Biochimica et Biophysica Acta, 440 (1976) 131–146

BBA 47110

FACTORS AFFECTING THE ADP/O RATIO IN ISOLATED CHLOROPLASTS

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(Received December 8th, 1975)

SUMMARY

(1) The effect of gradual disruption of the outer membrane of intact chloroplasts on CO_2 fixation, electron transport and phosphorylation was investigated. The results suggested that whilst ferricyanide and substrate amounts of ADP enter intact chloroplasts only very slowly, methyl viologen rapidly penetrates the outer membrane.

(2) Preparations of intact pea chloroplasts had an ATP-consuming reaction which resulted in decreased ADP/O ratios when noncyclic electron transport was measured after disruption of the outer membrane. The ATP-consuming reaction was removed into the supernatant after washing the disrupted chloroplasts. The resulting washed chloroplasts gave ADP/O ratios of 1.5—1.6 for ferricyanide and 1.9—2.0 for methyl viologen.

(3) Preparations of intact spinach chloroplasts had lower activity of the ATP-consuming reaction and gave similar ADP/O ratios to washed pea chloroplasts. The ADP/O ratios of spinach chloroplasts did not alter significantly after washing.

(4) An investigation of the effect of various assay conditions on the ADP/O ratio showed that the phosphate concentration was critical in obtaining optimal values for ADP/O ratio. Decreasing the phosphate concentration below 10 mM decreased the ADP/O ratio significantly.

(5) It is suggested that the maximum ADP/O ratio of chloroplasts is 2.0 but that lower values can be obtained in the presence of an ATP-consuming reaction, under suboptimal assay conditions or where the chloroplasts are structurally damaged.

INTRODUCTION

Many recent reports have suggested that the noncyclic pathway of electron transport in chloroplasts has two sites of phosphorylation [1–5]. The two sites, one in the region of the photo-oxidation of water and the other associated with plastoquinone, result in the inward translocation of four protons per pair of electrons transported ($H^+/2e = 4$). However, the number of molecules of ATP produced per pair of electrons transported (ATP/2*e*), which depends on $H^+/2e$ and H^+/ATP ratios, is still controversial.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Tricine, *N*-tris(hydroxyethyl)methylglycine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; MES, 2-(*N*-morpholino)-ethanesulphonic acid.

Carmeli [6, 7] has measured H^+/ATP ratios of two for the chloroplast ATPase. Measurement of the dark proton efflux after steady state also gave a value of two [8]. ATPase-induced amine uptake again suggests an H^+/ATP ratio of 2 [9]. In conjunction with two sites of phosphorylation this would result in ATP/2*e* ratios of 2, i.e. sufficient ATP for CO_2 fixation in the absence of cyclic phosphorylation. However, Junge and coworkers [10] obtained values of 3 for the H^+/ATP ratio from studies of flash-induced proton uptake, which would result in ATP/2*e* ratios of 1.33, i.e. insufficient ATP for CO_2 fixation.

Many workers have measured the ATP/2*e* ratio directly in isolated chloroplasts. Good's group [4, 5] and Trebst and Reimer [11] obtained ATP/2*e* ratios of 1.0–1.2 with isolated spinach chloroplasts by measuring the rate of ATP formation and the rate of electron transport. West and Wiskich [1] and Reeves and Hall [3] have measured ADP/O (equivalent to ATP/2*e*) ratios of 1.3–1.7 in tightly coupled chloroplasts using polarographic measurement of photosynthetic control cycles. Heber [12, 13] has suggested from quantum requirements of intact chloroplasts that the coupling of phosphorylation to electron transport is flexible and results in ATP/2*e* ratios of 1.2–1.3.

The ATP/2*e* ratio would be decreased if some uncoupled chloroplasts (or an uncoupled pathway of electron transport) is present in the chloroplast preparation. Some workers [2, 3, 14] have subtracted the "basal" or nonphosphorylating rate of electron transport to allow for this possibility, although the procedure is probably not valid [3]. The ATP/2*e* ratio would be increased if cyclic phosphorylation were contributing to ATP formation but not to measured electron transport. The work of Reeves and Hall [3] suggests that cyclic phosphorylation does not occur simultaneously with noncyclic phosphorylation. Finally, any ATP-consuming reaction would decrease the ATP/2*e* ratio by effectively decreasing the rate of ATP formation.

