide phenylhydrazone (Heytler, 1963).

The alternative explanation of the behavior of Class II acceptors, namely that they accept electrons before the site or sites of phosphorylation seems very improbable. Indophenols, in common with other uncouplers, inhibit the post-illumination phosphorylation of the "X_E" state of chloroplasts (Hind and Jagendorf, 1963). In this case the indophenol is not added until after the electron transport has ceased. Therefore the question of where DCPIP accepts electrons is not relevant to the uncoupling property.

Electron Transport and Phosphorylation with "Class III" Acceptors

Oxidized-p-Phenylenediamine (PD_{ox})

Electron transport and phosphorylation were greatly stimulated when PD_{ox} was the acceptor as Saha (1970) had found (Figure 6). At about 5 x 10^{-5}M concentration the phosphorylation was about twice the ferricyanide phosphorylation rate and remained constant. On the other hand the electron transport rate increased until the PD_{ox} concentration was about 6 x 10^{-4}M. The non-phosphorylating electron transport increased in the same manner as phosphorylating electron transport with about the same very high rate.

The uncoupler methylamine decreases somewhat the maximal rate of electron transport observed in the presence of PD_{ox}. This is undoubtedly an expression of the tendency of amine at higher pH values to inhibit electron transport. The P/e_2 ratio dropped with increasing PD_{ox} concentrations to half of the ratio in the system with ferricyanide alone. No further effects on the electron transport or phosphorylation rates or the P/e_2 ratio were observed when the PD_{ox} concentration was increased from about 5 x 10^{-4}M to 10^{-3}M.

The high rate of electron transport and phosphorylation with the halved phosphorylation efficiency does not seem to be due to an uncoupling effect of PD_{ox} or its decomposition products since the P/e_2 ratio is constant with increasing PD_{ox} concentrations once the system reaches the maximum electron transport rate. In contrast, phosphorylation is totally eliminated in the PD_{ox} system if enough methylamine is added (Saha, 1970). In any case, the increase in phosphorylation rate could not be associated with NH_3 derived from the hydrolysis of the diimide PD_{ox}, since the amount of ammonia which could come from this source does not increase phosphorylation (see Table 1). Ammonia plus p-benzoquinone does not differ much from p-benzoquinone itself and any small difference seems to be in the expected direction of a decrease in phosphorylation.

The possibility of cyclic phosphorylation, i.e., phosphorylation associated with an unmeasured cyclic electron flow, was largely eliminated since the experiments were conducted in the presence of ferricyanide (0.4 μmole/ml) and there is considerable evidence that cyclic electron flows do not occur in such an oxidizing medium. Furthermore the absorbance change was linear during the short period of these experiments and it has been shown that under these conditions absorbance changes are a true measure of the non-cyclic electron transport which results in PD_{ox} reduction (Saha, 1970).

Oxidized 2,3,5,6-Tetramethylphenylenediamine (oxidized diaminodurene or DAD_{ox}).

DAD_{ox} reduction has the same characteristics as PD_{ox} reduction although the rates of electron transport and phosphorylation are slightly lower (Figure 7).

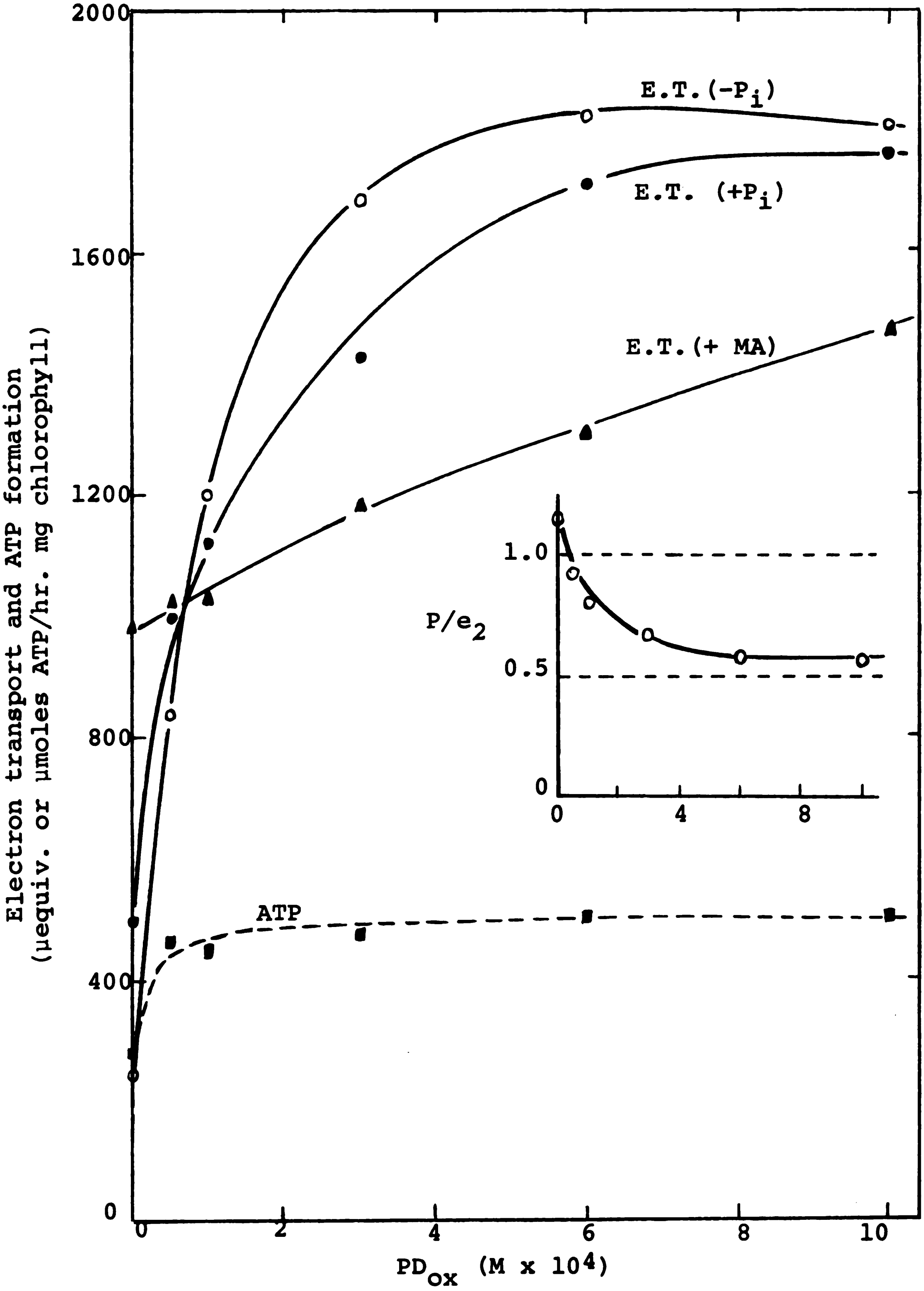


Figure 6. Electron transport, ATP formation and their P/e_2 ratio with oxidized p-phenylenediamine (PD_{ox}) as the electron acceptor.

Table 1

Effect of low concentrations of NH_4Cl on photophosphorylation with p-benzoquinone as electron acceptor.

Conc. of Quinone (M)	pH	Photophosphorylation[a] Control	NH_4Cl x 10^{-4}M	NH_4Cl 2 x 10^{-4}M
	6.0	21	19	18
1 x 10^{-4}	7.0	158	169	160
	8.0	294	276	256
	6.0	26	18	18
2 x 10^{-4}	7.0	138	150	134
	8.0	320	316	284

[a] µmoles ATP/hr.mg chlorophyll.

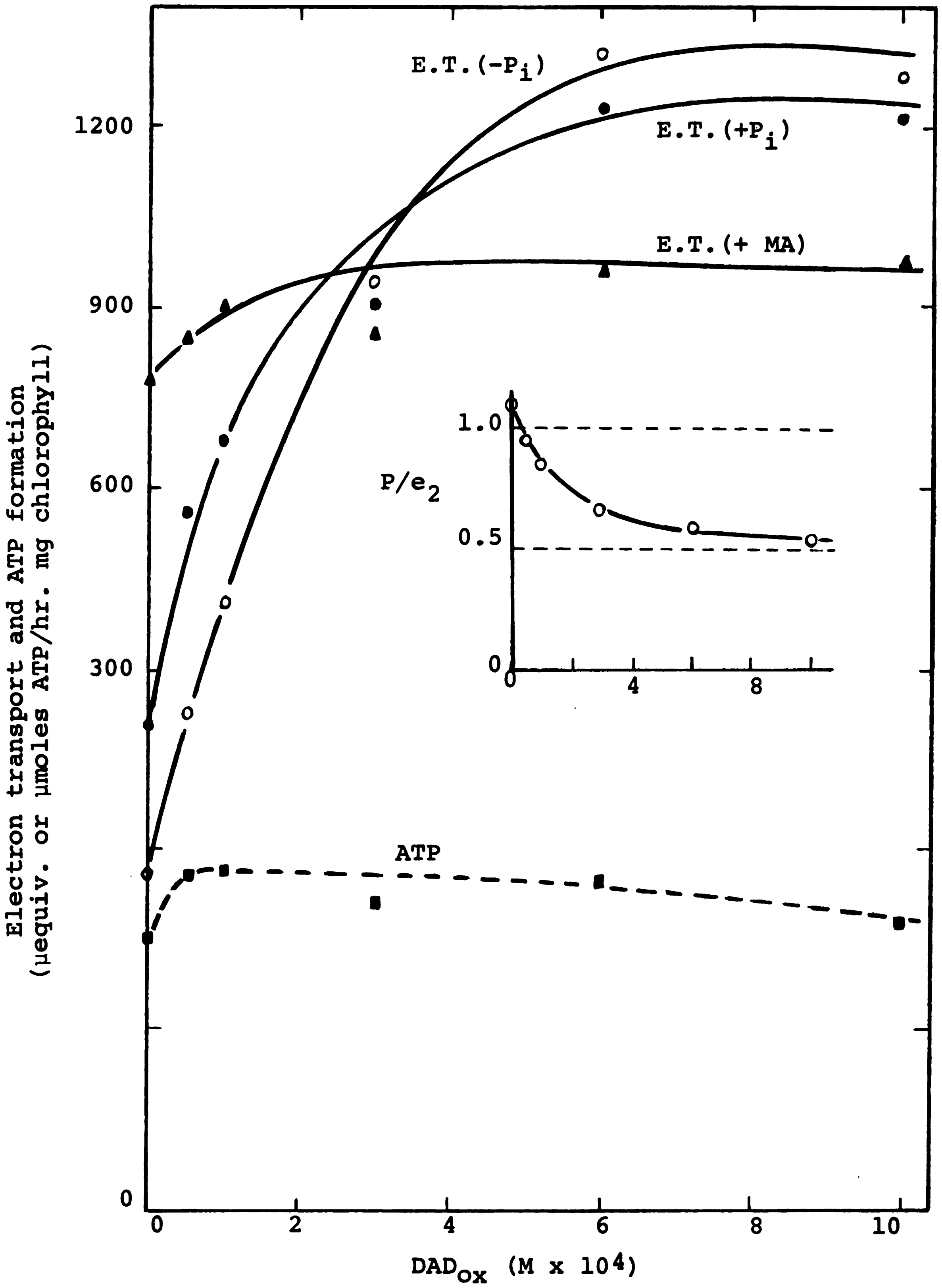


Figure 7. Electron transport, ATP formation and P/e_2 ratio, with oxidized diaminodurene (DAD$_{ox}$) as the electron acceptor.

