Two Sites for Adenosine Triphosphate Formation in Photosynthetic Electron Transport Mediated by Photosystem I. Evidence from Digitonin Subchloroplast Particles*

Joseph Neumann,† Charles J. Arntzen, and Richard A. Dilley

ABSTRACT: Two types of photophosphorylation, one supported by phenazine methosulfate and one by electron transport from reduced dichlorophenolindophenol ($DPIPH_2$) to methylviologen, have been studied in photosystem I digitonin subchloroplast particles. Phenazine methosulfate supported ATP formation is inhibited by a wide range of uncouplers, but it is resistant to the action of amines or nigericin. ATP formation supported by electron transport from $DPIPH_2$ to methylviologen is also inhibited by several uncouplers, including *m*-chlorocyanocarbonyl phenylhydrazone, citrate, and atebrin, but is *stimulated* by amines or nigericin at saturating light intensities. Electron transport from $DPIPH_2$ to methylviologen is stimulated by all the uncouplers tested including amines and nigericin. These results are explained on the basis of an hypothesis that electron transport from $DPIPH_2$ to methylviologen supports ATP formation at two sites (A and B) on the linear pathway between the two photosystems, each with different properties. Only one of these sites (site A) participates in ATP formation catalyzed by phenazine methosulfate.

In digitonin system I subchloroplast particles site B is inhibited by amines whereas site A is not. The net stimulation of ATP formation in the $DPIPH_2$ system by amines or nigericin is a result of an increased rate of electron transport through site A (which is not effected by amines or nigericin) due to a release by these compounds of a limiting step in electron flow at coupling site B. The use of a new inhibitor of photoreactions 2,3-dimethyl-5-hydroxy-6-phytolbenzoquinone provided evidence for two sites of oxidation of reduced dichlorophenolindophenol by the photosynthetic electron-transport chain, one preceding the ATP-forming sites and the other bypassing phosphorylation sites.

Electron transfer from H_2O to NADP and its accompanying noncyclic phosphorylation are known to be inhibited by DCMU[1] (Vernon and Avron, 1965). Ascorbate and DPIP can release the inhibition of NADP reduction by serving as an alternative hydrogen donor (Vernon and Zaugg, 1960) and this system supports ATP formation (Losada *et al.*, 1961). However, ascorbate and DPIP can support ATP formation even in the absence of an acceptor system (Trebst and Eck, 1961; Keister, 1963). In addition, it has been found that the ratio ATP/2e in a system of electron transport from $DPIPH_2$ to NADP can vary considerably and can be lower than 0.2 (Avron, 1964; Gromet-Elhanan, 1967). On the basis of these and other observations, several workers maintain that ATP formation in the presence of $DPIPH_2$ (whether an acceptor for electrons is present or absent) is of the cyclic type, with DPIP serving as a carrier, which is alternatively reduced and oxidized by the electron-transport chain, in an analogous way to the PMS cyclic phosphorylation. According to this formulation, the site of ATP formation is located on the "cyclic" part of the electron transport chain, between "X" and the linear pathway (Avron and Neumann, 1968).

However, it has been observed in several laboratories that noncyclic electron transport from $DPIPH_2$ to either NADP or MV is stimulated by various uncouplers (Keister, 1965; Trebst and Pistorius, 1967; Izawa *et al.*, 1967), indicating that this linear electron transport is coupled to an ATP-forming site. We reinvestigated this problem in digitonin photosystem I subchloroplast particles which are a simpler experimental system than chloroplasts. ATP formation in these and similar preparations has been studied before (Wessels, 1964; Arnon *et al.*, 1968; Anderson and McCarty, 1969; Nelson *et al.*, 1970). Our experiments support the concept that electron transport from $DPIPH_2$ to MV supports ATP formation at *two sites*, both of which are located on the linear-transport pathway. One of these sites is common to $DPIPH_2$ and PMS supported phosphorylation. The two sites have a different sensitivity to uncouplers and differ in other properties.

Materials and Methods

Chloroplasts were isolated from lettuce leaves, *Lactuca sativa* var. romaine. Leaves (100 g) were homogenized for 10 sec in a Waring blender in 130 ml of grinding media of 0.4 M sorbitol–0.1 M Tricine (pH 7.8)–0.07 M sodium ascorbate–2 mg/ml (final concentration) of bovine serum albumin. The homogenate was transferred through 12 layers of gauze and centrifuged for 7 min at 1000*g*. The pellet was resuspended in 10 ml of 0.4 M sorbitol–0.02 M Tricine (pH 8.0)–0.01 M KCl. The chlorophyll concentration was diluted to 2.3–2.7 mg/ml. NaCl at 1 M was added to the chloroplast suspension, to a final concentration of 0.1 M and a 4% digitonin solution was added dropwise to a final concentration of 0.55%. The suspension was incubated for 30–60 min at 0°, centrifuged for 30 min at 30,000*g*, and the precipitate discarded. The subchloro-

* Contribution No. 395 from the C. F. Kettering Research Laboratory, Yellow Springs, Ohio. *Received July 22, 1970.* This research supported in part by the National Science Foundation Grant No. G88432 to R. A. D.

† On leave from the Department of Botany, Tel-Aviv University, Tel-Aviv, Israel.

[1] Abbreviations used are: DPIP, 2,6-dichlorophenolindophenol; MV, methylviologen; DCMU, 3-(3,4-dichlorophenyl-1,1-dimethylurea); CCP, *m*-chlorocyanocarbonyl phenylhydrazone; PMS, phenazine methosulfate; DAD, diaminodurol (2,3,5,6-tetramethyl-*p*-phenylendiamine); SCP, subchloroplast particles; DMHPB, 2,3-dimethyl-5-hydroxy-6-phytolbenzoquinone; Tricine, *N*-tris(hydroxymethyl)methylglycine.

