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EVIDENCE FOR TWO PHOSPHORYLATION SITES ASSOCIATED WITH THE ELECTRON TRANSPORT CHAIN OF CHLOROPLASTS

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SUMMARY

1. Photophosphorylation associated with ferricyanide reduction in isolated pea chloroplasts was studied with the aim of determining the number of phosphorylation sites in the non-cyclic pathway of photosynthetic electron flow.

2. Improved methods of chloroplast isolation enabled photosynthetic control ratios as high as 6.6 to be measured. ADP/O ratios were in the range 1.22–1.66, *i.e.* clearly greater than 1.0 which should be the maximum value if there is one phosphorylation site.

3. Addition of ADP during simultaneous measurement of oxygen evolution and pH changes stimulated O_2 evolution, but not proton production. It is reasoned that one phosphorylation site in non-cyclic electron flow would stimulate proton production whereas two sites would not.

4. Inhibition of the Hill reaction rate by ATP is reported, confirming earlier observations. An explanation for this observation and also the observation that during noncyclic reduction of ferricyanide, State 4 rates of oxygen evolution are slower than Hill reaction rates, is offered in terms of the chemiosmotic hypothesis.

INTRODUCTION

For some time it has been accepted that there is one phosphorylation, or energy-conserving, site associated with non-cyclic photosynthetic electron flow, but recent reports have suggested that there may be two phosphorylation sites[1–3]. There are several methods for determining the number of photosynthetic phosphorylation sites. For example Schwartz[3], using a sensitive pH electrode, obtained a value of 2.0 for the ratio of translocated protons to transported electrons (*i.e.*$\rightarrow H^+/e$ ratio) during the reduction of both ferricyanide and $NADP^+$ by spinach chloroplasts. Equating each proton-translocating site to a phosphorylation site[4], Schwartz claimed this as evidence for two sites, although there may be some doubt concerning the interpretation of these results[5]. Similarly Jagendorf and Uribe[2], and Crofts[6] have suggested that the initial alkalinity, followed by acidification, observed on illumination of

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Abbreviations: ferricyanide, $K_3Fe(CN)_6$; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; TES, *N*-tris(hydroxymethyl))methyl-2-aminoethanesulphonic acid.

chloroplasts in the presence of ferricyanide, indicated two proton translocating sites.

Measurement of mole ratio of ATP formed, to oxidant reduced, (P/2*e* ratio) also enables the number of phosphorylation sites to be determined. There have been reports of the P/2*e* ratio exceeding 1.0, indicating more than one phosphorylation site[1,7–9]. Often the absolute values of the P/2*e* ratios do not greatly exceed 1.0 and several authors[1,10] have adjusted the values. The adjustment was made on the claim that phosphorylating and non-phosphorylating electron flow were different and occurring simultaneously in chloroplasts. These adjusted P/2*e* ratios have been criticized by Del Campo *et al.*[11], who present a strong case for the ratio $P/2e = 1.0$ and for one phosphorylation site.

Measurement of the ratio ATP/NADPH during non-cyclic photophosphorylation and reduction of $NADP^+$, with chloroplasts prepared by the method of Jensen and Bassham[12], also gave values greater than 1.0 (ref. 13).

The demonstration of photosynthetic control[14] enabled ADP/O ratios for non-cyclic photophosphorylation to be measured in a manner similar to that used to measure ADP/O ratios in phosphorylating mitochondria. No correction of the values (which ranged from 1.0 to 1.3) were made and as there was no evidence which indicated that these values were an over estimate, it was suggested that there are two photosynthetic phosphorylation sites. It is the purpose of this communication to present further evidence supporting two sites of photosynthetic phosphorylation. The evidence and interpretation is not dependent on the chemiosmotic, or any other, particular hypothesis of phosphorylation.