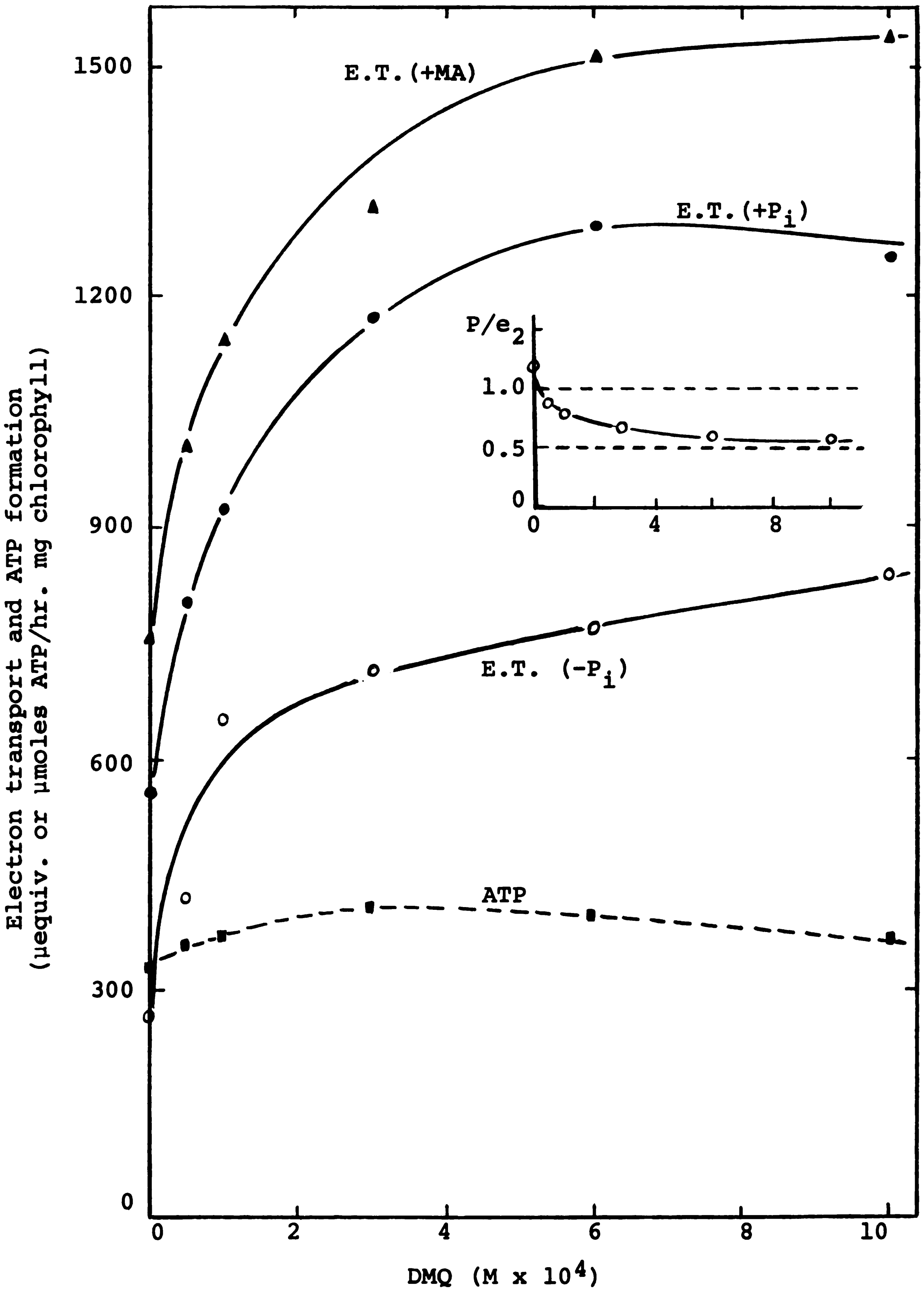


Figure 8. Electron transport, ATP formation and the P/e_2 ratio, with 2,5-dimethylquinone (DMQ) as the electron acceptor.

2.5-Dimethylquinone (DMQ)

DMQ reduction also has similar characteristics. However, electron transport with DMQ is considerably slower than with PD_{ox} and the rates are considerably increased by the addition of ADP + P_i or by an uncoupling amount of methylamine (Figure 8). Clearly DMQ exhibits properties which are intermediate between the properties of PD_{ox} and the properties of Class I acceptors such as ferricyanide. In this connection it should be mentioned that p-benzoquinone also exhibits intermediate characteristics but is somewhat closer to the behavior of ferricyanide than is DMQ (see Tables 2 and 3).

General Definition of a New Category
Class III Acceptors

Until the work of Saha and the investigations reported here, the only well recognized categories of electron acceptors were those designated Class I and Class II. Other acceptors exhibiting different characteristics, e.g., p-benzoquinone had been studies but only in an unsystematic and desultory fashion. On the basis of this work a new category of acceptors, Class III, is described. Oxidized p-phenylenediamine (PD_{ox}) is the prototype and typical member of this class. Class III acceptors are defined as those whose reduction occurs as follow:

1. Electron transport rates are very high whether or not ADP+P_i are present.

2. These high rates of electron transport are associated with high rates of phosphorylation, as high as or higher than the rates observed with Class I acceptors.

3. At concentrations which give maximal rates of electron transport and phosphorylation, the phosphorylation efficiency (P/e_2) is one half the value observed with Class I acceptors.

Class III acceptors include PD_{ox}, DAD_{ox}, oxidized N,N,N',N'-tetramethylphenylenediamine, oxidized 2-methyl-p-phenylenediamine, 2,5-dimethylquinone (DMQ), p-benzoquinone, 2,6-dichloro-p-benzoquinone, etc. These acceptors are relatively non-polar and lipophilic. As already mentioned, the quinones are somewhat intermediate between Class I and Class III.

In the study of the effect of pH on electron transport and phosphorylation, DAD_{ox} was chosen because it was more stable at acidic pH than PD_{ox}. It can be seen that the pH optima for electron transport and phosphorylation is different in DAD_{ox} system in contrast to the ferricyanide system where they are the same (Figure 9). The high electron transport rate has a pH optimum around 7.5 similar to the pH optimum of uncoupled electron transport (Good, _et al._, 1966). In contrast the phosphorylation has a pH optimum around pH8.2 similar to the pH optimum for electron

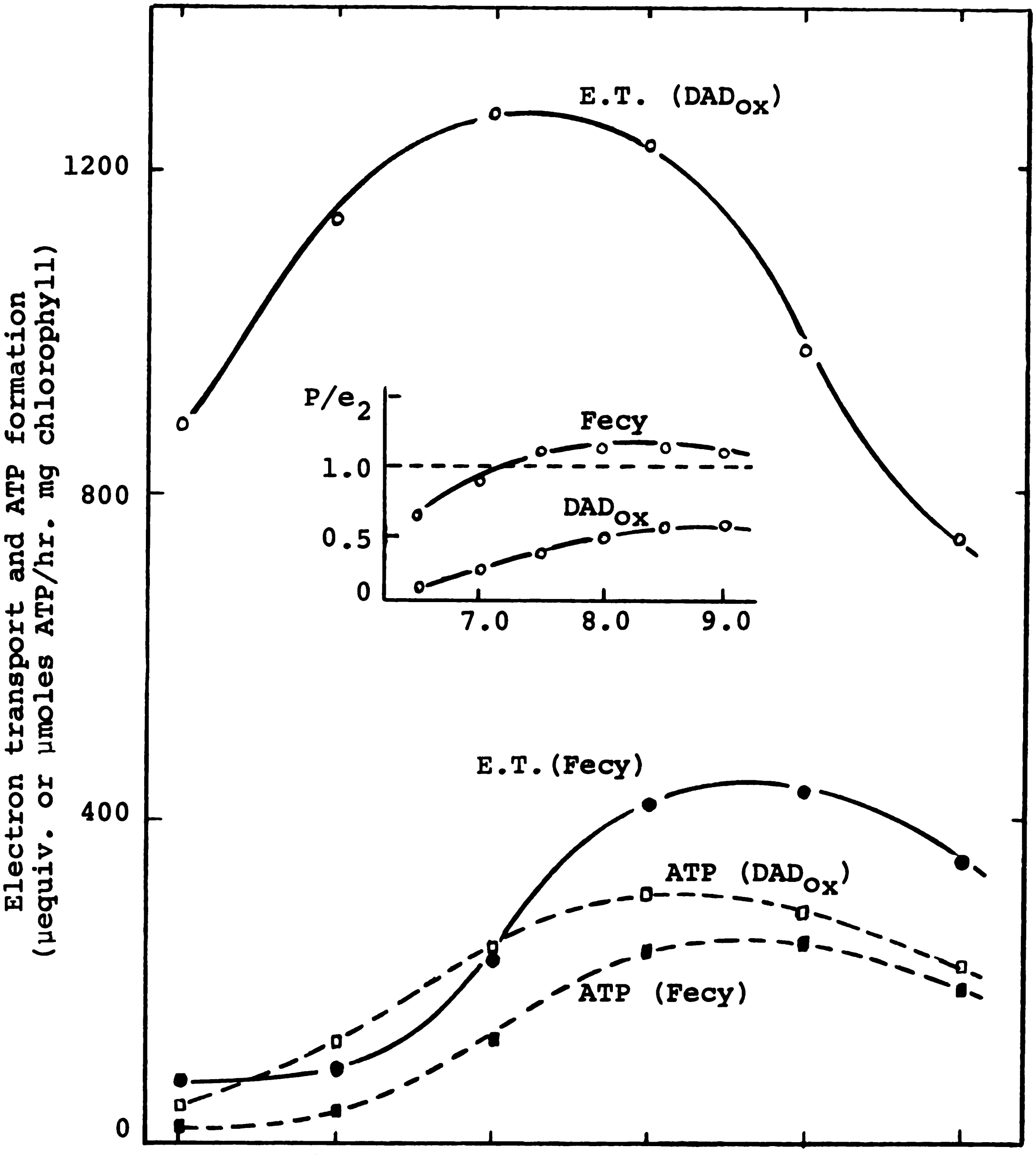
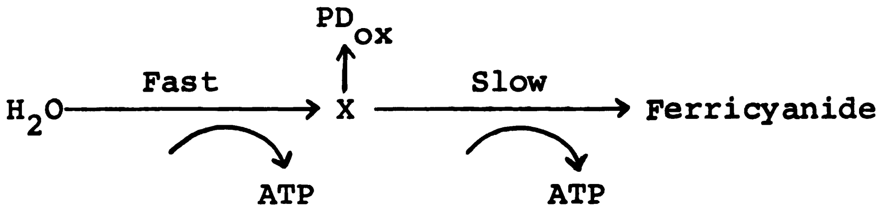


Figure 9. The effect of pH on electron transport and ATP formation in DAD$_{ox}$ and ferricyanide reducing systems.

transport and phosphorylation in ferricyanide reducing system. At low pH the electron transport and phosphorylation with DAD_{ox} reduction are many times faster than with ferricyanide reduction whereas at high pH most of the differences disappear. There is thus a much broader pH optimum for phosphorylation. PD_{ox} showed similar behavior with the peak around pH 8.2. This experiment contrasts with the observations of Saha (1970) who showed that in the PD_{ox} system phosphorylation was the same from pH 7.0 to 8.5. (The differences might be due to the different buffer used. The Bis-Tris-propane buffer used by Saha may have had inhibitory effects at the higher pH values. Certainly his phosphorylation rates at pH 8.0-8.5 were not significantly increased above the rates in ferricyanide system, unlike the situation with Tricine or TAPS as buffer).

Because at all pH values the electron transport to Class III acceptors is also different from the electron transport to Class I acceptors, and because this difference is not due to total uncoupling, one is compelled to believe that there is some fundamental difference in the electron transport pathway. Clearly the reduction of Class I acceptors involves some rate-limiting steps not involved in the reduction of Class III receptors. With the further consideration that the phosphorylation efficiency (P/e_2) with Class III acceptors is only half of the efficiency with Class I acceptors, a picture of two sequential sites of phosphorylation emerges. According to the model in the scheme below, Class III acceptors intercept electrons between the two sites. As the concentration of Class III acceptors increases the slow step is by-passed so that the rate of electron transport increases. However, at the same time one of the two sites of phosphorylation is also bypassed. Therefore, the electron traverses half as many phosphorylation sites and the P/e_2 falls to half its previous value.



Comparison Between Ionic and Non-ionic Form of Quinones as Acceptors

Since all of the Class I acceptors are highly polar, lipid-insoluble, in contrast to the Class III acceptors, we suspected that the solubility difference accounted for the differences associated with their reduction. To test this hypothesis, we compared electron transport and phosphorylation between two closely related substance, p-benzoquinone and p-benzoquinonesulfonate (The redox potentials at pH 8.0 are 0.22 V and 0.27 V, respectively). Quinonesulfonate is an anion and highly polar whereas quinone is non-ionic, relatively non-polar and quite soluble in most organic substances.

Table 2

Electron acceptor class as determined by lipid sobility (i). (Reaction mixture contained: tricine buffer (pH 7.8), 40 mM; chloroplasts with 10 μg chlorophyll per ml for Experiment I, 20 μg per ml for Experiment II).

