PHOTOPHOSPHORYLATION IN CHLOROPLASTS AND IN DIGITONIN SUBCHLOROPLAST PARTICLES

A thesis submitted to the
University of Adelaide
as a requirement for the degree of

DOCTOR OF PHILOSOPHY

by

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ADDENDUM

In experiments described in Fig.II, Chapter III and Table IV, Chapter VIII, ^{32}Pi equivalent to 31,000 and 41,000 cpm respectively were added to the reaction mixtures.

CONTENTS

SUMMARY

1. Chloroplasts were isolated from pea (*Pisum sativum* var. Green Feast) and spinach (*Spinacea oleracea* L.) leaves. The chloroplasts exhibited photosynthetic control with ferricyanide (FeCN) and methyl viologen (MV) as electron acceptors, giving control ratios ranging from 3.0-6.0. ADP/O ratios were also determined, and ranged from 1.3-1.9 with FeCN and MV.

2. Using the quinone analog, 2,5,dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) as an inhibitor of non-cyclic electron flow, it was possible to determine the sites of FeCN reduction on the photosynthetic electron flow chain. Two sites of FeCN reduction are postulated, one phosphorylating and the other non-phosphorylating. From this, it was deduced that the energy transducing sites are located between the two sites of FeCN reduction.

3. Phenazine methosulphate (PMS) is generally accepted as being a cyclic electron acceptor. However, it was found to catalyze a light-induced oxygen uptake under red light illumination which was DCMU sensitive . It also catalyzes aerobic phosphorylation, under red light illumination, and this was completely inhibited by DCMU. These observations indicate that PMS was catalyzing pseudo-cyclic electron flow. True cyclic phosphorylation could

be observed in the presence of DCMU, when an exogenous reductant (e.g. ascorbate or NADH) was present to reduce PMS non-enzymically. It was suggested that PMS can catalyze both cyclic and pseudocyclic phosphorylation simultaneously, as $P/2e^-$ determinations, gave values above 2.0

4. The effect of some inhibitors of electron flow and uncouplers of photophosphorylation on the various photochemical activities of chloroplasts and digitonin subchloroplast particles were studied, and their sites of action determined as much as possible.

5. Subchloroplast particles were prepared by treatment with digitonin. Heavy particles (10,000 and 30,000 xg) which contained only Photosystem II and light particles (50,000 and 14,000 xg) with Photosystem I activity only were obtained. The Photosystem I particles, although exhibiting no light-induced proton uptake activity, were capable of catalyzing cyclic photophosphorylation with PMS, at rates comparable to whole chloroplasts. The effect of uncouplers on cyclic photophosphorylation in these particles was studied and the relevance of these results to the chemiosmotic hypothesis is discussed.

6. An attempt was made to characterise and study the light-induced redox changes of the cytochromes present in chloroplasts, Photosystem II and Photosystem I subchloroplast particles.

7. There is controversy concerning phosphorylation accompanying electron flow from $DCPIPH_2$ to MV or NADP in the presence of DCMU. This arises from the effects of uncouplers and cyclic photophosphorylation which may accompany the non-cyclic electron flow. Thus, electron flow from $DCPIPH_2$ to MV in chloroplasts and Photosystem I particles has been reinvestigated. Although there is no doubt that non-cyclic electron flow is stimulated by uncouplers any ATP formed may be a result of simultaneous cyclic phosphorylation. A possible explanation of the effects of uncouplers on non-cyclic flow is proposed.

DECLARATION

The investigations described in this thesis were carried out in the Department of Botany, University of Adelaide, from May 1969 to September 1972. One paper was written and three more are in preparation:

1. Light-induced redox changes of cytochrome b_{559}: by J.M. Anderson, Than-Nyunt and N.K. Boardman (in press, Arch. Biochem. Biophys.)
2. Phenazine methosulphate as a pseudo-cyclic and cyclic electron acceptor: by Than-Nyunt and J.T. Wiskich (manuscript in preparation).
3. Phosphorylating and non-phosphorylation sites of ferricyanide reduction by isolated pea chloroplasts: by Than-Nyunt and J.T. Wiskich (manuscript in preparation).
4. Studies on the effects of 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone on isolated pea chloroplasts: by Than-Nyunt and J.T. Wiskich (manuscript in preparation).

To the author's belief and knowledge, this thesis contains no material previously submitted for a degree in any University by the author or by any other person, except where due reference is made in the text.

Than-Nyunt

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ABBREVIATIONS

ADP	Adenosine diphosphate
As	Arsenate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
CCCP	Carbonyl cyanide-m-chlorophenylhydrazone
CRS	Cytochrome c reducing substance
D_{50},D_{144}	Digitonin 50,000 and 144,000 xg fractions
DAD	Diaminodurene
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCMU	3-(3^1,4^1-dichlorophenyl)-1,1-dinethylurea
DCPIP,$DCPIPH_2$	Oxidised and reduced 2,6-dichlorophenol-indophenol
DNP	2,4,Dinitrophenol
DPC	Diphenylcarbazide
E'o	Oxidation-reduction potential
EGTA	Ethylene glycol-bis-(β-anino-ethyl ether) N,N'-tetra-acetic acid
FCCP	Carbonyl cyanide-p-trifluromethoxyphenyl hydrazone
FeCN	Ferricyanide
FMN	Flavin mononucleotide
FRS	Ferredoxin reducing substance
HEPES	N-2-Hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HOQNO	2-Alkyl-4-hydroxyquinoline-N-oxide

MES	2(Morpholino) ethane sulphonic acid
MV	Methyl viologen
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NOQNO	2-Noryl-4-hydroxyquinoline-N-oxide
Pi	Inorganic orthophosphate
PMF	Protonmotive force
PMS	Phenazine methosulphate
PSI,PSII	Photosystems I and II
SCP	Subchloroplast particles
TCPIP	2,3,6-Trichlorophenol-indophenol
TES	N,Tris (hydroxymethyl) methyl-2-amino ethane sulphonic acid
TMPD	N,N,N',N'-tetra methyl-p-phenylenediamine
TRICINE	N,Tris (hydroxy methyl) methyl glycine
TRIS	Tris (hydroxymethyl) amino methane

CHAPTER I

GENERAL INTRODUCTION

A. GENERAL BACKGROUND

Photosynthesis may be broadly defined as the utilisation of solar energy by plants and photosynthetic bacteria for synthesising carbohydrates. In green plants, the known enzymatic reactions concerned with the actual synthesis of carbohydrates are driven by ATP and reduced nicotinamide adenosine dinucleotide phosphate ($NADPH_2$). The pathway for the reduction of CO_2 into carbohydrates, commonly known as the Calvin cycle, was elucidated by Calvin and his associates (1957) in *Chlorella*, and requires the photochemical production of four equivalents of reducing agents ($2NADPH_2$) and 3 equivalents of a molecule supplying energy (3ATP) for one molecular equivalent of CO_2. Since the chloroplasts of photosynthetic cells contain all the photosynthetic pigments, and were observed to produce starch and oxygen upon illumination (see Arnon, 1966), it was thought for many years that photosynthetic reactions in green plants were entirely located in chloroplasts. However, this view was largely abandoned after Hill (1939) showed that isolated chloroplasts could evolve oxygen but could not assimilate CO_2. When the very sensitive $^{14}CO_2$ technique became available (Ruben and Kamen, 1940, 1941), the assimilation of CO_2 by chloroplasts was reinvestigated, but again without success (Fager, 1952, 1954). However, Arnon, Allen and Whatley (1954),

using different experimental techniques, were able to observe the reduction of CO_2 to the level of carbohydrates, with a simultaneous evolution of oxygen, by spinach chloroplasts under illumination. This was later confirmed by other workers in this field (Gibbs and Cynkin, 1958 and Tolbert, 1958). In these early experiments, isolated chloroplasts fixed CO_2 at very low rates. Improvement in the observed rates of fixation came when chloroplasts were isolated rapidly, and in medium more complex than the simple salt media previously used (Walker, 1964). It was also observed that addition of some intermediates of the carbon cycle stimulated the rates of CO_2 fixation (Walker, 1964, 1965). Subsequently, rates comparable to that of the intact leaf were obtained (Bucke, Walker and Baldry, 1966 and Jensen and Bassham, 1966). The latter authors obtained rates of up to 155μmoles CO_2 fixed/mg chll/hr without the addition of any intermediates of the carbon cycle. The isolated chloroplasts used to obtain high rates of CO_2 fixation had their outer membrane intact. The importance of an intact outer membrane and the composition of the isolating medium, to obtain high rates of CO_2 fixation with isolated chloroplasts, is discussed by Walker and Crofts (1970).

At the same time isolated chloroplasts were used to study the reactions which generate the energy rich compounds that are formed prior to, and are essential for, the conversion of CO_2 into organic compounds. Light-induced ATP formation was discovered by Frenkel (1954) in bacterial chromatophores, and independently in chloroplasts

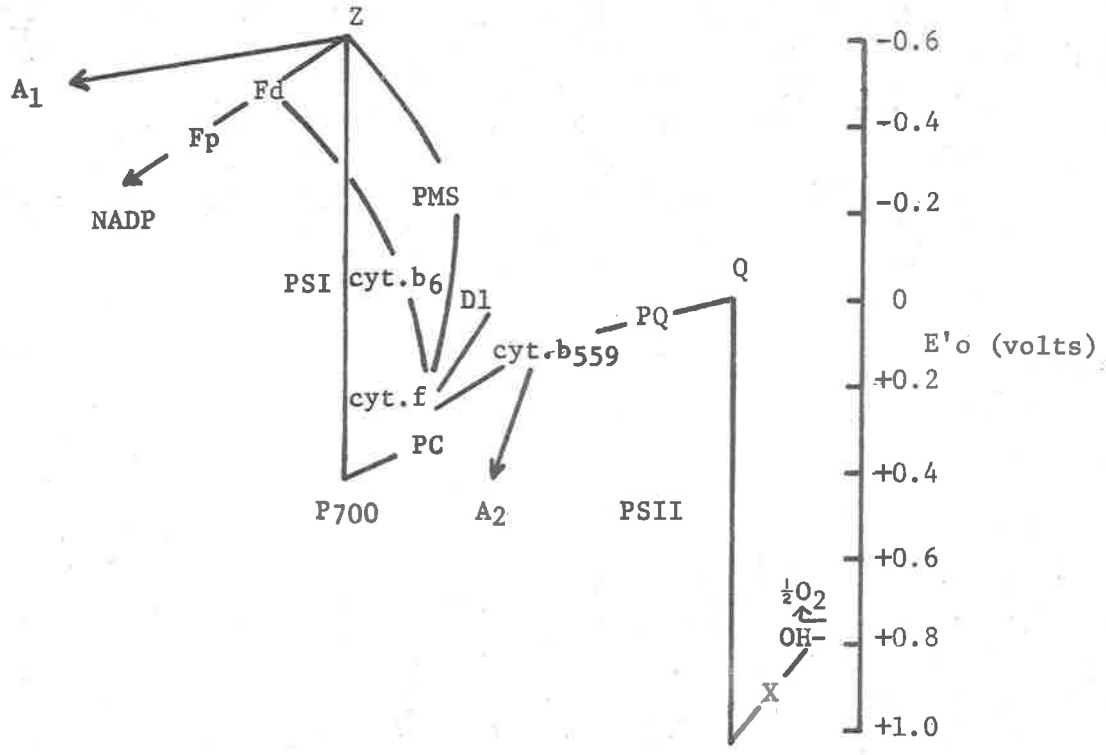


FIGURE I. Scheme for light-induced electron flow in chloroplasts. Artificial electron acceptors (A_1,A_2) receive electrons after PSII or after PSI. Artificial electron donors (D_1) feed electrons into PSI. PQ-plastoquinone, PC-plastocyanin, Fd-ferredoxin, Fp-ferredoxin-NADP reductase (Boardman, 1968).

by Arnon and his associates (1954). The latter authors referred to this process as photosynthetic phosphorylation, to distinguish it from the respiratory or oxidative phosphorylation which occurs in mitochondria. The discovery of photosynthetic phosphorylation was later confirmed and extended to other photosynthetic organisms (see also Introduction of Chapter III).

B. THE PHOTOSYNTHETIC ELECTRON FLOW CHAIN

It is generally accepted that photosynthesis in higher plants and algae involves the operation of two photochemical systems catalyzed by light (Hill and Bendall, 1960; Duysens and Amesz, 1967 and Boardman, 1968). Fig.I outlines such a scheme (Boardman, 1968). Quanta of light energy absorbed by pigments in Photosystem II are transferred to the trapping pigment. Electrons from water are transferred through 'X' to 'Q', which are respectively the primary oxidant and primary reductant of Photosystem II. From 'Q', electrons are transferred down a potential gradient through the various electron carriers to pigment P_{700}. Energy absorbed by Photosystem I is used to transfer electrons from P_{700} to 'Z', the primary reductant of this photosystem. Reduction of NADP by electrons from 'Z' occurs via ferredoxin and the flavoprotein, ferredoxin-NADP reductase. Support for the 'Z' scheme has come from enhancement studies with isolated chloroplasts. It was found that the rate of NADP reduction was greater when 714nm light

was supplemented by a background light of 650nm, than when 714nm light was used alone (Govindjee, Govindjee and Hoch, 1964). A similar enhancement effect was observed in the NADP and cytochrome c reduction by lettuce chloroplasts, by Avron and Ben-Hayyim (1969). Further support came from the physical separation of the two photosystems by digitonin (Boardman and Anderson, 1964 and Anderson and Boardman, 1966) to yield Photosystem II and Photosystem I particles. The former particles were capable of Hill activity with trichlorophenol indophenol (TCPIP) or ferricyanide (FeCN) as electron acceptor, but rates of NADP reduction were considerably lower. The Photosystem I particles displayed no Hill activity, but reduced NADP when supplied with an artificial electron donor and ferredoxin, ferredoxin-NADP reductase. This indicated that the co-operation of both photosystems were needed to reduce NADP.

I. Components of the photosynthetic electron flow chain

(a) 'Q' - the primary reductant of Photosystem II

As shown in Fig.I, 'Q' is closely associated with Photosystem II. Upon illumination of oxygen-evolving organisms with actinic light mainly absorbed by Photosystem II, an increase in fluorescence of chlorophyll was observed. When strong actinic light mainly absorbed by Photosystem I was superimposed, the high fluorescence yield was rapidly decreased to approximately its original level. These observations led Duysens and Sweers (1963) to postulate that

the fluorescence changes were brought about by the oxidation-reduction state of an intermediate located between the two photosystems. 'Q' was thought to be a quinone, such as plastoquinone (PQ) or a related compound (Duysens and Sweers, 1963), but spectral changes attributable to the oxidation or reduction of plastoquinone were not correlated with the chlorophyll fluorescence changes. In the presence of DCMU, the reduction of 'Q' (increase in chlorophyll a fluorescence) still occurs, but not its oxidation by Photosystem I light (Duysens and Sweers, 1963). On the other hand, the reduction by Photosystem II but not the oxidation by Photosystem I, of plastoquinone was inhibited by DCMU (Amesz, 1964) indicating that PQ was not identical to 'Q'. Kok and Cheniae (1966) equilibrated chloroplasts at various redox potentials using ferrocyanide-ferricyanide mixtures, and from the variation of the fluorescence rise with redox, they estimated the E'_o of 'Q' to be +180mV.*

(b) Plastoquinone

The first evidence for plastoquinone A functioning in photosynthesis was from the studies of Bishop (1959) with heptane-extracted chloroplasts. Hill activity in these extracted chloroplasts were restored on addition of the lipid fraction, which was shown to contain plastoquinone A. Vernon and Zaugg (1960) showed that NADP reduction by DCPIP plus ascorbate was not affected by the removal of plastoquinone A. Therefore,

*Footnote: Knaff and Arnon (1969) reported a new chloroplast component, C-550, which was photoreduced by PSII, and this was later confirmed (Erixon and Butler, 1971a). Erixon and Butler (1971b) also observed that when C-550 was in the reduced state, the fluorescence yield was high and that the low temperature photooxidation of cytochrome b_{559}, which could serve as an electron donor for C-550 reduction was inhibited. Therefore, they suggested that C-550 may be equivalent to 'Q', the primary reductant of PSII.

plastoquinone A must function at a site preceding the point of electron donation by DCPIP plus ascorbate, probably near Photosystem II. Other evidence for the involvement of plastoquinone in electron flow was provided by the light-induced redox changes of PQ in isolated chloroplasts. Klingenberg, Muller, Schmidt-Mende, and Witt (1965) observed that PQ was reduced by red light and re-oxidised in the dark. When PQ was extracted from chloroplasts, no redox changes were observed, but on addition of synthetic plastoquinone A (PQ-A) the redox changes were restored (Stiehl and Witt, 1968). Indirect evidence which throws light on the location of PQ on the electron flow chain comes from the studies of Bohme and Cramer (1971). They observed that the quinone analog, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) inhibited the reduction of cytochrome f by Photosystem II light and the oxidation of cytochrome b_{559} by Photosystem I light. These inhibitions were reversed upon the addition of PQ-A, indicating that it mediates electron flow from cytochrome b_{559} to cytochrome f.

(c) b-type cytochromes

Hill and Bendall (1960) located cytochrome b_6 close to Photosystem II. On the other hand, Arnon (1967) placed it in a cyclic electron flow pathway near Photosystem I. Rumberg (1965) observed that cytochrome b_6 was reduced by Photosystem II light and oxidised in the dark, therefore he concluded that electrons were transferred from

cytochrome b_6 to cytochrome f. He later modified his conclusion and placed cytochrome b_6 on a cyclic pathway instead of in series with cytochrome f, to account for the observation that cytochrome f was reduced more quickly than cytochrome b_6 was oxidised (Rumberg, 1966). Hind and Olson (1966) also observed the reduction of cytochrome b_6 by far red (Photosystem I) light. (See also Chapter VII for a more detailed discussion).

(d) Cytochrome f

Hill and Scarisbrick (1951) first reported the presence of cytochrome f in chloroplasts, and it has since been shown that it is photooxidised by far red light and reduced by red light (Duysens and Amesz, 1962 and Avron and Chance, 1966). In *Chlamydomonas reinhardii*, the reduction of NADP by water was not observed when either cytochrome$_{553}$ (cytochrome f) or plastocyanin were absent (Gorman and Levine, 1966). Therefore it appears that cytochrome$_{553}$ (cytochrome f) and plastocyanin lie in a series in the electron flow chain of *Chlamydomonas reinhardii*. However, Kok (1963) observed that plastocyanin was photooxidised as rapidly as cytochrome f, suggesting that both can interact directly with P_{700}. A similar conclusion was reached by Hind and Olson (1966), when they observed that reoxidation of cytochrome b_6 upon far red light illumination did not lead to the reduction of cytochrome f.

(e) Plastocyanin

This was first isolated by Katoh (1960) from *Chlorella*. Later, Katoh, Suga, Shiratori and Takamiya (1961) showed that several species of higher plants contained it and that it was localised in the chloroplast. Plastocyanin is necessary for NADP reduction by artificial electron acceptors in sonicated chloroplasts (Katoh and Takamiya, 1965), and in Photosystem I particles (Vernon, Shaw and Ke, 1966 and Wessels, 1966). Evidence for the location of plastocyanin near P_{700} comes from the studies of Vernon, Ke and Shaw (1967) and Hind (1968) with plastocyanin-depleted systems. The former authors showed that plastocyanin was required for rapid turnover of P_{700} and stimulated the dark reduction of P_{700} in the presence of DCPIP and ascorbate. Hind showed that oxidation of cytochrome f by Photosystem I was stimulated by plastocyanin in Triton X-100 treated chloroplasts. However, Kok, Rurainski and Harmon (1964) and Elstner (1969) thought that both plastocyanin and cytochrome f could serve as electron donors to Photosystem I.

(f) P_{700}

The presence of P_{700} was first observed by Kok (1956), in spinach chloroplasts and algae. He later partially purified it from chloroplasts mostly devoid of chlorophyll and determined its redox potential (Kok, 1961). Titration with ferrocyanide-ferricyanide

mixtures indicated that it was a single electron carrier having a E'_o of approximately +0.43mV. It was thought to be a specially modified chlorophyll a. This conclusion was also reached by Rumberg and Witt (1964) and Witt, Rumberg, Schmidt-Mende, Siggel, Skerra, Vater and Weikard (1965).

(g) 'Z' - the primary acceptor of Photosystem I.

The existence of the primary electron acceptor (a strong reductant) in Photosystem I, has been known for several years, but it has not yet been isolated. Arnon (1965) proposed that ferredoxin could fulfil this role, after finding that it had a redox potential approximately equal to the hydrogen electrode (Tagawa and Arnon, 1962). A similar conclusion was reached by Kassner and Kamen (1967). Malkin and Bearden (1971) have reported an electron paramagnetic resonance signal characteristic of reduced ferredoxin at 25°K, after illumination at 77°K. This signal was observed in chloroplasts from which soluble ferredoxin had been removed. Consequently these authors suggested that chloroplasts contain a bound ferredoxin that may serve as the primary acceptor. However, calculations of the redox potential of the primary acceptor by Kok, Rurainski and Owens (1965); Zweig and Avron (1965) and Black, Jr. (1966) have values between -0.5V and -0.7V. A substance which was required for the reductions of NADP and MV was isolated from spinach; this was called "ferredoxin

reducing substance", (FRS) and was thought to be the primary acceptor (Yocum and San Pietro, 1969). A similar substance called the "cytochrome c reducing substance" (CRS) had been reported earlier by Fujita and Murano (1967). They observed that treatment with polar solvents, which solubilised CRS drastically inhibited NADP reduction by $DCPIPH_2$. Therefore, they suggested that CRS could be the primary reductant of Photosystem I. Immunological studies with a water soluble substance isolated from lyophilised chloroplasts after treatment with diethyl ether, indicated that the substance could be similar to CRS and FRS (Regitz, Berzborn and Trebst, 1970). A spectral component (P430) with a band around 430nm and kinetically different from P_{700} was observed by Hiyama and Ke (1971 a,b). They found that an artificial dye (TMPD) was photo-reduced by P430, and that photooxidised P_{700} was directly reduced by the reduced dye ($TMPDH_2$). These authors thought that P430 could be the primary electron acceptor of Photosystem I. The E'_o of P430 was estimated by them to be about -0.5V.

II. Photosynthetic phosphorylation

Three types of photophosphorylation are known, namely, cyclic, non-cyclic and pseudo-cyclic.

(a) Cyclic photophosphorylation

This type of phosphorylation does not involve the production or consumption of oxygen. Since ATP formation is coupled to a cyclic flow of electrons, no net change in exogenous electron donor or acceptor occurs. Certain redox cofactors can catalyze cyclic phosphorylation (e.g. phenazine methosulfate, pyocyanine or $DCPIPH_2$) but these are non-physiological catalysts. However, Arnon (1966) thought that ferredoxin was the endogenous electron acceptor in cyclic phosphorylation

(b) Non-cyclic photophosphorylation

In contrast to cyclic photophosphorylation, this type of photophosphorylation is stoichiometrically coupled with light-induced electron flow from water to NADP (or FeCN) and the concomitant evolution of oxygen. Moreover, electron flow in this system is "controlled" by phosphorylation, so that under phosphorylating conditions the rate of electron flow is greatly increased.

(c) Pseudocyclic photophosphorylation

Pseudocyclic photophosphorylation depends on the presence of molecular oxygen and involves the reduction of an autooxidizable electron acceptor which is re-oxidised by the oxygen evolved during electron flow. Therefore, there is no net change in the

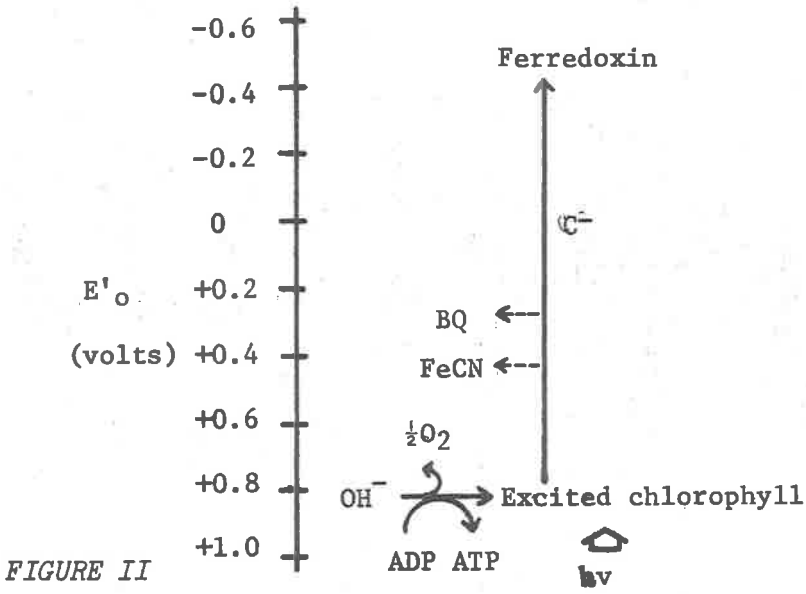


FIGURE II

(a) Scheme for non-cyclic photophosphorylation in chloroplasts. BQ - benzoquinone.

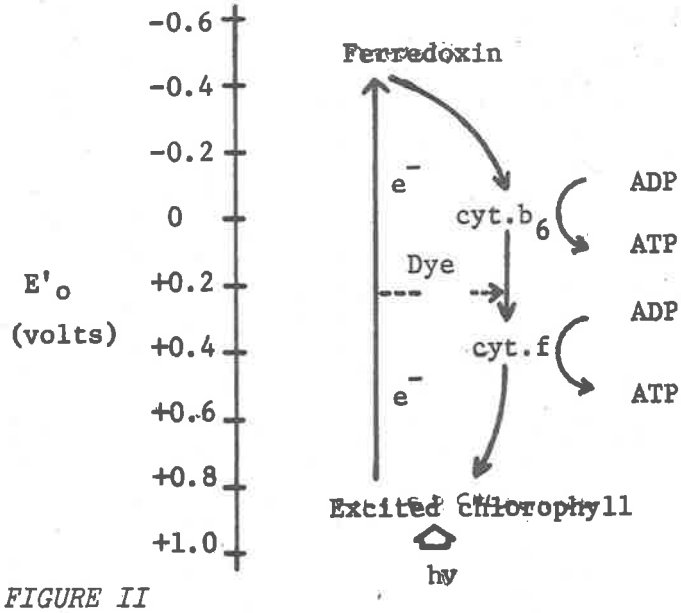


FIGURE II

(b) Scheme for cyclic photophosphorylation in chloroplasts (Arnon, 1967).

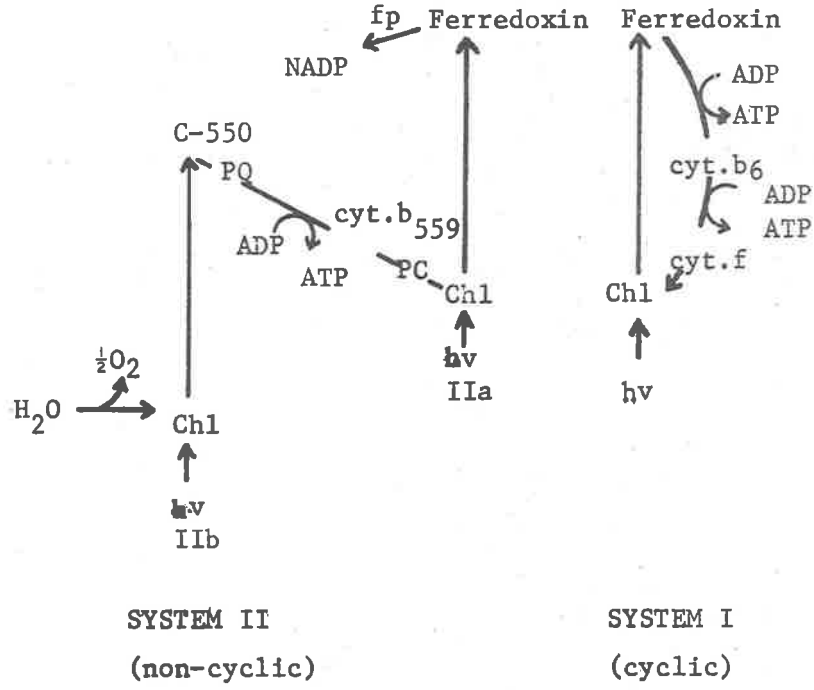


FIGURE III. Scheme for three light reactions in plant photosynthesis. System II consists of two "short wavelength" light reactions (IIb and IIa) operating in series and linked by a "dark" electron transport chain associated with non-cyclic phosphorylation. Parallel to System II is System I, consisting of a "long wavelength" light reaction linked to another dark electron transport chain associated with cyclic phosphorylation. PQ-plastoquinone, PC-plastocyanin, fp-ferredoxin-NADP reductase (Knaff and Arnon, 1969).

amount of electron acceptor and the net effect is equivalent to cyclic photophosphorylation. However, if the endogenous catalase activity of the chloroplasts were inhibited by azide or cyanide, there would be an uptake of oxygen. This reaction, in which oxygen is the electron acceptor, is often called the Mehler reaction (Mehler, 1951).

Arnon (1967) proposed an alternative scheme in which Photosystem II is involved in non-cyclic electron flow from water to NADP and incorporating non-cyclic phosphorylation, whereas Photosystem I was involved only in cyclic phosphorylation (Fig.II). Later, Knaff and Arnon (1969) modified this hypothesis and forwarded a concept involving three light reactions. Photosystem II in this new scheme is comprised of two, rather than one, short-wavelength light reactions (IIa and IIb), which operate in series and are linked by an electron flow chain which includes C_{550}, cytochrome b_{559} and plastocyanin. Parallel to Photosystem II is situated the long-wavelength light reaction of Photosystem I (Fig.III).

At the present time, experimental data favours the 'Z' scheme (Boardman, 1968 and Hind and Olson, 1968), whereas objections could be raised to Arnon's scheme. Levine (1969) observed that red and far red light have antagonistic effects on the redox state of cytochrome f and P_{700}, but these effects are lost when these

components have been affected by mutation. He concluded that 'Q', cytochrome b_{559}, cytochrome$_{553}$ (cytochrome f) and P_{700} must lie on the non-cyclic electron flow chain from water to NADP and discounted the simple scheme that contains chlorophyll, ferredoxin, ferredoxin-NADP reductase and NADP as its components as envisaged by Arnon (1967). It is thermodynamically possible for an electron from water to be moved to the level of ferredoxin with a single quantum of light (Ross and Calvin, 1967), but not with a simultaneous phosphorylation as proposed by Arnon (1967) because of the generally accepted low quantum efficiency of photosynthesis (Duysens, 1958 and Kok, 1966).

C. THE ENERGY CONSERVATION HYPOTHESES

It is now well established that electron flow in chloroplasts and mitochondria is coupled to phosphorylation. The mechanism by which these two processes are coupled is still unknown, but four hypotheses have been proposed to explain this coupling.

I. The chemical hypothesis (Slater, 1953) and its variants (Chance and Williams, 1956 and Lehninger, Wadkins, Cooper, Devlin and Gamble, 1958) proposed that a substance C (X or I) combines with the electron carrier during oxidation-reduction reactions to form a high energy compound A~C which subsequently undergoes phosphorylsis in the presence of ADP and Pi to form ATP as summarised below:

$$AH_2 + B + C \longrightarrow A\sim C + BH_2 \ldots\ldots\ldots (1)$$

$$A\sim C + Pi + ADP \longrightarrow ATP + A + C \ldots (2)$$

Although it is not required that respiratory carriers be phosphorylated, the theory does invoke some direct participation of respiratory catalysts in the formation of 'high-energy' intermediates, (Pullman and Schatz, 1967). No evidence is yet available that suggests such intermediates do exist. However, Slater, Lee, Berden and Wegdam (1970 a,b) recently reported the presence of two high-energy forms of cytochrome b which are joined to the same ligand X in phosphorylating submitochondrial particles. They suggested that this dimer is linked to ATP synthesis.

The mechanisms whereby A∿C, in the presence of Pi and ADP, is converted to ATP still remains unknown. Equation (2) is usually considered to consist of two partial reactions (Pullman and Schatz, 1967), involving the formation of a phosphorylated intermediate C∿P.

$$A\sim C + Pi \longrightarrow C\sim P + A \ldots\ldots\ldots (3)$$

$$C\sim P + ADP \longrightarrow ATP + C \ldots\ldots\ldots (4)$$

The search for such an intermediate has been intensive (see Chance, Lee and Mela, 1967 for a list of proposed intermediates), and the failure to isolate one could be because it would probably occur in low concentration, would probably be membrane bound and might well be highly labile, especially in aqueous media (Greville, 1969).

II. The second hypothesis was proposed by Boyer (1965). According to this hypothesis, energy made available by electron flow is conserved as a conformational change in the protein of a respiratory carrier; this change is then in some manner coupled to a phosphorylation reaction.

III. The chemiosmotic theory (Mitchell, 1961, 1966) originated from the suggestions of Lundegardh (1945) and Robertson and Wilkins (1948), from studies on salt accumulation by plant tissues, and Davies and Ogston (1950) from studies on acid secretion by gastric mucosa; that charge separation across membranes could be related to phosphorylation (Robertson, 1960 and Davies, 1961). However, these ideas on coupling mechanisms in oxidative phosphorylation were not generally accepted, largely because (apart from other difficulties) they failed to eliminate the necessity for a chemical coupling step between electron transport and phosphorylation (the synthesis of ATP would presumably have to be chemically coupled to the electron transport system (Mitchell, 1966).

Mitchell's (1966) hypothesis has four basic requirements:

1. A membrane bound reversible ATPase which translocates protons across mitochondrial and chloroplast membranes.
2. The electron transport chain of mitochondria and chloroplasts must be arrayed in such a way that protons are translocated during electron flow (i.e. a vectoral arrangement of chain members).

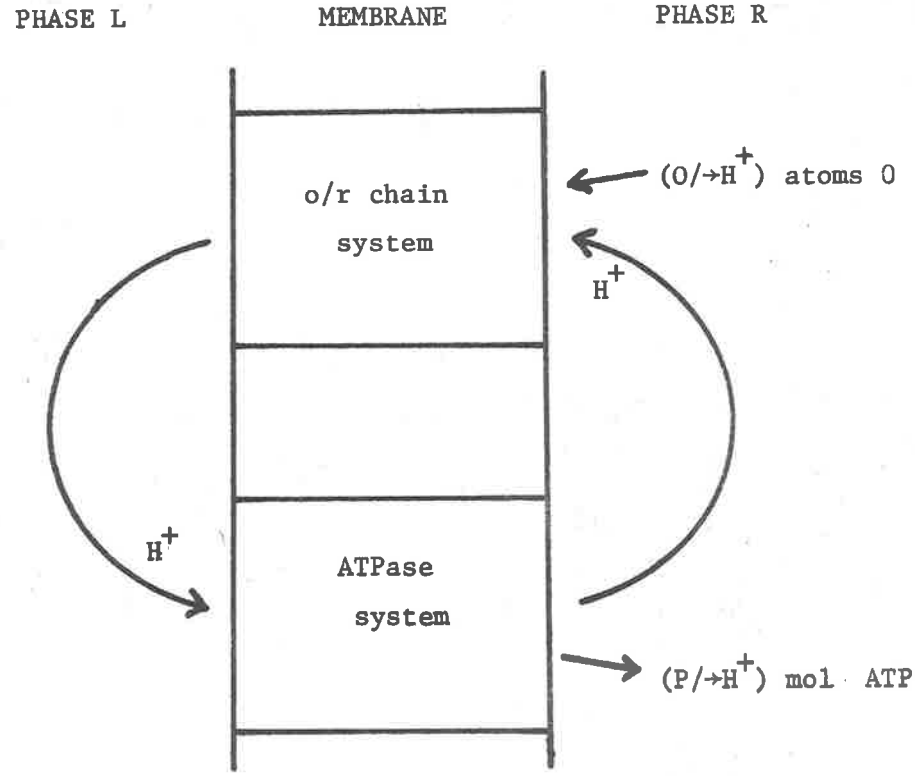


FIGURE IV. Stoichiometry of chemiosmotic coupling. The circulation of one proton is caused by the utilization of a certain number ($O/\rightarrow H^+$) of oxygen atoms, and causes the synthesis of a certain number ($P/\rightarrow H^+$) of ATP molecules. The P/O quotient is the product ($P/\rightarrow H^+$) x ($\rightarrow H^+/O$) (Mitchell, 1966).

3. A specific exchange-diffusion system that couples proton translocation to that of anions and cations, across the membrane.
4. That the coupling membranes (containing 1, 2 and 3), which are considered to be the inner membrane (cristae) of the mitochondria and the thylakoid membrane of chloroplasts, are permeable to water and virtually impermeable to protons and most other ions.

The chemiosmotic hypothesis can be summarised in Fig.IV. The proton current generated by the proton-translocating oxido-reduction reactions of the respiratory or photosynthetic electron transport chain, is considered to drive the reversible anisotropic ATPase (hydrodehydration system). According to the hypothesis, the region(s) of energy transduction in the respiratory or photo-synthetic electron transport system, corresponds to the region(s) of folding into proton-translocating oxido-reduction loop(s).

Since the membrane is impermeable to ions (4), the translocation of protons across the membrane creates not only a proton gradient, but also an electrical gradient (i.e. a membrane potential). Mitchell refers to this as the proton motive force (PMF).

A rise in pH was observed when unbuffered or weakly buffered suspensions of chloroplasts were illuminated, indicating the inward translocation of protons (Jagendorf and Hind, 1963 and Neumann and

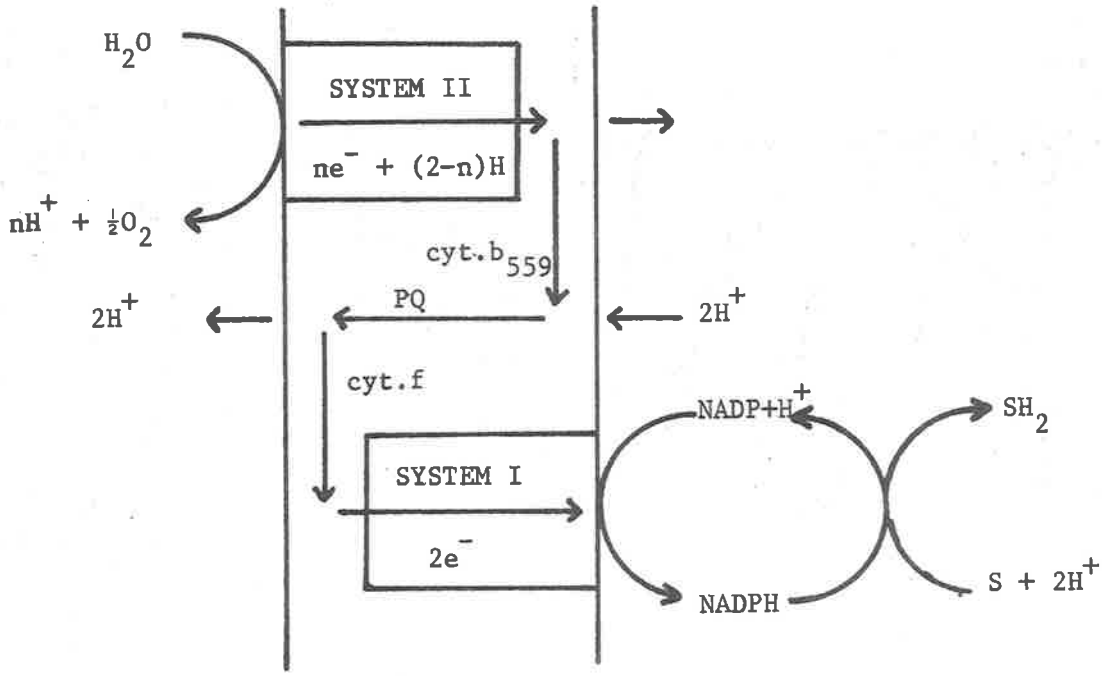


FIGURE V. Possible proton translocating oxido-reduction system for non-cyclic photophosphorylation in chloroplasts, giving $\rightarrow H^+/2e^- = 4$, 3, or 2, corresponding to $P/2e^- = 2$, 1.5 or 1.0 with ATPase II, according to whether n = 2, 1 or 0 respectively (Mitchell, 1966).

Jagendorf, 1964). Thus, Mitchell (1966) proposed that the electron transport chain in chloroplasts is arranged in such a way that protons are translocated inwards through the phosphorylating thylakoid membrane (Fig.V). The translocation of protons through the phosphorylating membrane is thought to be via one of the components of the electron transport chain which can accept both an electron and a proton. Plastoquinone would fulfil such a role (Friend and Redfearn, 1963), and it is positioned next to cytochrome b_{559} such that it can accept an electron from cytochrome b_{559} and a proton from the outside. On oxidation, it would donate the electron to cytochrome f and release the proton on the inside of the membrane. That is, an alternating chain of electron carriers and proton carriers are required by this hypothesis.

IV. The fourth hypothesis proposed by Williams (1961, 1969), favours the separation in space of the products of reactions, the so-called dislocated reactions in which oxidising (molecular oxygen) and reducing groups (hydrogen atom) are located at opposite ends of the chain and usually transferred through a co-enzyme, so that the spatial relationship between the catalysts should be maintained in the products.

This hypothesis (as applied to mitochondria) requires four basic conditions to be satisfied:

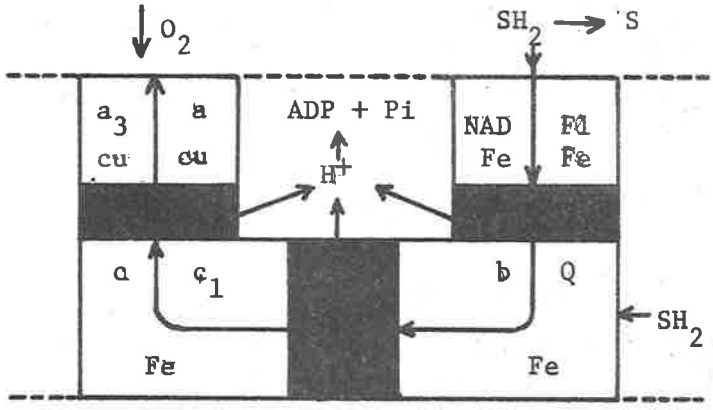


FIGURE VI(a). A diagram showing many of the features of oxidative phosphorylation. Shaded areas are regions of restricted electron transfer. The entire system is in the membrane. The ex t relationship of the different components within each block is not known and could change with conditions (Williams, 1969).

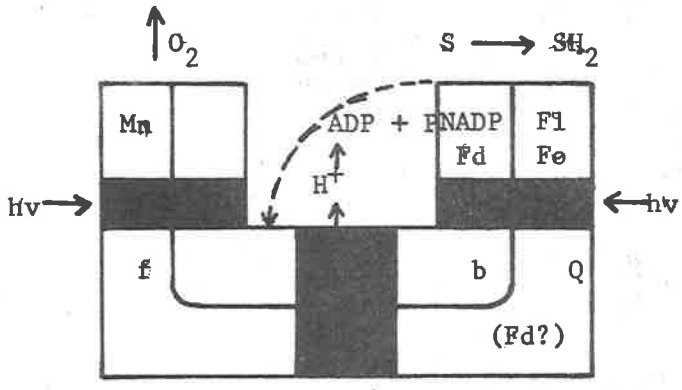


FIGURE VI(b). The photosynthetic electron flow chain in chloroplasts. Shaded areas are the poor electron transfer regions which contain chlorophyll. The broken line indicates cyclic electron flow pathway (Williams, 1969).

1. Electron transport is initiated by the reaction of molecular oxygen with cytochrome oxidase.
2. The electron which is transported comes from the hydrogen atom through the dehydrogenases, separation of electrons and protons occurring where the electron transport chain meets the hydrogen atom transport chain of the dehydrogenases.
3. The electrons must be accepted by molecular oxygen and react with water to form hydroxyl ions.
4. The energy from the oxidation of hydrogen by oxygen is not lost as heat but is consumed in the condensation reaction ADP + Pi + $H^+ \longrightarrow$ ATP.

The generated protons and hydroxyls ions are separated in space, but unlike the chemiosmotic hypothesis, this charge separation is not across the membrane. The proton is assumed to be in high local concentration in the lipid region near the ATPase. This low pH tends to drive the polymerisation of phosphate, with the extraction of water, yielding ATP and H_3O^+. This reaction could not occur if the region near the ATPase is accessible to water or OH^-. Fig.VI summarises the proposed scheme for oxidative phosphorylation.

A scheme for photosynthetic phosphorylation essentially similar to the scheme for oxidative phosphorylation was also proposed (Fig. VIb). The light reactions are separated by an array of electron carriers with a site of phosphorylation located between them. A cyclic electron transport pathway is also indicated which requires electron transport from ferredoxin to cytochrome b.

It is now accepted that there is a connection between phosphorylation and proton translocation. However, the controversy lies in whether the proton flux is a pre-requisite for phosphorylation (Mitchell, 1966 and Williams, 1969), or simply an alternative to phosphorylation (chemical). There seems to be increasing evidence that the proton flux is directly linked to light-induced electron flow in chloroplasts (Schwartz, 1971) and a pH gradient induced by acid-base treatment of chloroplasts has been shown to generate ATP in the dark (Hind and Jagendorf, 1963 and Jagendorf and Uribe, 1966). Mitchell and Moyle (1965, 1967) and Chance and Mela (1966) showed that a proton flux was associated with electron flow in mitochondria.

D. THE PRESENT STUDY

There is considerable controversy as to the number of coupling or energy transducing sites on the photosynthetic non-cyclic electron flow pathway (Avron and Neumann, 1968 and Walker and Crofts, 1970). It was first thought that there was only one site of energy transduction ($P/2e^- = 1$) but recent reports have indicated the presence of an additional site. Previous reports on $P/2e^-$ determinations have used either broken or washed, swollen chloroplasts (Leech, 1963). However, chloroplasts, with their outer membranes intact (Class I, Spencer and Unt, 1965), have been prepared which appear to be controlled by their energetic state, analogous to respiratory control in mitochondria (West and

Wiskich, 1968 and Kraayenhof, Groot and van Dam, 1969). These chloroplasts gave ADP/O ratios (equivalent to $P/2e^-$ ratios) much greater than 1.0 (West and Wiskich, 1968 and 1972 in press). Chloroplasts capable of exhibiting similar photosynthetic control were prepared and used to study photophosphorylation in an endeavour to elucidate the number and location of the energy transducing sites on the non-cyclic electron flow pathway.

Similar chloroplasts were used to study the effects of electron flow inhibitors and uncouplers on photophosphorylation. The action of uncouplers are discussed in terms of the chemiosmotic hypothesis. Their effects are further applied in an effort to resolve the question of phosphorylation during non-cyclic electron flow from $DCPIPH_2$ to MV.

Subchloroplast particles were prepared using a slightly modified method of Anderson and Boardman (1966). Cyclic photophosphorylation in Photosystem I subchloroplast particles was studied in detail to try and further the understanding of the mechanism of photophosphorylation.

Finally, an attempt was made to characterise the cytochromes and study their light-induced redox changes in whole chloroplasts, and in Photosystem II and Photosystem I subchloroplast particles.

CHAPTER II

MATERIALS AND METHODS

A. PLANT MATERIAL

Pea (*Pisum sativum* var. Green Feast) seedlings were grown in Vermiculite and their leaves harvested when they were 2-5 weeks old (depending on the time of the year). Spinach (*Spinacea oleracea* L.) leaves were purchased locally and used within 24 hours (being stored at 3°C in the meantime).

B. ISOLATION OF CHLOROPLASTS

1. *Unwashed chloroplasts*

Freshly harvested pea leaves or locally purchased spinach leaves were chilled at 3°C and homogenised in a Waring blender for approximately 7 sec. in a medium containing 0.4M sucrose, 1mM EGTA; 5mM $MgCl_2$; 40mM TES buffer, pH7.3 and 5mg/ml bovine serum albumin. The homogenate thus obtained was filtered through a layer of "Miracloth" and the filtrate centrifuged at 6000 rpm for 30 sec. in a Sorvall RC2 refrigerated centrifuge. The resultant chloroplast pellet was suspended in 0.4M sucrose; 0.5mM $MgCl_2$ and 30mM Tricine buffer, pH7.5 containing 5mg/ml bovine serum albumin. The whole procedure was carried out at 3°C and the chloroplast suspension kept in ice during subsequent experiments.

2. *Sonication of chloroplasts*

Chloroplasts were broken using an MSE 100W ultrasonic disintegrator. The probe was pre-cooled to 0.5°C and the vessel containing the chloroplast suspension (0.4M sucrose; 0.5mM $MgCl_2$ and 30mM Tricine buffer, pH7.5) was placed in an ice bath to minimise heating effects during disintegration.

3. Washed chloroplasts

The 6000 rpm pellet from (1) was suspended in approximately 50ml of 0.4M sucrose and recentrifuged at 1000 xg for 5 min. This pellet was resuspended in 0.4M sucrose containing 5mg/ml bovine serum albumin.

4. Osmotically shocked chloroplasts and chloroplast fragments

The 6000 rpm pellet from (1) was suspended in 1mM Tris-HCl buffer, pH8.0 and allowed to stand for 10 min. at 4°C. The suspension was then centrifuged at 1000 xg for 5 min. and the resultant pellet resuspended in a medium containing 0.4M sucrose; 0.5mM $MgCl_2$ and 30mM Tricine buffer, pH7.5 and 5mg/ml bovine serum albumin (*chloroplasts*). The supernatant from the above centrifugation was then recentrifuged at 20,000 xg for 10 min. and pellet resuspended in the same resuspending medium as above (*chloroplast fragments*).

C. PREPARATION OF DIGITONIN SUBCHLOROPLAST PARTICLES

Digitonin subchloroplast particles were prepared according to the method of Anderson and Boardman (1966) with a few modifications. The chloroplast pellet obtained by the above method (1) were suspended in a medium containing 0.2M sucrose; 50mM NaCl; 50mM KCl and 10mM Tricine buffer, pH8.0. Solid digitonin or 4% digitonin solution was added to a final concentration of 0.5% and the suspension incubated at 3°C for 30 min. with continuous stirring. The suspension was then diluted 3-fold with the same medium and subjected to differential centrifuging at 1000, 10,000 and 30,000 xg for 10, 10 and 30 min. respectively on the Sorval RC2 centrifuge; 50,000 and 144,000 xg for 30 and 60 min. respectively on the Beckman Spinco L-2 ultracentrifuge using the Ti50 rotor. The pellets were resuspended in a medium containing 0.2M sucrose; 0.5mM $MgCl_2$ and 30mM Tricine buffer, pH7.5. The 50,000 and

144,000 xg pellets were mildly sonicated to fully resuspend them in the medium. Photosystem II activity in these particles was assayed by following FeCN reduction at 420nm or oxygen evolution using a Rank oxygen electrode. Photosystem I activity was measured by following the reduction of NADP by the DCPIP-ascorbate couple (Vernon and Zaugg, 1960) at 340nm. (For details, see under "*Assays*"). Saturating amounts of partially purified Ferredoxin/Ferredoxin-NADP reductase (San Pietro and Lang, 1958) had to be added to accomplish NADP reduction. The 50,000 and 144,000 xg pellets had no Photosystem II activity present in them when assayed by the above methods, and are thus designated Photosystem I particles.

D. OXYGEN EVOLUTION MEASUREMENTS

Oxygen evolution was measured polarographically in a sealed perspex vessel, of varying volume, using a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, England), connected to a 1mV Varian graphic recorder (Model G-14; Varian Associates, Palo Alto, California, U.S.A.). The electrode, incorporated into the base of the reaction vessel is composed of a Ag-AgCl electrode encircling a platinum point which is in immediate contact with a teflon membrane (0.0005"), thus effectively sealing the lower chamber.

E. pH MEASUREMENTS

pH changes were measured according to the method of Chance and Nishimura (1967) using a Philips combined electrode (Model Ca 14/02) connected to a Beckman Research pH meter and recorder. The pH changes were calibrated by adding known amounts of standard acid at the end of each experiment. The vessel used for pH measurements was the same as for oxygen measurements.

During both oxygen and pH measurements the reaction mixture was vigorously stirred with a magnetic stirrer at the base of the vessel. The contents of the chamber were maintained at a temperature of 25°C by a circulating water bath. The oxygen concentration of air-saturated medium (100%) at 25°C was taken as 250µM.

F. ^{32}Pi INCORPORATION

Photophosphorylation was measured using the ^{32}Pi incorporation technique of Nielsen and Lehninger (1954), as modified by Avron (1960). Radioactivity was determined with a G-M tube connected to an Echo Scaler (type N529C).

The reaction mixture was placed in test tubes mounted on a test tube rack and placed in an aquarium. For dark treatments, the test tubes were wrapped in aluminium foil. Water in the aquarium was maintained at 25°C.

The light source for these experiments was from a Rank Aldis slide projector with a 150W quartz iodine lamp and a Wratten No.29 red filter, giving an intensity of about 8.72×10^5 ergs.cm^{-2}.sec^{-1} at the reaction vessel.

G. REDUCED MINUS DIFFERENCE SPECTRA OF CYTOCHROMES IN ISOLATED CHLOROPLASTS

Reduced minus oxidised difference spectra were recorded on a Cary model 14R recording spectrophotometer, fitted with a Cary model 1462 scatter transmission attachment which contained an RCA type 6217 photomultiplier. The 0 - 0.1 slide wire was used. For measurements at 20°C, the appropriately treated chloroplasts were incubated in the dark for 5 min. at 0°C. The chloroplast suspension (0.6ml per 1cm cuvette)

contained 75µg chl1/ml, and were suspended in a solution containing 62 parts by volume of glycerol and 38 parts of buffer. Reducing and oxidising agents were added in 20µl aliquots. These agents were; 0.2M potassium ferricyanide; 0.2M hydroquinone and 0.2M sodium ascorbate. For measurements at liquid nitrogen temperature, the chloroplasts were suspended in the same solution as used for measurements at 20°C. The cuvette assembly of Bonner (1961), as modified by Boardman and Highkin (1966) to suit the optical system of the Cary, was used. To determine the difference spectra at 77°K, the sample was frozen under a dim green light and the difference spectrum recorded. The photooxidation of cytochrome b_{559} was determined by illuminating the sample and reference cuvettes for 3 min. at 650nm in the Cary spectrophotometer (with 3mm slits), and the difference spectra recorded again. The decrease in absorbance at 557nm, upon illumination, was a measure of the photo-oxidation of cytochrome b_{559}.

H. LIGHT-INDUCED REDOX CHANGES OF CYTOCHROMES ~~FROM~~ IN ISOLATED CHLOROPLASTS

Light-induced absorbance changes at room temperature were measured with an Aminco-Chance dual-wavelength difference spectrophotometer (American Instrument Corporation, Silver Springs, Md., U.S.A.), fitted with a side illumination attachment. Actinic light was provided by a 650W tungsten iodine lamp, and passed through a 3cm layer of water and interference filters (half width 7-10mm). A Corning 2-64 filter was used in conjunction with a 714 filter. The reference wavelength used was 570nm.

I. ASSAYS

Photoreduction of FeCN and NADP was assayed spectrophotometrically using the molar extinction coefficients 1.05×10^3 and 6.2×10^3 respectively. The cuvette was suspended on a perspex rack in a water bath maintained at 25°C and illuminated; thus light was filtered through 2cm of water and a Wratten No.29 red filter. In the case of NADP reduction, saturating amounts of partially purified Ferredoxin/NADP reductase (San Pietro and Lang, 1958) was also added.

Chlorophyll concentration was determined by the method of Arnon (1949). ADP concentration was determined enzymically using the method of Wiskich, Young and Biale (1964).

J. REAGENTS

All reagents used were of the highest possible grade (Analytical reagents) available. Biochemicals were obtained from the British Drug House, Ltd., Poole, England; Sigma Chemical Co., St. Louis, Mo., U.S.A. and Calbiochem Inc., Los Angeles, California, U.S.A. Dio-9, DBMIB, DCMU and Nigericin were gifts from Dr. F.A. Smith, Department of Botany, University of Adelaide, South Australia; Dr. N.K. Boardman, Division of Plant Industry, C.S.I.R.O., Canberra; Dr. C.B. Osmond, Research School of Biological Sciences, A.N.U., and Dr. K.R. West, School of Pharmacy, S. A. Institute of Technology, Adelaide.

CHAPTER III

NON-CYCLIC, PSEUDO-CYCLIC AND CYCLIC PHOTOPHOSPHORYLATIONS IN ISOLATED PEA CHLOROPLASTS. ONE OR TWO SITES OF PHOSPHORYLATION ON THE NON-CYCLIC ELECTRON FLOW PATHWAY.

INTRODUCTION

Photophosphorylation was first reported in spinach chloroplasts by Arnon, Whatley and Allen (1954) and in bacterial chromatophores by Frenkel (1954). Later, Arnon, Whatley and Allen (1958) found that ATP formation was stoichiometrically coupled to the reduction of NADP or FeCN, with an associated evolution of oxygen. This photophosphorylation was termed "non-cyclic" because of the unidirectional path of the electron flow. Such a system involves the reduction of an oxidant and the oxidation of water and either one or both photosystems. Another type of photophosphorylation not associated with the evolution of oxygen nor with a net change in concentration of external electron donor or acceptor, has been termed "cyclic" photophosphorylation. Menadione and FMN were the first cofactors of "cyclic" photophosphorylation (Whatley, Allen and Arnon, 1955 and Arnon, Whatley and Allen, 1955) but subsequently PMS was found to be the more effective (Jagendorf and Avron, 1958 and Hill and Walker, 1959). It was shown by Nakamoto, Krogmann and Vennesland (1959) and Nakamoto and Vennesland (1960) that FMN and menadione-catalyzed pseudo-cyclic phosphorylation rather than cyclic, because of their dependence on molecular oxygen.

Pseudo-cyclic electron flow utilises the same pathway as non-cyclic electron flow, but the reduced oxidant is reoxidised by the oxygen evolved from the oxidation of water, and it also involves both photosystems. More recently, Schwartz (1967) has suggested that PMS may act as a non-cyclic electron acceptor (but at a reduced efficiency) as well as a cyclic electron acceptor.

The determination of the ratio of ATP produced to the number of electron pairs transferred ($P/2e^-$) in non-cyclic photophosphorylation, allows a calculation of the possible number of phosphorylation sites. $P/2e^-$ ratios of about 1.0 have been reported with FeCN (Arnon, Whatley and Allen, 1958; Avron, Krogmann and Jagendorf, 1958 and Stiller and Vennesland, 1962), NADP (Arnon, Whatley and Allen, 1958) and DCPIP (Shen, Yang, Shen and Yin, 1963). However, Winget, Izawa and Good (1965) obtained $P/2e^-$ ratios of 1.2 to 1.3 with either FeCN or NADP as electron acceptors, but Del Campo, Ramirez and Arnon (1968) were not able to confirm these results. Izawa and Good (1968) and Horton and Hall (1968) have reported studies on photophosphorylation in which they consistently obtained $P/2e^-$ ratios greater than 1.0. Another way of determining the number of sites of phosphorylation uses the polarographic method as applied to mitochondria (see Estabrook, 1967). With this method West and Wiskich (1968) demonstrated that Class I chloroplasts (Spencer and Unt, 1965) were capable of photosynthetic control (analogous to respiratory control of Chance and Williams, 1956), and with

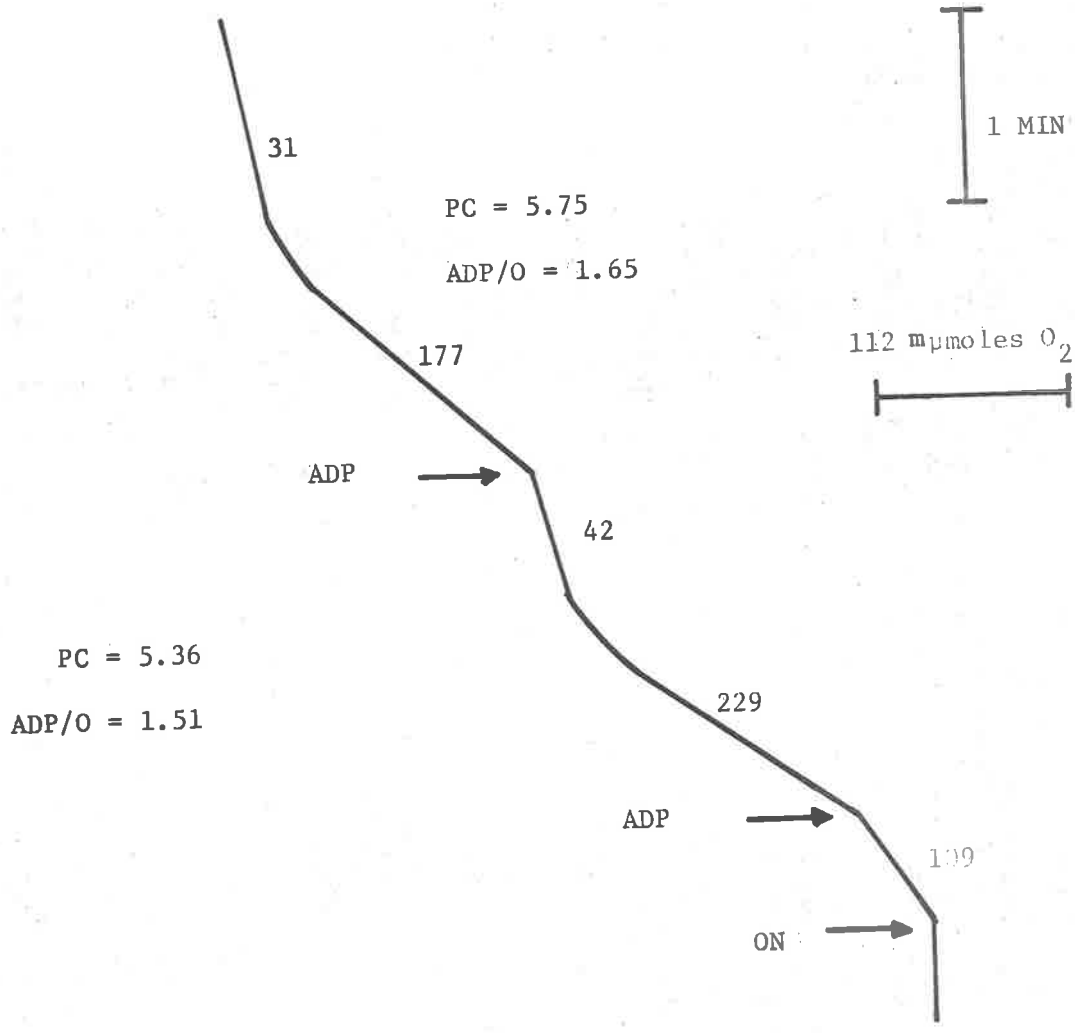


FIGURE 1. Photosynthetic control by isolated pea chloroplasts with FeCN as electron acceptor. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 13mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and chloroplasts equivalent to 43μg chlorophyll. The two additions of ADP were added to a final amount of 0.475 μmole.

these chloroplasts ADP/O ratios higher than 1.0 were frequently obtained. These results were confirmed by Kraayenhof, Groot and van Dam (1969) and Hall, Reeves and Baltscheffsky (1971), the latter authors obtaining ratios ranging from 1.4 to 2.1 using FeCN, NADP or MV as electron acceptors.