It is the purpose of this communication to present further evidence that the ATP/2*e* ratio in chloroplasts is greater than 1.33 but that decreased ATP/2*e* ratios can be apparent in preparations of intact chloroplasts as a result of ATP-consuming reactions, or as a result of certain assay conditions.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* L var. Massey Gem) were grown for 2–3 weeks in vermiculite in a glasshouse. Spinach plants (*Spinacia oleracea* var. True Hybrid 102) were grown in soil for 4–6 weeks.

60–80 g of pea shoots or de-ribbed spinach leaves were ground in a Polytron blender for 2–3 s in 200 ml of ice-cold medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 20 mM NaCl, 0.5 mM P_i, 2 mM D-isoascorbate, 50 mM 2-(*N*-morpholino)-ethanesulphonic acid (MES) buffer and 0.4 % bovine serum albumin, adjusted to pH 6.2 with HCl. The brei was squeezed through a double layer of miracloth containing a layer of cotton wool and the filtrate was centrifuged at $2000 \times g$ for 30 s in an MSE Super Minor centrifuge. The chloroplast pellet was rinsed once with a medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 20 mM NaCl, 0.5 mM P_i, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid (HEPES) buffer and 0.4 % bovine serum albumin, adjusted to pH 6.7 with HCl. The pellet was resuspended in

1 ml of the same medium using a glass rod wrapped with cotton wool. All procedures were carried out at 0 °C using chilled solutions and apparatus.

Evolution or uptake of oxygen was measured with a Rank oxygen electrode connected to a Rikadenki B-261 recorder. The vessel was illuminated by light from a Rank-Aldis 150 W projector filtered through a Wratten 29 red filter to give a light intensity of $2 \cdot 10^5$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1} at the centre of the reaction vessel. The vessel was maintained at 20 °C.

Oxygen concentration in the reaction media was determined by the method of Robinson and Cooper [15]. ADP concentration was determined enzymically [16]. Chlorophyll was determined from 80 % acetone extracts by the method of Arnon [17]. ADP production by chloroplasts was measured enzymically with pyruvate kinase and lactate dehydrogenase. Chloroplasts were added to a 1 cm cuvette containing 0.4 M (intact chloroplasts) or 0.1 M (shocked or washed chloroplasts) sucrose, 5 mM $MgCl_2$, 10 mM P_i, 50 mM HEPES (pH 7.6), 0.5 mM phospho*enol*pyruvate, 0.3 mM NADH, 0.05 mg pyruvate kinase and 0.05 mg lactate dehydrogenase in a final volume of 2.2 ml. The reaction was started by addition of ATP to a final concentration of 2.5 mM and the reaction rate was determined from the decrease in absorbance at 340 nm. The enzyme solutions were freed of ammonium sulphate by dialysis.

The percentage of intact chloroplasts was determined by the ferricyanide method [18] by measuring oxygen evolution in the presence of 1.3 mM ferricyanide and 5 mM NH_4Cl. Three types of chloroplasts are referred to in the text. "Intact" chloroplasts were assayed in reaction media containing 400 mM sucrose, which is sufficient to prevent rupture of the outer envelope membranes. "Shocked" chloroplasts were assayed in media containing 100 mM sucrose, which results in rupture of most of the outer envelope membranes. "Washed" chloroplasts were prepared by diluting the chloroplast suspension 20-fold with cold distilled water followed by addition of an equal amount of a medium containing 500 mM sucrose, 10 mM P_i, 5 mM $MgCl_2$ and 50 mM HEPES (pH 7.6) after 15 s. The resulting suspension was centrifuged at $2000 \times g$ for 2 min and the chloroplast pellet resuspended as described above. The resulting chloroplast suspension lacked the outer envelope membranes. Washed chloroplasts were assayed in media containing 100 mM sucrose.

The terminology of electron transport is adapted from that of Chance and Williams [19]. State 2 refers to electron transport in the presence of an electron acceptor and P_i but with no added ADP (Hill reaction rate). State 3 refers to the stimulated rate after addition of ADP and State 4 to the decreased rate observed after all the ADP has been phosphorylated to ATP. ADP/O ratios were calculated from oxygen electrode traces without correction for "nonphosphorylating" rates [2, 3, 14].