TABLE I: Effect of Uncouplers on Electron Flow from $DPIPH_2$ to MV and the Accompanying ATP Formation.[a]

Addition	Electron Flow (% of Control)	ATP Formation (% of Control)
None	100	100
Gramicidin D 0.2 μM	171	6
CCP 13 μM	152	17
Atebrin 17 μM	310	24
Triton-X-100 0.01%	163	0
Citrate 80 mM	126	44
NH_4Cl 5 mM	280	219
Methylamine 15 mM	287	238
Nigericin 0.17 μM	208	294
Valinomycin 0.5 μM	98	58
Valinomycin 0.5 μM + NH_4Cl 5 mM	362	21

[a] Values for the controls: O_2 uptake of 1160 μmoles per mg of chlorophyll per hr; ATP formation, 48 μmoles/mg of chlorophyll per hr. The reaction mixture contained the following in μmoles per total volume of 1.5 ml: sodium Tricine 25, (pH 8.0), NaCl 25, $MgCl_2$ 5, NaP_i 5, ADP 2, DPIP 0.4, ascorbate 2.5, MV 1.25, NaN_3 1.25, DCMU 0.03. In the nigericin reactions, NaCl was replaced by KCl. In addition, each reaction contained ^{32}P (10^6 cpm) and SCP equivalent to 11.5 μg of chlorophyll. The samples were illuminated for 1 min with white light which had been filtered through a yellow filter (Corning 3-68) and a solution of $Cu(NO_3)_2$. The light intensity at the surface of the vessel was 6×10^5 ergs cm^{-2} sec^{-1}. Electron flow was assessed by measuring oxygen uptake. ATP formation was measured by ^{32}P incorporation.

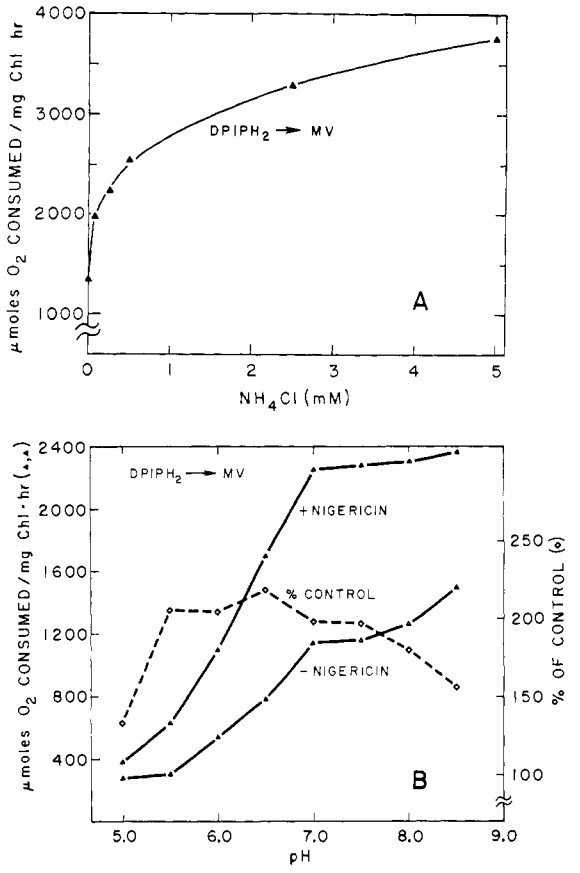

FIGURE 1: Effect of NH_4Cl and nigericin on electron transport from $DPIPH_2$ to MV. (A) Effect of NH_4Cl. The reaction mixture contained the following in μmoles in a total volume of 1.5 ml: sodium tricine, 25 (pH 8.0); DPIP, 0.4; ascorbate, 2.5; MV, 1.25; NaN_3, 1.25; DCMU, 0.03. In addition it contained SCP equivalent to 10 μg of chlorophyll. Otherwise experimental conditions as in Table I. (B) Effect of nigericin. Reaction mixture as in A, except for the replacement of NaCl and Tricine with 3 μmoles of KCl and a mixture of 50 μmoles of Tricine and 50 μmoles of maleic acid at the specified pH. SCP equivalent to 5 μg of chlorophyll was added. Otherwise experimental conditions as in Table I.

plast particles (SCP) were precipitated with protamine sulfate as described previously (Nelson *et al.*, 1970) or by centrifugation for 60 min at 90,000*g*. The particles were resuspended in 15 ml of 0.4 M sorbitol–0.02 M Tricine (pH 7.8)–0.01 M KCl–5 mg/ml of bovine serum albumin (final concentration). The chlorophyll concentration obtained was approximately 0.25 mg/ml. The particles were frozen in liquid nitrogen and kept at −70° with no loss of activity for at least 2 months.

Chlorophyll concentration was measured according to Arnon (1949). ATP formation was measured according to Nielsen and Lehninger (1955) as modified by Avron (1960). Oxygen uptake was measured by a Gilson KM-C oxygraph with a YSI Clark electrode in a temperature-controlled reaction vessel at 20°. Changes in pH were measured with Leeds and Northrup Model 124138 microelectrodes. A yellow Corning filter (3-68) was placed between the light source and the sample, to eliminate artifactual responses in both the O_2 and pH measurements. Light intensity was measured by a Kettering Yellow Springs Instruments radiometer, Model 65.

DPIP was purified according to the procedure of Savage (1957) as modified and brought to our attention by Dr. R. Cammack of Cambridge University. DAD was a generous gift from Dr. A. Trebst from the University of Gottingen. All other chemicals were of analytical grade.

A plastoquinone analog, 2,3-dimethyl-5-hydroxy-6-phytolbenzoquinone (DMHPB) was kindly provided by Dr. Karl Folkers, for studies on its inhibition of ATP formation.

Results

Uncouplers are known to stimulate electron flow while inhibiting ATP formation. Izawa *et al.* (1967) have studied in detail the reaction $DPIPH_2$ → MV and its accompanying phosphorylation. They have observed a marked stimulation of electron flow from $DPIPH_2$ to MV which is inconsistent with a low ATP/2e in this system (with no addition of uncouplers). We studied the effect of uncouplers on this reaction in digitonin SCP which is a simpler experimental system than chloroplasts. As shown in Table I, uncouplers also stimulate electron transport from $DPIPH_2$ to MV in the digitonin SCP. Interestingly, NH_4Cl and nigericin, which were shown not to inhibit PMS-supported ATP formation in SCP (Nelson *et al.*, 1970), did stimulate electron transport from $DPIPH_2$ to MV. In light of this discrepancy, the effect of these two compounds was studied in further detail. (The effect of uncouplers on ATP formation, as shown in Table I, will be discussed in a later section.) The extent of stimulation of electron flow as a function of NH_4Cl concentration is shown in Figure 1A. NH_4Cl (5 mM) increases the O_2 uptake from 1340 μmoles per mg of chlorophyll per hr to 3720. The combination of nigericin and K^+ causes the largest stimulation in electron transport