In this chapter, results will be presented which indicate that:

(a) there are two sites of phosphorylation (energy transduction) on the non-cyclic electron flow pathway,

(b) there are two sites of FeCN reduction on the non-cyclic electron flow pathway,

(c) the two sites of energy transduction are located between the two sites of FeCN reduction,

(d) PMS catalyzes both pseudo-cyclic and cyclic photophosphorylation, which can occur simultaneously.

RESULTS

A. FeCN AS THE ELECTRON ACCEPTOR

Photosynthetic control with FeCN has been demonstrated in isolated pea chloroplasts (Fig.I). The rate of oxygen evolution was stimulated more than 2-fold by the addition of ADP (referred to as State 3 Rate). The rate subsequently slows down (referred to as State 4 Rate) upon exhaustion of ADP. Fig.I also shows that this State 3, State 4 cycle could be repeated by further addition of ADP, after the initial ADP added had been phosphorylated. From the photosynthetic control data, ADP/O ratios (moles

TABLE I. Calculated ADP/O and photosynthetic control ratios using pea and spinach chloroplasts with FeCN as the electron acceptor. The reaction mixture was the same as in Fig.I. Experiments with pea chloroplasts contained 44µg chlorophyll, whereas spinach chloroplasts contained 56µg chlorophyll. Most of the variations occurred between different preparations of chloroplasts.

Chloroplasts	ADP Additions	Photosynthetic control	ADP/O Ratios
1. Pea	1st	3.70	1.33
2. Pea	1st	4.00	1.38
	2nd	5.00	1.60
3. Pea	1st	5.50	1.50
	2nd	5.90	1.65
4. Pea	1st	4.90	1.33
5. Spinach	1st	3.80	1.48
	2nd	4.00	1.40

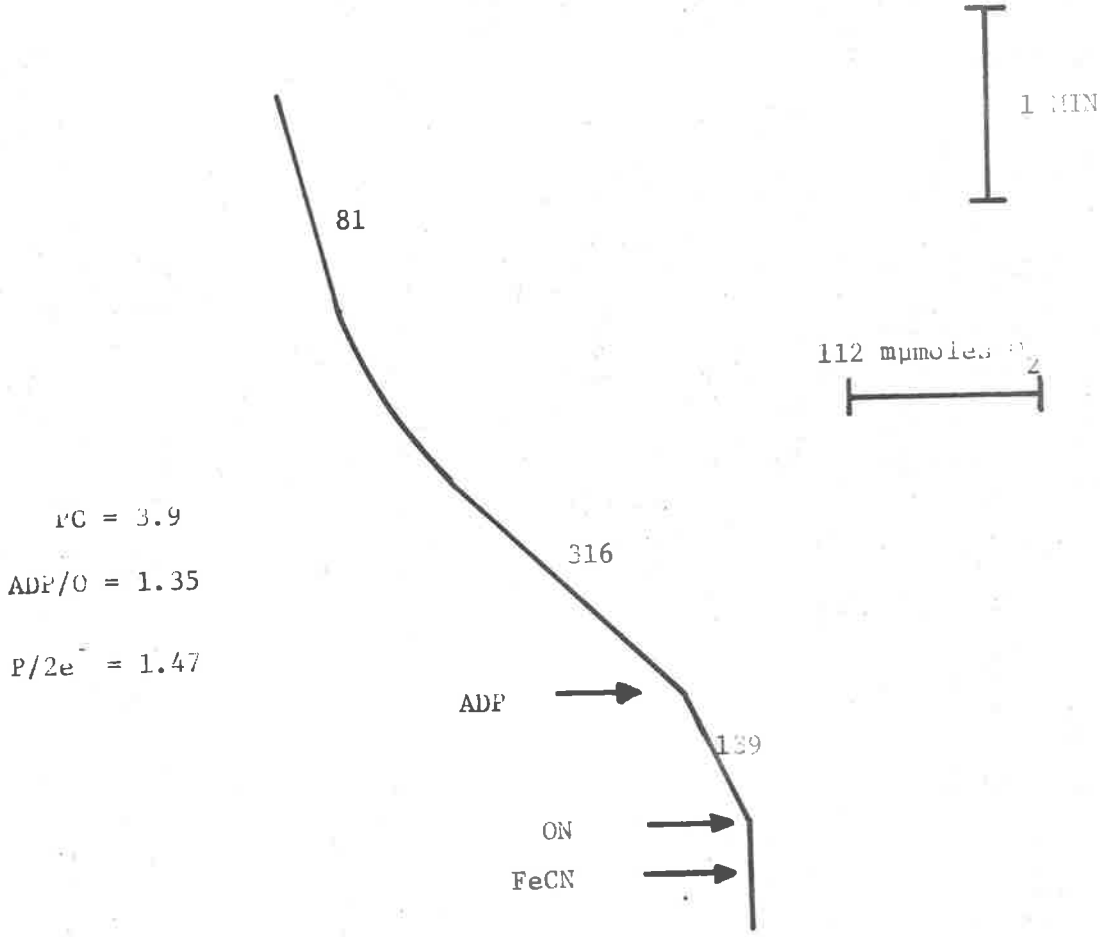


FIGURE II. Photosynthetic control, ADP/O and P/$2e^-$ ratios obtained with pea chloroplasts using FeCN as the electron acceptor. The reaction mixtures were the same as in Fig.I. ADP for ADP/O and P/$2e^-$ determinations were added to a final amount of 0.475 μmole and 2.375 μmole respectively. P, the amount of ATP^{32} formed was determined according to the procedure of Avron (1960) as described in Chapter II. $2e^-$, the amount of FeCN reduced in the presence of ADP and Pi was determined by following the absorbance changes at 420nm. The samples were illuminated for 2 min and reactions terminated by adding 20% trichloroacetic acid.

of ADP esterified per atom of O_2 evolved) can be calculated (Estabrook, 1967). Table I shows the values of photosynthetic control and ADP/O ratios thus calculated.

Usually, $P/2e^-$ ratios (moles of phosphate esterified per pair of electrons transferred) have been used to calculate the number of possible phosphorylation sites associated with non-cyclic photophosphorylation. Since West and Wiskich (1968) successfully applied the polarographic method for the calculation of possible phosphorylation sites to chloroplasts (a much faster and easier method) an attempt was made to see if the two methods would yield similar results. A preparation of isolated pea chloroplasts was made and the ADP/O and $P/2e^-$ ratios were determined.

Fig.II shows the polarographic oxygen electrode trace of oxygen evolution with a photosynthetic control ratio of 3.9 and an ADP/O ratio of 1.35. Included in the same figure is the calculated $P/2e^-$ ratio of 1.47, (P being the amount of ATP^{32} formed and $2e^-$ the ADP and Pi stimulated rate of FeCN reduction). These figures indicate a good agreement between the two techniques.

In view of the fact that broken chloroplasts could also show photosynthetic control (Telfer and Evans, 1971), a study of the effect of various treatments on photosynthetic control and ADP/O ratios was made. Chloroplast preparations which showed good control and ADP/O ratios were subjected to ultrasonic disintegration and osmotic shock. Different fractions were separated by centrifuging and various photochemical activities of these different

TABLE II. Effect of ultrasonic disintegration and osmotic shock on the various activities of chloroplasts. The reaction mixture was the same as in Fig.I except the chlorophyll content, which was 82µg in these experiments.

Treatments	Electron Flow (µmoles O_2/mg chll/hr)			Photosynthetic control ratio	ADP/O ratio
	Hill Reaction	State 3	State 4		
Control	47	122	30	2.9	1.73
5 sec. sonication	51	119	45	2.7	1.44
10 sec. sonication	60	119	51	2.4	1.16
20 sec. sonication	68	100	75	1.3	0.93
Osmotically shocked chloroplasts	44	100	23	3.00	1.16
Chloroplast fragments	56	111	42	2.0	1.16

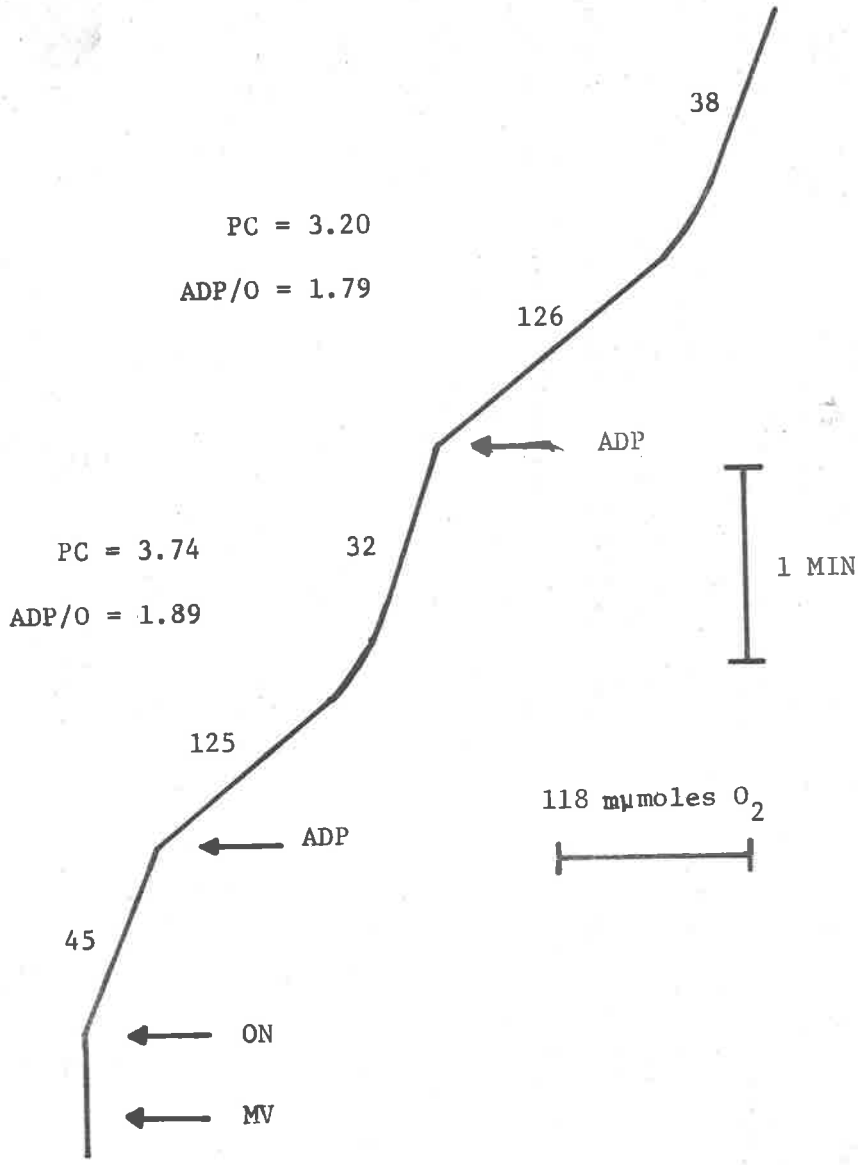


FIGURE III. Photosynthetic control by pea chloroplasts with MV as electron acceptor. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 13mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 40mM HEPES buffer, pH7.5; 64μM MV, 0.93mM azide and chloroplasts equivalent to 71μg chlorophyll. The two additions of ADP were added to a final amount of 0.492μmole.

preparations were measured. It can be seen (Table II) that sonication affected the photosynthetic control and ADP/O ratios quite drastically, while the basal and State 4 rates of electron flow increased (see West and Wiskich, 1968). The State 3 rate was only slightly affected indicating that the lower photosynthetic control ratio was due to the faster State 4 rate. Low ADP/O ratios were also observed with chloroplasts subjected to osmotic shock. However, the basal, State 3 and State 4 rates of electron flow were not greatly affected, suggesting that the osmotic shock affected the chloroplasts differently from sonication. The chloroplast fragments prepared from osmotically shocked chloroplasts showed low photosynthetic control and ADP/O ratios. These fragments showed activity similar to that of sonicated chloroplasts, namely fast basal and (State 4) rates of electron flow with the (State 3) rates only slightly affected.

B. MV AS THE ELECTRON ACCEPTOR

Photosynthetic control could also be demonstrated using MV as the electron acceptor, by monitoring the oxygen uptake in the presence of azide, an inhibitor of catalase. Fig.III shows that the rate of oxygen uptake was stimulated by ADP, in a similar fashion to the oxygen evolving system using FeCN as the electron acceptor. Hall, Reeves and Baltscheffsky (1971), using spinach chloroplasts which had been rapidly isolated in isotonic media, observed photosynthetic control when the preparation was assayed in slightly hypotonic medium. They obtained photosynthetic control

TABLE III. Calculated ADP/O and photosynthetic control ratios using pea chloroplasts with MV as the electron acceptor. The reaction mixture was the same as in Fig.III, except that experiments 1 and 2 contained chloroplasts equivalent to 47µg and 30µg chlorophyll respectively.

Experiments	ADP Additions	Photosynthetic control	ADP/O Ratios
1.	1st	4.1	1.66
	2nd	3.1	1.42
2.	1st	7.9	1.80

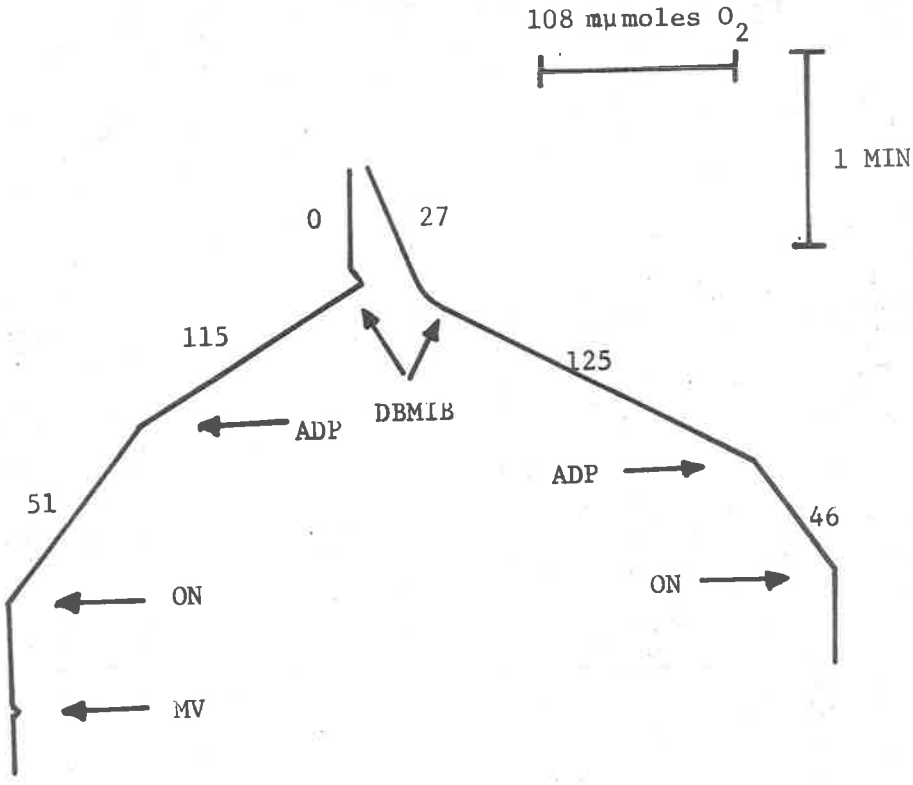


FIGURE IV. DBMIB inhibition of State 3 rate of electron flow with MV and FeCN as electron acceptors. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 17mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 40mM HEPES buffer, pH7.5; 64μM MV and 0.93μM azide or 1.5mM FeCN and pea chloroplasts equivalent to 110μg chlorophyll. The final amount of ADP present was 1μmole. DBMIB was added to a final concentration of 1μM. The numbers along the traces are in μmoles O_2/mg chll/hr.

TABLE IV. Effect of DBMIB on the Hill reaction rate in the absence and presence of uncouplers. Reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 64μM MV and 0.93mM azide or 1.5mM FeCN and 110μg chlorophyll. 21mM KCl was also present with nigericin.

Additions	Oxygen Evolution/Uptake (μmoles/mg chll/hr)
I FeCN	44
+ DBMIB 1μM	29
+ Nigericin 0.43μM	257
+ Nigericin + DBMIB	35
+ CCCP 8.4μM	82
+ CCCP + DBMIB	29
II MV	43
+ DBMIB 1μM	0
+ Nigericin 0.43μM	236
+ Nigericin + DBMIB	0
+ CCCP 8.4μM	78
+ CCCP + DBMIB	0

ratios of 5.0 - 8.0 and ADP/O ratios of 1.6 - 1.8, with MV as the electron acceptor. Telfer and Evans (1971) had also observed photosynthetic control using broken spinach chloroplasts, but the control ratios obtained by them were very low (1.7 - 2.7), as were the ADP/O ratios of about 1.0.

Table III shows the calculated ADP/O and photosynthetic control ratios with MV as electron acceptor. It can be seen that photosynthetic control ratios were similar to those obtained using FeCN as electron acceptor, but ADP/O ratios were marginally different, those obtained with MV being higher.

C. SITES OF FeCN REDUCTION AND LOCATION OF THE TWO SITES OF PHOSPHORYLATION (ENERGY TRANSDUCTION) ON THE NON-CYCLIC ELECTRON FLOW PATHWAY

Figure IV shows the State 3 rate with MV (measured by the rate of oxygen uptake in the presence of azide) was completely inhibited by DBMIB (1μM). On the other hand, the State 3 rate of oxygen evolution with FeCN was not completely inhibited. The same differential effect of DBMIB on the two systems could be observed with nigericin-uncoupled Hill reaction rates (Table IV). The nigericin-uncoupled Hill reaction rate with MV was completely inhibited by DBMIB, whereas that with FeCN was again only partially inhibited. Similar effects were observed when CCCP was used to uncouple the Hill reaction (Table IV). These results indicate that DBMIB inhibits electron flow (coupled or uncoupled) completely in the presence of MV, but only partially in the case of FeCN (see Trebst

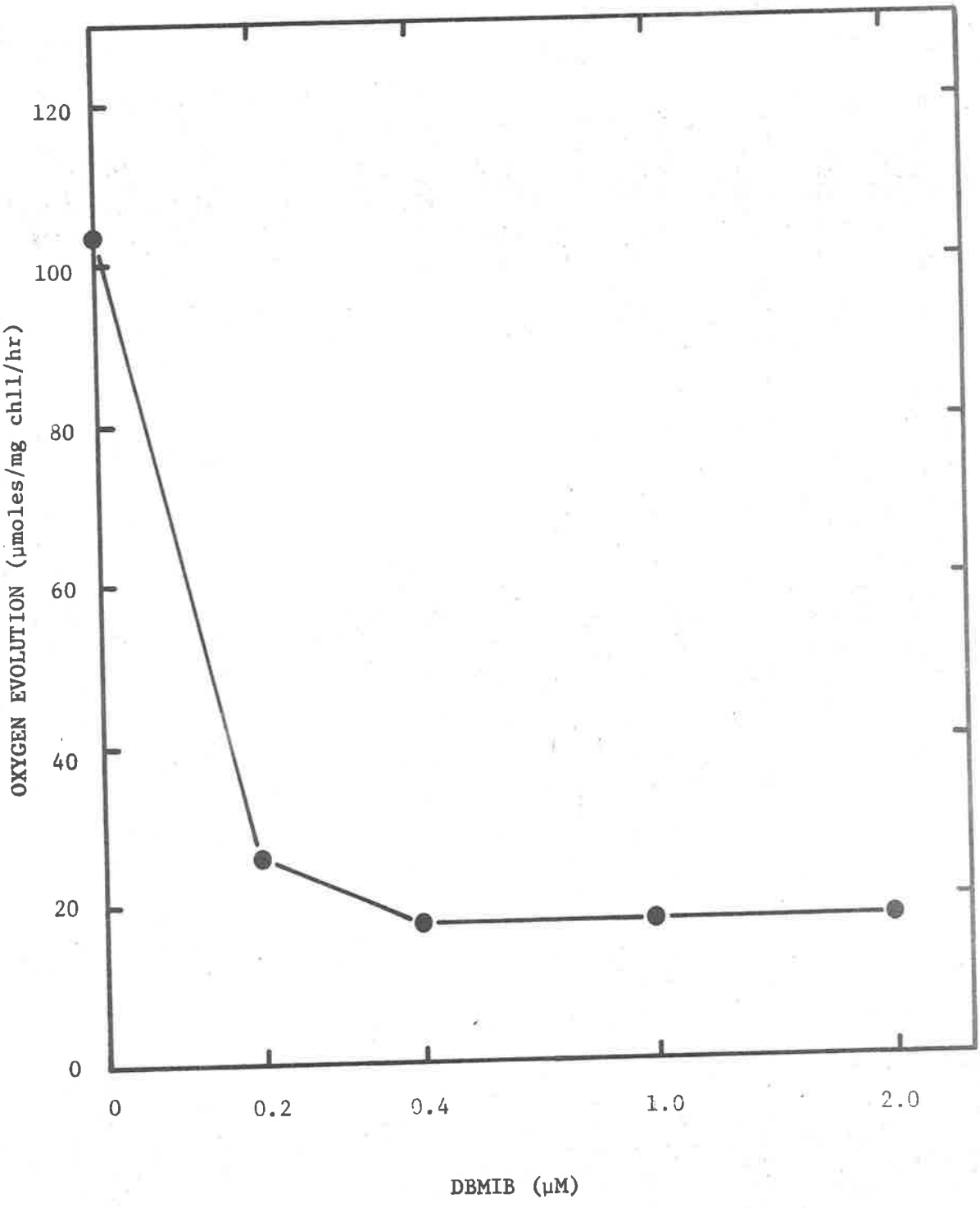


FIGURE V. Effect of DBMIB on the State 3 rate of electron flow with FeCN as electron acceptor. The experimental conditions were the same as in Fig.V, except that the amount of ADP present was 0.5µmole.

TABLE V. Effect of uncouplers and ADP on the DBMIB-inhibited Hill reaction rate. Reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 21mM KCl; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and 120µg chlorophyll. In the reaction with ADP, 17µM K_2HPO_4-KH_2PO_4 buffer, pH7.6 replaced KCl. The presence of phosphate buffer had no effect on the Hill reaction rate.

Additions	Electron Flow (µmoles O_2/mg chll/hr)
Control	42
+ DBMIB 1µM	27
+ DBMIB + Nigericin 0.43µM	27
+ DBMIB + Nigericin + Valinomycin 2.6µM	15
+ DBMIB + ADP 0.5µmole	27

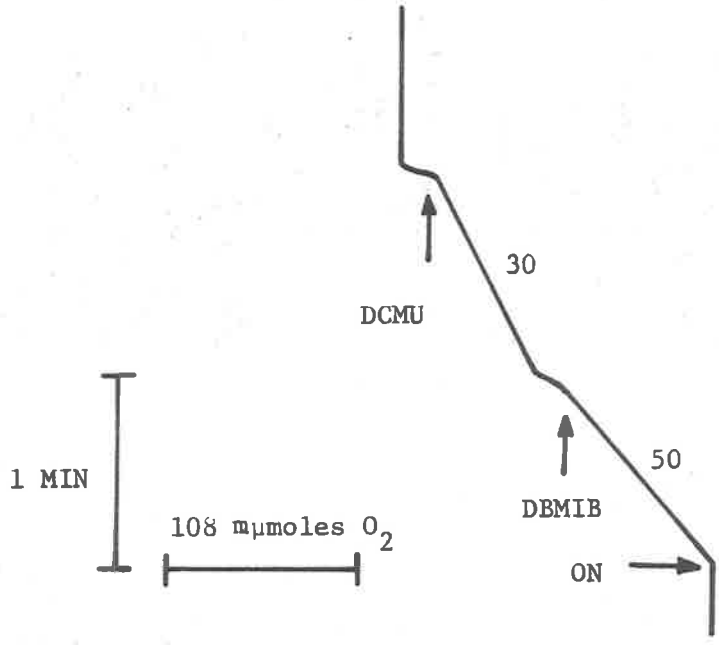


FIGURE VI. Effect of DCMU on the DBMIB-insensitive Hill reaction with FeCN. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and pea chloroplasts equivalent to 110μg chlorophyll. DBMIB and DCMU were added to a final concentration of 1μM and 4.2μM respectively. The numbers along the traces are in μmoles O_2/mg chl1/hr.

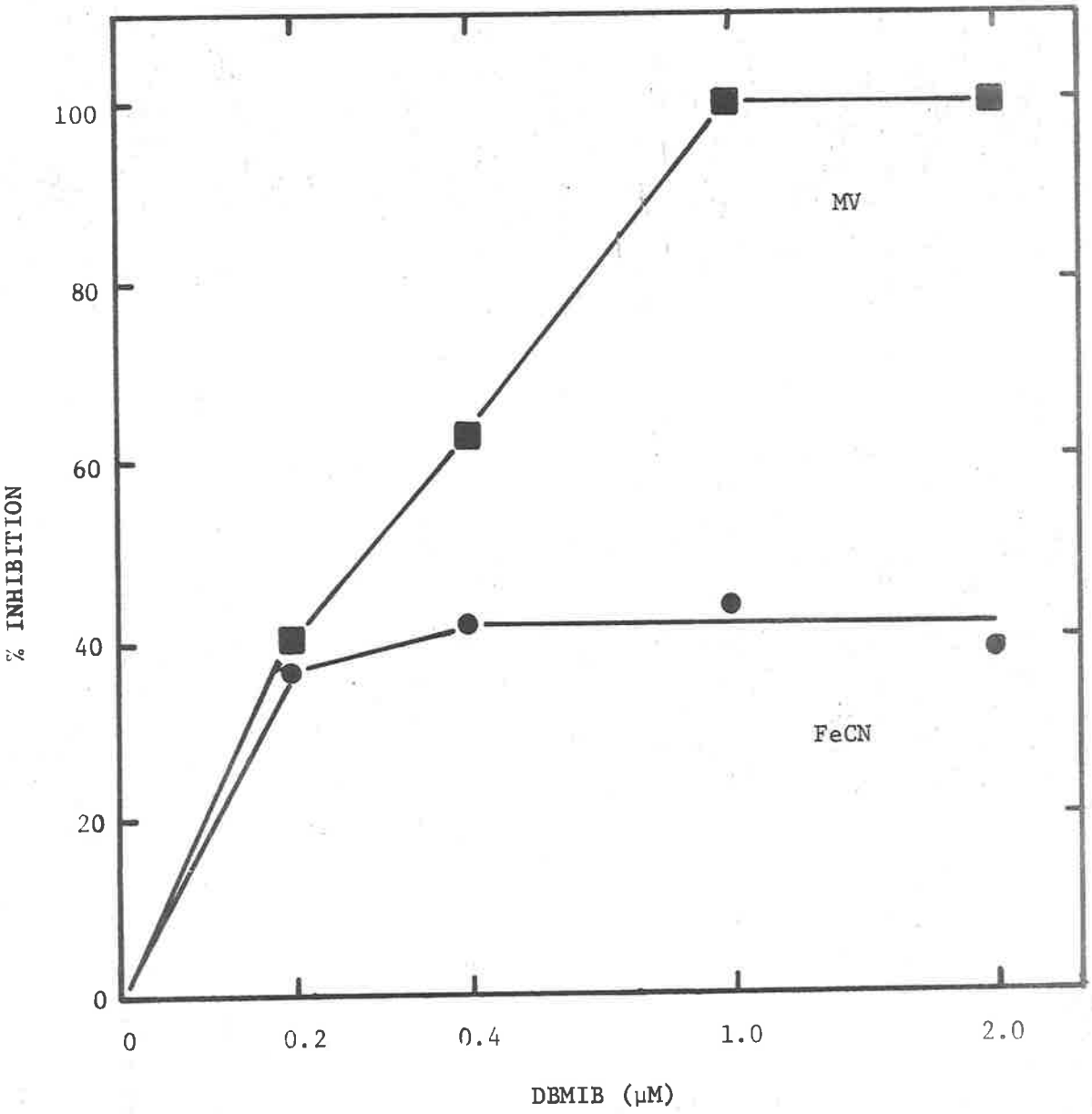


FIGURE VII. Effect of DBMIB on the Hill reaction rate with MV and FeCN. The experimental conditions were the same as in Fib.VII. MV was added to a final concentration of 64μM together with 0,93mM azide. 100% electron flow for MV and FeCN are 43 and 51 μmoles O_2/mg chll/hr, respectively.

et al., 1970). The inhibition of the State 3 rate of electron flow with FeCN did not increase as higher concentrations of DBMIB were used, as shown in Fig.V . The residual electron flow with FeCN suggests that FeCN was able to accept electrons from a point on the electron flow pathway preceding the site of inhibition of DBMIB.

Table V shows the inhibition of the Hill reaction by DBMIB and the effect of nigericin, nigericin plus valinomycin on this inhibited electron flow. It is evident that the residual rate of electron flow in the presence of DBMIB was insensitive to uncouplers, suggesting that it is non-phosphorylating. This was confirmed by the lack of stimulatory effect by ADP on this electron flow (Table V).

Figure VI shows that DCMU completely inhibited the DBMIB-insensitive rate of electron flow with FeCN. This places the non-phosphorylating site of FeCN reduction between the sites of inhibition of DCMU (Duysens, 1964) and DBMIB (Bohme and Cramer, 1971). The second site of FeCN reduction which is capable of phosphorylation and is sensitive to DBMIB must be located between the site of DBMIB inhibition and Photosystem I.

DBMIB inhibited the Hill reaction rate with MV more strongly than with FeCN. Figure VII shows that 1μM DBMIB completely inhibited the Hill reaction with MV (cf. Trebst et al., 1970). The Hill reaction with FeCN was only partially inhibited by 1μM DBMIB and higher concentrations did not inhibit further (Fig.VII). ATP inhibition of the Hill reaction has been reported (Neumann and

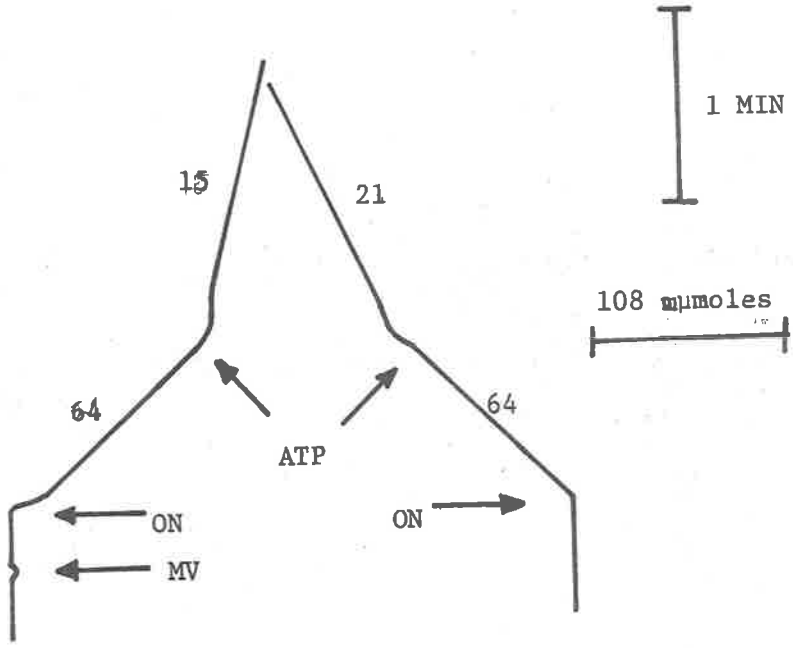


FIGURE VIII. ATP inhibition of the Hill reaction rate with MV and FeCN. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 64μM MV and 0.93mM azide or 1.5mM FeCN and pea chloroplasts equivalent to 110μg chlorophyll. ATP was added to a final amount of 0.4μmole. The numbers along the traces are in μmoles O_2/mg chll/hr.

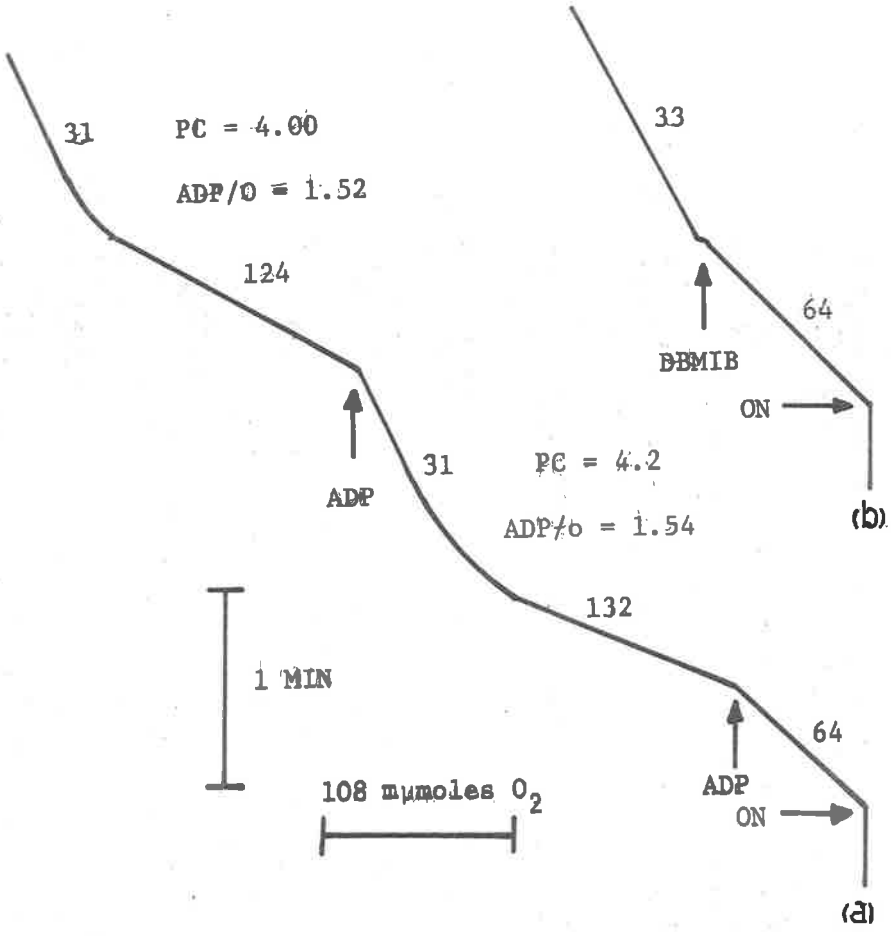


FIGURE IX. Photosynthetic control by pea chloroplasts with FeCN as electron acceptor and inhibition of the Hill reaction rate by DBMIB. The experimental conditions were the same as in Fig.V. The final amount of ADP added each time was 0.5 μmole. DBMIB was added to a final concentration of 1μM. The numbers along the traces are in μmoles O_2/mg chll/hr.

TABLE VI. Calculated ADP/O ratios with FeCN and MV as electron acceptors. Reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 17mM K_2HPO_4-KH_2PO_4 buffer, pH7.6; 40mM HEPES buffer, pH7.5; 1.5mM FeCN or 64μM MV and 0.93mM azide and 110μg chlorophyll. ADP was present to a final amount of 0.5μmole. Corrected ADP/O ratios are calculated by subtracting the electron flow in the presence of (a) 1μM DBMIB and (b) 0.42mM ATP.

Electron Acceptor	Electron Flow (μmoles O_2/mg chll/hr)			ADP/O Ratios		
	Hill Reaction	+DBMIB	+ATP	Uncorrected	Corrected(a)	Corrected(b)
FeCN	64	33	31	1.51	2.00	1.91
FeCN	64	33	31	1.52	2.02	1.93
FeCN	64	33	31	1.52	2.02	1.93
MV	64	0	16	1.72	-	1.96
MV	64	0	16	1.56	-	1.76

Jagendorf, 1968 and Telfer and Evans, 1971) and Fig.VIII shows the effect of ATP on the Hill reaction rate with both FeCN and MV. ATP inhibited the Hill reaction in both cases but considerably more in the case of MV. The ATP-inhibited Hill reaction rate of oxygen evolution with FeCN was similar to the DBMIB-inhibited Hill reaction rate. This suggests that DBMIB, like ATP, inhibits that part of electron flow which is coupled to phosphorylation.

Fig.IX(a) shows a typical polarographic tracing of oxygen evolution with FeCN as electron acceptor. Oxygen evolution in the presence of Mg^{++} and Pi was stimulated by the addition of ADP. This State 3 rate subsequently slows down after phosphorylation of the added ADP. The calculated ADP/O ratio is 1.51. Fig.IX(b) shows the inhibition by DBMIB of oxygen evolution in the presence of Mg^{++} and Pi. The DBMIB inhibited rate of oxygen evolution was not stimulated by ADP. This has been shown earlier to be the non-phosphorylating electron flow with FeCN (Table VI). When a correction was made for this non-phosphorylating electron flow in the control experiment, an ADP/O ratio of 2.00 was obtained. Table VI shows the uncorrected ADP/O ratios with FeCN and MV as electron acceptors. It also includes the ADP/O ratios, corrected for electron flow in the presence of 1μM DBMIB (correction a) and ATP (correction b). The uncorrected ratios with FeCN are lower than those with MV, but when both FeCN and MV ratios are corrected by method b, the ratios became comparable. Since DBMIB inhibits MV reduction completely it is impossible to apply correction (a) to the uncorrected ratios.

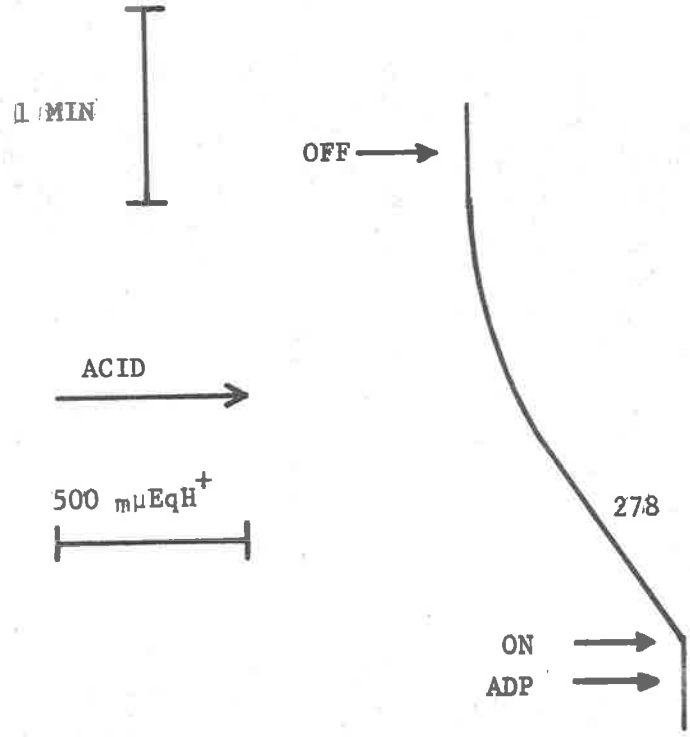
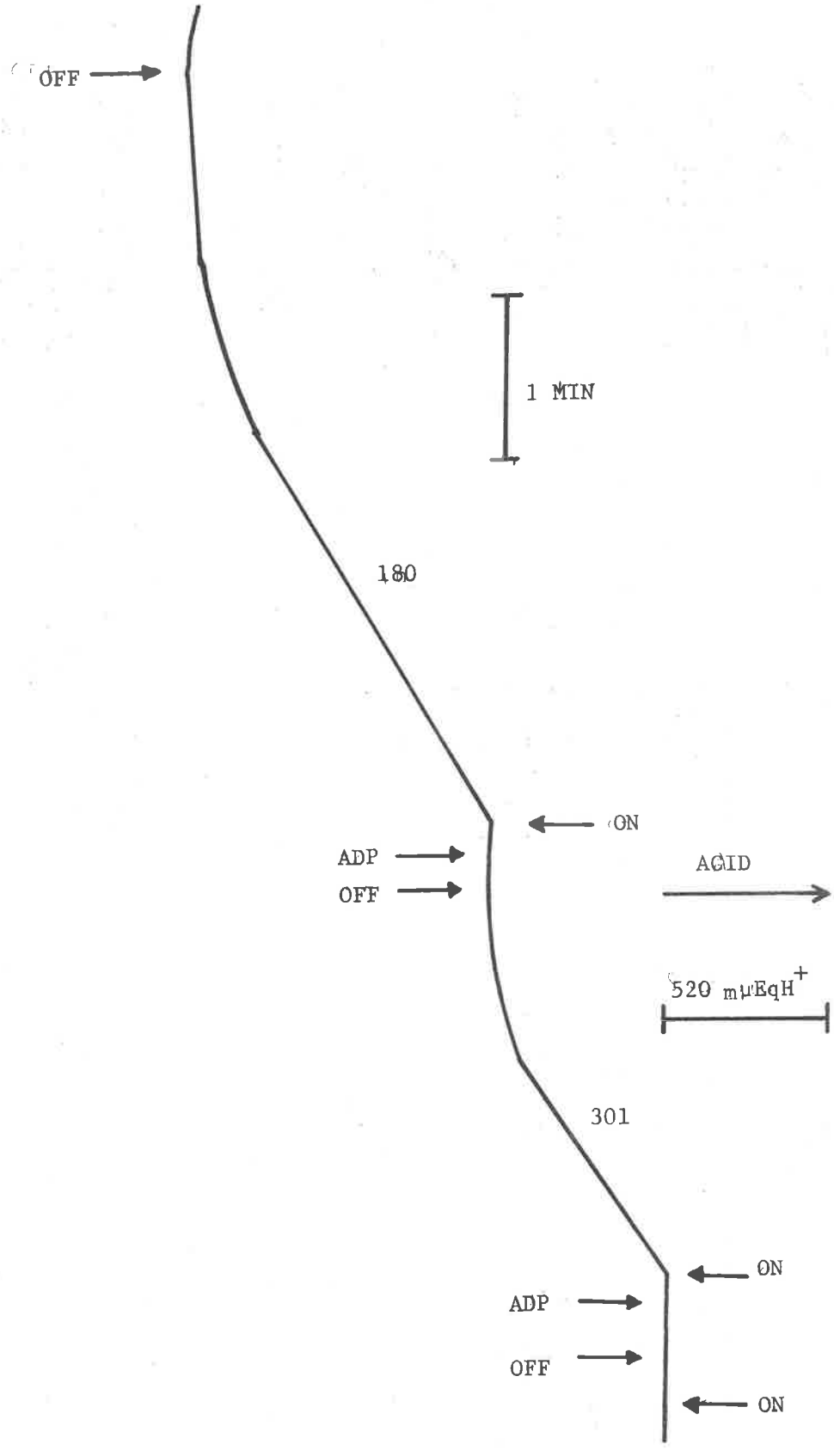


FIGURE X. PMS-catalyzed "cyclic" photophosphorylation in spinach chloroplasts. The reaction mixture contained 26.5mM sucrose; 4mM $MgCl_2$; 13mM K_2HPO_4-KH_2PO_4 buffer, pH8.0; 16μM PMS and 75μg chlorophyll. ADP was added to a final amount of 0.697 μmole. The numbers along the trace are expressed in μEqH^+/mg chll/hr.

FIGURE XI. PMS-catalyzed "cyclic" photophosphorylation in spinach chloroplasts. The experimental conditions were the same as in Fig.XI. The final amount of ADP added each time was 0.697 μmole. The numbers along the trace are expressed in μEqH^+/mg chll/hr.


OFF
1 MIN
180
ON
ADP
OFF
ACID
520 mμEqH+
301
ON
ADP
OFF
ON

D. PMS-CATALYZED PHOTOPHOSPHORYLATIONS AND LIGHT-INDUCED PROTON UPTAKE

Photophosphorylation catalyzed by PMS under aerobic conditions is shown in Fig.X. It was measured by following pH changes in the reaction medium. For every molecule of ADP phosphorylated, approximately one proton is consumed, causing an increase in pH (Chance and Nishimura, 1967). Thus ATP formation can be measured by following the associated alkalinisation of the medium.

Fig.XI shows that there was no change in pH when the reaction mixture was illuminated in the presence of PMS alone; ADP was then added during the subsequent dark period. When the reaction mixture was illuminated again, an increase in pH was observed which was maintained until all the ADP was phosphorylated. A second addition of ADP was added and the same effect was observed. This result shows that phosphorylation occurs during electron flow catalysed by PMS.

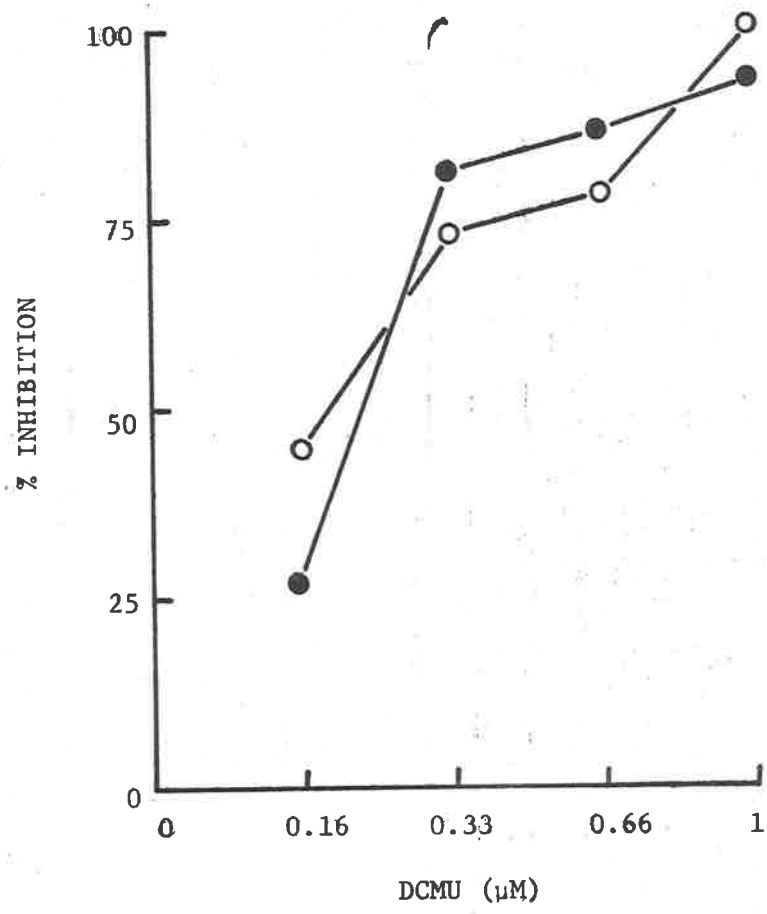


FIGURE XII. DCMU inhibition of State 3 rate of electron flow with FeCN and "cyclic" photophosphorylation with PMS. The reaction mixture for non-cyclic electron flow contained 26.5mM sucrose; 4mM $MgCl_2$; 10mM K_2HPO_4-KH_2PO_4 buffer, pH7.5 and 1.6mM FeCN. The reaction mixture for "cyclic" photophosphorylation contained 26.5mM sucrose; 4mM $MgCl_2$; 10mM K_2HPO_4-KH_2PO_4 buffer, pH8.0 and 16μM PMS. The final amount of ADP present in both media was 0.697 μmole. Spinach chloroplasts were added equivalent to 75μg chlorophyll. Open and closed circles represent "cyclic" photophosphorylation and State 3 rate of electron flow respectively.

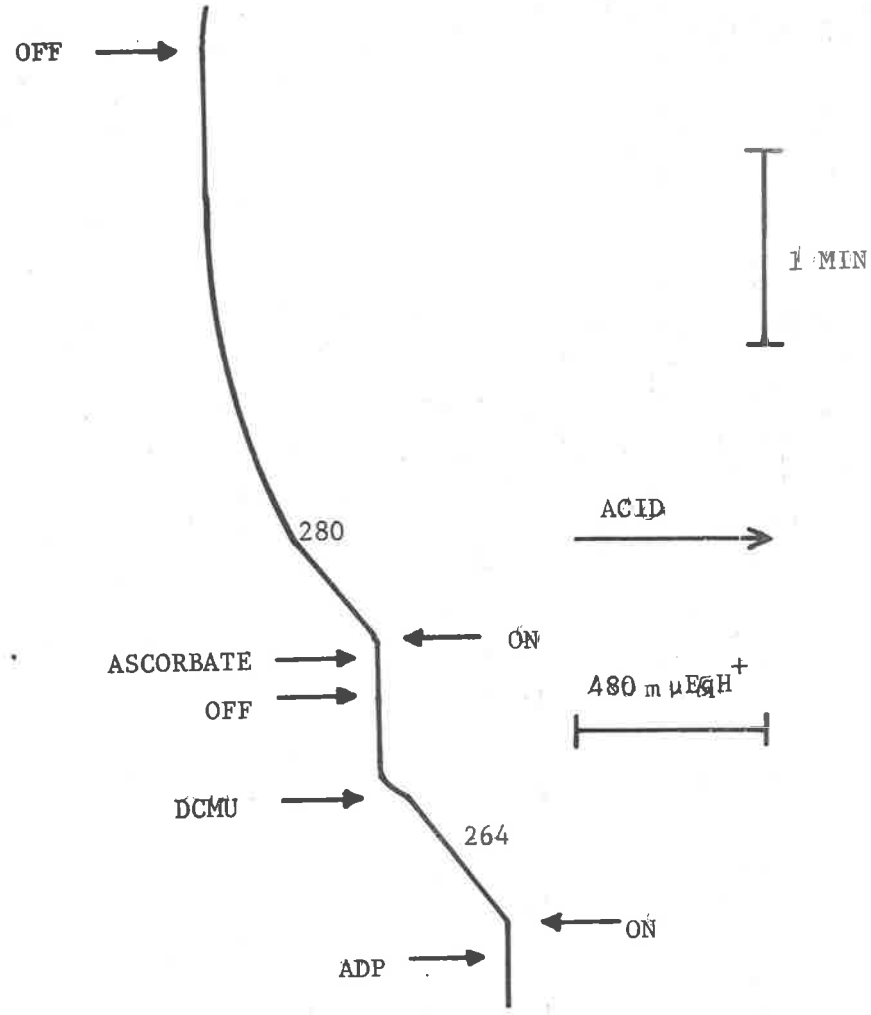
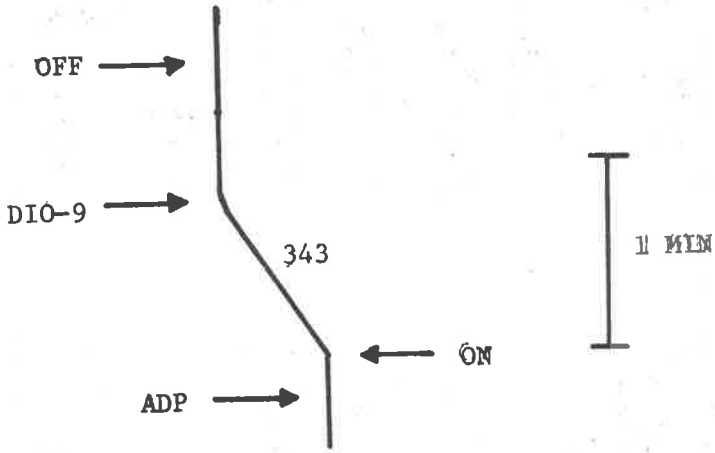


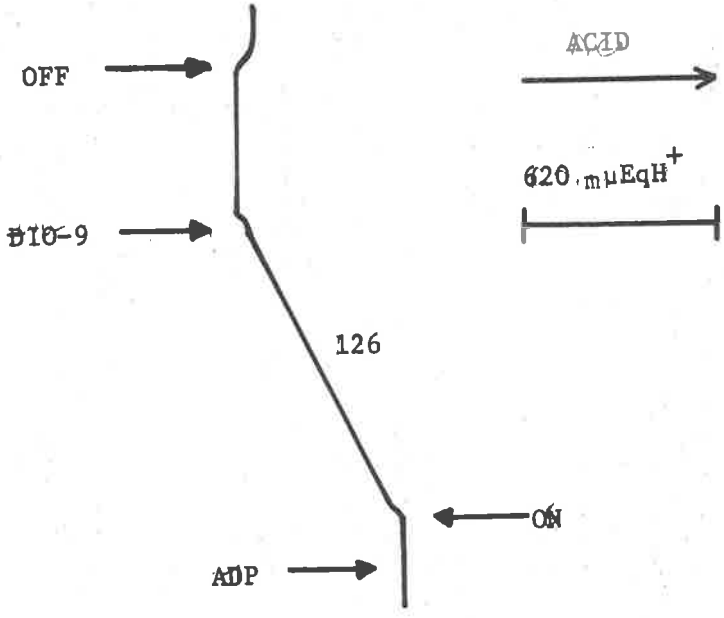
FIGURE XIII. Inhibition by DCMU of "cyclic" photophosphorylation catalyzed by PMS and true cyclic photophosphorylation with PMS plus ascorbate. The reaction mixture contained 26.5mM sucrose; 4mM $MgCl_2$; 10mM K_2HPO_4-KH_2PO_4 buffer, pH8.0; 61μM PMS and 75μg chlorophyll. Final amount of ADP added was 0.697 μmole. Concentrations of DCMU and ascorbate were 1.3μM and 6.4mM respectively. The numbers along the trace are in μEqH^+/mg chl1/hr.

FIGURE XIV(a). Inhibition of PMS-catalyzed "cyclic" photophosphorylation by Dio-9. The experimental conditions were the same as in Fig.XI. Dio-9 was added to a final concentration of 33µg/ml. Gas phase: air.

FIGURE XIV(b). Inhibition of PMS-ascorbate catalyzed cyclic photophosphorylation by Dio-9. The reaction mixture contained 25.6mM sucrose; 4mM $MgCl_2$; 10mM K_2HPO_4-KH_2PO_4 buffer, pH8.0; 16µM PMS; 1.3µM DCMU; 6.4mM ascorbate and 162µg chlorophyll. Dio-9 was added to a final concentration of 33µg/ml. Gas phase: nitrogen. The numbers along the traces are in μEqH^+/mg chl1/hr.



(a)



(b)

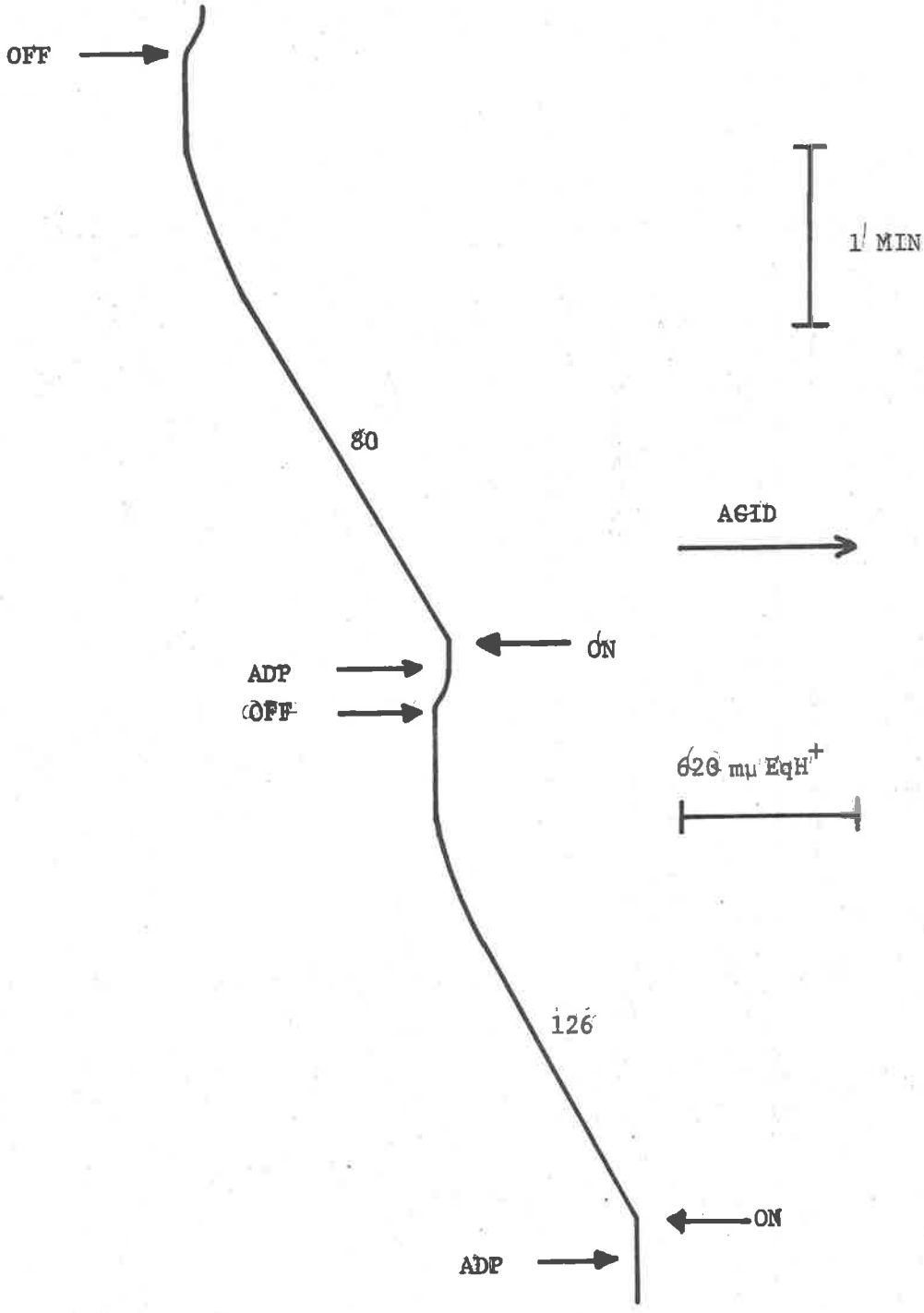


FIGURE XV. Cyclic photophosphorylation catalyzed by PMS-ascorbate in the presence of DCMU. The experimental conditions were the same as in Fig.XIV(b).

Fig.XII shows the effect of DCMU on PMS-catalyzed photophosphorylation and coupled non-cyclic electron flow with FeCN as the electron acceptor. It is seen that 1μM DCMU inhibited the PMS-catalyzed photophosphorylation by 93%. This inhibition of PMS-catalyzed photophosphorylation by DCMU can be relieved by the addition of an external reductant, e.g. ascorbate (Fig.XIII). The pH changes observed in these experiments are due only to phosphorylation, as indicated by the inhibition of these pH changes by Dio-9, an energy-transfer inhibitor (McCarty et al., 1965) in Fig.XIV.

The inhibition of PMS-catalyzed photophosphorylation by DCMU suggests that when PMS was reduced by Photosystem II, the phosphorylation is not cyclic in nature but rather pseudo-cyclic. The true cyclic photophosphorylation catalyzed by PMS was evident only when Photosystem II was blocked by DCMU and ascorbate used to reduce PMS non-enzymically. Similar observations on the DCMU sensitivity and requirement of ascorbate have been reported by Jagendorf and Margulies (1960), Schwartz (1967) and Hauska et al. (1970).