METHODS

2–3-week-old pea seedlings (*Pisum sativum* L. cv. Green Feast) were grown as previously described[14]. 10 g of leaves were ground in either a Waring Blendor or Sorvall Omnimixer for 3–4 s., in 100 ml of an ice-cold medium containing 0.4 M sucrose, 1 mM $MgCl_2$, 30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) – KOH buffer (pH 7.3) and 0.5% bovine serum albumin. In some experiments the concentration of sucrose was reduced from 0.4 to 0.25 M. The brei was filtered through a single layer of muslin or "miracloth" and centrifuged for 2–3 min at 300 × *g* in a Sorvall RC2 refrigerated centrifuge. A chloroplast pellet was formed by centrifuging the resulting supernatant. The centrifuge was accelerated from rest to 4000 × *g* and back to rest in an elapsed time of 4 min; chloroplasts were resuspended in 2 ml of 0.4 M or 0.25 M sucrose containing 0.5% bovine serum albumin and stored in an ice bath.

The assay medium contained, 0.25 M sucrose, 10 mM K_2HPO_4–KH_2PO_4, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) buffer, 5 mM $MgCl_2$ (adjusted to pH 7.6 or pH 8.0 with KOH). The pH of ADP was adjusted with KOH to correspond to that of the assay medium used. The concentration of ADP was assayed enzymically[14].

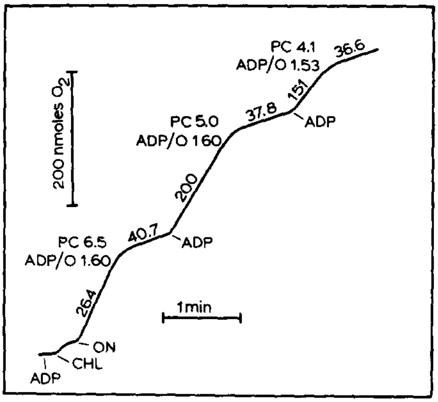
The apparatus and method for measurement of O_2 evolution and pH changes was similar to that described previously[14], except that the reaction vessel was constructed of perspex (lucite). Calibration of pH traces was achieved by adding standardized alkali at the completion of each experiment and back titrating with standard acid. The oxygen electrode used was calibrated by the method of Robinson and Cooper[15].

RESULTS

Light-induced electron flow in isolated chloroplasts is coupled to phosphorylation in a manner analogous to oxidative phosphorylation in isolated mitochondria.

Fig. 1 shows a polarographic tracing of O_2 evolution by isolated pea chloroplasts during the reduction of ferricyanide. The rate of O_2 evolution is governed by the presence or absence of ADP. Three additions of ADP were made, the first before the light was turned on. The rate of O_2 evolution in the presence of ADP is rapid and is termed the State 3 rate and the rate of O_2 evolution after ADP has been phosphorylated is slow and is termed the State 4 rate. This control of oxygen evolution by the level of phosphate acceptor (ADP) in the medium has been defined as photosynthetic control[14]. The ratio State 3/State 4 is known as the photosynthetic control ratio and together with an ADP/O ratio has been calculated for each addition of ADP.

Coupling appears to be tighter when ADP is added prior to illumination, as the control ratio calculated for the first addition of ADP was greater than that for the second addition of ADP. The concentration of the third addition of ADP was insufficient to show optimal photosynthetic control.



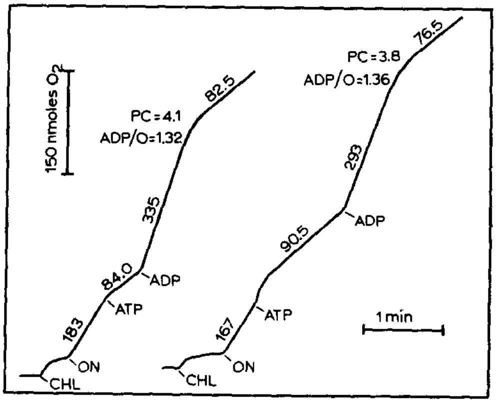


Fig. 1. Polarographic tracing of O_2 evolution by pea chloroplasts showing phosphorylation of ADP (added before chloroplasts, CHL) followed by further addition of ADP. O_2 evolution is indicated by an upward deflection of the trace. The medium (2.33 ml) contained 250 mM sucrose, 10 mM potassium phosphate, 5 mM $MgCl_2$, 10 mM HEPES-KOH, 1.3 mM ferricyanide and chloroplasts at a chlorophyll concentration of 67 µg/ml. The pH was 7.6. The first two additions of ADP were to a concentration of 0.18 mM and the third addition to a concentration of 0.09 mM. Numbers along trace are nmoles O_2/min. PC = photosynthetic control ratio.

