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THE RELATIONSHIP OF CYCLIC AND NON-CYCLIC ELECTRON FLOW PATTERNS WITH REDUCED INDOPHENOLS TO PHOTOPHOSPHORYLATION

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SUMMARY

Optimal cyclic photophosphorylation with reduced indophenols under anaerobic conditions was shown to require a critical redox balance. Over-reduction inhibited this phosphorylation; addition of oxidizing agents like ferricyanide, air, ferredoxin or ferredoxin *plus* triphosphopyridine nucleotide relieved the inhibition.

When ascorbate and indophenol served as the electron donor couple for TPN^+ reduction, only the amount of TPNH formed was dependent on the concentration of TPN^+. The phosphorylation observed in this system was dependent only on the concentration of indophenol, and on the ability of reduced indophenol to mediate cyclic photophosphorylation. The cyclic electron flow with reduced indophenol was shown to operate simultaneously with the non-cyclic electron flow to TPN^+. It was concluded that there was no phosphorylation site in the non-cyclic electron flow between ascorbate–indophenol and TPN^+ and that the phosphorylation observed in this case was due only to cyclic photophosphorylation with the reduced indophenols.

In the light of these results, a working hypothesis with two different sites for cyclic and non-cyclic photophosphorylation is suggested.

INTRODUCTION

VERNON AND ZAUGG[1] observed that spinach chloroplasts poisoned by 3(3,4-dichlorophenyl)-1,1-dimethylurea could photoreduce TPN^+ on addition of ascorbate and a catalytic amount of DCIP. In 1961 LOSADA, WHATLEY AND ARNON[2] reported that in the above system the photoreduction was also coupled to ATP formation and so concluded that this was a non-cyclic photophosphorylation. However, in 1961 TREBST AND ECK[3] found that catalytic amounts of reduced DCIP mediated cyclic photophosphorylation. This observation was confirmed in other laboratories[4–6]. The question then arose as to which electron flow system is responsible for the ATP formation in this system (see GROMET-ELHANAN AND AVRON[7]).

Recently it was shown that, under certain conditions, $P/2e^-$ values exceeding 1 could be obtained when TPN^+ was photoreduced by ascorbate and DCIP and that

Abbreviations: CMU, 3(4-chlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenol-indophenol; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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the amounts of ATP formed in this non-cyclic system were the same as those formed in the cyclic system with reduced DCIP (refs. 8–10). These results indicated that all the ATP formed in the non-cyclic system could be accounted for by cyclic photophosphorylation. However, they did not exclude the possibility that, when the non-cyclic system operated, ATP formation was coupled to TPN^+ reduction and that only the ATP in excess of TPNH was due to cyclic photophosphorylation.

A clarification of this controversial point was sought through studies of cyclic photophosphorylation with reduced DCIP and of the relationships between the cyclic and non-cyclic systems. A preliminary account of this work has been published[21].

METHODS

The chloroplast fragments used in this communication were of the P_{1s} type prepared according to WHATLEY AND ARNON[11] from spinach or lettuce leaves. Chlorophyll was determined according to ARNON[12].

Reactions were run in conventional Warburg vessels with one side-arm in a Warburg bath maintained at 20° with continuous shaking and illuminated from below. The vessels were equilibrated with argon or air as indicated for 10 min in the dark. Time course experiments were run in erlenmeyer flasks closed with special stoppers as described by GROMET-ELHANAN AND AVRON[7]. ATP was measured after deproteinization with 0.1 vol. of 30% perchloric acid as described by AVRON[13].

TPNH was measured directly, without prior centrifugation, in a Cary spectrophotometer in cuvettes with a 1-mm light path. In the time course experiments TPNH was measured by a method developed in this laboratory (BEN HAYYIM, AVRON AND GROMET-ELHANAN, in preparation). It includes deproteinization with KOH, neutralization to pH 8.5 and centrifugation. TPNH was then assayed on an aliquot of the supernatant by following the drop in absorbance at 340 mμ, after addition of diaphorase in the presence of benzyl-viologen. ATP was determined on aliquots of the same supernatant after acidification with 1 vol. of 0.5 M HCl.

