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THE STOICHIOMETRY (ATP/$2e^-$ RATIO) OF NON-CYCLIC PHOTOPHOSPHORYLATION IN ISOLATED SPINACH CHLOROPLASTS

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SUMMARY

1. The stoichiometry of non-cyclic photophosphorylation and electron transport in isolated chloroplasts has been re-investigated. Variations in the isolation and assay techniques were studied in detail in order to obtain optimum conditions necessary for reproducibly higher ADP/O (equivalent to ATP/$2e^-$) and photosynthetic control ratios.

2. Studies which we carried out on the possible contribution of cyclic phosphorylation to non-cyclic phosphorylation suggested that not more than 10% of the total phosphorylation found could be due to cyclic phosphorylation.

3. Photosynthetic control, and the uncoupling of electron transport in the presence of NH_4Cl, were demonstrated using oxidised diaminodurene as the electron acceptor. A halving of the ADP/O ratio was found, suggesting that electrons were being accepted between two sites of energy conservation, one of which is associated with Photosystem I and the other associated with Photosystem II.

4. ATP was shown to inhibit State 2 and State 3 of electron transport, but not State 4 electron transport or the overall ADP/O ratio, thus confirming its activity as an energy transfer inhibitor. It is suggested that part of the non-phosphorylating electron transport rate (State 2) which is not inhibited by ATP is incapable of being coupled to subsequent phosphorylation triggered by the addition of ADP (State 3). If the ATP-insensitive State 2 electron transport is deducted from the State 3 electron transport when calculating the ADP/O ratio, a value of 2.0 is obtained.

5. The experiments reported demonstrate that there are two sites of energy conservation in the non-cyclic electron transfer pathway: one associated with Photosystem II and the other with Photosystem I. Thus, non-cyclic photophosphorylation can probably produce sufficient ATP and NADPH "*in vivo*" to allow CO_2 fixation to proceed.

INTRODUCTION

Since the discovery of photosynthetic phosphorylation in isolated chloroplasts[1], the question of the number of molecules of ATP produced per pair of electrons trans-

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; PMS, phenazine methosulphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

ported in non-cyclic phosphorylation (the ATP/$2e^-$ or ADP/O ratio) has been a matter of some controversy[2–5]. This problem is closely linked to the role of cyclic electron transport and phosphorylation in photosynthesis. The fixation of CO_2 in photosynthesis requires the production of 3 molecules of ATP and 2 molecules of NADPH for one molecule of CO_2 fixed and one molecule of O_2 evolved (an ATP/$2e^-$ ratio of 1.5). As carefully isolated chloroplasts can fix CO_2 in the absence of added ATP, at rates approaching those found *in vivo*[6–8], the chloroplast must have the ability to produce sufficient NADPH and ATP for efficient CO_2 fixation.

If non-cyclic photophosphorylation produces only one molecule of ATP per molecule of NADPH, then the extra ATP must, of necessity, be produced within the chloroplast by a process other than non-cyclic photophosphorylation, most likely by cyclic photophosphorylation. If however, the ADP/O ratio of non-cyclic photophosphorylation is 2.0 then cyclic photophosphorylation need not play a major role in CO_2 fixation.

Previously reported results showed that carefully isolated chloroplasts will fix CO_2 and have reproducibly high ADP/O ratios (1.4–1.7) and photosynthetic control ratios (4–6), the latter ratio being indicative of well-coupled chloroplasts carrying out photophosphorylation efficiently[9]. In order to discover whether these ratios were truly a reflection of non-cyclic photophosphorylation, studies were made of the possibility of there being a contribution by cyclic phosphorylation to ATP formation, and of there being some non-phosphorylating electron flow occurring during phosphorylating non-cyclic electron transport.

METHODS

Chloroplasts were isolated that could fix CO_2 at rates of 40–60 μmoles CO_2/mg chlorophyll per h. Spinach was obtained commercially from Covent Garden. The chloroplast isolation technique was based on that of Walker[6]. Washed spinach leaves were pre-illuminated by a 60-W incandescent lamp on iced water for 30 min before use. 50 g deribbed leaf blades were cut briefly into about 2-cm^2 pieces with a sharp knife and placed in a cooled perspex grinding vessel, 6 cm $\times$ 5 cm $\times$ 25 cm deep. 200 ml of freshly prepared grinding medium, cooled until it was a slushy consistency, were poured on top of the leaves. The grinding medium contained 400 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM isoascorbic acid, 0.4% (w/v) bovine serum albumin and 50 mM 2-(*N*-morpholino)ethanesulphonic acid, adjusted to pH 6.5 (at room temperature). The leaves were ground for 3 s with a Polytron PT 20 with a PT 35 head (Willems, Kinematica GbmH, Lucerne, Switzerland) at a speed setting of 3.5. The resultant slurry was squeezed through two layers of butter muslin (cheese cloth) and the filtrate poured through eight more layers of muslin. The final filtrate was centrifuged in an MSE Super Minor bench centrifuge with pre-cooled head. Rapid acceleration up to 4000 $\times g$, followed by braking by hand, allowed the total centrifugation time to be less than 1 min. The supernatant was discarded, and the pellet was gently resuspended with the aid of cotton wool and a glass rod in about 1 ml of solution consisting of 400 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.4% (w/v) bovine serum albumin and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) adjusted to pH 7.5 (at room temperature). All procedures were carried out at 0–4 °C and the resultant chloroplast