Fig. 2. O_2 evolution by chloroplasts showing the effect of ATP on chloroplast activity. The medium (2.13 ml) contained 250 mM ~~sucrose~~, 10 mM potassium phosphate, 5 mM $MgCl_2$, 10 mM HEPES-KOH, 1.4 mM ferricyanide and chloroplasts at a chlorophyll concentration of 125 µg/ml. The pH was 7.6. In Curve A, ATP (0.18 mM) was added during the Hill reaction, followed by phosphorylation of 0.25 mM ADP. Curve B shows a similar experiment except that the concentration of ATP was increased to 0.88 mM. The increase in O_2 concentration observed immediately on addition of ATP in this experiment was due to dissolved O_2 in the added ATP. Numbers along trace are nmoles O_2/min.

TABLE I

CALCULATED ADP/O AND PHOTOSYNTHETIC CONTROL RATIOS

Conditions of experiments were similar to those described for Fig. 1.

Experiment	*ADP/O ratio*	*Control raito*
1	1.45	4.0
	1.63	4.6
2	1.22	4.1
	1.28	4.1
3	1.55	5.0
	1.62	4.1
	1.62	3.7
4	1.66	6.6
	1.60	6.0
5	1.55	5.8
6	1.50	4.1
7	1.43	5.2

Table I summarizes the results of several such experiments. Photosynthetic control ratios and ADP/O ratios are higher than those reported previously[14]. ADP/O ratios are clearly greater than 1.0 although they do not approach 2.0 which should be the maximum value if there are two photosynthetic phosphorylation sites.

Fig. 2 shows two experiments in which ATP was added to tightly coupled chloroplasts during electron flow in the absence of ADP, *i.e.* during the Hill reaction. The Hill reaction rate was reduced approx. 50% when ATP was added. The concentration of exogenous ATP needed to produce a large inhibition of the Hill reaction rate was approximately the same as the concentration of ADP needed to demonstrate high photosynthetic control ratios. Addition of larger amounts of ATP did not increase the inhibition (Fig. 2, Curve B). The ability of chloroplasts to phosphorylate ADP efficiently and to exhibit coupling was not impaired by the prior addition of ATP (Fig. 2) as ADP/O and control ratios comparable to those reported in Table 1, were observed on subsequent addition of ADP.

Fig. 3 shows an experiment in which both O_2 evolution and pH changes were monitored simultaneously during ferricyanide reduction. The chloroplasts were tightly coupled, as the addition of ADP gave a rapid State 3 rate of O_2 evolution followed by a slow State 4 rate and hence relatively high control ratio. When ferricyanide is reduced, there is a net production of protons according to the equation:

$$2\,H_2O + 4\,Fe(CN)_6^{3-} \xrightarrow{\text{Light}} O_2 + 4\,Fe(CN)_6^{4-} + 4\,H^+ \qquad (1)$$

Thus, in Fig. 3 the rate of proton release during the Hill reaction (454 nequiv H^+/min) is approximately equivalent to the rate of O_2 evolution (108 nmoles O_2/min). When