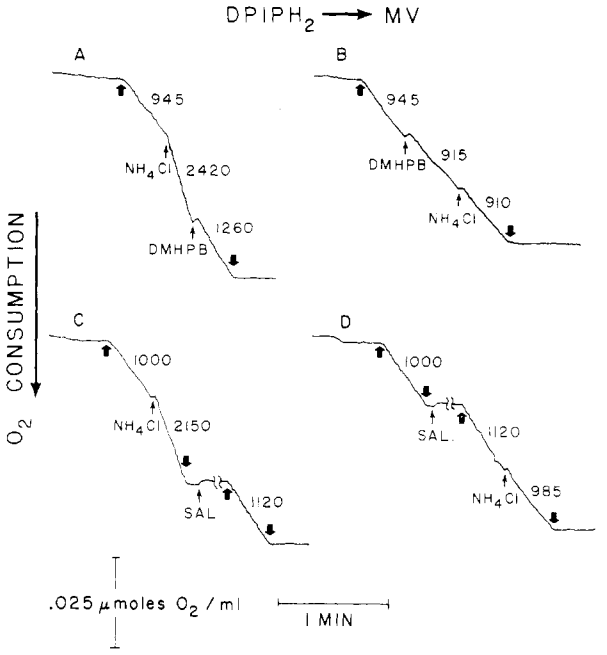

FIGURE 2: Effect of DMHPB and salicyaldoxime on electron flow from $DPIPH_2$ to MV. The reaction mixture was identical with that of Figure 1A, except that 50 μmoles of Tricine were added and SCP, equivalent to 5 μg of chlorophyll. DMHPB was added at 0.46 mM, NH_4Cl at 5 mM, and salicylaldoxime at 33 mM, where indicated. Other experimental conditions are as in Table I.

near pH 6.5 (Figure 1B). This result is consistent with the hypothesis that nigericin plus K^+ uncouples electron flow by dissipating the proton gradient (Shavit *et al.*, 1968), since the latter has a similar pH optimum (Neumann and Jagendorf, 1964). Stimulation of electron transport from $DPIPH_2$ to MV by amines or nigericin and lack of effect of these compounds on PMS phosphorylation (in digitonin SCP) indicated the

ELECTRON DONATION SITE	DONORS OXIDIZED
1	$DPIPH_2$
1a	PMSH
2	$DPIPH_2$

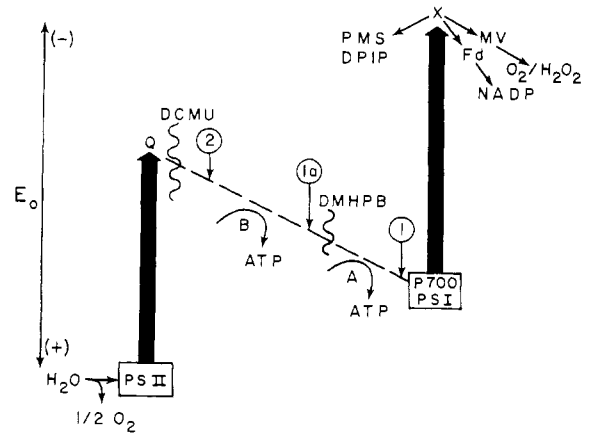

FIGURE 3: A scheme of photosynthetic electron transport and ATP formation.

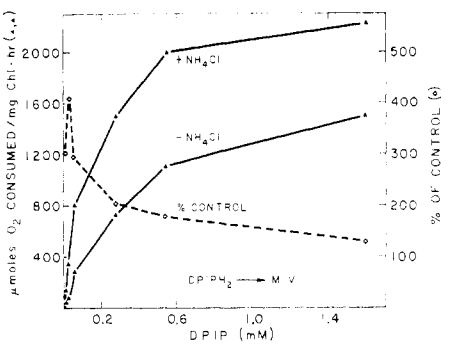

FIGURE 4: Effect of NH_4Cl on electron flow from $DPIPH_2$ to MV at various concentrations of $DPIPH_2$. The reaction mixture was identical with that of Figure 1A, except for DPIP which was added as indicated. NH_4Cl was added at 5 mM. Otherwise experimental conditions are as in Table I.

existence of two coupling sites, for which more evidence will be presented below.

It is very likely that $DPIPH_2$ can be oxidized at a site in the photosynthetic electron transport chain, which bypasses ATP formation (Avron and Neumann, 1968). However, the effect of uncouplers in stimulating electron transport from $DPIPH_2$ to MV would support the concept that $DPIPH_2$ *may also* be oxidized at a site which precedes a coupling site. Further evidence to support the idea of two sites for $DPIPH_2$ oxidation is provided by the use of a new inhibitor of electron transport, the quinone analog 2,3-dimethyl-5-hydroxy-6-phytolbenzoquinone (DMHPB). This compound has recently been synthesized by K. Folkers *et al.* (unpublished results, 1970). This inhibitor has no effect on basal electron transport from $DPIPH_2$ to MV but it inhibits markedly the uncoupled electron transport in the presence of NH_4Cl (Figure 2A) and correspondingly in the presence of the inhibitor, NH_4Cl does not stimulate electron transport (2B). Salicylaldoxime has similar properties (Figure 2C,D). The inhibition of uncoupled electron transport but lack of effect on basal electron flow from $DPIPH_2$ to MV was also found in the presence of other uncouplers (Arntzen *et al.*, 1971). A possibility that stimulation of electron flow from $DPIPH_2$ to MV is not caused by uncoupling, but perhaps by increasing the permeation of $DPIPH_2$ at its site of oxidation is inconsistent with the observation that inhibition of electron transport occurred only in the presence of uncouplers. In chloroplasts (Arntzen *et al.*, 1971), the inhibitor blocked electron flow from H_2O to MV to the same extent in the presence or absence of NH_4Cl. In addition, phosphorylation catalyzed by linear electron flow from H_2O to MV, and phosphorylation catalyzed by $DPIPH_2$ and MV, or by PMS were all inhibited to approximately the same extent with varying amounts of DMHPB. These results may be interpreted as is shown schematically in Figure 3. DMHPB, which interacts with a component in the linear electron transport chain, does not effect electron donation by $DPIPH_2$ at site 1. Since very low ATP/2e values are observed with normal electron flow from $DPIPH_2$ to MV, it can be assumed that most electrons enter the chain at site 1. Electron donation by $DPIPH_2$ at site 2, which supports ATP formation, is very low in coupled plastids but rapid in the presence of an uncoupler, and it is inhibited by DMHPB. Since the inhibitor also blocks PMS phosphorylation, its point of action would be after the site of electron donation by PMSH.