pellet was kept on ice. Total preparation time from cutting the leaves was about 4 min. The chloroplasts when isolated were "Type A, complete" in the classification of Hall[10]. These chloroplasts are capable of fixing CO_2 but are impermeable to added ADP, ATP and ferricyanide (see for example Walker[11], Heldt[12] and Heber and Santarius[13]). Thus, in order to study the effect of adenine nucleotides and electron acceptors, the chloroplast reactions were followed in a hypotonic medium containing 0.1 M sorbitol. This low osmolarity ruptured the chloroplast envelope, and the resultant reaction medium contained chloroplasts of "Type C, broken" in Hall's classification[10]. We have shown that these experimental conditions are optimum for measuring ADP/O ratios (refs 9 and 14).

The chlorophyll concentration was measured by the technique of Arnon[15].

Oxygen uptake and evolution was continuously monitored on a Rank Bros., Cambridge, oxygen electrode[16]. The illumination system consisted of two Prinz "Low Line" projectors with 300-W tungsten bulbs. Light was passed through a 3-cm water heat filter and a red filter (Cinemoid No. 5a, Rank Strand Electric Co., London WC2) transmitting light between 540 and 740 nm. The light intensity in the cell was $8.8 \cdot 10^4$ ergs$\cdot s^{-1} \cdot cm^{-2}$. The temperature was maintained at 15 °C by a Churchill Thermo-circulator. Radioactive inorganic phosphate was obtained from the Radiochemical Centre, Amersham, Bucks. Incorporation of the radioactive phosphate into [^{32}P]ATP was measured by the technique of Hagihara and Lardy[17].

For the measurement of endogenous inorganic phosphate and adenine nucleotide a perchloric acid extraction[18] was carried out on chloroplast pellets resuspended in water to rupture the chloroplast envelope. Inorganic phosphate analysis was carried out by the method of Fiske and SubbaRow[19], as modified by Allen[20]. Endogenous nucleotides were assayed enzymatically using Boehringer Test Kits, (Boehringer Corp. (London) Ltd) the methods being based on those of Adam[18, 21].

All chemicals used were of the highest grade commercially available from B.D.H Chemicals, Boehringer and Sigma Chemical Co. Diaminodurene was a gift of Professor A. Trebst, Ruhr University, Bochum, Germany.

RESULTS

Initial experiments were carried out in detail to optimise the components of the grinding and resuspending media and the method of isolation of the chloroplasts, and also to optimise the reaction conditions for the assay procedures. The conditions used in the experiments presented here resulted in reproducibly high photosynthetic control ratios, *i.e.* low States 2 and 4 and high State 3, and were correlated with uniformly high ADP/O ratios[9]. Details of these experiments can be found in ref. 14. Table I shows typical results for three different electron acceptors. The terminology used for the various "states" of electron transport is that of Chance and Williams[22]. State 2 refers to the electron transport rate in the presence of electron acceptor and inorganic phosphate, but with no added phosphate acceptor, *i.e.* ADP, present. State 3 is the phosphorylating rate of electron transport (ADP added), and State 4 the phosphate acceptor-depleted rate after the ADP has all been converted to ATP. State 4 is lower than State 2 due to ATP inhibition of electron transport (see later). The photosynthetic control ratio is defined as State 3/State 4. Table I shows that similar values of photosynthetic control and ADP/O ratios were obtained with three

TABLE I

A COMPARISON OF NON-CYCLIC ELECTRON TRANSPORT AND PHOSPHORYLATION WITH THREE ELECTRON ACCEPTORS

Oxygen evolution, photosynthetic control ratios and ADP/O ratios were measured in an oxygen electrode. The reaction mixture contained, in a total volume of 2.0 ml: chloroplasts equivalent to 100 μg chlorophyll; 100 mM sorbitol; 50 mM HEPES (pH 7.5); 4 mM $MgCl_2$; 20 mM NaCl; 10 mM K_2HPO_4; 2 mM EDTA and either 2.5 mM $K_3Fe(CN)_6$ or 2 mM $NADP^+$ *plus* saturating amounts of spinach ferredoxin (approximately 100 μg) or 0.2 mM methyl viologen *plus* 2.0 mM NaN_3. ADP (250 nmoles) was added to the reaction mixture during State 2, in order to give one photosynthetic control cycle. An uncoupled rate was obtained by adding NH_4Cl (10 μmoles) during State 4.

	Electron transport (μmoles O_2/mg chlorophyll per h)		
	Ferricyanide	*Methyl viologen*	*$NADP^+$*
State 2	22	24	11
State 3	55	59	38
State 4	10	12	7
State 4 + NH_4Cl	60	61	44
Photosynthetic control	5.5	4.9	5.4
ADP/O ratio	1.5	1.7	1.7

commonly used non-cyclic acceptors, *viz.* potassium ferricyanide, methyl viologen and $NADP^+$. The ADP/O ratio found with ferricyanide as electron acceptor was usually slightly lower than that found with either of the other acceptors, and the rate of electron transport with $NADP^+$ was usually lower than that found with methyl viologen or ferricyanide. Concentrations of ferricyanide from 10 mM down to 0.2 mM gave similar ADP/O ratios, in agreement with the report of Saha and Good[5].