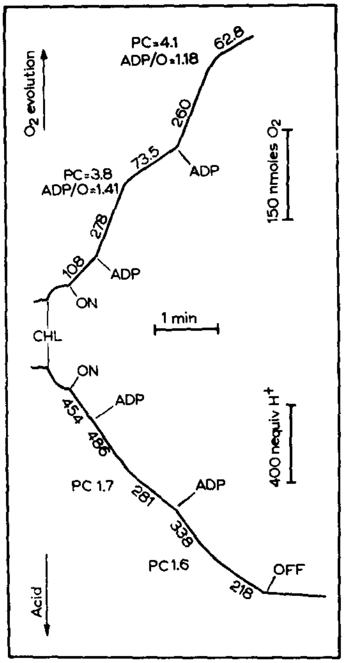


Fig. 3. Simultaneous recording of O_2 evolution and changes in pH during light-induced reduction of ferricyanide. The medium (2.13 ml) contained 250 mM sucrose, 10 mM potassium phosphate, 5 mM $MgCl_2$, 10 mM HEPES–KOH, 1.3 mM ferricyanide and chloroplasts at a chlorophyll concentration of 134 μg/ml. The pH was 7.6. ADP was added to a concentration of 0.16 mM. Numbers along the O_2 electrode trace are nmoles O_2/min and along the pH trace are nequiv H^+/min.

ADP was added, the rate of O_2 evolution increased, but the rate of proton production did not match this increase as some protons were consumed during the phosphorylation of ADP according to the equation:

$$P_i + ADP + n\,H^+ \rightarrow ATP \qquad (2)$$

Fig. 4 (Curve A) shows an experiment similar to that described in Fig. 3 except that the pH was adjusted to 8.04 before beginning the experiment. At pH values above 8.0 the value of n in Eqn 2 above is unity[16,17]. Although the chloroplasts were tightly coupled and the addition of ADP more than doubled the rate of O_2 evolution, there was no corresponding increase in the rate of acidification. The rate of acidification actually declined, and decreased further, as the rate of O_2 evolution indicated a return to non-phosphorylating conditions. The control ratio calculated from the pH trace (2.1) was consequently lower than that calculated from the oxygen electrode trace (5.7). A second addition of ADP was made, stimulating O_2 evolution and also the rate of proton production.

In a second experiment of this type, (Fig. 4, Curve B) ADP was added to the chloroplasts before the light was turned on. As is usual under these conditions (see ref. 14) the State 3 rate of O_2 evolution is somewhat faster than the State 3 rate obtained on adding ADP during the Hill reaction. None the less, the initial State 3 rate