PMS was also able to support cyclic photophosphorylation under anaerobic conditions, in the presence of DCMU (Fig.XV), but the rate of phosphorylation was only about 50% of the rate observed under aerobic conditions. The amount of oxidised PMS under aerobic conditions would be considerably more than under anaerobic conditions, due to the oxidation of reduced PMS by oxygen; thus there would be more oxidised PMS to accept electrons. On the other hand, under anaerobic conditions more PMS would be in the reduced state due to

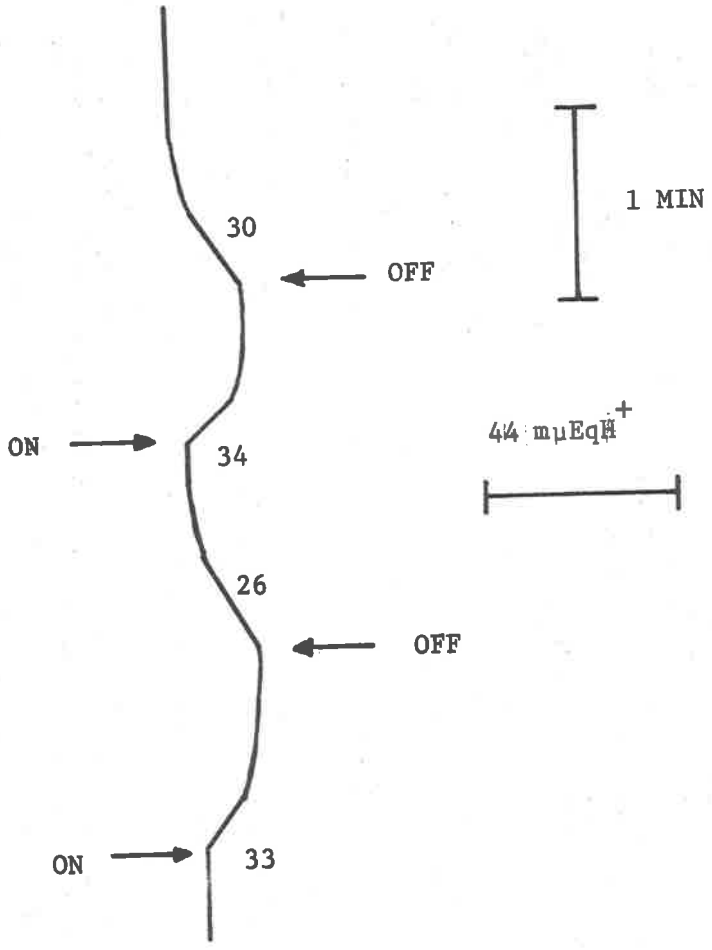
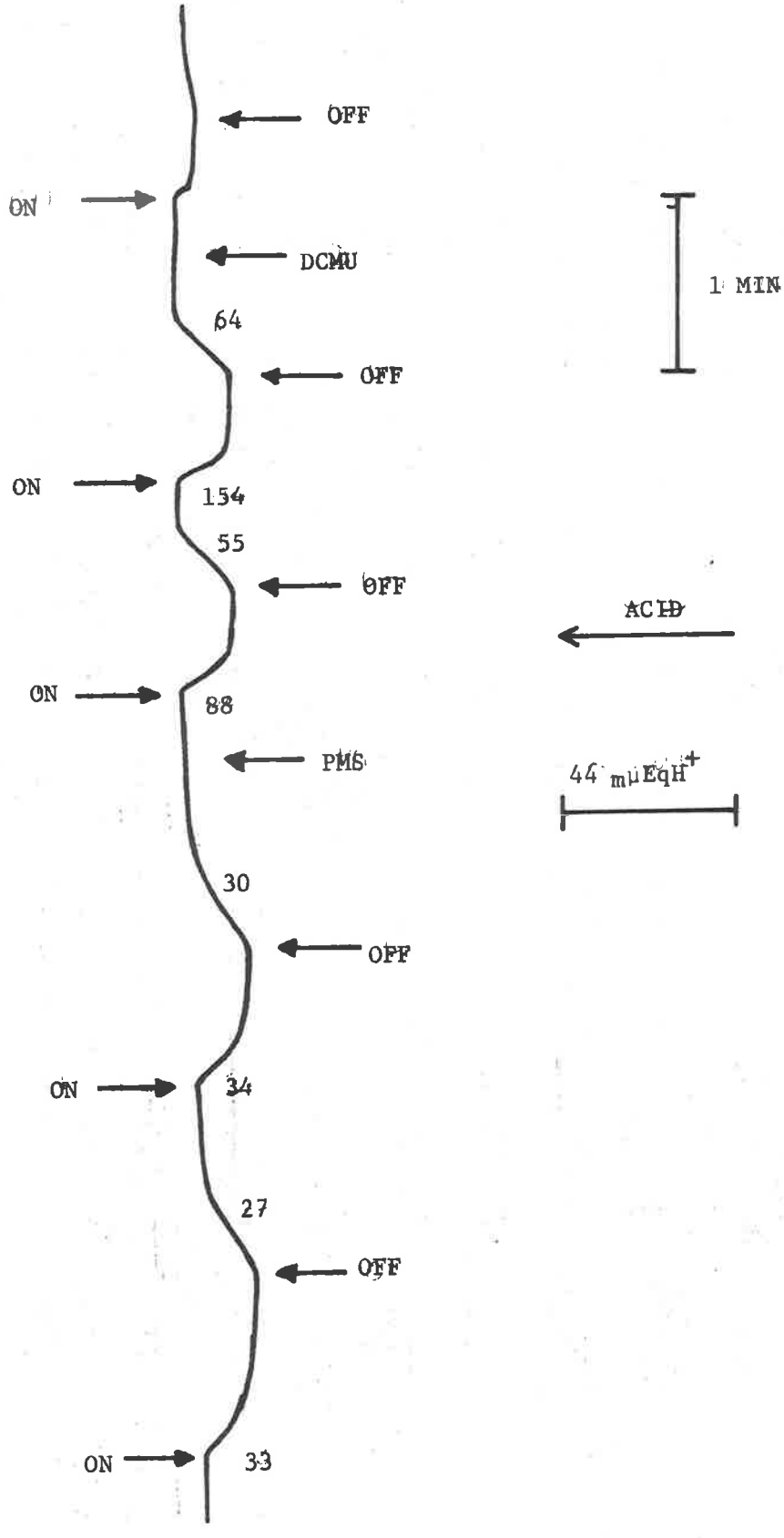


FIGURE XVI. Reversible light-induced proton uptake by spinach chloroplast in the absence of an electron acceptor. The reaction misture contained 0.4M sucrose and washed chloroplasts equivalent to 59 g chlorophyll. Initial pH of the reaction mixture was 6.59. The numbers along the trace are in $M\mu EqH^+$/min.

FIGURE XVII. Light-induced proton uptake by spinach chloroplasts, and the effect of DCMU. The reaction mixture contained 0.4M sucrose and washed chloroplasts equivalent to 59μg chlorophyll. Concentrations of PMS and DCMU were 16μM and 6.5μM respectively. Initial pH of the reaction mixture was 6.59. Gas phase: air. The numbers along the traces are in $m\mu Eq H^+/min$.


OFF
ON
DCMU
64
OFF
ON
154
55
OFF
ON
88
PMS
30
OFF
ON
34
27
OFF
ON
33
1 MIN
ACID
44 mμEqH+

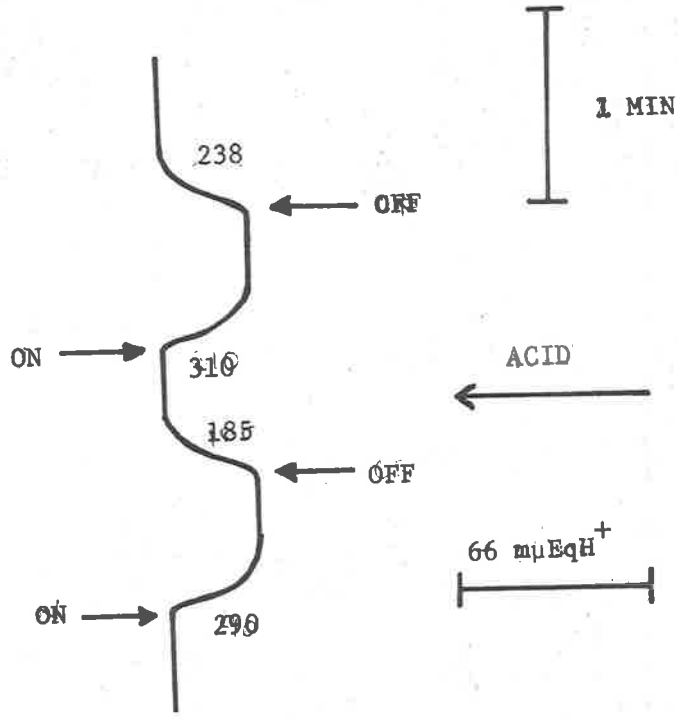


FIGURE XVIII. Light-induced proton uptake by spinach chloroplasts in the presence of DCMU. The reaction mixture contained 0.4M sucrose; 6.5μM DCMU; 16μM PMS; 1.6mM ascorbate and washed chloroplasts equivalent to 59μg chlorophyll. Initial pH of the reaction mixture was 6.55. Gas phase: nitrogen. The numbers along the trace are in $M\mu EqH^+$/min.

excess ascorbate, thus limiting the amount of electron acceptor and hence the cyclic electron flow.

Jagendorf and Hind (1963) observed a pH rise when isolated chloroplasts were suspended in a slightly or unbuffered medium and illuminated. This suggests that protons are translocated into the chloroplasts under illumination; this inward translocation is soon balanced by leakage of protons out of the chloroplast, thus establishing a steady state (Fig.XVI). The protons stored during this illumination diffuse out of the chloroplast when the light is turned off. The initial rate of proton translocation inwards (R_f) is faster than the efflux of protons in the dark (R_d). When PMS was added to the above suspension and illuminated, an increase in the rate of the pH rise was observed. This increase in the rate of the pH rise, in the presence of PMS was probably due to stimulated electron flow, i.e. cyclic electron flow. This pH rise was completely inhibited by DCMU, as seen in Fig.XVII. DCMU inhibited not only the increase in pH due to PMS but also the cofactor-less light-induced pH rise. This effect was also observed by Neumann and Jagendorf (1964). A light-induced pH rise with PMS in the presence of DCMU could be seen, if ascorbate was added to the medium (Fig.XVIII). The second pH rise with PMS, in the presence of DCMU is dependent on cyclic electron flow. The PMS + ascorbate catalyzed pH rise was much faster than that catalyzed by PMS alone in the absence of DCMU. It could be due to a faster turnover of components in this restricted pathway.

FIGURE XIX. Light-induced proton uptake by spinach chloroplasts under white light illumination. The reaction mixture contained 0.4M sucrose and 59µg chlorophyll. Concentration of PMS was 16µM. Gas phase: air. Initial pH of the reaction mixture was 6.52. The numbers along the trace are in $m\mu EqH^+/min$.

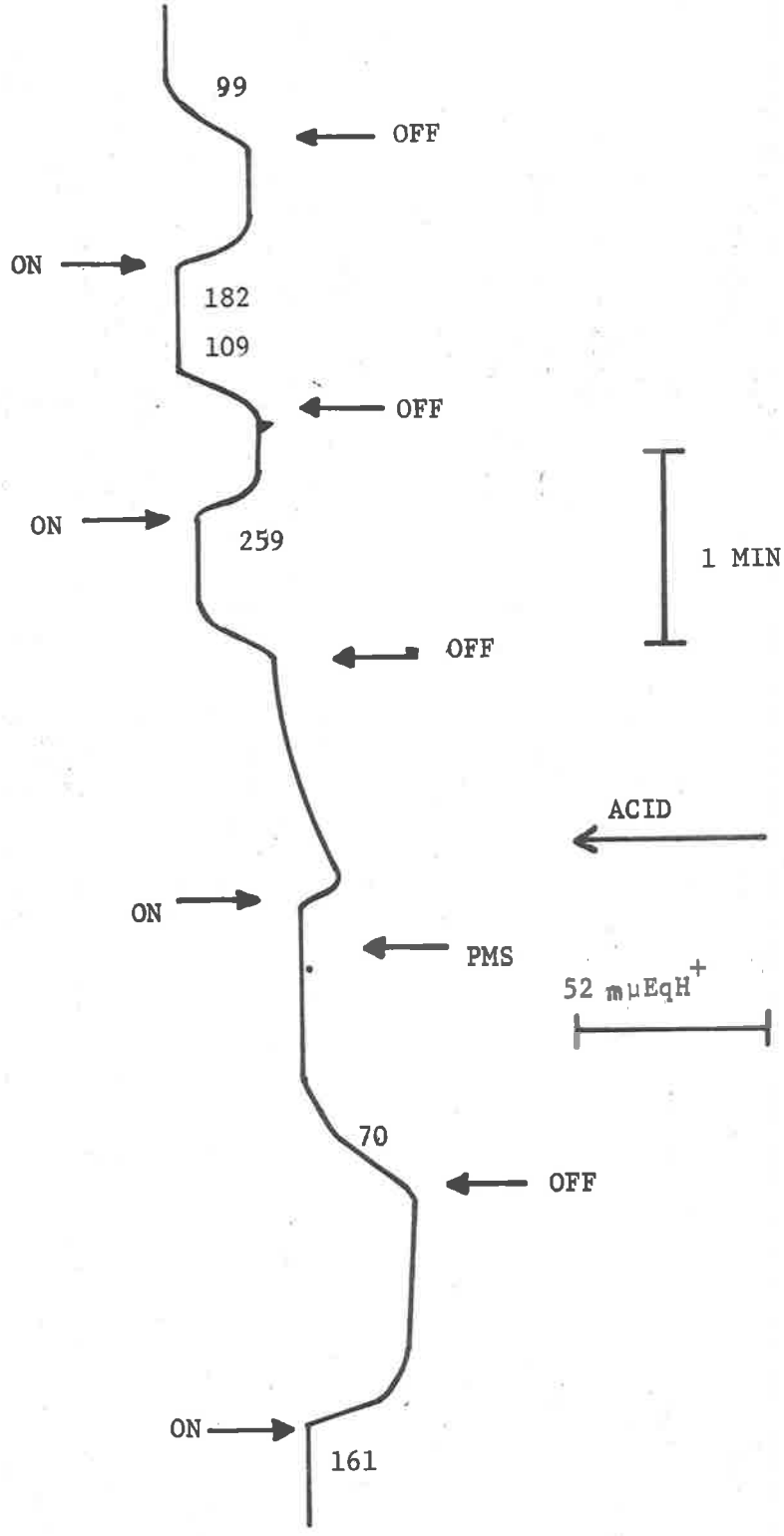
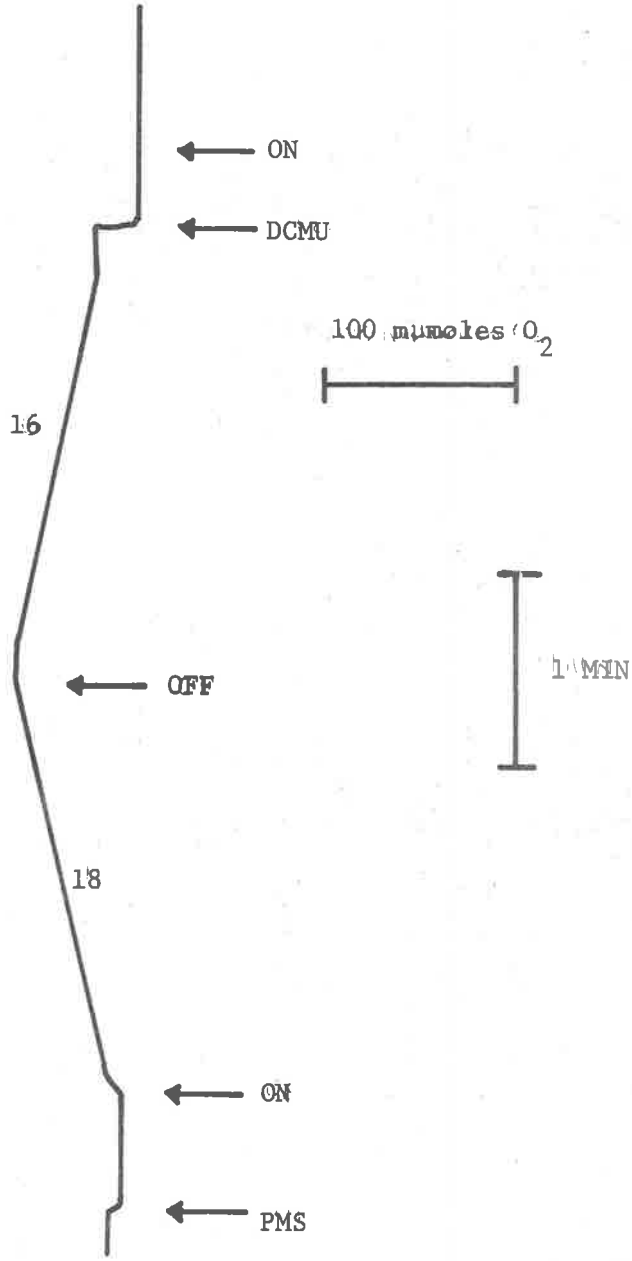

99
OFF
ON
182
109
OFF
ON
259
1 MIN
OFF
ACID
ON
PMS
52 mμEqH+
70
OFF
ON
161

FIGURE XX. Light-induced oxygen uptake by spinach chloroplasts in the presence of PMS. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5 and chloroplasts equivalent to 78µg chlorophyll. Concentration of DCMU was 1.9µM. The numbers along the trace are in µmoles O_2/mg chl/hr.


ON
DCMU
100 mμmoles O_2
16
1 MIN
OFF
18
ON
PMS

Jagendorf and Margulies (1960) observed that PMS (under aerobic conditions) was converted to pyocyanine by white light. Fig.XIX shows a pH trace with PMS added before illumination. The first illumination induced a slight rise in pH which decreased in the light to a steady level and decreased again when the light was turned off. Subsequent illuminations produced the typical reversible light-induced pH shift.

Fig.XX shows that PMS was able to catalyze a light-induced oxygen uptake. A slow rate of oxygen evolution was seen when the light was turned off, presumably due to the breakdown of hydrogen peroxide. DCMU inhibition of this light-induced oxygen uptake in the presence of PMS, indicated that PMS was acting as a Hill oxidant. The reaction can be represented by the following reaction sequence:

$$2PMS_{ox} + H_2O \xrightarrow{light} 2PMS_{red} + \tfrac{1}{2}O_2$$

$$2PMS_{red} + 2H^+ + O_2 \longrightarrow 2PMS_{ox} + H_2O_2$$

$$\text{Net result: } H_2O + \tfrac{1}{2}O_2 \xrightarrow{light} H_2O_2$$

If catalase was present in the reaction medium before illumination in the chloroplast suspension, no uptake of oxygen was observed: the oxygen consumed during the oxidation of reduced PMS was balanced by the oxygen liberated from the breakdown of hydrogen peroxide.

In view of the fact that PMS can catalyze pseudo-cyclic and cyclic electron flows, $P/2e^-$ ratios were determined (1) to determine

TABLE VII. $P/2e^-$ ratios obtained with phenazine methosulphate (PMS) as electron acceptor. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 10mM K_2HPO_4-KH_2PO_4 buffer, pH7.6; 40mM HEPES buffer, pH7.5; 50μM azide; 66μM PMS, 530,000 cpm ^{32}Pi and spinach chloroplasts equivalent to 55μg chlorophyll. ADP was added to a final amount of 5 μmoles. The variations occurred between different preparations of chloroplasts.

Additions	Electron Flow/Uptake (μg atoms O/mg chl/hr)	ATP^{32} Formation (μmoles/mg chl/hr)	$P/2e^-$ Ratios
Experiment I			
1. Control	108	182	1.68
2. +DCMU (3.3μM)	0	10	
Experiment II			
1. Control	90	237	2.60
2. +DCMU (3.3μM)	0	29	

whether the two types of electron flow could operate simultaneously, and (2) the contribution of each type to the amount of ATP formed during electron flow with PMS. Electron flow was determined from the rate of oxygen uptake catalyzed by PMS in the presence of ADP and Pi. Phosphorylation was measured by the incorporation of ^{32}Pi into ATP. Table VII shows the calculated $P/2e^-$ ratios thus obtained. It can be seen that the ratios are around 2.0, indicating that the bulk of the ATP formed could come from the pseudo-cyclic pathway, but some cyclic electron flow operates simultaneously in the presence of PMS under red light illumination.

DISCUSSION

A. NON-CYCLIC PHOTOPHOSPHORYLATION

Previously reported $P/2e^-$ ratios of around 1.0 have been obtained using broken or washed chloroplasts which contain a very high quantity of swollen chloroplasts (Leech, 1963). Avron and Neumann(1968) suggested that "the intactness of isolated chloroplasts has little or no importance in their ability to catalyze photophosphorylation". However, West and Wiskich (1968) had demonstrated photosynthetic control with chloroplast preparations containing a higher proportion of intact chloroplasts (Class I of Spencer and Unt, 1965), and obtained ADP/O ratios greater than 1.0 (average 1.5; West, 1969). This report came out shortly after the suggestions of Izawa and Good (1968) and Horton and Hall (1968) that the number of

phosphorylation sites could be more than 1.0, since they obtained ATP/$2e^-$ ratios greater than 1.0 consistently. This was questioned by Walker and Crofts (1970), who claimed that the situation was complicated by the possible presence of cyclic ATP production. The contribution from cyclic photophosphorylation could be ruled out when FeCN was used as the electron acceptor, since it has been shown that FeCN inhibits all types of cyclic photophosphorylation (Winget, Izawa and Good, 1965).

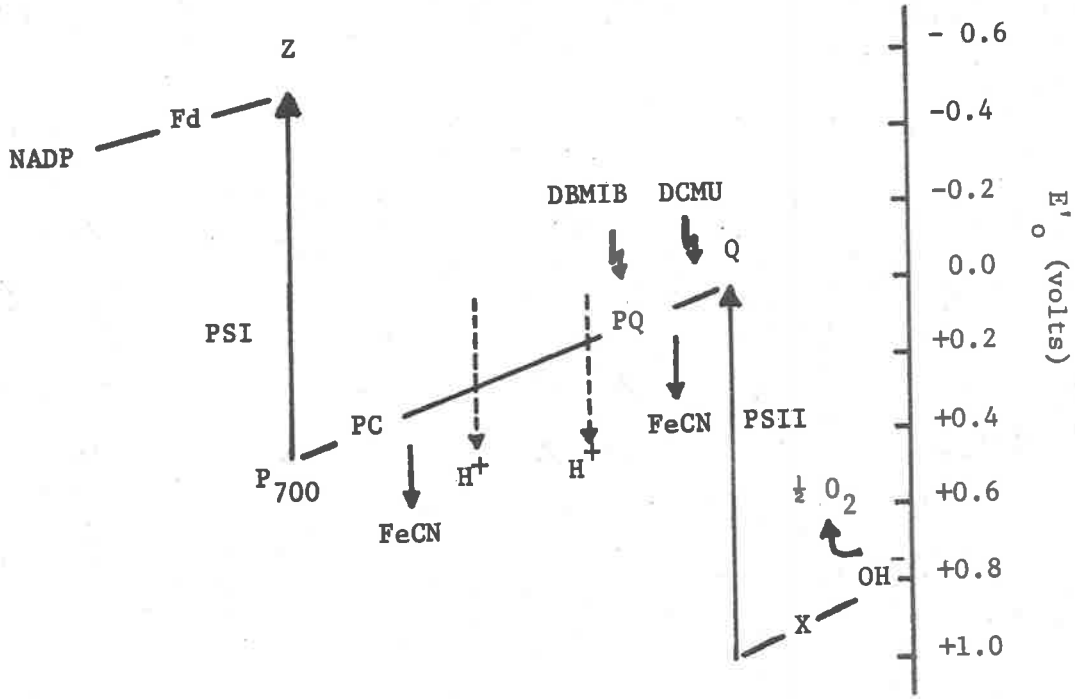
The use of FeCN as an electron acceptor presents some problems, as it could possibly accept electrons at more than one point on the photosynthetic electron flow pathway (Duysens and Amesz, 1967 and Boardman, 1968) depending on the experimental conditions. If some electrons are accepted before any of the proposed energy transducing sites, then the P/$2e^-$ and ADP/O ratios that have been deduced from these experiments are lower than actual ratios. The data on DBMIB inhibition of electron flow with FeCN supports the above idea. DBMIB inhibited the electron flow with FeCN only partially, whereas electron flow with MV was completely inhibited (Fig.VII). The DBMIB-insensitive electron flow with FeCN was not affected by uncouplers or ADP, suggesting that this residual electron flow was not coupled to phosphorylation (Table V). The lack of stimulatory effect of ADP on the DBMIB-inhibited electron flow has also been reported by Trebst, Harth and Draber (1970). This residual electron flow in the presence of DBMIB was completely inhibited by DCMU,

suggesting that FeCN was accepting electrons from somewhere between the DCMU (Duysens, 1964) and DBMIB (Bohme and Cramer, 1971) inhibition sites.

FeCN was also able to accept electrons at a point beyond the DBMIB inhibition site, as indicated by the sensitivity of the State 3 rate with FeCN. Measurements of $P/2e^-$ and ADP/O ratios (Forti, 1968; Hall, Reeves and Baltscheffsky, 1971; Izawa and Good, 1968; Horton and Hall, 1968 and West and Wiskich, 1968), and results in Table VII, indicate that there are two energy transducing sites operative during non-cyclic electron flow. Therefore both these energy transducing sites of phosphorylation must be located between the site of DBMIB inhibition and the second site of FeCN reduction.

ADP/O ratios with FeCN have been lower than those obtained with MV. It is now evident that the contribution to oxygen evolution from the first site of FeCN reduction (which is non-phosphorylating) is the cause of these lower ADP/O ratios. When this non-phosphorylating electron flow was subtracted from the State 3 rate, ADP/O ratios with FeCN became comparable to those obtained with MV (Table VI). In the calculations (Table VI), the rate of electron flow in the presence of 1μM DBMIB has been taken as the non-phosphorylating rate of electron flow. This rate was approximately equal to the State 4 (Fig.IXa) or ATP-inhibited rate (Fig.VIII) with FeCN as electron acceptor. The

latter rate has also been used to correct for non-phosphorylating electron flow (also Table VI). Similarly, Horton and Hall (1968) and Izawa and Good (1968) have employed the ATP-inhibited electron flow rate as the basal rate to calculate $P/2e^-$ ratios and obtained ratios of 2.0 and above. However, it must be stressed that the equivalence of DBMIB-inhibited rate with the ATP or ADP-inhibited rates is valid only for chloroplasts showing good coupling and control, because ATP or ADP would not inhibit uncoupled electron flow as does DBMIB (Table IV). It is therefore proposed that the correction for non-phosphorylating electron flow in the presence of 1μM DBMIB is a more valid one.



Scheme for photosynthetic electron flow showing the proposed sites of ferricyanide reduction between the two photosystems.

To explain the data, a scheme is proposed in which there are 2 sites of FeCN reduction; one near Photosystem II, but after the site of DCMU inhibition, and the other site near Photosystem I. The two energy transducing sites have been placed between the DBMIB inhibition site and Photosystem I, assuming that DBMIB inhibits near plastoquinone, as envisaged by Bohme and Cramer (1971).* It is interesting to note that Neumann et al. (1971) have also proposed two sites of phosphorylation between the two photosystems, using another quinone analog, 2,3-dimethyl-5-hydroxy-6-phytol-benzoquinone, as an inhibitor of electron flow. It is also assumed that FeCN reduction does not require the participation of Photosystem I as indicated by enhancement studies (Avron and Ben-Hayyim, 1969).

That the capacity to demonstrate photosynthetic control was not the sole property of intact (Class I) chloroplasts was further shown by Telfer and Evans (1971), when they demonstrated photosynthetic control with broken chloroplasts. However, the control ratios they obtained were very low (1.7 - 2.4) compared to those presented in Tables I and III. Even their "whole" chloroplasts do not show comparable control ratios. Thus, although an intact outer membrane may not be essential to demonstrate good photosynthetic control, intactness of the outer membrane would help retain its natural environment including the soluble proteins necessary of carbon dioxide fixation. Chloroplasts lose their ability to fix carbon dioxide fixation when subjected briefly to osmotic shock and electron microscopy showed this to be correlated to the loss of the outer

*Footnote: The potential span between 'Q' and plastoquinone has to accomodate the sites of DCMU inhibition and FeCN reduction as well as cytochrome b_{559}, therefore it seems quite unlikely that a proton translocating loop is situated between the first site of FeCN reduction and the DBMIB site of inhibition.

membrane (Walker and Crofts, 1970). Similar treatment and ultrasonic disintegration have been shown to affect the photosynthetic control and ADP/O ratios, the latter treatment having a more drastic effect (Table II).

B. PSEUDO-CYCLIC AND CYCLIC PHOTOPHOSPHORYLATION

The DCMU-sensitivity of the PMS catalyzed photophosphorylation demonstrates that PMS can be reduced by Photosystem II. The reduced PMS, instead of reducing an intermediate in the cytochrome chain, as would be expected of cyclic electron acceptors (Boardman, 1968), was being re-oxidised by oxygen, thus acting as a pseudo-cyclic electron acceptor. PMS has generally been accepted as a cyclic electron acceptor because only low rates of oxygen exchange were observed to be coupled with high rates of phosphorylation (Krall et al., 1961). However, Schwartz (1967) also found considerable rates of oxygen exchange catalyzed by PMS, and that DCMU inhibited this reaction. He came to the conclusion that PMS acts as a Hill oxidant, but at a reduced efficiency. This is in agreement with the oxygen uptake activity observed in the presence of PMS (Fig. XX); this oxygen uptake was also sensitive to DCMU. The view that PMS is primarily a pseudo-cyclic electron acceptor is further supported by the evidence that PMS-catalyzed photophosphorylation, under aerobic conditions, was inhibited to about the same extent by varying concentrations of DCMU, as was the coupled electron transport with FeCN.

Jagendorf and Margulies (1960) and Hauska et al. (1970) observed

that CMU and DCMU inhibited PMS-catalyzed phosphorylation in red light, but not under white light. The former authors also showed that white light reduced PMS non-enzymically, thus explaining why DCMU has no effect in this system.

DCMU inhibition of this phosphorylation was relieved by adding either ascorbate or NADH. This indicates that PMS could not be reduced to catalyze phosphorylation when Photosystem II was blocked by DCMU. If PMS catalyzes a truly cyclic phosphorylation under these conditions, it should be able to catalyze it after the initial reduction by Photosystem II that occurs before DCMU inhibition. However the phosphorylation is unable to proceed in the presence of DCMU unless an exogenous reductant is added. This second phosphorylation which is insensitive to DCMU would be the true cyclic phosphorylation. Jagendorf and Margulies (1960) also found that ascorbate relieved CMU inhibition of the PMS-catalyzed phosphorylation. The phosphorylation catalyzed by PMS in digitonin subchloroplast particles is the same as when Photosystem II is blocked by DCMU in intact chloroplasts. That the phosphorylation in these particles is very resistant to DCMU indicates that this PMS catalyzed phosphorylation is truly cyclic in nature (see Chapter VII).

The stimulation of the light-induced pH rise by PMS was thought to be due to increased cyclic electron flow, since PMS has previously been considered a cyclic electron acceptor. DCMU inhibition of this increase in the rate of pH rise further suggests that PMS does not catalyze a truly cyclic electron flow under these conditions.

The DCMU inhibition indicates that when Photosystem II is blocked, PMS can no longer catalyze a cyclic phosphorylation. Assuming a $P/2e^-$ ratio of 2.0 for non-cyclic phosphorylation (i.e. two energy transducing sites), $P/2e^-$ ratios determined with PMS would indicate any contribution from cyclic photophosphorylation. Results in Table VII indicate that pseudo-cyclic and cyclic electron flow could operate simultaneously. Pseudo-cyclic and cyclic electron flow was apparent when Photosystem II reduces PMS and cyclic electron flow alone was apparent when Photosystem II was inhibited (or absent), and an exogenous reductant was present to reduce PMS non-enzymically. However, difficulties arise when an effort is made to determine the relative contribution of each phosphorylating pathway or to compare the relative rates of each pathway. Under aerobic conditions with PSII operating (as in Table VI) the flow of electrons from PSII tends to keep the chain reduced and hence limit cyclic flow. This effect is aggravated by the slow overallreaction with PMS as compared to that with methyl viologen. On the other hand, chemical reduction of PMS (e.g. ascorbate) tends to keep the PMS completely reduced and limits the amount of acceptor available for cyclic flow.

CHAPTER IV

EFFECT OF UNCOUPLERS ON THE PHOTOCHEMICAL ACTIVITIES OF ISOLATED CHLOROPLASTS

INTRODUCTION

Uncouplers have been considered to stimulate electron flow while inhibiting photophosphorylation (Good, Izawa and Hind, 1960). Mitchell (1966) and Chappell and Crofts (1966) proposed that uncouplers could act by transporting protons across membranes, dissipating the energy gradient in the process. It has also been considered that uncouplers act by hydrolyzing "high energy" intermediates (Slater, 1966).

This chapter will describe studies on the effects of some uncouplers on the various photochemical activities of isolated chloroplasts.

A. NH_4Cl AND VALINOMYCIN

RESULTS

Ammonia (Krogmann, Jagendorf and Avron, 1959) and other amines (Good, 1960 and Hind and Whittingham, 1963) have been found to be uncouplers of photophosphorylation. NH_4Cl uncoupling of the Hill reaction was found by Good (1960) and Hind and Whittingham (1963) to be pH-dependent, but Stiller (1965) has shown that the uncoupling effect is relatively pH-independent, if correction is made for the change in concentration of NH_3 with pH. This is in

line with Crofts' (1967) proposal that the active species of amine in uncoupling is the uncharged species, NH_3. McCarty (1969) found NH_4Cl to be a poor uncoupler of photophosphorylation in subchloroplast particles prepared by sonication but the effect was greatly enhanced by the addition of valinomycin. Neumann and Jagendorf (1964) found that NH_4Cl inhibited the reversible light-induced pH rise in chloroplasts.

Valinomycin was found by Murray and Begg (1959) to be a potent uncoupler of oxidative phosphorylation. It has also been found to increase the K^+ ion permeability of mitochondrial (Moore and Pressman, 1964 and Chappell and Crofts, 1965) and chloroplast membranes (Shavit, Dilley and San Pietro, 1968 and Junge and Schmid, 1971).

Avron and Shavit (1965) found that valinomycin had no effect on the photoreactions tested by them, up to concentrations of 10μM, but at higher concentrations it acted as a weak uncoupler. McCarty (1970) observed that valinomycin stimulated H^+ uptake, as well as the formation of X_E in subchloroplast particles obtained by sonication or digitonin treatments. Keister and Minton (1970) found that valinomycin, in the absence of KCl, acts as an energy transfer inhibitor similar to Dio-9, phloridzin and synthalin, in spinach chloroplasts. However unlike the other energy transfer inhibitors, the inhibition by valinomycin was only partially reversed by uncouplers. Similar observations have been made by Karlish and Avron (1970) who found that valinomycin stimulated the light-induced H^+ uptake and H/e^- ratio.

TABLE I. NH_4 uncoupling of State 3 rate of oxygen evolution with FeCN as electron acceptor in pea chloroplasts. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 13mM K_2HPO_4-KH_2PO_4 buffer, pH7.6; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and 33µg chlorophyll. ADP was added to a final amount of 0.475 µmole.

Additions	Electron Flow (µmoles O_2/mg chl1/hr)
Control	268
NH_4Cl 0.88mM	268
NH_4Cl 1.77mM	329
NH_4Cl 3.52mM	355

TABLE II. NH_4Cl uncoupling of the Hill Reaction with FeCN as electron acceptor in pea chloroplasts. The reaction mixture contained the same components as in Table I, except that the phosphate buffer was absent.

Additions	Electron Flow (μmoles O_2/mg chll/hr)
Control	120
NH_4Cl 0.95mM	250
NH_4Cl 1.90mM	298
NH_4Cl 3.77mM	346
NH_4Cl 9.30mM	332

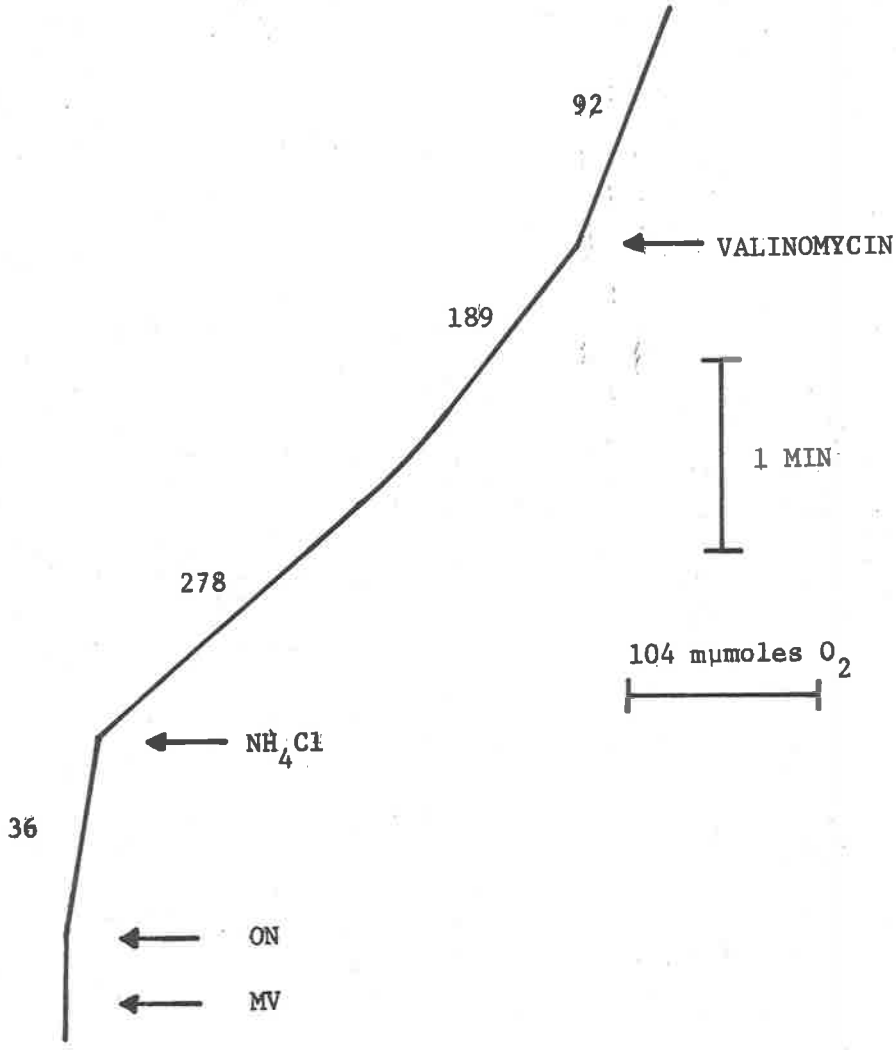


FIGURE I. Effect of NH_4Cl and valinomycin on electron flow with MV as electron acceptor. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 64μM MV; 0.93mM and pea chloroplasts equivalent to 26μg chlorophyll. Concentrations of NH_4Cl and valinomycin were 3.8mM and 4.7μM respectively.

TABLE III. Effect of NH_4Cl on the Hill Reaction and State 3 rates with MV as the electron acceptor in pea chloroplasts. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 64μM MV; 0.93mM azide and 58μg chlorophyll. 13mM K_2HPO_4-KH_2PO_4 buffer and 0.475 μmole ADP were also present during State 3 rates.

Additions	Oxygen Uptake (μmoles/mg chll/hr)
Hill Reaction	100
+ NH_4Cl	249
State 3	175
+ NH_4Cl	209

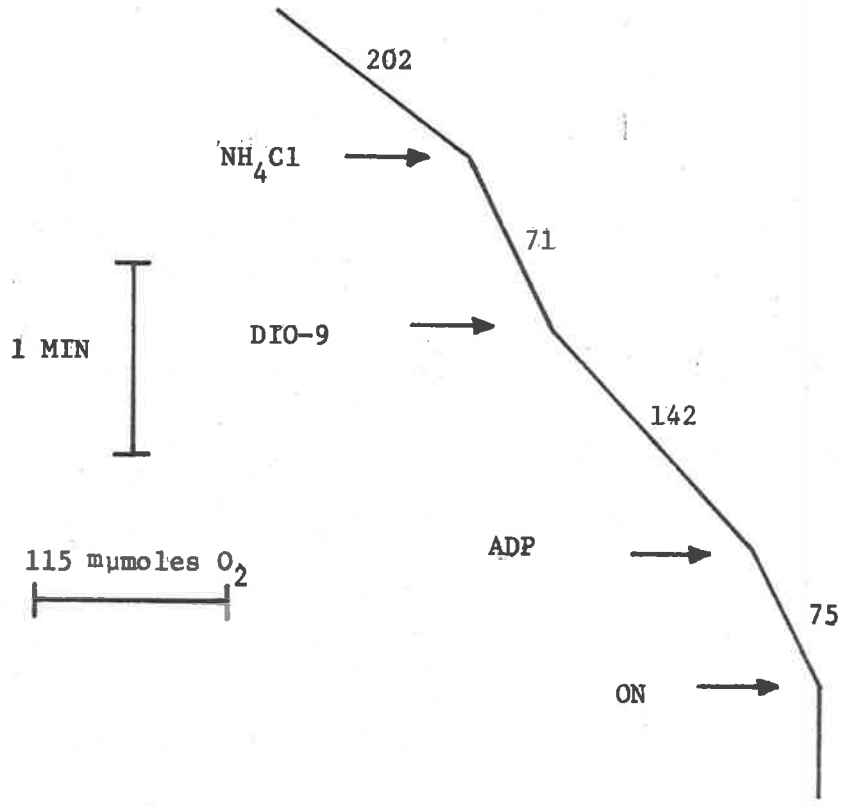


FIGURE II. Inhibition of the State 3 rate of electron flow by Dio-9. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 13mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 40mM HEPES buffer, pH7.5; 1.5mM FeCN a pea chloroplasts equivalent to 46μg chlorophyll. ADP was added to a final amount of 0.475 mole. Concentrations of Dio-9 and NH_4Cl were 21μg/ml and 1.7mM respectively. The numbers along the traces are in moles O_2/mg chll/hr

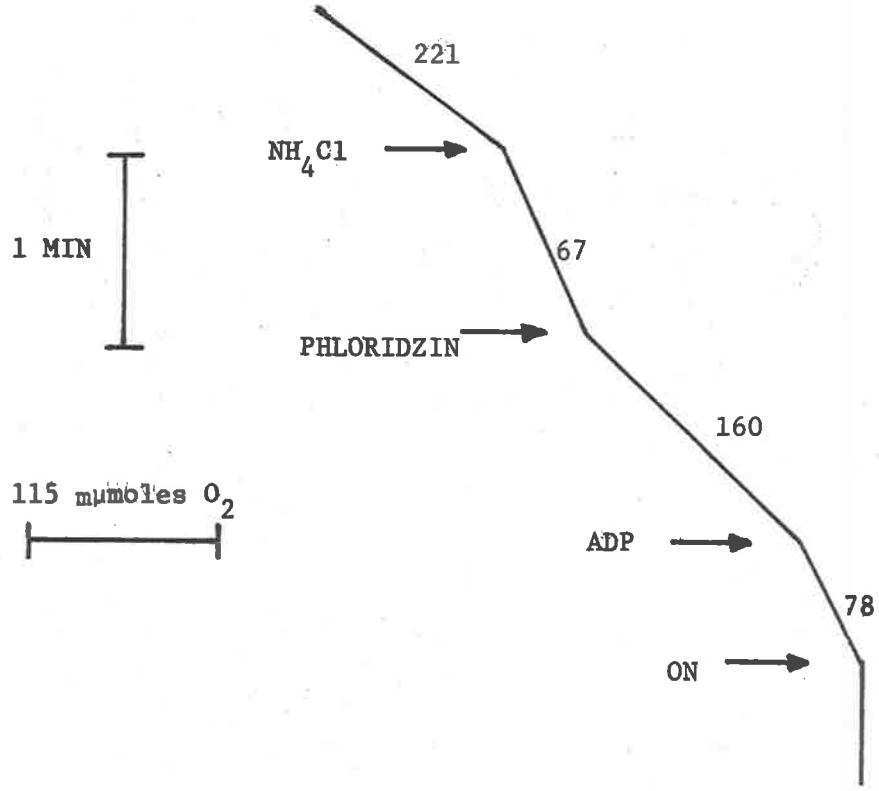


FIGURE III. Inhibition of the State 3 rate of electron flow by phloridzin. The reaction mixture was the same as in Fig.II. Phloridzin and NH_4Cl were added to a final concentration of 0.54mM and 1.7mM respectively. The numbers along the traces are in μmoles O_2/mg chl1/hr.

Table I shows that the State 3 rate with FeCN as electron acceptor was stimulated considerably by NH_4Cl, indicating that in these chloroplasts, electron transport was controlled by phosphorylation. The same concentration of NH_4Cl stimulated the Hill reaction rate much more than the State 3 rate (Table II). The largest stimulation was attained at 3.77mM NH_4Cl concentration, the stimulated rate decreasing as the concentration of the uncoupler was increased. This decrease in stimulation of the Hill reaction could be due to swelling of chloroplasts in the presence of NH_4Cl (Packer and Crofts, 1967) or to damage of the electron flow chain by high concentrations of the uncoupler. Fig.I shows that oxygen uptake by reduced MV was stimulated by the addition of NH_4Cl. This stimulated rate of oxygen uptake decreased with time and the addition of valinomycin had no effect on it. Similar stimulatory effects of other uncouplers were observed in the Hill reaction and State 3 rates with MV as the electron acceptor (Table III).

Phloridzin and Dio-9, the energy transfer inhibitors (Izawa, Winget and Good, 1966 and McCarty, Guillory and Racker, 1965) inhibited State 3 rates with FeCN as electron acceptor (Fig.II), but the inhibition was relieved by the addition of NH_4Cl. Phloridzin also inhibited the State 3 rate with MV as the electron acceptor, and, as observed with FeCN, this inhibition was also relieved by NH_4Cl (Fig.III). McCarty, Guillory and Racker (1965) and Izawa, Winget and Good (1966) have reported similar findings.

TABLE IV. Effect of NH_4Cl and Valinomycin on cyclic photophosphorylation in pea chloroplasts. The reaction mixture contained 0.4M sucrose; 8.7mM $MgCl_2$; 14mM K_2HPO_4-$KH_2PO_4^*$; 3.4μM DCMU: 17μM PMS; 3.4mM ascorbate and 47μg chlorophyll. ADP was added to a final amount of 0.475 μmole. *pH of Pi buffer, 8.0.

Additions	ATP formation (μ Eq H^+/mg chll/hr)
Control	496
NH_4Cl 0.7mM	175
NH_4Cl 1.4mM	125
Valinomycin 1.7μM	328
NH_4Cl 0.7mM + Valinomycin 1.7μM	0

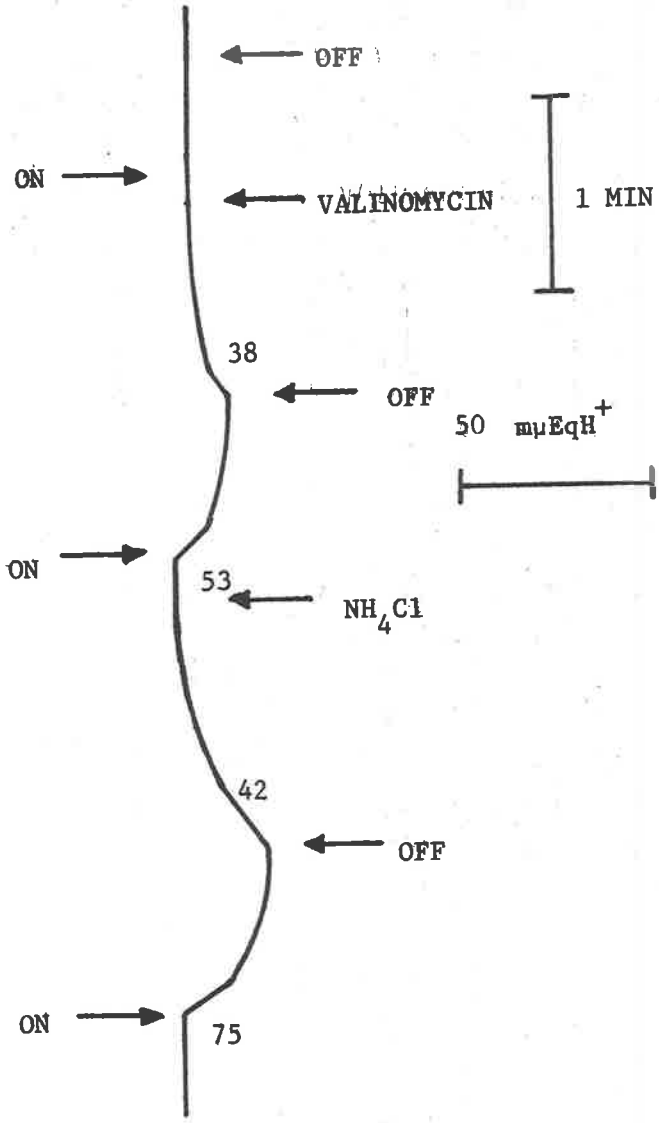


FIGURE IV. NH_4Cl and valinomycin on the cofactor-less light-induced proton uptake in pea chloroplasts. The reaction mixture contained 0.4M sucrose; 6.5mM KCl and washed chloroplasts equivalent to 66μg chlorophyll. Concentrations of NH_4Cl and valinomycin were 0.65mM and 1.6μM respectively. The numbers along the traces are in $m\mu EqH^+$/min.

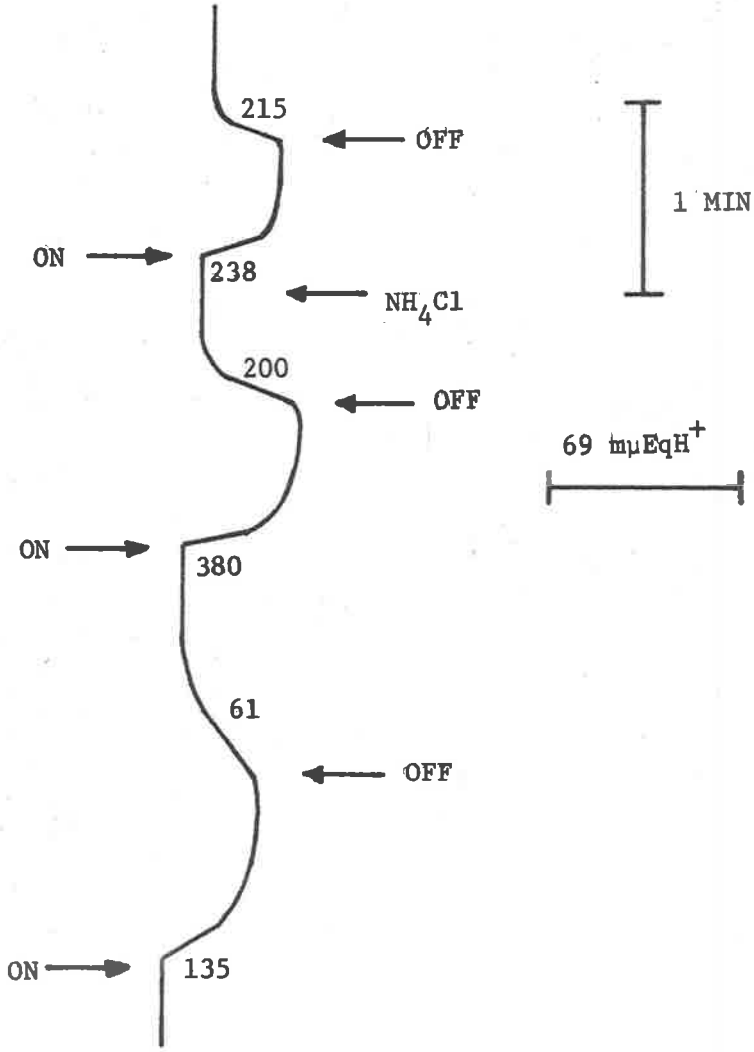


FIGURE V. NH_4Cl on the PMS-catalyzed light-induced proton uptake. The reaction mixture was the same as in Fig.IV. Concentrations of PMS and NH_4Cl were 15μM and 1.3mM respectively. The numbers along the traces are in $m\mu EqH^+$/min.

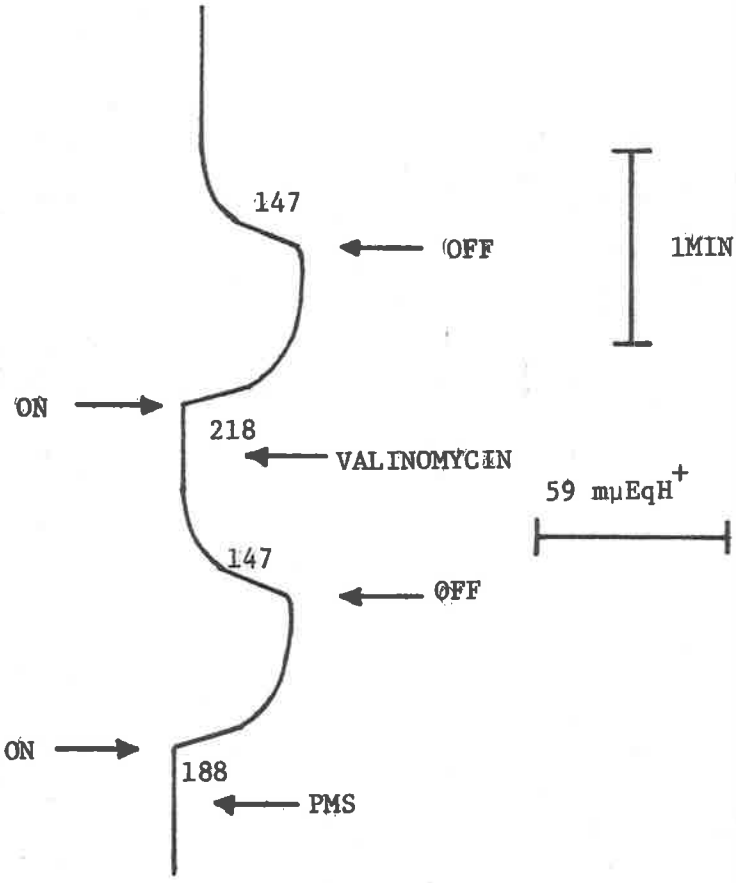


FIGURE VI. Valinomycin on the PMS-catalyzed light-induced proton uptake. The reaction mixture was the same as in Fig.IV. Concentrations of PMS and valinomycin were 15μM and 1.6μM respectively. The numbers along the sides are in $m\mu EqH^+$/min.

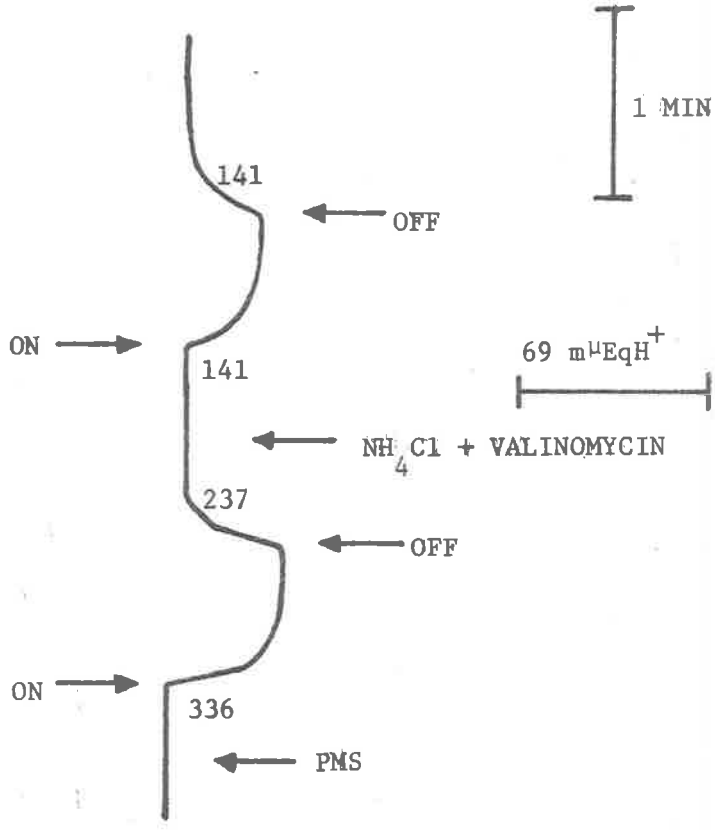


FIGURE VII. Effect of NH_4Cl and valinomycin on the PMS-catalyzed light-induced proton uptake. The reaction mixture was the same as in Fig.V. Concentration of valinomycin was 1.6μM.

NH_4Cl also uncouples PMS-catalyzed cyclic photophosphorylation (Table IV). This uncoupling was greatly enhanced by the addition of valinomycin. The antibiotic on its own in the absence of K^+ ions, inhibited cyclic photophosphorylation catalyzed by PMS, and basal and coupled electron flow with FeCN. Synergistic uncoupling of cyclic photophosphorylation by NH_4Cl and valinomycin, as well as inhibition of photophosphorylation by valinomycin in the absence of K^+ ions, has been demonstrated (McCarty, 1969 and Keister and Minton, 1970).

When chloroplasts suspended in lightly-buffered or unbuffered media were illuminated, a pH rise was observed (Jagendorf and Hind, 1963). NH_4Cl inhibited the extent of this light-induced H^+ uptake in the absence of a cofactor (Fig.IV), The rate of formation (k_f) of this pH rise was also inhibited, but the rate of decay (k_d) did not appear to be affected. Jagendorf and Neumann (1965) observed similar inhibitory effects, but also found that NH_4Cl accelerated the rate of decay. When valinomycin was added in addition to NH_4Cl, the light-induced H^+ uptake was completely abolished. NH_4Cl also inhibited the H^+ uptake in the presence of PMS (Fig.V). Valinomycin on its own did not affect the extent of the H^+ uptake in the presence of PMS (Fig.VI), but when present with NH_4Cl, H^+ uptake was inhibited to a greater degree than it was by NH_4Cl alone (Fig.VII). Stimulation of the extent of H^+ uptake in the presence of pyocyanine, by valinomycin has been observed by Karlish and Avron (1971) at pH8. At a lower

pH (6.3) they found that it did not stimulate the extent of H^+ uptake, but the influx and efflux of H^+ ions were stimulated over the entire pH range used.

DISCUSSION

The Hill reaction rate was stimulated progressively by increasing concentrations of NH_4Cl, reaching a maximum around 4mM, after which the stimulated rate slowly declined (Table II). Crofts (1967) proposed that the active and uncharged species NH_3, entered the chloroplast freely and was protonated by the H^+ taken up during electron flow under illumination. Thus the concentration of H^+ inside the chloroplast was lowered and the H^+ gradient dissipated, causing increased H^+ uptake and electron flow. However, as the NH_4^+ ions accumulate inside the chloroplast, they could set up a "back pressure" of electric potential and limit the rate of H^+ uptake, resulting in decreased electron flow. Packer and Crofts (1967) claimed that NH_4^+ ions did not freely permeate chloroplast membranes; therefore it was thought that the addition of valinomycin could alleviate this "back pressure" by rendering the chloroplast membrane more permeable to NH_4^+ ions. However, it has been shown that valinomycin did not relieve the inhibition (Fig.I), indicating that it may not have been caused by accumulation of NH_4^+ ions inside the chloroplasts. Alternatively, the inhibition could be due to structural

damage as a consequence of swelling in the presence of high concentrations of NH_4Cl (Deamer and Packer, 1967).

NH_4Cl also uncoupled the State 3 rate of oxygen evolution (Table I) and cyclic photophosphorylation catalyzed by PMS (Table IV). The uncoupling of cyclic photophosphorylation by NH_4Cl was enhanced by valinomycin (Table IV) and was independent of added K^+ ions. Such synergistic uncoupling has been reported in chloroplasts and subchloroplast particles prepared by sonication (McCarty, 1969). These results are interpreted to indicate that NH_4Cl uncouples by dissipating the H^+ gradient (Neumann and Jagendorf, 1964), resulting in net accumulation of NH_4^+ ions inside the chloroplast (Crofts, 1967). These NH_4^+ ions then move out in the presence of valinomycin, causing the collapse of the membrane potential. Since NH_4Cl on its own is quite an effective uncoupler, it seems unlikely that the membrane potential plays a large role in phosphorylation in chloroplasts. This synergistic uncoupling was also reflected in the inhibition of the light-induced H^+ uptake with PMS (Fig.V), and was also independent of K^+ ions. It is reasonable, therefore, to assume that valinomycin renders the chloroplast membrane permeable to NH_4^+ ions. Similar observations on the valinomycin induced permeability of the mitochondrial membrane to NH_4^+ ions have been reported (Cockrell, 1969). Montal, Chance and Lee (1970) have also observed that the extent of respiration-dependent H^+ uptake in submitochondrial particles was progressively inhibited by increasing concentrations of NH_4Cl in the presence of valinomycin.

The proton pump in these submitochondrial particles operated in the same direction as in chloroplasts, i.e. activation of electron flow in submitochondrial particles resulted in proton uptake by these particles (Mitchell and Moyle, 1965 and Chance and Mela, 1967).

Therefore, it is concluded that NH_4Cl at high concentrations causes inhibition of electron flow and is due to structural effects on the electron flow chain.