Cyclic and non-cyclic photophosphorylation

Certain co-factors, *e.g.* phenazine methosulphate (PMS) and vitamin K_3 (menadione or 2-methyl-1,4-naphthoquinone) are known to induce high rates of cyclic photophosphorylation with no concomitant oxygen evolution or uptake. Cyclic photophosphorylation is normally measured in systems where non-cyclic electron flow is inoperative. The effects of adding either of these two cyclic co-factors to a system undergoing non-cyclic electron transport are shown in Figs 1 and 2.

The effect of PMS on electron transport (Fig. 1a) was similar to that of a mild uncoupler, in that it stimulated electron transport rates, the maximum stimulation occurring with the lowest electron transport rate, *i.e.* State 4. This greater stimulation of State 4 than of State 3 caused a small reduction in photosynthetic control, as shown in Fig. 1b. PMS also caused a slight, but reproducible, decrease in the ADP/O ratio. These results are similar to recently published results of Demidov *et al.*[23], where it has been suggested that PMS partially bypasses a rate-limiting step of non-cyclic electron transport. Their results were obtained from measurement of pH changes and $P/2e^-$ ratios.

The addition of vitamin K_3 to chloroplasts carrying out non-cyclic phosphoryla-

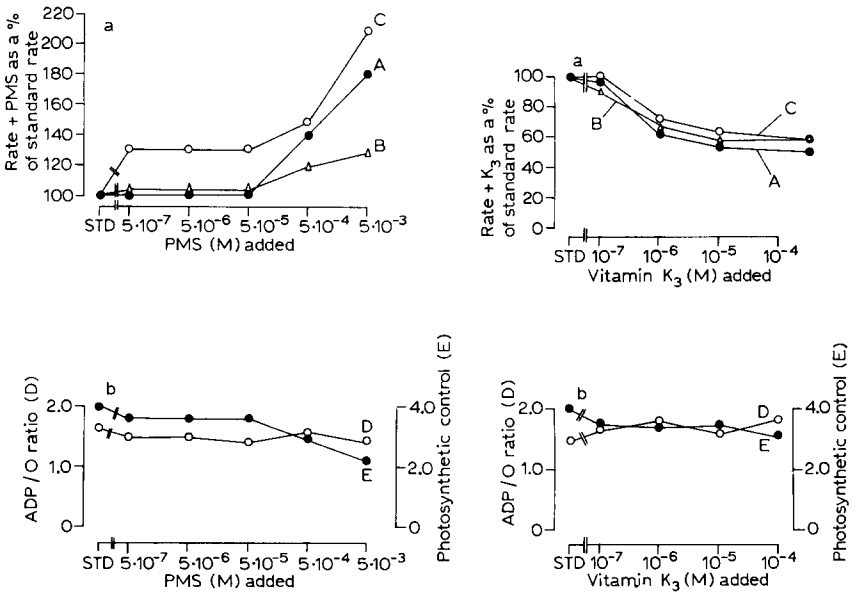

Fig. 1. The effect of PMS on non-cyclic electron transport and phosphorylation. Oxygen evolution, photosynthetic control ratios and ADP/O ratios were measured in an oxygen electrode. The reaction mixture contained, in a total volume of 2.0 ml: chloroplasts equivalent to 100 μg chlorophyll; 100 mM sorbitol; 50 mM HEPES (pH 7.5); 4 mM $MgCl_2$; 20 mM NaCl; 10 mM K_2HPO_4; 2 mM EDTA; 2.5 mM $K_3Fe(CN)_6$, and PMS as indicated. ADP (250 nmoles) was added to the reaction mixture in a small volume during State 2, in order to give one photosynthetic control cycle. Curve A, State 2; Curve B, State 3; Curve C, State 4; Curve D, ADP/O ratio; Curve E, photosynthetic control ratio.

Fig. 2. The effect of vitamin K_3 on non-cyclic electron transport and phosphorylation. Details as in Fig. 1 except that PMS was replaced by vitamin K_3. Curve A, State 2; Curve B, State 3; Curve C, State 4; Curve D, ADP/O ratio; Curve E, photosynthetic control ratio.

tion slightly decreases all rates of electron transport (Fig. 2a) and as all the rates were decreased in a similar manner, there was no major change in the photosynthetic control (Fig. 2b). The ADP/O ratio found in the presence of vitamin K_3 was routinely about 10% higher than that found in the control, several experiments giving an average ADP/O ratio *plus* vitamin K_3 of 1.65 compared with 1.50 in the absence of vitamin K_3. This increase may be due to a small contribution of cyclic phosphorylation induced by the cyclic co-factor.

A summary of the effect of cyclic and pseudo-cyclic co-factors added to chloroplasts carrying out non-cyclic electron transport and phosphorylation is given in Table II. These co-factors gave the same results if they were present in the reaction medium from the start of the experiment, before the light was turned on, or if they were added during State 2. The table shows that there was very little effect on the ADP/O ratios found, and thus they were not apparently inducing any cyclic phosphorylation.

A further experiment with PMS is shown in Fig. 3. This type of experiment was originally carried out by Avron and Jagendorf[24], with broken, washed chloroplasts; they demonstrated that in the presence of a non-cyclic electron acceptor and a cyclic

TABLE II

A SUMMARY OF THE EFFECT OF ADDING VARIOUS CYCLIC AND PSEUDO-CYCLIC CO-FACTORS TO CHLOROPLASTS CARRYING OUT NON-CYCLIC ELECTRON TRANSPORT AND PHOSPHORYLATION

Details as in Fig. 1, except that the co-factors indicated were added instead of PMS.

