Photosynthetic Control by Isolated Pea Chloroplasts

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Isolated pea chloroplasts undergo both cyclic and non-cyclic electron flow. Both processes are coupled to photophosphorylation. During non-cyclic flow the rate of oxygen production showed ADP-governed 'photosynthetic control' analogous to respiratory control of isolated mitochondria. Measurements of ADP/O and photosynthetic control ratios yielded values of 1.1-1.3 and 2.5-5.7 respectively. 'Photosynthetic control' was shown to be dependent on the intactness of the chloroplasts.

It is known that isolated plant mitochondria are capable of oxidative phosphorylation and that the rate of substrate oxidation is dependent on the concentration of phosphate acceptor present in the medium (Wiskich & Bonner, 1963). Chance & Baltscheffsky (1958) have defined this dependence as 'respiratory control'. Demonstration of 'respiratory control' on a polarographic oxygen-electrode trace permits measurement of respiratory control ratios and of ADP/O ratios. The value of these ratios may be a measure of the integrity of the phosphorylating membranes.

Photosynthetic phosphorylation, where a light-dependent reduction of electron acceptors by isolated chloroplasts is 'coupled' to ATP production, has also been demonstrated (see reviews by Hill, 1965; Avron, 1967). Several reports have clearly shown that the inclusion of phosphate and a phosphate-acceptor system stimulates the rate of the Hill reaction with ferricyanide (Arnon, Whatley & Allen, 1958; Avron, Krogmann & Jagendorf, 1958; Good, 1960) and with NADP (Davenport, 1960) as electron acceptors.

However, though these reports show coupling between electron transfer and photosynthetic phosphorylation, they do not provide evidence of 'control'. To demonstrate 'control' it is essential to establish that the rate of electron transfer is not only stimulated by the addition of phosphate acceptor, but also that the rate is decreased on exhaustion of phosphate acceptor, and can be stimulated again by further addition.

It is the purpose of this paper to describe a method for the isolation of relatively intact chloroplasts from pea leaves, and to show that both cyclic and non-cyclic electron flow are coupled to phosphorylation. It is further shown that oxygen production during non-cyclic electron flow is subject to 'control' in a manner analogous to 'respiratory control'.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* L. var. Green Feast) were grown in vermiculite in a glasshouse. Leaves (10 g.) from 2-3-week-old plants were placed in the cold-room at 0-5° for 5 min. and then blended for 5 sec. in 100 ml. of 0.4 M-sucrose containing MgCl₂ (1.0 mM), EDTA (disodium salt) (1.0 mM), tris(hydroxymethyl)methylethanesulphonic acid (30 mM) and bovine serum albumin (0.5%), pH 7.2. The brei was filtered through muslin and centrifuged for 2-3 min. at 300 g_{av.} in a Sorvall RC2 refrigerated centrifuge to remove coarse debris and the supernatant was centrifuged again at 1000 g_{av.} for 10 min. The resulting pellet, resuspended in 10 ml. of 0.4 M-sucrose containing bovine serum albumin (0.5%), was layered over 1.0 M-sucrose and centrifuged again at 1000 g_{av.} The chloroplasts were then suspended in 2 ml. of 0.4 M-sucrose containing bovine serum albumin (0.5%) and kept in an ice bath.

The reaction vessel (5 ml.) consisted of a glass cell and a Teflon plug with three holes to permit simultaneous measurement of oxygen concentration [Titron (Melbourne, Vic., Australia) oxygen electrode connected to a 1 mV Honeywell recorder] and of pH [Titron combined pH electrode connected to a Beckman Research pH-meter and recorder] and to admit reactants. The pH changes measured were calibrated by adding known amounts of standard acid or alkali at the end of each experiment. The reaction mixture was vigorously stirred with a magnetic stirrer, and maintained at 25° by a gasket of circulating water. The oxygen concentration of the medium was lowered by bubbling oxygen-free nitrogen through it before the addition of chloroplasts.