B. CCCP AND VALINOMYCIN

RESULTS

Heytler and Pritchard (1962) found CCCP to be a potent inhibitor of PMS-catalyzed cyclic photophosphorylation as well as of oxidative phosphorylation in mitochondria. Bamberger, Black, Fenson and Gibbs (1963) showed that the uncoupler had no effect on NADP reduction at concentrations as high as 10^{-5}M, whereas phosphorylation was inhibited at 10^{-7}M. De Kiewiet (1965) found CCCP to be a powerful inhibitor of both photophosphorylation and oxygen evolution, the latter activity affected only at higher concentrations. Similar observations were also made by Plengridhya and Burris (1965). De Kiewiet, Hall and Jenner (1965) proposed that the site of inhibition of CCCP on the Hill reaction was at the oxygen evolving reaction centre. Katoh and San Pietro (1967), studying the electron transport system of *Euglena* chloroplasts, observed an inhibition of ascorbate-supported NADP reduction by

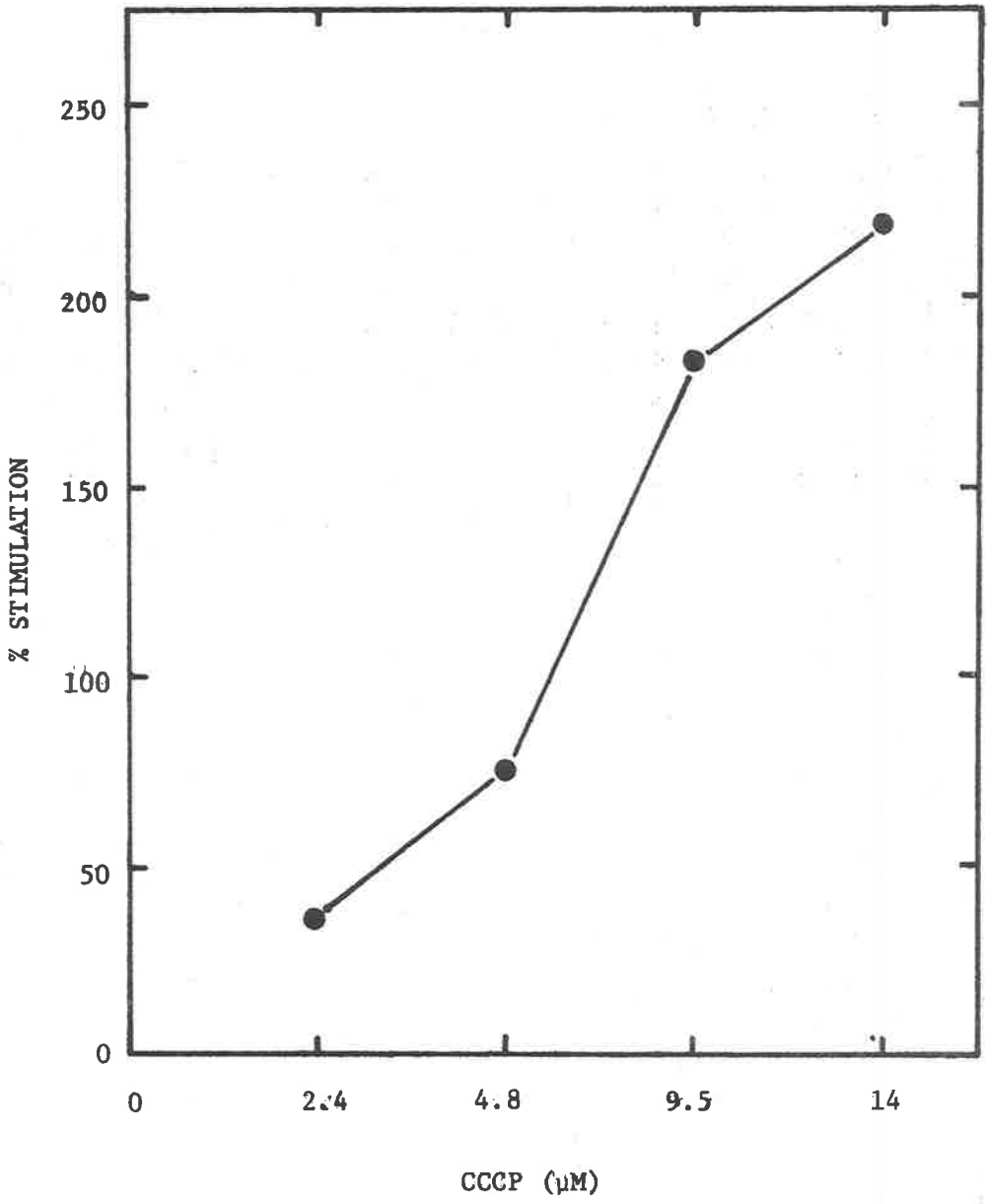


FIGURE VIII. The stimulation of electron flow by CCCP. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and pea chloroplasts equivalent to 43μg chlorophyll. Control rate of electron flow was 73 μmoles O_2/mg chll/hr.

CCCP, a similar effect to that observed with classical inhibitors of oxygen evolution such as DCMU and O-phenanthroline (Cheniae, 1970). The complexity of the action of CCCP on photosynthetic electron transport was further revealed by the observations of Mantai (1969) with trypsin-treated spinach chloroplasts. He found that CCCP inhibited the trypsin-uncoupled electron transport and this inhibition was concentration dependent. However, in untreated chloroplasts, CCCP (up to concentrations of 33μM) uncoupled the electron transport and did not inhibit electron flow. Mantai (1969) concluded that CCCP, as well as acting as an efficient uncoupler, could also act as an electron transport inhibitor at another site, under certain conditions.

Data presented by Renger (1969) and Itoh, Yamashita, Nishi, Konishi and Shibata (1969) suggested that the site of inhibition by CCCP was located on the oxidizing side of PSII. This was further supported by Homann (1971) who studied the actions of CCCP on the fluorescence properties and electron transport of EDTA or methylamine-uncoupled chloroplasts. This author found that CCCP acted at two points in these chloroplasts: on the reducing side of PSI and on the oxidising side of PSII.

Fig.VIII shows the stimulation of oxygen evolution by CCCP during Hill reaction with FeCN as the electron acceptor. Contrary to the observations of De Kiewiet, Hall and Jenner (1965) that 50% inhibition of oxygen evolution was obtained with 4.5μM CCCP, it was found that CCCP in concentrations of up to 14μM, stimulated oxygen

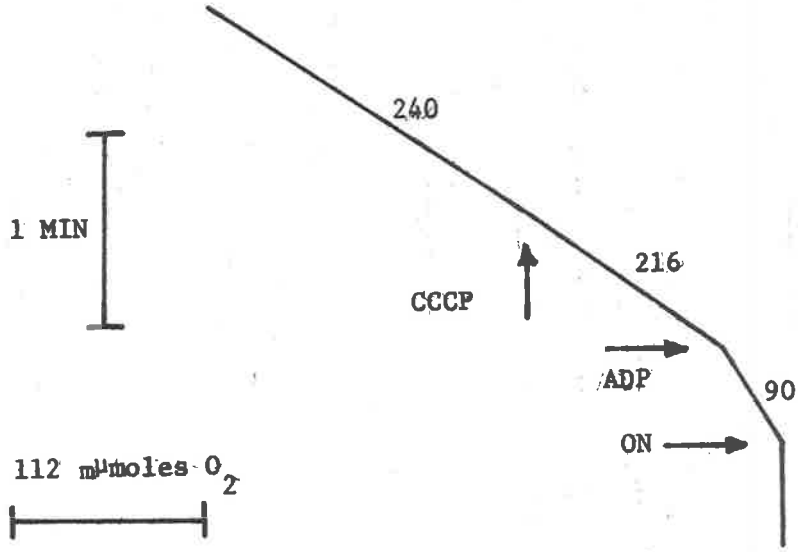


FIGURE IX. Uncoupling of State 3 rate of electron flow by CCCP. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 13mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and pea chloroplasts equivalent to 43μg chlorophyll. ADP was added to a final amount of 0.475 μmole. The concentration of CCCP was 2.2μM. The numbers along the traces are in μmoles O_2/mg chll/hr.

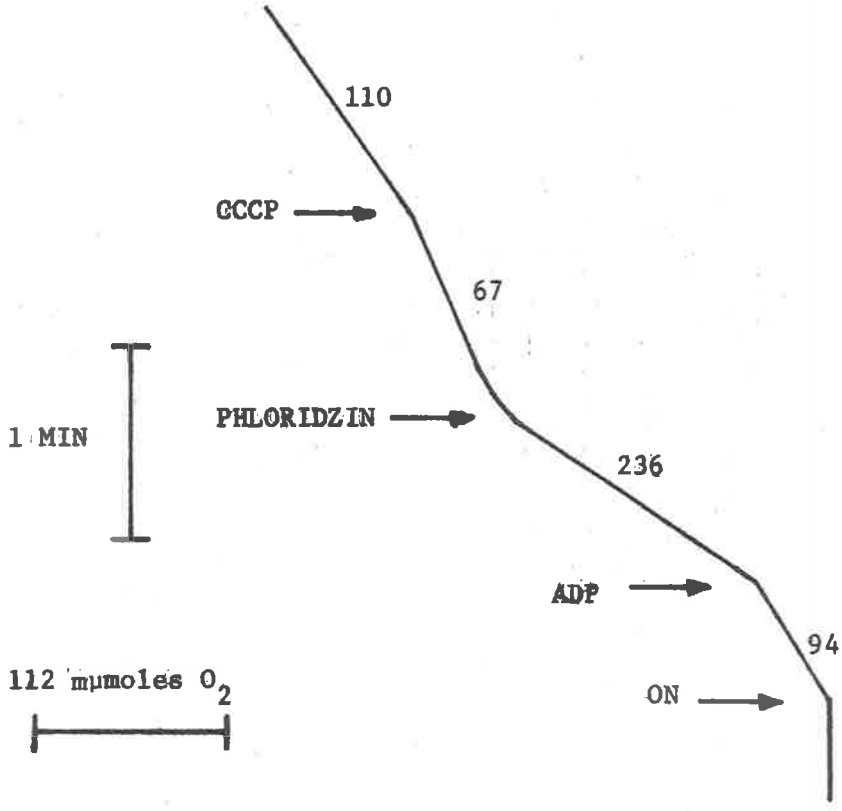


FIGURE X. Effect of CCCP on the phloridzin-inhibited State 3 rate of electron flow. The reaction mixture was the same as in Fig. IX. ADP was added to a final amount of 0.475 μmole. Concentrations of phloridzin and CCCP were 0.54mM and 2.2μM respectively.

TABLE V. Uncoupling of the Hill Reaction and State 3 rates by suboptimal concentration of CCCP and Valinomycin. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 21mM KCl; 1.5mM FeCN and 44µg chlorophyll. ADP was added to a final amount of 0.475 µmole and 17mM K_2HPO_4-KH_2PO_4 buffer, pH7.6, where specified.

Additions	Electron Flow (µmoles O_2/mg chl1/hr)
Hill Reaction	73
CCCP 2.37µM	99
CCCP + Valinomycin 2.30µM	271
State 3	216
CCCP 2.20µM	240
CCCP + Valinomycin 2.30µM	302

TABLE VI. Effect of CCCP and Valinomycin on cyclic photophosphorylation in pea chloroplasts. The reaction mixture contained the same components as in Table IV, except that 21mM KCl was also present.

Additions	ATP formation (μ Eq H^+/mg chll/hr)
Control	495
CCCP 0.34μM	282
Valinomycin 1.73μM	328
CCCP + Valinomycin	0

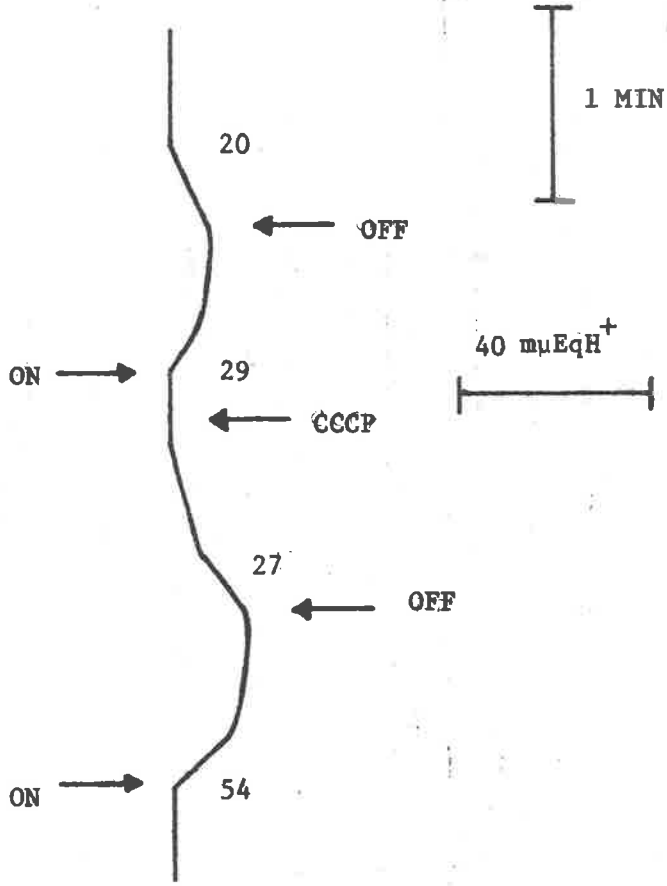


FIGURE XI. CCCP on the cofactor-less light-induced proton uptake. The reaction mixture contained 0.4M sucrose; 6.5mM KCl and washed pea chloroplasts equivalent to 66μg chlorophyll. Concentration of CCCP was 1.6μM. The numbers along the traces are in $m\mu EqH^+$/min. Initial pH of reaction mixture was 6.1.

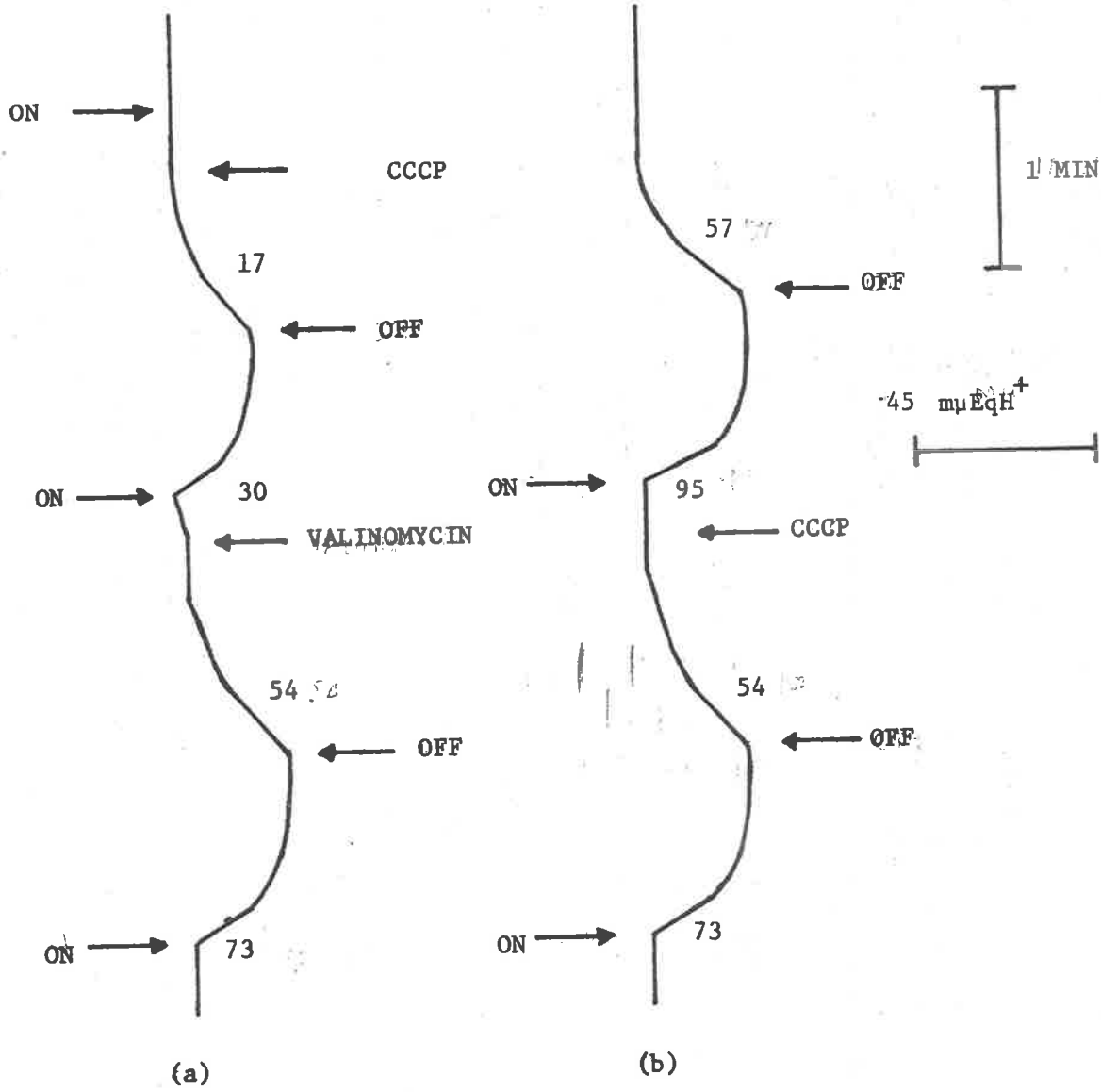


FIGURE XII. Valinomycin and CCCP on the cofactor-less light induced proton uptake. The reaction mixture was the same as in Fig.XI. Concentrations of valinomycin and CCCP were 1.6μM and 0.15μM respectively. The numbers along the traces are in $m\mu EqH^+$/min. Initial pH of reaction mixture was 6.1.

evolution. Mantai (1969) also observed stimulation of DCPIP reduction by CCCP concentrations of up to 33μM.

State 3 rates of oxygen evolution were stimulated by CCCP (Fig.IX) which also relieved the inhibition of State 3 rate by phloridzin (Fig.X). Izawa, Winget and Good (1966) found that the inhibition of coupled electron transport by phloridzin was completely eliminated when methylamine, atebrin or FCCP was present. The uncoupling of State 3 and the Hill reaction rates by suboptimal concentration of CCCP was greatly enhanced by valinomycin (Table V). Von Stedingk (1970) observed that FCCP, at an ineffective concentration almost completely inhibited photophosphorylation with PMS when valinomycin was present. This synergistic effect of low concentrations of CCCP with valinomycin was also observed in PMS-catalyzed cyclic photophosphorylation, as shown in Table VI. CCCP alone, at the concentration used, inhibited the phosphorylation only 40%, while valinomycin on its own was not effective. However when the two were added together the phosphorylation was completely inhibited.

The cofactor-less, light-induced pH rise in pea chloroplasts was also inhibited by CCCP at 1.6μM, as shown in Fig.XI. Valinomycin on its own inhibited this light-induced pH rise slightly (Fig.XII). but when CCCP was added the pH rise was completely abolished. This synergistic effect was observed at a CCCP concentration which had no effect on the extent of this pH rise (Fig. XII). Karlish and Avron (1970) found that FCCP up to 0.1μM concentration increased the rate

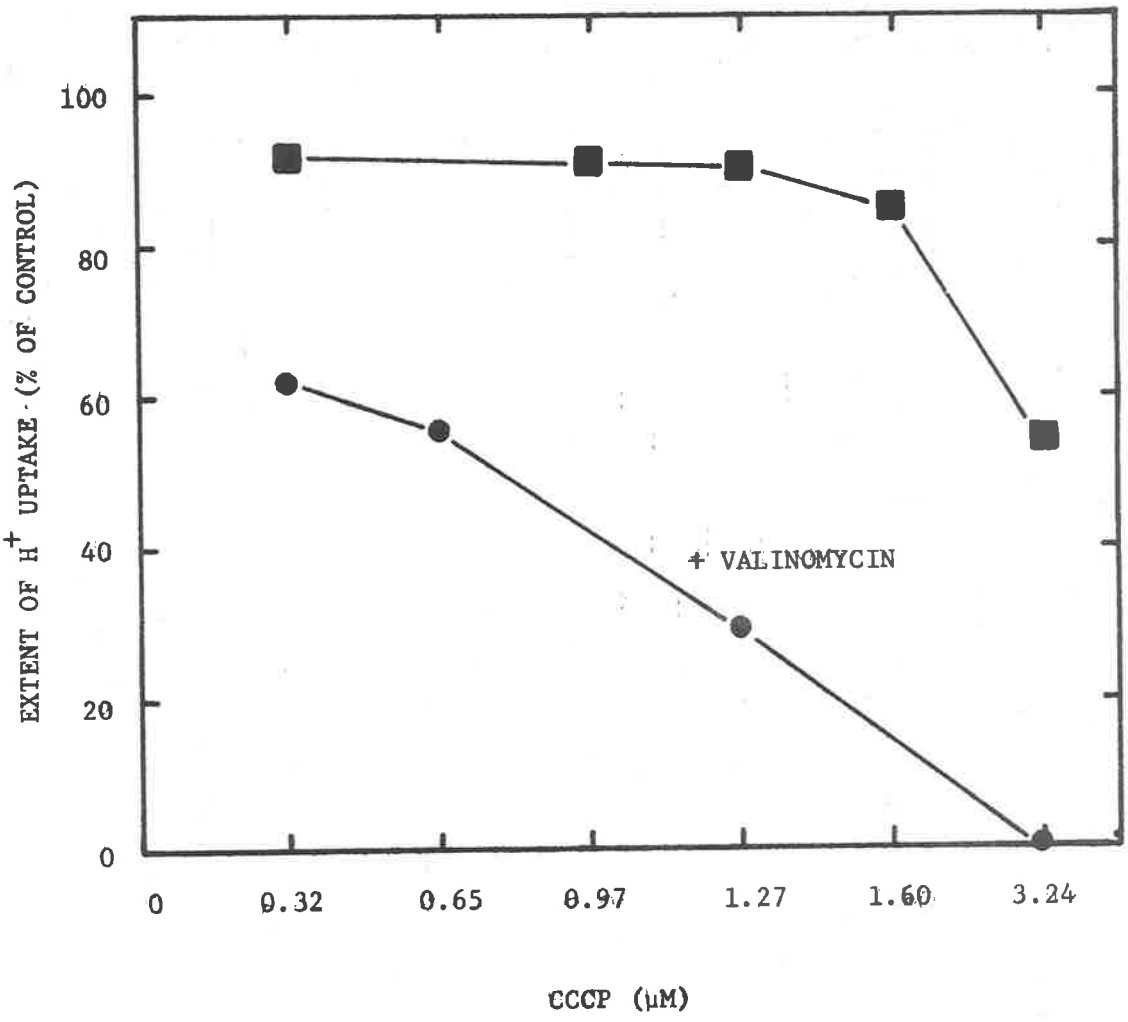


FIGURE XIII. Effect of CCCP and valinomycin on the extent of PMS-catalyzed light-induced H^+ uptake. The reaction mixture was similar to that used in Fig.XI. Concentration of PMS was 15μM. Extent of H^+ uptake in the control was 0.561 μEqH^+/mg chl1.

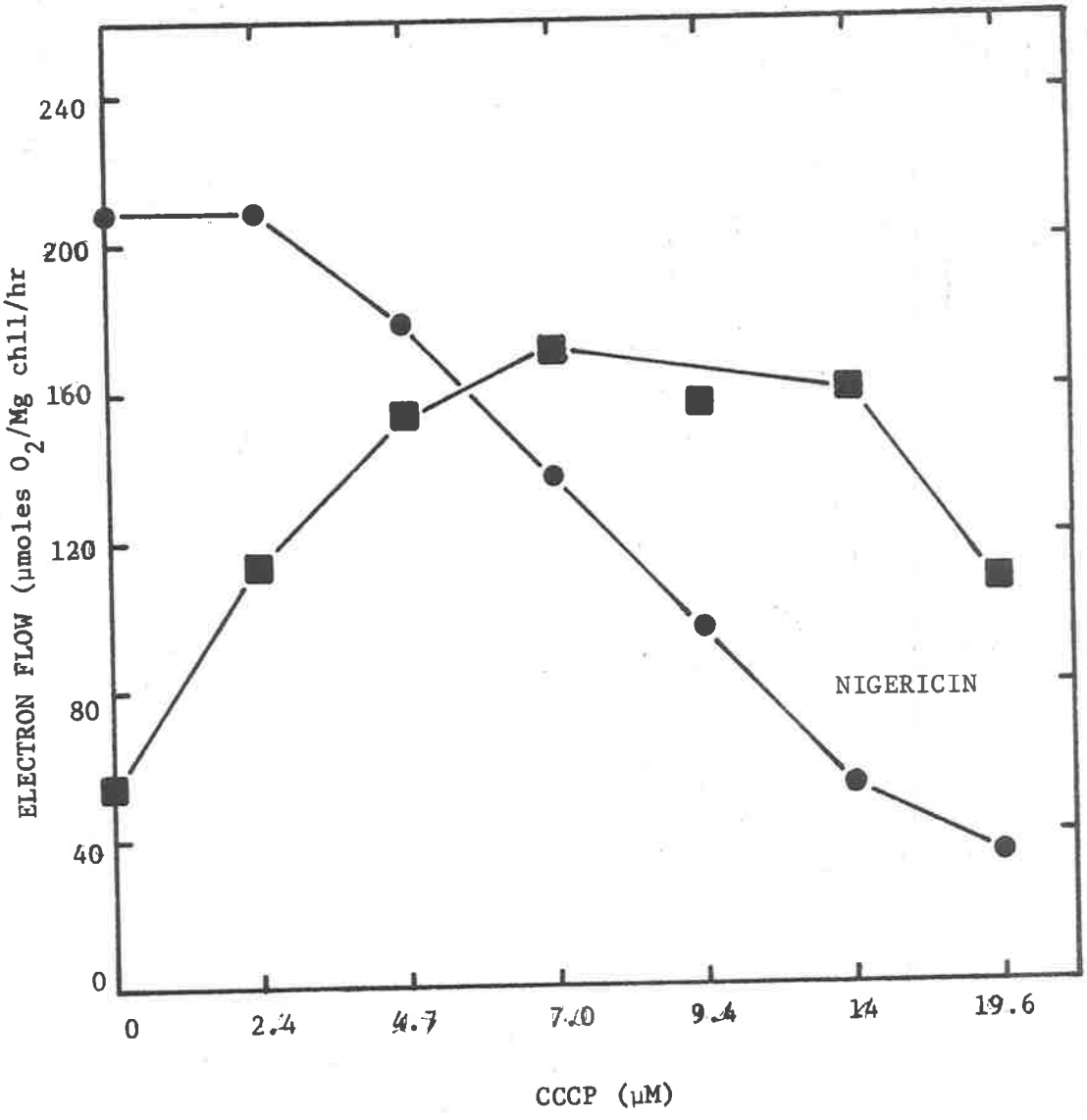


FIGURE XIV. Effect of CCCP on the rate of electron flow in the absence and presence of nigericin. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 23mM KCl; 1.5mM FeCN and pea chloroplasts equivalent to 68µg chlorophyll.

of formation and decay of proton uptake, and 0.06μM FCCP which had no effect on the light-induced proton uptake with pyocyanine, became very effective in inhibiting the proton uptake in the presence of valinomycin. Von Stedingk (1970) observed the same synergistic effect with 0.4μM FCCP and valinomycin on the light-induced proton uptake with PMS. This effect on the proton uptake with PMS was also investigated in the present study and the results are shown in Fig.XIII. As can be seen, CCCP at concentrations which do not uncouple fully become very effective in the presence of 1.6μM valinomycin and K^+ ions, complete inhibition of the proton uptake was obtained with 3.2μM CCCP, which on its own inhibited the proton uptake by only 55%

CCCP has been found to be an inhibitor of electron transport in Tris-treated chloroplasts (Itoh, Yamashita, Nishi, Konishi and Shibata, 1969) and in chloroplasts uncoupled by EDTA-treatment or by addition of methylamine (Kimimura, Katoh, Ikegani and Takamiya, 1971 and Homann, 1971). Fig.XIV shows the effect of CCCP at varying concentrations on oxygen evolution by pea chloroplasts in the absence and presence of nigericin. CCCP stimulated oxygen evolution in control chloroplasts, reaching a peak at 7μM concentration, the stimulated rate decreasing at higher concentrations. In contrast to the control, oxygen evolution in nigericin-uncoupled chloroplasts decreased sharply as the concentration of CCCP increased. It seems that in these uncoupled chloroplasts, the concentration of CCCP

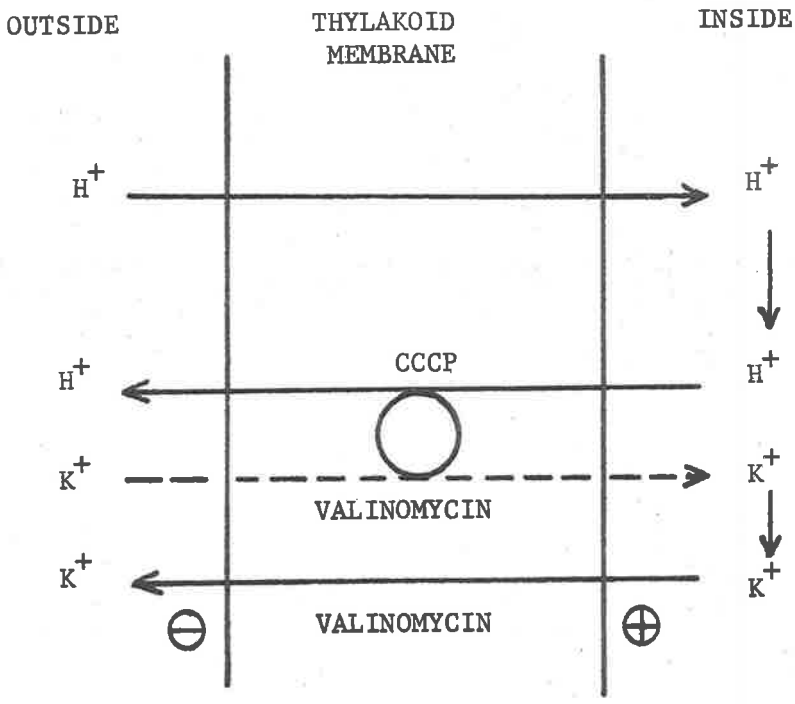
TABLE VII. CCCP on non-cyclic electron flow mediated by Photosystem I. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 21mM KCl; 4.2μM DCMU; 25μM DCPIP; 3.4mM ascorbate; 70μM MV; 70μM azide and 69μg chlorophyll.

Additions	Electron Flow (μmoles O_2/mg chll/hr)
Control	41
Nigericin 0.46μM	289
Nigericin + CCCP 9.2μM	285
CCCP 9.2μM	276

needed to inhibit electron transport has been lowered. The results of Itoh, Yamashita, Nishi, Konishi and Shibata (1969), Kimimura, Katoh, Ikegami and Takamiya (1971) and Homann (1971) indicated that CCCP could be inhibiting on the oxidizing side of PSII. An attempt was made to see if CCCP would inhibit electron transport mediated by PSI only. Table VII shows the effect of CCCP on electron transport from $DCPIPH_2$ to MV, in the presence of DCMU (Duysens, 1964). CCCP was found to have no inhibitory effect on the electron transport already stimulated by nigericin, whereas in the absence of nigericin the electron transport was greatly enhanced. This indicates that CCCP does not affect electron transport mediated by PSI and that PSII could be the site of inhibition of CCCP. This is in agreement with the results of the above mentioned authors.

DISCUSSION

Uncoupling of the Hill reaction and State 3 rates by CCCP suggests that the chloroplast membranes have become "leaky" to protons in the presence of CCCP, resulting in fast equilibration of protons across the membrane and subsequent stimulation of electron flow. In the case of the State 3 rate in which the rate of electron flow is controlled by phosphorylation, CCCP removed this control and stimulated electron flow by causing a collapse of the proton gradient (Mitchell, 1966). This shows that even in State 3 the rate of electron flow is limited by the rate of



SCHEME I. Proposed synergistic uncoupling of photophosphorylation by CCCP and valinomycin in chloroplasts. See text for explanation.

phosphorylation which could be restricted by the turnover of the ATPase or the rate of penetration of ADP to the ATPase. Pre-incubation of chloroplasts with ADP before illumination gave a State 3 rate which was faster than that obtained after illumination (West and Wiskich, 1968), which may indicate that penetration is limiting.

CCCP also inhibits cyclic photophosphorylation and the extent of light-induced proton uptake (see Jagendorf and Neumann, 1967), which could indicate that a proton gradient, set up in the light, may contribute towards supporting ATP synthesis. Experiments of Hind and Jagendorf (1963) and Neumann and Jagendorf (1964) indicate a close correlation between the pH gradient and X_E: a high energy intermediate state formed during illumination which subsequently was able to support ATP formation in the dark, when supplied with ADP and Pi. Further support for this proposal comes from the observations of Jagendorf and Uribe (1966) who found that ATP was formed by chloroplasts when the proton gradient was created artificially in the dark. The synergistic effect of CCCP and valinomycin suggests that a membrane potential may also contribute to phosphorylation. In the presence of both CCCP and valinomycin, the thylakoid membrane would become permeable to both K^+ and H^+ ions causing rapid exchange of these ions across the membrane, similar to Mitchell and Moyle's (1967) and Chappel and Haarhoff's observations with mitochondria. Scheme I illustrates the proposed

mode of action of the synergistic effect of CCCP and valinomycin on chloroplasts. Upon illumination of the chloroplasts, a proton gradient and a membrane potential are set up across the thylakoid membrane. When CCCP and valinomycin are present, the proton gradient is abolished due to the exchange of internal H^+ for external K^+ ions. The K^+ ions thus accumulated inside would move outwards by the action of valinomycin, thereby causing a dissipation of the membrane potential. This is consistent with the chemiosmotic hypothesis, which postulates that energy is conserved in an electro-chemical gradient across a coupling membrane.

Apart from its uncoupling effect on photophosphorylation in chloroplasts, the data presented indicates that CCCP inhibits electron transport under certain conditions. That the site of this inhibition by CCCP is located close to PSII, is indicated by its inhibition of oxygen evolution in nigericin-uncoupled chloroplasts, and its lack of inhibitory effect on electron transport from $DCPIPH_2$ to MV which is mediated solely by PSI. These results are in agreement with the results of earlier authors who postulated that CCCP inhibits electron transport on the oxidising side of PSII (Itoh, Yamashita, Nishi, Konishi and Shibata, 1969; Kimimura, Katoh, Ikegami and Takamiya, 1971 and Homann, 1971). It is well established that CCCP acts as a proton carrier in mitochondrial and chloroplast membranes (Mitchell and Moyle, 1967;

see also Results of this Chapter). By virtue of this property, CCCP could prevent the primary charge separation by PSII or the charge separation after the splitting of water before oxygen evolution. The former idea seems more reasonable as Kimimura, Katoh, Ikegami and Takamiya (1971) observed a decrease in the steady-state fluorescence level (see also Itoh, Yamashita, Nishi, Konishi and Shibata, 1969) indicating that 'Q' the fluorescence quencher, remained in the oxidising and quenching state (Duysens and Sweers, 1963). Heath and Hind (1969) and Cheniae and Martin (1970) found that Mn^{2+} deficiency, while impairing the oxygen evolving system, did not greatly impair the primary photochemical charge separation by PSII, suggesting that the observations of Kimimura, Katoh, Ikegami and Takamiya (1971) may be explained by assuming that CCCP inhibits the photo-reduction of 'Q'. Whatever the mechanism of inhibition may be, it needs high concentrations of CCCP or uncoupled chloroplasts. The important feature of the inhibition is that it is real at low concentrations of CCCP but can be masked by its uncoupling effects (Fig.X).

C. ARSENATE

RESULTS

Crane and Lipmann(1953) found that arsenate stimulated the respiratory activity of washed mitochondria in the absence of Pi.

TABLE VIII. Effect of phosphate and Arsenate on the Hill Reaction in the absence and presence of ADP. The reaction mixture was similar to that used in Table II. Chloroplasts were added to the equivalent of 46µg chlorophyll. ADP was present to a final amount of 0.475 µmole, where specified.

Additions	Electron Flow (µmoles O_2/mg chl1/hr)
Control	67
Pi 4.3mM	78
Pi 8.7mM	78
Pi 13mM	78
As 4.3mM	75
As 8.7mM	75
Pi 4.3mM + ADP	149
Pi 13mM + ADP	160
As 4.3mM + ADP	141
As 8.7mM + ADP	156

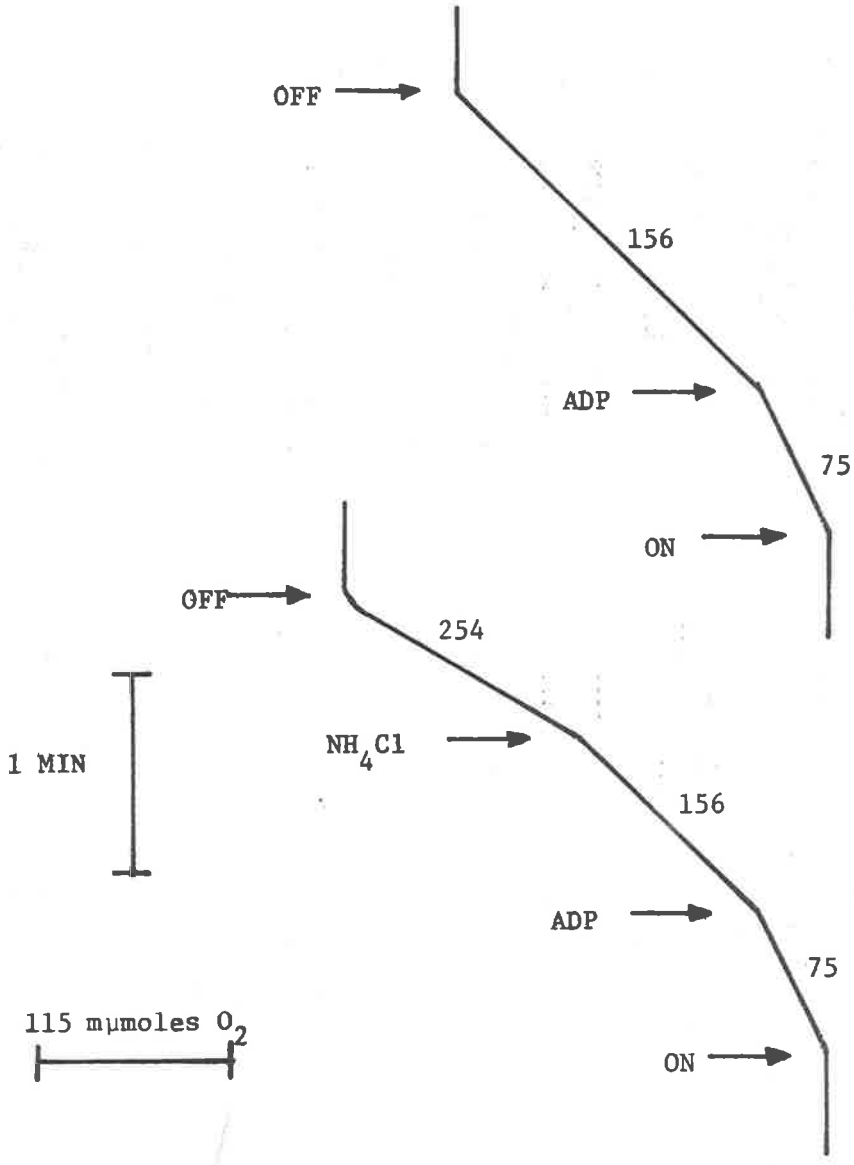
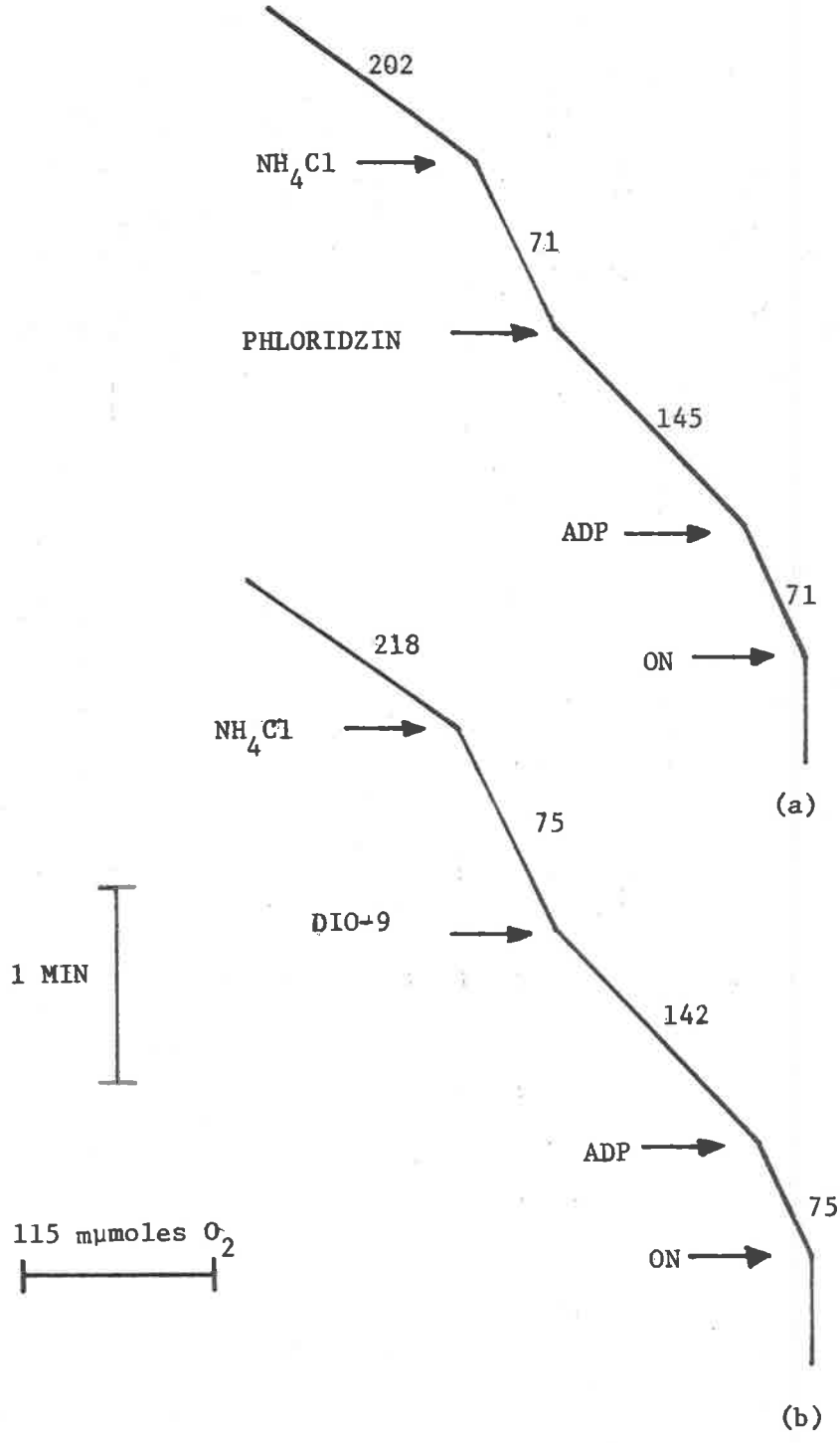


FIGURE XV. Effect of arsenate on electron flow in the presence of ADP. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, 8.7mM arsenate; 1.5mM FeCN and pea chloroplasts equivalent to 46μg chlorophyll. ADP was added to a final amount of 0.475 μmole. Concentration of NH_4Cl was 1.7mM. The numbers along the traces are in μmoles O_2/mg chll/hr.

They proposed that arsenate uncoupled aerobic phosphorylation by substituting for Pi. Borst and Slater (1961) came to the same conclusion. Estabrook (1961) observed that the arsenate-stimulated respiration was further stimulated by addition of ADP, but inhibited by oligomycin. Since the DNP-stimulated respiration was insensitive to oligomycin, he concluded that arsenate and DNP reacted at different sites on the electron transport chain. Huijung and Slater (1961) also suggested that DNP and arsenate reacted at different sites with two different high-energy non-phosphorylated intermediates. Avron and Jagendorf (1959) found that arsenate could substitute for Pi in photophosphorylation and also in the stimulation of FeCN reduction during Hill reaction, but these effects were dependent on Mg^{2+} and ADP. They suggested that an ADP-arsenate complex was formed and rapidly hydrolyzed.

Table VIII shows the effect of arsenate on oxygen evolution during the Hill reaction. It can be seen that arsenate stimulated oxygen evolution to about the same extent as Pi. In the presence of ADP and Mg^{2+}, arsenate, as did Pi, stimulated oxygen evolution, but this stimulated rate was maintained and did not slow down as the State 3 rate did with Pi (Fig.XV). Thus with arsenate, the State 3 and State 4 rates as seen with Pi were not achieved. When an uncoupler like NH_4Cl was added during State 3, the rate of oxygen evolution was further stimulated. The same effect was observed when NH_4Cl was added to the reaction medium containing

FIGURE XVI. Effect of phloridzin and Dio-9 on electron flow in the presence of ADP and arsenate. The reaction mixtures were similar to that used in Fig.XV. Concentrations of phloridzin and Dio-9 were 0.54mM and 21µg/ml. ADP was added to a final amount of 0.475 µmole. The numbers along the trace are in µmoles O_2/mg chll/hr.


202
NH4Cl
71
PHLORIDZIN
145
ADP
71
ON
(a)
218
NH4Cl
75
DIO-9
142
ADP
75
ON
(b)
1 MIN
115 mμmoles O2

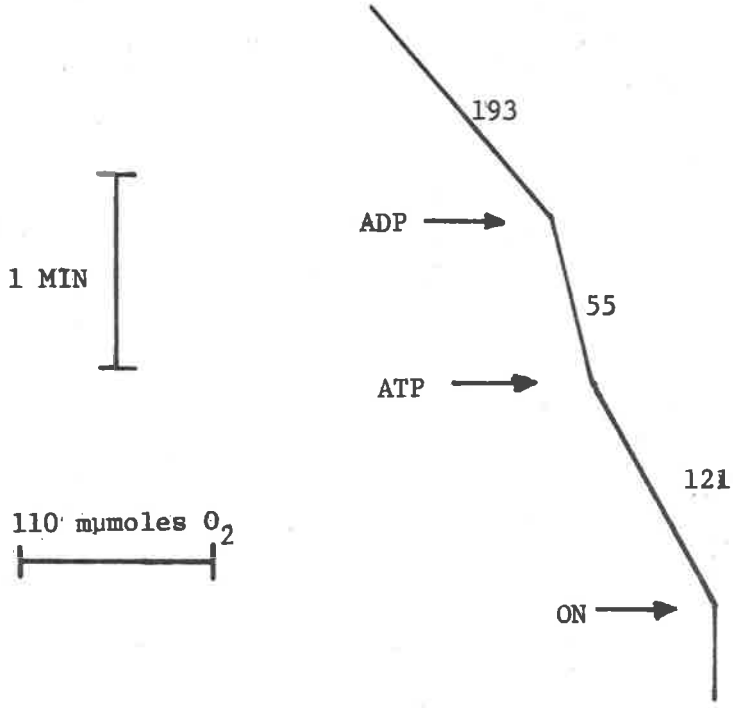


FIGURE XVII. The inhibition of the Hill reaction rate by ATP. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 9mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and pea chloroplasts equivalent to 30μg chlorophyll. ATP and ADP were added to a final amount of 0.22mM and 0.475 μmole respectively. The numbers along the traces are in μmoles O_2/mg chl1/hr.

arsenate, ADP and Mg^{2+}. This indicates that electron transport under these conditions is subject to similar control as it is under phosphorylating conditions (i.e. in the presence of Pi, ADP and Mg^{2+}).

Phloridzin, an energy transfer inhibitor (Izawa, Winget and Good, 1966) inhibited the oxygen evolution rate in the presence of arsenate, ADP and Mg^{2+}. Izawa, Connolly, Winget and Good (1966) have made similar observations, and also found that the inhibition by phloridzin could be completely reversed by the addition of uncouplers methylamine, atebrin and FCCP. The arsenate and ADP stimulated oxygen evolution was also inhibited by another energy transfer inhibitor Dio-9, as shown in Fig.XVI. This has also been observed by McCarty, Guillory and Racker (1965). The inhibition by both the above energy transfer inhibitors was relieved by the addition of NH_4Cl, supporting Izawa, Winget and Good's (1966) observations with phloridzin.

Fig.XVII shows ATP inhibition of oxygen evolution during the Hill reaction. Avron, Krogmann and Jagendorf (1958) found that low concentrations of ADP inhibited, rather than stimulated, FeCN reduction. They traced this inhibitory effect to the ATP formed during illumination of the chloroplasts. This inhibition by ATP was independent of the presence of Mg^{2+} and/or Pi. It can be seen in Fig.XVII that the inhibition of oxygen evolution by ATP was reversed when ADP was added, indicating that the

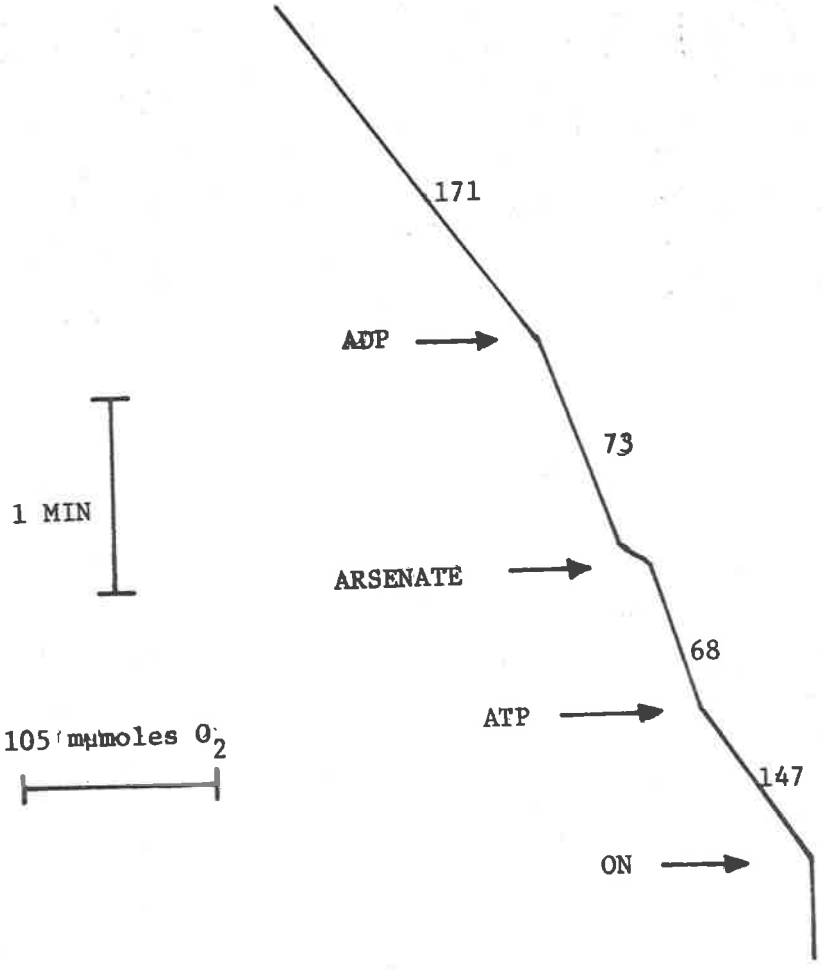


FIGURE XVIII. The effect of arsenate and ADP on the ATP-inhibited Hill reaction rate. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and chloroplasts equivalent to 30μg chlorophyll. Concentration of arsenate was 8.7mm. ATP and ADP were added to a final amount of 0.22mM and 0.475 μmole respectively.

phosphorylative activity of these chloroplasts had not been impaired. Telfer and Evans (1971) have also observed an inhibition with ATP and ADP in broken spinach chloroplasts. Arsenate alone did not have any effect on the ATP inhibition of the Hill reaction; ADP was needed to overcome the ATP inhibition (Fig.XVIII). It is also interesting to note that arsenate did not induce ATPase activity in these chloroplasts as oxygen evolution would have been stimulated if ATP hydrolysis had occurred. Thus it seems that chloroplasts are quite different in this respect as arsenate can induce ATPase activity in mitochondria (see Wadkins, 1960; Azzone and Ernster, 1961 and Ter Welle and Slater, 1967). Jagendorf (1958) also was not able to observe arsenolysis of ATP in spinach chloroplasts.

DISCUSSION

The stimulation of oxygen evolution during the Hill reaction, by arsenate in the presence of ADP, was similar to the stimulation of oxygen evolution by Pi and suggests that arsenate could replace Pi in photophosphorylation. This is in agreement with earlier observations of Jagendorf (1958) and Avron and Jagendorf (1959) although these authors did not find any stimulation of non-phosphorylating FeCN reduction by arsenate. It is evident from Table VIII that both arsenate and Pi stimulated oxygen evolution, but this stimulation did not increase with increasing concentrations

of arsenate and Pi. On the other hand, Schwartz (1965) did not observe any stimulation of oxygen evolution by arsenate when ADP was added, although FeCN reduction was stimulated when ADP was added to a medium containing arsenate. This lack of stimulation of oxygen evolution by arsenate in the presence of ADP, observed by Schwartz (1965) is difficult to explain, since with pea chloroplasts, arsenate had a stimulatory effect in the presence of ADP irrespective of whether FeCN reduction or oxygen evolution was monitored. The slight stimulatory effect of both Pi and arsenate on the Hill reaction in pea chloroplasts may be due to the presence of some endogenous ADP still present in these chloroplasts (see Hall, Reeves and Baltscheffsky, 1971).

The pseudo-State 3 rate (arsenate and ADP) was maintained, unlike the State 3 rate (with Pi) which decreases upon exhaustion of added ADP. This is an indication of ADP being regenerated in this system, probably due to the spontaneous breakdown of an arsenate-ADP complex as suggested by Avron and Jagendorf (1959).

Equal sensitivity of arsenate plus ADP stimulated and Pi plus ADP stimulated rates of oxygen evolution, to energy transfer inhibitors, Dio-9 and phloridzin, together with the relief of such inhibition by the uncoupler NH_4Cl, indicates the similarity between the two systems. This is reminiscent of Chan, Thomas and Wadkins' (1969) observations that the formation of an arsenylated component in rat liver mitochondria was inhibited by oligomycin and DNP.

Estabrook (1961) had already shown that oligomycin (an inhibitor of oxidative phosphorylation and the associated respiration) inhibited the arsenate stimulated rate of respiration in the presence of ADP. The main difference between the arsenate and Pi systems is that with Pi a stable compound (ATP) is formed, whereas arsenate forms an unstable AS ADP complex, which is rapidly hydrolysed. Thus the responses produced by arsenate are in fact not strictly true uncoupling effects, as the reaction

$$I \sim ADP + AsO_4 \longrightarrow I + ADP \sim AsO_4 \xrightarrow{H_2O} ADP + AsO_4$$

(after Avron and Jagendorf, 1959) is only a partial reaction of photophosphorylation. Therefore, it must be concluded that arsenate can participate in the partial reactions of photophosphorylation, differing only in the terminal steps, where an unstable high energy complex is produced instead of a high energy stable compound.

There is no evidence in this work to suggest the presence of an unstable arsenylated intermediate (other than ADP ~ As) as proposed by Ter Welle and Slater for mitochondrial phosphorylations (see Pullman and Schatz, 1967 for discussion and references).

CHAPTER V

EFFECT OF ELECTRON FLOW INHIBITORS ON THE PHOTOCHEMICAL ACTIVITIES OF ISOLATED CHLOROPLASTS AND DIGITONIN SUB-CHLOROPLAST PARTICLES

INTRODUCTION

Electron flow inhibitors are compounds which react with the components of the electron transfer chain, and are useful in determining the sequence of these components. Some of these inhibitors were selected and their effects on the various photochemical reactions of photosynthesis in isolated chloroplasts studied in an attempt to:

(a) determine their sites of action on the presently known electron flow chain of photosynthesis,

(b) use their known effects to resolve some differences of opinion concerning some electron flow pathways.

A. ANTIMYCIN A

RESULTS

Antimycin A has been widely used as an inhibitor of the respiratory chain electron transport in mitochondria. Its site of action on the respiratory chain has been located between cytochromes b and c, (Chance, 1958 and Estabrook, 1962). Whatley, Allen and Arnon (1959) did not find any inhibition of Vitamin K and FMN-catalyzed phosphorylations in chloroplasts,

by antimycin A in the μM concentration range. Bamberger, Black, Fewson and Gibbs (1963) found that antimycin A at 100μM concentration inhibited phosphorylation without inhibiting NADP reduction. Tagawa, Tsujimoto and Arnon (1963) observed that antimycin A inhibited ferredoxin-catalyzed cyclic phosphorylation but not other types of phosphorylation, and suggested that it inhibited cytochrome b_6 which participates in the ferredoxin-catalyzed cyclic pathway. On the other hand, Izawa, Connolly, Winget and Good (1966) found that at low light intensities all types of phosphorylation were inhibited. They thought that antimycin A inhibited coupled electron flow, as well as electron flow uncoupled by exogenous uncouplers, but not basal electron flow. Hind (1968) reported that antimycin A inhibited phosphorylation at pH8.3 and stimulated NADP reduction at pH7, thus acting in a manner characteristic of uncouplers. Antimycin A was found by Drechsler, Nelson and Neumann (1969) to be an uncoupler of photophosphorylation and an inhibitor of electron flow, the latter effect being observed at lower concentrations. They also observed that cyclic photophosphorylation catalyzed by both PMS and ferredoxin was inhibited.

Oxygen evolution during Hill reaction with FeCN as electron acceptor was not inhibited significantly by antimycin A, but

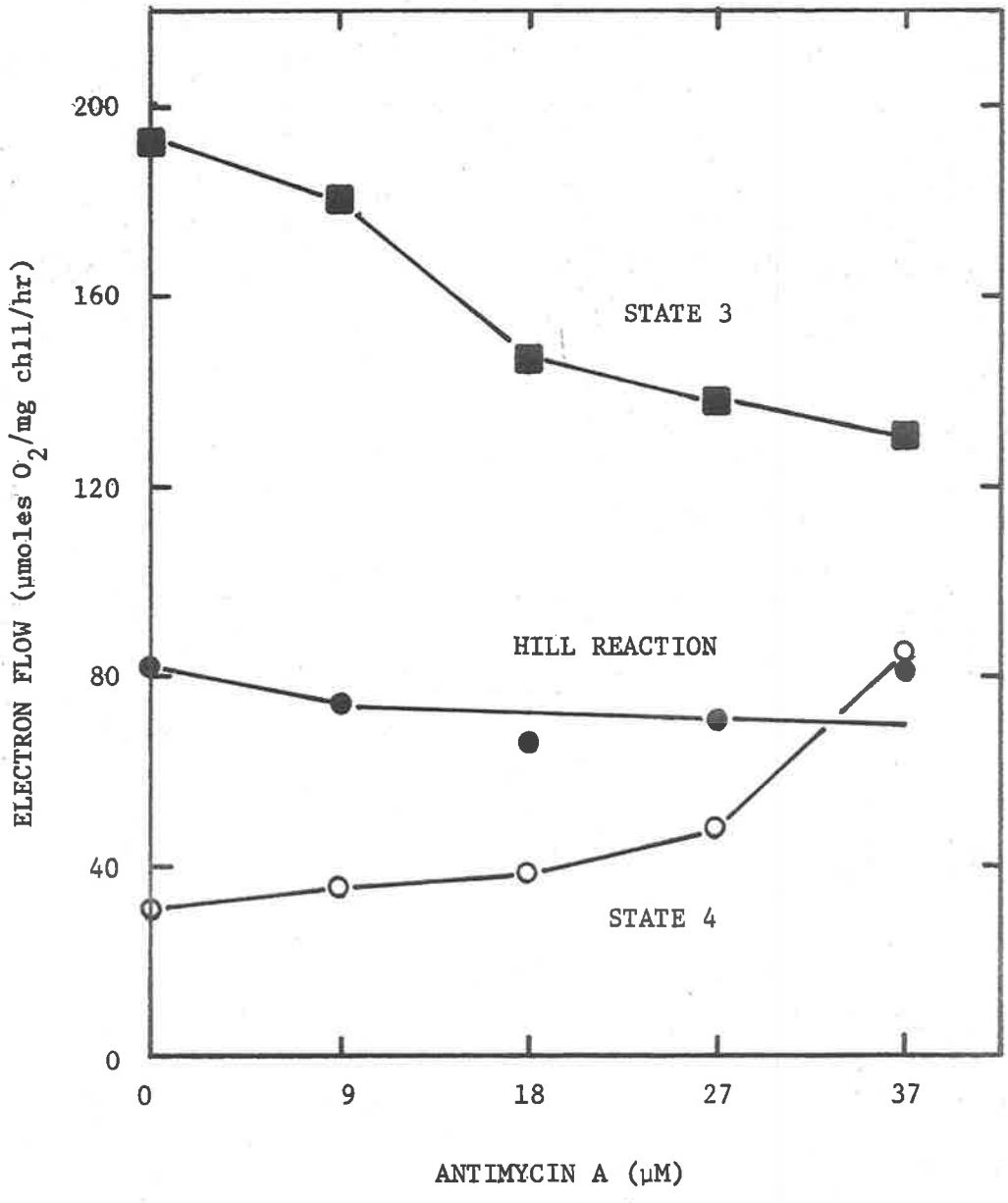


FIGURE I. Effect of antimycin A on non-cyclic electron flow in pea chloroplasts. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 10mM KH_2PO_4; 40mM HEPES buffer, pH7.5 and chloroplasts equivalent to 150µg chlorophyll.

FIGURE II. Effect of antimycin A on photosynthetic control and ADP/O ratios. The reaction mixture was the same as used in Fig.I. The closed and open circles denote photosynthetic control and ADP/O ratios respectively.