	Co-factor added to reaction:				
	PMS	*Vitamin K_3*	*FMN*	*Ferredoxin*	*Methyl viologen*
E_0'	+0.080 V	−0.01 V	−0.219 V	−0.430 V	−0.466 V
Concentrations used in the experiments	$5 \cdot 10^{-7}$–$5 \cdot 10^{-3}$ M	10^{-7}–10^{-4} M	$5 \cdot 10^{-7}$–$5 \cdot 10^{-3}$ M	50 μg–5 mg per reaction mixture	$5 \cdot 10^{-7}$–$5 \cdot 10^{-3}$ M
Concentration normally used for cyclic phosphorylation	10^{-4} M	10^{-4} M	10^{-4} M	2 mg per reaction mixture	$2 \cdot 10^{-4}$ M
Effect on State 2	Increased	Decreased	None	None	None
Effect on State 3	Increased	Decreased	Decreased	None	None
Effect on State 4	Increased	Decreased	None	None	None
Effect on photosynthetic control	Decreased	Decreased	Decreased	None	None
Effect on ADP/O	Decreased	10% increase	None	None	None

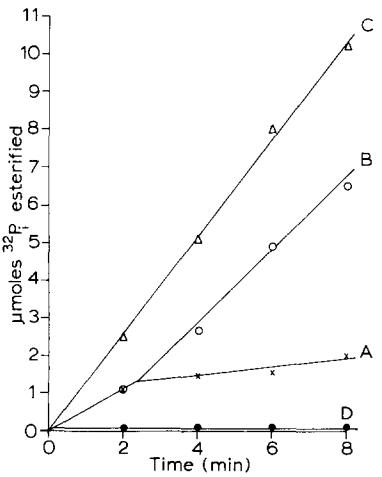

Fig. 3. Measurement of PMS-induced cyclic phosphorylation in chloroplasts. The reaction was carried out aerobically in an oxygen electrode vessel. The reaction mixture contained, in a total volume of 2.0 ml: chloroplasts equivalent to 100 µg chlorophyll; 100 mM sorbitol; 50 mM HEPES (pH 7.5); 4 mM $MgCl_2$; 20 mM NaCl; 10 mM K_2HPO_4 + carrier-free $^{32}P_i$; 2 mM EDTA, and 2.5 mM ADP. $K_3Fe(CN)_6$ sufficient to allow non-cyclic electron flow for 2.5 min; PMS, 10^{-4} M; and DCMU, 10^{-6} M, were added as indicated. Curve A, with $K_3Fe(CN)_6$; Curve B, with $K_3Fe(CN)_6$ and PMS; Curve C, with PMS; Curve D, with $K_3Fe(CN)_6$, PMS and DCMU.

co-factor, non-cyclic phosphorylation occurred preferentially until the non-cyclic electron acceptor (*i.e.* ferricyanide) was depleted, whereupon cyclic phosphorylation started. In the experiment shown here the same conclusions can be drawn with the type of chloroplasts used in this series of experiments. Curve A shows [^{32}P]ATP formation with sufficient ferricyanide present to allow electron transport for approx. 2.5 min. Curve C shows the ATP formation when PMS alone is present. When both ferricyanide and PMS are present (Curve B) phosphorylation occurs at the non-cyclic rate until the ferricyanide is all reduced, whereupon cyclic phosphorylation starts. Curve D shows that there is no phosphorylation in the presence of ferricyanide, PMS and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in red light, showing that PMS cannot induce cyclic phosphorylation in this aerobic system. Jagendorf and Margulies[25], and Hauska *et al.*[26] have concluded that PMS can catalyse cyclic photophosphorylation only in a "poised" system, *e.g.* in red light under aerobic conditions, and in the presence of DCMU ascorbate needs to be added to the reaction mixture if ATP formation is to be detected; however, in white light the addition of ascorbate is not necessary. It is interesting to note that in the results reported by Avron and Jagendorf[24] with chloroplasts that were devoid of stroma and envelopes, there was no phosphorylation when all the ferricyanide had been reduced and there was no PMS present (*cf.* Curve A). Curve A indicates that in our preparations of chloroplasts, which retain their stroma, there is some endogenous phosphorylation, but as shown it is only about 10% of the non-cyclic phosphorylation rate.

Oxidised diaminodurene as an electron acceptor

Saha *et al.*[27] have recently introduced a new classification of electron acceptors for non-cyclic electron transport. The electron acceptors used previously in this report,

ferricyanide, methyl viologen and NADP, are termed Class I acceptors, since they are water soluble. Another class of electron acceptors, called Class III, are lipid-soluble compounds that show very high rates of electron transport and phosphorylation. In their report Saha *et al.*[27] found little stimulation of the non-phosphorylating rate of electron transport with Class III acceptors by the addition of either the constituents of the phosphorylating system (ADP and P_i) or by an uncoupler (methylamine); however, they did show a halving of the ATP/$2e^-$ ratio when using these acceptors, and suggested that the electron acceptor, being lipid soluble, dissolved in the membrane and accepted electrons between two phosphorylating sites of the non-cyclic electron transfer chain.