RESULTS

Cyclic phosphorylation with reduced DCIP under argon

In an attempt to get a clear-cut demonstration of which electron flow chain, the cyclic or non-cyclic, is responsible for the ATP formation observed when TPN^+ is photoreduced with the ascorbate–DCIP couple serving as the electron donor system, it was particularly important to compare the cyclic system with reduced DCIP under conditions as close as possible to those of the non-cyclic system. In this system, under argon and with excess ascorbate, no ATP formation could be demonstrated with any DCIP concentration when TPN^+ and ferredoxin were omitted from the reaction mixture. Under air, in the same system, there was always ATP formation (Table I).

As a consequence, possible conditions for obtaining cyclic photophosphorylation under argon were investigated over a wide range of concentrations of reduced DCIP. As can be seen from Fig. 1, cyclic, CMU-resistant photophosphorylation could be demonstrated under argon as well as under air; but under argon, special conditions were necessary for the establishment of this photophosphorylation. Thus, when

TABLE I

COMPARISON OF PHOTOPHOSPHORYLATION WITH DCIP AND EXCESS ASCORBATE IN THE PRESENCE AND ABSENCE OF TPN^+ AND FERREDOXIN

The reaction mixture contained, in a final volume of 3.0 ml, chloroplast fragments containing 0.5 mg of chlorophyll; CMU, $2 \cdot 10^{-5}$ M; and the following in μmoles: Tris buffer (pH 8.0), 80; $MgCl_2$, 5; ADP, 10; $K_2H^{32}PO_4$, 10; ascorbate, 20; and, where indicated, TPN^+, 4 μmoles; ferredoxin, 0.1 mg; and DCIP as indicated. The reaction was run for 15 min at an illumination of 30000 lux.

Additions	*Gas phase*	*ATP formed (μmoles)*
DCIP (0.2 μmole), TPN^+, ferredoxin	Argon	4.1
DCIP (0.2 μmole)	Argon	0.1
DCIP (0.2 μmole), TPN^+, ferredoxin	Air	3.3
DCIP (0.2 μmole)	Air	3.2
DCIP (1.5 μmoles), TPN^+, ferredoxin	Argon	7.1
DCIP (1.5 μmoles)	Argon	0.1
DCIP (1.5 μmoles), TPN^+, ferredoxin	Air	6.5
DCIP (1.5 μmoles)	Air	7.0

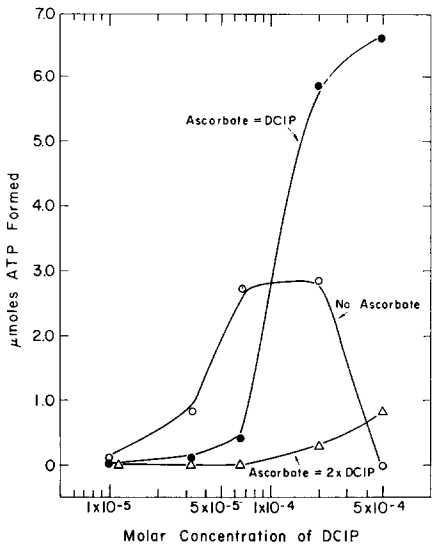

Fig. 1. Cyclic photophosphorylation under argon as a function of DCIP and ascorbate concentration. Experimental conditions as in Table I, except that ascorbate and DCIP were added as indicated.

stoichiometric amounts of ascorbate were added at each DCIP concentration, there was ATP formation with 2 and $5 \cdot 10^{-4}$ M DCIP but not with the lower DCIP concentrations used. However, when ascorbate at a concentration only twice that of the DCIP was added, ATP formation was already completely inhibited.