Electron acceptor[a] (mM)	Assay of electron transport	Electron transport[b] $-P_i$	Electron transport[b] $+P_i$	ATP formation[b]	P/e_2
<u>Experiment I</u>					
Fecy(0.4)	Fecy reduction	240	350	177	1.01
QS(0.05)+Fecy(0.4)	"	216	331	157	0.96
Quinone(0.05) + Fecy(0.4)	"	482	614	205	0.67
<u>Experiment II</u>					
Fecy(0.4)	Fecy reduction	---	289	164	1.14
QS(0.26)+Fecy(0.4)	"	---	271	128	0.95
QS(0.26)	Titration of the hydroquinone	---	283	156	1.10
Quinone(0.26) + Fecy(0.4)	Fecy reduction	---	760	285	0.75
Quinone(0.26)	Titration of the hydroquinone	---	637	207	0.65

[a] Fecy, ferricyanide; QS, quinonesulfonate.

[b] μequiv. (or μmoles ATP)/hr. mg chlorophyll.

Table 3

Electron acceptor class as determined by lipid solubility (ii) (Reaction conditions as in Table 3).

Class of electron acceptor[a]	Acceptor[b] (mM)	Rate of electron transport $-P_i$	$+P_i$	Rate of phosphorylation[c]	P/e_2
I	Fecy(0.4)	240	350	177	1.01
	QS(0.05) +Fecy(0.4)	216	338	160	0.95
II	QS(0.10) +Fecy(0.4)	225	366	163	0.90
	QS(0.3) +Fecy(0.4)	291	414	156	0.75
III	Quinone(0.05) +Fecy(0.4)	482	614	205	0.67
	Quinone(0.1) +Fecy(0.4)	497	640	208	0.65
	Quinone(0.3) +Fecy(0.4)	556	644	196	0.61

[a] See text for explanation.

[b] Fecy, ferricyanide; QS, quinonesulfonate.

[c] μequiv.(or μmoles ATP)/hr. mg chlorophyll.

Table 2 shows that the addition of quinonesulfonate to the ferricyanide reducing system causes almost no change in the characteristics of the electron transport and phosphorylation processes. Quinonesulfonate in the absence of ferricyanide is reduced in a very similar manner. On the other hand the reduction of quinone itself, shows many of the characteristics of the reduction of Class III acceptors, non-phosphorylating and phosphorylating electron transport are both increased markedly, ATP formation is also increased and the efficiency of phosphorylation (P/e_2) falls. However, the increase in the rates of electron transport and the decrease of the P/e_2 are not as marked as with PD_{ox} and therefore, as mentioned before, p-benzoquinone is somewhat intermediate between Class III and Class I. Table 3 somewhat extends the observations of Table 2. Quinonesulfonate exhibits some tendency to uncouple as shown by a slight decrease in the rate of phosphorylation when the concentration of quinonesulfonate is increased. However the same concentrations of quinone always result in an increased electron transport, increased phosphorylation and lowered P/e_2. Thus p-benzoquinone behaves much as does 2,5-dimethylquinone (DMQ). (See Figure 8).

These experiments confirmed the suggestion that the distinctive characteristics of Class I and Class III acceptors are related to their lipid solubilities.

The Effect of Class I and Class III Acceptors on the X_E Stage of Chloroplasts

The characteristic high rates of non-phosphorylating and phosphorylating electron transport with Class III acceptors can be understood if Class III Acceptors are uncouplers. To test this possibility we looked for an effect of Class I and Class III acceptors on the post-illumination ATP formation capability (X_E) of chloroplasts. Neither ferricyanide nor PD_{ox} has an effect on chloroplasts post-illumination phosphorylation that is characteristic of uncouplers (Table 4). These results virtually eliminate the possibility of PD_{ox} being an uncoupler. Whatever the cause of the high rate of electron transport and phosphorylation with PD_{ox}, it seems unlikely that it is uncoupling. On the other hand, the low post-illumination ATP formation in the presence of DAD_{ox} indicates that the substance may have some energy-transfer inhibitor or uncoupler action. As is already known, the uncoupler methylamine severely eliminated phosphorylation.

Therefore, PD_{ox} system which showed a rapid electron transport and has half the phosphorylation efficiency of the ferricyanide system does not seem to involve an uncoupling mechanism. At very least, if PD_{ox} is an uncoupler, it must be a site-specific uncoupler which uncouples one of two sites of phosphorylations by a mechanism which does not

Table 4

Effect of various electron acceptors and methylamine on the post-illumination ATP formation (X_E).

Addition (mM)	Expt. No.	ATP formed (n moles)	ATP/chlorophyll (mole/mole)	Inhibitory effect (%) of additive
none	a	11.6	0.11	-
	b	12.4	0.11	
Fecy (1.8)	a	14.4	0.13	0
	b	12.5	0.11	
PD_{ox}(0.68) + Fecy(1.8)	a	12.0	0.11	0
	b	10.8	0.10	
DAD_{ox}(0.68)+ Fecy(1.8)	a	7.3	0.07	35
	b	8.5	0.08	
MA (6.8)	a	2.2	0.02	80

Chloroplasts (100 μg chlorophyll) were illuminated for 30 sec in 2 ml reaction medium containing 0.1 M sucrose, 0.05 M NaCl, 2 x 10^{-3} M $MgCl_2$, 0.01 M MES-NaOH buffer, pH 6.0, and 10^{-5} M pyocyanin. Immediately after shutting off the light 1 ml of a strongly buffered ADP+P_i mixture containing the indicated additives (0.1 M tricine-NaOH, pH8.2; 0.002 M ADP; 0.01 M P_i and the additives) was quickly injected into the suspension to initiate ATP formation. The dark ATP formation was terminated after 30 sec by adding 1 ml of 10% perchloric acid. [$^{32}P_i$]ATP was determined by the method described under Materials and Methods.

involve dissipation of the high-energy state (p.15). Clearly this kind of "uncoupling" is most easily pictured as the interception of the electron flow before one of the sites of phosphorylation. Thus the results raise the possibility of PD_{ox} accepting electrons between two phosphorylation sites.

The Effect of Light Intensities on Electron Transport and Phosphorylation with Class I and Class III Acceptors

The Class III acceptors, PD_{ox} and DAD_{ox}, require high light intensities for saturation of the reactions. The faster rate of electron transport in the PD_{ox} system requires higher light intensities than the slower rate in the DAD_{ox} system. The very slower rates with ferricyanide require very much lower light intensities for saturation (Figure 10). When the rate, R, versus R/I is plotted (Figure 11), it may be seen that the quantum efficiencies of electron transport with PD_{ox},DAD_{ox} and ferricyanide are all about the same, whereas quantum efficiencies of phosphorylation with Class III acceptors were almost exactly half of the quantum efficiency of phosphorylation with Class I acceptor. The results of other experiments using low light intensities are plotted directly as rate versus intensity (Figure 12). From the initial slopes of the lines it can be seen that the quantum efficiencies of electron transport are similar regardless of the acceptors used, whereas the quantum efficiencies of phosphorylation with DAD_{ox} and PD_{ox} show precisely

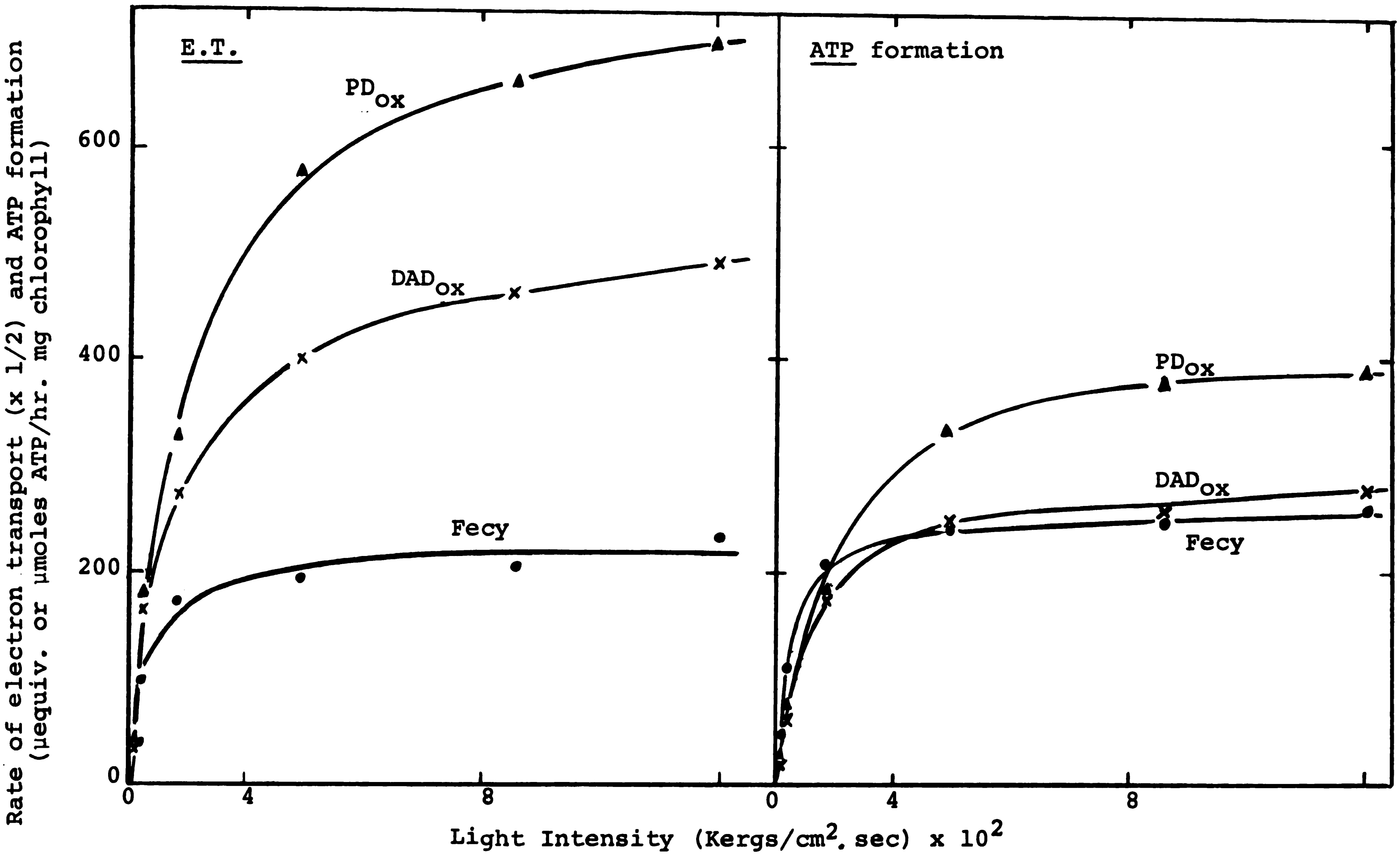


Figure 10. The effect of light intensities on electron transport and phosphorylation with PD$_{ox}$, DAD$_{ox}$ and potassium ferricyanide as electron acceptors.