Honeycutt and Krogmann (1970) have recently presented evidence for two sites for $DPIPH_2$ oxidation in *Anabaena*

TABLE II: Effect of NH_4Cl on Two Types of ATP Formation in Various Fractions.[a]

	ATP Formation (μmoles/mg of Chlorophyll per hr)			
	$DPIPH_2 \rightarrow MV$		PMS	
1 Fraction	Control	$+NH_4Cl$	Control	$+NH_4Cl$
30,000–50,000*g*	95	220	440	400
50,000–90,000*g*	91	294	587	582
90,000–140,000*g*	215	657	560	
All combined (protamine sulfate)	85	102	535	535
2 Concn of Chlorophyll during Digitonin Incubation (mg/ml)				
2.0	45	175	619	670
0.8	30	127	392	417
0.3	25	121	368	392

[a] In experiment 1, the chlorophyll concentration during digitonin incubation was 2.25 mg/ml. In experiment 2, the SCP were precipated by centrifugation for 1 hr at 90,000*g* (under the conditions of this experiment no chlorophyll remained in the supernatant). The reaction mixture for PMS phosphorylation contained the following in μmoles in a total volume of 3.0 ml: sodium Tricine (pH 8.0) 50, NaCl 50, $MgCl_2$ 10, NaP_i 10, ADP 4, PMS 0.15, sodium ascorbate 20, and DCMU 0.06. In addition it contained ^{32}P (10^6 cpm) and SCP equivalent to 10–20 μg of chlorophyll. Illumination for 2 min was provided by white light at an incident light intensity of 5×10^5 ergs cm^{-2} sec^{-1}. The reaction mixture for DPIP phosphorylation was described in Table I, except for the conditions of illumination which were the same as that for PMS phosphorylation. NH_4Cl when added was at 5 mM.

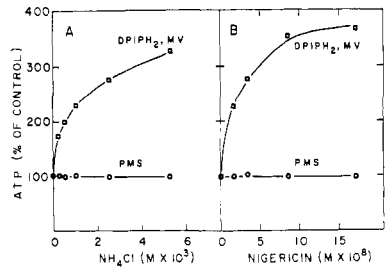

FIGURE 5: Effect of NH_4Cl and nigericin on ATP formation in the presence of $DPIPH_2$ and MV, or PMS. (A) Effect of NH_4Cl. The reaction mixture for the $DPIPH_2$ to MV reaction was as in Table I. The reaction for PMS phosphorylation, and the conditions of illumination were as described in Table II. SCP equivalent to 18 μg of chlorophyll was added. The control rates for DPIP and PMS phosphorylation were 133 and 753 μmoles per mg of chlorophyll per hr, respectively. (B) Effect of nigericin. The reaction mixture was as in A, except that 50 μmoles of KCl was present instead of NaCl and SCP equivalent to 11 μg of chlorophyll was added. The control rates for DPIP and PMS phosphorylation were 52 and 448 μmoles per mg of chlorophyll per hr, respectively.

variabilis membrane fragments, with different affinities for the dye. To establish optimal conditions for electron donation to site 2 under our experimental conditions, a comparison of the stimulatory effect of NH_4Cl on electron transport (O_2 uptake with MV) was made over a range of $DPIPH_2$ concentrations. The stimulated rate (donation at both sites 1 and 2) as compared to the basal electron flow (donation primarily at site 1) should give an indication of the affinities of the two sites for $DPIPH_2$. As can be seen in Figure 4, the highest stimulation is obtained at a low $DPIPH_2$ concentration (25 μM). The rate of electron flow at the maximum *relative* stimulation is 340 μmoles of O_2 uptake/mg of chlorophyll per hr, which is only 15% of the maximal rate obtained at the highest $DPIPH_2$ concentration tested, therefore the decrease in the relative stimulation at $DPIPH_2$ concentration above 25 μM is not the result of an inherent limited capacity to transport electrons. The maximal stimulation at low $DPIPH_2$ indicates that site 2 has a higher affinity for the reduced dye than site 1.

In light of the fact that PMS phosphorylation in digitonin SCP is insensitive to NH_4Cl (Nelson *et al.*, 1970), whereas this compound stimulated electron flow from $DPIPH_2$ to MV, it was of interest to test the effect of NH_4Cl on ATP formation accompanying the latter reaction. Surprisingly, as seen in Figure 5A, while NH_4Cl indeed had no effect on PMS phosphorylation it stimulated markedly the phosphorylation which takes place in the presence of DPIP + ascorbate and MV. NH_4Cl (5 mM) increased the rate of ATP formation from 133 (μmoles/mg of chlorophyll per hr) to 432. The same pattern of stimulation of ATP formation in the presence of $DPIPH_2$ and MV was seen over a range of nigericin concentrations (Figure 5B). To exclude the possibility that PMS phosphorylation and $DPIPH_2$ phosphorylation are catalyzed by various populations of particles, the digitonin subchloroplast particles were separated into several fractions by differential centrifugation. As can be seen in the results of Table II, essentially the same pattern was obtained in the different fractions: NH_4Cl did not affect PMS phosphorylation but stimulated markedly $DPIPH_2$ phosphorylation. The same pattern has also been obtained when the ratio of chlorophyll to digitonin in the incubation mixture was changed over a wide range; a procedure which affects the extent of separation between the two photosystems (unpublished results).

The stimulation of ATP formation in the presence of DPIP plus ascorbate and MV by NH_4Cl is a property of digitonin SCP only. In chloroplasts, in a typical experiment, the following per cent of inhibition was obtained by 5 mM NH_4Cl, for the various reaction systems: $H_2O \rightarrow MV$, 87; $DPIPH_2 \rightarrow MV$ (in the presence of DCMU), 85; PMS, 64; and $H_2O \rightarrow$ Fe-CN, 94. It should also be noted that among a wide range of uncouplers tested with the SCP, only amines and nigericin stimulated ATP formation in the $DPIPH_2$ to MV system whereas all the other uncouplers were inhibitory (Figure 5A,B, Table I).