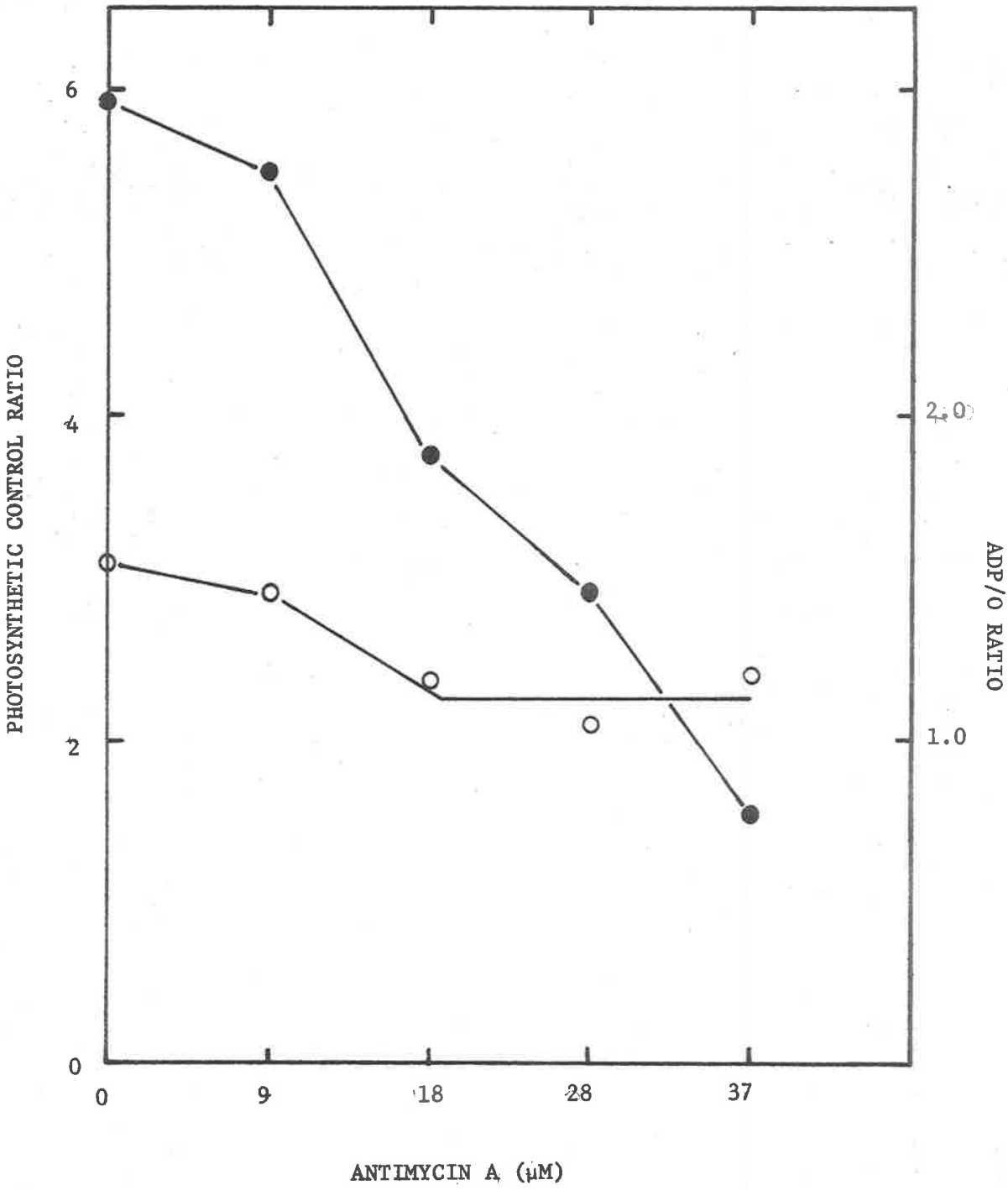

PHOTOSYNTHETIC CONTROL RATIO
ADP/O RATIO
6
4
2
0
2.0
1.0
0
9
18
28
37
ANTIMYCIN A (μM)

TABLE I. Effect of antimycin A on NH_4Cl or methylamine-uncoupled electron flow in pea chloroplasts. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 1.5mM FeCN and 90µg chlorophyll.

Additions	Electron Flow (µmoles O_2/mg chl1/hr
Control	39
+ antimycin A 33µM	45
NH_4Cl 3.6mM	268
+ antimycin A	143
NH_4Cl 7.1mM	270
+ antimycin A	145
methylamine 0.0055%	287
+ antimycin A	144

TABLE II. Effect of antimycin A on electron flow in digitonin subchloroplast particles. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 0.33mM FeCN or 25μM DCPIP; 3.4mM ascorbate; 10μM NADP and excess ferredoxin/ferredoxin-NADP reductase. PSII and PSI subchloroplast particles equivalent to 10μg and 22μg chlorophyll were added for FeCN and NADP reduction respectively. FeCN and NADP reductions were followed by the changes in O.D. at 420 and 340nm respectively; the cuvettes were illuminated for 1 min. as described in Chapter II.

Experiments	Electron Flow (μmoles FeCN or NADP reduced/mg chl1/hr)
Experiment I (FeCN)	
1. Control	118
2. + antimycin A 6.6μM	79
3. + antimycin A 13.3μM	39
4. + antimycin A 20μM	14
Experiment II (NADP)	
1. Control	100
2. + antimycin A 67μM	115

the coupled electron flow (State 3) was inhibited increasingly with increasing concentrations of the inhibitor (Fig.I). On the other hand, the State 4 rate of oxygen evolution was progressively stimulated by increasing concentrations of antimycin A (Fig.I). The photosynthetic control ratio decreased as the concentration of antimycin A increased, but the ADP/O ratios were only slightly affected (1.55 to 1.10) as shown in Fig.II. In contrast to the relative insensitivity of the Hill reaction to antimycin A inhibition, the NH_4Cl and methylamine-uncoupled rate of oxygen evolution was much more sensitive to the inhibitor (Table I). A similar effect of antimycin A on uncoupled electron flow has been reported (Izawa, Connolly, Winget and Good, 1966 and Drechsler, Nelson and Neumann, 1969).

The effect of antimycin A on the electron flow in digitonin Photosystem II (PSII) subchloroplast particles (SCP) was also studied, and the results shown in Table II. The degree of inhibition by the same concentration of antimycin A was greater in these subchloroplast particles than in intact chloroplasts. However, antimycin A has no inhibitory effect on the non-cyclic electron flow from $DCPIPH_2$ to NADP in digitonin Photosystem I (PSI) subchloroplast particles (Table II). These results indicate a site of action of antimycin A close to PSII.

FIGURE III. Antimycin A inhibition of cyclic photophosphorylation in pea chloroplasts and digitonin subchloroplast particles. The reaction mixture contained 0.4M sucrose; 7.8mM $MgCl_2$; 12.5mM K_2HPO_4-KH_2PO_4 buffer, pH8.0; 15µM ~~PSM~~ PMS; 3mM ascorbate; 3µM DCMU and chloroplasts and SCP equivalent to 60µg and 34µg chlorophyll respectively. Control rates for chloroplasts and SCP were 256 and 350 μEqH^+/mg chl1/hr.

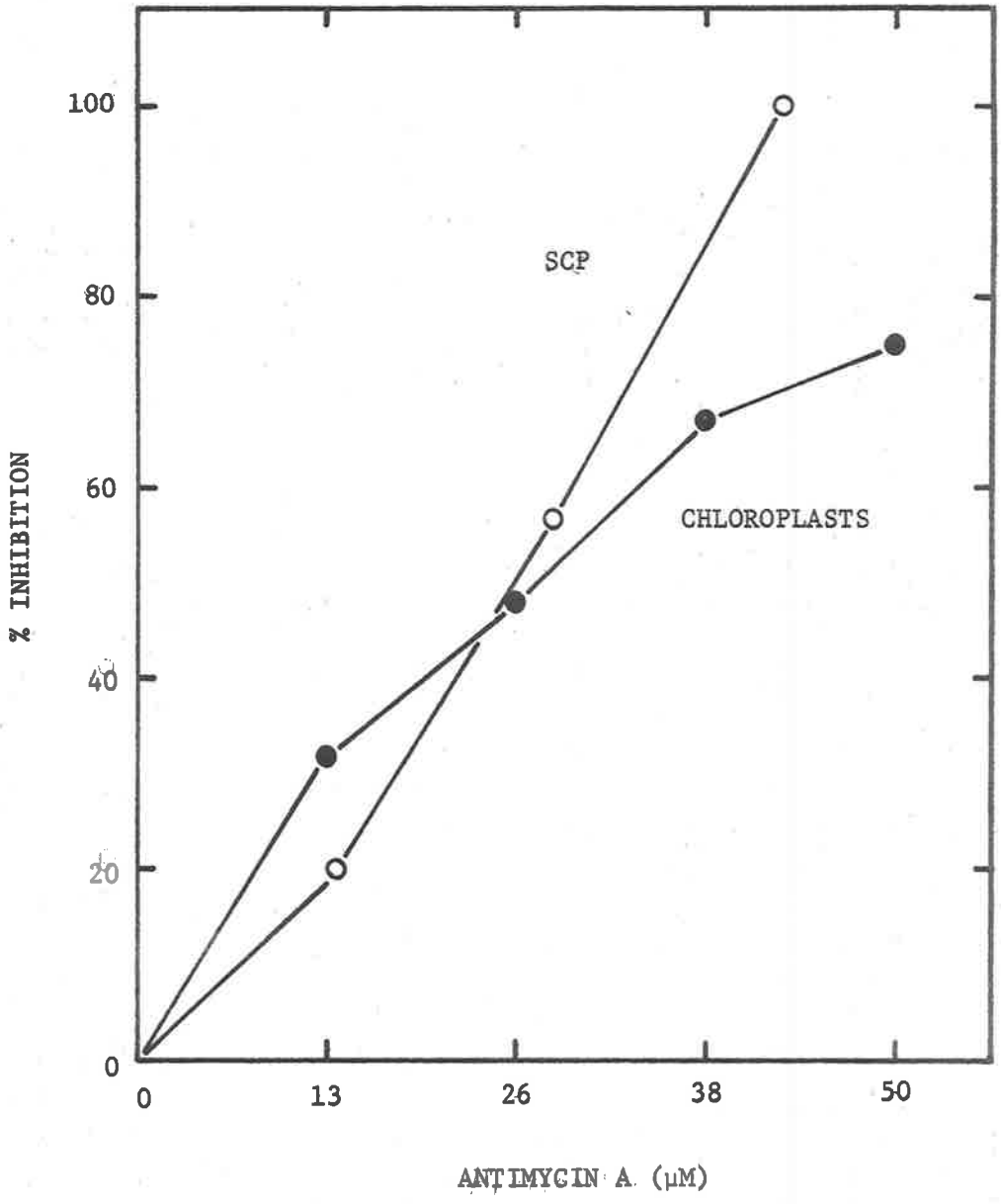
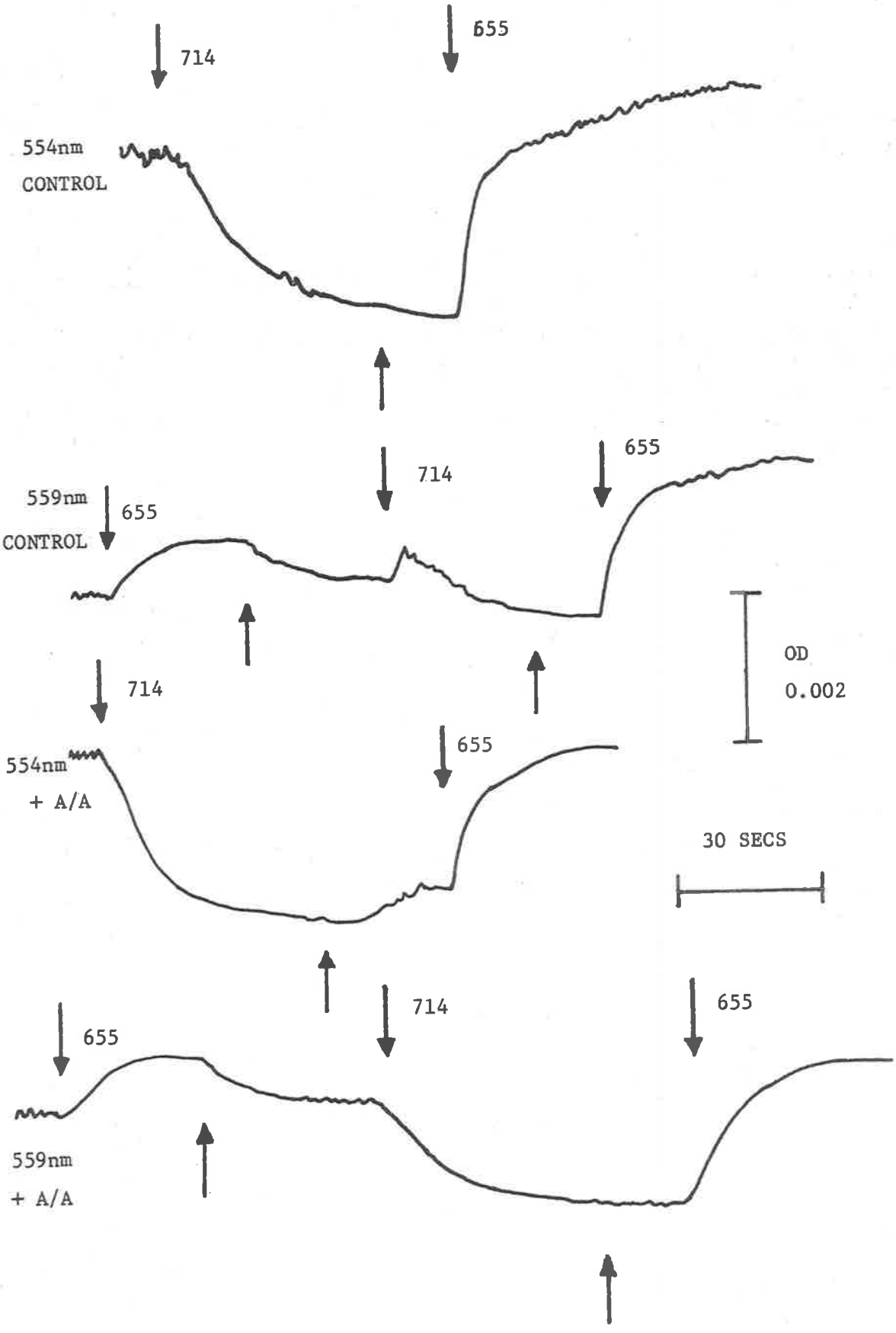

100
80
60
40
20
0
% INHIBITION
SCP
CHLOROPLASTS
0
13
26
38
50
ANTIMYCIN A (μM)

FIGURE IV. Antimycin A on the light-induced redox changes of cytochromes f and b_{559} in pea chloroplasts. The reaction mixture contained 30mM Tricine buffer, pH7.6; 50mM KCl and 50mM NaCl. Concentrations of chlorophyll, 75µg/ml and antimycin A, 57µM. Intensities of actinic light were 3×10^4 ergs/cm^{-2}/sec^{-1} at 655nm and 3.45×10^4 ergs/cm^{-2}/sec^{-1} at 714nm respectively. The reference wavelength was 570nm.


714
655
554nm
CONTROL
714
655
559nm
655
CONTROL
714
655
OD
0.002
554nm
+ A/A
30 SECS
714
655
655
559nm
+ A/A

Cyclic photophosphorylation in intact and digitonin PSI subchloroplast particles was also inhibited by antimycin A (Fig.III). The phosphorylation in PSI subchloroplast particles seemed to be more sensitive to antimycin A than phosphorylation in intact chloroplasts, 44μM antimycin A inhibiting the phosphorylation completely in PSI subchloroplast particles while 50μM inhibited the reaction only 75% in intact chloroplasts. Drechsler, Nelson and Neumann (1969) had previously reported the inhibition of PMS-catalyzed cyclic photophosphorylation by antimycin A in chloroplasts. These results suggest another site of inhibition of antimycin A, probably on the cyclic electron flow pathway.

It is well established that antimycin A inhibits between cytochrome b and cytochrome c on the mitochondrial electron flow chain (Chance, 1958 and Estabrook, 1962). Drawing an analogy to the situation in mitochondria, one would expect antimycin A to inhibit the oxidation of a b-type cytochrome in chloroplasts. Fig.IV shows the light-induced absorbance changes of cytochrome b_{559} in pea chloroplasts in the absence and presence of antimycin A. The reduction of cytochrome b_{559} by 655nm actinic light was not affected by antimycin A, but the oxidation by 714nm actinic light was stimulated. Hind (1968) had reported a similar effect of antimycin A on cytochrome b_{559} oxidation by PSI light in spinach chloroplasts. Thus, it seems as if antimycin A has a different effect in chloroplasts than it does in mitochondria.

DISCUSSION

The inhibition of electron flow by antimycin A in intact chloroplasts seems to be dependent on uncoupling agents (e.g. NH_4Cl and methylamine), as shown by data in Table I. Chloroplasts were found to swell in the presence of NH_4Cl and methylamine (Deamer & Packer, 1967 and Izawa, 1965), and this could facilitate the entry of the inhibitor into the chloroplast. This view is supported by Dreschler, Nelson and Neumann (1969) who observed that the degree of inhibition by antimycin A was dependent on the time of preincubation with the inhibitor.

Data displayed in Fig.II shows that although the photosynthetic control ratios are progressively lowered by increasing concentrations of the inhibitor, the ADP/O ratios are not severely affected. The lowering of the photosynthetic control ratios is caused by the faster State 4 rates, suggesting that ATPase activity is induced by antimycin A. The presence of an active ATPase is also reflected in the lowering of the ADP/O ratio from 1.55 to approximately 1.15 by 37μM antimycin A, but it is thought that the uncoupling action by antimycin A is too slight to be considered significant. Drechsler, Nelson and Neumann (1969) also termed antimycin A an uncoupler upon observing that the inhibitor lowered the $P/2e^-$ from approximately 1.0 to 0.2 at a concentration of 30μM; which is a slightly

higher concentration than needed for inhibiting electron flow. A similar lowering of $P/2e^-$ was reported by Hind (1968) in spinach chloroplasts.

Antimycin A also inhibits electron flow in digitonin PSII subchloroplast particles (Table II), but has no inhibitory effect on the electron flow from $DCPIPH_2$ to NADP in digitonin PSI subchloroplast particles (Table II). This suggests that the inhibitor has a site of inhibition near PSII. Results in Chapter VII show that the PSII subchloroplast particles have cytochrome b_{559} and cytochorme f associated with them (see also Boardman, 1968). An attempt to determine whether antimycin A would inhibit the oxidation of cytochrome b_{559} was made, but as shown in Fig.IV the oxidation was actually stimulated rather than inhibited. Hind (1968) reported similar observations in spinach chloroplasts, using FMN as the electron acceptor and attributed this to the uncoupling action of antimycin A. The stimulation of the oxidation of cytochrome b_{559} by antimycin A may indeed be a reflection of the uncoupling property of antimycin A, but it is clear that it does not have the same affect on cytochrome b_{559} as it has on cytochrome b in mitochondria.

Although antimycin A had no inhibitory effect on the electron flow from $DCPIPH_2$ to NADP in digitonin PSI subchloroplast particles, cyclic photophosphorylation catalyzed by PMS in these particles,

and in intact chloroplasts, was inhibited. Drechsler, Nelson and Neumann (1969) also found that PMS-catalyzed cyclic photophosphorylation was sensitive to antimycin A and in addition observed that ferredoxin-catalyzed cyclic photophosphorylation was sensitive. However Tagawa, Tsujimoto and Arnon (1963) claimed that antimycin A inhibited only ferredoxin-catalyzed cyclic photophosphorylation due to the specific participation of cytochrome b_6 in this pathway. The inhibition of PMS-catalyzed photophosphorylation by antimycin A in both chloroplasts and digitonin PSI subchloroplast particles, as well as the presence of cytochrome b_6 in these particles (Chapter VII), suggests the participation of cytochrome b_6 in cyclic photophosphorylation and that this is the site of action of antimycin A.

B. 2-ALKYL-4-HYDROXYQUINOLINE N-OXIDE (HOQNO)

RESULTS

HOQNO and NOQNO have been shown to be inhibitors of electron flow in cell free preparations of heart muscle and bacterial chromatophores (Lightbrown and Jackson, 1956 and Jackson and Lightbrown, 1958). The latter authors considered the site of inhibition of HOQNO in heart muscle mitochondria to be between cytochromes b and c. Smith and Baltscheffsky (1959) observed that 1μM HOQNO completely inhibited the light-induced

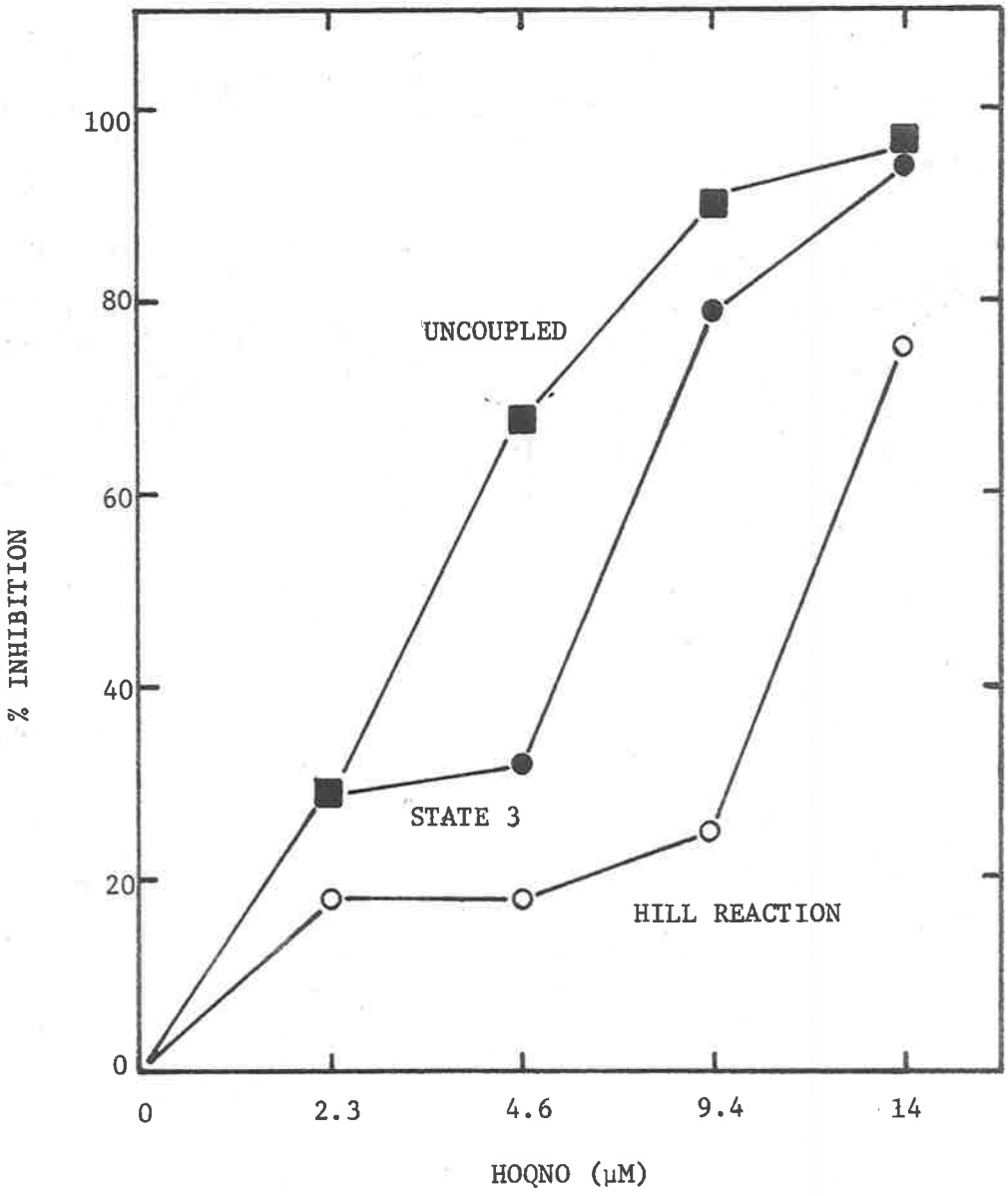


FIGURE V. Effect of HOQNO on the Hill reaction, State 3 and NH_4Cl-uncoupled electron flow in pea chloroplasts. The reaction mixture was similar to that used in Fig.I, except that chloroplasts were added to the equivalent of 50μg chlorophyll. Control rates of the Hill reaction, coupled and uncoupled electron flow are 60, 107 and 189 μmoles O_2/mg chl/hr respectively.

photophosphorylation in extracts of the photosynthetic bacterium, *Rhodospirillum rubrum*. In addition, they found that it produced changes in the light-dependent difference spectrum and attributed this to an increased oxidation of cytochrome c_2 and a decreased oxidation of the b-type cytochrome. Avron (1961) found that FeCN reduction and its associated phosphorylation were the most sensitive reactions catalyzed by swiss-chard chloroplasts. PMS-catalyzed cyclic phosphorylation was the most resistant to the inhibitor. Izawa, Connolly, Winget and Good (1966) observed that HOQNO inhibited 3 types of electron flow - coupled, non-phosphorylating and uncoupled. At low light intensities, PMS-catalyzed cyclic phosphorylation was insensitive to this inhibitor. They proposed the site of HOQNO inhibition to be before the branching off of the basal electron flow by-pass.

Oxygen evolution by isolated chloroplasts in the presence of FeCN was inhibited by HOQNO (Fig.V). This inhibition increased with ascending concentrations of the inhibitor, but a lag in the inhibition was evident at lower concentrations. Coupled electron flow, as well as the NH_4Cl uncoupled electron flow, was also sensitive to the inhibitor. The degree of inhibition observed in the two latter systems was greater than in the Hill reaction system. Similar inhibitions have been observed in swiss-chard (Avron, 1961) and spinach chloroplasts (Izawa, Connolly, Winget

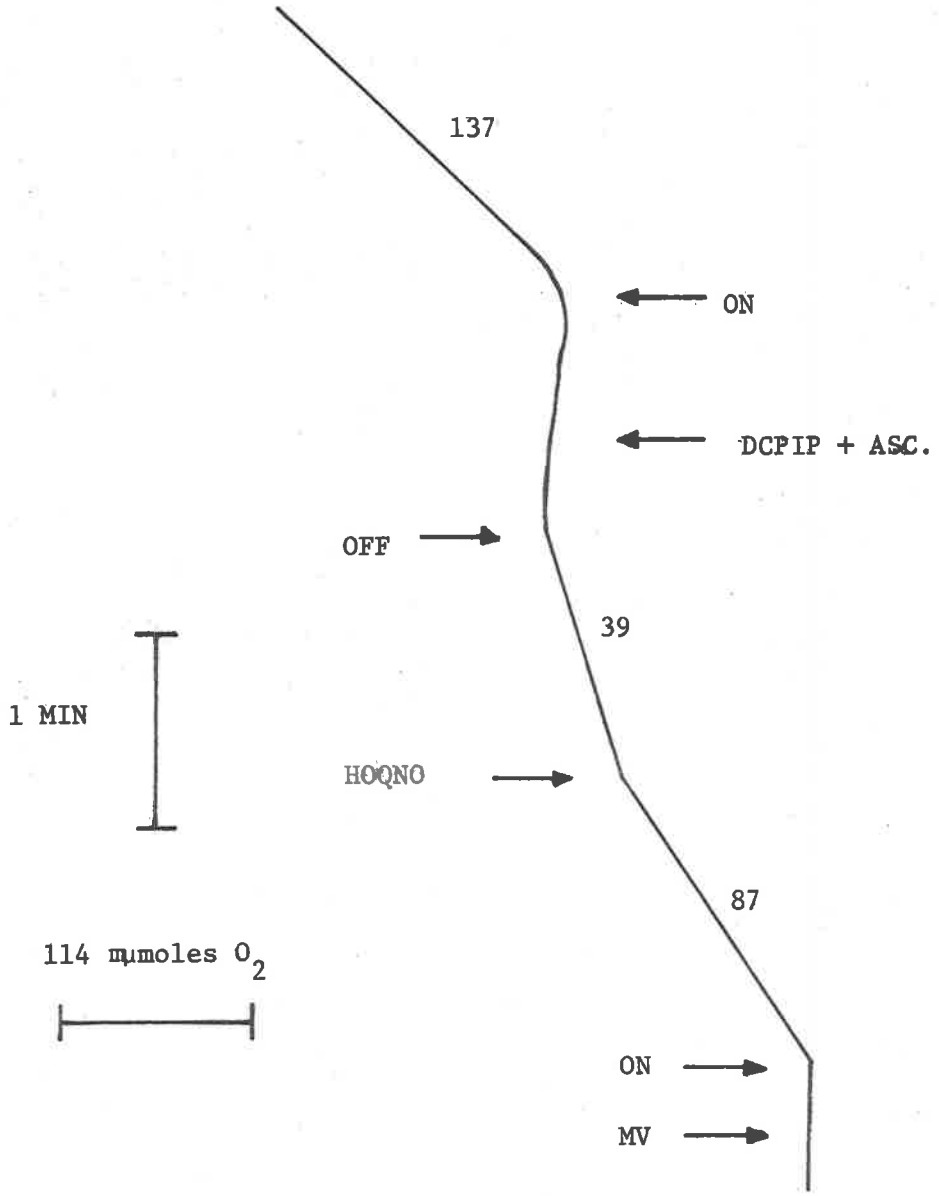


FIGURE VI. Effect of the electron donor couple, DCPIP plus ascorbate on HOQNO-inhibited electron flow with MV as electron acceptor. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 64µM MV; 0.93mM azide and pea chloroplasts equivalent to 50µg chlorophyll. Concentrations of HOQNO, 13mM; DCPIP, 25µM and ascorbate, 4.3mM. The numbers along the traces are in µmoles O_2/mg chl1/hr.

TABLE III. Inhibition of electron flow in digitonin PSII subchloroplast particles by HOQNO. The reaction mixture was the same as in Table I. Subchloroplast particles equivalent to 75µg chlorophyll was added.

Additions	Electron Flow (µmoles O_2/mg chll/hr)
Control	40
+ HOQNO 2.4µM	9
+ HOQNO 4.7µM	4
+ HOQNO 9.4µM	0

TABLE IV. Effect of HOQNO on cyclic photophosphorylation in pea chloroplasts. The reaction mixture contained 0.4M sucrose; 8.7mM $MgCl_2$; 14mM K_2HPO_4-KH_2PO_4 buffer, pH7.6; 3.4μM DCMU; 17μM PMS; 3.4mM ascorbate and 88μg chlorophyll. ADP was added to a final amount of 0.420 μmole.

Additions	ATP Formation (μ Eq H^+/mg chll/hr)
Control	262
+ HOQNO 1.6μM	234
+ HOQNO 6.3μM	237

and Good, 1966). HOQNO also inhibited electron flow in digitonin PSII subchloroplast particles as seen in Table III. These particles are more sensitive to the inhibitor than intact chloroplasts, 9.4μM HOQNO completely inhibiting oxygen evolution whereas the same concentration inhibited only 25% in intact chloroplasts. Avron (1961) did not find substantial difference in sensitivity to the inhibitor between intact and fragmented chloroplasts.

The effect of HOQNO on the Hill reaction can also be studied using MV as an electron acceptor. The electron flow in this case is measured by the oxygen uptake upon the oxidation of reduced MV in the presence of azide (a catalase inhibitor). Fig.VI shows that oxygen uptake was inhibited by HOQNO, but this inhibition was completely relieved by the addition of the electron donor couple DCPIP-ascorbate. This indicates that HOQNO is inhibiting somewhere between the point of entry of electrons from the donor couple, and PSII.

Cyclic photophosphorylation catalyzed by PMS in intact chloroplasts (Table IV) was only slightly inhibited by HOQNO. Insensitivity of PMS-catalyzed photophosphorylation has also been reported in swiss-chard chloroplasts (Avron, 1961).

DISCUSSION

The data on the Hill reaction rate, uncoupled and coupled electron flow (Fig.V) indicates that HOQNO is an inhibitor of

electron flow. A similar conclusion was reached by Avron (1961) after observing the inhibition of electron flow and the accompanying phosphorylation, using different electron acceptors. Izawa, Connolly, Winget and Good (1966) placed the site of HOQNO inhibition near DCMU and before the by-pass of the basal electron flow pathway, because of its inhibition of basal, coupled and uncoupled electron flow. Inhibition of electron flow from water to MV by HOQNO was relieved by adding the DCPIP-ascorbate couple (Fig.VI). This limits the site of action of HOQNO to between the point of electron donation by the electron donor couple, and PSII. Avron and Shavit (1965) came to the same conclusion after observing that inhibition of NADP reduction by NOQNO could be reversed by adding the DCPIP-ascorbate couple. Inhibition of oxygen evolution in PSII subchloroplast particles by HOQNO further suggests a site of action close to PSII. These results also suggest a similarity in action between HOQNO and DCMU. Such a suggestion has also been forwarded by Avron (1967).

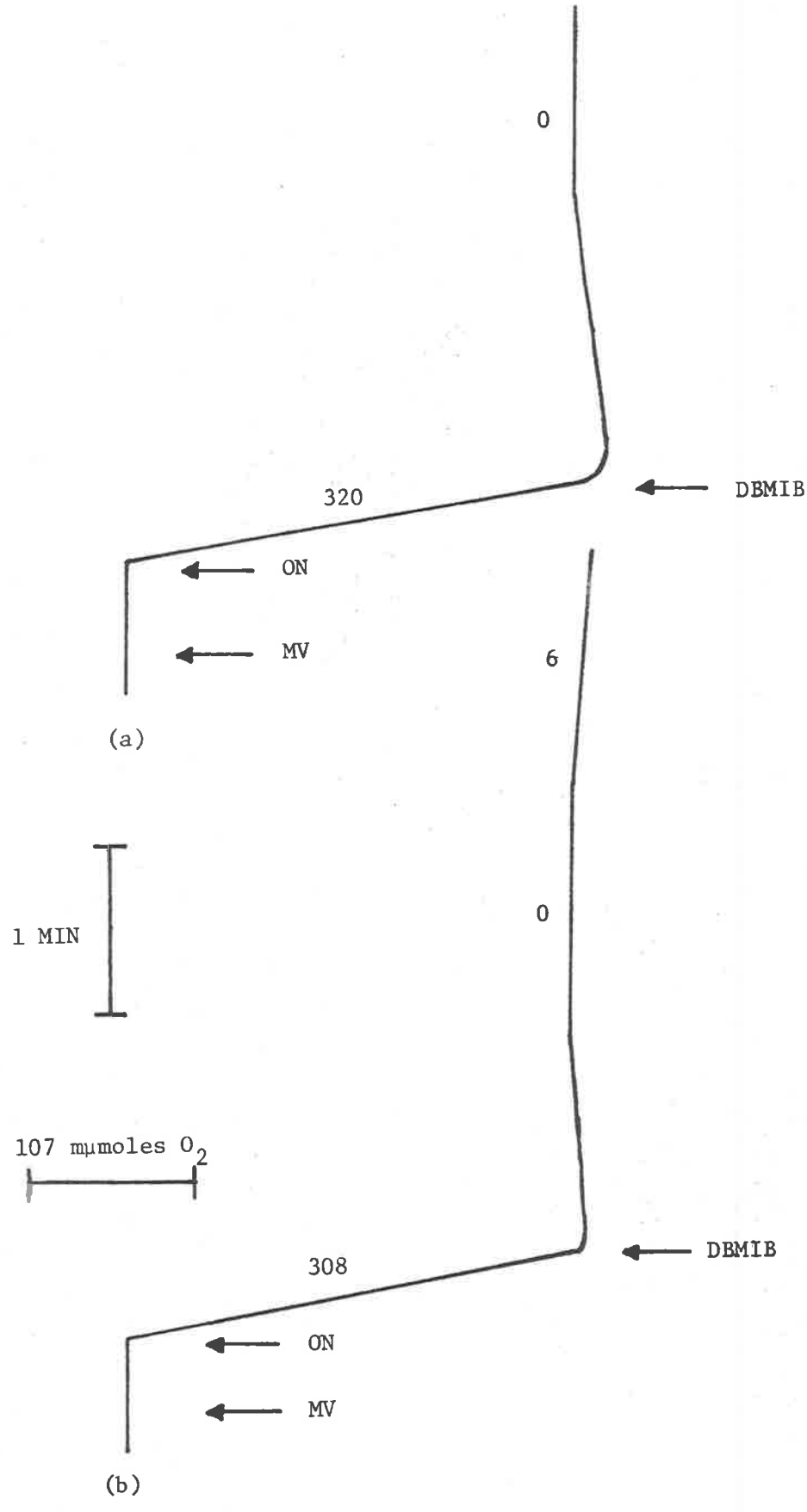
Cyclic photophosphorylation in intact chloroplasts (Table IV) was only slightly inhibited by HOQNO, further strengthening the argument that HOQNO inhibits at a site close to PSII. The fact that the reduction of cytochrome f by PSII is inhibited by HOQNO, whilst its oxidation by PSI is unaffected (Hind and Olson, 1966 and Avron and Chance, 1966), shows its site of action to be removed from PSI.

C. 2,5-DIBROMO-3-METHYL-6-ISOPROPYL-P-BENZOQUINONE (DBMIB)

RESULTS

Trebst, Harth and Draber (1970), first reported the effects of the quinone analog, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), on electron flow in spinach chloroplasts. They observed that it inhibited the Hill reaction rate, as well as coupled and uncoupled electron flow with anthraquinone-2-sulfonate and methyl viologen (MV) as electron acceptor. On the other hand, the same reactions with ferricyanide (FeCN) as electron acceptor were only partially inhibited. NADP reduction with water as electron donor was also inhibited (50% with 0.8μM DBMIB). The photo-reduction of NADP became resistant, when water was replaced by diaminodurene (DAD) or 2,6,dichlorophenolindophenol (DCPIP), plus ascorbate as electron donor. Trebst, Harth and Draber (1970), suggested that because of its structure, DBMIB could be an antagonist of plastoquinone, thus acting between the two photosystems. Bohme, Reimer and Trebst (1971), confirmed the observations of Trebst, Harth and Draber (1970) and in addition found that exogenous plastoquinone relieved the inhibition by DBMIB of non-cyclic electron flow and cyclic photophosphorylation. Bohme and Cramer (1971) observed that DBMIB prevented the reduction of cytochrome f by Photosystem II and the oxidation of cytochrome b_{559} by Photosystem I.

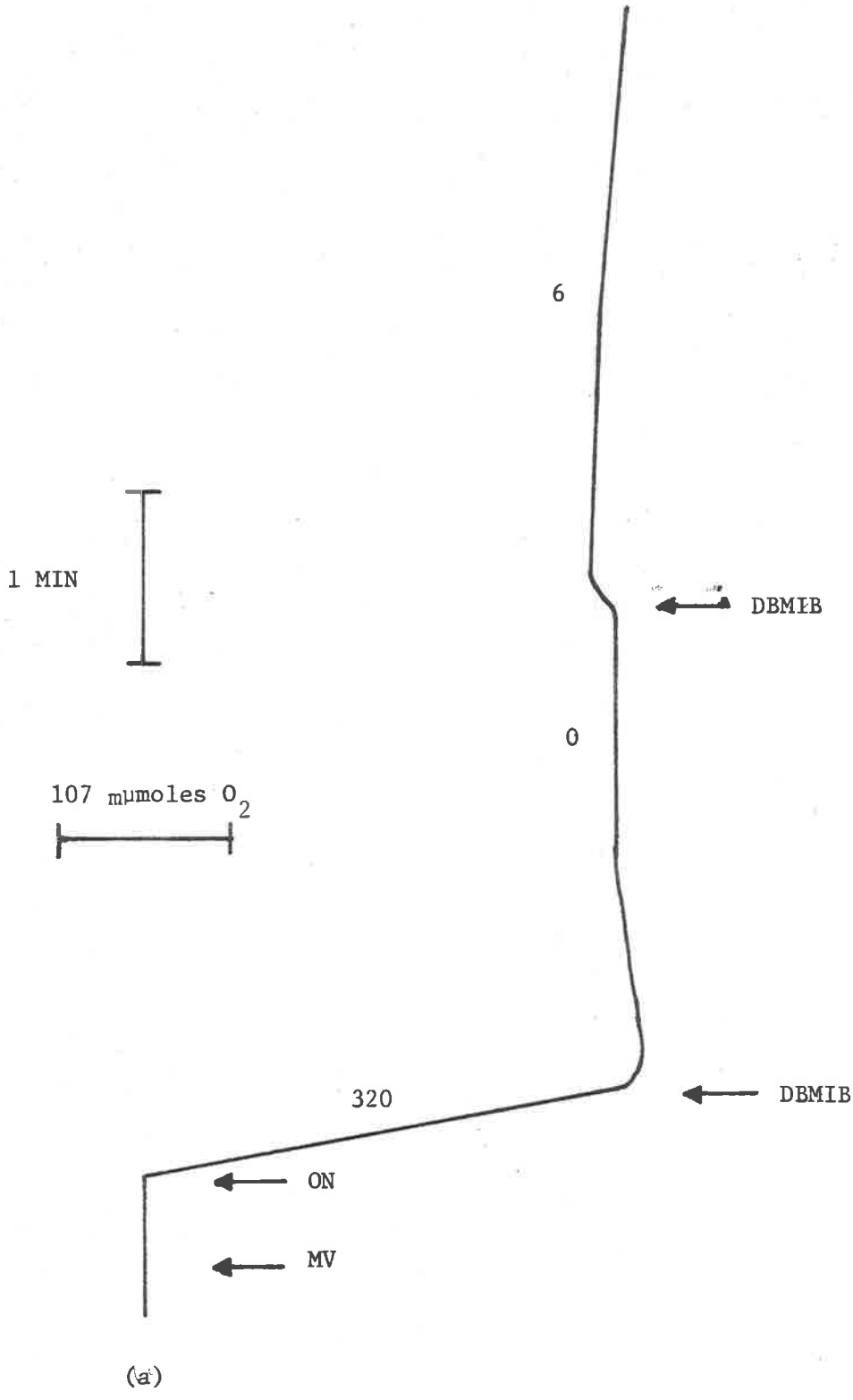
FIGURE VII. Effect of DBMIB on the nigericin-uncoupled electron flow with MV as electron acceptor. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 64μM MV; 0.93mM azide; 21mM KCl; 0.46μM nigericin and pea chloroplasts equivalent to 55μg chlorophyll. Concentrations of DBMIB (a) 1μM and (b) 4.6μM. The numbers along the traces are in μmoles O_2/mg chl/hr.


0
320
DBMIB
ON
MV
6
(a)
1 MIN
0
107 mμmoles O_2
308
DBMIB
ON
MV
(b)

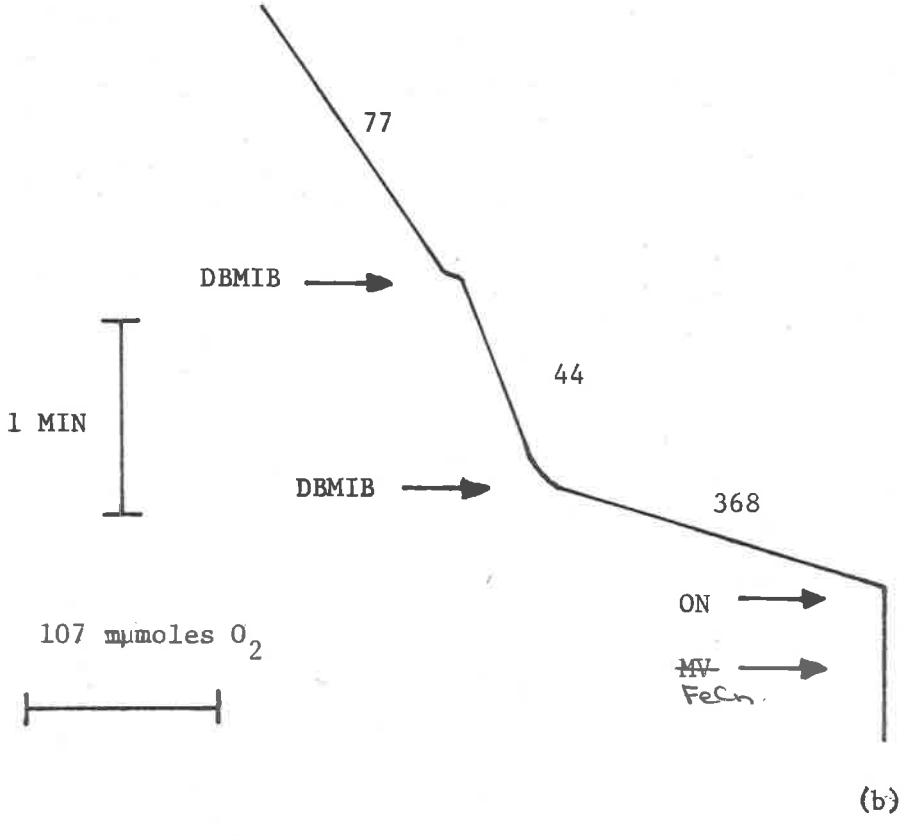
It has been shown that DBMIB is a potent inhibitor of non-cyclic electron flow (Trebst, Harth and Draber, 1970 and Chapter III). At a concentration of 1μM, DBMIB completely inhibited electron flow catalyzed by MV but only partially with FeCN as electron acceptor, regardless of whether it was coupled or uncoupled. The insensitive part of electron flow with FeCN has been shown to be due to the presence of a site of FeCN reduction preceding the site of DBMIB inhibition (Chapter III). In contrast, Bohme, Reimer and Trebst (1971) interpreted that DBMIB does not inhibit the reduction of FeCN by Photosystem II and only affects that portion reduced by Photosystem I. This conclusion was reached after observing that FeCN and DCPIP reductions were inhibited partially in intact chloroplasts by DBMIB but became completely insensitive in sonicated chloroplasts.

Fig.VII shows the effect of DBMIB on electron flow with MV as the electron acceptor. DBMIB (1μM) completely inhibited electron flow, but at a higher concentration (4.6μM), it exhibited an entirely different effect. Electron flow was completely inhibited initially but resumed again at a decreased rate. The same effect could be observed by inhibiting electron flow completely with 1μM DBMIB, then adding 4.6μM DBMIB as shown in Fig.VIII(a). 1μM DBMIB inhibited electron flow with FeCN only partially, and when 4.6μM DBMIB was added, the inhibition by 1μM DBMIB was relieved

FIGURE VIII. Effect of DBMIB on the nigericin-uncoupled electron flow with MV and FeCN as electron acceptor. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 64μM MV and 0.93mM azide or 1.5mM FeCN; 21mM KCl; 0.46μM nigericin and pea chloroplasts equivalent to 55μg chlorophyll. The two additions of DBMIB were 1μM and 4.6μM respectively. The numbers along the traces are in μmoles O_2/mg chl/hr.

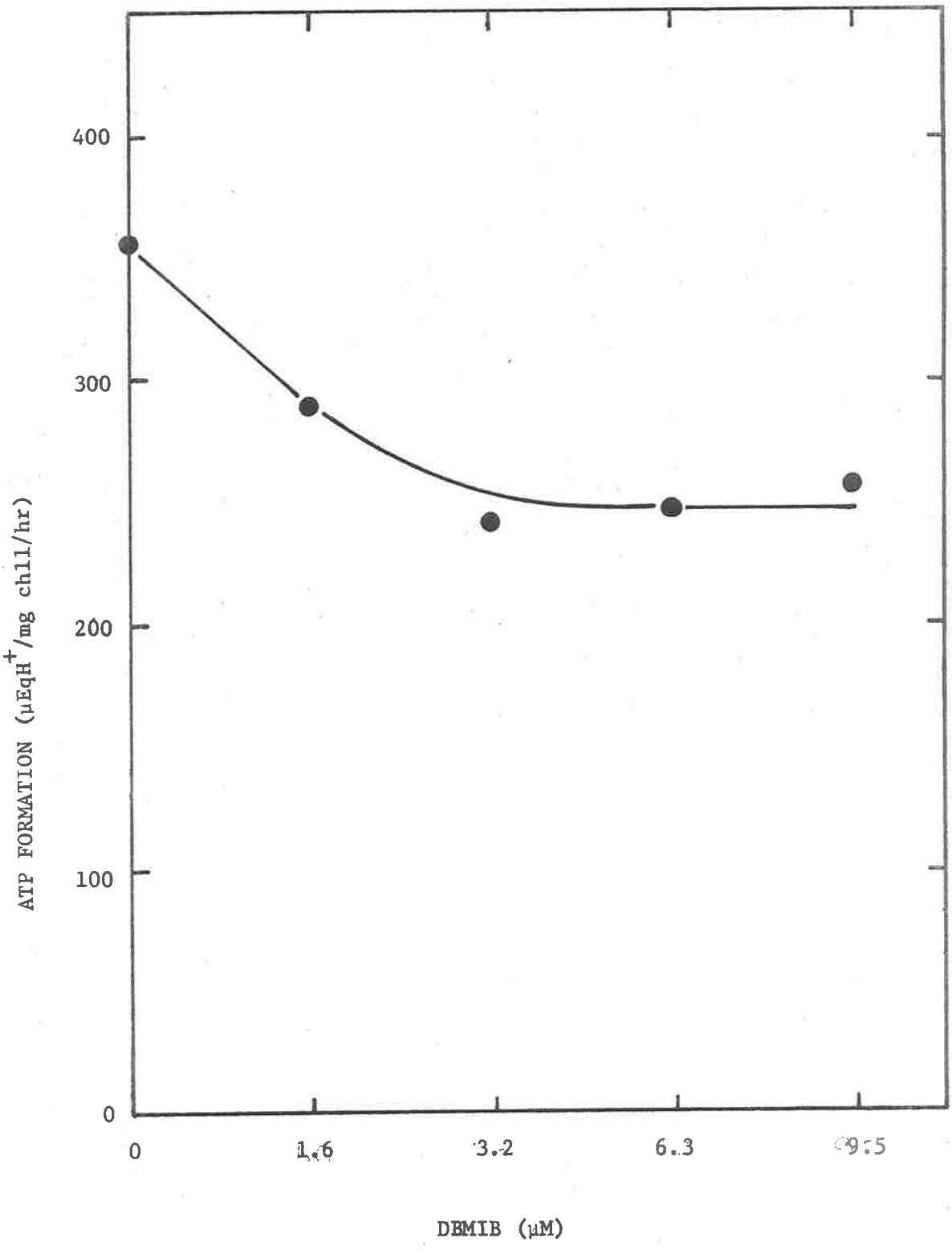


(a)



(b)

FIGURE IX. Effect of DBMIB on cyclic photophosphorylation in pea chloroplasts. The reaction mixture contained 0.4M sucrose; 7.8mM $MgCl_2$; 12.5mM K_2HPO_4-KH_2PO_4 buffer, pH8.0; 15μM PMS; 3mM ascorbate; 3μM DCMU and chloroplasts equivalent to 84μg chlorophyll.


400
300
200
100
0
ATP FORMATION (μEqH+/mg chl1/hr)
0
1.6
3.2
6.3
9.5
DBMIB (μM)

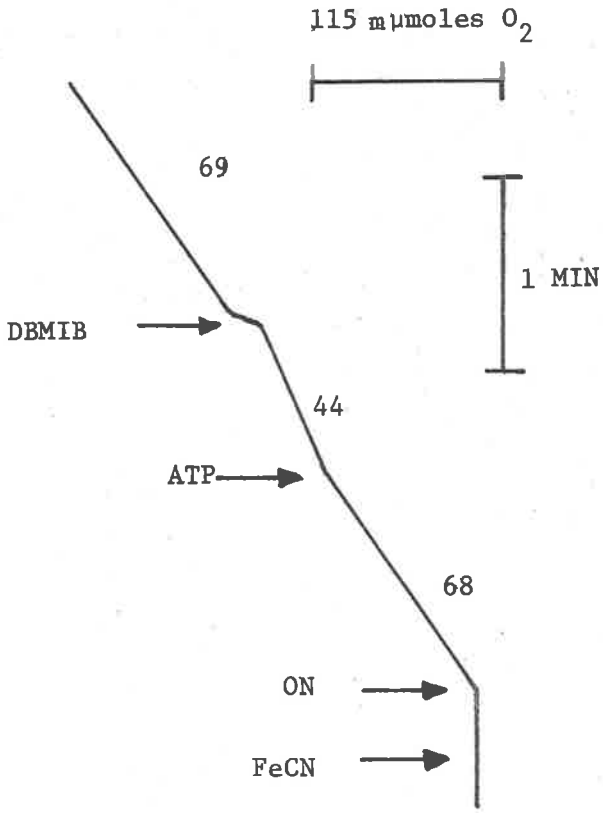


FIGURE X. The DBMIB stimulation of ATP-inhibited Hill reaction rate with FeCN as the electron acceptor. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 17mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 40mM HEPES buffer, pH7.5; 1.5mM and chloroplasts equivalent to 65μg chlorophyll. The concentration of DBMIB added was 4.3μM. ATP was added to a final amount of 1 μmole. The numbers along the trace are in μmoles O_2/mg chl1/hr.

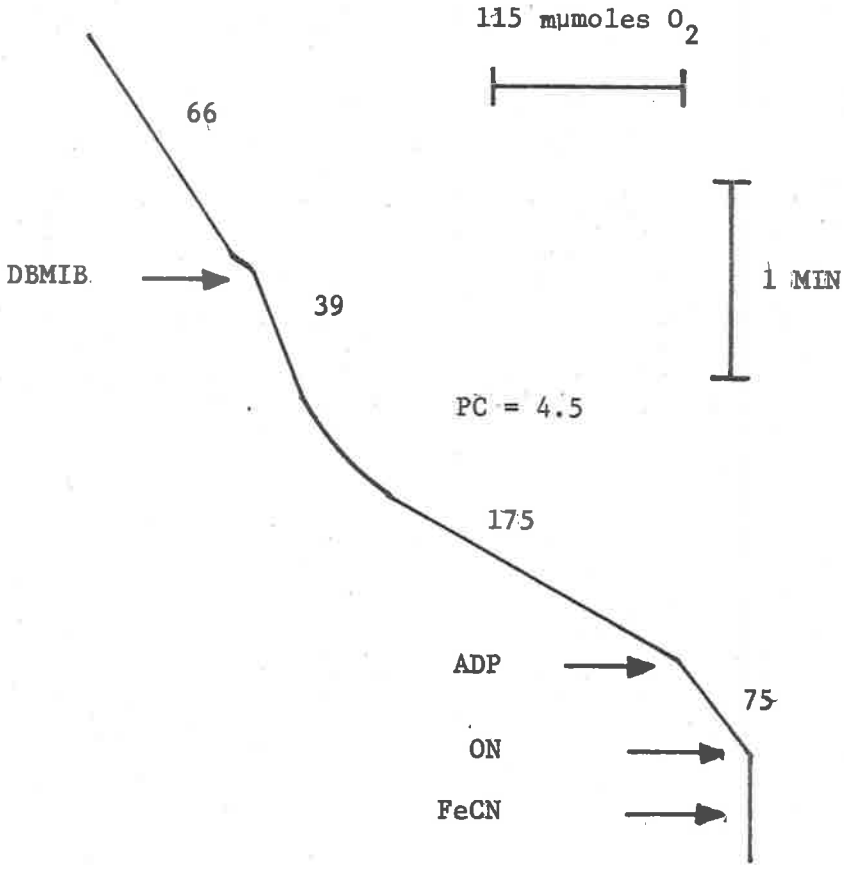


FIGURE XI. The DBMIB stimulation of State 4 rate of oxygen evolution with FeCN as electron acceptor. The reaction mixture was the same as in Fig.X. The concentration of DBMIB added was 4.3μM. ADP was added to a final amount of 0.42 μmole. The numbers along the trace are in μmoles O_2/mg chll/hr.

(Fig.VIII(b). This means that DBMIB at high concentrations could by-pass its own inhibition site by functioning as a hydrogen carrier, similar to plastoquinone (Friend and Redfearn, 1963).

In addition to being an inhibitor of non-cyclic electron flow, DBMIB also inhibits (or uncouples) cyclic photophosphorylation catalyzed by PMS (Fig.IX). However, higher concentrations are needed to inhibit cyclic photophosphorylation relative to the concentration needed to completely inhibit non-cyclic electron flow. The degree of inhibition does not increase with increasing concentrations of DBMIB. These results are not consistent with the observations of Bohme, Reimer and Trebst (1970) who found that PMS-catalyzed cyclic photophosphorylation was completely insensitive to DBMIB. Whether DBMIB was acting as an inhibitor or uncoupler under these conditions was not clear. However, results in Fig.X and XI would suggest that DBMIB was acting as a weak uncoupler at these high concentrations. Fig.X shows ATP inhibition of oxygen evolution during Hill reaction with FeCN; this inhibition was relieved by the addition of 4.6μM DBMIB. A polarographic tracing of oxygen evolution with FeCN as electron acceptor, exhibiting a State3/State 4 transition with ADP was shown in Fig.XI. When DBMIB (4.6μM) was added during State 4, a stimulation of the State 4 rate was observed. These

TABLE V. Effect of DBMIB, CCCP and nigericin on electron flow from $DCPIPH_2$ to MV in pea chloroplasts. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH 7.5; 35μM DCPIP; 4.63mM ascorbate; 64μM MV; 0.97mM azide; 3.4μM DCMU and 40μg chlorophyll. 21mM KCl was also added with nigericin.

Additions	Electron Flow (μmoles O_2/mg chl/hr)
Control	32
DBMIB 2.3μM	52
DBMIB 4.6μM	97
DBMIB 9.2μM	203
CCCP 9.2μM	203
Nigericin 0.46μM	224

effects are characteristic of uncouplers, suggesting that DBMIB also possesses uncoupling properties.

Non-cyclic electron flow from $DCPIPH_2$ to MV or NADP is known to be stimulated by uncouplers (Wessels, 1964; Keister, 1965 and Izawa, Connolly, Winget and Good, 1966). CCCP and nigericin plus K^+ ions stimulated the rate of electron flow from $DCPIPH_2$ to MV considerably (Table V). Similarly, DBMIB stimulated the electron flow from $DCPIPH_2$ to MV, and at a concentration of 9.2μM, the extent of stimulation was comparable to that with CCCP or nigericin. Therefore, it seems as if DBMIB was acting in a similar manner to CCCP and nigericin under these conditions. The report that 1μM DBMIB had no effect on the NADP reduction by $DCPIPH_2$ (Bohme, Reimer and Trebst, 1971) is consistent with these observations.

DISCUSSION

DBMIB was shown to inhibit non-cyclic electron flow completely when electron acceptors like MV or NADP are used, the reduction of which requires both photosystems (Trebst, Harth and Draber, 1970; see also Chapter III). On the other hand, when FeCN was used as the electron acceptor, electron flow was only partially inhibited (Trebst, Harth and Draber, 1970; see also Chapter III). Bohme, Reimer and Trebst (1971) observed that the reductions of FeCN and DCPIP became totally insensitive to DBMIB in sonicated spinach

chloroplasts. The partial inhibition of FeCN reduction by DBMIB in intact chloroplasts has been shown to be due to the presence of another site of FeCN reduction preceding the DBMIB site of inhibition (Chapter III). Katoh and Takamiya (1965) have shown that electron flow chain was interrupted somewhere between the two photosystems by sonication. Therefore, it seems that sonication has caused FeCN and DCPIP to be reduced at a site(s) preceding the DBMIB site of inhibition.

Results in Fig.VII indicated that in addition to being an inhibitor of non-cyclic electron flow, DBMIB was able to substitute as a hydrogen carrier similar to plastoquinone (Friend and Redfearn, 1963). Low concentrations (up to 1μM) only inhibited electron flow with MV (see also Trebst, Harth and Draber, 1970), but high concentrations of DBMIB were needed to overcome this inhibition and allow electron flow to resume again. Thus, DBMIB must be by-passing its own site of inhibition. The rate of electron flow with MV, in the presence of a high concentration of DBMIB was slow compared to the corresponding rate of electron flow with FeCN. This could be due to a non-enzymic reduction of FeCN by DBMIB, whereas electrons have to traverse a longer chain of intermediates to MV.

DBMIB was also found to inhibit cyclic photophosphorylation with PMS. This is contrary to the observations of Bohme, Reimer and Trebst (1971), who found that DBMIB (10μM) did not have any inhibitory

effect on PMS-catalyzed cyclic photophosphorylation, but inhibited menadione-catalyzed cyclic photophosphorylation. On the basis of these differential inhibitions, they concluded that plastoquinone does not participate in PMS-catalyzed cyclic system. Bohme, Reimer and Trebst (1971) also observed partial reversal of DBMIB inhibition of menadione-catalyzed cyclic photophosphorylation by plastoquinone. The inhibition of PMS-catalyzed cyclic photophosphorylation by DBMIB (Fig.IX) could not be due to participation of plastoquinone in the system, but rather due to a weak uncoupling effect of DBMIB. This conclusion was drawn by analogy from the stimulation of State 4 and ATP-inhibited Hill reaction rates by DBMIB (Figs. X and XI). This idea was also supported by the stimulation of the electron flow from $DCPIPH_2$ to MV by high concentrations of DBMIB (Table V). CCCP and nigericin also stimulated this electron flow, therefore, DBMIB under these conditions was acting similarly to these uncouplers. The concentration of DBMIB used by Bohme, Reimer and Trebst (1971) was too low to observe a stimulation of NADP reduction by $DCPIPH_2$.

Therefore, it is concluded that DBMIB acts as an inhibitor of non-cyclic electron flow, presumably at the site of function of plastoquinone (see Bohme and Cramer, 1971), between the two photosystems. In addition, it possesses weak uncoupling properties at high concentrations, higher than needed for complete inhibition of non-cyclic electron flow, and is also able to act as an intermediate in non-cyclic electron flow, by-passing its own site of inhibition.

CHAPTER VI

PHOTOSPHOPHORYLATION IN DIGITONIN SUB-CHLOROPLAST PARTICLES - Δ pH OR Δ E? RELATIONSHIP OF THE 518-520nm ABSORBANCE CHANGE TO PHOTOPHOSPHORYLATION IN CHLOROPLASTS AND PHOTOSYSTEM I SUB-CHLOROPLAST PARTICLES.

INTRODUCTION

Electron flow in chloroplasts, mitochondria and bacterial chromatophores has been shown to be closely associated with a proton translocating system (Neumann and Jagendorf, 1964; Jagendorf and Uribe, 1966; Mitchell and Moyle, 1967 and von Stedingk and Baltscheffsky, 1966). Mitchell (1961 and 1966) has also postulated that electron transport is coupled to phosphorylation. According to this hypothesis, the movement of protons across the membranes creates a pH gradient and a membrane potential, the two components together making up the proton motive force (P.M.F.). Under conditions where the pH gradient (Δ pH) is reduced, the membrane potential (Δ E) could become the major part of the proton motive force. Whereas the proton translocation is easily detectable as the change in pH of the suspending medium, the detection and identification of a membrane potential is considerably more difficult. Junge and Witt (1968) proposed that light-induced absorbance changes at 515nm, due to chlorophyll b, reflect an electrical potential across the thylakoid membrane. Strichartz and Chance (1972) have

confirmed that these light-induced absorbance changes reflect membrane potential in chloroplasts. A similar correlation has been proposed for the light-induced "carotenoid shift" in chromatophores, by Fleischman and Clayton (1968) and Jackson and Crofts (1969).