These results indicated that under argon the cyclic phosphorylation with reduced DCIP was dependent on a very strict redox balance, so that a slight overreduction was enough to cause inhibition[10,14]. The absence of ATP formation with

DCIP below $2 \cdot 10^{-4}$ M, even in the presence of only stoichiometric amounts of ascorbate (Fig. 1), could be explained by the presence of some residual reducing power in the chloroplast preparation (see line 1, Table IV). Indeed, when no ascorbate was added (Fig. 1), there was ATP formation with 3.3 and $6.7 \cdot 10^{-5}$ M DCIP. Obviously, the reducing power of the chloroplast preparation was enough to reduce these amounts of DCIP (0.1 or 0.2 μmole) which catalysed cyclic, CMU-resistant phosphorylation; and the addition of more reducing power (ascorbate) was already enough to cause complete inhibition of this phosphorylation.

The reducing power of the chloroplast preparation was probably too low to reduce the higher amounts of DCIP. Thus, the cyclic photophosphorylation in the absence of added ascorbate dropped to about 50% of its value in the presence of stoichiometric ascorbate with $2 \cdot 10^{-4}$ M DCIP and was completely abolished with $5 \cdot 10^{-4}$ M DCIP (where the blue color of oxidized DCIP did not disappear). On the other hand, with DCIP lower than $3.3 \cdot 10^{-5}$ M, no ATP was formed, even without the addition of ascorbate (Fig. 1). It would seem that in this case the reducing power of the chloroplast preparation was already too high and caused over-reduction (see Table III). Since air counter-balanced the over-reduction caused by excess ascorbate under argon (Table I), it was of interest to test whether other oxidants could act in the same way and whether with the addition of oxidants, ATP would be obtained even with DCIP concentrations below $3.3 \cdot 10^{-5}$ M.

A critical redox balance for optimal cyclic phosphorylation with reduced DCIP under argon

Two different types of oxidants were tested: (1) oxidants which could oxidize ascorbate non-enzymically due to the difference in their redox potentials, and (2) oxidants which would counter-balance the effect of excess ascorbate through their ability to serve as electron acceptors in a chlorophyll-dependent electron flow from ascorbate–DCIP.

Table II illustrates the effect of adding an oxidant of the first type—ferricyanide—to an over-reduced DCIP system. It relieved the inhibition by excess ascorbate as long as its concentration was enough to leave a stoichiometric amount of ascorbate and DCIP. With 0.2 μmole DCIP, where a stoichiometric amount of ascorbate was already inhibitory (Fig. 1), ferricyanide relieved the inhibition only

TABLE II

REVERSAL BY FERRICYANIDE OF THE INHIBITION BY OVER-REDUCTION OF CYCLIC PHOTOPHOSPHORYLATION WITH DCIP UNDER ARGON

Experimental conditions as in Table I, but with 3.0 μmoles of ascorbate. In addition the complete system contained in μmoles: DCIP, 1.0; ferricyanide, 4.0.

Treatment	*ATP formed* (μmoles)
Complete	5.1
Minus ferricyanide	0.4
Minus ascorbate	0.1
Minus DCIP	0.2
Complete with 5 μmoles ascorbate	0.3
Complete with 5 μmoles ascorbate and 8 μmoles ferricyanide	5.4

when it oxidized all the ascorbate added. When ferricyanide was replaced by ferrocyanide, no phosphorylation occurred. These results indicated that the inhibition of cyclic phosphorylation with reduced DCIP by excess ascorbate under argon was indeed due to an over-reduction of the system.

TABLE III

REVERSAL BY FERREDOXIN OF THE INHIBITION BY OVER-REDUCTION OF CYCLIC PHOTOPHOSPHORYLATION WITH DCIP UNDER ARGON

Experimental conditions as in Table I, without TPN^+. DCIP, ascorbate and ferredoxin were added as indicated.