Chloroplasts were broken in an MSE 100 W ultrasonic disintegrator. The probe was pre-cooled to 0-5° and the vessel containing chloroplasts [in 0.4 M-sucrose containing bovine serum albumin (0.5%)] was placed in an ice bath to minimize heating effects during disintegration.

Light (from a 100 W tungsten source) was passed through a 10 cm. water bath and reached the vessel at an intensity of 6.22×10^{-2} cal./cm.²/min.

Chlorophyll concentration was determined by the method of Arnon (1949); ADP concentration was determined enzymically (Wiskich, Young & Biale, 1964). The data for oxygen evolution are presented as 'percentage saturation'.

with oxygen. The oxygen concentration of air-saturated medium (100%) is taken as 250 μM .

Biochemicals were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Carbonyl cyanide *m*-chlorophenylhydrazone was a gift from Dr P. G. Heytler (E. I. du Pont de Nemours and Co., Wilmington, Del., U.S.A.).

RESULTS

Examination under the phase-contrast microscope showed that most of the chloroplasts had an intact outer membrane (class I chloroplasts in the terminology of Spencer & Unt, 1965). Electron-microscope studies have confirmed this finding (K. R. West & J. T. Wiskich, unpublished work). Even though most of the chloroplasts were intact, they were capable of supporting a light-induced pH shift similar to that shown by Jagendorf & Hind (1963) and by Neumann & Jagendorf (1964) for 'broken' spinach chloroplasts. The pH shift was activated by cofactors of cyclic photosynthetic phosphorylation and was detected as an increased alkalinity in the medium (Fig. 1). The addition of Triton X-100, an uncoupling agent (Neumann & Jagendorf, 1964), completely abolished the light-induced pH shift.

In a medium containing phosphate, phosphate acceptor and Mg^{2+} ions, cyclic photosynthetic phosphorylation was demonstrated by following pH changes. [For every molecule of ADP phosphorylated approximately one proton is consumed, causing an increase in pH (Chance & Nishimura, 1967).] Fig. 2 shows the light-induced pH shift

Fig. 1. Light-induced pH rise by pea chloroplasts in an unbuffered sucrose medium (400 mM). The additions were phenazine methosulphate (20 μM) and Triton X-100 (0.26%). The chloroplasts were present at a chlorophyll concentration of 0.31 mg./ml.

when the light is turned on in the presence of phenazine methosulphate; ADP was added during the subsequent dark period. When the system was illuminated again, the pH increase was maintained until all the ADP was phosphorylated. A second addition of ADP duplicated this effect. This result shows that ATP was formed during cyclic electron flow (see Karlisch & Avron, 1967).

Non-cyclic electron flow with ferricyanide as electron acceptor was demonstrated by monitoring oxygen evolution during the Hill reaction. Fig. 3 shows that the amount of oxygen evolved was governed by the amount of ferricyanide present. The light was turned off and on again (curve B in Fig. 3) after all the ferricyanide had been reduced, showing that ferricyanide was not reoxidized in the dark. Although these intact chloroplasts were capable of non-cyclic electron flow with ferricyanide

Fig. 2. pH tracing demonstrating cyclic phosphorylation, with phenazine methosulphate (PMS) (16 μM) as a cofactor. The medium (1.5 ml.) contained sucrose (265 mM), tris-phosphate buffer, pH 7.3 (6.6 mM), EDTA (0.66 mM), MgCl_2 (0.66 mM) and chloroplasts at a chlorophyll concentration of 0.5 mg./ml. ADP (0.1 mM) was added to initiate phosphorylation and 5 $\mu\text{l.}$ of 0.1 N-HCl (500 m-equiv. of H^+) to measure the buffering capacity of the medium.

Fig. 3. Polarographic tracing of oxygen production by pea chloroplasts. Curves A and B show oxygen production at ferricyanide concentrations of 2.2 mM and 1.1 mM respectively. The medium (4.5 ml.) contained sucrose (280 mM), EDTA (0.67 mM), MgCl_2 (0.67 mM), tris-phosphate buffer, pH 7.3 (3.4 mM), and chlorophyll at a chloroplast concentration of 0.11 mg./ml.