Figs 4 and 5 show the effect of oxidised diaminodurene as electron acceptor in the well-coupled chloroplasts used in this work. Unlike the report of Saha *et al.*[27], Fig. 4 shows that photosynthetic control and uncoupling can both be demonstrated at all levels where oxidised diaminodurene stimulates the electron transport rate. The ADP/O ratio (Fig. 5) shows a halving in the same way as did the ATP/$2e^-$ ratio of Saha *et al.*[27] but we measure a higher initial, 1.6, and therefore final, 0.7, value. Thus these experiments are essentially in agreement with those of Saha *et al.*[27], but we have shown photosynthetic control to exist with a Photosystem II associated phosphorylation site.

The effect of endogenous and added adenine nucleotides

The inhibition of electron transport by ATP (ref. 28) and its action as an energy transfer inhibitor[29,30], have been previously reported. ADP has also been shown to inhibit electron transport (in the absence of P_i) to a certain degree[31]. The inhibition of

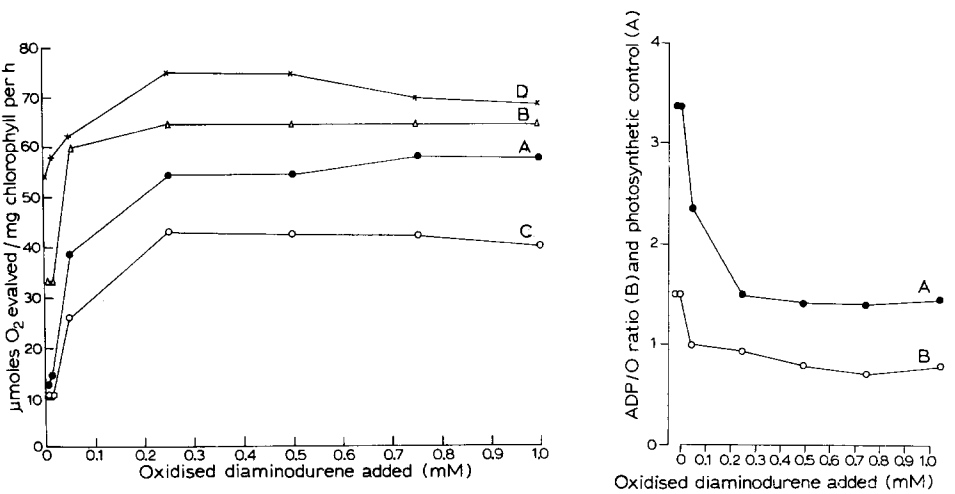

Fig. 4. The effect of oxidised diaminodurene on non-cyclic electron transport and phosphorylation. Rates of oxygen evolution were measured as in Fig. 1, except that PMS was replaced by diaminodurene. The diaminodurene was maintained in an oxidized form by the presence of the 2.5 mM $K_3Fe(CN)_6$. Curve A, State 2; Curve B, State 3; Curve C, State 4; Curve D, uncoupled rate (+10 µmoles NH_4Cl).

Fig. 5. The effect of oxidised diaminodurene on phosphorylation. The photosynthetic control ratios (Curve A) and ADP/O ratios (Curve B) were calculated from the data shown in Fig. 4.

State 2 electron transport by ADP can easily be demonstrated in chloroplasts washed free of endogenous P_i. This process, however, requires the rupture of the chloroplast envelope during preparation, which is likely to damage the chloroplast phosphorylating system. The chloroplasts used in our experiments were not broken during preparation and still contained endogenous P_i so that ADP inhibition was not seen. If these chloroplasts, containing endogenous P_i, have ADP added during State 2, there is a small stimulation of the rate of electron transport, due to a slow rate of endogenous phosphorylation; this is followed by a rate slower than the original State 2, due to inhibition of electron transport by the ATP which is formed. The effect of added ATP on electron transport is shown in Fig. 6. State 2 shows a linear decrease with increasing ATP concentration saturating at about $5 \cdot 10^{-5}$ M. State 3 showed a linear decrease from 10^{-5} M up to the highest ATP concentrations used, $5 \cdot 10^{-2}$ M. State 4 was unaffected by added ATP.