	Additions			*ATP formed* (*μmoles*)
	DCIP (*μmole*)	*Ascorbate* (*μmole*)	*Ferredoxin* (*μg*)	
1	0.2	—	—	2.70
2	0.2	—	25	3.00
3	0.2	—	100	2.82
4	0.2	0.2	—	0.27
5	0.2	0.2	25	1.10
6	0.2	0.2	100	3.18
7	0.03	—	—	0.07
8	0.03	—	100	0.14
9	0.03	—	200	0.78
10	0.03	—	300	0.90

Table III illustrates the effect of adding an oxidant of the second type—ferredoxin—which by itself is unable to oxidize ascorbate or DCIP non-enzymically. The addition of ferredoxin did not affect cyclic phosphorylation with reduced DCIP when the system was not over-reduced (lines 1–3) but, in an over-reduced system, optimal ATP formation could be obtained only when enough of the oxidant was added to restore proper balancing (lines 4–6). With 0.03 μmole of DCIP the cyclic phosphorylation was found to be dependent on the addition of ferredoxin even in the absence of added ascorbate (lines 7–9). It can therefore be concluded that in this case the absence of phosphorylation (line 7 and Fig. 1) was indeed due to over-reduction by the reducing power present in the chloroplast preparation and that the added oxidant could counter-balance this over-reduction. With 0.1 μmole of DCIP it was found that the phosphorylation obtained in the absence of ascorbate and ferredoxin (0.8 μmole, see Fig. 1) could be raised up to 2.5 μmoles ATP formed by the addition of ferredoxin. The phosphorylation obtained with the low concentrations of DCIP was always much lower than with higher amounts of DCIP, and the addition of more ferredoxin did not further stimulate it (lines 9–10). The ATP formed in all the experiments illustrated in Table III was not due to cyclic phosphorylation with ferredoxin, since the amount of ferredoxin used gave no phosphorylation by itself. Also antimycin A at a concentration of 10 μg/ml did not inhibit the phosphorylation, while according to TAGAWA, TSUJIMOTO AND ARNON[15] 3 μg of antimycin per ml were found to inhibit completely the cyclic phosphorylation with ferredoxin.

Since ferredoxin was able to counter-balance the over-reduction by ascorbate

TABLE IV

REVERSAL BY TPN^+ AND FERREDOXIN OF THE INHIBITION BY OVER-REDUCTION OF CYCLIC PHOTOPHOSPHORYLATION WITH DCIP UNDER ARGON

Experimental conditions as in Table I, except that ascorbate was added only as indicated. Where indicated the following were added: DCIP, 0.2 μmole; TPN^+, 4 μmoles; ferredoxin, 0.1 mg.

Additions	*ATP formed (μmoles)*	*TPNH formed (μmoles)*	*P/2e⁻*
1. TPN^+, ferredoxin	0.1	0.5	—
2. DCIP	2.7	—	—
3. DCIP, ascorbate (0.2 μmole)	0.2	—	—
4. DCIP, ascorbate (0.2 μmole), TPN^+, ferredoxin	3.6	0.6	6.0
5. DCIP, ascorbate (20 μmoles), TPN^+, ferredoxin	3.7	3.1	1.2

of the cyclic system with DCIP under argon (Table III), it was interesting to test whether ferredoxin in the presence of TPN^+ would have the same effect (Table IV). In the presence of CMU, which eliminated water as an electron donor[16], and with no addition of another electron donor, ATP formation with TPN^+ and ferredoxin was completely inhibited. There was, however, always a residual photoreduction of about 0.5 μmole of TPN^+, probably due to the reducing power present in the chloroplast preparation (line 1). This reducing power was enough to reduce the amount of DCIP added and enabled the operation of cyclic photophosphorylation with the reduced DCIP (line 2). The addition of stoichiometric amounts of ascorbate over-reduced this system and completely inhibited the cyclic photophosphorylation (line 3). When TPN^+ and ferredoxin were added to this inhibited system, ATP formation was restored (line 4). In this case the reducing power of both the chloroplast preparation and the added ascorbate was enough only for the photoreduction of 0.6 μmole of TPN^+, while the amount of ATP formed was 6 times higher. The high $P/2e^-$ ratio of 6 clearly indicated that the ATP formation was not coupled to TPN^+ reduction but was mediated by the cyclic DCIP system. When excess ascorbate was added to serve as the electron donor for the non-cyclic electron flow to TPN^+ (line 5), TPN^+ reduction was raised about 5-fold but the amount of ATP formed remained the same as in the cyclic system (line 4) resulting in a $P/2e^-$ ratio of 1.2.