Fig. 4. Polarographic tracing of oxygen production showing 'photosynthetic control' by pea chloroplasts with ferricyanide (2.3 mM) as an electron acceptor. The medium (4.4 ml.) contained sucrose (275 mM), K_2HPO_4 - KH_2PO_4 buffer, pH 7.2 (6.8 mM), $MgCl_2$ (3.4 mM), EDTA (0.68 mM), tris-HCl buffer, pH 7.2 (6.8 mM), and chloroplasts (Chl) at a chlorophyll concentration of 0.06 mg./ml. ADP was added at a concentration of 0.096 mM and carbonyl cyanide *m*-chlorophenylhydrazone (CCP) at 4 μ M. PC, Photosynthetic control ratio. Numbers along the trace are μ M-oxygen/min.

as electron acceptor, measurement of phosphorylation by pH changes was complicated by the release of protons during ferricyanide reduction. However, if non-cyclic electron flow in these chloroplasts was coupled to phosphorylation, the rate of oxygen evolution would be affected by the presence or absence of ADP.

Fig. 4 shows a polarographic oxygen-electrode trace of oxygen evolution by pea chloroplasts with ferricyanide as the electron acceptor. It can be seen that the first addition of ADP stimulated the rate of oxygen evolution and that the stimulated rate subsequently decreased. This effect was repeated by a subsequent addition of ADP. This is interpreted as 'photosynthetic control' by isolated chloroplasts, analogous to 'respiratory control' shown by isolated mitochondria. In the convention of Chance & Williams (1955) the ADP-stimulated rate is referred to as state III and the lower rate after all the ADP has been phosphorylated is referred to as state IV. Photosynthetic control ratios (equivalent to respiratory control ratios; Chance & Baltscheffsky, 1958) can be derived from

Table 1. Calculated ADP/O and photosynthetic control ratios

Conditions of experiments were the same as described for Fig. 4. The numbers in parentheses indicate successive additions of ADP. Unless stated otherwise all estimations were made at pH 7.2.

Expt. no.	ADP/O ratio	Photosynthetic control ratio
1	1.36 (1)	2.5
	1.30 (2)	2.5
2	1.06 (1)	2.4
	1.04 (1)	2.4
	1.08 (1)	2.2
3	1.27 (1)	3.5*
	1.21 (2)	2.3
	1.15 (3)	2.4
4	1.21 (1)	2.0 (pH 8.0)
	1.05 (1)	1.5 (pH 8.3)
	1.22 (1)	2.6
	1.32 (1)	3.0
5	1.08 (1)	2.8
	1.18 (2)	2.8
	1.18 (1)	4.9*

* ADP was added before addition of chloroplasts, as for curve B in Fig. 5.

the state III/state IV ratio. Fig. 4 also shows that the addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone during state IV photosynthesis gives a continuing stimulation of oxygen production. The short period of rapid oxygen evolution observed immediately on illumination (Fig. 4) may be due to endogenous phosphate acceptor. This initial effect was not evident under non-phosphorylating conditions (Fig. 3). Photosynthetic control data also permitted the calculation of ADP/O ratios (equivalent to P/2e ratios).

Table 1 shows values calculated for the ADP/O and photosynthetic control ratios in a number of experiments. The photosynthetic control ratio varied considerably among experiments, but was also dependent on the time of addition of ADP. Addition of ADP before and after illumination is shown in Fig. 5. Photosynthetic control ratios as high as 4.9 were obtained when ADP was added before illumination, whereas addition of ADP after illumination normally gave values lower than 3.

The suggestion that the demonstration of photosynthetic control requires a chloroplast preparation containing a high proportion of intact chloroplasts was tested in the following way. A chloroplast suspension that initially showed a high photosynthetic control ratio was subjected to ultrasonic disintegration for periods of 5, 10 and 15 sec. Table 2 shows the effect of disintegration on the Hill reaction rate (before addition of ADP), state III

rate, state IV rate, photosynthetic control ratio and ADP/O ratio. A striking decrease in both photosynthetic control and ADP/O ratios occurred, and the Hill reaction rate and state IV rate increased sharply. The state III rate was not markedly affected by disintegration, and therefore only the increased state IV rate led to a loss of photosynthetic control. This result shows that, though the rate of the Hill reaction with broken chloroplasts may be stimulated by ADP, 'photosynthetic control' will not necessarily be evident. It should be noted that the Hill reaction rates and state IV rates (Table 2) are not comparable.