To account for the data shown so far and for those to be presented below we suggest the following hypothesis (Figure 3): (a) two sites for ATP formation on the linear pathway between the DCMU-sensitive step and P_{700}; (b) multiple sites of donation of electrons to the electron-transport chain. PMSH is oxidized by a carrier whose location precedes coupling site A, but $DPIPH_2$ whenever it supports phosphorylation is oxidized at a locus preceding coupling site B. In addition, $DPIPH_2$ can be oxidized at a site proximal to P_{700}, bypassing both coupling sites; (c) in the absence of uncoupler, oxidation of $DPIPH_2$ at site 2 is slow and limited by the coupling step

TABLE III: The Light-Induced Proton Shift in Chloroplasts and SCP.

	PMS-Mediated Proton Shift		% Reduction by Detergent Treatment SCP/ Chloroplast	$DPIPH_2$, MV Mediated Proton Shift		% Reduction by Detergent Treatment SCP/ Chloroplast
	Chloroplast	SCP		Chloroplast	SCP	
Rate (μmoles/mg of chlorophyll per hr)	540	26	5	230	53	23
Extent (μmoles/mg of chlorophyll)	1.13	0.15	13	0.42	0.13	31

[a] Chloroplasts were isolated as described in Methods. They were once washed in sorbitol 0.4 M–Tricine 0.02 M (pH 7.8)–KCl 0.01 M, and resuspended in the SCP resuspension medium. The reaction mixture contained, in 3 ml, 50 μmoles of KCl, 2 μmoles of dithioerythritol, and 0.03 μmole of DCMU. It also contained either 0.15 μmole of PMS or 0.8 μmole of DPIP plus 2.5 μmoles of MV plus 2.5 μmoles of NaN_3. The initial pH of the reaction was brought to pH 6.0. Illumination was provided by white light transferred through a yellow filter (Corning No. 3-69) providing an intensity of 4×10^5 ergs cm^{-2} sec^{-1}.

at site B; and (d) in digitonin-SCP, ATP formation at site A only (PMS phosphorylation), is resistant to uncouplers which dissipate the proton gradient (amines and nigericin plus K). The last two assumptions can explain the stimulation of ATP formation by amines or nigericin in the $DPIPH_2$ system. Under conditions (high light and presence of acceptor) where the limiting step in this reaction is at coupling site B, NH_4Cl will inhibit ATP formation at site B, but allow faster electron transport through the remaining electron-transport chain, including site A which is coupled to ATP formation and not affected by NH_4Cl. If the rate of electron flow through this site is more than doubled (by NH_4Cl or nigericin) the net effect would be an increased rate of ATP formation in comparison with the control (Figure 5 and Table I). According to the scheme in Figure 3, any uncoupler which inhibits ATP formation in digitonin SCP at site A (PMS phosphorylation) like CCP (Nelson *et al.*, 1970) or NH_4Cl plus valinomycin (Hauska *et al.*, 1970a; Neumann *et al.*, 1970) should inhibit ATP formation supported by electron transport from $DPIPH_2$ to MV. However, these compounds may also inhibit ATP formation directly at site B. Thus, of the two coupling sites for ATP synthesis supported by electron flow from $DPIPH_2$ to MV, site B is affected by proton dissipating agents (NH_4Cl and nigericin) whereas site A *in digitonin SCP* has undergone a change which makes it resistant to these agents. More direct evidence for this hypothesis is presented in the results of Table III. These results show that the proton gradient supported by PMS decreased more in SCP (in comparison to chloroplast) than the proton gradient supported by electron flow from $DPIPH_2$ to MV, although the latter is also significantly decreased.

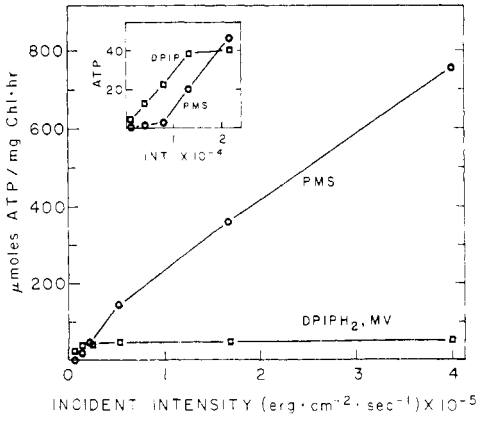

FIGURE 6: Effect of light intensity on ATP formation in the presence of $DPIPH_2$ and MV or PMS. The reaction mixtures for DPIP and PMS phosphorylation were as described in Tables I and II, respectively. Illumination was for 2 min with white light at the specified intensity. SCP equivalent to 25 μg of chlorophyll was added.

Consistent with the scheme presented in Figure 3 are the results showing the different effect of light intensity on ATP formation in the presence of PMS as compared to that in the presence of $DPIPH_2$ and MV (Figure 6). At an incident light intensity of about 2×10^4 erg cm^{-2} sec^{-1} the rate of the reaction with $DPIPH_2$ reached saturation and was constant, whereas the rate of PMS phosphorylation steadily increased at all intensities tested. On the other hand, at low light intensities (up to 1.6×10^4 ergs cm^{-2} sec^{-1}) the rate of $DPIPH_2$ phosphorylation was significantly higher than that with PMS. Actually at several points at the low light intensities the rate of ATP formation with $DPIPH_2$ was twice that with PMS, as would be expected if $DPIPH_2$ is oxidized at a site preceding two coupling sites.

Simultaneous measurements of electron flow and ATP formation in the presence of $DPIPH_2$ and MV are presented in Figure 7. Although the ratio ATP/2e is low presumably because a large portion of the $DPIPH_2$ is oxidized at site 1 and bypasses both phosphorylation sites, NH_4Cl over a wide range of light intensities (between 6×10^4 and 6×10^5 ergs cm^{-2} sec^{-1}) stimulates both electron transport and ATP formation approximately to the same extent. Such parallel stimulation is inconsistent with the hypothesis that the site of ATP formation in the presence of $DPIPH_2$ is located on the cyclic pathway, *i.e.*, that portion of the chain which links the reductant "X" with an electron carrier which is located between the two photosystems (Gromet-Elhanan, 1967). If that would have been the case, stimulation of electron flow from $DPIPH_2$ to MV along the linear pathway should have inhibited ATP formation and *vice versa*.