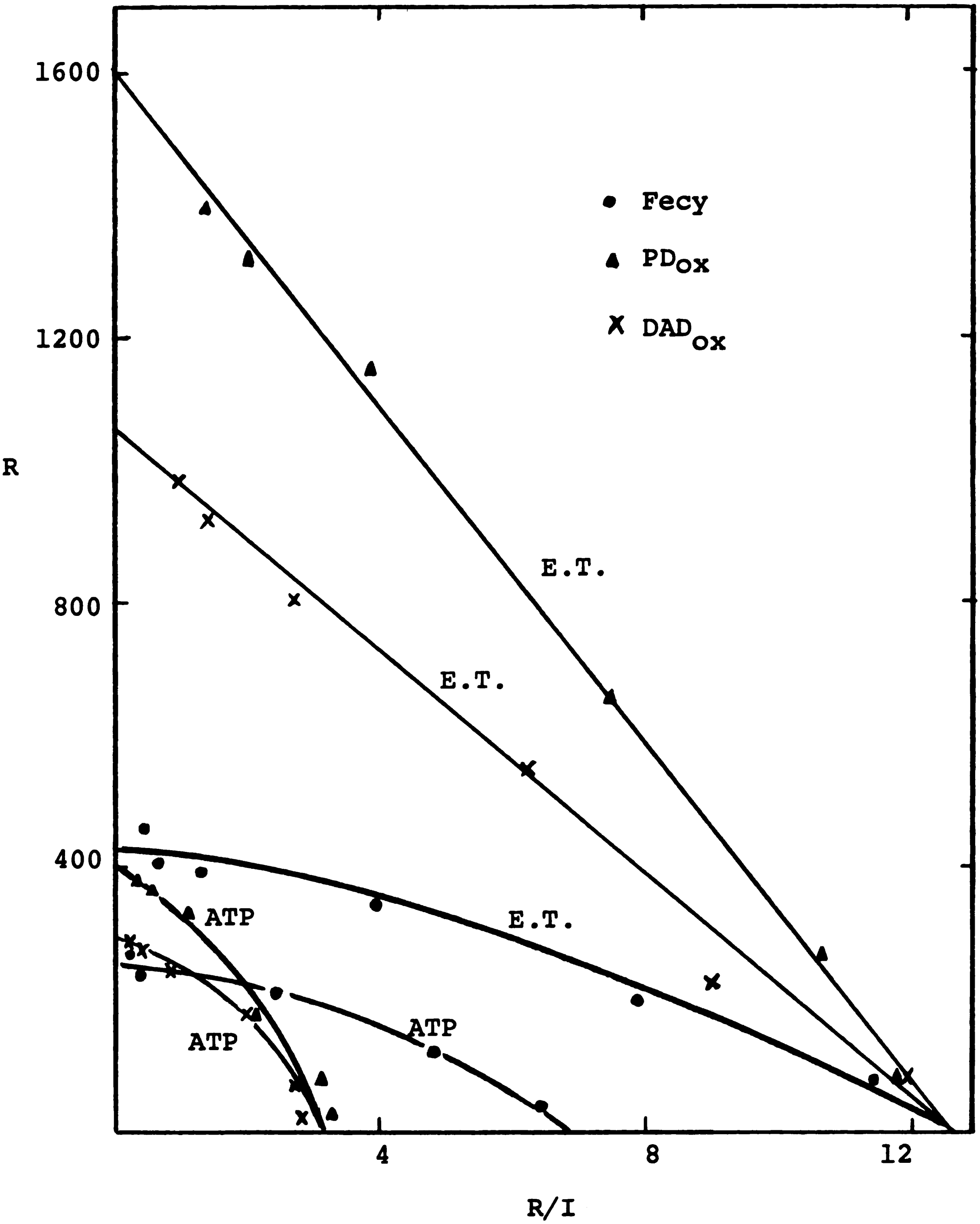


Figure 11. Plots of rate versus rate/intensities (R vs R/I) with PD_{ox}, DAD_{ox} and ferricyanide as electron acceptors. In this type of plot the intercept of the line in the R/I axis is a measure of the quantum efficiency of the process, and the intercept on the R-axis represents the maximal rate of the rate-limiting dark reaction.

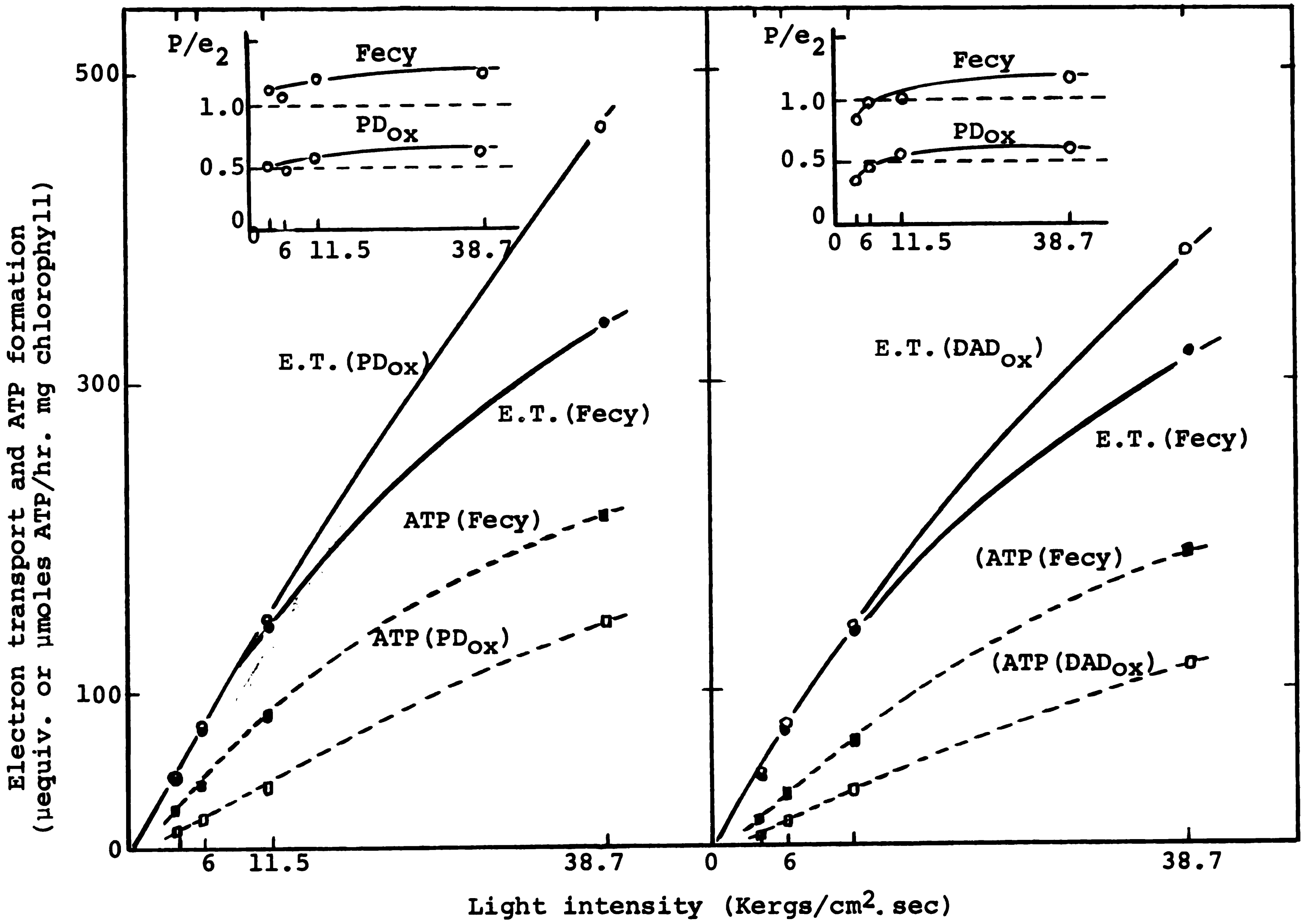


Figure 12. Electron transport and ATP formation at low light intensities with PD$_{ox}$, DAD$_{ox}$ and potassium ferricyanide as electron acceptors.

half the efficiency observed with ferricyanide. These results are consistent with the half P/e_2 ratio observed with Class III acceptors in Figures 6, 7, and 8.

These experiments taken together show that the decrease in phosphorylation efficiency to one half with Class III acceptors occurs at very high and low light intensities. Considering the great range in rates of phosphorylation at these different intensities, this 1:2 difference in phosphorylation efficiency is not likely to be fortuitous.

Saha (1970) has reported that the half phosphorylation efficiency with PD_{ox} occurred at pH 7.0 and that the difference from ferricyanide reduction decreased to essentially none at pH 8.0. The present experiments were carried out at pH 8.2 and showed the half phosphorylation efficiency as described. In this respect the results are therefore in conflict with the earlier observations.

The Effect of DCMU on Electron Transport and Phosphorylation with DAD_{ox} and Ferricyanide as Acceptors at High Light Intensities

DCMU (diuron) is an inhibitor thought to prevent the oxidation of reduced Q. We investigated the effect of DCMU concentration on the inhibition of electron transport and the consequent phosphorylation. It can be seen that both electron transport and phosphorylation (Figure 13) in the DAD_{ox} system are more sensitive than in the ferricyanide system.

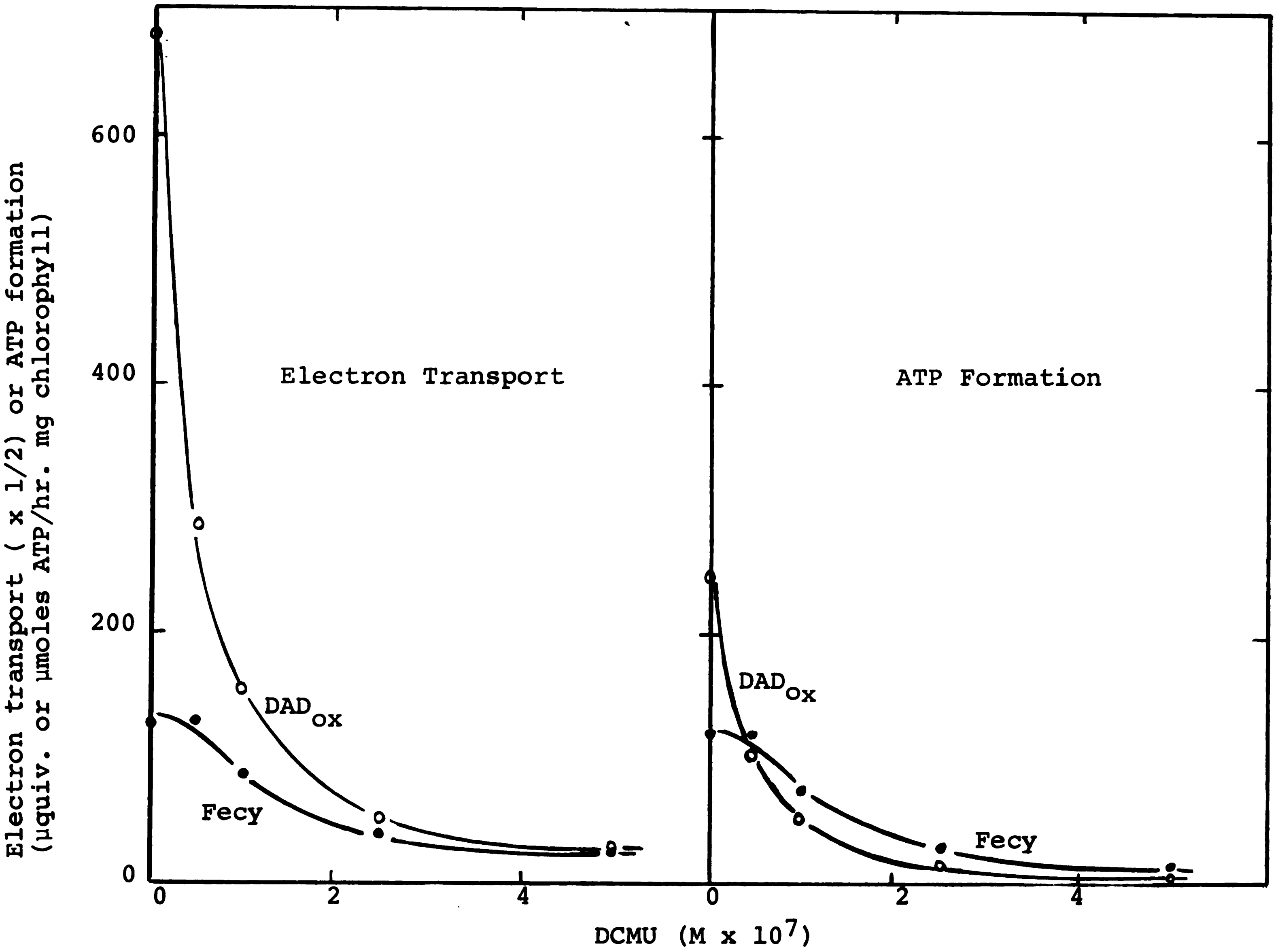


Figure 13. The effect of DCMU on electron transport and ATP formation with DAD_{ox} and ferricyanide as electron acceptors at high light intensity.

The Effect of DCMU on Electron Transport and Phosphorylation at Different Light Intensities

On the basis of Figure 13 one might conclude that the pathway of ferricyanide reduction is less easily blocked by DCMU than the pathway of DAD_{ox} reduction. However, an alternative explanation might be in the fact that ferricyanide reduction is so much slower. In other words the ferricyanide reduction might be severly limited by some slow reactions not directly sensitive to DCMU. In this case the low concentration of DCMU would have relatively little effect. For this reason we investigated DCMU inhibition with PD_{ox} and with ferricyanide over a range of light intensities.