DISCUSSION

Isolated mitochondria can, under certain circumstances, phosphorylate ADP without evidence of

Fig. 5. Polarographic tracing of oxygen production by pea chloroplasts. The composition of the medium was the same as in Fig. 4 except that the chloroplasts were present at a chlorophyll concentration of 0.04 mg./ml. Trace A shows the standard method of assay for photosynthetic control; ADP (0.102 mM) was added after the chloroplasts (Chl) had been added and illuminated. Trace B shows the effect of adding ADP before adding the chloroplasts (Chl) and before illumination. PC, Photosynthetic control ratio. Numbers along the trace are μM -oxygen/min.

respiratory control. However, 'respiratory control' and ADP/O ratios close to the expected maximum values are regarded as indicators of intact mitochondria. D. L. Millard (personal communication) has shown that plant mitochondria showing good respiratory control are morphologically intact when observed with the electron microscope. These data suggest that the isolated mitochondria closely resemble those of the intact cell. Similar data have not been reported for isolated chloroplasts and it has been difficult to get some measure of biochemical integrity. Jensen & Bassham (1966) and Walker (1965) have suggested that the rate of carbon dioxide fixation by isolated chloroplasts is related to the intactness of the preparation. Chloroplasts with an intact outer membrane (termed 'class I chloroplasts' by Spencer & Unt, 1965) were capable of fixing carbon dioxide at a rate 60% of that shown by intact leaves (Jensen & Bassham, 1966). 'Photosynthetic control' may reflect the integrity of the chloroplasts, as the preparations used in this work were mostly class I chloroplasts. This information suggests that these isolated chloroplasts resembled those of the intact cell and were fully functional, but data on rates of carbon dioxide fixation to confirm this are not yet available.

Most studies of ATP synthesis by isolated chloroplasts have used 'broken' chloroplasts, consisting mainly of swollen fragments of grana from broken chloroplasts (Turner, Black & Gibbs, 1962; Neumann & Jagendorf, 1964; Karlisch & Avron, 1967), or at best a '1000g fraction' (Kroghmann, Jagendorf & Avron, 1959; Stiller & Vennesland, 1962; Winget, Izawa & Good, 1965), which Leech (1963) has shown contains a very heterogeneous chloroplast population.

The technique for isolation of chloroplasts described in the present paper yields a preparation with many intact chloroplasts. The light-induced pH shift and phosphorylation previously reported for 'broken chloroplasts' during cyclic electron flow was observed (Figs. 1 and 2). Phosphorylation during non-cyclic electron flow was detected by

Table 2. *Effect of ultrasonic disintegration on chloroplast activity*

Conditions for assay were the same as for Fig. 4. Activity was measured immediately after the period of disintegration. ADP was added after illumination, as in Fig. 4.

Duration of ultrasonic disintegration (sec.)	Rate ($\text{m}\mu\text{moles of O}_2/\text{min.}$)				
	Hill reaction rate	State III rate	State IV rate	Photosynthetic control ratio	ADP/O ratio
0	22.9	54.0	9.5	5.7	1.28
5	30.3	58.0	21.5	2.7	1.00
10	34.7	56.0	29.3	1.9	0.88
15	37.8	52.5	31.3	1.7	0.58

measuring pH changes, but, as mentioned above, quantitative evaluation of these pH changes during ferricyanide reduction proved difficult.

The demonstration of 'photosynthetic control' (Fig. 4) has not been previously reported. In the work described here it appeared that morphological integrity rather than the time taken between cell disruption and assay was the critical factor. An alternative method of isolation (small scale) that shortened the preparation time to 13 min. (as compared with 35–40 min. with the standard method) did not improve the photosynthetic control ratios. The data of Table 2 also indicate the importance of 'intactness' of chloroplasts in demonstrating 'photosynthetic control'.