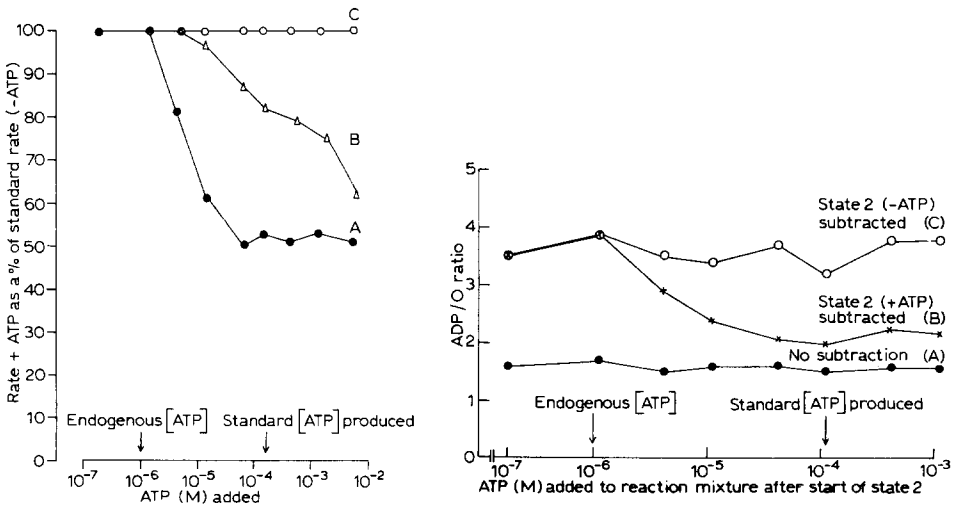

Fig. 6. The effect of ATP on electron transport and phosphorylation. Details as in Fig. 1, except that PMS was replaced by ATP. The maximum amount of endogenous ATP found in a chloroplast preparation, and the amount of ATP produced by one standard cycle of photosynthetic control, are shown. Curve A, State 2; Curve B, State 3; Curve C, State 4.

Fig. 7. The effect of ATP on the ADP/O ratio, calculated with and without the subtraction of basal rates. The data were taken from the experiment shown in Fig. 6. The ADP/O ratios were calculated, (A) without any subtraction of a basal rate; (B) with the State 2+ATP rate subtracted as a basal rate, and (C) with State 2−ATP subtracted as a basal rate.

The effect of ATP on the ADP/O ratio is shown in Fig. 7. It has been suggested by Izawa and Good[32] that the electron transport rate which is found in the presence of ADP and absence of P_i in the reaction mixture, (the "basal" rate), remains uncoupled during the phosphorylation which is initiated by the addition of P_i. It was suggested that this nonphosphorylating 'basal' rate of electron transport was the same as that which is insensitive to energy transfer inhibitors (*e.g.* phloridzin) and was not capable of being coupled to ATP formation; subtraction of this "basal" rate from the overall rate of electron transport would give an accurate reflection of the ADP/O ratio. Fig. 7 shows that there is no effect of ATP (an energy transfer inhibitor) on the

overall ADP/O ratio measured when there is no subtraction of a "basal" rate (Curve A). Curve B shows that if the "basal" rate is subtracted and is taken to be that rate which is unaffected by an energy transfer inhibitior (ATP), the calculated ADP/O ratio becomes approximately 2.2 at the higher concentrations of added ATP. At lower concentrations of ATP the curve tends towards that found when the "normal" State 2 (*i.e.* no added ATP) is substracted as the "basal" rate in the calculation of the ADP/O ratio (Curve C). These data would seem to suggest that that part of the State 2 which is not inhibited by ATP does indeed remain uncoupled from phosphorylation during State 3, and that the ADP/O ratio is 2.0.

As a further test for this, the effect of ATP was compared using ferricyanide and NADP as the electron acceptors. These two acceptors routinely resulted in different electron transport rates and slightly different ADP/O ratios, using the same chloroplast preparation (Table I). However, as can be seen in Table III, the effect of ATP on both systems was similar. In both cases there was approximately a 40% reduction in State 2 due to the addition of ATP, and the ADP/O ratio, calculated with the subtraction of the State 2 (*plus* added ATP) rate, was just over 2.0. This lends support to the suggestion[32], that there is a part of the electron transport chain which is not coupled to phosphorylation and that there are indeed two sites of phosphorylation on the non-cyclic electron transport pathway.

As the adenine nucleotides showed such a marked effect on electron transport and phosphorylation, the levels of endogenous nucleotides and P_i were assayed in typical chloroplast preparation (Table IV). These levels correspond with other estimations on isolated spinach and pea chloroplasts[13,33–35]. The endogenous nucleotides levels are not high enough to have any marked effect on electron transport or phosphorylation (see also Figs 6 and 7), but the P_i level is great enough to allow some phosphorylation in the absence of added phosphate.

TABLE III

A COMPARISON OF THE EFFECT OF ATP ON NON-CYCLIC PHOSPHORYLATION AND ELECTRON TRANSPORT

The reaction conditions were as in Table I, except that 250 nmoles ATP were added during State 2.

	Electron transport (*μmoles O_2/mg chlorophyll per h*)	
	Ferricyanide	*$NADP^+$*
State 2	32	16
State 2 + ATP	18	9
State 3	65	47
State 4	16	13
State 4 + NH_4Cl	76	54
Photosynthetic control	4.0	3.7
ADP/O ratio (no subtraction)	1.5	1.7
ADP/O (State 2 − ATP subtracted)	3.8	3.5
ADP/O (State 2 + ATP subtracted)	2.3	2.1

TABLE IV

ENDOGENOUS CONTENT OF ADENINE NUCLEOTIDES AND P_i IN A TYPICAL CHLOROPLAST PREPARATION

Details as in Methods.

	Content (nmoles/mg chlorophyll)
P_i	3600
AMP	4.5
ADP	9.0
ATP	15.6

DISCUSSION

There are two views on the number of phosphorylating sites associated with non-cyclic electron transport, and the two alternatives suggest different roles for cyclic phosphorylation in photosynthesis. If there are two sites of phosphorylation on the non-cyclic electron transfer chain, then cyclic phosphorylation *in vivo* would produce ATP predominantly for processes other than CO_2 fixation[36]. However, if there is only one site of phosphorylation associated with non-cyclic electron flow, then cyclic phosphorylation would be obligatory for CO_2 fixation, as well as producing ATP for other metabolic processes[37].