In subchloroplast particles prepared by sonication, McCarty (1968) found that photophosphorylation occurred under conditions where the light-induced proton uptake was inhibited by NH_4Cl. Later McCarty (1969), after observing that NH_4Cl was a poor uncoupler of photophosphorylation in subchloroplast particles and that valinomycin greatly enhanced the inhibition of photophosphorylation by NH_4Cl, concluded that phosphorylation in chloroplasts was dependent upon a pH gradient (see Jagendorf and Uribe, 1966) whereas a membrane potential may be required for phosphorylation in subchloroplast particles. Nelson, Drechsler and Neumann, (1970)found that subchloroplast particles prepared from lettuce by incubation with digitonin did not exhibit any light-induced proton uptake activity, yet were able to phosphorylate at high rates. Neumann, Ke and Dilley (1970), using subchloroplast particles prepared by the same method, looked at the possible relationship of the 515nm absorbance changes to phosphorylation. After observing differential effects by some uncouplers (e.g. NH_4Cl and nigericin plus K^+) on the two processes, they came to the conclusion that there was no correlation between the two processes. On the other hand, Hauska, McCarty and Olson (1970) observed that in spinach subchloroplast

particles obtained by digitonin treatment (Anderson and Boardman, 1966) ATP formation was possible under conditions where the light-induced proton uptake was totally inhibited; however, under conditions where the proton uptake and the absorbance increase at 518nm were inhibited by valinomycin and NH_4Cl, phosphorylation was completely abolished. When the uncoupling of photophosphorylation by valinomycin and NH_4Cl was reversed by the addition of aliphatic amines, the absorbance change at 518nm was restored as well. From these results they concluded that there was a correlation between phosphorylation and the light-induced 518nm absorbance change in subchloroplast particles.

Subchloroplast particles have been prepared from peas using the slightly modified digitonin method of Anderson and Boardman (1966), and these particles do not show any light-induced proton uptake activity. However, they are capable of phosphorylating at rates comparable to chloroplasts. The effect of various uncouplers of photophosphorylation on the ATP formation by the particles has been studied. An attempt has also been made to measure the 518-520nm absorbance change in chloroplasts as well as in digitonin subchloroplast particles to determine any relationship between the phosphorylation and these absorbance changes. The effect of various electron transport inhibitors and uncouplers on the absorbance change has been studied also.

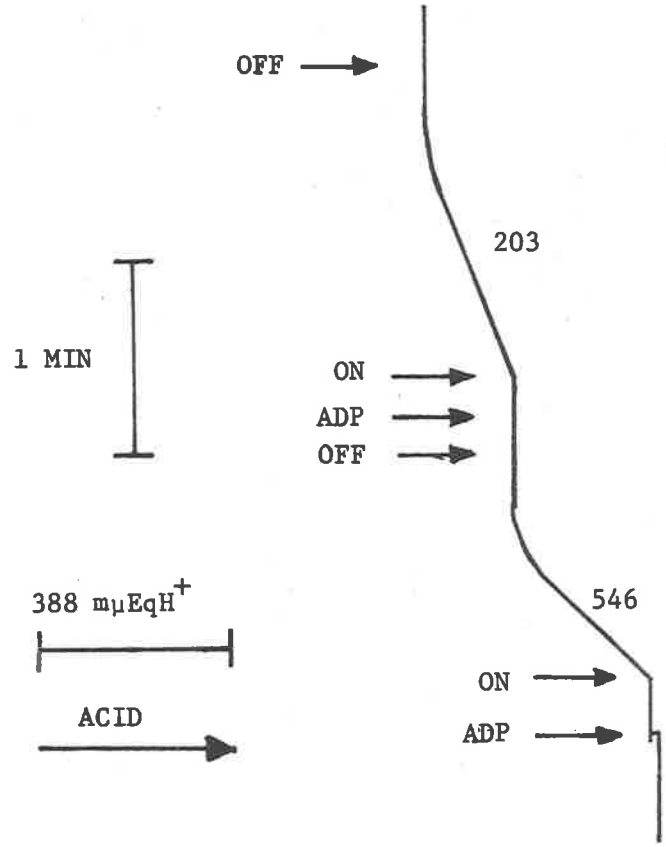


FIGURE I. Cyclic photophosphorylation catalyzed by PMS in digitonin PSI subchloroplast particles. The reaction mixture contained 0.4M sucrose; 7.8mM $MgCl_2$; 12.5mM K_2HPO_4-KH_2PO_4 buffer, pH8.0; 15μM PMS; 3mM ascorbate; 3μM DCMU and SCP equivalent to 42μg chlorophyll. The two additions of ADP contained 0.463 μmole. The numbers along the trace are in μEqH^+/mg chll/hr.

TABLE I. Electron flow and cyclic photophosphorylation in digitonin subchloroplast particles (SCP). The reaction mixture for electron flow contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 2.38mM FeCN or 25μM DCPIP; 3.4mM ascorbate; 10μM NADP and excess ferredoxin/ferredoxin-NADP reductase. In Experiment I SCP equivalent to 75μg (10,000xg); 150μg (30,000xg); 42μg (50,000xg) and 32μg (144,000xg) chlorophyll were added; in Experiment II 41μg (50,000xg) and 35μg (144,000xg) chlorophyll were added respectively. The reaction mixture for cyclic photophosphorylation contained 0.4M sucrose; 6.7mM K_2HPO_4-KH_2PO_4 buffer, pH7.6; 8.5mM $MgCl_2$; 3.4μM DCMU; 17μM PMS and 3.4mM ascorbate. SCP were added to the same amount as in electron flow measurements. ADP was added to a final amount of 0.492 μmole. NADP reduction was followed by the increase in O.D at 340nm; the cuvettes were illuminated for 1 min as described in Chapter II.

Fractions	Electron Flow μmoles O_2/mg chll/hr or NADP reduced/mg chll/hr	ATP_+ formation μEqH^+/mg chll/hr
Experiment I		
1. 10,000xg	78	-
2. 30,000xg	45	-
3. 50,000xg	0	357
4. 144,000xg	0	468
Experiment II		
1. 50,000xg	289	384
2. 144,000xg	317	333

TABLE II. Effect of digitonin fragmentation on the light-induced H^+ uptake. The reaction mixture contained 0.4M sucrose; 6.5mM KCl; 16μM PMS and washed pea chloroplasts or Photosystem I SCP equivalent to 66μg, 42μg, and 32μg chlorophyll respectively. 3.4mM ascorbate to reduce PMS non-enzymically, was also added to the reaction mixture with SCP. Measurements with SCP were carried out under anaerobic conditions to prevent a pH change due to the oxidation of reduced PMS. This was acheived by bubbling N_2 slowly into the reaction medium.

	H^+ Uptake	
	Extent $m\mu EqH^+/mg$ chll	Rate "on" $m\mu EqH^+/min$
Chloroplasts	691	380
50,000xg SCP	0	0
144,000xg SCP	0	0

TABLE III. Effect of digitonin fragmentation on photophosphorylation. The reaction mixture contained the same components as in Table I, except that pea chloroplasts and Photosystem I SCP equivalent to 48µg, 42µg and 35µg chlorophyll were added in these experiments.

		ATP Formation μEqH^+/mg chl1/hr
1.	Chloroplasts	497
2.	50,000xg SCP	581
3.	144,000xg SCP	552

A. ABSENCE OF THE LIGHT-INDUCED PROTON UPTAKE AND PRESENCE OF CYCLIC PHOTOPHOSPHORYLATION IN DIGITONIN-PSI SUBCHLOROPLAST PARTICLES.

Subchloroplast particles (SCP) prepared by digitonin treatment lost their capacity to exhibit the reversible light-induced proton uptake activity, whereas the capacity to phosphorylate was not impaired (Nelson, Drechsler and Neumann, 1970). Fig.I shows a pH trace demonstrating cyclic photophosphorylation catalyzed by PMS in SCP. The requirement of a reductant of PMS in these SCP has been shown in Chapter III, when red light was used for illumination (see also Hauska, McCarty and Racker, 1970). No exogenous reductant was necessary when white light was used (Jagendorf and Margulies, 1960). These SCP are similar to the particles prepared by Anderson and Boardman (1966), as they are devoid of Photosystem II activity, but different from those prepared by Nelson, Drechsler and Neumann (1969) which contained an appreciable amount of Photosystem II activity (Table I). The effect of digitonin treatment on the light-induced proton uptake and phosphorylation in chloroplasts is shown in Table II and III. It is clear that digitonin treatment abolished the light-induced proton uptake whereas these particles were still able to phosphorylate at rates comparable to chloroplasts.

Anderson and Boardman (1966) have shown that the light particles (D_{50} and D_{144}) have a high chlorophyll a to chlorophyll b ratio

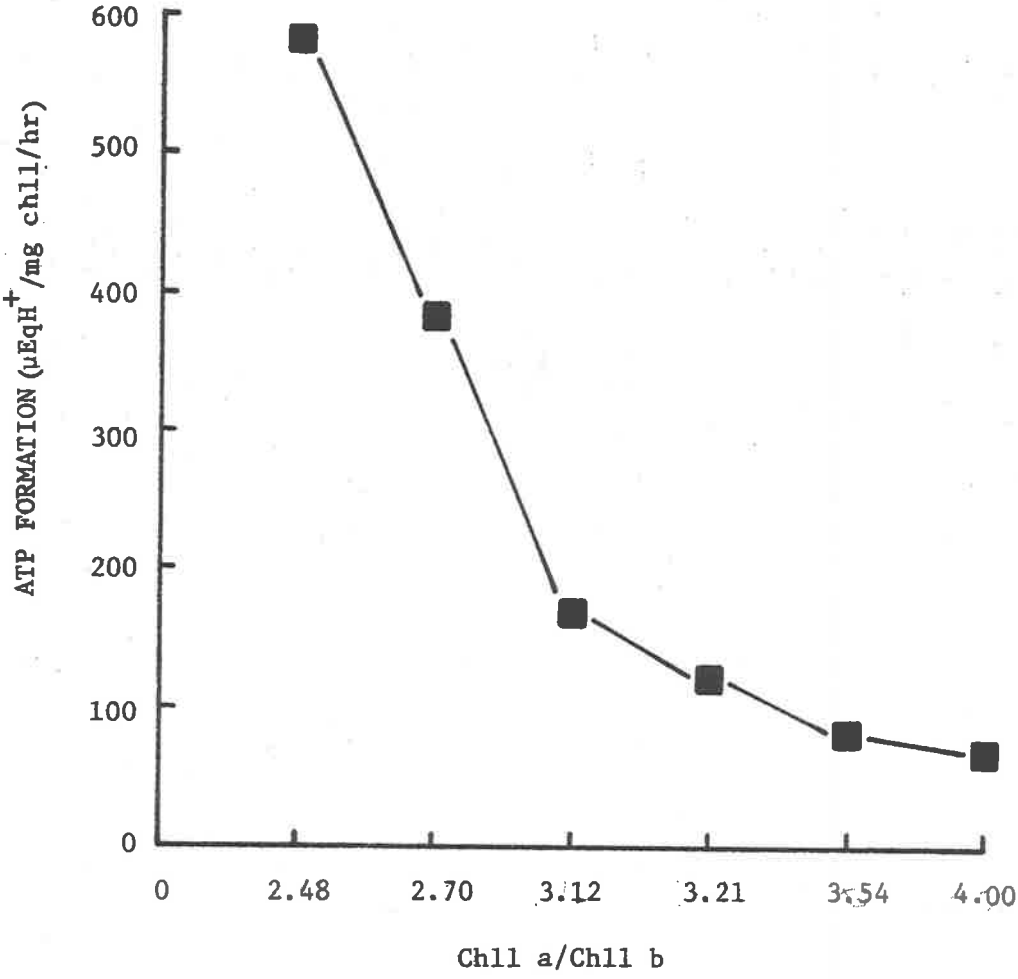


FIGURE II. The relationship between chlorophyll a to chlorophyll b ratio and phosphorylation in digitonin PSI subchloroplast particles. The experimental conditions were the same as in Fig.I.

TABLE IV. Synergistic effect of NH_4Cl and valinomycin on photophosphorylation in chloroplasts and PSI subchloroplast particles. Experimental conditions were the same as those described for Table I. Pea chloroplasts and SCP were added to an equivalent of 48 and 32μg chlorophyll respectively.

Additions	ATP formation, % of control
Chloroplasts	100
NH_4Cl 0.7mM	35
NH_4Cl 1.4mM	25
NH_4Cl 0.7mM + Val. 1.7μM	0
SCP	100
NH_4Cl 0.7mM	64
NH_4Cl 0.7mM + Val. 3.2μM	36

100% chloroplasts = 497 μEqH^+/mg chll/hr

100% SCP = 468 μEqH^+/mg chll/hr

relative to intact chloroplasts. Arnon, Tsujimoto, McSwain and Chain (1968), following the same procedure, obtained SCP with similarly high chlorophyll a to chlorophyll b ratios. These particles also carried out cyclic photophosphorylation with PMS, at low rates (approximately 50 µmoles ATP/mg chll/hr). Hauska, McCarty and Olson (1970) using the same procedure, and Nelson, Drechsler and Neumann (1970) using a different procedure, have shown that digitonin SCP were able to phosphorylate at high rates. Fig.II shows the effect of the chlorophyll a to chlorophyll b ratio in digitonin SCP on photophosphorylation. It was evident that the rates of photophosphorylation by these SCP decreased as the ratio of chlorophyll a to chlorophyll b increased. This suggests that as the particles became more enriched in chlorophyll a (reaction centre I), their ability to phosphorylate declined.

B. EFFECT OF UNCOUPLERS ON PHOTOPHOSPHORYLATION IN DIGITONIN PSI SUBCHLOROPLAST PARTICLES

Photosphosphorylation in SCP prepared by sonication (McCarty, 1968, 1969) and digitonin-treatment (Nelson, Drechsler and Neumann, 1970; Hauska, McCarty and Olson, 1970 and Neumann, Ke and Dilley, 1970) was found to be resistant to inhibition by NH_4Cl. FCCP (Nelson, Drechsler and Neumann, 1970), CCCP (Hauska, McCarty and Olson, 1970) and atebrin (Nelson, Drechsler and Neumann, 1970) were also found to be effective in inhibiting the photophosphorylation in SCP. Table IV shows the synergistic uncoupling of

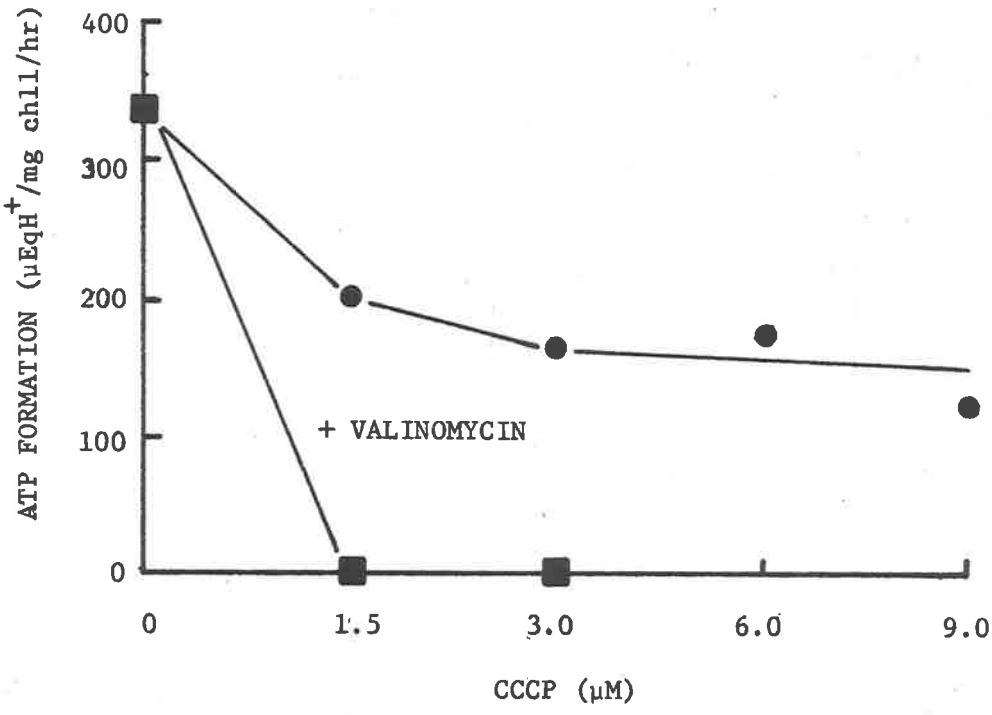


FIGURE III. Effect of CCCP on cyclic photophosphorylation in digitonin PSI ~~chl~~ sub chloroplast particles. The experimental conditions were the same as in Fig.I, except that SCP were added equivalent to 41μg chlorophyll. Concentration of valinomycin, 1.5μM and KCl, 21mM.

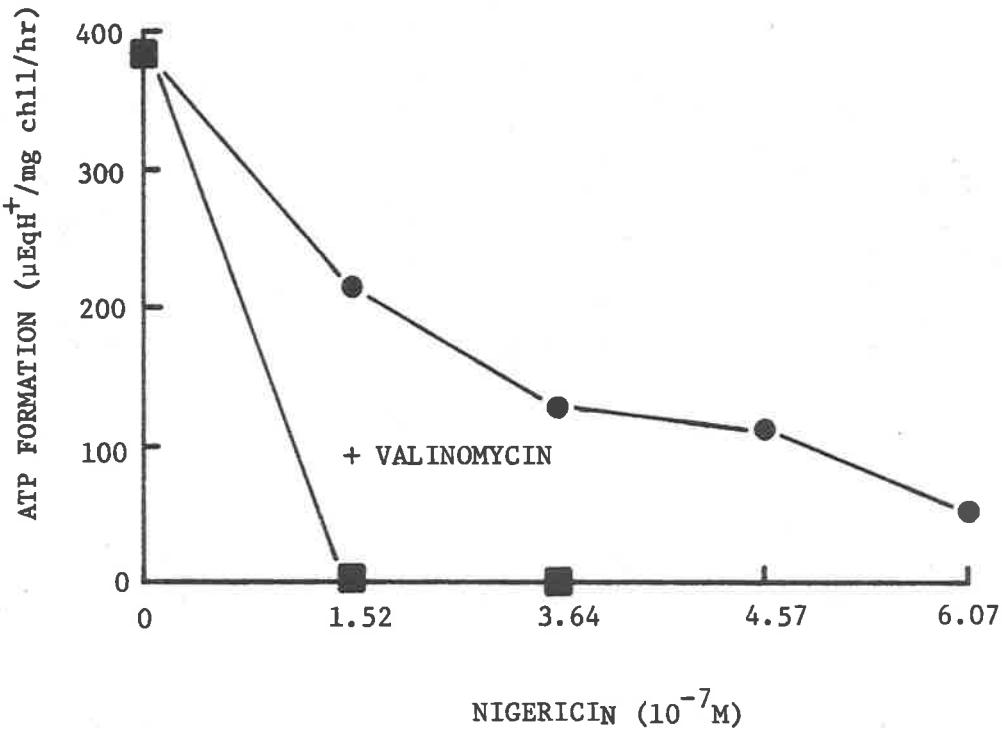
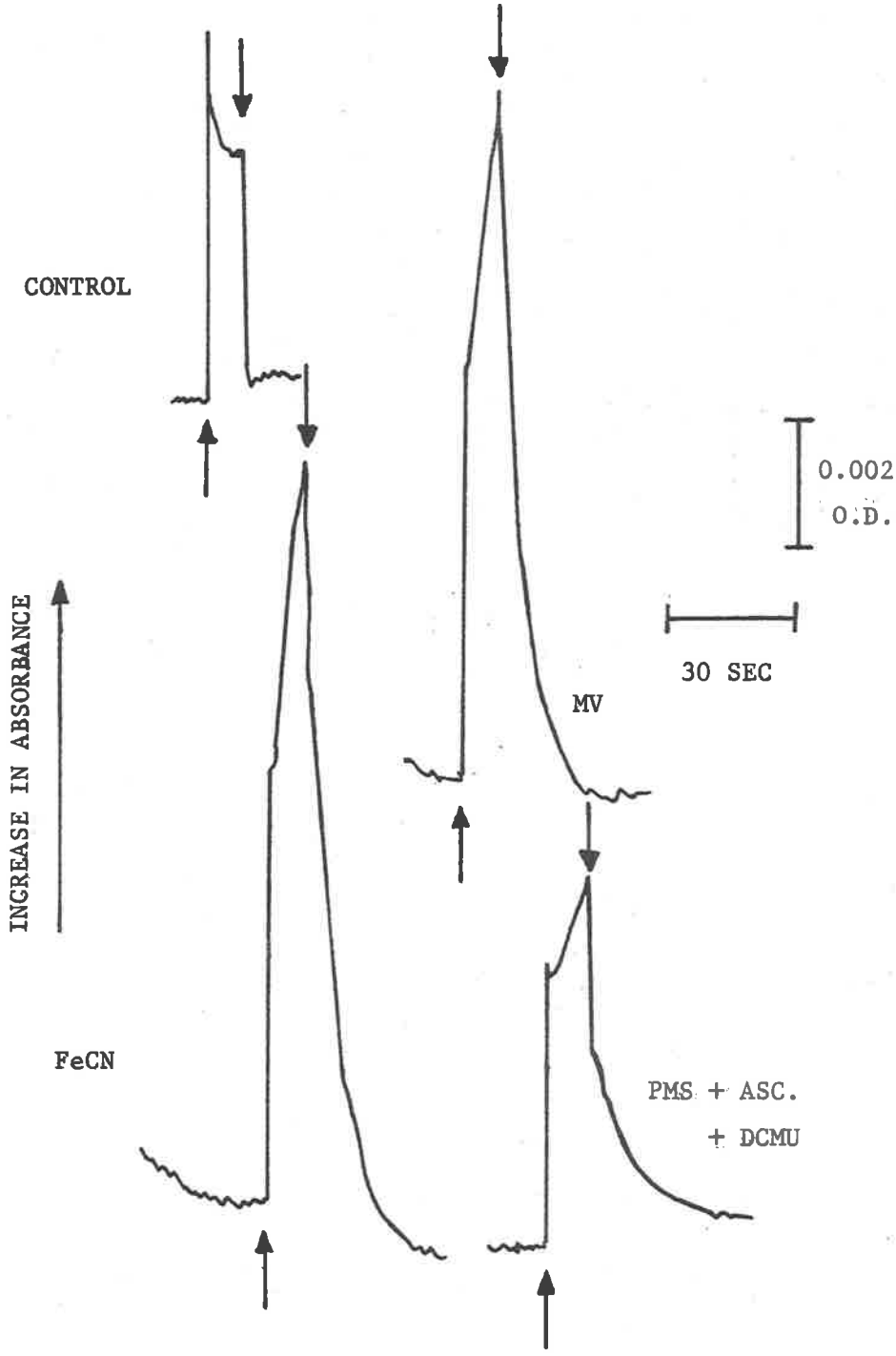


FIGURE IV. Effect of nigericin on cyclic photophosphorylation in digitonin PSI subchloroplast particles. The experimental conditions were the same as in Fig.III. Concentration of valinomycin, 1.5μM and KCl, 21mM.

photophosphorylation in SCP and chloroplasts by NH_4Cl and valinomycin. It can be seen that NH_4Cl, at a concentration which uncoupled photophosphorylation in chloroplasts quite effectively, had little effect on photophosphorylation in SCP. The presence of valinomycin greatly increased the uncoupling of photophosphorylation in SCP by NH_4Cl, consistent with the observations of Hauska, McCarty and Olson (1970). CCCP on its own was quite effective in uncoupling photophosphorylation in SCP (Fig.III) and when valinomycin was present with CCCP, photophosphorylation was completely abolished. Nigericin and K^+ ions, which uncoupled photophosphorylation in chloroplasts (Shavit, Dilley and San Pietro, 1968) was reported to be quite ineffective on phosphorylation in SCP (Nelson, Drechsler and Neumann, 1970 and Neumann, Ke and Dilley, 1970), but became effective when valinomycin was also present (Hauska, McCarty and Olson, 1970). Fig.IV shows the effect of nigericin and K^+ ions in the absence or presence of valinomycin on photophosphorylation in SCP. Valinomycin was seen to greatly enhance the uncoupling of photophosphorylation by nigericin, consistent with the earlier observations.

FIGURE V. The light-induced absorbance change at 518nm in pea chloroplasts in the absence and presence of electron acceptors. Chloroplasts were suspended in 30mM Tricine, pH7.6; 50mM KCl and 50mM NaCl. Concentration of chlorophyll, 75μg/ml; FeCN, 1.5mM; MV and azide, 64μM and 0.85mM; PMS, 28μM; ascorbate, 2.8mM and DCMU, 2.8μM. Light intensity; Corning filter 2-64 (>600nm), 1 x 10^6 ergs/cm^{-2}/sec^{-1}. Light "on" and "off" responses are indicated by arrow upwards and downwards respectively.


CONTROL
0.002
O.D.
30 SEC
INCREASE IN ABSORBANCE
MV
FeCN
PMS + ASC.
+ DCMU

518 - 540nm

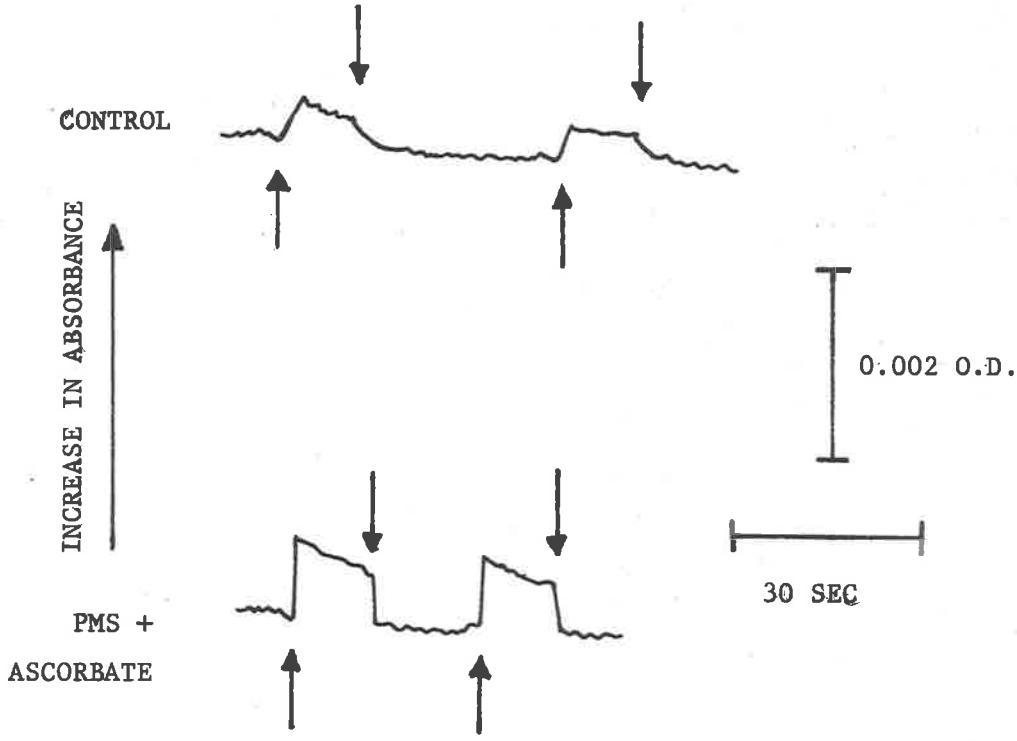


FIGURE VI. The light-induced absorbance change at 518nm in digitonin PSI subchloroplast particles. The experimental conditions were the same as in Fig.V. Concentrations of chlorophyll, 50µg/ml; PMS, 28µM; ascorbate, 2.8mM and DCMU, 218µM. Light "on" and "off" responses are indicated by arrow upwards and downwards respectively.

C. *THE 518-520nm ABSORBANCE CHANGE IN CHLOROPLASTS AND DIGITONIN PSI SUBCHLOROPLAST PARTICLES*

Fig.V shows the 518nm absorbance change in pea chloroplasts under continuous illumination, in the absence and presence of electron acceptors. The signal in the absence of electron acceptors consists of two components, a fast component and a slow component which attains a steady-state level. In the presence of electron acceptors, the signal consisted of the fast component, and a slow component which did not attain a steady-state level during the 10 sec illumination period. The presence of the signal with FeCN and MV suggests that both PSII alone, and PSII plus PSI were able to produce the signal. DCMU completely abolished the signal, but it could be reactivated upon the addition of PMS plus ascorbate. This confirms that PSI was also capable of producing the signal. Similar conclusions have been reached by Rubinstein (1965) and Govindjee and Govindjee (1965), after studies of the 520nm absorbance change in *Chlorella* cells.

The 518nm absorbance change can also be observed in digitonin PSI SCP (Fig.VI). The signal became more pronounced upon the addition of PMS and ascorbate, and consisted of the fast and the slow component which attained a steady-state level. This was different to the signal produced by PMS plus ascorbate in DCMU-inhibited chloroplasts, in that the slow component which did not attain a steady-state level was absent. Hauska, McCarty and Olson (1970) and Murata and Fork (1971) have reported the need of an electron acceptor in order to observe the signal in digitonin and French pressure cell PSI SCP respectively.

FIGURE VII. Effect of CCCP on the light-induced absorbance change at 518nm in digitonin PSI sub-chloroplasts. The experimental conditions were the same as in Fig.V. Concentrations of chlorophyll, (a) 50µg/ml; (b) 75µg/ml; CCCP, 10µM; PMS, 28µM; ascorbate, 2.8mM and DCMU, 2.8µM (a); 64µM MV and 0.85mM azide (b).

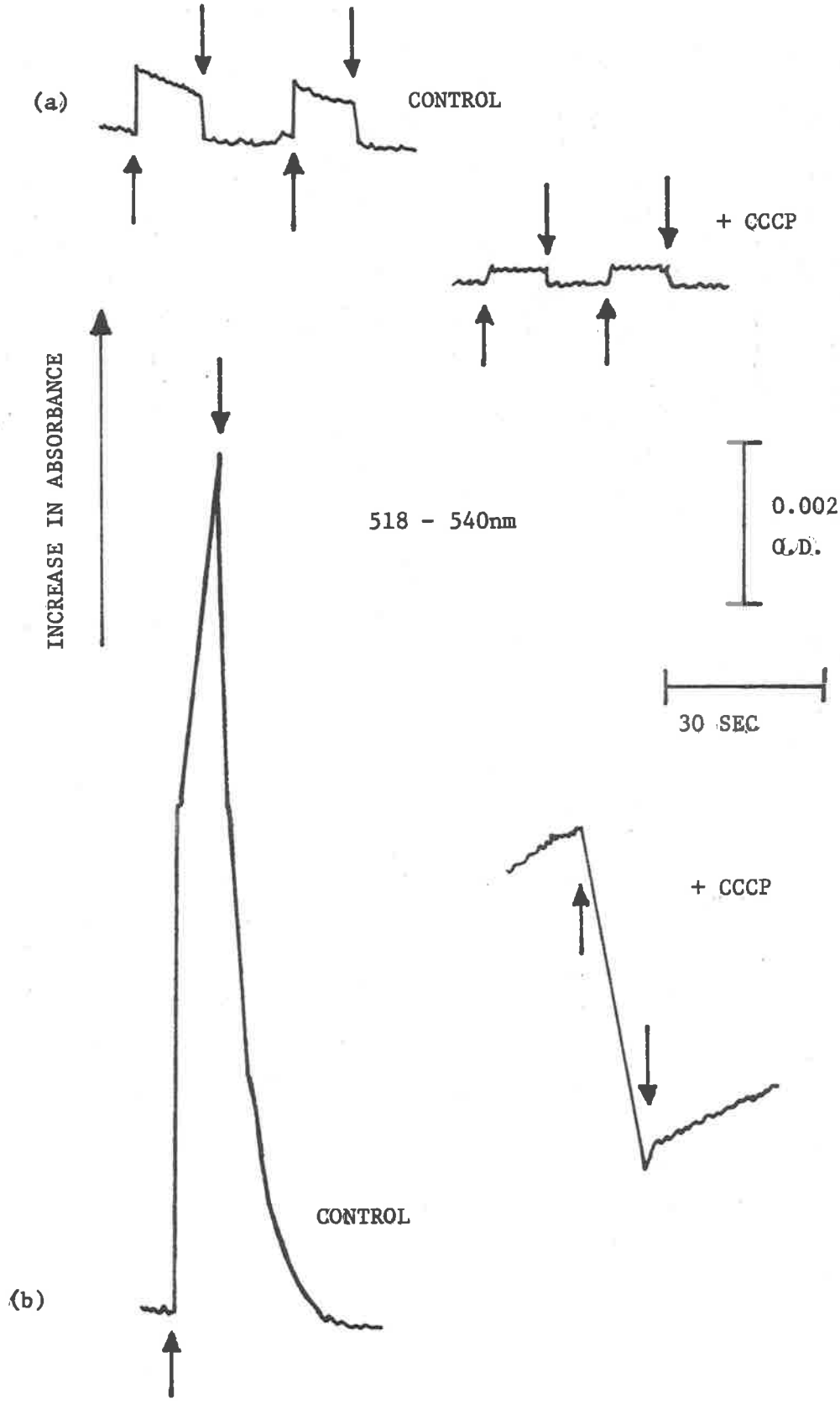

(a)
CONTROL
+ CCCP
INCREASE IN ABSORBANCE
518 - 540nm
0.002
O.D.
30 SEC
+ CCCP
CONTROL
(b)

FIGURE VIII. Effect of nigericin on light-induced absorbance change at 518nm in digitonin PSI subchloroplast particles and chloroplasts. The experimental conditions were the same as in Fig.VII. Concentration of nigericin, 0.57μM and KCl, 21mM.

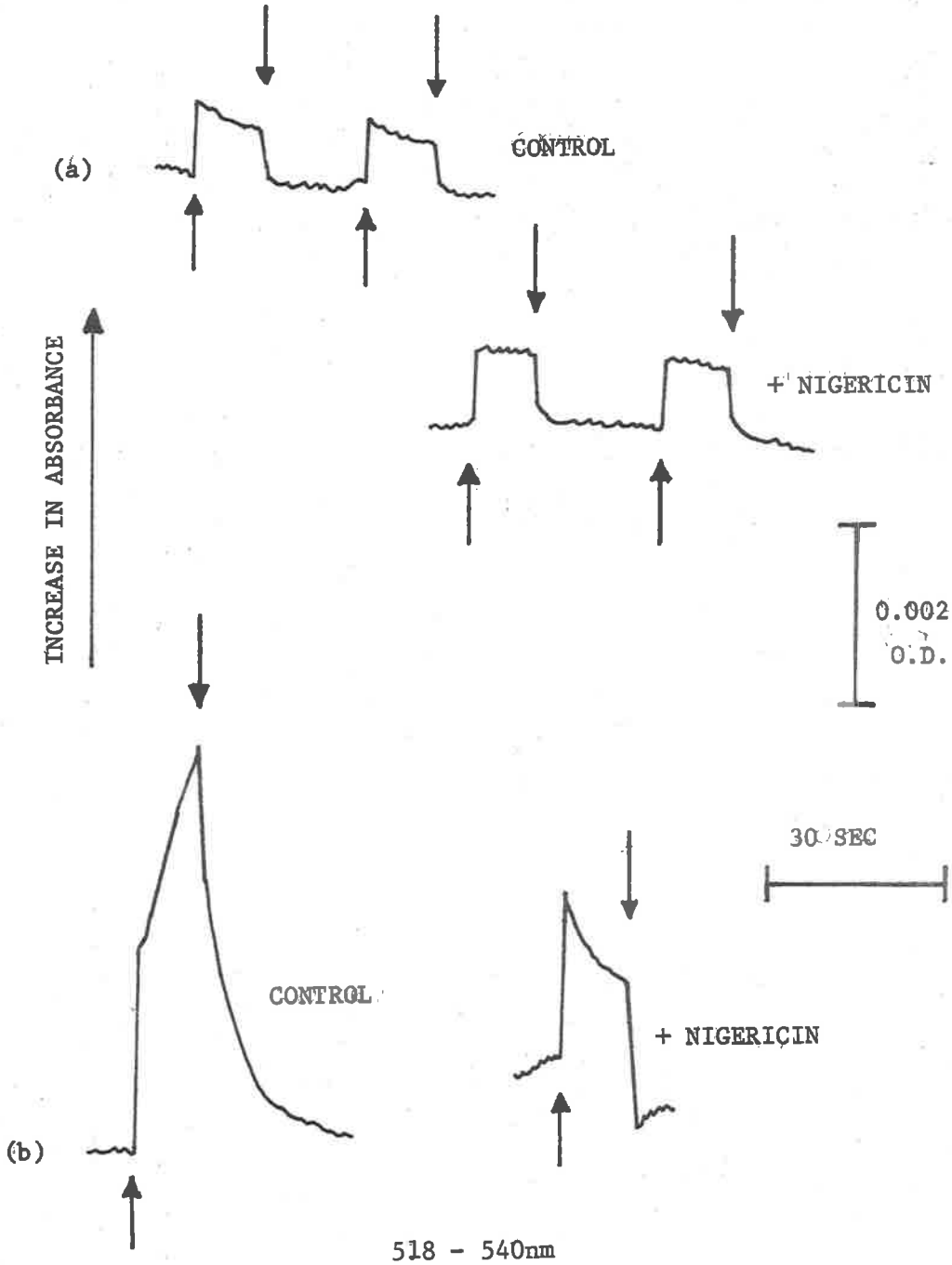

(a)
CONTROL
+ NIGERICIN
INCREASE IN ABSORBANCE
0.002
O.D.
30 SEC
CONTROL
+ NIGERICIN
(b)
518 - 540nm

TABLE V. Effect of phosphorylating agents on the 518nm absorbance change in chloroplasts under continuous illumination. The reaction mixture contained 50mM KCl; 50mM NaCl; 5mM $MgCl_2$; 30mM Tricine buffer, pH7.5; 42μM MV and 75μg/ml chlorophyll. 10mM K_2HPO_4-KH_2PO_4 buffer, pH7.6 and ADP to a final amount of 0.5 μmoles were added where specified. Other experiment conditions were the same as in Table IV.

Additions	O.D at 518nm/mg chll
Control	0.15
+ ADP and Pi	0.08

TABLE VI. Effect of electron transport inhibitors on the 518nm absorbance change in chloroplasts in the absence or presence of electron acceptors. The reaction mixture contained 50mM KCl; 50mM NaCl; 30mM Tricine buffer, pH7.5; 42μM MV or diquat and 75μg/ml chlorophyll. The reaction mixture was illuminated for 10 sec by red light (Corning filter 2-64) of intensity 1 x 10^6 ergs/cm^{-2}/sec^{-1}. The changes in absorbance at 518nm were recorded on a full chart scale of 0.0223 O.D. Reference wavelength 540nm.

Additions	O.D. at 518nm/mg chll
Control	0.08
DCMU 5.6μM	0
DBMIB 5.6μM	0
+ MV	0.13
+ MV + DCMU 5.6μM	0
+ MV + DBMIB 5.6μM	0.01
+ Diquat	0.13
+ Diquat + DCMU 5.6μM	0
+ Diquat + DBMIB 5.6μM	0.01

Neumann, Ke and Dilley (1970) and Hauska, McCarty and Olson (1970) observed that phosphorylating agents inhibited the extent of the signal in chloroplasts and digitonin SCP respectively. The same effect was investigated in the present study in chloroplasts and the results are shown in Table V. It can be seen that the extent of the signal in chloroplasts was inhibited by approximately 50% upon the addition of ADP and Pi.

Electron transport inhibitors DCMU and DBMIB (see Chapter V) completely inhibited the signal in chloroplasts in the absence of electron acceptors. However DBMIB was slightly less effective in the presence of electron acceptors while DCMU was equally effective (Table VI). The uncouplers CCCP and nigericin plus K^+ ions also affected the signal in chloroplasts. Unlike the observations of Rumberg and Siggel (1968) and Neumann, Ke and Dilley (1970), who found that CCCP accelerated the dark decay of the signal, CCCP completely reversed the signal, i.e. a negative signal was observed (Fig.VII). On the other hand, CCCP only inhibited the signal in SCP and did not have the dramatic effect observed in the chloroplasts (Fig.VII). Hauska, McCarty and Olson (1970) and Murata and Fork (1971) have also observed CCCP inhibition of the signal in digitonin and French pressure cell SCP respectively. Nigericin and K^+ ions also had different effects on the signal in chloroplasts and SCP. In chloroplasts, nigericin abolished the slow phase of the signal without affecting the fast phase (Fig.VIII), but had no effect on the signal in SCP.

Similar observations have been reported by Neumann, Ke and Dilley (1970) for the signal induced in SCP by the repetitive flash technique.

DISCUSSION

Subchloroplast particles prepared by digitonin treatment and containing only PSI activity have been shown to be able to phosphorylate at rates comparable to intact chloroplasts (Fig.I and Table I). These SCP did not exhibit any light-induced proton uptake activity, consistent with the observations of Nelson, Drechsler and Neumann (1970) and Neumann, Ke and Dilley (1970), but contrary to Hauska, McCarty and Olson (1970) who found residual proton uptake activity in their SCP. According to the chemiosmotic hypothesis (Mitchell, 1966) proton translocation across a coupling membrane creates a pH gradient (ΔpH), and a membrane potential (ΔE), which together constitute the electrochemical potential gradient needed to support phosphorylation. It was also postulated that ΔE could play a major role in phosphorylation under conditions where ΔpH was reduced. Since these SCP did not exhibit any light-induced proton uptake activity but were able to phosphorylate at high rates, ΔE could be supporting phosphorylation in SCP. This idea is supported by the observed effect of various uncouplers and ionophores on photophosphorylation in SCP. NH_4Cl and nigericin plus K^+ ions, which do not affect ΔE

but dissipate ΔpH (Neumann and Jagendorf, 1964 and Shavit, Dilley and San Pietro, 1968), were not very effective in uncoupling photophosphorylation in SCP. When valinomycin was also present, photophosphorylation was completely uncoupled. Valinomycin on its own was ineffective (Table III and Fig.IV). Such synergistic uncoupling by valinomycin and NH_4Cl or nigericin plus K^+ ions, of photophosphorylation in SCP has previously been reported (McCarty, 1969; Nelson, Drechsler and Neumann, 1970 and Neumann, Ke and Dilley, 1970).

These results suggest that complete uncoupling of photophosphorylation in SCP requires dissipation of both the proton gradient and the membrane potential. It is quite possible that a miniscule proton uptake activity (undetectable by conventional glass pH electrodes) was still present in these SCP. This activity may be part or all of the charge separation across the thylakoid membrane of the SCP. However, it is quite obvious that such a small ΔpH would not be able to support photophosphorylation in SCP. McCarty (1968), found that photophosphorylation in SCP obtained by sonication was not sensitive to NH_4Cl, whereas the light-induced pH rise was sensitive to the uncoupler. He concluded that the light-induced pH rise could not be the driving force for photophosphorylation in SCP. Later, McCarty (1969) reported that NH_4Cl became an effective uncoupler of photophosphorylation in SCP when valinomycin was also present. He further concluded that photophosphorylation in SCP was not dependent on a pH gradient, but that

a membrane potential was needed for photophosphorylation in SCP. CCCP which affects both ΔpH and ΔE (Mitchell and Moyle, 1967) was quite effective in uncoupling photophosphorylation in SCP (Fig.V). Hauska, McCarty and Olson (1970) found that 10μM CCCP completely abolished photophosphorylation in SCP. However, valinomycin was seen to increase the sensitivity of photophosphorylation in SCP to suboptimal concentrations of CCCP, lending further support to the view that ΔE plays a large role in photophosphorylation in SCP.

Fig.II shows that in SCP, the ability to phosphorylate diminishes as the ratio of chlorophyll a to chlorophyll b increases. A high chlorophyll a to chlorophyll b content means that these SCP are enriched in PSI. According to presently accepted photosynthetic electron flow schemes (Arnon, 1967; Boardman, 1968 and Hauska, McCarty and Racker, 1970), the site of phosphorylation on the cyclic electron flow pathway is envisaged to be located between 'X' (or P430) (Hiyama and Ke, 1971) and the site at which cyclic flow feeds into the cytochrome chain. If the above assumption is correct, the variation in the chlorophyll a to b ratio should not affect the phosphorylating activity of the SCP. Results shown in Fig.II are not consistent with the location of a phosphorylation site (energy transduction site) on the cyclic pathway. Thus, the simplest explanation would be to place the site of energy transduction on the non-cyclic

pathway, so that it is common to both non-cyclic and cyclic pathways. Such a scheme has been forwarded by Neumann, Arntzen and Dilley (1970), who used different results to support their idea.

The 518nm absorbance change has been measured in chloroplasts in the absence and presence of electron acceptors (Fig.V). In contrast to the control, the presence of electron acceptors introduced a component which does not attain a steady-state level, in addition to the fast component present in the control This component may be due to increased electron flow in the presence of electron acceptors; in the absence of added acceptor, the electron flow would be limited to the reduction of endogenous primary electron acceptors. The relationship of the signal to electron flow and proton uptake was also indicated by its inhibition by electron transport inhibitors (Table VI). These results suggest that the signal observed under these conditions was not the same as that induced by laser pulses (Junge and Witt, 1968; Jackson and Crofts, 1969; Avron and Chance, 1966 and Hildreth, Avron and Chance, 1966) which is too rapid to be generated by the electron transport process (Duysens, 1964). Furthermore, the laser-induced "on" response is insensitive to electron transport inhibitors (Hildreth, Avron and Chance, 1966). Inhibitory effects of antimycin A and HOQNO have been reported on the carotenoid shift in bacterial chromatophores; this was thought to indicate a membrane

potential (Fleischmann and Clayton, 1968). Larkum and Bonner (1972) have also reported the inhibition of the 518nm signal in chloroplasts, in the absence of electron acceptors, by DCMU, but found antimycin A and HOQNO effective only at high concentrations. The possibility of the slow phase being linked to proton uptake activity was indicated by its absence in SCP (Fig.VI), which have been shown to have no detectable proton uptake activity (Table I; Nelson, Drechsler and Neumann, 1970 and Neumann, Ke and Dilley, 1970). Further support for this relationship can be drawn from the result in Fig.VII which shows that nigericin abolished the slow phase of the signal in chloroplasts without affecting the steady-state level, whereas the signal in SCP was not affected at all. Therefore, the steady-state signal in both chloroplasts and SCP could be indicative of a membrane potential since a ΔpH dissipating agent such as nigericin did not affect it. On the other hand, CCCP which dissipates both ΔpH and ΔE (Mitchell and Moyle, 1967) inhibited the 518nm signal in both chloroplasts and SCP. Surprisingly, CCCP induced a negative signal in chloroplasts. Similar negative absorbance changes in bacterial chromatophores have been reported to be induced by KOH pulses in the dark in the presence of FCCP (Jackson and Crofts, 1969). FCCP and nigericin plus valinomycin also inhibit the 518nm signal in chloroplasts (Larkum and Bonner, 1972), and NH_4Cl and valinomycin in SCP (Hauska, McCarty and Olson, 1970). Recently, Strichartz and

Chance (1972) were able to produce a membrane potential by the use of concentrated salt solutions; they further showed that these salt additions induced absorbance changes at 520nm, similar to those produced by light. They also found that valinomycin stimulated the 520nm signal induced by KCl, whereas nigericin had no effect, valinomycin presumably increasing the permeability of the chloroplast membrane to K^+ ions.

Hauska, McCarty and Olson (1970) looked at the possible relationship of the 518nm absorbance change to phosphorylation in SCP. They observed that under conditions where the pH rise was completely inhibited, SCP were still able to phosphorylate. However, if both the pH rise and the 518nm absorbance change were inhibited, phosphorylation was completely abolished. They further observed that phosphorylating agents reduced the extent of the absorbance change by approximately 30%. As shown in Table V, phosphorylating agents were also found to inhibit the extent of the absorbance change in chloroplasts, consistent with the results of Hauska, McCarty and Olson (1970) in SCP.

Data on the effect of nigericin, CCCP and phosphorylating agents indicates that the 518nm absorbance change reflects a membrane potential and suggests a relationship between the absorbance change and phosphorylation. Such a relationship was postulated by Junge and Witt (1968) and supported by Hauska, McCarty and Olson (1970). Neumann, Ke and Dilley (1970) however, concluded that there

was no relationship between the two phenomena, basing their conclusion on the differential effects of nigericin on the 515nm absorbance change induced by flash techniques and steady-state phosphorylation. Junge and Witt (1968) and Jackson and Crofts (1971) have indicated that the flash-induced fast responses of the 515nm signal in chloroplasts, and the carotenoid shift in bacterial chromatophores, correspond to the electrical field generated by charge separation between the reaction centers and primary electron acceptors. Furthermore, Jackson and Crofts (1971) have shown that the fast response was not affected by CCCP or nigericin plus valinomycin, whereas both inhibited the slow phase. In view of these observations, the argument forwarded by Neumann, Ke and Dilley (1970) may not be valid.

CHAPTER VII

CYTOCHROMES OF ISOLATED CHLOROPLASTS AND DIGITONIN SUB-CHLOROPLAST PARTICLES

INTRODUCTION

Chloroplasts have been found to contain at least three cytochromes: cytochrome f and cytochrome b_6, with α -bands at 554nm and 563nm respectively (Davenport and Hill, 1952 and Hill, 1954), and another b-type cytochrome with an α -band at about 559nm at 20°C and 557nm at 77°K (Lundegardh, 1962 and Boardman and Anderson, 1967). The latter b-type cytochrome was named cytochrome b_{559} by Boardman and Anderson (1967) to differentiate it from the soluble cytochrome b_3 of Hill and Scarisbrick (1951). Cramer and Butler (1967) also detected two b-type cytochromes which they designated b_{560} and b_{563} respectively. They concluded that b_{560} was situated between Photosystem I and II, and b_{563} on the electron-accepting side of Photosystem I. Cytochrome b_6 which was chemically reduced was shown to be photooxidised by Photosystem I light (Knaff, 1972). He concluded that cytochrome b_6 functions as an electron carrier in a cyclic electron flow pathway. Cytochromes f and b_{559} were in the reduced state in the isolated chloroplast, while cytochrome b_6 was oxidised. Boardman and Anderson (1967)

calculated the ratios of the cytochromes present in mature spinach chloroplasts to be 1mole of cytochrome f for 2moles of cytochrome b_6 and 2moles of cytochrome b_{559}. From cytochrome determinations with subchloroplast particles obtained by digitonin treatment, they came to the conclusion that cytochrome f and cytochrome b_6 are associated with Photosystem I, and cytochrome b_{559} with Photosystem II. The participation of cytochrome b in the two light reactions of photosynthesis has also been inferred from earlier studies on the green alga *Chlamydomonas reinhardii*, and various mutants of it (Chance, Schleyer and Legallais, 1966; Levine, Gorman, Avron and Butler, 1966; Levine and Gorman, 1966 and Hildreth, 1968). Other studies with *Euglena* (Olson and Smillie, 1963 and Ikegami, Katoh and Takamiya, 1970), the blue-green alga, *Anacystis* (Olson and Smillie, 1963) and spinach chloroplasts (Rumberg, 1966 and Hind and Olson, 1968), further suggest such participation. Thus the existence of cytochrome b in the photosynthetic electron flow chain is well established, but its position relative to known components of the chain is still a point of conjecture. A recent report by Ben-Hayyim and Avron (1970) on the light-induced absorbance changes of cytochromes b and f in isolated lettuce chloroplasts, indicates that both are located on that part of the electron transport chain joining the two Photosystems. They concluded that cytochrome b precedes cytochrome f in the

electron transport chain and is nearer to Photosystem II. On the other hand, Hiller, Anderson and Boardman (1971) were not able to detect any absorbance changes due to cytochrome b_{559} in untreated spinach leaves or in isolated chloroplasts. Cytochrome f however, was photooxidised by far-red light and reduced by red light. Photooxidation of cytochrome b_{559} was observed only after treatment of leaves, or isolated chloroplasts, with CCCP. A slow photoreduction of cytochrome b_{559} was observed in aged mutant pea chloroplasts in red light. From these results, they concluded that cytochrome b_{559} is not located between the two light reactions, but on a side path from Photosystem II.

More recently, Cramer, Fan and Bohme (1971) concluded that cytochrome b_{559} in chloroplasts existed in two forms; a high potential and a low potential form, differentiated on the basis of hydroquinone and ascorbate reducibility. The high potential form which is hydroquinone reducible was thought to be the most abundant form in chloroplasts, the two forms being interconvertible. Subsequently Wada and Arnon (1971) reported the presence of three forms of cytochrome b_{559} which have the same α-peak at 559nm, but were distinguishable by the difference in oxidation-reduction potentials. The high potential form was reducible by hydroquinone, the middle potential form reducible by ascorbate (but not by hydroquinone) and the low potential form

reducible by dithionite but not by ascorbate. The high potential form was thought to be the predominant one and was strongly correlated with PSII activity, contrary to Cramer, Fan and Bohme (1971) conclusion that it was the low potential form (probably the mid potential form) which was located on the electron flow chain between the two photosystems.

The presence of a low potential b_{559} (reducible by dithionite but not ascorbate) in untreated spinach chloroplasts has also been reported (Erixon, Lozier and Butler, 1972).

Earlier Bendall (1968) mentioned the presence of two low potential b-type cytochromes in pea chloroplasts reducible by dithionite, but not by ascorbate. From studies on the kinetics of the reduction of cytochrome b_6 by dithionite, he concluded that it is composed of two components with α-bands at 559 and 563nm respectively. The 559nm component would presumably be the low potential form of cytochrome b_{559}.

Avron and Chance (1966) forwarded evidence for an energy transducing site before cytochrome f. More recently Hildreth (1968) suggested that the phosphorylation site is localised between cytochrome b_{559} and cytochrome f. Isolated chloroplasts (pea and spinach) which are tightly coupled were used to study the light-induced redox changes of cytochromes in an endeavour to find out the role of cytochrome b_{559} in phosphorylating and

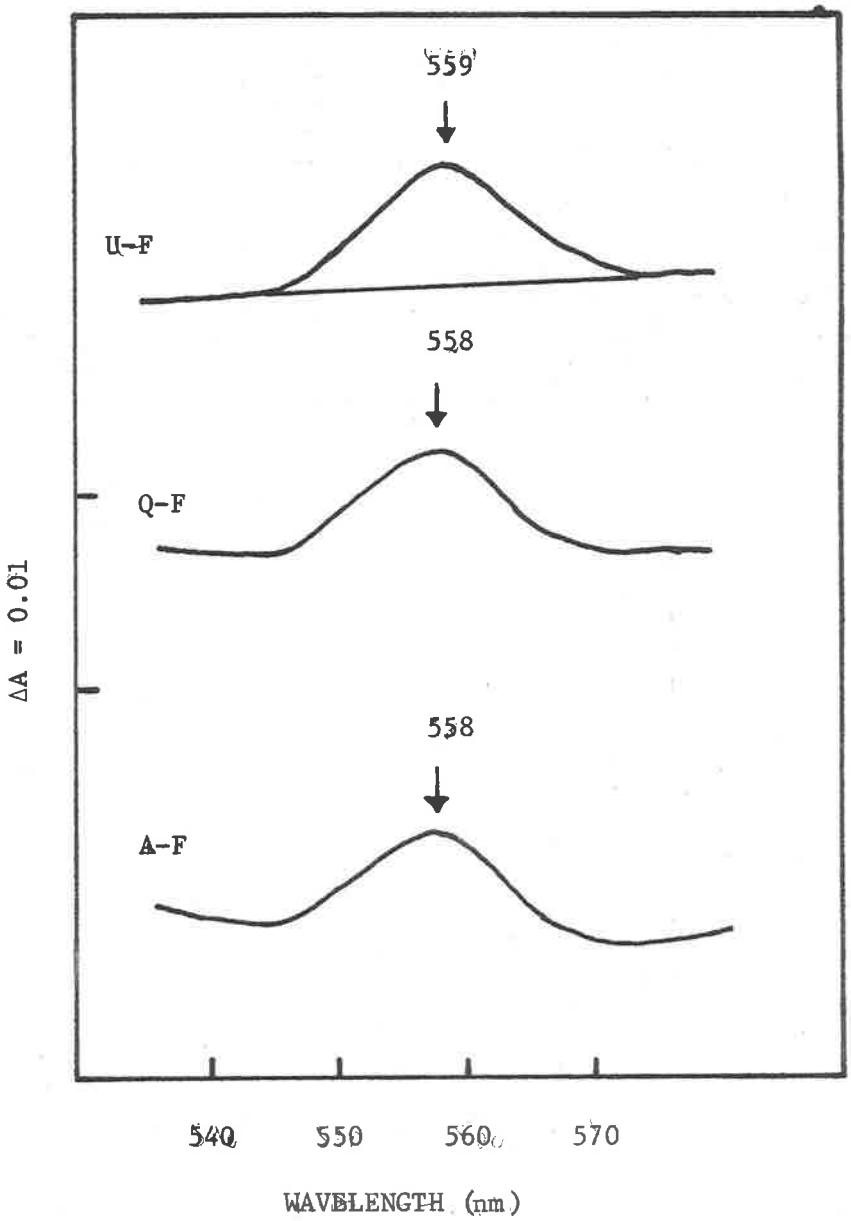
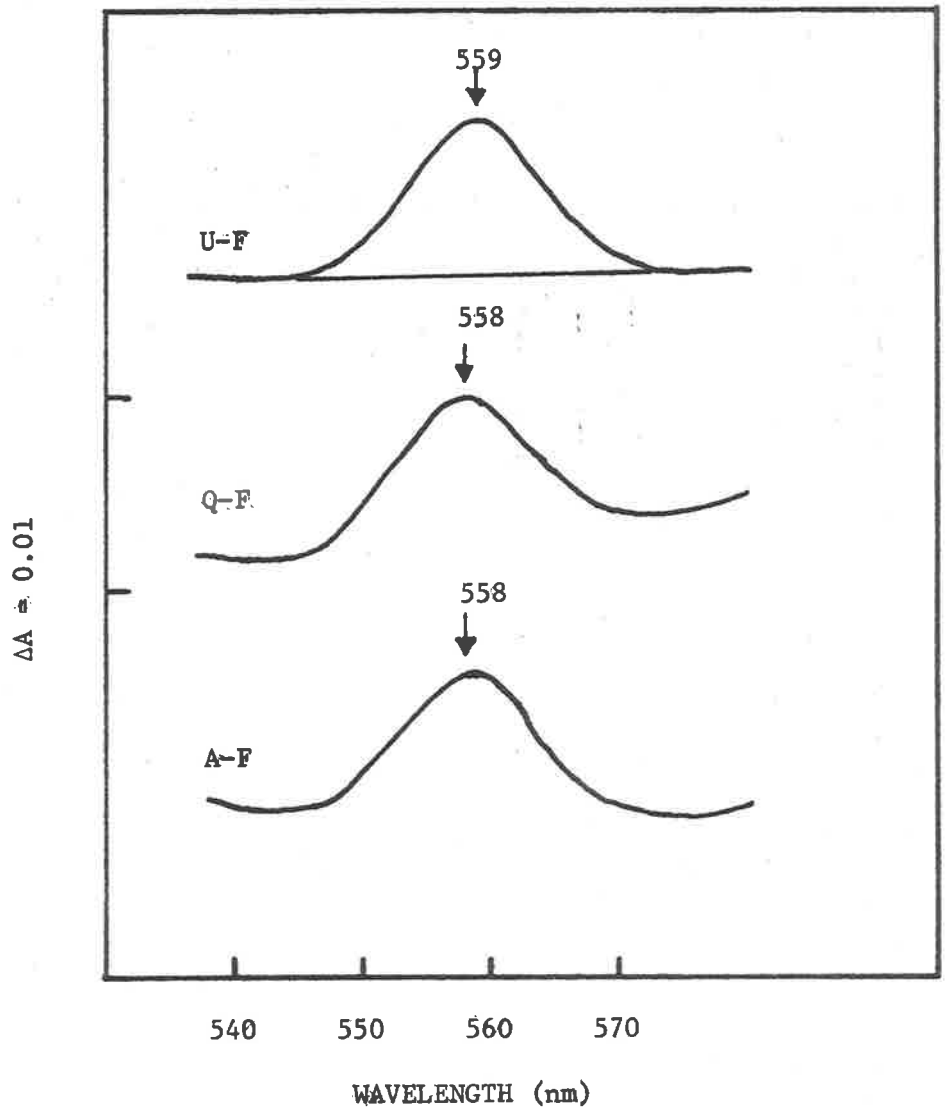


FIGURE I. Reduced minus oxidised difference spectra of pea chloroplasts at 20°C. (A-F) - ascorbate-reduced minus ferricyanide oxidised; (Q-F) - hydroquinone reduced minus ferricyanide and (U-F) - untreated minus ferricyanide oxidised. Chlorophyll concentration, 75µg/ml; optical path-length, 1cm.

FIGURE II. Reduced minus oxidised difference spectra of spinach chloroplasts at 20°C. (A-F)-ascorbate-reduced minus ferricyanide oxidised; (Q-F)-hydroquinone reduced minus ferricyanide and (U-F)-untreated minus ferricyanide oxidised. Chlorophyll concentration, 360µg/ml; optical path-length, 2mM.


559
U-F
558
Q-F
558
A-F
ΔA = 0.01
540
550
560
570
WAVELENGTH (nm)

non-phosphorylating electron flow conditions. The effect of CCCP on the light-induced redox changes of cytochrome b_{559} was also studied. Studies on the effect of antimycin A, an inhibitor of mitochondrial electron flow, on photosynthetic electron flow indicated the presence of two sites of inhibition (see Chapter V). One site of inhibition was deduced to be on the non-cyclic electron flow pathway and the other on the cyclic electron flow pathway catalyzed by PMS. Therefore, the effect of antimycin A on the light-induced redox changes of cytochrome b_{559} was studied to find out if the inhibitor would affect cytochrome b_{559} in a similar manner that it does cytochrome b in mitochondria (see Chance, 1958 and Estabrook, 1962). No attempt was made to study the effect of antimycin A on the light-induced redox changes of cytochrome b_6 in chloroplasts because of the difficulty involved in observing its light-induced redox changes (Cramer and Butler, 1967 and Ben-Hayyim and Avron, 1970).