It can be seen from Figure 14 and Table 5 that electron transport and phosphorylation in the PD_{ox} reducing system are inhibited by DCMU to the same extent at all light intensities: in this experiment the inhibition was about 70%. In contrast the sensitivity of ferricyanide reduction to DCMU was a function of light intensity. At low light intensities ferricyanide reduction was equally sensitive but with ferricyanide at high light intensity the 65% inhibition fell to 25%. Other electron transport inhibitors such as CMU and Atrazine, which inhibit at about the same site as DCMU (Izawa and Good, 1965) were also tested. They too inhibited PD_{ox} reduction equally at all light intensities

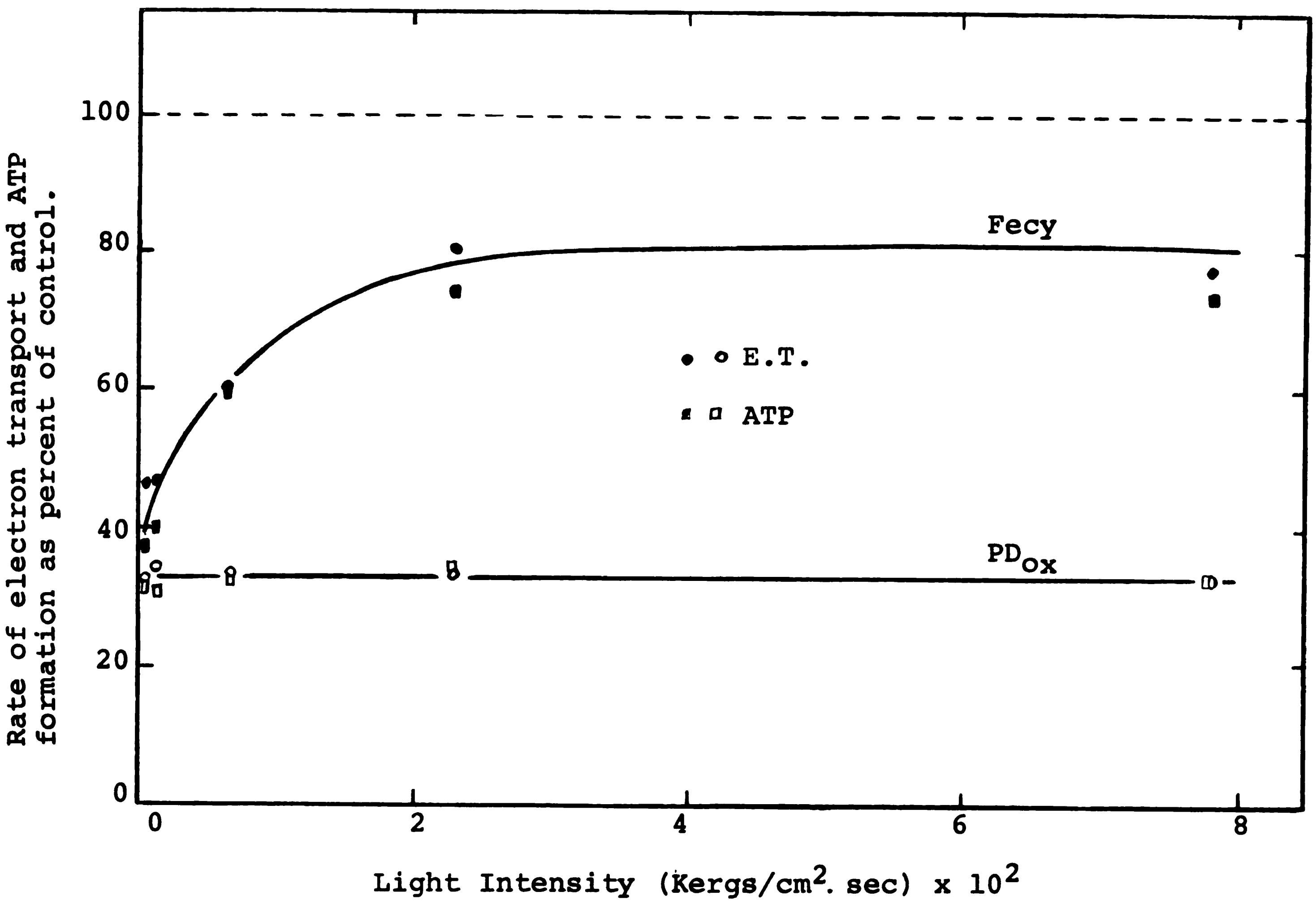


Figure 14. Inhibition of PD$_{ox}$ and Ferricyanide reduction by 3 x 10^{-8} M DCMU as a function of the light intensity.

Table 5

Effect of DCMU (3 x 10^{-8}M) on electron transport and photophosphorylation with ferricyanide and PD_{ox} as electron acceptors at different light intensities.

		Electron transport[c]			Photophosphorylation[c]		
Electron acceptor[a]	Light intensity[b]	control	+DCMU	(% inhibition)	control	+DCMU	(% inhibition)
Fecy	780	442	345	(22)	259	190	(26)
	230	354	286	(19)	200	149	(25)
	67	330	198	(40)	176	104	(41)
	13	154	72	(57)	80	32	(59)
	6	72	32	(56)	38	14	(63)
PD_{ox}	780	1500	486	(18)	384	124	(68)
	230	1140	388	(66)	303	105	(65)
	67	650	213	(67)	177	57	(68)
	13	169	58	(66)	42	13	(68)
	6	70	24	(63)	17	5	(68)

[a] Concentration of acceptor: 4 x 10^{-4} M Potassium ferricyanide, 6 x 10^{-4} M PD_{ox} (with 0.4 µmoles/ml ferricyanide).

[b] Kergs/cm^2, sec (λ= 550-700 nm).

[c] µequiv.(or µmoles ATP)/hr. mg chlorophyll.

Table 6

Effect of CMU (3 x 10^{-7}M) and atrazine (10^{-7}M) on the photoreduction of ferricyanide and PD_{ox} at various light intensities.

		Ferricyanide reduction[b]			PD_{ox} reduction[b]		
Inhibitor	Light intensity[a]	control	+DCMU	(% inhibition)	control	+DCMU	(% inhibition)
	780	464	384	(17)	1459	632	(57)
	230	432	335	(12)	940	505	(49)
CMU	67	365	230	(27)	585	285	(51)
	13	129	75	(42)	153	78	(49)
	6	71	39	(45)	70	31	(56)
	780	464	347	(25)	1454	500	(66)
Atrazine	230	432	274	(36)	940	410	(58)
	67	365	186	(49)	585	239	(59)
	13	129	69	(47)	153	53	(65)
	6	71	40	(43)	70	28	(60)

[a] Kergs/cm^2. sec (λ = 550-700 nm)

[b] μequiv./hr. mg chlorophyll. The assay conditions were as in Table 5.

while they inhibited ferricyanide reduction much less at high light intensities (Table 6).

The implication of this inhibition data, particularly the implications of the differences encountered at different light intensities will be presented in detail in the Discussion.

The Effect of KCN on Electron Transport and Phosphorylation with Ferricyanide and PD_{ox} as Electron Acceptor

It has long been known that KCN inhibits photosynthesis at the level of CO_2 reduction. At concentrations of about 10^{-3} M KCN inhibits catalase and phenol oxidase activity (Trebst, et al., 1963). With higher concentration of KCN (about 10^{-2} M) effects on the Hill reaction have been observed. Early work of Bishop and Spikes (1955) showed that when chloroplasts were incubated in 0.03 M KCN, almost complete inhibition occurred after about one hour. Cyanide seems to remove the copper from plastocyanin (Katoh, 1960) and therefore it has been thought to inhibit the Hill reaction at the plastocyanin site (Trebst, 1963; Trebst and Elstner, 1967). Later work of Katoh and San Pietro (1966) on isolated plastocyanin showed that oxidized plastocyanin was more sensitive to KCN and to salicyladoxime than was the reduced form. The reaction of cyanide with plastocyanin was a slow process.

Since ferricyanide is thought to accept electrons either at the level of P700 or after photosystem I, inhibition at plastocyanin or any site that is close to P700 should inhibit ferricyanide reduction and be much less likely to inhibit PD_{ox} reduction. Investigations on the effect of KCN on ferricyanide and PD_{ox} reduction were made in an attempt to show such a selective inhibition.

The effect of incubation of chloroplasts with ferricyanide, KCN, KCN + ferricyanide on ferricyanide (Figure 15) and PD_{ox} (Figure 16) reduction were studied. A very low concentration of ferricyanide was used in combination with KCN during the incubation to oxidize any components, e.g., plastocyanin, cytochrome-f or P700 that might be affected, on the assumption that the oxidized forms of these components might be more sensitive to cyanide. It can be seen in Figure 15 that after 30 min of incubation the ferricyanide reducing system was almost completely (90%) inhibited by 3×10^{-5} M ferricyanide + 0.03 M KCN. KCN alone caused a slow inhibition although this effect was accelerated after 30 min of incubation. Ferricyanide alone caused slow and progressive inhibition but the inhibitory effect was less than KCN alone. Phosphorylation was affected slightly more than electron transport but this difference might be because the chloroplasts were also slightly uncoupled as a result of this incubation.

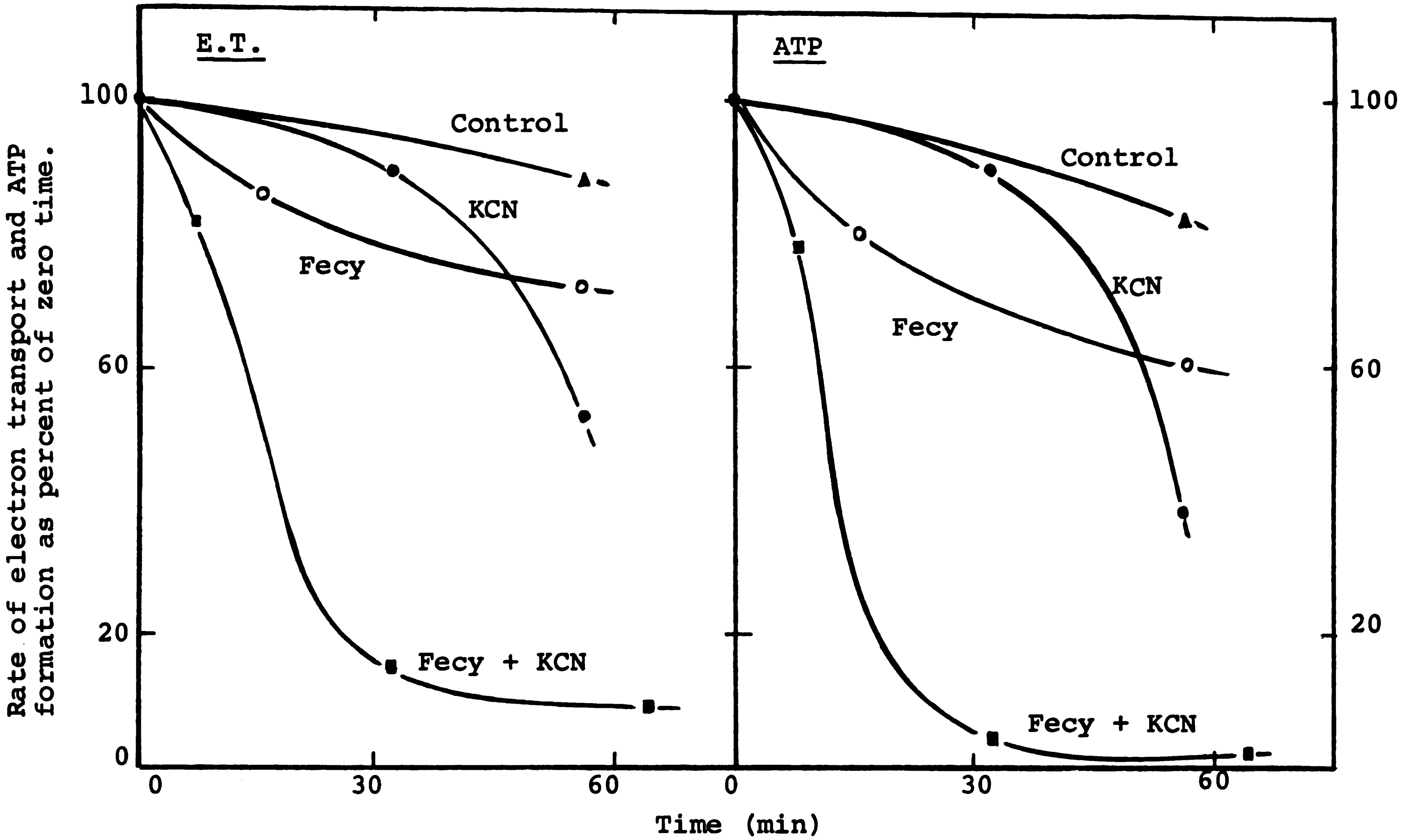


Figure 15. The inhibitory effects of ferricyanide, KCN and KCN plus ferricyanide on the ferricyanide reducing system.
Incubation medium: 0.083 M tricine with 0.03 M KCN and 3 x 10^{-5} potassium ferricyanide, final pH 7.8. For control. 0.083 M tricine plus KOH to give also pH 7.8, chlorophyll 133 µg/ml. Incubations were carried out in the dark in sealed test tubes at 0° C. Chloroplasts suspended in the incubation medium (0.3 ml) were pipetted into 2.0 ml reaction medium. Thus the concentration of KCN was diluted to 4.5 x 10^{-3} M during the incubation.