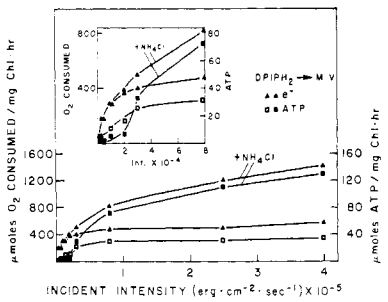

FIGURE 7: Effect of light intensity on simultaneous electron transport from $DPIPH_2$ to MV and ATP formation. Reaction conditions as in Table I. SCP equivalent to 17 μg of chlorophyll was added. NH_4Cl at 5 mM was added where indicated.

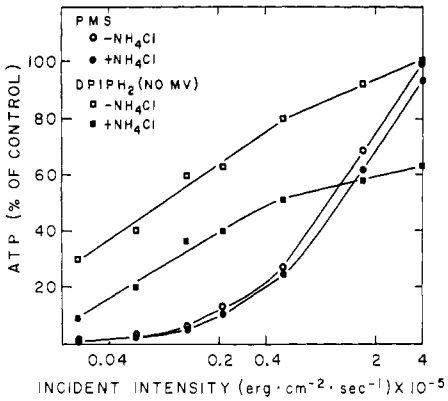

FIGURE 8: Effect of light intensity on ATP formation in the presence of $DPIPH_2$ or PMS. The reaction mixtures for DPIP and PMS phosphorylation was as described in Tables I and II, respectively, except that in the DPIP reaction mixture, *no* MV and no NaN_3 were present. Illumination was for 2 min with white light at the specified intensity. In the DPIP experiment SCP equivalent to 25 μg of chlorophyll was added and in the PMS experiment SCP equivalent to 17 μg of chlorophyll was added. NH_4Cl at 5 mM was added where indicated. The rates for DPIP and PMS phosphorylation at maximal light intensity (4×10^5 ergs cm^{-2} sec^{-1}) were 34 and 463 μmoles per mg of chlorophyll per hr, respectively.

It should be noted that at low light intensities NH_4Cl did not stimulate electron transport (inset of Figure 7), presumably because the rate-limiting step has been shifted from coupling site B to another locus along the e^- transport chain (turnover of P_{700}?). Under these conditions, NH_4Cl *inhibited* ATP formation in the DPIP system (inset of Figure 7). This is consistent with the formulation presented in Figure 3 and with the assumption that site B (unlike site A) is sensitive to amine uncoupling. Another means of causing a rate limitation on electron transport is the deletion of MV from the reaction mixture (Figure 8). In the absence of MV, NH_4Cl caused an inhibition of ATP formation at all light intensities. As expected, the observed maximal inhibition was approximately 50%, indicating that amine did not effect phosphorylation at site A. It should be emphasized that PMS phosphorylation was not affected by NH_4Cl at any light intensity (Figure 8). It is of interest that unlike "XH" in chloroplasts, in digitonin SCP "XH" can be oxidized at a relatively high rate by oxygen with no addition of MV, resembling membrane fragments from *Anabaena variabilis* (Honeycutt and Krogmann, 1970).

The effect of temperature on the two types of photophosphorylation is shown in Figure 9. Raising the temperature from 10 to 30° increases the rate of ATP formation in the $DPIPH_2$ reaction by 120% whereas the PMS phosphorylation is only increased by 32%. NH_4Cl, which had a marginal effect on the PMS phosphorylation throughout the whole temperature range measured, stimulated the $DPIPH_2$ phosphorylation, especially at the higher temperature. This is consistent with the idea that electron flow from $DPIPH_2$ to MV is using more electron carriers than PMS, and that a step in the vicinity of $DPIPH_2$ oxidation has a higher temperature dependency.

Discussion

The working hypothesis we suggest to explain our results and other documented observations is summarized in Figure 3. According to this scheme there are two coupling sites for ATP formation between the DCMU sensitive carrier and P_{700}. Several workers reported ATP/2e ratios greater than 1 (Winget *et al.*, 1965; Horton and Hall, 1968; West and Wiskich, 1968; Forti, 1968; but see Del Campo *et al.*, 1968). We have performed several measurements of ATP/2e ratio in chloroplasts, where H_2O served as the electron donor and MV as the electron acceptor and found values between 1.2 and 1.3, (unpublished results), which would indicate the existence of two coupling sites. In the system, $DPIPH_2 \rightarrow MV$, the ATP/2e ratio is meaningless if ATP formation is supported either by a cyclic electron flow (Kok, 1963) or if there are multiple sites of electron donation, some of which bypass the coupling sites.

It is well documented that several compounds including $DPIPH_2$ can donate electrons to a carrier located between the two photosystems and the oxidized compound can be subsequently reduced by a system I reaction, thus giving rise to cyclic electron flow, as shown schematically in Figure 3. The question unresolved at the present is whether the reduction of exogenous compounds like DPIP is *required* for ATP formation or whether it can be replaced by any electron acceptor (oxygen, methylviologen and oxygen, ferredoxin and NADP, etc.). According to the proponents of the hypothesis that ATP formation with DPIP is cyclic, the role of DPIP is to bridge a gap between two electron carriers which are located between X and the electron-transport chain, and the site of ATP formation is located on this chain (Gromet-Elhanan, 1967, 1968; however, see Trebst and Pistorius, 1969).

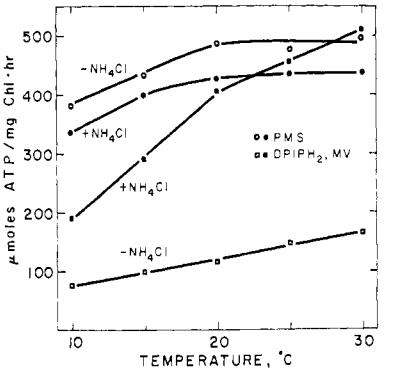

FIGURE 9: Effect of temperature on ATP formation in the presence of $DPIPH_2$ and MV or PMS. The reaction mixtures for DPIP and PMS phosphorylation as described in Tables I and II, respectively. Illumination was for 2 min with white light. SCP equivalent to 18 μg of chlorophyll was added. NH_4Cl at 5 mM was added where specified. The reaction mixture was equilibrated for 2 min prior to illumination at the specified temperature.