The high ADP/O ratios found in carefully isolated chloroplasts[3,9,38–41] could be due to either two sites of phosphorylation on the non-cyclic electron flow chain, or to one site of phosphorylation and a contribution from endogenous cyclic phosphorylation. The low level of effect of added cyclic co-factors on the ADP/O ratio (Table II) and the demonstration that PMS catalysed photophosphorylation does not occur in the presence of added ferricyanide (Fig. 3), suggests that the contribution of cyclic phosphorylation under these conditions is small, and that there are two sites of ATP formation on the non-cyclic electron transfer pathway. The apparent endogenous cyclic phosphorylation seen in Fig. 3, Curve A, after the complete reduction of electron acceptor, was only about 10% of the normal non-cyclic rate, and this may well represent the amount of cyclic phosphorylation occurring in our non-cyclic system. This is in reasonable agreement with the estimate of Izawa and Good[32] of about 5–7%.

The suggestion that there are two sites of phosphorylation coupled to non-cyclic electron flow is further supported by the data obtained with oxidised diaminodurene as the electron acceptor, and from the effect of ATP. The use of oxidised diaminodurene as an electron acceptor gives a halving of the ADP/O ratio, as occurred in the original work of Saha *et al.*[27]. However, the demonstration of photosynthetic control and NH_4Cl uncoupling, which was not shown by Saha *et al.*[27], is important in showing a coupled electron transport for a Photosystem II catalysed phosphorylation.

The ATP data presented supports the theory of Izawa and Good[32] that the energy transfer inhibitor-insensitive part of the non-phosphorylating rate of electron transfer (*i.e.* the "basal" rate or that part of the State 2 uninhibited by ATP) is in-

capable of being coupled to subsequent phosphorylation initiated by the addition of ADP. That part of the State 2 electron transport that is sensitive to ATP inhibition results in coupled electron transport upon the addition of ADP. The permanently uncoupled electron transport may be a consequence of the rupture of the chloroplast envelope by osmotic shock, necessary to allow the penetration of ADP[12], and subsequent damage to the lamellar system[32]. Alternatively the uncoupled electron transport may be present *in vivo*. The ADP/O ratios calculated by the subtraction of the ATP-inhibited State 2 as a "basal" rate were generally 2.0–2.4. This may well be due to an ADP/O ratio of 2.0 and a small contribution of ATP by cyclic phosphorylation, approximately 10% of the non-cyclic phosphorylation.

If the ADP/O ratio is maximally 2.0 *in vivo* then cyclic phosphorylation would not be necessary for CO_2 fixation to proceed. This has indeed been suggested by several authors[42–44]. A recent paper on cyclic phosphorylation in isolated chloroplasts that fixed CO_2 (Schurmann *et al.*[45]) suggested that CO_2 fixation did need cyclic phosphorylation. This work was partly based on studies using antimycin A as a cyclic electron flow inhibitior. However, other reports[46–48] have shown antimycin A to stimulate CO_2 fixation, uncouple phosphorylation[49], and to act as an energy transfer inhibitor[50], so that the data are confusing. Also the chloroplasts used by Schurmann *et al.*[45] would appear to be freely permeable to added ATP (which chloroplasts retaining their outer membrane are not[12]) and the chloroplasts fixed CO_2 at rates of 4–12 μmoles CO_2/mg chlorophyll per h; this is rather low compared with *in vivo* rates of about 100 μmoles CO_2 /mg chlorophyll per h, and compared to the usually reported *in vitro* rates of 40–80 μmoles CO_2/mg chlorophyll per h[51]. From these considerations it would appear that the chloroplasts used by Schurmann *et al.*[45] were not very close to the *in vivo* state, and care should be taken in interpreting their results.

In conclusion this work suggests that carefully isolated chloroplasts, which may approximate those found *in vivo*, show two sites of phosphorylation on the non-cyclic electron transfer pathway, one associated with Photosystem II and the other with Photosystem I, and that if cyclic phosphorylation does occur during non-cyclic phosphorylation, it is not more than about 10% of the non-cyclic rate.

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REFERENCES

1 Arnon, D. I., Allen, M. B. and Whatley, F. R. (1954) *Nature* 174, 394–396
2 Arnon, D. I., Whatley, F. R. and Allen, M. B. (1958) *Science* 127, 1026–1034
3 Winget, G. D., Izawa, S. and Good, N. E. (1965) *Biochem. Biophys. Res. Commun.* 21, 438–443
4 Del Campo, F. F., Ramirez, J. and Arnon, D. I. (1968) *J. Biol. Chem.* 243, 2805–2809
5 Saha, S. and Good, N. E. (1970) *J. Biol. Chem.* 245, 5017–5021
6 Walker, D. A. (1964) *Biochem. J.* 92, 22c–23c
7 Jensen, R. G. and Bassham, J. A. (1966) *Proc. Natl Acad. Sci. U.S.* 56, 1095–1107
8 Kalberer, P. P., Buchanan, B. B. and Arnon, D. I. (1967) *Proc. Natl Acad. Sci. U.S.* 57, 1542–1549
9 Reeves, S. G., Hall, D. O. and West, J. (1972) in *Proc. 2nd Int. Congr. Photosynth. Res.* (Forti, G., Avron, M. and Melandri, A., eds), Vol. 2, pp. 1357–1369, Dr W. Junk N.V. Publishers, The Hague