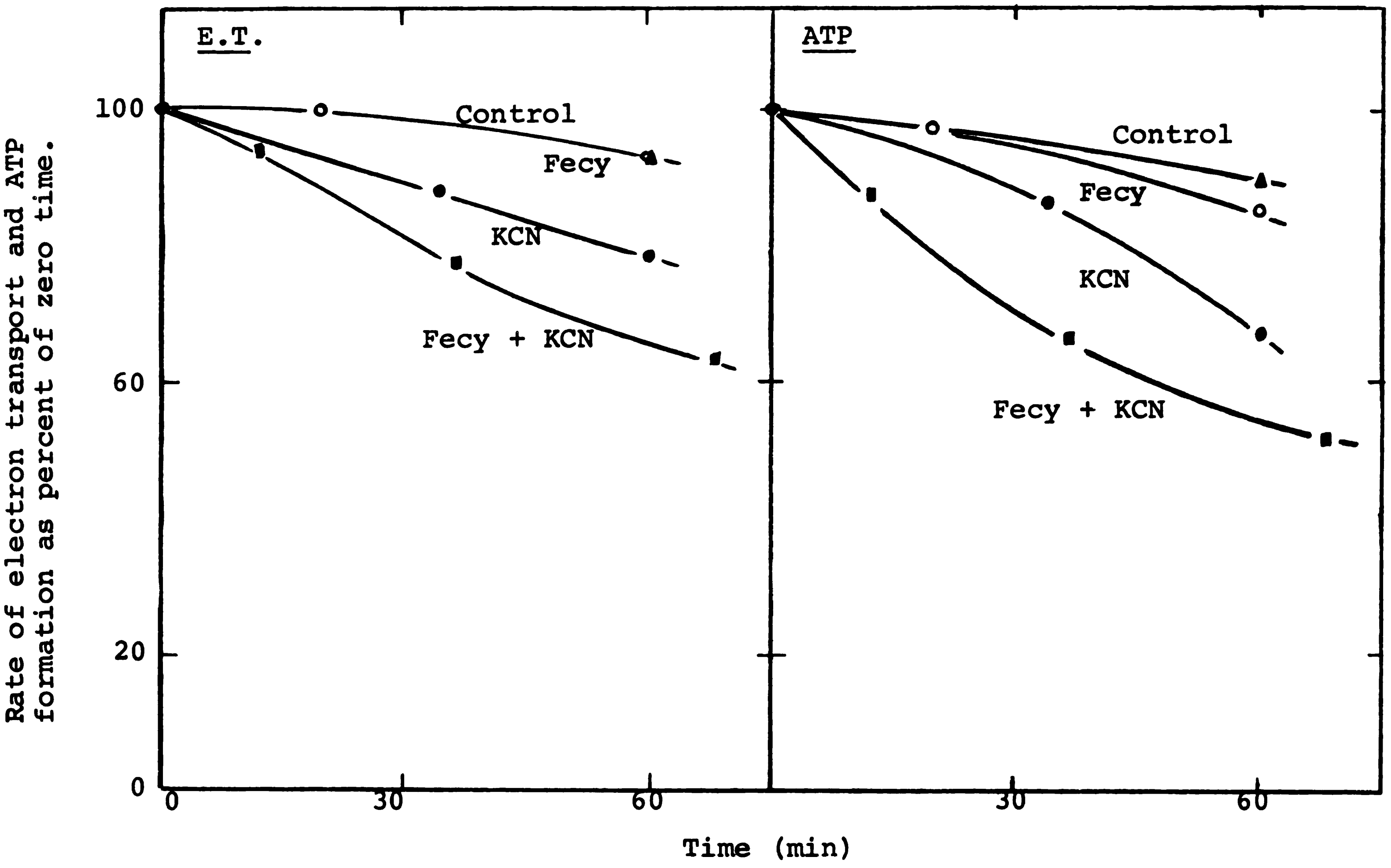


Figure 16. The inhibitory effects of ferricyanide, KCN and KCN plus ferricyanide on the PD_{ox} reducing system. The reaction conditions were as described in Figure 15.

In contrast, the PD_{ox} reducing system was much less sensitive to cyanide. Incubation with ferricyanide + KCN gave slightly stronger inhibition than with KCN alone. After approximately 30 min of incubation the inhibitory effect on electron transport was less than 15% while the effect on phosphorylation was less than 25%. Incubation with ferricyanide alone did not inhibit PD_{ox} reduction at all (Figure 16).

The incubations were also done at pH 7.4 as well as pH 7.8. At the lower pH a much longer incubation time (about 90 min) was required to completely inhibit the ferricyanide reducing system.

These results show a significant selectivity in the inhibition of the ferricyanide reducing system. The inhibition is increased by the oxidative property of ferricyanide during incubation. The main site of inhibition must be after the PD_{ox} reducing reaction site. The partial inhibition of the PD_{ox} system can be viewed as a possibility that there is a secondary site of KCN inhibition in the PD_{ox} reduction pathway. The experiments were also done at lower KCN concentration, e.g., 0.02 M. A longer incubation time was then required but the two systems were inhibited in the same selective manner as observed at the higher concentrations.

The effect of KCN inhibition in a short period can be observed. In the results given in Table 7, chloroplasts

Table 7

Reversibility of KCN Inhibition.[a]

Conditions	Rate of electron transport[b]	% of control	Rate of phosphorylation[b]	% of control	P/e_2
Before washing chloroplasts					
Fecy as acceptor					
control	510	100	320	100	1.25
KCN-treated	25	5	3	1	0.23
PD_{ox} as acceptor					
control	1410	100	377	100	0.54
KCN-treated	890	63	170	45	0.38
After 2 x washing[c]					
Fecy as acceptor					
control	510	100	300	100	1.18
KCN-treated	315	62	178	593	1.13
PD_{ox} as acceptor					
control	1450	100	384	100	0.53
KCN-treated	1015	70	230	60	0.45

[a] Incubation medium for KCN-treatment (45 min); 0.083 M tricine-0.03 M KCN, 5 x 10^{-5} M ferricyanide, pH 7.8. For control; 0.083 M tricine-KOH, pH 7.8.

[b] µequiv. (or µmoles ATP)/hr. mg chlorophyll.

[c] Chloroplasts (both control and KCN-treated) were washed twice at 0°C with the washing medium described under Materials and Methods.

were incubated in the same manner as Figure 15 at 0°C for 45 min. Again electron transport and phosphorylation with ferricyanide were almost completely inhibited. These chloroplasts were then washed twice with the standard HEPES-NaOH washing medium, pH 7.4. Assay of electron transport showed that washing caused the ferricyanide reducing system to recover from an inhibition level of 5% of control to 60% of the control rate. The very much smaller inhibition of the PD_{ox} reducing system was hardly releived at all by washing. This is therefore another indication that the inhibition of the PD_{ox} reducing system is by a different mechanism. Thus these results indicate the second site of KCN inhibition which is irreversible and common to both systems. If so, the inhibition mechanism which only affects the ferricyanide reducing site within 45 minutes may be almost totally reversible. However, an experiment in which the chloroplasts were washed twice after a prolonged incubation (e.g., 3 hours) in the same manner as Table 7 showed no recovery of activity.

KCN Inhibition of Other Non-cyclic Electron Transport Systems

At this moment PD_{ox} is the best Class III acceptor known. All other Class III acceptors are in some degree intermediate between Class I and Class III in terms of the effects on electron transport and phosphorylation rates.

Table 8

Effect of KCN on non-cyclic electron transport and photophosphorylation.[c]

System[a]	Rate of electron transport[b]	% of control	Rate of phosphorylation[b]	% of control	P/e_2
MV(0.1 mM)					
control	510	100	296	100	1.16
KCN-treated	75	15	17	6	0.45
Fecy(0.4 mM)					
control	491	100	255	100	1.04
KCN-treated	53	11	7	3	0.29
PD_{ox}(0.6 mM)+Fecy(0.4mM)					
control	2140	100	482	100	0.40
KCN-treated	1750	82	258	60	0.30
DAD_{ox}(0.6 mM)+Fecy(0.4 mM)					
control	1500	100	322	100	0.43
KCN-treated	996	67	110	35	0.22
DMQ(0.6 mM)+Fecy(0.4 mM)					
control	1114	100	297	100	0.53
KCN-treated	600	53	86	29	0.29
Quinone(0.4 mM)+Fecy(0.4 mM)					
control	930	100	251	100	0.54
KCN-treated	250	27	20	8	0.16
QS(0.4 mM)+Fecy(0.4 mM)					
control	358	100	157	100	0.88
KCN-treated	33	9	4	3	0.26

[a] The pH of the assay medium was 8.2 except for systems for quinone and quinonesulfonate (QS) in which the pH was 7.8.

[b] μequiv./hr. mg chlorophyll.

[c] Conditions for KCN-treatment and control were as in Table 7.

A further investigation was made on the effect of KCN on the reaction of various alleged Class I and Class III acceptors.

The reductions of the typical Class I acceptors ferricyanide and methylviologen were similarly and strongly inhibited by KCN. The reductions of the "Class III" acceptors DAD_{ox}, DMQ and p-benzoquinone were all more inhibited than the reduction of PD_{ox}. Furthermore, the degree of inhibition in all instances corresponded to the intermediate nature of the acceptor (Table 8). Thus when one ranks the acceptors in decreasing Class III character and increasing Class I character by either the criterion of KCN inhibition or by the criterion of electron transport and phosphorylation behavior, one finds the same series: PD_{ox}, DAD_{ox}, DMQ, p-benzoquinone followed by p-benzoquinone sulfonate and all other ionic Class I acceptors.

Inhibition of Cyclic Phosphorylation by KCN

As pointed out in the Introduction, it has not yet been established whether "cyclic" phosphorylation occurs at the same site as non-cyclic phosphorylation (that is before cytochrome-f) or at some entirely different site which is involved only in the cyclic process. The cyclic system is a rapid process as shown by the high phosphorylation rate compared with the non-cyclic system. There is no apparent (i.e., net) electron transport. The site of electron entry is believed to be between the two photosystems since the cyclic process can be insensitive to DCMU. Information from experiments with mutants of *Chlamydomonas reinhardi* has suggested that PMS-catalyzed cyclic phosphorylation requires plastocyanin and P700. *In vivo* electron flow in the presence of DCMU indicates the involvement of cytochrome 553, P700 but not cytochrome-b559 (Levine, 1969). In isolated chloroplasts the cyclic electron flow can be changed to a system I non-cyclic process in the presence of a photosystem I acceptor such as methylviologen and a donor such as the reduced form of DAD or $DCPIPH_2$. The high rate of phosphorylation still remains and this high rate is associated with even higher rate of electron transport. In fact, cyclic phosphorylation probably simply represents the activity of some substances which can accept electrons from photosystem I when oxidized and indirectly donate electrons to photosystem I when reduced. Thus if DAD or DCPIP are kept reduced such as by an excess of ascorbate and an electron acceptor such as methylviologen is added there is a non-cyclic flow. But if DAD or $DCPIPH_2$ are allowed to become partially oxidized they begin to accept electrons themselves and thus catalyze a cyclic electron flow. Since cyclic phosphorylation is a property of photosystem I, an inhibition which is close to P700 should somehow affect cyclic electron flow and therefore cyclic phosphorylation. If, indeed, KCN inhibits electron transport at plastocyanin and the site of electron entry is before or at plastocyanin, we should be able to see the same inhibitory effect as is seen with ferricyanide reduction. If the site of electron entry is after plastocyanin, no severe inhibition should be detected.