These results can be interpreted in two different ways: (a) That TPN^+ and ferredoxin relieved the inhibition by over-reduction of the cyclic system with DCIP not only with the stoichiometric amounts of ascorbate (line 4) but also with a great excess of ascorbate (line 5). The fact that the phosphorylation was the same in both cases, although the amount of TPN^+ reduced was different, might indicate that there is no site of phosphorylation coupled to the open non-cyclic electron flow from ascorbate–DCIP to TPN^+ and that all the ATP formed in this case was due to cyclic phosphorylation[8,9]. (b) That when a great excess of ascorbate was present, the addition of TPN^+ and ferredoxin did not relieve the inhibition of the cyclic system, and the phosphorylation (which happened to be to the same extent as the cyclic phosphorylation) was tightly coupled to TPN^+ reduction[17]. Only with high DCIP concentrations where $P/2e^-$ values exceeding 1 were reported[8–10] could the inhibition of the cyclic system be overcome, and then the ATP in excess of TPNH could be due to cyclic phosphorylation.

The relationship between ATP and TPNH formation with ascorbate–DCIP as the electron donor couple

If the first interpretation is correct then the phosphorylation in the system with excess ascorbate (Table IV, line 5) should show the same dependence on an optimum concentration of DCIP as the cyclic system itself (Fig. 1 and Tables III and IV). If the second interpretation is correct, then photophosphorylation and photoreduction should follow a parallel line, as in any coupled system, independent of DCIP concentration up to the concentration of DCIP where the inhibition of the cyclic system will be overcome.

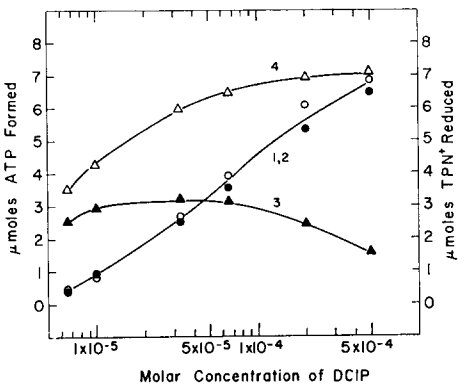

Fig. 2. ATP and TPNH formation under argon with ascorbate–DCIP as the electron donor as a function of DCIP and TPN^+ concentration. Experimental conditions as in Table I, except that ferredoxin (0.1 mg) was included in all the reaction mixtures. Curves 1 and 3 – TPN^+, 4 μmoles. Curves 2 and 4 – TPN^+, 8 μmoles. ○, ● – ATP formation; △, ▲ – TPNH formation.

In order to test these possibilities the relationship between phosphorylation and photoreduction, when ascorbate and DCIP serve as the electron donor couple for TPN^+ reduction, were studied as a function of both DCIP and TPN^+ concentrations. As can be seen from Fig. 2, the photophosphorylation was found to be completely independent of the TPN^+ concentration and changed only as a function of DCIP concentration (curves 1 and 2). With DCIP concentrations above $4 \cdot 10^{-5}$ M, ATP formation was in excess of TPN^+ reduction when 4 μmoles of TPN^+ were used. However, with DCIP concentrations below $4 \cdot 10^{-5}$ M ATP formation did not run parallel to TPN^+ reduction but decreased very rapidly, resulting in $P/2e^-$ values as low as 0.2 or even 0.1 (with $6.7 \cdot 10^{-6}$ M DCIP and 4 or 8 μmoles of TPN^+, respectively). This decrease in phosphorylation could not be explained on the grounds of a poorly coupling activity in the chloroplast preparation used[17], since in the same experiment values of $P/2e^- \geqslant 1$ were obtained with higher DCIP concentrations (curves 1 and 3). Also, with water as the electron donor to TPN^+, the same chloroplast preparation gave a $P/2e^-$ of approx. 1. When the values of ATP formation in this system were compared with those obtained with the same amounts of DCIP under optimal conditions for the cyclic system (Fig. 1 and Tables II–IV), they were found to be similar and, moreover, never exceeded those observed in the cyclic system. It can, therefore, be concluded that the phosphorylation observed in the non-cyclic system of Fig. 2 was dependent only on the ability of reduced DCIP to mediate cyclic photophosphorylation.