10 Hall, D. O. (1972) *Nat. New Biol.* 235, 125–126
11 Walker, D. A. (1965) *Plant Physiol.* 40, 1157–1161
12 Heldt, H. W. (1969) *FEBS Lett.* 5, 11–14
13 Heber, U. and Santarius, K. A. (1970) *Z. Naturforsch.* 25, 718–728
14 Reeves, S. G. (1972) Ph.D. Thesis, University of London
15 Arnon, D. I. (1949) *Plant Physiol.* 24, 1–15
16 Delieu, T. and Walker, D. A. (1972) *New Phytol.* 71, 201–226
17 Hagihara, B. and Lardy, H. A. (1960) *J. Biol. Chem.* 235, 889–894
18 Adam, H. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 539–543, Academic Press, New York and London
19 Fiske, C. H. and SubbaRow, Y. (1925) *J. Biol. Chem.* 66, 375–400
20 Allen, R. J. (1940) *Biochem. J.* 34, 858–865
21 Adam, H. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 573–577, Academic Press, New York and London
22 Chance, B. and Williams, G. R. (1956) *Adv. Enzymol.* 17, 65–134
23 Demidov, E. D., Krupenko, A. N., Kulakov, A. A. and Bell, L. N. (1972) *FEBS Lett.* 21, 307–310
24 Avron, M. and Jagendorf, A. T. (1959) *J. Biol. Chem.* 234, 1315–1320
25 Jagendorf, A. T. and Margulies, M. (1960) *Arch. Biochem. Biophys.* 90, 184–195
26 Hauska, G. A., McCarty, R. E. and Racker, E. (1970) *Biochim. Biophys. Acta* 197, 206–218
27 Saha, S., Ouitrakul, R., Izawa, S. and Good, N. E. (1971) *J. Biol. Chem.* 246, 3204–3209
28 Avron, M., Krogmann, D. W. and Jagendorf, A. T. (1958) *Biochim. Biophys. Acta*, 30, 144–153
29 Shavit, N. and Herscovici, A. (1970) *FEBS Lett.* 11, 125–128
30 Reeves, S. G. and Hall, D. O. (1972) in *Proc. 6th Int. Congr. Photobiol., Bochum, Germany* (Schenk, G. O., ed.), Abstr. No. 284
31 Walz, D., Schuldiner, S. and Avron, M. (1971) *Eur. J. Biochem.* 22, 439–444
32 Izawa, S. and Good, N. E. (1968) *Biochim. Biophys. Acta* 162, 380–391
33 Frackowiack, B. and Kaniuga, Z. (1971) *Biochim. Biophys. Acta* 226, 360–365
34 Nobel, P. S., Chang, D. T., Wang, C., Smith, S. S. and Barcus, D. E. (1969) *Plant Physiol.* 44, 655–661
35 Strottman, H. (1972) in *Proc. 2nd Int. Congr. Photosynth. Res.* (Forti, G., Avron, M. and Melandri, A., eds), Vol. 2, pp. 1319–1327, Dr W. Junk N.V. Publishers, The Hague
36 Raven, J. A. (1969) *New Phytol.* 63, 1089–1113
37 Simonis, W. and Urbach, W. *Annu. Rev. Plant Physiol.*, in the press
38 Forti, G. (1968) *Biochem. Biophys. Res. Commun.* 32, 1020–1024
39 Horton, A. A. and Hall, D. O. (1968) *Nature* 218, 386–388
40 Miginiac-Maslow, M. and Moyse, A. (1969) in *Progr. Photosynth. Res.* (Metzner, H., ed.), Vol. 3, pp. 1203–1212, H. Laupp, Tübingen
41 West, K. R. and Wiskich, J. T. (1968) *Biochem. J.* 109, 527–532
42 Kandler, O. and Tanner, W. (1966) *Ber. Deutsch. Bot. Ges.* 79, 48–57
43 Gimmler, H., Neimanis, S., Eilmann, I. and Urbach, N. (1970) *Z. Pflanzenphysiol.* 64, 358–369
44 Raven, J. A. (1969) *J. Exp. Bot.* 22, 420–433
45 Schurmann, P., Buchanan, B. B. and Arnon, D. I. (1971) *Biochim. Biophys. Acta* 267, 111–124
46 Schacter, B. and Bassham, J. A. (1972) *Plant Physiol.* 49, 411–416
47 Ellyard, P. (1967) Ph.D. Thesis, Cornell University
48 Champigny, M. L., Mathieu, Y. and Miginiac-Maslow, M. (1972) in *Proc. 2nd Int. Congr. Photosynth. Res.* (Forti, G., Avron, M. and Melandri, A., eds), Vol. 2, pp. 1909–1916, Dr W. Junk N.V. Publishers, The Hague
49 Dreschler, Z., Nelson, N. and Neumann, J. (1969) *Biochim. Biophys. Acta* 189, 65–73
50 Izawa, S., Connolly, T. N., Winget, G. D. and Good, N. E. (1969) *Brookhaven Symp. Biol.* 19, 169–184
51 Cockburn, W., Walker, D. A. and Baldry, C. W. (1968) *Plant Physiol.* 43, 1415–1418