With the above criteria in mind, the effect of KCN on the cyclic and related system I non-cyclic processes were investigated. Table 9 shows that the cyclic system utilizing pyocyanin is very sensitive to cyanide. The DAD and $DCPIPH_2$ systems, either cyclic or non-cyclic, were also very sensitive to KCN. Indeed all of these systems were as sensitive to KCN as the ferricyanide reducing system.

The above experiments indicate that the site of electron entry from these three donors is indeed at or before plastocyanin. However, it does not establish whether or not the phosphorylation is involved in the same non-cyclic pathway site as ferricyanide supported phosphorylation. It is interesting that even though this electron system I flow seemed to share a portion of non-cyclic pathway with the ferricyanide reducing system, it is a much faster reaction.

PMS-catalyzed cyclic phosphorylation exhibited different characteristics. With 10^{-5} M PMS the cyclic process was also strongly inhibited by KCN either in red light or white light. With 10^{-4} M PMS the KCN inhibition

Table 9

Effect of KCN on photosystem I-dependent reactions.

System	DCMU 3×10^{-8}M	Rate of phosphorylation[a] control	Rate of phosphorylation[a] treated	Rate of electron transport[a] control	Rate of electron transport[a] treated
Pyocyanin (cyclic)	-	830	66		
DAD (cyclic)	+	415	trace		
DAD→MV	+	606	trace	2880	14
DAD (cyclic)	+	90	trace	408	12
DAD→MV	+	475	trace	2740	87
$DCPIPH_2$ (cyclic)	+	44	0		
$DCPIPH_2$ →MV	+	44	0		

[a] μequiv. (μmoles ATP)/hr. mg chlorophyll

Chloroplasts were pre-treated with KCN as described in Table 7. Concentrations of electron carriers used; 1×10^{-5} M pyocyanin, 6×10^{-4} M DAD, 6×10^{-4} M $DCPIPH_2$, 1×10^{-4} M MV, 5×10^{-3} M ascorbate. The pH of the reaction medium was 8.2.

Table 10

Effect of KCN on PMS-mediated cyclic photophosphorylation.

PMS (M)	Light	DCMU 3×10^{-8}M	Photophosphorylation rate[a] control	KCN-treated
1×10^{-5}	Red	-	473	61
		+	13	trace
	White	-	563	32
		+	245	trace
1×10^{-4}	White	+	640	125
2×10^{-4}	Red	-	650	442
		+	200	40
	White	-	820	505
		+	650	463

[a] μmoles ATP/hr. mg chlorophyll.

Chloroplasts were pre-treated with KCN as described in Table 7.

was slightly decreased. At a still higher concentration of PMS (2×10^{-4} M) cyclic phosphorylation was only partially inhibited under red light in the absence of DCMU. Under white light either in the presence or absence of DCMU the phosphorylation was also partially inhibited (Table 10). The strong inhibition in the presence of DCMU under red light could be due to the requirement for reduced PMS which is obtained by the photoreduction of PMS by white light.

DISCUSSION

Characteristics of Electron Transport with Class III Acceptors

Three classes of electron acceptors can be distinguished by their different characteristics in electron transport and phosphorylation. Class I acceptors, e.g., ferredoxin-NADP$^+$, ferricyanide and methylviologen are reduced in a reaction in which electron transport and phosphorylation are relatively tightly coupled. In other words the rate of electron transport can be enhanced under phosphorylation conditions and uncoupling of phosphorylation increases the electron transport rate (Figures 2, 3). Class II acceptors such as DCPIP are uncouplers as well as acceptors. They enhance the electron transport rate at low pH without phosphorylation. At higher pH values phosphorylation is still appreciable (Figures 4, 5). Class III acceptors as exemplified by the quinonediimide, PD_{ox} and DAD_{ox} give high electron transport whether or not ADP + P_i are present. Phosphorylation efficiency is half of the phosphorylation efficiency observed with Class I acceptors, although the phosphorylation rate is higher (Figures 6 and 7).

The differences in these three types of acceptors which are responsible for the variations in electron transport and phosphorylation seem to be primarily physical.

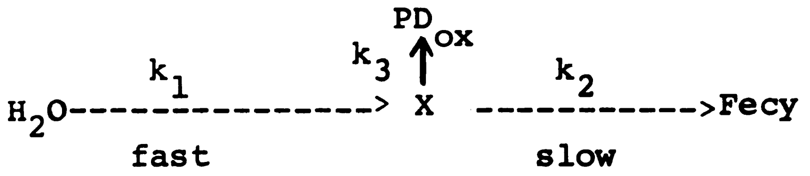
Class I acceptors are highly ionic, lipid-insoluble compounds whereas Class III acceptors are non-ionic and lipid-soluble. Due to these characteristics it is reasonable to postulate that the differences in lipid-solubility account for the ability to accept electrons at different sites. The membrane-soluble Class III acceptors might be able to permeate into the thylakoid membrane thereby accepting electron before the site accessible to ionic acceptors. By intercepting electrons at different positions, different reaction steps become rate-determining; Class III acceptors clearly accept electrons before Class I acceptors and before the site of the major limitation of rate of the reduction of Class I acceptors (see Figures 6 and 7).

The characteristics of the behavior of Class III acceptors may also depend on their molecular structure in other ways and not only on their lipid solubilities. Furthermore, chemical differences between quinonediimides (e.g., PD_{ox}) and quinones may in part, determine the nature of the electron transport and phosphorylation processes. Compare Figures 6 and 7 to Figure 8. Certainly low potential lipid-soluble oxidants such as menadione do not seem to be typical Class III acceptors (data not presented). Possibly such substances are incapable of accepting electrons at the site accessible to the Class III lipid-soluble acceptors for thermodynamic reasons.

Two main lines of evidence support the concept that PD_{ox} accepts electrons before the site which reacts with ferricyanide:

1. <u>The differences in the reaction rates of the two systems</u>.

It is well-known that the electron transport pathway from water to Class I acceptors such as ferricyanide involves a severe rate-limiting step before cytochrome-f (v_{max} = 1000 μequiv/hour.mg chl). From the very high rate of PD_{ox} reduction (v_{max} = 2500 μequiv/hour.mg chl) and from the differences in coupling phenomena, one is forced to believe that PD_{ox} intercepts electrons before they enter the major rate-limiting step involved in ferricyanide reduction. (See Scheme 1).



$$H_2O \xrightarrow[\text{fast}]{k_1} \overset{\overset{PD_{ox}}{k_3\uparrow}}{X} \xrightarrow[\text{slow}]{k_2} \text{Fecy}$$

Scheme 1

In this scheme k_1, k_2 and k_3 signify the apparent rate constants of the corresponding reaction steps. X represents the PD_{ox} reduction site.

The kinetics of the fast reduction of "A pool" by Q followed by a slow reaction for the oxidation of A have been shown by Kok, <u>et al</u>. (1966) and Forbush and Kok (1968). Let us examine the possibility that PD_{ox} accepts electrons from A either directly (i.e., X = A in the above scheme) or indirectly via an unknown carrier situated near A. We assume that the rate of the electron transfer step $A \longrightarrow PD_{ox}$ is much faster than the rate of the reduction of A by Q ($k_3 \gg k_1$), so that in the PD_{ox} reducing system the rate-limiting step lies in the step $A \longrightarrow Q$. Would this hypothesis be compatible with the known kinetics of the electron transfer reaction between Q and A? The reduction of A by Q has been shown by Forbush and Kok to be second order, $dA/dt = k_1[Q]_{red} \times [A]_{ox}$, with the rate constant $k_1 = 165$ $[\text{pool unit}]^{-1}.\text{sec}^{-1}$. One "pool unit" is defined as $[Q]_{total}$ which is probably equivalent to 1/400 chl. Since we assume $K_3 \gg k_1$, the pool A must stay largely oxidized during the steady state reduction of PD_{ox}; hence $[A]_{ox} \approx [A]_{total}$. The $[A]_{total}$ is equivalent to 1/7 of $[Q]_{total}$ (Forbush and Kok, 1968). An approximate value for $[Q]_{red}$ at the steady state could be extrapolated from the early fluorescence data of Kok (1963). The data indicate that approximately 1/2 of the total Q stays reduced when chloroplasts are reducing ferricyanide at near-saturating light intensities. Doubling the electron transport rate by the addition of ammonia lowers the $[Q]_{red}/[Q]_{total}$ ratio to 1/3. Since PD_{ox} enhances the rate by almost another factor of 2, one may reasonably infer the ratio to be around 1/4 to 1/5 in the PD_{ox} reducing system. By substituting all these values into the above rate equation one obtains the theoretical steady-state rate of the electron transfer reaction $Q \longrightarrow A$ during PD_{ox} reduction, 2400 - 3000 μequiv/hour.mg chl, which is in good agreement with the observed rate of PD_{ox} reduction, 1500 - 2500 μequiv/hour • mg chl. Thus the available kinetic data do seem compatible with the hypothesis that PD_{ox} intercepts the electrons from photosystem II at or near A, and the rate limiting step of this system occurs between Q and A. This hypothesis will be examined further in the following section from an entirely different angle.

2. Differences in the sensitivity to electron transfer inhibitors.

a. DCMU

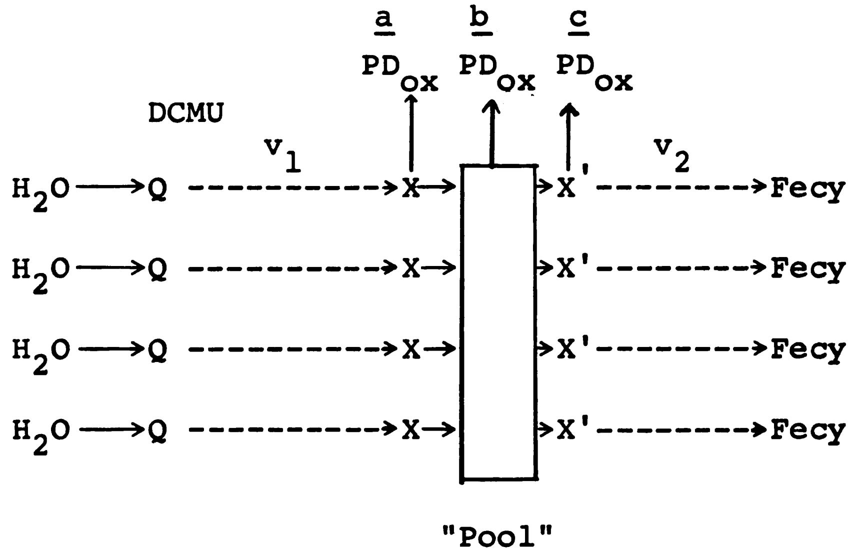
When the supply of electrons is used very slowly because of some limiting reactions, a decrease or increase in the supply does not change the reaction rate very much. Thus when a somewhat lower light intensity supplies electrons more slowly to the ferricyanide reducing system the overall rate of ferricyanide reduction is not much changed. On the other hand, the much faster PD_{ox} reducing system, which makes more demand on the electron supply, is more sharply changed by a decrease in the electron supply (See Figure 10). For the same reason it is not surprising that inhibition by DCMU, which also limits the supply of electrons, was found to be more pronounced in the fast PD_{ox} reduction than in the slow ferricyanide reduction.

When the effects of the electron transport inhibitor DCMU (Figure 14) or CMU and Atrazine (Table 6) were examined at different light intensities, a further interesting difference between the reductions of Class I and Class III acceptors was noted. PD_{ox} reduction was similarly inhibited at all light intensities whereas ferricyanide reduction was inhibited less severely at higher light intensities. Moreover the inhibitions of PD_{ox} reduction and ferricyanide reduction were identical at low light intensity.

What can be the meaning of equal inhibition at all light intensities in terms of the mechanisms of electron transport and inhibition?

It has been recognized that many of the components of the electron transport system in chloroplasts (cytochrome-f, plastocyanin, P700, etc.) are in a 1 to 1 to 1 ratio (see Introduction). This and other evidence (Kok, et al., 1970) suggest that there may be separate chains of carriers down which the electrons are passed. If there is no possibility of a cross-transfer of an electron from one chain to another, inhibition of a chain anywhere between the point of entry and point of exit of the electrons will make this whole chain to be inoperative. Since light absorption and the consequent charge separation would be a function of such a separate electron transport chain, a block at any level of the chain would simply make a portion of the chlorophyll photochemistry inert. This would be a tantamount to removing a portion of the chloroplasts and, clearly, the inhibition would be the same at all light intensities.

For this reason it would seem that electrons are transported from water to PD_{ox} by a mechanism which involves some kind of units which constitute separate transport chains. Between these chains no cross-transfer of electrons occurs. DCMU then either block or does not block some of the many chains. See pathway a of Scheme 2 below.