An alternative view is that ATP is formed on the linear pathway and the type of acceptor which oxidizes reduced X (whether DPIP, ferredoxin, and NADP, etc.) will affect ATP formation *only* by affecting the rate of electron flow. In view of the low ATP/2e in the presence of $DPIPH_2$ and MV, this formulation would require the operation of two sites for $DPIPH_2$ oxidation. The latter had been supported by several workers (Vernon and Avron, 1965; Trebst and Pistorius, 1965; Schwartz, 1966; Yamashita and Butler, 1968; Honeycutt and Krogmann, 1970). Oxidation of $DPIPH_2$ by a carrier on the linear pathway, and preceding a phosphorylation site, is supported by the observation that $DPIPH_2$ oxidation is stimulated by uncouplers and by the addition of phosphorylating reagents (Keister, 1965; Trebst and Pistorius, 1967; Izawa *et al.*, 1967, and also the present work, Table I and Figures 1 and 4). This hypothesis is also supported by the action of inhibitors or treatments which inhibit to the same extent noncyclic electron transport (from H_2O to an acceptor) and $DPIPH_2$ supported phosphorylation, without inhibiting to a large extent $DPIPH_2$ oxidation (Trebst *et al.*, 1963; Jones and Kok, 1966; Neumann and Dreschler, 1967; Arntzen *et al.*, 1970).

It is at present unclear whether enough potential energy is available between the DCMU-sensitive site and P_{700} to support the formation of two molecules of ATP. In an alternative formulation, site A can be placed on the cyclic pathway (between X and the electron-transport chain). However, such a formulation would be inconsistent with the parallel increase in electron transport and ATP formation in SCP, caused by NH_4Cl, at high light intensities (Figure 7). It is also inconsistent with our findings that addition of MV, at high light intensity, markedly stimulates ATP formation (compare Figures 7 and 8). It is unlikely that the effect of MV is due to the prevention of overreduction of DPIP as has been found under an atmosphere of argon (Gromet-Elhanan, 1967) since we did not find an effect of MV on ATP formation at an incident light intensity below 10^4 ergs cm^{-2} sec^{-1} where overreduction should have been more severe. A location of one of the two ATP forming sites on the cyclic pathway is also inconsistent with the same extent of inhibition of noncyclic electron flow and PMS and $DPIPH_2$ supported phosphorylation by various electron-transport inhibitors.

After this work was completed, a paper by Hauska *et al.* (1970b) was published describing phosphorylation studies with digitonin system I particles prepared from spinach. In this paper a scheme was suggested in which the carrier diaminodurol (DAD) served both as a carrier on the cyclic pathway and as a carrier of a noncyclic pathway, in which DAD reduced cytochrome b_6. Both pathways share the same site for ATP formation, and the noncyclic pathway supported a very low ATP/2e (0.1) for reasons which are not elaborated. The reduction of cytochrome b_6 by DAD is inconsistent with the published oxidation reduction potentials (+0.3 V for DAD, 0.0 or −0.18 for cytochrome b_6) for these compounds (Michaelis *et al.*, 1939; Hill, 1954; Hill and Bendall, 1967; Fan and Cramer, 1970). From the work of Hauska *et al.* (1970a,b) and our own experiments, we would suggest that DAD can donate electrons both at site 1a supporting high rates of ATP formation and at site 1, where it by passes the sites of ATP formation. With DAD phosphorylation we obtained only marginal effects with NH_4Cl.

The mode of action of digitonin in reducing the H^+ pump catalyzed by PMS and the related insensitivity of ATP formation to some of the proton-dissipating agents (amines or nigericin) in this system is at the present not understood. The link between proton pump and ATP formation can be disrupted also by sonication (Dreschler *et al.*, 1968; McCarty, 1968) and recently we were able to prepare particles with similar characteristics by the use of the French press. It is possible that even in chloroplasts the proton pump is primarily site specific (at site B) and only indirectly linked to site A, although so far there is no experimental evidence for this notion. It is of some interest to reiterate the observation of Fan and Cramer (1971) that the carriers localized at the terminal end of system I, *i.e.*, plastocyanin, cytochrome *f*, P_{700}, and cytochrome b_6 are electron carriers, therefore making unlikely their participation in hydrogen ion transfer.

Acknowledgment

The skillful and conscientious technical assistance of Mrs. Joan Dybas Schneiders is highly appreciated. We would like to thank Dr. D. L. Keister for critically reading this manuscript.

References

Anderson, M. M., and McCarty, R. E. (1969), *Biochim. Biophys. Acta 189*, 193.

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

Arnon, D. I., Tsujimoto, H. Y., McSwain, B. D., and Chain, R. K. (1968), *in* Comparative Biochemistry and Biophysics of Photosynthesis, K. Shibata, A. Takamiya, A. T. Jagendorf, and R. C. Fuller, Ed., Tokyo, University of Tokyo Press, p 113.

Arntzen, C. J., Neumann, J., and Dilley, R. A. (1971), *J. Bioenerg. 1* (in press).

Avron, M. (1960), *Biochim. Biophys. Acta 40*, 257.

Avron, M. (1964), *Biochem. Biophys. Res. Commun. 17*, 430.

Avron, M., and Neumann, J. (1968), *Annu. Rev. Plant Physiol. 19*, 137.

Del Campo, F. F., Ramirez, J. M., and Arnon, D. I. (1968), *J. Biol. Chem. 243*, 2805.

Drechsler, Z., Neumann, J., and Ben-Shaul, Y. (1968), *Isr. J. Chem. 6*, 130.

Fan, H. N., and Cramer, W. A. (1971), *Biochim. Biophys. Acta* (in press).

Forti, G. (1968), *Biochem. Biophys. Res. Commun. 32*, 1020.

Gromet-Elhanan, Z. (1967), *Biochim. Biophys. Acta 131*, 526.

Gromet-Elhanan, Z. (1968), *Arch. Biochem. Biophys. 123*, 447.

Hauska, G. A., McCarty, R. E., and Olson, J. S. (1970a), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 151.

Hauska, G. A., McCarty, R. E., and Racker, E. (1970b), *Biochim. Biophys. Acta 197*, 206.