The amount of TPNH formed in the presence of ascorbate and DCIP was, however, found to be dependent on TPN^+ concentration and was always much higher when the TPN^+ concentration was doubled (curves 3 and 4). When water served as the electron donor, both ATP and TPNH formation were dependent on TPN^+ concentration (Table V).

TABLE V

THE EFFECT OF TPN^+ CONCENTRATION ON ATP AND TPNH FORMATION UNDER ARGON WITH WATER AS THE ELECTRON DONOR

Experimental conditions as in Table I, except that CMU and ascorbate were omitted and ferredoxin (0.1 mg) was included.

Additions	*ATP formed* (*μmoles*)	*TPNH formed* (*μmoles*)
TPN^+, 4 μmoles	3.0	3.4
TPN^+, 8 μmoles	5.4	7.8

The results illustrated in Fig. 2 were, therefore, not consistent with the assumption[17] that the photophosphorylation is coupled to the non-cyclic electron flow from ascorbate–DCIP to TPN^+.

The location of the site of ATP formation in cyclic photophosphorylation

A possible explanation of the results summarized above could be suggested by assuming that there was no ATP-forming site in the non-cyclic electron flow to TPN^+ with ascorbate–DCIP as the electron donor and that the observed ATP formation was coupled to the cyclic electron flow with reduced DCIP at a site which was not

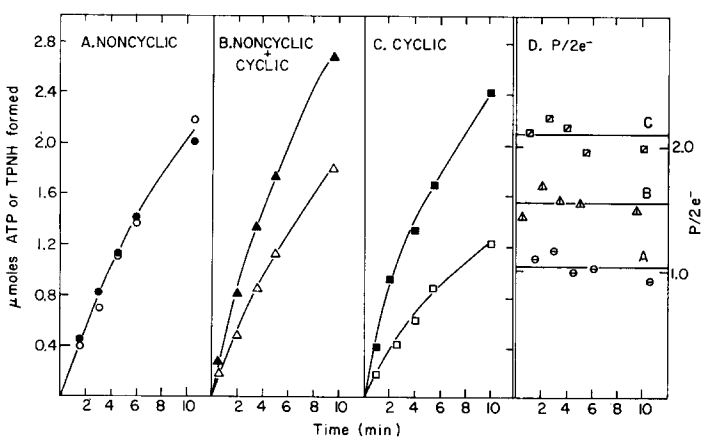

Fig. 3. Time course of ATP and TPNH formation under argon with water or ascorbate–DCIP or both together as the electron donor. Reaction mixture A contained, in a final volume of 6.0 ml, chloroplast fragments containing 0.5 mg of chlorophyll; 0.1 mg of ferredoxin and the following in μmoles: Tris buffer (pH 8.0), 160; $MgCl_2$, 10; ADP, 10; $K_2H^{32}PO_4$, 10; TPN^+, 3. Reaction B contained, in addition to the above reagents, 20 μmoles ascorbate and 1.2 μmoles DCIP and reaction mixture C contained also $2 \cdot 10^{-5}$ CMU. ●, ▲, ■ – ATP formation; ○, △, □ – TPNH formation. The designation non-cyclic or cyclic in A, B and C defines only the type of the photophosphorylating system and not the type of the electron flow pathway.

shared by the non-cyclic flow. The results would then be composed of a cyclic phosphorylation with reduced DCIP superimposed on a non-cyclic photoreduction of TPN^+ by ascorbate–DCIP. This suggestion would imply that the cyclic and non-cyclic systems could operate simultaneously.