RESULTS

A. CYTOCHROMES OF ISOLATED CHLOROPLASTS

Reduced minus oxidised difference spectra of pea and spinach chloroplasts at room temperature are shown in Fig.I and II. The untreated minus FeCN oxidised (U-F) was identical with the hydroquinone reduced minus FeCN oxidised (Q-F), indicating that the

TABLE I. Concentration of cytochromes b_{559} and f in spinach and pea chloroplasts. Chlorophyll concentration was 37 μg/ml.

Difference Spectrum	Concentration of reduced cytochrome b_{559} (μM)	Concentration of reduced cytochrome f (μM)
Spinach		
U-F	0.330	0.158
Q-F	0.310	0.130
A-F	0.318	0.120
Pea		
U-F	0.276	0.120
Q-F	0.240	0.127
A-F	0.280	0.110

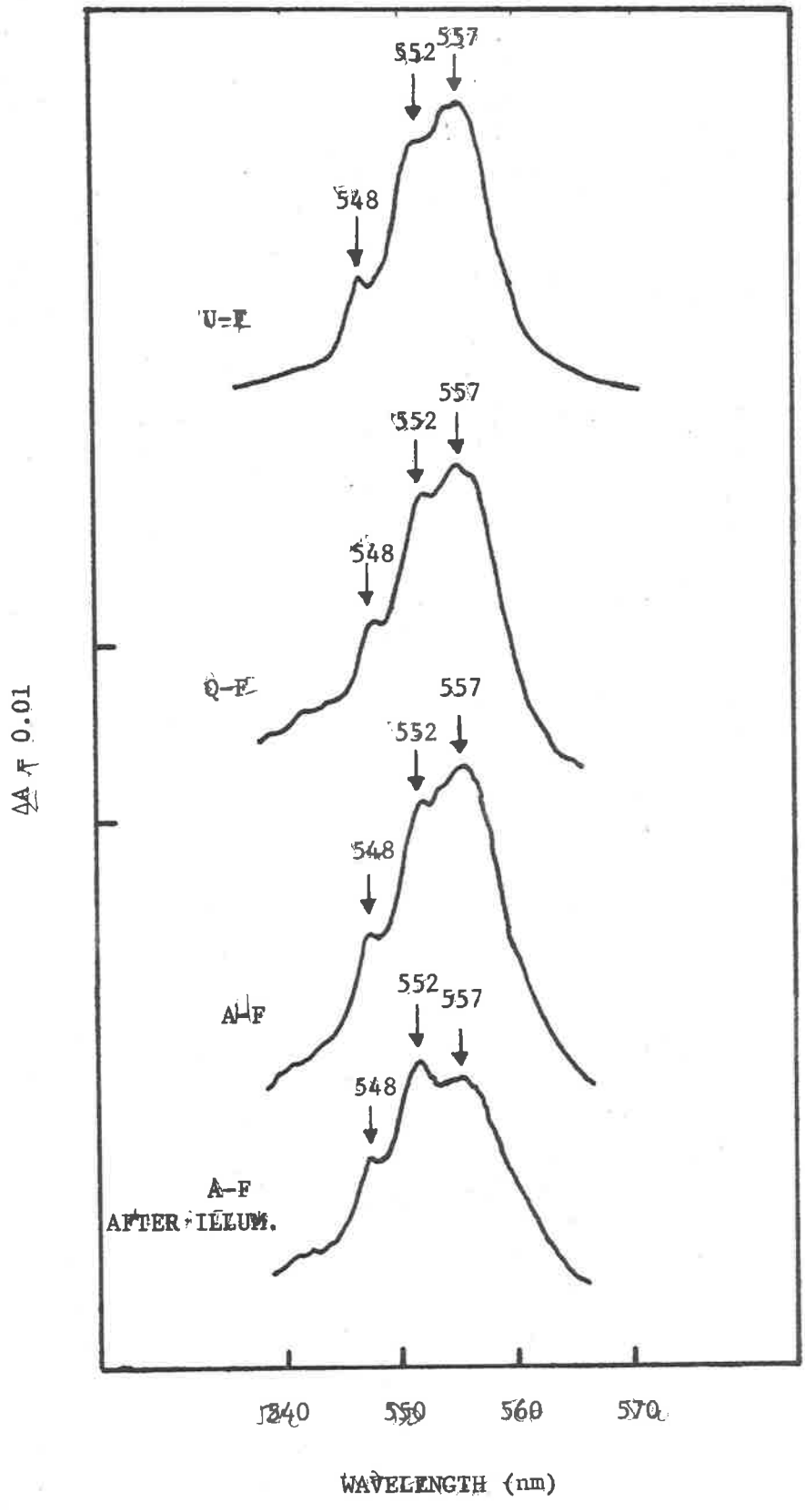
cytochromes are normally in the reduced state in these chloroplasts. The ascorbate reduced minus FeCN oxidised (A-F) spectrum was also the same as the (U-F) spectrum. The absorption maximum of the (U-F) spectrum was at 559nm and that of the (Q-F) and (A-F) spectra was at 558nm. Table I shows the amount of reduced cytochrome b_{559} and cytochrome f calculated from these readings. For each spectrum, absorbance difference were read at 559nm and 554nm relative to a baseline joining the troughs about 572nm and 543nm. The absorbance of cytochrome f at 559nm was taken as one third of its absorbance at 554nm, and the absorbance of cytochrome b_{559} at 554nm was taken as one-half of its absorbance at 559nm. These estimates were made on the basis of the difference spectra of a preparation of cytochrome f (Boardman and Anderson, 1967) and of Photosystem II fragments devoid of cytochromes f and b_6 (Boardman, 1972, in press, Biochim. Biophys. Acta). Molar extinction coefficients (reduced minus oxidised) of 2.2×10^4 for cytochrome f (Forti, Bertole and Zanetti, 1967) and 2.0×10^4 for cytochrome b_{559} (Boardman and Anderson, 1967) were used. The following equations were derived:

$$\text{cytf } (\mu M) = 53.1\ A_{554} - 24.3\ A_{559}$$

$$\text{cyt}b_{559}\ (\mu M) = 58.4\ A_{559} - 18.3\ A_{554}$$

When the (A-F) difference spectrum of cytochromes in spinach chloroplasts were measured at 77°K, three peaks were observed at

FIGURE III. Reduced minus oxidised difference spectra at 77°K. Chlorophyll concentration and optical path-length as for Fig.II.


552
557
548
U-F
557
552
548
Q-F
557
552
548
A-F
552
557
548
A-F
AFTER ILLUM.
ΔA F 0.01
540
550
560
570
WAVELENGTH (nm)

547, 552 and 557nm as shown in Fig.III. Boardman and Anderson (1967) observed a splitting of the α-band of cytochrome f, at 77°K, into two bands with maxima at 548 and 552nm. Thus, the three peaks observed in the (U-F) and (A-F) spectra at 77°K are probably due to cytochrome f (547 and 552nm) and cytochrome b_{559} (557nm). The peak positions in liquid nitrogen temperature are shifted 2-3nm towards the violet region when compared with room temperature spectra. Similar observations have been reported in pea chloroplasts by Bendall and Sofrova (1971). When the frozen reference and sample cuvettes were illuminated by 650nm actinic light, at 77°K for 3 mins, the bands attributed to cytochrome f (547 and 552nm) remained unchanged, whereas the 557nm band of b_{559} decreased in height. This indicates that cytochrome b_{559} rather than cytochrome f was photooxidised at liquid nitrogen temperature. Similar photooxidations of cytochrome b_{559} at liquid nitrogen temperature have previously been reported (Knaff and Arnon, 1969; Floyd, Chance and De Vault, 1971; Boardman, Anderson and Hiller, 1971; Erixon and Butler, 1971 and Bendall and Sofrova, 1971).

Hill (1954) first detected the presence of cytochrome b_6 with an α-peak at 563nm, in chloroplasts and this has since been confirmed (Boardman and Anderson, 1967; Bendall, 1968 and Ben-Hayyim and Avron, 1970). The absence of cytochrome b_6 (561-563nm) in (A-F) spectra in Fig.I and II indicates that cytochrome b_6 was not reduced by ascorbate (James and Leech, 1964).

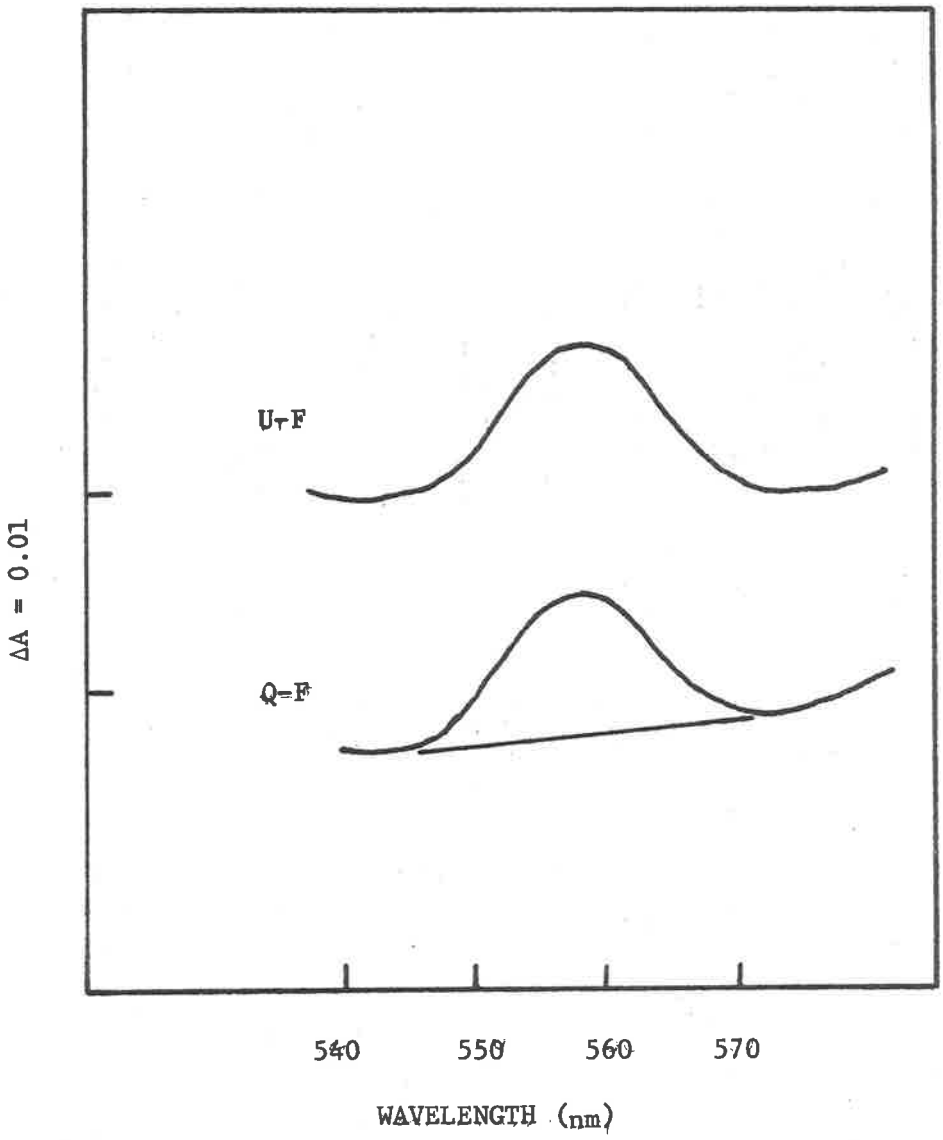


FIGURE IV. Untreated minus ferricyanide (U-F) and hydroquinone minus ferricyanide (Q-F) difference spectra of spinach chloroplasts. Chlorophyll concentration, 75µg/ml; optical path-length, 1cm.

TABLE II. Effect of CCCP and FCCP on the redox state of cytochromes in spinach and pea chloroplasts at room temperature. Chlorophyll concentration 82μM; CCCP, 10μM and FCCP, 3.3μM.

Difference Spectrum	Treatment	Concentrations of reduced cytochrome b_{559} (μM)	
		Spinach	Pea
U-F	Nil	0.26	0.28
Q-F	Nil	0.25	0.27
U-F	CCCP	0.26	0.28
Q-F	CCCP	0.26	0.26
U-F	Nil	0.24	0.24
U-F	FCCP	0.25	0.21
Q-F	FCCP	0.24	0.21

1. *Effect of CCCP and FCCP on the redox state of cytochrome b_{559} in isolated chloroplasts*

In control spinach or pea chloroplasts, at least 95% of the cytochrome b_{559} was in the reduced state, and an untreated minus ferricyanide (U-F) difference spectrum of the chloroplasts was similar to the hydroquinone minus ferricyanide (Q-F) difference spectra (Fig.IV). Table II shows the effects of dark preincubation of chloroplasts for 5 min at room temperature with CCCP (10μM) or FCCP (3.3μM) on the redox state of cytochrome b_{559}. Untreated minus ferricyanide and hydroquinone minus ferricyanide difference spectra were recorded at room temperature for control and incubated chloroplasts.

It is apparent from Table II that incubation of spinach or pea chloroplasts with CCCP did not influence the amount of reduced form of cytochrome b_{559} (high potential) in the dark adapted chloroplasts. Care was taken to ensure that the chloroplasts remained either in the dark or in dim green light, since cytochrome b_{559} was readily photooxidised in CCCP-treated chloroplasts. CCCP also had a negligible effect on the amount of cytochrome b_{559} reducible by hydroquinone indicating that CCCP did not cause a dark conversion of cytochrome b_{559} (high potential) to a form of lower potential.

Similar results were obtained when spinach chloroplasts were incubated with FCCP, but pea chloroplasts in the presence of

TABLE III. Photooxidation of cytochrome b_{559} in spinach chloroplasts at 77°K. Chloroplasts were incubated with 10μM CCCP and, or 3.3μM DBMIB for 5 min at 20°C, then made to 70% glycerol (V/V). An hydroquinone minus ferricyanide difference spectrum of the treated chloroplasts was then recorded in the dark at 77°K and again after illumination in the Cary spectrophotometer at 650nm for 3 min. Chlorophyll concentration was 375μg/ml. The decrease in absorbance at 557nm on illumination was a measure of the photooxidation of cytochrome b_{559}.

Treatment	Δ557nm x 10^4		
	Before Illumination	After Illumination	Difference
Nil	168	107	61
CCCP	150	88	62
DBMIB	160	98	62
DBMIB + CCCP	154	83	71

FCCP consistently showed a decrease of 10-20% in the amount of reduced cytochrome b_{559}.

It was shown previously that cytochrome b_{559} (high potential) is photooxidised at 77°K by light absorbed by Photosystem II (Knaff and Arnon, 1969a; Floyd, Chance and De Vault, 1971; Boardman, Anderson and Hiller, 1971; Bendall and Sofrova, 1971 and Erixon and Butler, 1971a). C-550, thought to be a component of PSII is the electron acceptor for the photo-oxidation of cytochrome b_{559} (high potential) and the extent of the photooxidation appears to be limited by the amount of C-550 in the chloroplast (Erixon and Butler, 1971b).

Treatment of spinach chloroplasts with 10μM CCCP for 5 min at room temperature before freezing to 77°K did not influence the low temperature photooxidation of cytochrome b_{559} by 655nm light (Table III). About 40% of cytochrome b_{559} (high potential) present in chloroplasts was photooxidised at 77°K, both in the presence and absence of CCCP.

B. *CYTOCHROMES OF DIGITONIN SUBCHLOROPLAST PARTICLES FROM SPINACH AND PEA*

1. *10,000 xg particles (PSII particles)*

Anderson and Boardman (1966) have shown that these particles are enriched in PSII activity. The difference spectra for cytochrome oxidation by FeCN, and successive reductions by

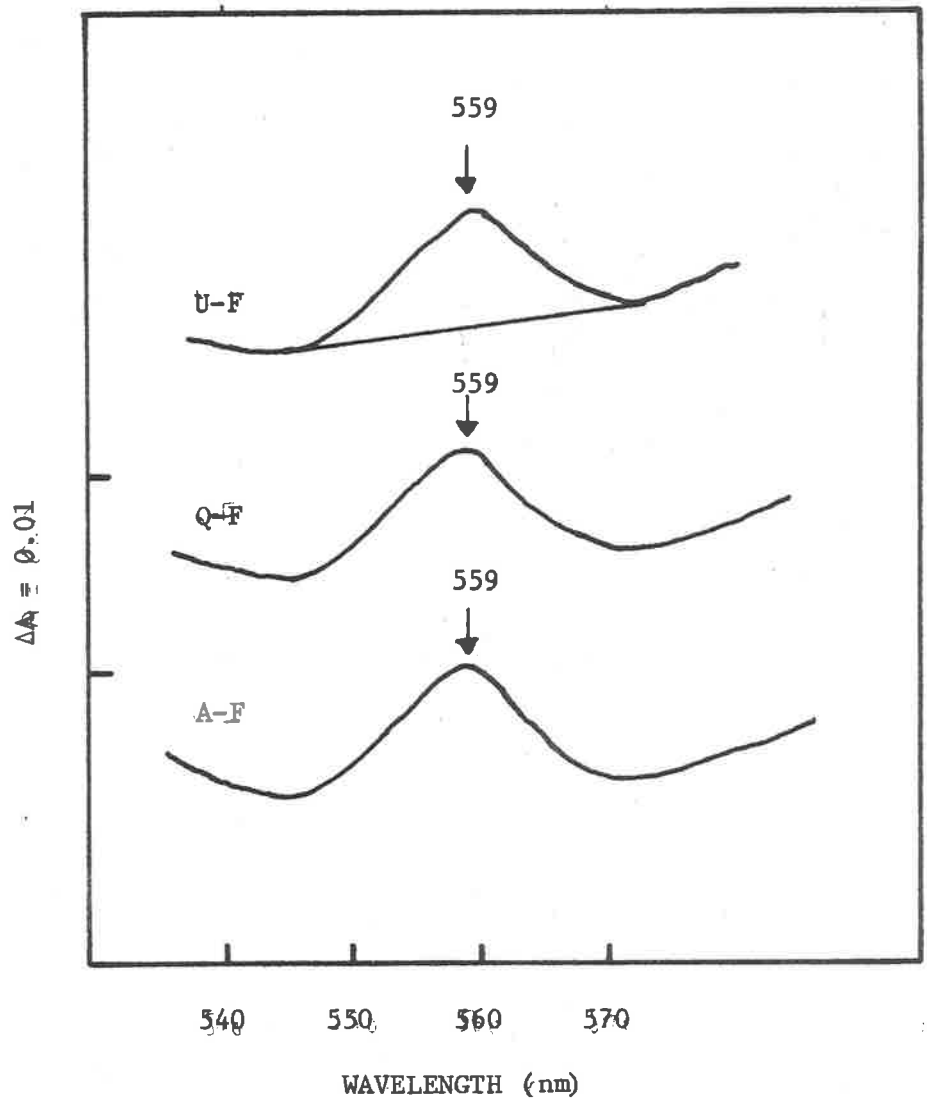


FIGURE V. Reduced <u>minus</u> oxidised difference spectra of 10,000 xg digitonin subchloroplast particles from pea at 20°C. Chlorophyll concentration, 75µg/ml; optical path-length, 1cm.

FIGURE VI. Reduced minus oxidised difference spectra of 10,000 xg digitonin subchloroplast particles from spinach at 20°C and 77°K. Chlorophyll concentration, 360µg/ml; optical path-length, 2mM.

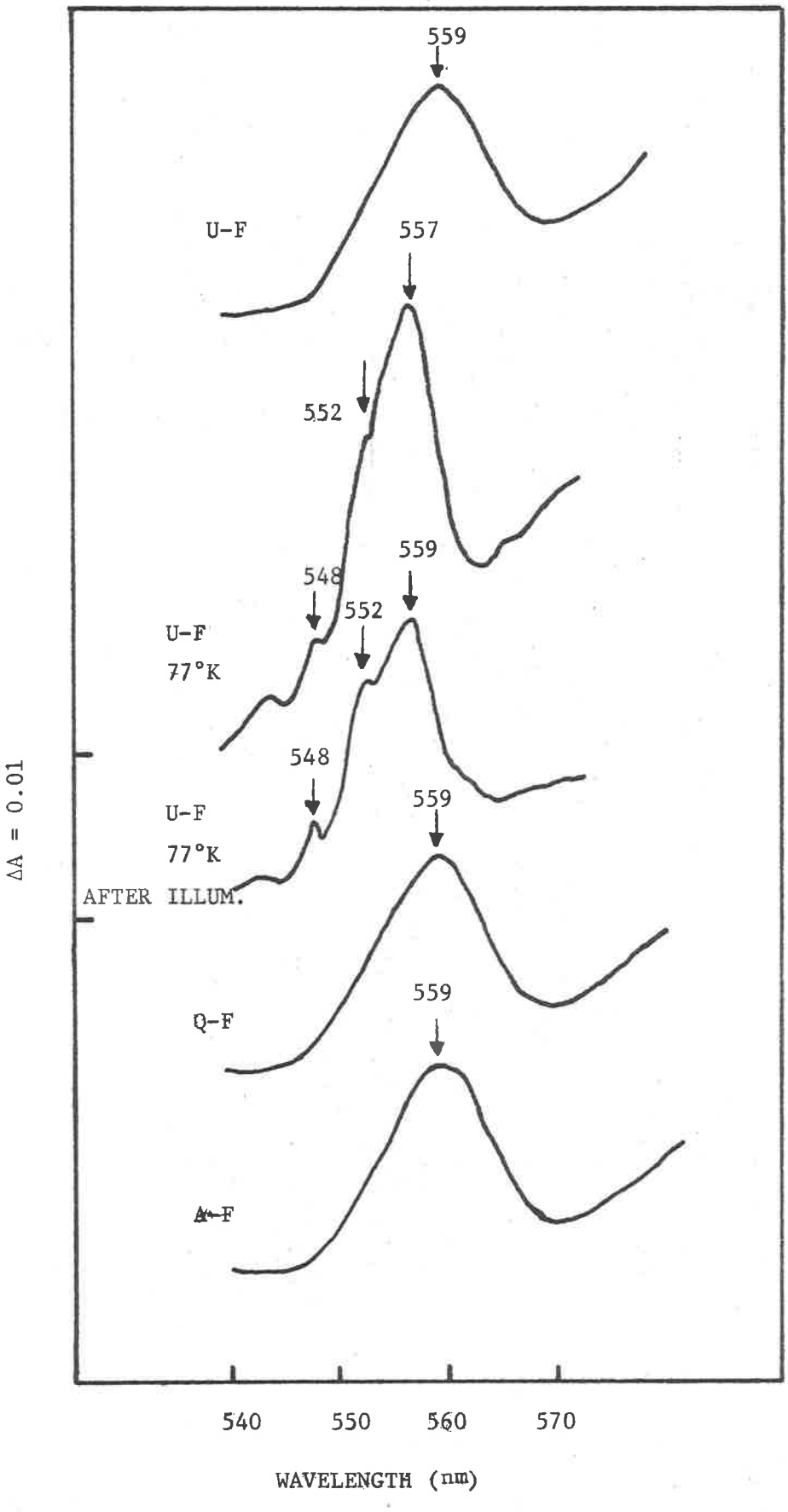

559
U-F
557
552
559
548
552
U-F
77°K
548
559
U-F
77°K
AFTER ILLUM.
559
Q-F
A-F
ΔA = 0.01
540
550
560
570
WAVELENGTH (nm)

TABLE IV. Photooxidation of cytochrome b_{559} in spinach PSII subchloroplast particles at 77°K. SCP were incubated with 10μM CCCP for 5 min at 0°C, then made up to 62% glycerol (V/V). The difference spectra were then recorded in the dark at 77°K and again after illumination in the Cary spectrophotometer at 650nm for 3 min. Chlorophyll concentration was 360μg/ml.

Treatment	Δ557nm x 10^4		
	Before Illumination	After Illumination	Difference
Control (U-F)	190	130	60
CCCP (Q-F)	60	40	20
CCCP (A-F)	140	90	50

hydroquinone and ascorbate, in pea subchloroplast particles are shown in Fig.V. The peak of the (U-F) spectrum was at 559nm and resembled the (Q-F) and (A-F) spectra. The (A-F) spectrum, showed no increase in the reduction of the cytochromes. This suggests that the cytochrome b_{559} present in this fraction was mainly of high potential and in the reduced state. Thus, these particles differ, with respect to redox state, from the PSII particles prepared by treatment of spinach chloroplasts with Triton X-100 (Ke, Vernon and Chaney, 1972). Cytochrome b_{559} of the Triton X-100 PSII particles are present in the oxidised state and are of mid potential (ascorbate reducible). Spinach SCP obtained by digitonin treatment also gave similar results (Fig.VI) except that the position of the maxima of (U-F), (Q-F) and (A-F) spectra are at 558nm (see also Boardman and Anderson, 1967). The (U-F) and (Q-F) spectra of spinach subchloroplast particles at 77°K showed a peak at 557nm, with a small shoulder at 552nm, which was less pronounced than that of chloroplasts, and another at 548nm (Fig.VI). This reflects the presence of more cytochrome b_{559} than cytochrome f in these particles. Similar observations were reported by Boardman and Anderson (1967) in dithionite reduced minus FeCN oxidised difference spectra in the 10,000 xg fraction. Photooxidation of cytochrome b_{559} at 77°K was also observed in these particles in the (U-F) spectrum, when illuminated by 650nm actinic light for 3 min (Table IV).

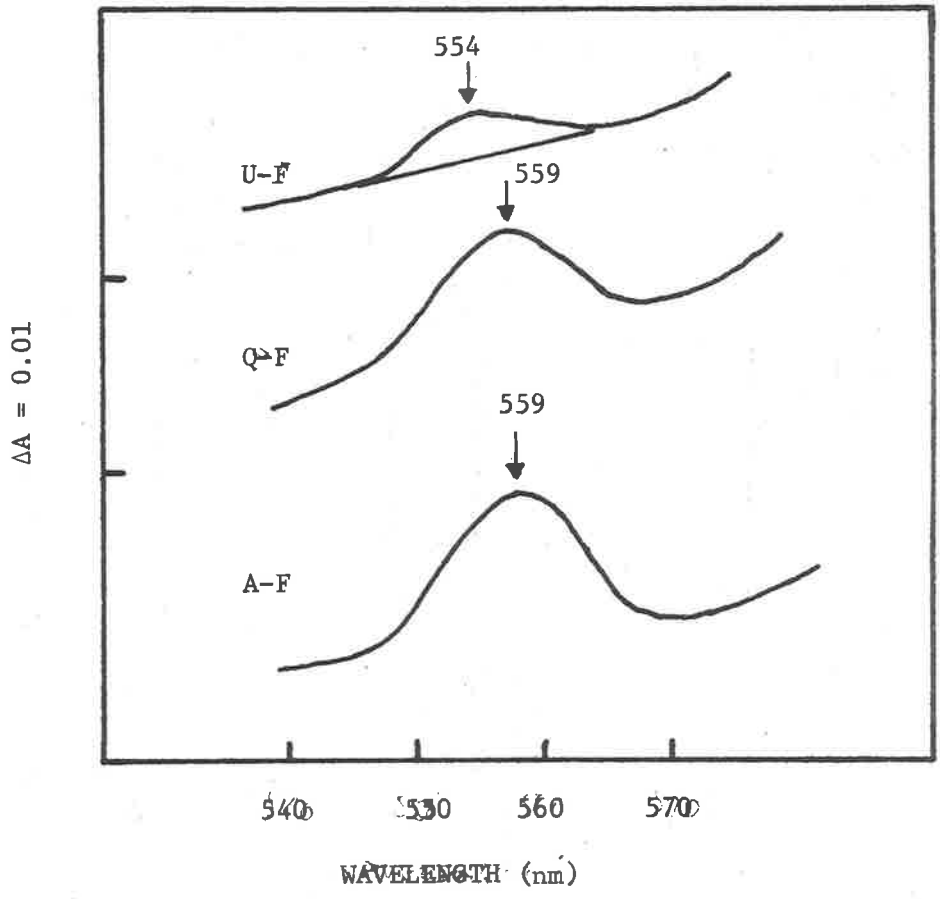


FIGURE VII. Reduced minus oxidised difference spectra of CCCP-treated 10,000 xg digitonin subchloroplast particles from spinach at 20°C. Chlorophyll concentration, 360µg/ml; optical path-length, 2mM.

(a) Effect of CCCP on the redox state of cytochrome b_{559} in 10,000 xg particles from spinach

Cytochrome b_{559} in PSII (10,000 xg) particles was mostly in the reduced state. Fig.VII shows that it was mainly in the high potential form. The effect of dark incubation of these particles with CCCP (10μM) for 3 min was shown in Fig.VII. The (U-F) spectrum showed very little cytochrome b_{559} in the reduced state, but the (Q-F) spectrum showed that more of it was reduced. There was also an increase in the (A-F) spectrum compared to the (Q-F) spectrum with respect to the amount of reduced cytochrome b_{559} present. This increase of the (A-F) over the (Q-F) spectrum suggests that some high potential cytochrome b_{559} had been converted to the mid-potential form. This is in agreement with the findings of Cramer, Fan and Bohme (1971) with FCCP-treated spinach chloroplasts.

Photooxidation of cytochrome b_{559} in CCCP-treated particles was also observed at 77°K (Table IV) when the (Q-F) difference spectrum was measured before and after illumination with 650nm actinic light for 3 min.

However, a direct comparison with untreated particles cannot be made, as a corresponding (Q-F) spectrum in the control was not illuminated. Nevertheless, this photooxidation must be the oxidation of the high potential cytochrome b_{559}. An enhanced photooxidation was observed in the (A-F) spectrum, suggesting

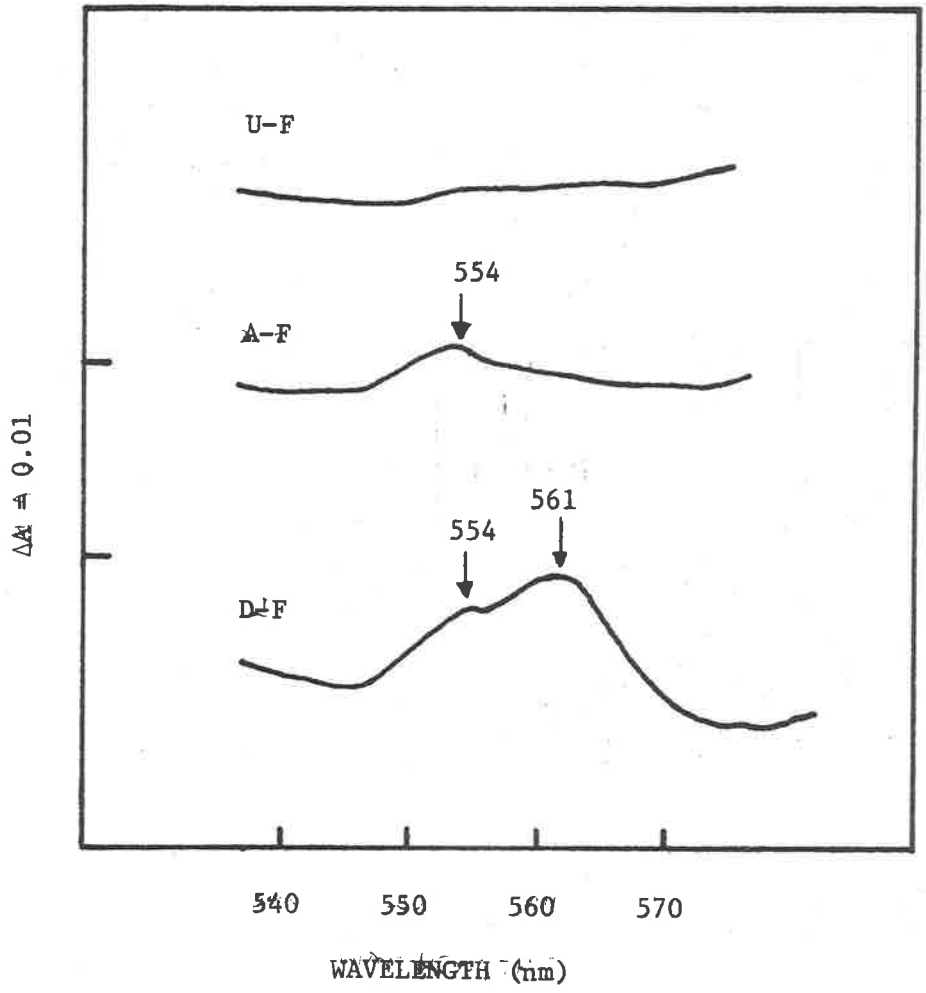


FIGURE VIII. Reduced <u>minus</u> oxidised difference spectra of 144,000 xg digitonin subchloroplast particles from pea. Chlorophyll concentration, 75µg/ml; optical path-length, 1cm.

that the mid-potential form of cytochrome b_{559} was also photo-oxidised at 77°K. This was evident from the data in Table IV which showed that the amount of cytochrome b_{559} photooxidised in the (A-F) spectrum was greater than that photooxidised in the (Q-F) spectrum. This observation is in contrast to the findings of Ke, Vernon and Chaney (1972) who did not observe any photooxidation of the mid-potential cytochrome b_{559} in Triton X-100 PSII particles.

2. *144,000 xg particles from pea chloroplasts (PSI particles)*

These particles do not contain any Photosystem II activity (Results in Chapter VI) as was the case for spinach PSI particles (Anderson and Boardman, 1966). The untreated minus FeCN oxidised (U-F) spectrum in these particles (Fig.VIII) resembled the base-line (untreated minus untreated) spectrum, indicating that the cytochromes present were in the oxidised state (see Boardman and Anderson, 1967). The ascorbate reduced minus FeCN oxidised (A-F) spectrum showed a peak at 554nm which suggests that this fraction is relatively free of high and mid-potential cytochrome b_{559}. Boardman and Anderson (1967) observed only a small hump at 557nm in the (A-F) spectrum at 77°K. The dithionite reduced minus FeCN oxidised spectrum showed a peak at 561nm and a pronounced shoulder at 554nm, suggesting that only cytochrome f and cytochrome b_6 are present.

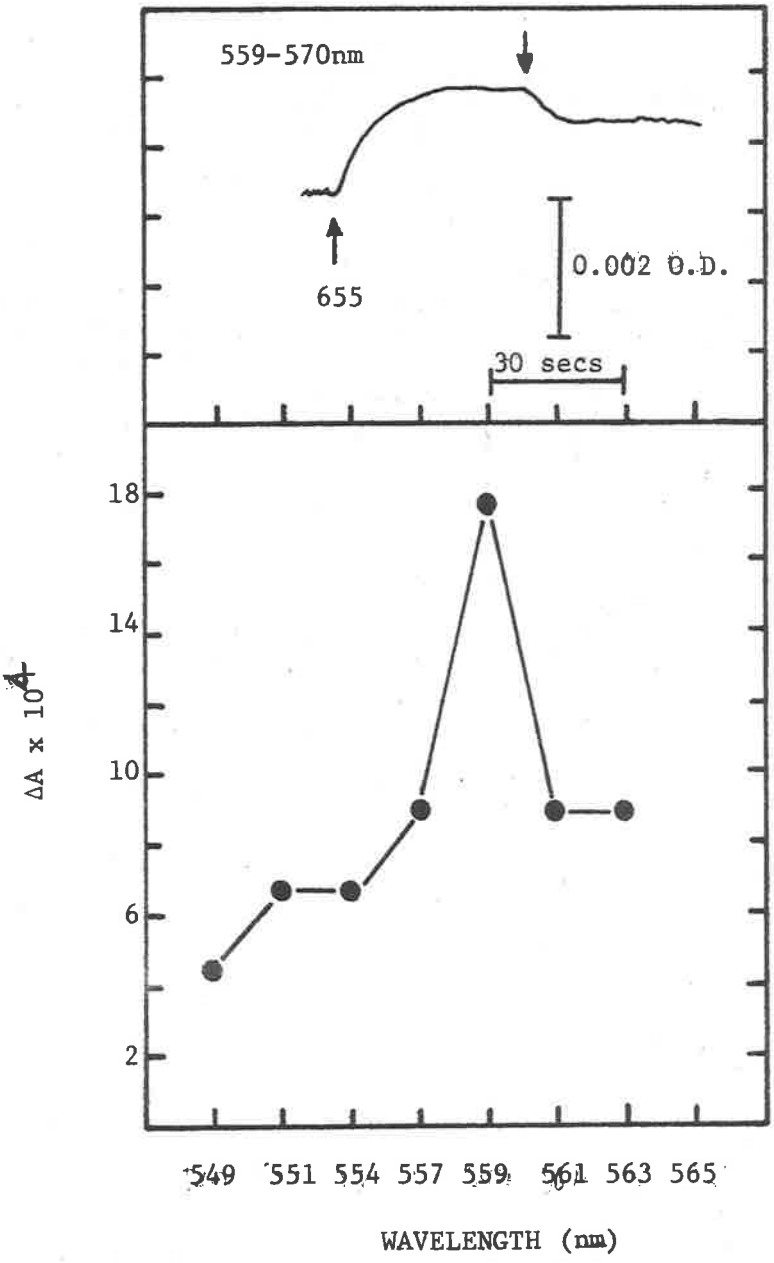


FIGURE IX. Light minus dark difference spectra of untreated pea chloroplasts. Illumination, 655nm, 3.05 x 10^3 ergs/cm^{-2}/sec^{-1}. Reference wavelength, 570nm. Light "on" and "off" response indicated by arrow pointing upwards and downwards. Chlorophyll concentration, 75µg/ml.

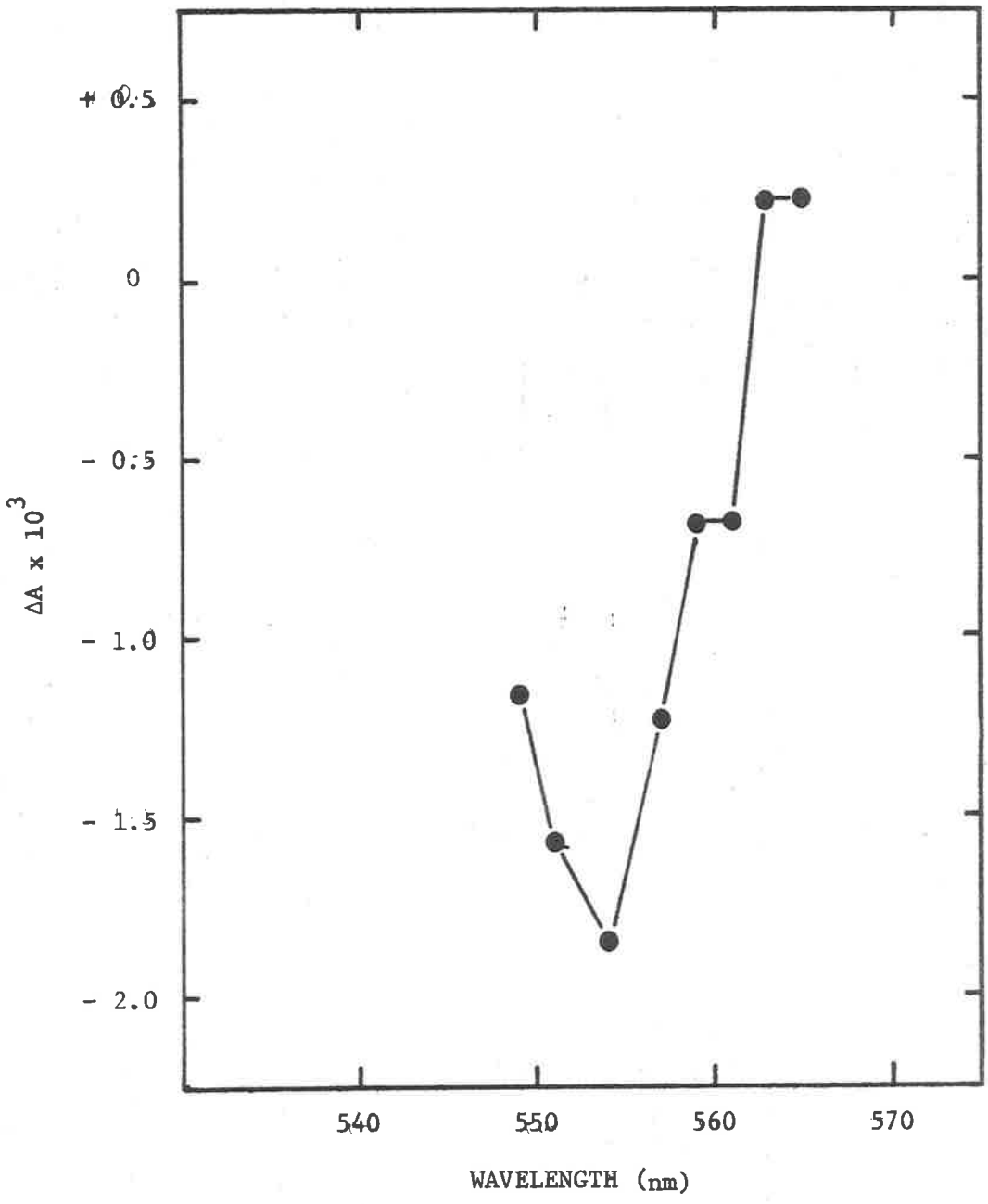


FIGURE X. Light <u>minus</u> dark difference spectra of untreated pea chloroplasts. Illumination, 714nm, 3.45 x 10^3 ergs/cm^{-2}/sec^{-1}. Reference wavelength, 570nm. Chlorophyll concentration, 75µg/ml.

C. *LIGHT-INDUCED REDOX CHANGES OF CYTOCHROMES*

1. *Chloroplasts*

Fig.IX shows the reduction of cytochrome b_{559} by actinic red light in the absence of an electron acceptor in pea chloroplasts. The light minus dark difference spectrum of the increase in absorbance (i.e. reduction), caused by illumination with 655nm actinic light, clearly shows that cytochrome b_{559} was reduced under these conditions (Fig.IX). There is no evidence for a reduction of cytochrome f. However, the difference spectrum for the far-red oxidation shows a minimum at 554nm and a small shoulder at 559nm, indicating that cytochrome f was oxidised to a greater extent than cytochrome b_{559} (Fig.X). These data are in agreement with previous reports that cytochrome f in isolated chloroplasts is mostly in the reduced state (Hill, 1954 and Boardman and Anderson, 1967).

A small transient increase in absorbance (i.e. reduction) was seen at 563nm when chloroplasts were illuminated by 714nm actinic light. This was probably due to the reduction of cytochrome b_6. Similar observations have been reported in spinach chloroplasts and mutant pea leaf (Cramer and Butler, 1967, and Hiller, Anderson and Boardman, 1971). However, Ben-Hayyim and Avron (1970) failed to observe any light-induced redox changes attributable to cytochrome b_6 in lettuce chloroplasts.

FIGURE XI. Effect of CCCP on the light-induced redox changes of cytochrome b_{559} in pea chloroplasts. Chloroplasts were suspended in 30mM Tricine buffer, pH7.6, containing 50mM KCl and 50mM NaCl. Chlorophyll concentration, 75µg/ml, CCCP, 10µM. Intensity of actinic light, 3×10^3 ergs$\cdot$cm$^{-2}\cdot$sec^{-1}. Reference wavelength, 570nm. Light "on" and "off" response indicated by arrow upwards and downwards respectively.

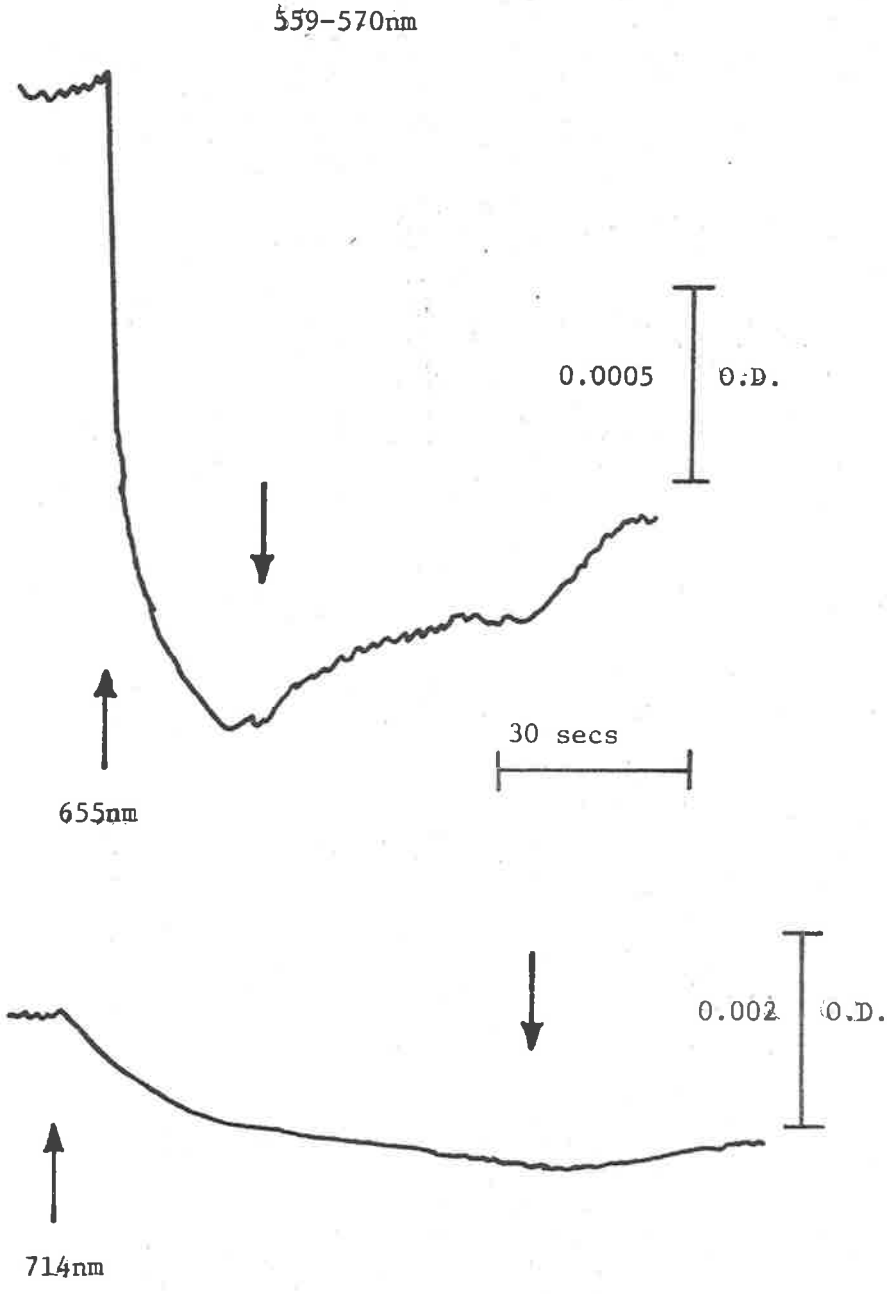
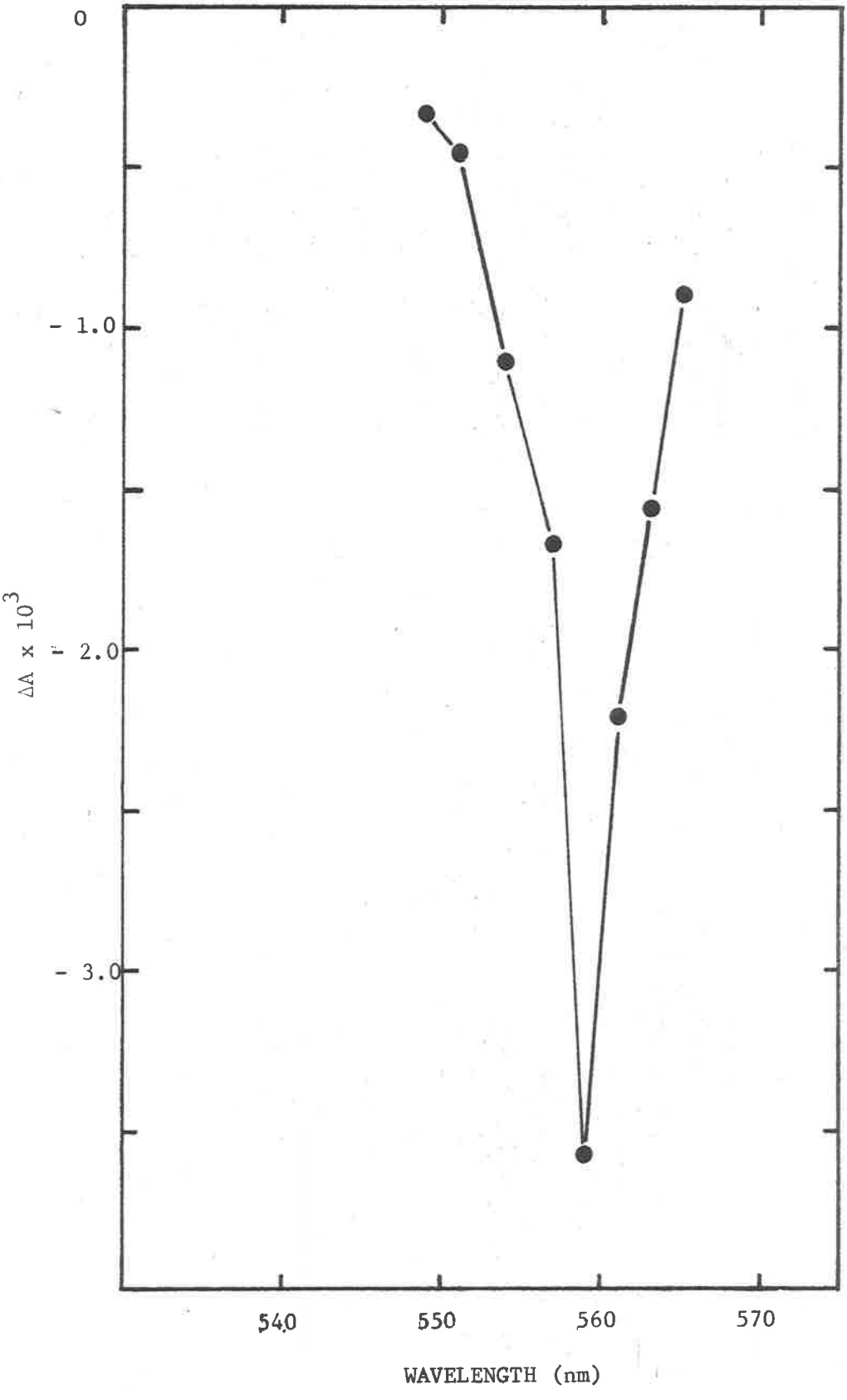

559-570nm
0.0005 O.D.
30 secs
655nm
0.002 O.D.
714nm

FIGURE XII. Light minus dark difference spectra of CCCP-treated pea chloroplasts. Experimental conditions were the same as in Fig.XI. Illumination, 655nm, 3.05×10^4 ergs $\cdot cm^{-2} \cdot sec^{-1}$.


0
- 1.0
- 2.0
- 3.0
ΔA x 10^3
540
550
560
570
WAVELENGTH (nm)

The substituted carbonyl cyanide phenylhydrazones are known to increase the photooxidation of cytochrome b_{559} by far-red light (Cramer and Butler, 1967; Hind, 1968 and Hiller, Anderson and Boardman, 1971) and red light (Hiller, Anderson and Boardman, 1971). Ben Hayyim and Avron (1970) found that FCCP caused a reversion from reduction to oxidation of cytochrome b_{559} by 640nm actinic light in lettuce chloroplasts. Cramer and Butler (1967) however, observed that in the presence of CCCP, cytochrome b_{559}, oxidised by far-red light was reduced by red light. Fig.XI shows the effect of CCCP on the light-induced redox changes of cytochrome b_{559} in pea chloroplasts. Consistent with previous reports, CCCP was found to increase the oxidation of cytochrome b_{559} by far-red light; in addition it inhibited the reduction of cytochrome b_{559} by red light and instead induced an oxidation. It can be seen that red light oxidised cytochrome b_{559} faster in the presence of CCCP than did far-red light (Fig.XI). Hiller, Anderson and Boardman (1971) have reported similar observations. The light minus dark difference spectrum of far-red oxidation in CCCP-treated chloroplasts, has a minimum at 559nm, indicating that cytochrome b_{559} was oxidised under these conditions (Fig.XII).

The inhibition of non-cyclic and cyclic electron flow, and phosphorylation, by the quinone analog, DBMIB, can be reversed by plastoquinone as shown by Bohme, Reimer and Trebst (1971). It has also been shown that DBMIB blocks the reduction of cytochrome

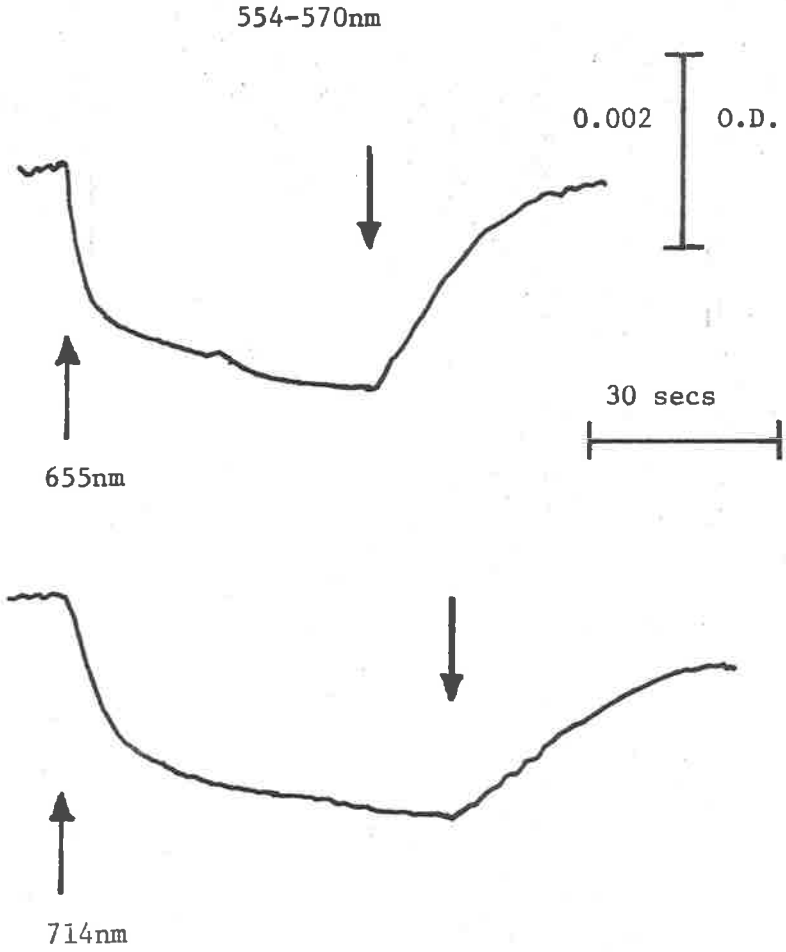


FIGURE XIII. Effect of DBMIB on the light-induced redox changes of cytochrome f in pea chloroplasts. Experimental conditions are the same as in Fig.XI. Concentration of DBMIB, 5μM. Light "on" and "off" response indicated by arrows upwards and downwards respectively.

TABLE V. Light-induced oxidation of cytochromes in pea chloroplasts. The chloroplasts were suspended in a medium containing 0.03M Tricine buffer, pH7.6; 0.05M KCl and 0.05M NaCl. Chlorophyll concentration, 75µg/ml; CCCP, 10µM and DBMIB, 5.68µM. Intensities of actinic light, 3.0×10^3 ergs $cm^{-2} sec^{-1}$; reference wavelength, 570nm.

Actinic Light (nm)	Extent of cytochrome oxidation (nmoles cm^{-3})					
	CCCP		CCCP + DBMIB		DBMIB	
	cyt.f	$cyt.b_{559}$	cyt.f	$cyt.b_{559}$	cyt.f	$cyt.b_{559}$
655	0.099	0.145	0.167	0.167	0.132	0
714	0.122	0.089	0.116	0.045	0.141	0

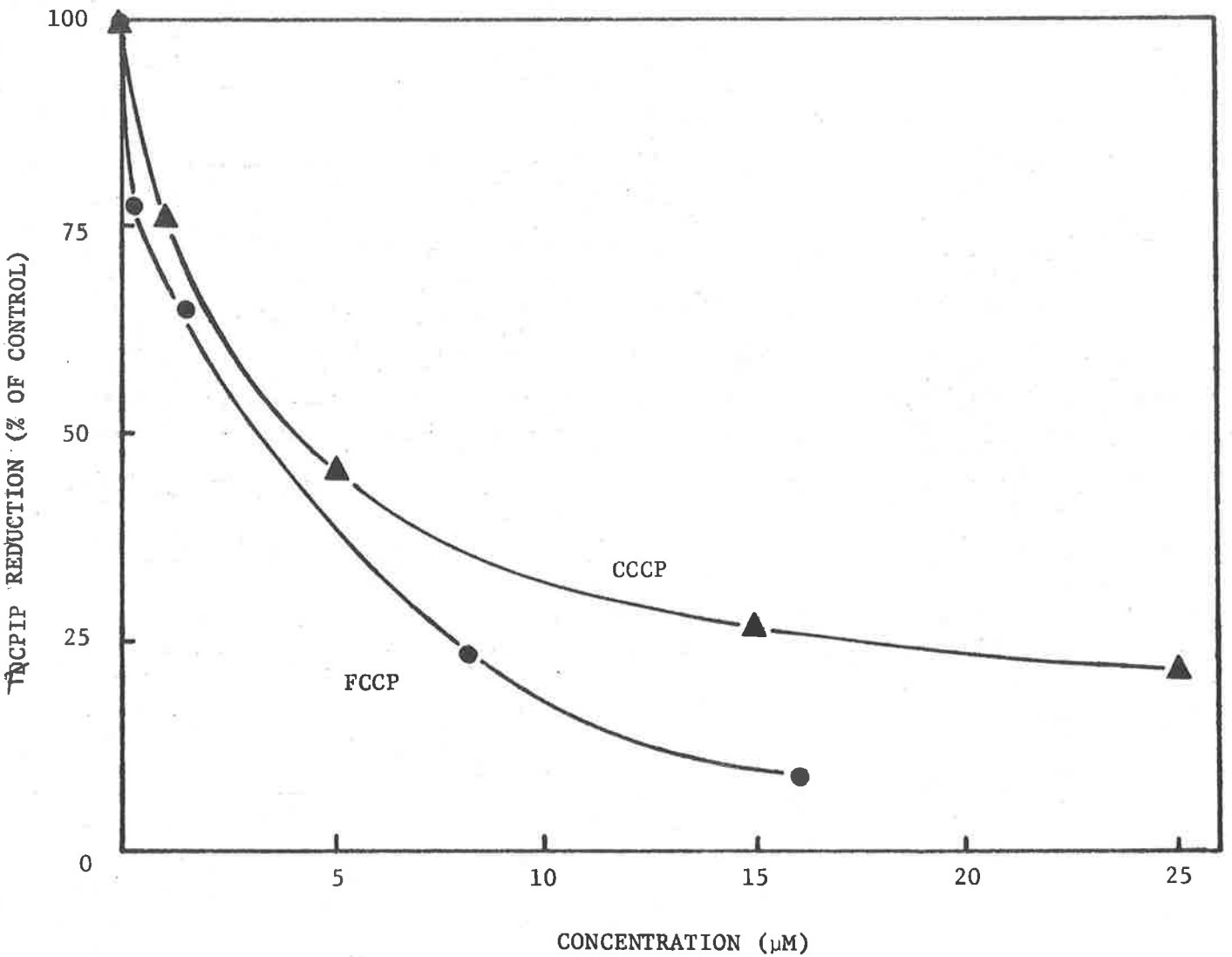
TABLE VI. Effect of FCCP and CCCP concentration on the photooxidation of cytochrome b_{559} in spinach chloroplasts with 655nm light. Chloroplasts were isolated and suspended in 0.3M sucrose; 10mM KCl and 50mM K_2HPO_4-KH_2PO_4 buffer, pH7.2. Chlorophyll concentration was 75µg/ml. Actinic light, 655nm; intensity, 3.0×10^3 ergs·cm^{-2}·sec^{-1}. Reference wavelength used was 540nm instead of 570nm. Extent of cytochrome b_{559} oxidation (nmoles cm^{-3}): with 3.3µM FCCP, 0.182 and with 10µM CCCP, 0.169.

FCCP (µM)	Extent of cyt.b_{559} oxidation (% oxidation with 3.3µM FCCP)	CCCP (µM)	Extent of cyt.b_{559} oxidation (% oxidation with 10µM CCCP)
13.8	87	66	71
6.6	85	33	100
3.3	100	10	100
1.15	76	5	94
0.66	66	1.5	91
0.33	37	0.5	65
0.16	32	0.2	32

f by Photosystem II and the photooxidation of cytochrome b_{559} by Photosystem I. Both these inhibitory effects were relieved by the addition of plastoquinone, implying that it mediates electron flow between cytochrome b_{559} and cytochrome f (Bohme and Cramer, 1971). The effect of DBMIB on the light-induced redox changes of cytochrome f and cytochrome b_{559} are shown in Fig.XIII. DBMIB inhibited the photoreduction of cytochrome b_{559} and cytochrome f by red light, and instead induced a photo-oxidation of cytochrome f. No redox changes due to cytochrome b_{559} were observed in the presence of DBMIB, nor did it have an effect on the far-red oxidation of cytochrome f. However, there was a marked stimulation of the rate of dark reduction of cytochrome f after illumination. The affect of DBMIB on the CCCP-induced oxidations of cytochrome b_{559} and cytochrome f was also studied. Table V shows that DBMIB had no effect on the oxidation of cytochrome b_{559} driven by red light, but it inhibited the oxidation driven by far-red light. The oxidation of cytochrome f by red and far-red light was not affected by DBMIB.