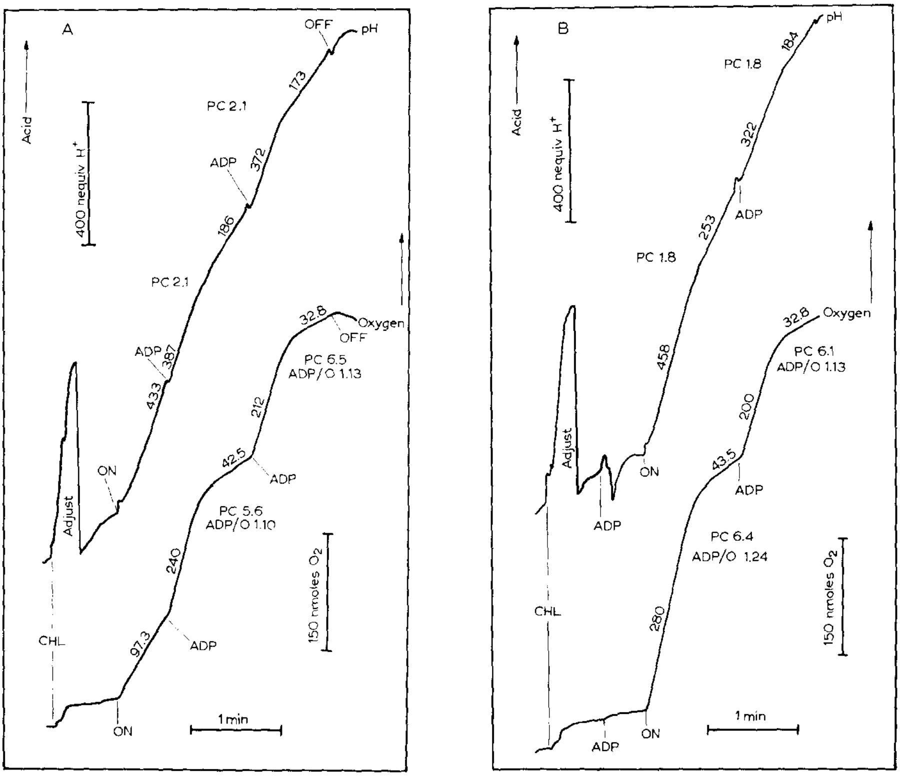


Fig. 4. O_2 evolution and pH changes at pH 8.0. ADP was added during the Hill reaction (A) and before illumination (B). ADP was added to a concentration of 0.14 mM (both additions) in A and a concentration of 0.14 mM (first addition) and 0.28 mM (second addition) in B. The medium (2.33 ml) was identical in each experiment and contained 250 mM sucrose, 10 mM potassium phosphate, 5 mM $MgCl_2$, 10 mM HEPES–KOH, 2.15 mM ferricyanide and chloroplasts at a chlorophyll concentration of 42 μg/ml. The initial pH was 8.04 and final pH 7.94. Numbers along the O_2 electrode trace are nmoles O_2/min and along the pH trace are nequiv H^+/min.

of proton production observed under these conditions (Curve B) is close to the Hill reaction rate (Curve A) and only slightly faster than the first State 3 rate in curve A thus confirming that ADP does not stimulate proton production. Note that the second addition of ADP in both experiments stimulated proton production. Precise agreement between the stoichiometry of O_2 evolution and proton production was not always achieved. The rate of proton production was often lower than expected. The assay medium in these experiments was sufficiently buffered so that the pH did not decline more than 0.1 pH unit from the initial pH (8.04). Changes in the buffering capacity or a small amount of O_2 uptake not linked to proton production may have been responsible for the discrepancy. Note that values of ADP/O ratios shown on Fig. 4 are lower than those given in Table I. However, the value of the ADP/O ratio is pH dependent and is maximal at pH 7.6 (Figs 1–3, Table I) and lower at pH 8.0 (Fig. 4) and pH 7.2 (ref. 14).

DISCUSSION

The experiment outlined in Fig. 4 is interpreted as indicating that there are two phosphorylation sites in the non-cyclic photosynthetic electron transport pathway. If there was one phosphorylation site, the initiation of phosphorylation would result in the consumption of one proton (Eqn 2 above) and the release of two protons for every two electrons transported (Eqn. 1 above). Hence the rate of acidification would increase, but only half as much as the rate of O_2 production. If there were two phosphorylation sites then two protons would be consumed for every two electrons transported (Eqn 2) and two protons would be released (Eqn 2). Thus the rate of acidification would not increase on initiation of phosphorylation, but should decline on exhaustion of ADP (Fig. 4).

The quantitative relationships of proton production and oxygen evolution are not important to the argument. The crucial point is that O_2 evolution is stimulated by the addition of ADP. This stimulation of O_2 evolution could have come only from phosphorylating chloroplasts. If there were one phosphorylation site, the rate of proton production would increase. However, if there were two phosphorylation sites any stimulated proton production would be exactly cancelled by the loss of protons in the phosphorylation reaction, *i.e.* there should be no increase in proton production on the initiation of phosphorylation, a prediction which is confirmed. We consider that this experiment together with the fact that ADP/O ratios are greater than 1.0 (Table I) provide convincing evidence for two photosynthetic phosphorylation sites. Hall *et al.*[18], have recently confirmed our earlier observations[14], that ADP/O ratios for ferricyanide-supported phosphorylation are greater than 1.0 and also measured ADP/O ratios up to 2.1 and 1.9 during the photoreduction of $NADP^+$ and methylviologen, respectively.

An explanation for the subsequent decrease (a slight decrease shown in Fig. 4 Curve A, greater decrease in other experiments Table II) in the rate of acidification after addition of ADP is not immediately apparent. It is probable that three types of chloroplasts contribute to the Hill reaction rate of oxygen evolution and acidification, namely (a) completely uncoupled; (b) partly (loosely) coupled; (c) completely (tightly) coupled chloroplasts.