The value of the photosynthetic control ratio obtained with chloroplasts stored in ice with bovine serum albumin as a stabilizing agent decreased very slowly with time. 'Photosynthetic control' was quickly lost in those preparations of pea chloroplasts that did not contain bovine serum albumin. This protection may perhaps indicate the release of an uncoupler that can bind with bovine serum albumin. Some degree of 'control' could be restored by recenterfuging with 0.1% cysteine. This result may support the suggestion of Newton (1962) that a functional thiol group is involved in photophosphorylation.

'Respiratory' or 'photosynthetic control' does not provide any direct indication of the number of phosphorylation sites because the rate-limiting site controls the rate of electron flow. However, the ADP/O ratios, as reported here, should reflect the number of phosphorylation sites. Evidence on the number of phosphorylation sites in photosynthesis has come previously from the measurement of P/2e ratios (Krogmann *et al.* 1959). Measurement of overall P/2e ratios less than unity suggest that there is only one phosphorylation site during non-cyclic electron flow (Arnon *et al.* 1958; Krogmann *et al.* 1959; Avron & Jagendorf, 1959; Stiller & Vennesland, 1962; Turner *et al.* 1962). However, Winget *et al.* (1965), Shavit & Avron (1967) and Horton & Hall (1968) have measured P/2e ratios greater than unity. Jagendorf & Uribe (1966) claim that their measurements of pH changes during ferricyanide reduction demonstrate that there are two phosphorylation sites.

The demonstration of 'photosynthetic control' and the calculation of ADP/O ratios provides new evidence on this point. All the values for the ADP/O ratio reported in Table 1 are greater than unity, suggesting that there is more than one phosphorylation site, but these values may not be true estimates of the ADP/O ratios owing to any one or more of a number of possibilities: (a) if some cyclic photophosphorylation was occurring to decrease the amount of available ADP, the meas-

ured ADP/O ratios would be higher than the true ADP/O ratio; (b) if the ADP added contained any AMP, and if AMP could be phosphorylated, the observed ADP/O ratio would be higher than the true ADP/O ratio; (c) if some reaction not associated with photophosphorylation decreased the amount of available ADP the observed ADP/O ratio would be higher than the true ADP/O ratio; (d) if there was an apparent low affinity for ADP the observed ADP/O ratio would be higher than the true ADP/O ratio; (e) if an adenosine triphosphatase were operating the observed ADP/O ratios would be lower than the true ADP/O ratios.

Initial experiments to test these various possibilities have indicated that (a), (b) and (d) do not contribute significantly. Adenosine triphosphatase activity has been detected, but its effect in these experiments is not known. Thus the evidence so far obtained tends to suggest that there is more than one site of phosphorylation in photosynthesis; however, the use of artificial electron acceptors may give misleading results.

If there is only one site of photophosphorylation and it is situated at about the plastoquinone level of the electron-transport chain, the photosynthetic control ratio may then be considered comparable with the respiratory control ratio obtained with succinate as substrate. Photosynthetic control ratios were dependent on the time of addition of ADP (i.e. before or after illumination). The reason is not clear, but it could be that illumination under non-phosphorylating conditions causes inhibition of electron transfer, as it is the initial state III rate that is most affected (see Fig. 5). Fig. 4 shows that state III rates decrease with continued illumination, where physical damage caused by continuous stirring may also be a contributing factor. It is not possible to distinguish between these possibilities.

According to the chemiosmotic hypothesis (Mitchell, 1966) control represents a back pressure on electron flow caused by a build up of a pH gradient or an electrochemical potential. Although photosynthetic control ratios may decrease with time, ADP/O ratios need not change. State III rates can be limited by rate of electron flow, by rate of entry of ADP or by rate of phosphorylation, whereas state IV rates are limited by the back leakage of the pH gradient or electrochemical potential. Thus changes in state IV rates need affect only the control ratio and not the ADP/O ratio.

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