Hill, R. (1954), *Nature (London) 174*, 501.

Hill, R., and Bendall, D. S. (1967), *in* Biochemistry of Chloroplasts, T. W. Goodwin, Ed., London, Academic, Vol. II, p 559.

Honeycutt, R. C., and Krogmann, D. W. (1970), *Biochim. Biophys. Acta 197*, 267.

Horton, A. A., and Hall, D. O. (1968), *Nature (London) 218*, 386.

Izawa, S., Connolly, T. N., Winget, G. D., and Good, N. E. (1967), *Brookhaven Symp. Biol. 19*, 169.

Jones, L. W., and Kok, B. (1966), *Plant Physiol. 41*, 1044.

Keister, D. L. (1963), *J. Biol. Chem. 238*, PC2590.

Keister, D. L. (1965), *J. Biol. Chem. 240*, 2673.

Kok, B. (1963), *in* Photosynthetic Mechanisms of Green Plants, B. Kok and A. T. Jagendorf, Ed., Washington,

D. C., National Academy of Sciences, National Research Council, p 35.
Losada, M., Whatley, F. R., and Arnon, D. I. (1961), *Nature (London) 190*, 606.
McCarty, R. E. (1968), *Biochem. Biophys. Res. Commun. 32*, 37.
Michaelis, L., Schubert, M. P., and Granick, S. (1939), *J. Amer. Chem. Soc. 61*, 1981.
Nelson, N., Drechsler, Z., and Neumann, J. (1970), *J. Biol. Chem. 245*, 143.
Neumann, J., and Drechsler, Z. (1967), *Plant Physiol. 42*, 473.
Neumann, J., and Jagendorf, A. T. (1964), *Arch. Biochem. Biophys. 107*, 109.
Neumann, J., Ke, B., and Dilley, R. A. (1970), *Plant Physiol.*, in press.
Nielsen, S. O., and Lehninger, A. L. (1955), *J. Biol. Chem. 215*, 555.
Savage, N. (1957), *Biochem. J. 67*, 146.
Schwartz, M. (1966), *Biochim. Biophys. Acta 112*, 204.
Shavit, N., Dilley, R. A., and San Pietro, A. (1968), *Biochemistry 7*, 2356.
Trebst, A., and Eck, H. (1961), *Z. Naturforsch. 16*, 455.
Trebst, A., Eck, H., and Wagner, S. (1963), *in* Photosynthetic Mechanism of Green Plants, B. Kok and A. T. Jagendorf, Ed., Washington, D. C., National Academy of Sciences, National Research Council, p 174.
Trebst, A., and Pistorius, E. (1965), *Z. Naturforsch. 20B*, 143.
Trebst, A., and Pistorius, E. (1967), *Biochim. Biophys. Acta 131*, 580.
Trebst, A., and Pistorius, E. (1969), *in* Progress in Photosynthesis Research, H. Metzner, Ed., Tubingen, Germany, University of Tubingen, Vol. III, p 1229.
Vernon, L. P., and Avron, M. (1965), *Annu. Rev. Biochem. 34*, 269.
Vernon, L. P., and Zaugg, W. S. J. (1960), *J. Biol. Chem. 235*, 2728.
Wessels, J. S. C. (1964), *Biochim. Biophys. Acta 790*, 640.
West, K. R., and Wiskich, J. T. (1968), *Biochem. J. 109*, 527.
Winget, G. D., Izawa, S., and Good, N. E. (1965), *Biochem. Biophys. Res. Commun. 21*, 438.
Yamashita, T., and Butler, W. L. (1968), *Plant Physiol. 43*, 1978.

Kinetic Relationships of Some Factors Affecting the Time of Onset of Mitochondrial Swelling*

Jerald L. Connelly,† Gary W. Evans, and Curtis H. Hallstrom‡

ABSTRACT: Evidence is presented which demonstrates that time of onset (T_0) of rat liver mitochondrial swelling is an appropriate and useful parameter in experiments designed to determine kinetic relationships between factors affecting T_0. The competitive inhibition of succinate-increased T_0 by malonate as shown by $1/T_0$ *vs.* 1/[succinate] plots provides a model for further kinetic experiments and, in addition, demonstrates the requirements for substrate oxidation in substrate-supported increased T_0. A competitive relationship between P_i, as swelling agent, and substrate, as protective agent, has been demonstrated.

Adenosine diphosphate, required in addition to respiratory substrate for optimum protection, acts independently of P_i concentration.

It is currently accepted that several energy-requiring mechanisms in the mitochondrion are closely, if not intimately related. These include, in addition to phosphorylation of ADP, ion movement across the mitochondrial membranes, and NADH–NADP transhydrogenation. Furthermore, it has been observed that these functions are often reflected by variations in the internal or gross structure of the mitochondrion. Such findings are not altogether surprising since the early studies of Raaflaub (1953), and many more recent studies, demonstrated that gross structural variation, swelling, and contraction, are inducible and controllable by agents such as ATP, ADP, P_i, and respiratory substrates, which are common to energy-producing and -requiring processes.

It is likely, then, that a thorough knowledge of the mechanisms involved in the control of mitochondrial structural change would contribute to the understanding of the related energy-linked processes. Accordingly, this laboratory has undertaken to ascertain the chemistry of the mechanisms involved in the mitochondrial processes which act to retard the swelling process. Connelly and Hallstrom (1967) reported that ADP and respiratory substrate have primary roles in processes which delay mitochondrial swelling under conditions in which oxidative phosphorylation is prevented by oligomycin. Previous reports (Connelly and Hallstrom, 1966a, 1967) indicated an inverse relationship between P_i concentration and time of onset (T_0) of swelling. These observations suggested that P_i may be encouraging a shorter T_0 by affecting, in some manner, the protective roles of substrate, ADP, or both. This

* From the Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota, Grand Forks, North Dakota. *Received August 31, 1970.* This work was aided by grants from the U. S. Public Health Service (GM-13080) and the National Science Foundation (GB-1658).

† This investigation was supported (in part) by a Public Health Service research career program award (1-K3-GM-7028-01) from the National Institute of General Medical Sciences. To whom correspondence should be addressed.

‡ U. S. Public Health Service Predoctoral Fellow. Present address: Central Research Laboratories, General Mills, Inc., Minneapolis, Minn.