RESULTS

Chloroplasts isolated from pea leaves as described in Methods were 70–90 % intact as judged by ferricyanide penetration. Rates of CO_2-dependent oxygen evolution in the absence of any added intermediates ranged from 20–60 μmol $\cdot$ mg chlorophyll$^{-1} \cdot$ h^{-1} but were increased to 70–130 μmol $\cdot$ mg chlorophyll$^{-1} \cdot$ h^{-1} by the addition of catalytic amounts of ATP. The stimulatory effect of ATP was not due to any effect on broken chloroplasts and will be discussed in detail in a forthcoming article. 5 h after isolation the chloroplast preparations retained more than 90 % of their initial CO_2-fixing activity.

Decreasing the osmolarity of suspensions of intact chloroplasts resulted in swelling and eventual rupture of the outer envelope. Fig. 1 shows that decreasing the sorbitol concentration of the reaction medium resulted in a decrease in the rate of CO_2-dependent oxygen evolution at sorbitol concentrations below 300 mM. At sorbitol concentrations below 50 mM, no CO_2-dependent oxygen evolution was detectable. The decrease in CO_2-dependent oxygen evolution was paralleled by increased ferricyanide penetration, indicating a decrease in the percentage of intact chloroplasts (Fig. 1).

Suspensions of intact chloroplasts exhibited O_2 evolution and photosynthetic control with ferricyanide, although the rates were very low and could be attributed to the small percentage of broken chloroplasts present in the preparation. The high concentrations of P_i and Mg^{2+} in the reaction medium for ferricyanide-dependent oxygen evolution (Fig. 2) eliminated the possibility of any CO_2-dependent oxygen

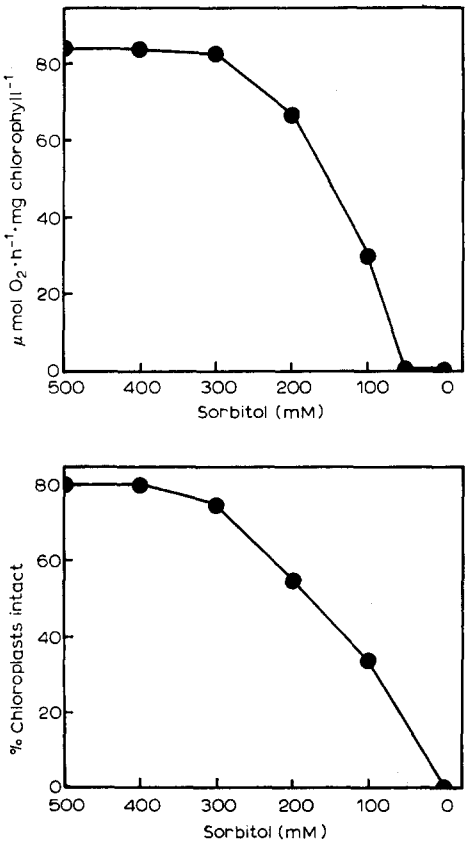

Fig. 1. Effect of sorbitol concentration on the rate of CO_2-dependent oxygen evolution by pea chloroplasts and on the percentage of chloroplasts intact. Oxygen evolution was measured in a medium containing varying sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES buffer (pH 7.6), 5 mM PP_i, 10 mM $NaHCO_3$, 0.4 mM ATP and chloroplasts equivalent to 96 μg chlorophyll in a total volume of 1.2 ml. Percentage of chloroplasts intact was measured in the same media by the ferricyanide method.