Experimental support for this explanation was obtained when the time course of ATP and TPNH formation was followed in three different systems: in the non-cyclic phosphorylation coupled to the non-cyclic electron flow from water to TPN^+ (Fig. 3A); in the cyclic phosphorylation superimposed on the non-cyclic electron flow from ascorbate–DCIP to TPN^+ (in the presence of CMU, Fig. 3C); and in the two together (in the absence of CMU, Fig. 3B). Although the $P/2e^-$ ratio was 1 in Fig. 3A, it was 2 in Fig. 3C from the start and throughout the time course. Also, as was already shown in Fig. 2, the $P/2e^-$ ratio of Fig. 3C was dependent on the concentration of DCIP. With concentrations around $3 \cdot 10^{-5}$–$6 \cdot 10^{-5}$ M this ratio was near 1 while with lower DCIP concentrations this ratio was much below 1 throughout the time course (not shown here). In all cases there was no indication for a transition from a non-cyclic phosphorylation with a $P/2e^-$ ratio of 1 to a cyclic phosphorylation later on in the time course, as was shown for ferredoxin $\pm$ TPN^+ by TAGAWA, TSUJIMOTO AND ARNON[15]. The $P/2e^-$ ratio when both phosphorylation systems were operating together (Fig. 3B) was in all cases in between the ratios observed with each of them separately (Fig. 3D).

DISCUSSION

Cyclic photophosphorylation with reduced DCIP under anaerobic conditions was shown to be inhibited by over-reduction with excess ascorbate in chromatophores[14] as well as in chloroplasts[10]. This over-reduction was found to be counterbalanced either by oxidants like ferricyanide which oxidized any excess ascorbate non-enzymically (Table II) or by oxidants like air or ferredoxin and TPN^+ which could not act on ascorbate directly (Tables I, III and IV). In the last system, as distinct from the system with ferricyanide, there was no need to add the oxidant at the same concentration as that of the excess ascorbate. It seems that the counterbalancing effect of ferredoxin and TPN^+ was dependent on the competing rates of the oxidation of DCIP *via* the light-induced electron flow to TPN^+ and the non-enzymatic reduction of DCIP by ascorbate. It was indeed observed that illumination changed the steady state of DCIP in this system from a completely reduced to a more oxidized one*.

The fact that oxidants like ferredoxin and TPN^+ induced a non-cyclic electron flow from ascorbate–DCIP raised the question whether the phosphorylation observed in their presence was coupled to this non-cyclic electron flow or to the cyclic electron flow catalysed by reduced DCIP. If ATP formation was indeed coupled to TPNH formation, as maintained by ARNON *et al.*[2,17], one would expect that the $P/2e^-$ ratio would be the same whether water or ascorbate–DCIP serves as the electron donor for TPN^+ reduction. It was, however, found that with ascorbate–DCIP as electron donor the phosphorylation changed as a function of DCIP concentration, resulting in $P/2e^-$ values not only well above the theoretical unity[8,9] but also as low as 0.1 (Fig. 2).

* Z. GROMET-ELHANAN AND M. AVRON, unpublished results.

Experiments with reported P/2e$^-$ values above 1 could be explained as suggested by TAGAWA, TSUJIMOTO AND ARNON[15], only if in a time course study a non-cyclic phosphorylation coupled to TPN$^+$ reduction with a P/2e$^-$ ratio of 1 were first observed, which was later on followed by a cyclic phosphorylation, resulting in an overall P/2e$^-$ ratio of more than 1. This explanation was overruled by the finding that in time course studies (Fig. 3) the P/2e$^-$ ratio was constant and significantly above 1 throughout the experiment, giving no indication for a lower ratio of P/2e$^-$ near 1 at the start. Also, the P/2e$^-$ values of 0.1 obtained with low concentrations of DCIP (Fig. 2) could be explained according to ARNON *et al.*[15,17], only if the chloroplast preparation used had a poor coupling activity, a condition which was not corroborated by the experimental results.