Table VI shows the effect of FCCP and CCCP concentrations on the extent of cytochrome b_{559} in 655nm actinic light. Reducing the concentration of CCCP from 10μM to 1.5μM caused only a slight decrease in the extent of the photooxidation of cytochrome b_{559}, but a decrease of FCCP from 3.3μM to 0.33μM lowered the cytochrome b_{559} photooxidation by two-thirds. High concentrations

FIGURE XIV. Inhibition of the Hill reaction of spinach chloroplasts by CCCP and FCCP. The reaction mixture (3ml) contained in µmoles: Tris-HCl buffer, pH7.8, 40; NaCl; 70 and TCPIP, 0.06. Chloroplasts were added equivalent to 16.5µg chlorophyll. Photoreduction of TCPIP was monitored continuously at 596nm in a Cary Model 14R recording spectrophotometer, fitted with a scattered transmission accessory. Actinic light was provided by a 650W tungsten iodine lamp and filtered through a 3cm layer of water and a 675nm interference filter. A light guide carried the light to the surface of the cuvette, at right angles to the measuring beam. The photomultiplier was protected with a 596nm interference filter.


100
75
50
25
0
DCPIP REDUCTION (% OF CONTROL)
CCCP
FCCP
5
10
15
20
25
CONCENTRATION (μM)

of CCCP (66μM) or FCCP (13.8μM) gave lower amounts of photo-oxidised cytochrome b_{559}, due possibly to some destruction of the cytochrome at high concentrations of the inhibitor.

Hiller, Anderson and Boardman (1971) suggested that the photooxidation of cytochrome b_{559} in CCCP-treated chloroplasts may be related to an inhibition of electron flow from water. The effect of CCCP and FCCP on the rate of the Hill reaction with TCPIP as electron flow is shown in Fig.XIV. The intensity of the actinic light was comparable to that used in the study of the redox changes of cytochrome b_{559}. At this low light intensity, the rate of electron flow is determined by the quantum flux and uncoupling of photophosphorylation does not result in a stimulation of electron flow. Electron flow was inhibited about 25% by 1μM CCCP and 0.33μM FCCP. At 25μM CCCP or 8.3μM FCCP, the rate of photoreduction of TCPIP was 20-25% of the control rate in the absence of the inhibitor. Fig.XIV shows that 50% inhibition of TCPIP reduction was observed with about 3μM FCCP and 4μM CCCP.

2. 10,000 xg particles

Although the composition of PSII subchloroplast particles, obtained by treatment with digitonin (Anderson and Boardman, 1966) and with Triton X-100 (Vernon, Ke, Mollenhauer and Shaw, 1969) had been previously investigated, it was only recently

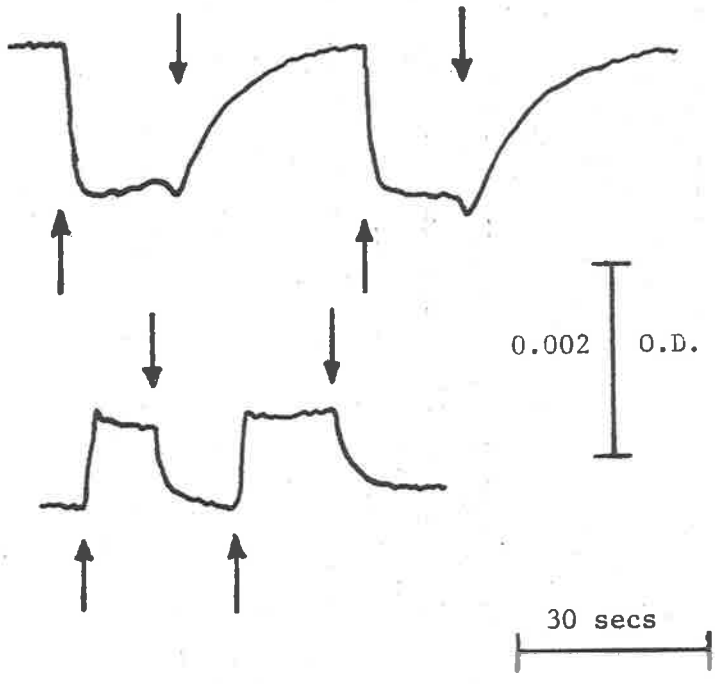
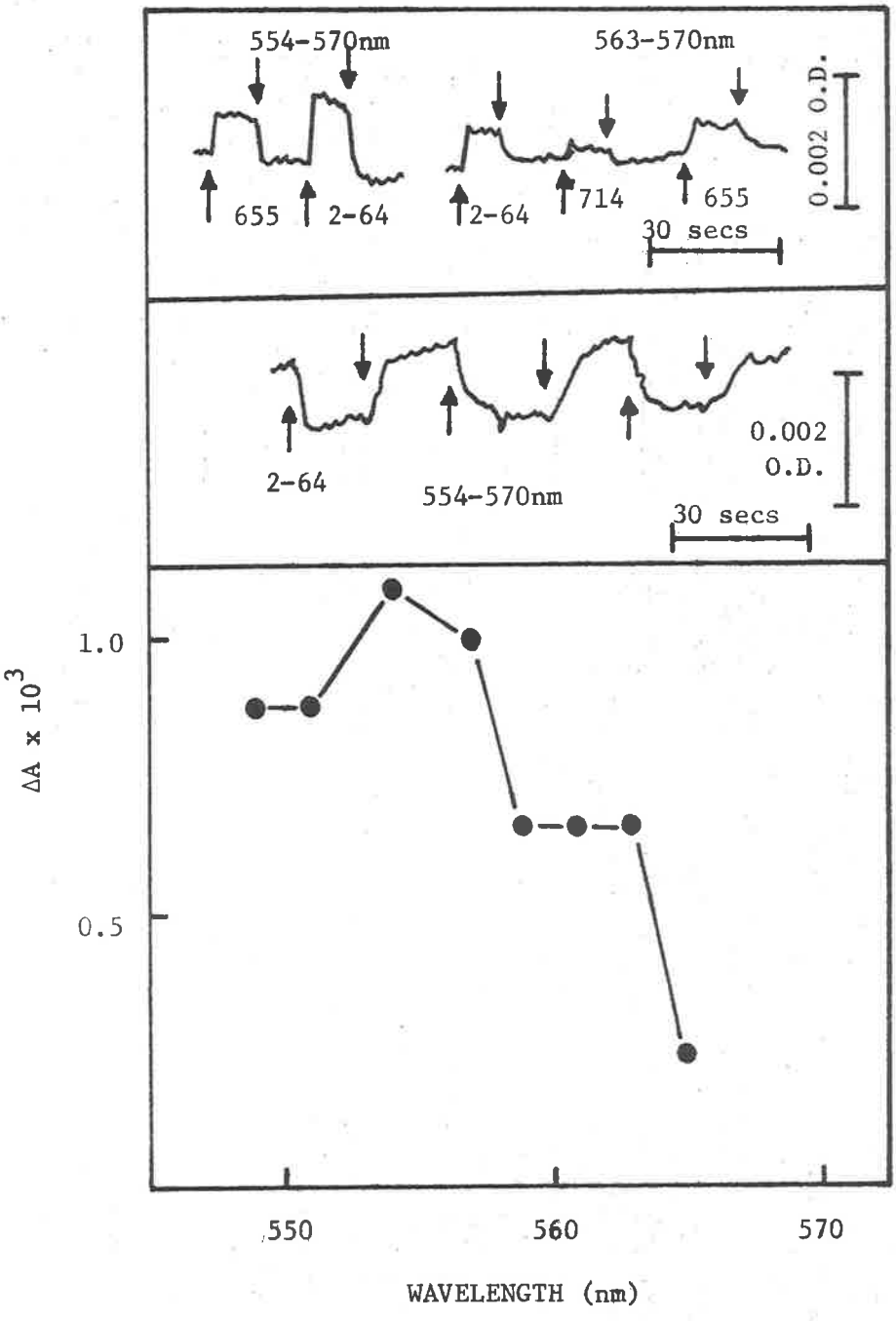


FIGURE XV. Light-induced redox changes of cytochrome b_{559} in digitonin 10,000 xg subchloroplast particles. SCP were suspended in 30mM Tricine buffer, pH7.6, containing 50mM KCl and 50mM NaCl. Chlorophyll concentration, 50µg/ml, CCCP, 10µM. Illumination, Corning filter 2-64 ($>$600nm), 1 x 10^6 ergs$\cdot$cm$^{-2}\cdot$sec^{-1}. Light "on" and "off" responses indicated by arrow upwards and downwards respectively. Reference wavelength, 570nm.

that Ke, Vernon and Chaney (1972) reported light-induced changes of cytochrome b_{559} in an enriched PSII Triton particle. These particles appear to be more enriched in this component than suggested by previously reported values (Boardman and Anderson, 1967 and Vernon, Ke, Mollenhauer and Shaw, 1969). Cytochrome b_{559} was photoreducec by red light, suggesting that it was in the oxidised state prior to illumination. The photoreduction of cytochrome b_{559} in these particles by red light was only partially inhibited by DCMU (10μM), at a concentration which completely inhibited DCPIP reduction by diphenylcarbazide (DPC). Reduced minus oxidised difference spectra of these particles confirm their data on light-induced redox changes.

Fig.XV shows the light-induced redox changes due to cytochrome b_{559} in pea digitonin PSII particles. It can be seen that actinic red light or high intensity red light (Corning Filter 2-64) caused an oxidation of cytochrome b_{559}; this effect was reversed in the dark. This was contrary to the observations of Ke, Vernon and Chaney(1972) with Triton PSII particles. Pre-incubation of the particles with CCCP in the dark, resulted in a reversion from photooxidation to photoreduction (Fig.XV). This indicates that cytochrome b_{559} was readily oxidised in the presence of CCCP (see Section A). No light-induced redox changes of cytochrome f were observed.

FIGURE XVI. Light minus dark difference spectra and light-induced redox changes of cytochromes in digitonin 144,000 xg subchloroplast particles. Reaction mixture was the same as for Fig.XV. Chlorophyll concentration, 50µg/ml. Light intensity, 655nm, 3.05×10^4 ergs/cm^{-2}/sec^{-1}; 714nm, 3.45×10^4 ergs/cm^{-2}/sec^{-1}; Corning filter 2-64, 1×10^6 ergs/cm^{-2}/sec^{-1}. Light minus dark difference spectra was obtained by illumination with Corning filter 2-64 light. Reference wavelength, 570nm.


554-570nm
563-570nm
0.002 O.D.
655
2-64
2-64
714
655
30 secs
2-64
554-570nm
0.002
O.D.
30 secs
1.0
0.5
ΔA x 10^3
550
560
570
WAVELENGTH (nm)

3. 144,000 xg particles

These particles have been shown to contain only PSI activity (Chapter VI and Anderson and Boardman, 1966). It has also been shown that cytochrome f and cytochrome b_6 are closely associated with it (Section B of this chapter and Boardman and Anderson, 1967). Figure XVI shows the light-induced redox changes, at 554 and 563nm, caused by illumination with high intensity red light (Corning Filter 2-64) and 655nm actinic light. The increase in absorbance at 554 and 563nm indicates that cytochrome f and cytochrome b_6 were reduced under these conditions. The light minus dark difference spectra caused by illumination with high intensity red light show a peak at 554nm and a shoulder at 563nm, suggesting that cytochrome f and cytochrome b_6 were reduced (Fig.XVI). When ascorbate was present in the medium to reduce cytochrome f, illumination caused an oxidation, confirming that cytochrome f was being reduced in the above experiments (Fig. XVI). No light-induced redox changes were observed at 563nm in the presence of ascorbate since it does not reduce cytochrome b_6. Even in the presence of dithionite, redox changes due to cytochrome b_6 were not evident.

DISCUSSION

The ascorbate-reduced minus FeCN-oxidised difference spectrum of chloroplasts revealed the presence of cytochrome f and cytochrome

b_{559}. The presence of cytochrome b_6 in spinach and lettuce chloroplasts has been reported by Boardman and Anderson (1967) and Ben-Hayyim and Avron (1970). In accordance with the results of Boardman and Anderson (1967) digitonin Photosystem II particles have more cytochrome b_{559} associated with them than cytochrome f, compared to chloroplasts (Fig.V and VI). On the other hand, digitonin PSI particles have very little high or mid-potential cytochrome b_{559} associated with them, but contain more cytochrome f and cytochrome b_6 and presumably low potential cytochromb b_{559} (Fig.VIII, see also Boardman and Anderson, 1967).

The association of low potential cytochrome b_{559} with cytochrome b_6 (Bendall, 1968) means that it is also associated with PSI particles. Boardman and Anderson (1967) observed two peaks in the dithionite reduced minus ascorbate reduced spectrum in spinach chloroplasts at 77°K at 557nm and 561nm. They thought that it was due to the splitting of the band due to cytochrome b_6. The peak at 557nm could be the low potential cytochrome b_{559} and not due to the splitting of the cytochrome b_6 band. Therefore, the presence of two species of cytochrome b_{559}, i.e. high potential and low potential *in vivo* seems quite certain. Erixon, Lozier and Butler (1972) suggested that the various forms of cytochrome b_{559} with a range of midpoint potentials could be due to the modification of the high potential

form by the various treatments. However, treatment of chloroplasts with digitonin did not convert the high potential form of cytochrome b_{559} in chloroplasts to a form of lower potential in PSII subchloroplast particles (Figs.V and VI). On the other hand, treatment with Triton X-100 caused the conversion of the native high potential form of cytochrome b_{559} into a mid-potential form (Ke, Vernon and Chaney, 1972).

A. LIGHT-INDUCED REDOX CHANGES

1. *Chloroplasts*

Light-induced redox changes in chloroplasts mainly reflected the presence of cytochrome f and cytochrome b_{559}. Although cytochrome b_{559} was reduced by red light, its oxidation by far-red light was very small or non-existent. In contrast, cytochrome f was readily oxidised by far-red light and reduced by red light. Fast oxidation of cytochrome b_{559} by far-red light was observed only in CCCP-treated chloroplasts (Fig.XI). In these chloroplasts, photooxidation of cytochrome b_{559} was also observed under red light illumination. This is consistent with the observations of Hiller, Anderson and Boardman (1971). These authors were unable to detect any light-induced redox changes attributable to cytochrome b_{559} in untreated or DCMU-treated spinach chloroplasts, and therefore concluded that cytochrome b_{559} could not be situated

between the two photosystems. However, they observed that cytochrome b_{559} was photooxidised by red light as well as far-red light in CCCP-treated chloroplasts, and proposed an electron flow scheme in which cytochrome b_{559} was connected to the oxidising side of Photosystem II. In order to explain the photooxidation in far-red light, they also included a link between cytochrome b_{559} and Photosystem I. On the other hand, Cramer and Butler (1967) and Ben Hayyim and Avron (1970) observed that cytochrome b_{559} was reduced by red light and oxidised by far-red light in spinach and lettuce chloroplasts respectively. Cramer, Fan and Bohme (1971) reported that incubation of lettuce chloroplasts for 5 min with FCCP (20μM) caused the oxidation of most of the cytochrome b_{559}, which was in the reduced state in untreated chloroplasts. The oxidised cytochrome was reducible with ascorbate but not with hydroquinone, indicating that treatment of chloroplasts with FCCP had converted a high potential cytochrome b_{559} to a form of lower potential.

Since both the photooxidation of cytochrome b_{559}, and its conversion from a high potential to a mid-potential form, are similarly dependent on pH, Cramer, Fan and Bohme (1971) concluded that it was the mid-potential form of cytochrome b_{559} which was oxidised by PSI and reduced by PSII. In addition, since Bohme

and Cramer (1971) observed that DBMIB inhibited the photo-oxidation of cytochrome b_{559} in spinach chloroplasts treated with FCCP, Cramer, Fan and Bohme (1971) proposed a scheme of electron flow in which the high and mid-potential forms of cytochrome b_{559} were interconvertible. The mid-potential form was shown as a component of the electron flow pathway between the two photosystems, but closer to PSII than plastoquinone. Table II shows that only a very small fraction of the high potential cytochrome b_{559} of spinach chloroplasts was converted to a mid potential form by dark incubation with CCCP or FCCP, at concentrations that were sufficient to cause substantial photooxidation of cytochrome b_{559} in both red and far-red light.

The inhibition of the PSI oxidation of cytochrome b_{559} by DBMIB (Table V), supports the conclusion of Cramer, Fan and Bohme (1971) that the mid-potential form of cytochrome b_{559} is photooxidised by PSI. DBMIB is an antagonist of plastoquinone (Bohme, Reimer and Trebst, 1970), and therefore, it seems reasonable that mid-potential cytochrome b_{559} is photooxidised by PSI via plastoquinone. The lack of inhibition of PSII oxidation of cytochrome b_{559} by DBMIB indicates that plastoquinone does not mediate this photooxidation.

A possible explanation of these results is that the high potential cytochrome b_{559} is photooxidised by PSII, whereas the

mid potential cytochrome b_{559} is reduced by PSII and oxidised by PSI, far-red light favouring the mid potential form and red light the high potential form.

Since photooxidation of cytochrome b_{559} by PSII occurs at liquid nitrogen temperature in untreated chloroplasts (Knaff and Arnon, 1969a; Floyd, Chance and De Vault, 1971; Boardman, Anderson and Hiller, 1971; Bendall and Sofrova, 1971 and Erixon and Butler, 1971a) and at room temperature in Tris-treated chloroplasts (Knaff and Arnon, 1969a,b), Hiller, Anderson and Boardman (1971) suggested previously that the photooxidation of cytochrome b_{559} in 655nm light in CCCP-treated chloroplasts may be related to the inhibitory effect of CCCP on electron flow from water. Fig.XIV shows a 25% inhibition of the Hill reaction with 1.5μM CCCP, a concentration sufficient to cause considerable photooxidation of cytochrome b_{559} in 655nm light (Table VI). It seems unlikely that this partial inhibition of electron flow is the prime cause of the photooxidation of cytochrome b_{559} in CCCP treated chloroplasts. However, the inhibition of cytochrome f reduction by PSII light in the presence of CCCP may be caused by this partial inhibition of electron flow by CCCP.

Boardman, Anderson and Hiller (1971) showed that high potential b_{559} was photooxidised at 77°K, and therefore, the

lack of effect of CCCP on this photooxidation is consistent with the finding that the redox potential of cytochrome b_{559} (high potential) was not lowered by dark incubation with CCCP.

The functional role of the high potential cytochrome b_{559} is difficult to assess. It seems that cytochrome b_{559} is closely associated with the primary reaction centre of PSII (Vernon, Shaw, Ogawa and Raveed, 1971) and can serve as an electron donor to PSII, (Erixon and Butler, 1971a). It has also been suggested that cytochrome b_{559} functions on the water-splitting side of PSII (Erixon, Lozier and Butler, 1972), and might function in the S states proposed by Kok, Forbush and McGloin (1970) to participate in the oxygen-evolving mechanism (Bendall and Sofrova, 1971). Photooxidation by PSII light of high potential b_{559} at liquid nitrogen temperature (Knaff and Arnon, 1969a; Floyd, Chance and De Vault, 1971; Boardman, Anderson and Hiller, 1971; Bendall and Sofrova, 1971; Erixon and Butler, 1971b and Table III) suggests cytochrome b_{559} may be an electron donor to PSII. However, this low temperature photooxidation of cytochrome b_{559} may be an artifact of these conditions where the strong oxidant of PSII may not be able to receive electrons from water (Erixon and Butler, 1971b). Therefore, emphasis should not be placed on the evidence obtained at low temperatures, but on the data obtained at physiological

temperatures. Under such conditions the reduction of cytochrome b_{559} by PSII light and its oxidation by PSI light has been observed (Levine, Gorman, Avron and Butler, 1966; Cramer and Butler, 1967; Ben Hayyim and Avron, 1970). Bohme and Cramer (1971) placed cytochrome b_{559} nearer PSII after observing that the oxidation of cytochrome b_{559} by PSI light was inhibited by a plastoquinone antagonist, 2,5,dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). Thus, the presence of a cytochrome b_{559} between the two photosystems seems quite certain. Later, Cramer, Fan and Bohme (1971) proposed a scheme for photosynthetic electron flow in which a"low"potential cytochrome b_{559} (reducible by ascorbate) with mid-point potential of +80mV is situated near PSII and placed plastoquinone between cytochrome b_{559} and cytochrome f. However, the presence of a low potential cytochrome b_{559} (reducible only by dithionite but not by ascorbate) would indicate that it could be the one reduced by PSII (see Erixon, Lozier and Butler, 1972), and not the"low"potential cytochrome b_{559} of Cramer, Fan and Bohme (1971) which is reducible by ascorbate. Studies on the effect of DBMIB on non-cyclic electron flow indicated the presence of energy transducing sites after the site of DBMIB inhibition. This is consistent with the scheme proposed by Bohme and Cramer (1972) in which an energy transducing site is located between

plastoquinone and cytochrome f. However, Ben Hayyim and Avron (1971) found that the presence of uncouplers or phosphorylating agents induced a more reduced steady-state of cytochromes b_{559} and f, and they concluded that a site of ATP formation is present between PSII and cytochrome b_{559}. The presence of energy transducing sites after cytochrome b_{559} was also suggested by Hildreth (1968) and Hind (1968) after observing the stimulation of cytochrome b_{559} oxidation by CCCP and antimycin A (see also Chapter V).

2. 10,000 xg particles

Light-induced redox changes in these particles were different from those observed in chloroplasts. In agreement with the data on reduced minus oxidised difference spectra, which showed very little cytochrome f present in the particles, no light-induced redox changes attributable to cytochrome f were observed. Contrary to the situation in chloroplasts, illumination with actinic red light or high intensity red light caused an oxidation of cytochrome b_{559}, instead of a reduction. Far-red light did not cause any light-induced redox changes. It has been shown in chloroplasts that CCCP caused a reversion from a reduction of cytochrome b_{559} by red light to an oxidation (Fig.XI). A similar effect in the opposite direction was observed when

digitonin PSII particles were treated with CCCP. A photo-reduction of cytochrome b_{559} by red light was observed in the presence of CCCP, indicating that the reduced cytochrome b_{559} had been oxidised in the dark. This dark oxidation in the presence of CCCP is difficult to explain, since no such effect of CCCP was observed in chloroplasts. Further work on the light-induced redox changes is needed before a definite conclusion can be reached.

The photooxidation of cytochrome b_{559} by PSII light occurs at liquid nitrogen temperature in untreated SCP (Table IV). Unlike chloroplasts, incubation of these particles with CCCP, caused some conversion of high potential cytochrome b_{559} to a low potential form (Table IV). Both the high potential (Q-F spectrum in Fig.VII) and mid-potential forms (A-F spectrum in Fig.VII) were photooxidised at liquid nitrogen temperature in these SCP by PSII light (Table IV). This photooxidation of mid-potential cytochrome b_{559} by PSII was not consistent with the observations of Ke, Vernon and Chaney (1972), who did not observe any such effect in their Triton PSII particles. They suggested that the photooxidation at liquid nitrogen temperatures could be a unique characteristic of high potential cytochrome b_{559}. However, the photooxidation of mid-potential cytochrome b_{559} at 77°K has been observed in Tris-treated chloroplasts by Erixon, Lozier and Butler (1972).

3. 144,000 xg particles (PSI particles)

The light-induced redox changes due to cytochrome f and cytochrome b_6 in these particles agree well with the data on reduced minus oxidised difference spectra, which showed that both the cytochromes were in the oxidised state (Fig.VIII and XVI). Although a fast reduction of cytochrome b_6 was observed under illumination by either red light (655nm) or high intensity red light (Corning filter 2-64), actinic far-red light was hardly able to reduce it at all, presumably due to the lower light intensity employed. In the case of cytochrome f, actinic and high intensity red light, as well as far-red light, were able to induce redox changes, although with far-red illumination changes were very small (Fig.XVI). Oxidation of cytochrome f by far-red light in the presence of an electron donor-acceptor system in digitonin PSI particles have been reported by Anderson, Fork and Amesz (1966). Vernon, Ke and Shaw (1967) isolated a PSI particle by treatment with Triton X-100. This particle had cytochromes f and b_6 associated with it, but they were unable to detect any light-induced redox changes. Only P_{700} underwent a change upon illumination. Thus, it appears that the PSI particle obtained by treatment with Triton X-100, similar to the PSII particle of Ke, Vernon and Chaney (1972) obtained by a similar method, is photochemically very different to digitonin subchloroplast particles.

CHAPTER VIII

ELECTRON FLOW FROM DCPIPH$_2$ TO MV IN INTACT CHLOROPLASTS AND DIGITONIN PSI SUBCHLOROPLAST PARTICLES. ABSENCE OF PHOSPHORYLATION SITE(S) ON THIS PATHWAY.

INTRODUCTION

Electron flow from water to NADP and the coupled ATP formation has been shown to be inhibited by DCMU (Vernon and Avron, 1965). This inhibition of NADP reduction was relieved when the electron donor couple DCPIP and ascorbate was added (Vernon and Zaugg, 1960). It has been reported that this system supports phosphorylation (Losada, Whatley and Arnon, 1961). However, DCPIP and ascorbate have been found to support phosphorylation even in the absence of an electron acceptor (Trebst and Eck, 1961 and Keister, 1965) and it was suggested that reduced DCPIP catalyzed a cyclic phosphorylation instead of non-cyclic phosphorylation. On the other hand, it has been observed that non-cyclic electron flow from DCPIPH$_2$ to either NADP or MV can be stimulated by uncouplers (Wessels, 1964; Keister, 1965 and Izawa, Connolly, Winget and Good, 1966), suggesting that this pathway supports phosphorylation. Recently, Neumann, Arntzen and Dilley (1971) reported that electron flow from DCPIPH$_2$ to MV supported phosphorylation at two sites, both of which were located on the non-cyclic pathway. Subsequently

TABLE I. Effect of uncouplers on the rate of electron flow from $DCPIPH_2$ to MV in pea chloroplasts. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 4.2μM DCMU; 25μM DCPIP; 4.65mM ascorbate; 70μM MV and 70μM azide. 21mM KCl was also present with nigericin. Chloroplasts were added equivalent to 30 and 44μg chlorophyll during experiments I and II respectively.

Additions	Oxygen Uptake (μmoles/mg chll/hr)
Experiment I	
1. Control	39
2. NH_4Cl (1.8mM)	90
3. NH_4Cl (9.5mM)	108
4. CCCP (2.3μM)	134
Experiment II	
1. Control	73
2. Nigericin (0.46μM)	398

TABLE II. Effect of uncouplers on the rate of electron flow from $DCPIPH_2$ to MV in digitonin PSI subchloroplast particles. The reaction mixture was the same as in Table I. Subchloroplast particles equivalent to 27µg chlorophyll were added.

	Additions	Oxygen Uptake (µmoles/mg chll/hr)
1.	Control	84
2.	NH_4Cl (1.85mM)	131
3.	CCCP (2.3µM)	130

TABLE III. Effect of ADP on the rate of electron flow from water or $DCPIPH_2$ to MV in pea chloroplasts. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 10mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 30mM HEPES buffer, pH7.5; 70μM MV and 70μM azide; 25μM DCPIP and 4.65mM ascorbate plus 4.2μM DCMU and 58μg chlorophyll. ADP was added to a final amount of 0.475 μmoles.

Additions	Electron Flow (μmoles O_2/mg chl/hr)
Hill reaction (H_2O)	100
State 3	163
Hill reaction ($DCPIPH_2$)	24
State 3	24

Shavit and Shoshan (1971) claimed that non-cyclic electron flow from DCPIP and ascorbate to NADP was coupled to a site of phosphorylation.

This Chapter will present evidence that the non-cyclic electron flow from $DCPIPH_2$ to MV need not be coupled to phosphorylation and proposes an alternative hypothesis to explain the stimulatory effect of uncouplers.

RESULTS

The electron flow from $DCPIPH_2$ to MV in chloroplasts, as measured by the oxygen uptake by reduced MV in the presence of azide, was stimulated by uncouplers (Table 1). NH_4Cl and CCCP stimulated the rate of oxygen uptake more than two fold, but the highest stimulation was observed with nigericin in the presence of K^+ ions. Similar stimulation has been observed by other authors (Wessels, 1964; Keister, 1965; Izawa, Connolly, Winget and Good, 1966 and Arntzen, Neumann and Dilley, 1971). The same reaction can be studied using digitonin PSI subchloroplast particles. Table II shows that NH_4Cl and CCCP stimulated electron flow from $DCPIPH_2$ to MV in these particles to approximately the same extent as they did in intact chloroplasts. These stimulatory effects could imply the presence of coupling sites on this electron flow pathway (Neumann, Arntzen and Dilley, 1971 and Shavit and Shoshan, 1971), but as shown in Table III, addition of ADP had no stimulatory effect on the rate of oxygen uptake during electron flow from $DCPIPH_2$ to MV

TABLE IV. ATP formation in three electron flow systems (H_2O or $DCPIPH_2$ to MV, and $DCPIPH_2$ in the absence of an electron acceptor). The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 13mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 30mM HEPES buffer, pH7.5; 50µM MV and 50µM azide; 30µM DCPIP and 3mM ascorbate plus 3µM DCMU. Chloroplasts equivalent to 78 and 53µg chlorophyll were added during Experiments I and II respectively. ADP was added to a final amount of 4 µmoles. The systems were illuminated for 2 min. and the reactions terminated by adding 20% trichloroacetic acid.

Electron Flow Systems	ATP^{32} Formation (µmoles/mg chll/hr)
Experiment I	
A. H_2O to MV	369
B. $DCPIPH_2$ to MV	88
Experiment II	
A. $DCPIPH_2$ to MV	78
B. $DCPIPH_2$	72

in chloroplasts. On the other hand, the electron flow from water to MV was stimulated greater than two-fold by the addition of ADP. ATP formation during non-cyclic electron flow from water or $DCPIPH_2$ to MV and cyclic electron flow catalyzed by $DCPIPH_2$ was also measured (Table IV). Phosphorylation during electron flow from water to MV was much greater than from $DCPIPH_2$ to MV. It can also be seen that the low rate of phosphorylation during non-cyclic electron flow from $DCPIPH_2$ to MV was similar to cyclic phosphorylation catalyzed by $DCPIPH_2$. This indicates that $DCPIPH_2$ is donating electrons after the sites of phosphorylation on the non-cyclic electron flow pathway. The same conclusion was reached by Avron (1964) after observing that phosphorylation associated with NADP reduction could be accounted for by the cyclic phosphorylation catalysed by DCPIP and ascorbate (see also Wessels, 1964).

Neumann and Jagendorf (1964) detected a pH shift with DCPIP, but it was only partially reversible. They attributed the irreversible part of the pH rise to the difference between the pK of the oxidised and reduced forms of the dye, the protons being retained inside the grana. It can be envisaged that the protons left inside the grana are limiting the rate at which $DCPIPH_2$ was being oxidised. This idea was prompted by the lack of phosphorylation in this system and the stimulatory effect of uncouplers. In a phosphorylating system, uncouplers act by transporting protons

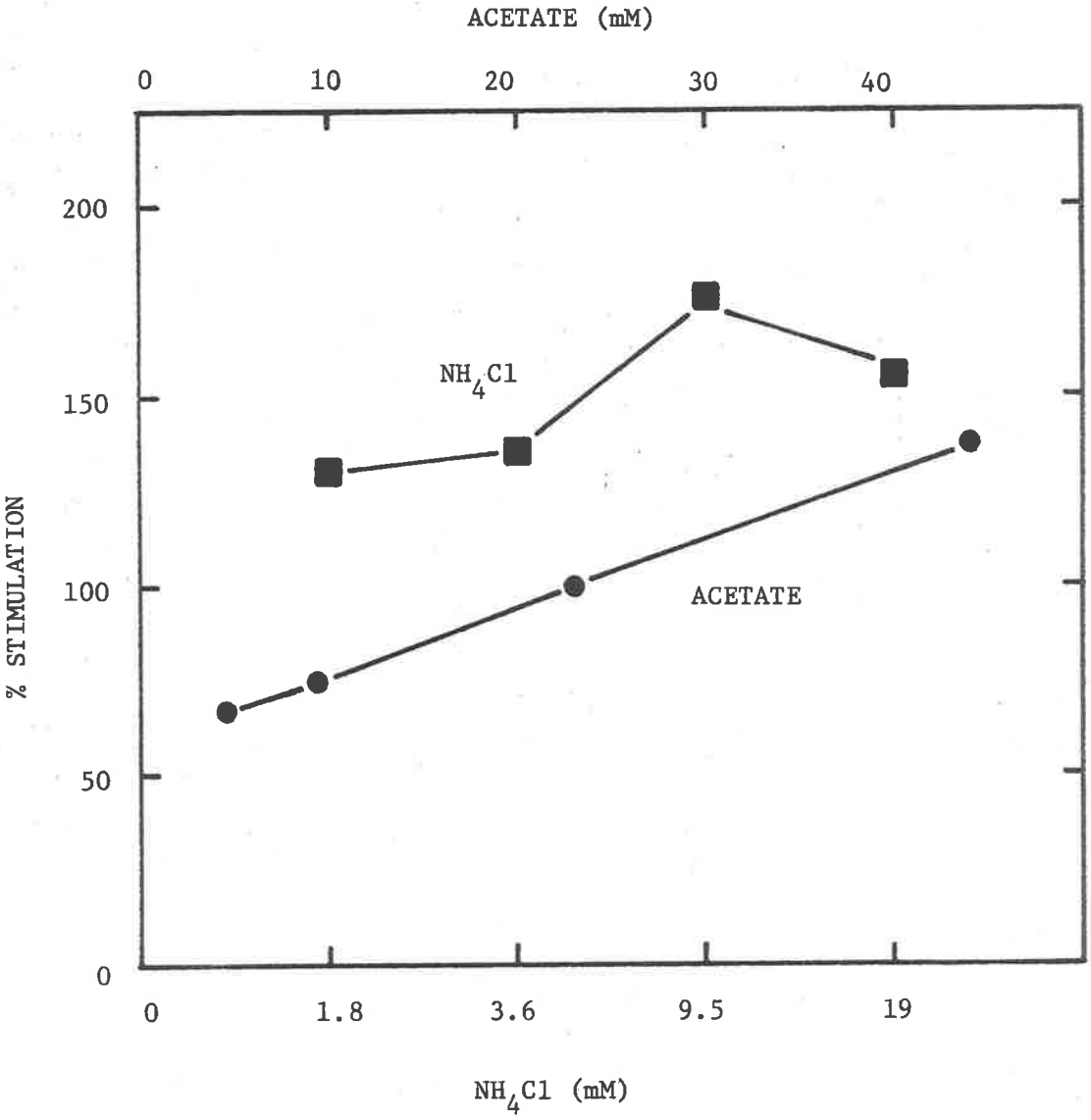


FIGURE I. Stimulation of electron flow from $DCPIPH_2$ to MV by NH_4Cl and acetate. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 40mM HEPES buffer, pH7.5; 4.6μM DCMU; 15μM DCPIP; 4.65mM ascorbate; 64μM MV; 0.93mM azide and pea chloroplasts equivalent to 29μg chlorophyll. Control rate of electron flow was 39 μmoles O_2/mg chll/hr.

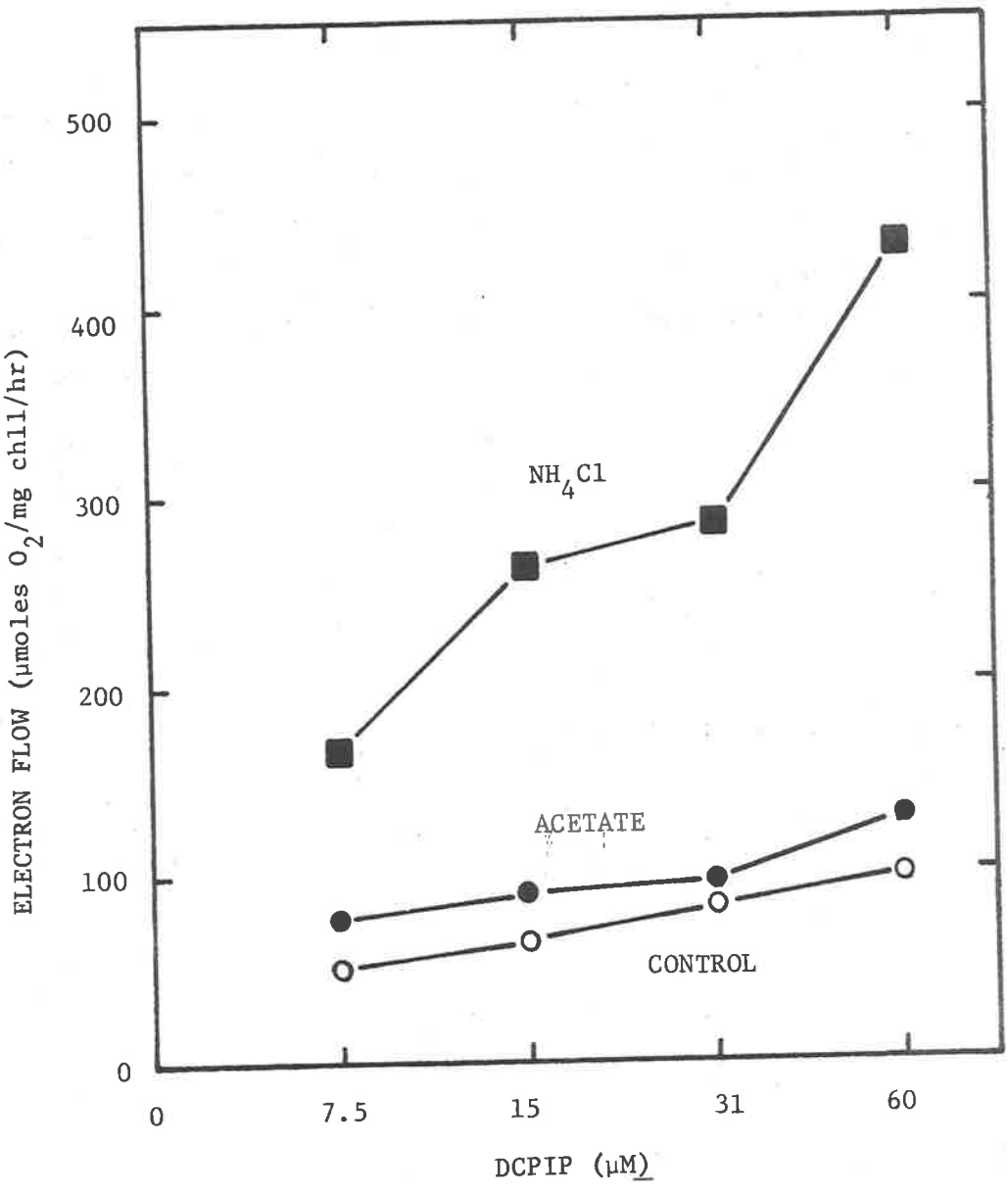


FIGURE II. Effect of NH_4Cl and acetate on electron flow from $DCPIPH_2$ to MV at various concentrations of $DCPIPH_2$. The reaction mixture was the same as used in Fig.I, except that pea chloroplasts were added equivalent to 43μg chlorophyll. Concentration of NH_4Cl, 1.8mM and acetate, 4.6mM.

FIGURE III. Effect of DNP on electron flow from $DCPIPH_2$ to MV at various pH. The reaction mixture contained 0.4M sucrose; 7.8mM $MgCl_2$; 4.6μM DCMU; 32μM DCPIP; 4.65mM ascorbate; 64μM MV; 0.93mM azide and pea chloroplasts equivalent to 81μg chlorophyll. 4.65mM MES buffer for pH5.5 and 4.65mM K_2HPO_4-KH_2PO_4 buffer for pH6.0-9.0. Concentration of DNP was 0.3mM.

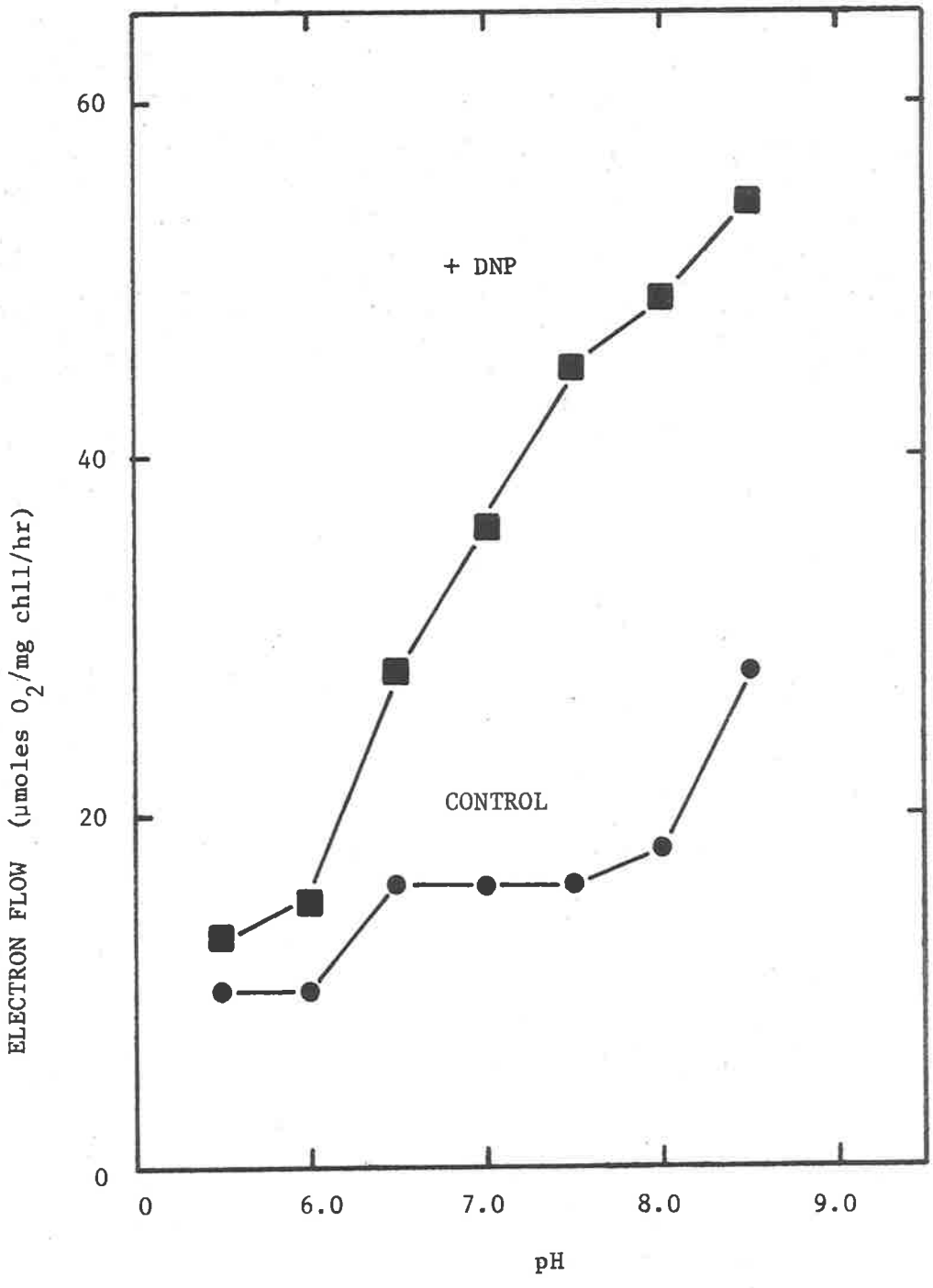

60
40
20
0
+ DNP
CONTROL
ELECTRON FLOW (μmoles O_2/mg chll/hr)
0
6.0
7.0
8.0
9.0
pH

TABLE V. Effect of acetate on Hill reaction and State 3 rates of electron flow with FeCN or MV as electron acceptors. The reaction mixture contained 0.33M sorbitol; 5mM $MgCl_2$; 1mM $MnCl_2$; 15mM K_2HPO_4-KH_2PO_4 buffer, pH7.5; 30mM HEPES buffer, pH7.5; 64μM MV and 0.93μM azide or 1.5mM FeCN and 32μg chlorophyll. ADP was added to a final amount of 0.475 μmoles.

Additions	Oxygen evolution/Uptake (μmoles/mg chll/hr)
1. Hill reaction (FeCN)	116
2. + Acetate (9mM)	121
3. Hill reaction (MV)	150
4. + Acetate	114
5. State 3 (FeCN)	292
6. + Acetate	292

TABLE VI. Effect of succinate and sulphate on electron flow from $DCPIPH_2$ to MV. The reaction mixture contained the same components as in Table I. Chloroplasts added were equivalent to 44µg chlorophyll.

Additions	Electron Flow (µmoles O_2/mg chll/hr)
Control	77
Sod. Succinate (4.6mM)	150
Sod. Succinate (9.2mM)	167
Sod. Sulphate (4.6mM)	77
Sod. Sulphate (9.2mM)	81
Sod. Sulphate (22.7mM)	0

from inside the membrane to the outside medium causing dissipation of the pH gradient and resulting in faster electron flow (Mitchell, 1966 and Good, Izawa and Hind, 1966). It has been shown that, although the electron flow from $DCPIPH_2$ to MV is not associated with phosphorylation, uncouplers have the same stimulatory effect, indicating the involvement of protons in this system. This could mean that uncouplers are acting in a similar manner, resulting in faster oxidation of $DCPIPH_2$ due to rapid efflux of protons from inside the membrane. The effect of the anion, acetate, on the rate of oxygen uptake during electron flow from $DCPIPH_2$ to MV lends support to this idea. Fig.I shows that acetate stimulates the rate of oxygen uptake progressively with increasing concentration, similar to NH_4Cl. The effect of acetate was specific for this system as shown in Table V. This indicates that acetate does not act as an uncoupler. Electron flow, at various concentrations of $DCPIPH_2$, was measured and the effect of acetate studied. The rate of oxygen uptake was stimulated increasingly as the concentration of $DCPIPH_2$ was increased (Fig.II). Essentially similar results were obtained with succinate but not sulphate, which was ineffective at low concentrations (10mM) and completely inhibited oxygen uptake at high concentrations (Table VI).

Electron flow from $DCPIPH_2$ to MV was measured over a range of pH (Fig.III). It can be seen that electron flow was slower under acidic

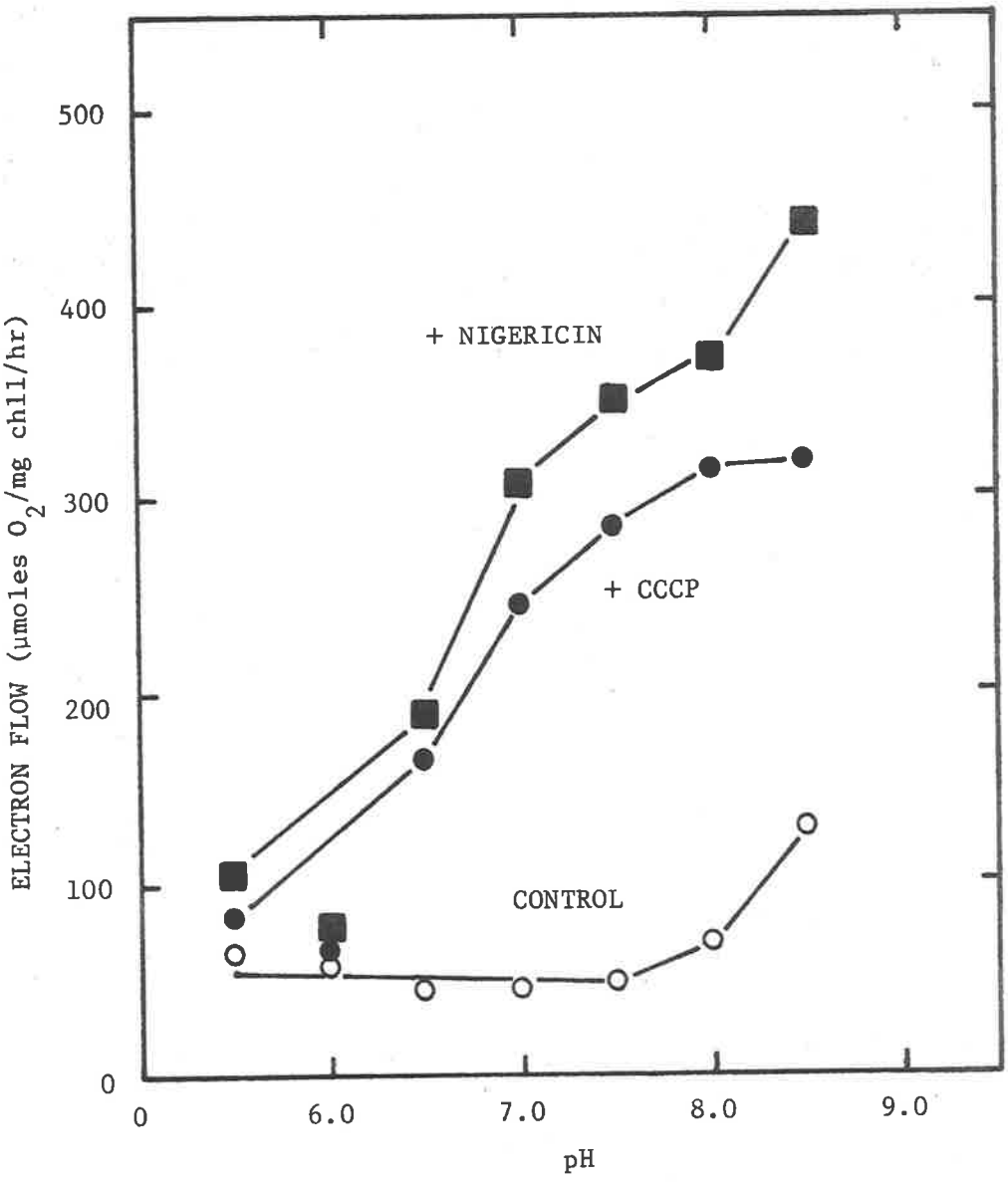


FIGURE IV. Nigericin and CCCP on electron flow from $DCPIPH_2$ to MV at various pH. The reaction mixture was the same as used in Fig.III, except that pea chloroplasts were added equivalent to 66µg chlorophyll. Concentrations of nigericin, KCl and CCCP were 0.46µM; 21mM and 9.2µM respectively.

conditions than under basic conditions. DNP stimulated electron flow over the whole range of pH, but more dramatically above pH7. This stimulation by DNP could mean that protons are being transported out of the thylakoid membrane, as DNP has been shown to render membranes more permeable to protons (Van Dam and Slater, 1967; Mitchell and Moyle, 1967; Carafoli and Rossi, 1967; Pressman, Harris, Jagger and Johnson, 1967 and Karlish, Shavit and Avron, 1969). The same stimulatory effect was observed when CCCP and nigericin together with K^+ ions were substituted for DNP (Fig.IV) supporting the above idea.

DISCUSSION

These data indicate that electron flow from $DCPIPH_2$ to MV is not coupled to phosphorylation. In contrast, Neumann, Arntzen and Dilley, (1971) observed a slow rate of ATP formation associated with this electron flow pathway, which they attributed to coupling at two sites of phosphorylation. It has also been observed that NADP reduction is stimulated by phosphorylating agents (Shavit and Shosham, 1971). However, no evidence of coupling during electron flow from $DCPIPH_2$ to MV was observed (Table III). Observations of phosphorylation on this electron flow pathway have also been made by Avron (1964) and Wessels (1964), but these authors attributed this to cyclic phosphorylation catalyzed by $DCPIPH_2$ and concluded that electron flow from $DCPIPH_2$ to NADP is not coupled to phosphorylation.

Uncouplers have previously been found to stimulate the electron flow from $DCPIPH_2$ to either MV or NADP, and this was thought to indicate coupling (Wessels, 1964; Keister, 1965; Izawa, Connolly, Winget and Good, 1966 and Neumann, Arntzen and Dilley, 1971). The stimulation of electron flow from $DCPIPH_2$ to MV, by uncouplers, has been confirmed here (Table I). However, contrary to previous conclusions, it is thought that the stimulatory effect does not indicate that this pathway is coupled to phosphorylation. The uncouplers may only be facilitating the efflux of protons held inside the thylakoid membrane during the change in pK following the oxidation of reduced DCPIP (Neumann and Jagendorf, 1964). This idea is supported by the differential stimulatory effects on this electron flow by the anions acetate and succinate. There is no evidence available that acetate and succinate uncouple photophosphorylation. This can only mean that these anions are transporting the protons from inside the membrane to the outside medium, resulting in increased electron flow. It is therefore concluded that reduced DCPIP donates electrons at a site which by-passes the sites of phosphorylation on the non-cyclic electron flow pathway.

Electron flow was observed to be slow under acidic conditions, but increased as the pH was raised. This is interpreted to mean that protons held inside the thylakoid membrane are able to diffuse out only slowly when the concentration of protons on the

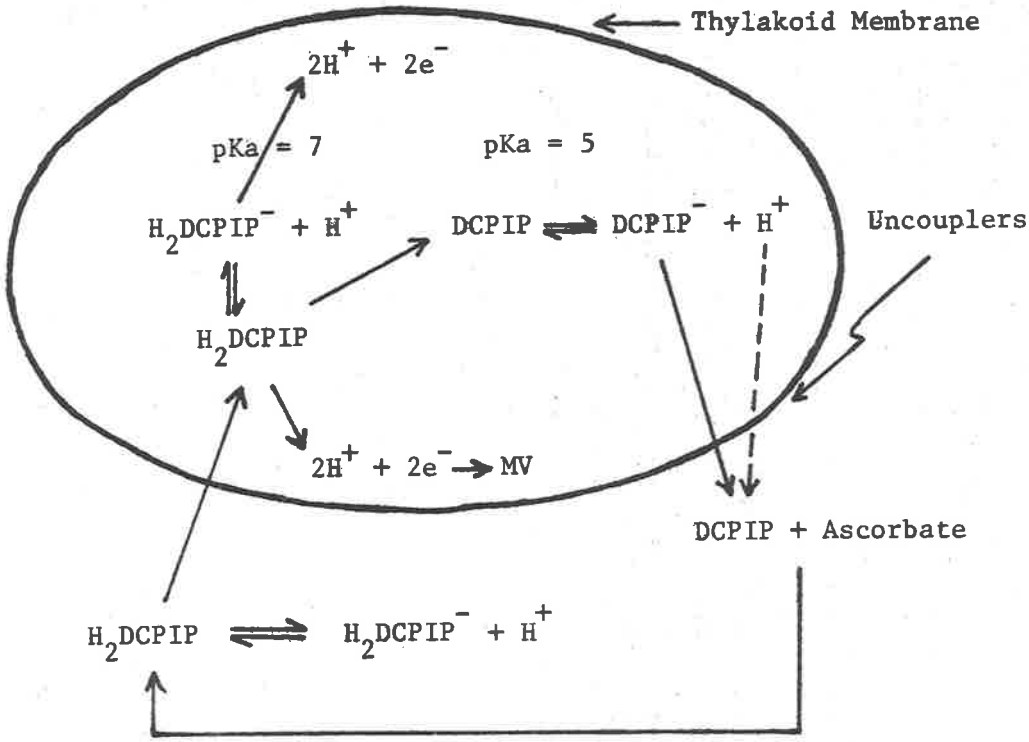


FIGURE V. Scheme for the oxidation of $DCPIPH_2$ by the photosynthetic electron flow chain and the possible action of uncouplers and anions on this electron flow.

outside is large (acidic conditions), but under more basic conditions the efflux of the protons is faster due to a lower proton concentration in the outside medium. The fact that proton carriers like DNP, CCCP and nigericin are more effective under basic conditions could be due to the greater proton gradient existing between the inside of the thylakoid membrane and the outside medium. The proton gradient between the inside of the membrane and the outside medium of pH6.0 would be small compared to that existing at an outside pH of 8.0. Fig.V represents a scheme to explain the effect of uncouplers and anions on the electron flow from $DCPIPH_2$ to MV.

CHAPTER IX

GENERAL DISCUSSION

$P/2e^-$ determinations with FeCN as electron acceptor have used mostly broken or swollen chloroplasts (Leech, 1963), which led Good, Izawa and Hind (1967) to suggest that electron flow in chloroplast preparations investigated to date have probably been significantly uncoupled. Hence, the low $P/2e^-$ ratios obtained (about 1.0). Recently, however, $P/2e^-$ (ADP/O) ratios higher than 1.0 have been obtained, suggesting that the true stoichiometry in non-cyclic electron flow is 2.0 (Izawa and Good, 1968; Horton and Hall, 1968 and West and Wiskich, 1968). These relatively high ratios (ADP/O) were obtained using Class I chloroplasts (Spencer and Unt, 1965) which exhibited photosynthetic control (West and Wiskich, 1968). More recently, Hall, Reeves and Baltscheffsky (1971) obtained even higher ADP/O ratios (1.4 - 2.1) using FeCN, MV and NADP as electron acceptors. The chloroplasts used by these authors, although they exhibited photosynthetic control were not Class I chloroplasts. Therefore, having an intact outer membrane need not be a pre-requisite for photosynthetic control in isolated chloroplasts. Thus improvement in the isolation technique, i.e. a very rapid separation of the chloroplasts from the cytoplasm, seems to be the reason for obtaining chloroplasts which are more "controlled" by their

energetic state. Results in Chapter III would support the idea that there are two sites of energy transduction and further indicate that the two sites are situated between the two photosystems. The difficulty that could arise when using FeCN as electron acceptor in determining $P/2e^-$ (ADP/O) ratios, because of the presence of a non-phosphorylating site of FeCN reduction, have also been pointed out. The presence of a non-phosphorylating site of FeCN reduction refutes the idea of the presence of a "basal" non-phosphorylating electron flow pathway which is parallel to a phosphorylating electron flow pathway as envisaged by Izawa and Good (1968). Using MV or NADP as electron acceptors would eliminate the presence of a non-phosphorylating electron flow observed in the case with FeCN.

Experiments of McCarty (1968 and 1969) with sonic SCP suggested that phosphorylation in these particles could be supported by the electrical gradient (ΔE) instead of the proton gradient (ΔpH). Digitonin SCP which had appreciable PSII activity but capable of cyclic photophosphorylation have also been reported (Nelson, Drechsler and Neumann, 1970). These particles did not show any light-induced proton uptake activity. The above authors suggested that a "high-energy" intermediate might be responsible for driving phosphorylation in these particles. Using essentially the same procedure as Anderson and Boardman (1966),

digitonin PSI subchloroplast particles were prepared and it was shown that they were capable of cyclic phosphorylation at rates comparable to whole chloroplasts, yet did not exhibit light-induced proton uptake activity. Effect of uncouplers and antibiotics (e.g. NH_4Cl and nigericin) which dissipate the proton gradient were not effective in inhibiting phosphorylation in these SCP. On the other hand, phosphorylation in these SCP was effectively inhibited by CCCP which affects both the proton gradient and the electrical gradient, thus suggesting that phosphorylation in these PSI subchloroplast particles could be supported by the electrical gradient.

The 515nm absorbance change in chloroplasts was thought to be an indicator of the electrical potential and related to phosphorylation (Junge and Witt, 1968). Results seem to suggest that there is a relationship between the absorbance change and phosphorylation in chloroplasts and PSI subchloroplast particles. Similar to phosphorylation in these SCP, proton gradient dissipating agents did not inhibit the 518nm absorbance change, whereas CCCP inhibited the absorbance change. The absorbance change in chloroplasts was similarly affected by these two types of agents, and in addition, phosphorylation itself also inhibited the absorbance change. However, more conclusive results might be forthcoming, if one were to use SCP with varying ability to phosphorylate. The extent of the 518nm absorbance change in

these different types of SCP would be expected to vary if a relationship between phosphorylation and the absorbance change really exists.

The participation of cytochromes in photosynthetic electron flow is well established (Bishop, 1971). Kinetic studies of cytochrome f oxidation clearly demonstrated that it functions between the two photosystems, and is situated close to PSI. Results in Chapter VII are in agreement with this idea. Cytochrome b_6 is thought to be involved in cyclic electron flow around PSI and its association with PSI particles has been shown (Boardman, 1970 and Chapter VII). However, the position of cytochrome b_{559} on the electron flow chain is still un-resolved (Ben Hayyim and Avron, 1970; Hiller, Anderson and Boardman, 1971; Wada and Arnon, 1971 and Cramer, Fan and Bohme, 1971). On the other hand, its association with PSII seems quite well established from studies with SCP (Boardman, 1970 and Vernon, Shaw, Ogawa and Raveed, 1971). Results in Chapter VII showed that cytochrome b_{559} could be reduced by PSII light, but the oxidation by PSI light was limiting. This may be due to the presence of phosphorylation sites after cytochrome b_{559} (see Chapter III). A similar suggestion has been forwarded by Hildreth (1968) and Hind (1968) to explain the accelerated cytochrome b_{559} oxidation by PSI light in the presence of uncouplers. The action of CCCP

on cytochrome b_{559} seems to be unique and may be due to the intrinsic properties of this uncoupler. Further studies with other uncouplers might help to resolve the situation.

Studies on the light-induced redox changes of cytochromes to date have used broken chloroplasts or conditions under which the chloroplasts would be swollen and partly uncoupled. However, the attempt to measure the redox changes of cytochromes in intact, tightly coupled chloroplasts was given up, due to the very small redox changes observed under conditions which would keep the chloroplasts intact. Therefore, it seems as if swollen or broken chloroplasts are needed to study the redox changes of cytochromes. Using such chloroplasts, the effect of phosphorylating agents on the redox states of cytochrome b_{559} have been reported, and gave conflicting results (Ben Hayyim and Avron, 1970 and Bohme and Cramer, 1972). Ben Hayyim and Avron (1970) found that these agents caused a more reduced steady-state of cytochrome b_{559}, indicating the presence of a phosphorylation site before cytochrome b_{559}. On the other hand, no indication of phosphorylation linked to either the reduction or oxidation of cytochrome b_{559} was observed by Bohme and Cramer (1972). Thus, the location of cytochrome b_{559} relative to the sites of phosphorylation on the electron flow chain still seems to be a matter of conjecture. However, results in Chapter III suggests that phosphorylation sites are situated between cytochrome b_{559} and PSI.

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