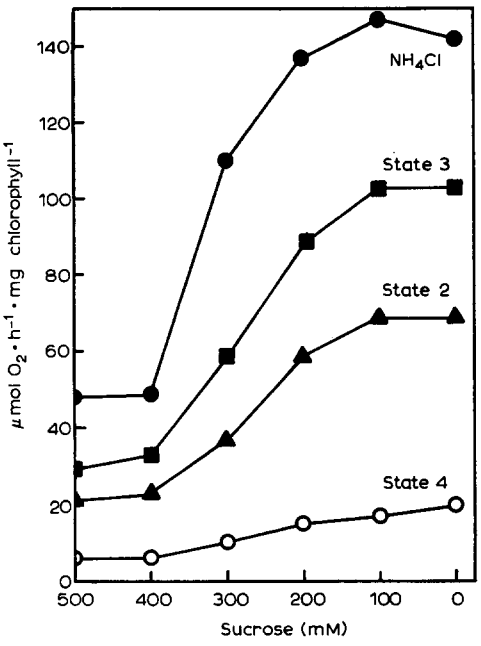

Fig. 2. Effect of sucrose concentration on rates of oxygen evolution by pea chloroplasts with ferricyanide. Oxygen evolution was measured in a medium containing varying sucrose, 10 mM P_i (K^+ salts), 5 mM $MgCl_2$, 50 mM HEPES (pH 7.6), 1.3 mM ferricyanide and chloroplasts equivalent to 71 μg chlorophyll in a total volume of 2.2 ml. ADP and NH_4Cl were added to final concentrations of 0.14 mM and 5 mM, respectively. State 2 = Hill reaction rate (−ADP), State 3 = phosphorylating rate (+ADP), State 4 = rate after exhaustion of added ADP.

evolution that is strongly inhibited by both P_i [20] and Mg^{2+} [21]. As the sucrose concentration of the reaction medium was decreased, rates of O_2 evolution with ferricyanide increased (Fig. 2). The photosynthetic control ratio (State 3/State 4) and the ADP/O ratio did not change significantly over the range of sucrose concentrations shown. These results confirm the very low permeability of the chloroplast envelope to ferricyanide [18], but give no indication of the permeability to ADP.

In contrast to ferricyanide, methyl viologen rapidly enters intact chloroplasts. The uncoupled rate of oxygen uptake with methyl viologen did not increase as the sucrose concentration decreased (Fig. 3). In addition, we have observed that methyl viologen rapidly inhibits CO_2-dependent oxygen evolution by intact chloroplasts, probably by competing with ferredoxin as an electron acceptor. Whilst the State 4 and uncoupled rates of O_2 uptake with methyl viologen did not change with decreasing sucrose concentration, both State 2 and State 3 rates increased (Fig. 3). The increase in State 2 rate was probably a result of endogenous ATP in the intact chloroplasts. ATP is known to inhibit coupled electron transport in broken chloroplasts [1, 3]. When the chloroplast envelope membranes are ruptured, internal ATP would be diluted throughout the reaction medium, thereby releasing electron transport from ATP inhibition. The level of adenine nucleotides in chloroplasts (25–30 nmol · mg chlorophyll^{-1}) [18], and the chloroplast volume (25 μl · mg chlorophyll^{-1}) [22] suggest that the internal concentration of ATP is in the order of 1 mM. Table I

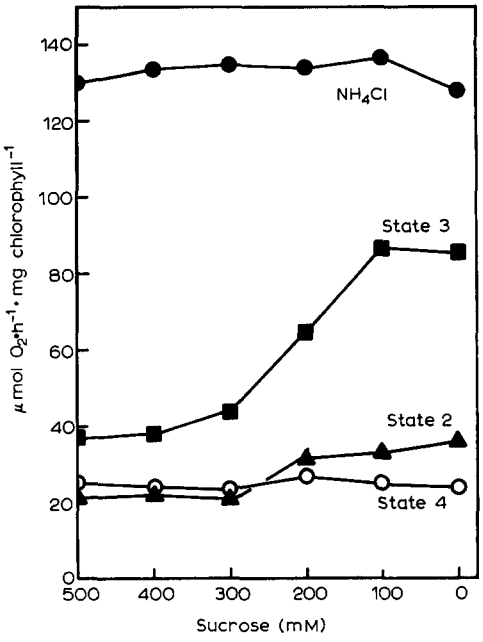

Fig. 3. Effect of sucrose concentration on rates of oxygen uptake by pea chloroplasts with methyl viologen. Oxygen uptake was measured as described in fig. 2, except that 4.5 mM NaN_3 and 0.3 mM methyl viologen replaced ferricyanide.

shows that 1 mM ATP inhibited the rate of ferricyanide-dependent oxygen evolution by 50 %. Oxygen uptake with methyl viologen was also inhibited by ATP in shocked or washed chloroplasts, but not in intact chloroplasts. The ATP-inhibited rate in shocked or washed chloroplasts was always very close to the State 2 rate in intact chloroplasts. From this it appears that internal adenine nucleotides can control electron transport in intact chloroplasts.

TABLE I

EFFECT OF ATP ON STATE 2 (HILL REACTION) ELECTRON TRANSPORT IN PEA CHLOROPLASTS

Oxygen evolution or uptake was measured as described for Figs. 2 and 3. ATP was added to a final concentration of 1 mM. FeCN, ferricyanide; MV, methyl viologen.