Any change in the rate of acidification on addition of ADP could come only

TABLE II

EFFECTS OF ADP ADDITION ON RATES OF O_2 EVOLUTION AND ACIDIFICATION AT DIFFERING pH VALUES

Conditions of experiments were similar to those described for Figs 3 and 4A.

Experiment	*pH*	*O_2 evolution (μmoles O_2/h/mg chlorophyll)*		*Control ratio*	*Acidification (μequiv H^+/h/mg chlorophyll)*	
		Hill reaction	*State 3*		*Hill reaction*	*State 3*
1	7.6	28.5	60.5	4.3	95.5	102.0
2	8.0	30.2	61.2	4.3	132.1	109.1
3	8.1	32.4	63.6	4.0	158.3	111.2

from partly coupled chloroplasts because uncoupled chloroplasts would maintain their rate of ferricyanide reduction (*i.e.* proton production) and tightly coupled chloroplasts would not contribute because the increased rate of acidification would be neutralised by proton consumption (Eqn 2) during phosphorylation.

It is suggested that the small inhibition of proton production is due to ATP formation by partly coupled chloroplasts. Prior to the addition of ADP these chloroplasts were contributing to proton production ("leaky" chloroplasts), but during phosphorylation they may phosphorylate ADP and become effectively coupled*.

Mitchell's chemiosmotic hypothesis of phosphorylation[4] allows for such an explanation. The Hill reaction rate of acidification is due to proton production from ferricyanide reduction (Eqn 1) and continues because of back leakage of inwardly translocated protons. If phosphorylation offers a lower resistance to the outward movement of the inwardly translocated protons, than does back leakage, phosphorylation would occur in preference to leakage. Thus some ferricyanide reduction observed during the Hill reaction is now coupled to phosphorylation, *i.e.* some proton release during the Hill reaction would be cancelled, by proton uptake in the phosphorylation reaction.

This effect is distinct from the initial inhibition of electron flow by ATP (Fig. 2). If the reduction in the rate of proton production during State 3 was due to ATP inhibition of electron flow, we would expect a similar inhibition of O_2 evolution. However, no such inhibition was observed, until most of the ADP had been phosphorylated, *i.e.* when the ATP/ADP ratio was high.

We suggest that the inhibition of the Hill reaction rate by ATP is due to the operation of a specific proton translocating ATPase as proposed by Mitchell. Minimal hydrolysis of ATP may increase the proton concentration inside the phosphorylating space. The increased proton concentration would increase the resistance to further inward translocation of protons by the electron transfer chain in the membrane. This increased resistance would in turn inhibit electron flow, *i.e.* ferricyanide reduction or oxygen evolution. A second addition of ADP would allow phosphorylation to begin again, releasing the inhibition stimulating proton production and oxygen evolution.

This interpretation explaining the inhibition of the Hill reaction rate by ATP and slow State 4 rate assumes changed chloroplast properties under phosphorylating conditions. If the interpretation is valid it is incorrect to assume that the Hill reaction is a separate non-phosphorylating path of electron flow[1]. Some electron flow is indeed uncoupled, but mathematical adjustment of $P/2e$ ratios is not warranted.

It should be noted that there is an apparent inconsistency in the results. While the results of the pH electrode tracing indicate that there are exactly two phosphorylation sites, the values for the ADP/O ratios obtained in the same experiment

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* The degree of inhibition of acidification, observed on addition of ADP during the Hill reaction, is pH dependent. At pH values of 8.0 and greater a decrease in the rate of acidification was observed. If our interpretation of this result is correct the degree of inhibition will depend on the number of uncoupled chloroplasts present in the preparation. A high percentage of uncoupled chloroplasts, *i.e.* a low photosynthetic control ratio should result in a relatively large decrease in the rate of acidification. At pH values less than 8.0, the value of n in Eqn 2 above is less than unity and we observe progressively greater stimulation of acidification on addition of ADP as the pH is lowered.

(Fig. 4) were less than 2.0. However, the ratio takes the total amount of O_2 into account, while the data of Fig. 4 (and our interpretation of it) takes only the extra (or added) H^+ into account.

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