Scheme 2

An alternative but rather minor modification of this model allows the electrons transported by the individual chains to be pooled in some reservoir ("pool"), provided that the rate of electron transfer (v_3) from this pool to PD_{ox} is so fast that the rate limiting step always resides in the step Q ⟶ pool at any light intensities (pathway b or c in Scheme 2). One might equate this "pool" with the well-known "pool A" of Kok (e.g., Kok, et al., 1967) and recall the kinetic arguments described in the preceding section.

What then is the meaning of the smaller inhibition at high light intensity observed when ferricyanide is reduced? Usually the answer is expressed (correctly) in a few words: at high light intensities a DCMU-insensitive reaction is rate-limiting. In this section, however, we need a more explicit explanation with reference to the structure of the electron transport system.

As already suggested, DCMU probably acts by inhibiting separate, non-communicating electron transport chains. If we postulate a pooling of the electrons traversing the individual chains after (or near) the PD_{ox} reducing site but before the ferricyanide reducing site, and if we make the very reasonable assumption that the utilization of electrons from the common pool for ferricyanide reduction is the slow, rate-determining process, the observed light intensity-dependence of the inhibition is predicted. Consider Scheme 2 again. When light intensity is very low the rate is determined almost entirely by the supply of electrons from photosystem II. Since DCMU blocks individual chains the supply of electrons is reduced in proportion to the number of the chains blocked. Since this rate of supplying electrons (v_1) at low light intensity controls all rates, the extent of inhibition is independent of how the electrons are used. Thus ferricyanide reduction and PD_{ox} reduction are equally inhibited at low light intensities. For reasons already given PD_{ox} reduction is also equally inhibited at high light intensities. (Even at high light intensities and regardless of the three possible PD_{ox} reduction sites, a, b, c in Scheme 2, the activities of the individual chains control the overall rate). The situation during ferricyanide reduction is quite different. Here at high light intensities v_2 becomes rate-determining. Because of the common pool of electrons, the photosystem II transport chains (before the pool) not blocked by DCMU have access to all of the chains (after the pool) reducing ferricyanide. Therefore at high light intensities the high velocity potential of the remaining unblocked chains (v_1) tends to compensate for the missing activity of the blocked chains.

b. KCN

From the hypothesis that PD_{ox} accepts electrons before the site of ferricyanide reduction, one would predict that any inhibition of the common part of the pathway would affect the fast PD_{ox} reduction as much as, or probably more than, the slow ferricyanide reduction. (This is the case with the DCMU inhibition at high light intensities discussed above). Any demonstration that the slow ferricyanide reduction can be more sensitive to an inhibitor than the fast PD_{ox} reduction suggests that ferricyanide reduction involves a site of inhibition not shared by PD_{ox} reduction. Thus the fact that ferricyanide reduction is much more sensitive to KCN than is PD_{ox} reduction (Figures 15, 16) strongly support the model proposed under the discussion of DCMU inhibition. PD_{ox} does indeed seem to accept electrons before the KCN-sensitive site and ferricyanide after this site.

The site of KCN inhibition is likely to be plastocyanin. Izawa's data (unpublished) show that KCN causes an irreversible decrease in the absorbance of 596 nm of isolated plastocyanin at pH 7.8. (KCN did not cause any absorbance change of isolated cytochrome-f.) The failure of other workers, e.g., Katoh and San Pietro (1966), to observe significant effects of KCN on plastocyanin absorbance in a short incubation time is possibly due to pH of their incubation medium. As mentioned above they looked for absorbance changes in phosphate buffer at pH 7.0. My inhibition data showed that incubation at pH 7.8 requires a markedly shorter incubation time than at pH 7.4.

Bishop and Spikes (1955) showed that 1.5×10^{-3} M ferricyanide decreased the inhibitory effect of 0.01 M KCN on the Hill reduction in rhubarb chard and sugar beet chloroplasts. Their result is contradictory to the present result that low concentrations of oxidant increased the inhibitory effect of KCN on spinach chloroplasts.

Speculation Concerning the Nature and Location of the Phosphorylation Sites

PD_{ox} probably has no uncoupling action on isolated chloroplasts (Table 4). Nevertheless the PD_{ox} reducing system has only one half of phosphorylation efficiency of ferricyanide system, both on the basis of P/e_2 value (Figure 6) and on the basis of the quantum efficiency (Figures 11, 12). Therefore it is likely that the PD_{ox} reducing system involves one of the two phosphorylation sites involved in the non-cyclic electron transport to ferricyanide reducing site. This analysis only applies with fairly high concentration of PD_{ox} when the electron transport reaches a maximum. The intermediate portion of P/e_2 curves (Figure 6) is easily visualized in terms of a low concentration of PD_{ox} accepting electrons at both sites. High concentration of PD_{ox} increases the acceptor concentration in the lipoidal membrane, thereby blocking the transport of electrons to the polar acceptor site. The interpretation of the high and constant rate of phosphorylation which occurred long before the increasing concentration of PD_{ox} have given the maximum rates or have decreased the P/e_2 to one half might be the coincidence of additive effect of both systems operating at different rates. The explanation of the halving of phosphorylation efficiency with PD_{ox} in terms of an interception of electrons between two sites of phosphorylation implies that the efficiency of these two sites are are equal. This is not easy to understand since the site of phosphorylation operative with PD_{ox} seems to be "loosely coupled". That is to say the rate of electron transport is independent of the presence or absence of phosphorylation. However, lacking a real understanding of the nature of the non-phosphorylation electron transport which occurs in chloroplasts to a lesser or greater extent with any acceptor, I shall not attempt to explain it.

It seems reasonable to postulate that the location of one of the non-cyclic phosphorylation sites is before (or somewhere near) the pool A of Kok. However, the data do not tell if the phosphorylation is coupled to the thermochemical processes of the dark reactions between the two photosystems or is due to the photoacts. We have so little knowledge of the electron transfer steps between water and photosystem II that it is impossible to form an estimate of the plausibility of a phosphorylation site in this region (Figures 17b and c). The data could be easily understood in terms of photoacts driving phosphorylation. Indeed Witt, et al., (1969) and others have so proposed. In their thinking phosphorylation is driven by a pH gradient resulting from a membrane potential which is generated by the primary photochemical charge separation (i.e., chemiosmotic hypothesis). On the other hand, the chemical coupling theory points out that the potential drop between Q and cytochrome-f is barely sufficient to produce 2 molecules of ATP with the transfer of a pair of electrons. If the stoichiometry is such that the transfer of a larger number of electrons, say 3 electrons, is required for the formation of an ATP molecule, this potential drop would probably suffice. (In fact, the actually observed P/e_2 ratio is $\leq$ 1.3.) However, it is beyond the scope of this paper to try to assess the plausibility and implausibility of the above two opposing theories.

The so-called cyclic photophosphorylation is obscure. Clearly "cyclic" electron transport is not a necessity since some phosphorylation with net electron transport seems to have almost identical characteristics. "Cyclic phosphorylation" therefore, would be better described as "system I phosphorylation". Figure 17 illustrates some of the possible electron transport pathways which could account for system I phosphorylation. In these models PMS or pyocyanine is reduced by photosystem I.

Figure 17 (a) which has the phosphorylation driven by the photoacts would easily explain the inhibition data and many other observations. The reduced PMS and pyocyanine or DAD or DCPIPH_2 reduce cytochrome-f or plastocyanin. Electron transport is inhibited by KCN which inactivates the plastocyanin. Higher concentrations of reduced PMS react directly with P700 so that the associated phosphorylation is not inhibited by KCN.

Figure 17 (b) has two phosphorylation sites one of which is involved in cyclic phosphorylation and both of which are involved in non-cyclic phosphorylation. It has yet another site which is used only when high PMS concentrations are present. Since the rate of phosphorylation with high

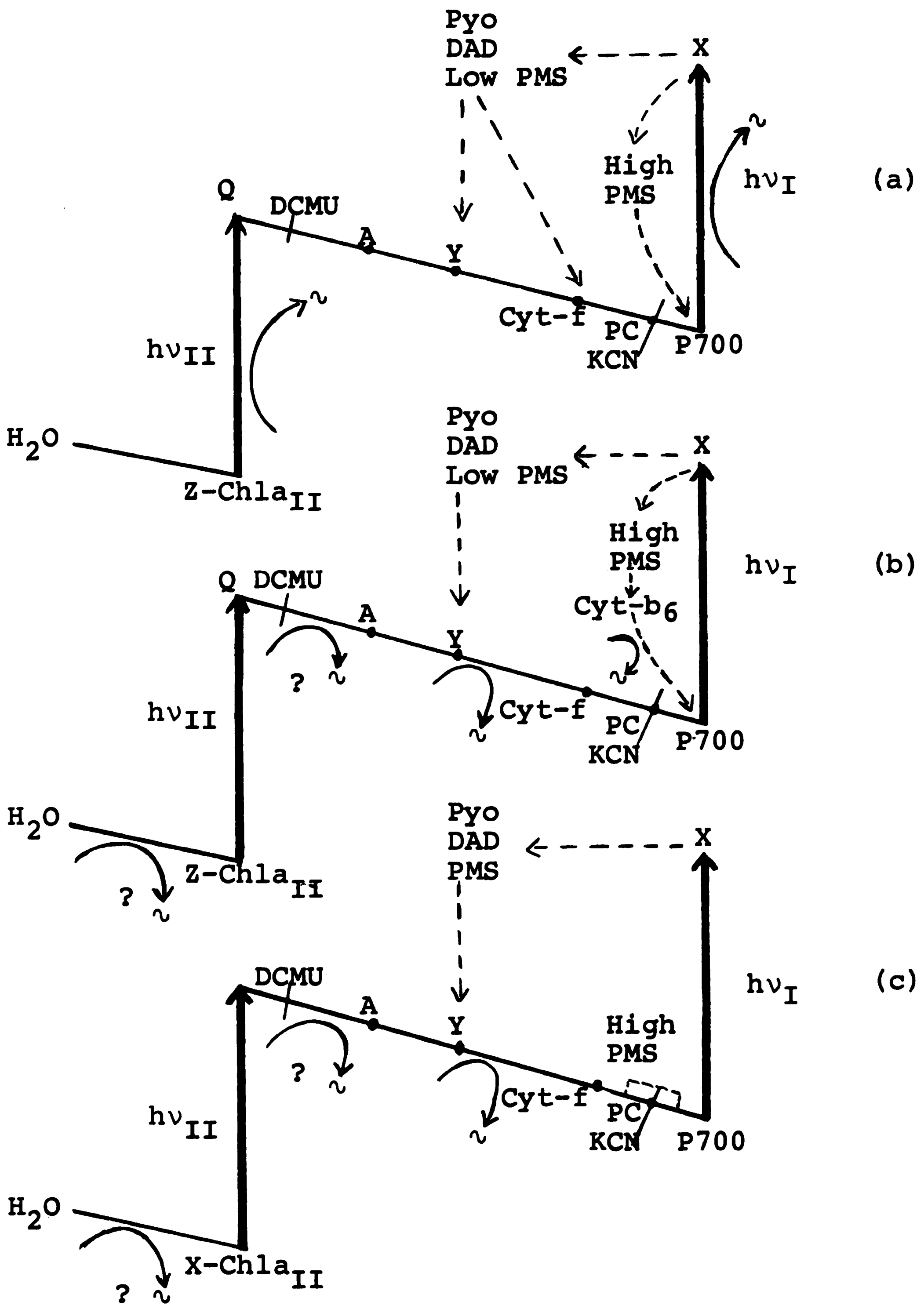


Figure 17. The scheme for non-cyclic and cyclic electron transport pathway with the possible phosphorylation sites.
The reaction A--->Y is assumed to be the rate-determining step in the non-cyclic pathway.

PMS + KCN is virtually the same as with low PMS and no KCN, it seems rather improbable that a totally different site is involved. It also seems unreasonable to postulate a site of phosphorylation simply to account for the characteristics of one set on unusual condition--high concentration of an artificial electron transport catalyst. Nevertheless, varieties of this model have been proposed by many investigators. In this model and in the model of Figure 17 (c), reduced pyocyanine, low concentrations of reduced PMS and DAD all donate electrons before the second site of phosphorylation. The phosphorylation electron transport is therefore sensitive to KCN. In Figure 17 (c) high PMS concentrations are considered to by-pass the block produced by the reaction of KCN with plastocyanin.

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