TREBST[18] observed that ascorbate–TMPD could replace ascorbate–DCIP as the electron donor for TPN$^+$ reduction. It was also reported[8,18] that with ascorbate–TMPD, TPN$^+$ reduction was not accompanied by phosphorylation. These results led TREBST AND PISTORIUS[19] to propose that electrons from the ascorbate–TMPD couple could enter the electron flow chain after the site of phosphorylation. According to this hypothesis the site of phosphorylation would still be one and the same for both cyclic and non-cyclic photophosphorylation.

He also tried to explain the different P/2e$^-$ values observed with different concentrations of DCIP by assuming that electrons from the ascorbate–DCIP couple could enter the chain at two different points, depending on the concentration of DCIP. With low concentrations of DCIP the electrons would enter the chain, as in the case of TMPD, after the site of phosphorylation, whereas with high concentrations of DCIP they would enter the chain before the site of phosphorylation[19].

The phosphorylation with ascorbate–DCIP as electron donor for TPN$^+$ reduction was, however, found to increase in a linear way with increasing DCIP concentrations (Fig. 2). There was no break in the curve and no indication of a sharp transition from a non-phosphorylating to a phosphorylating system with increasing DCIP concentrations. Furthermore, it has been recently reported by SCHWARTZ[20], that he observed both phosphorylation and photoreduction when ascorbate–TMPD served as electron donor for TPN$^+$ reduction. This observation was confirmed in this laboratory*, and it was also found that whenever a phosphorylation was observed in this system with TMPD, DCIP or other compounds, they were also found to catalyse cyclic phosphorylation.

A different hypothesis which can explain these results is presented in Fig. 4. According to this hypothesis there are two different sites of ATP formation in photophosphorylation One site is located on the non-cyclic pathway of electron flow prior to the point of entry of electrons from reduced DCIP. The other is located on the pathway of electron flow which is exclusive for the cyclic system and not shared by the non-cyclic one. As distinct from some previously published schemes[2,17], there is no site of phosphorylation on the non-cyclic pathway of electron flow after the point of entry of electrons from reduced DCIP.

The results with ascorbate–DCIP as electron donor for TPN$^+$ reduction can be explained by the simultaneous operation of both cyclic and non-cyclic electron flows (Fig. 3). The non-cyclic electron flow results in TPN$^+$ reduction, while the cyclic flow

* Unpublished observations.

results in ATP formation. Since photoreduction and phosphorylation are in this case (unlike the system from water to TPN^+) due to different electron flow patterns, this explains the absence of coupling between them, the dependence of the phosphorylation only on DCIP concentration and the dependence of only the photoreduction

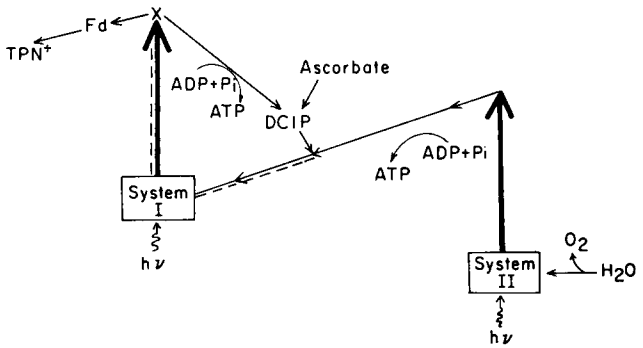

Fig. 4. Schematic representation of the phosphorylation sites in cyclic and non-cyclic photophosphorylation. The dotted line represents the part of the electron flow which is shared by both cyclic and non-cyclic systems.

on TPN^+ concentration (Fig. 2). The ability of the cyclic system with reduced DCIP to operate concomitantly with the non-cyclic system differentiates it from the system with ferredoxin in air[15] where according to ARNON, TSUJIMOTO AND MCSWAIN[17] the "cyclic phosphorylation will occur only under conditions when non-cyclic photophosphorylation is excluded".

The scheme in Fig. 4 specifies one phosphorylation site in the cyclic electron flow chain. This does not exclude the possibility of additional phosphorylation sites, since as long as the $P/2e^-$ value of the cyclic system is unknown, there is no way of determining the actual number of phosphorylation sites in this system.

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