Acceptor	Chloroplasts	Rate of oxygen evolution or uptake (μmol O_2 · mg chlorophyll^{-1} · h^{-1})			(%) Inhibition by ATP
		State 2	+ATP	+NH_4Cl	
FeCN	Intact	30	14	54	53
FeCN	Shocked	107	54	252	50
FeCN	Washed	95	47	241	51
MV	Intact	11	12	150	−8
MV	Shocked	19	11	153	42
MV	Washed	21	12	160	43

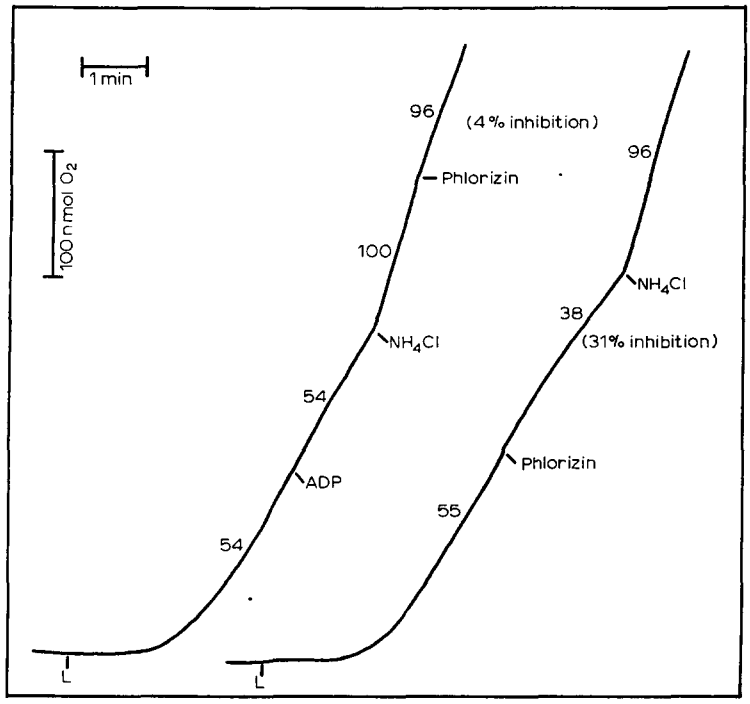

Fig. 4. Oxaloacetate-dependent oxygen evolution by pea chloroplasts. Oxygen evolution was measured in a medium containing 400 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES buffer (pH 7.6), 10 mM D,L-glyceraldehyde, 2 mM oxaloacetate and chloroplasts equivalent to 100 μg chlorophyll in a total volume of 2.2 ml. ADP, phlorizin and NH_4Cl were added to final concentrations of 0.2 mM, 1 mM and 10 mM, respectively. Numbers along the traces are μmol O_2 · mg chlorophyll^{-1} · h^{-1}.

The increase in State 3 rate of oxygen uptake with decreasing sucrose concentration can be attributed to increased penetration of ADP as the envelope membranes become swollen or ruptured. This suggests that ADP enters intact chloroplasts slowly, and is in agreement with the work of Heber and Santarius [18]. The slow penetration of ADP explains the lack of any stimulation by ADP of oxaloacetate-dependent oxygen evolution (Fig. 4). The reduction of oxaloacetate to malate by intact chloroplasts does not appear to involve any adenine nucleotide-dependent reactions but is stimulated by uncouplers [13]. If substrate amounts of ADP were able to rapidly enter the chloroplasts the rate of oxaloacetate-dependent oxygen evolution would be expected to increase upon addition of ADP.

The increased photosynthetic control ratios as the sucrose concentration was decreased were due to the increase in State 3 rate of oxygen uptake. In intact chloroplast suspensions, the ADP/O ratio with methyl viologen was low (Fig. 6) due to the high percentage of intact chloroplasts which cannot phosphorylate exogenous ADP but contribute to oxygen uptake. Decreasing the sucrose concentration to 100 mM ruptured the outer envelope membranes allowing ADP to penetrate resulting in ADP/O ratios of 1.2–1.5 (Table II and Figs. 5 and 6). In many preparations of pea chloroplasts the ADP/O ratio was significantly lower than 2 even after osmotic shock.

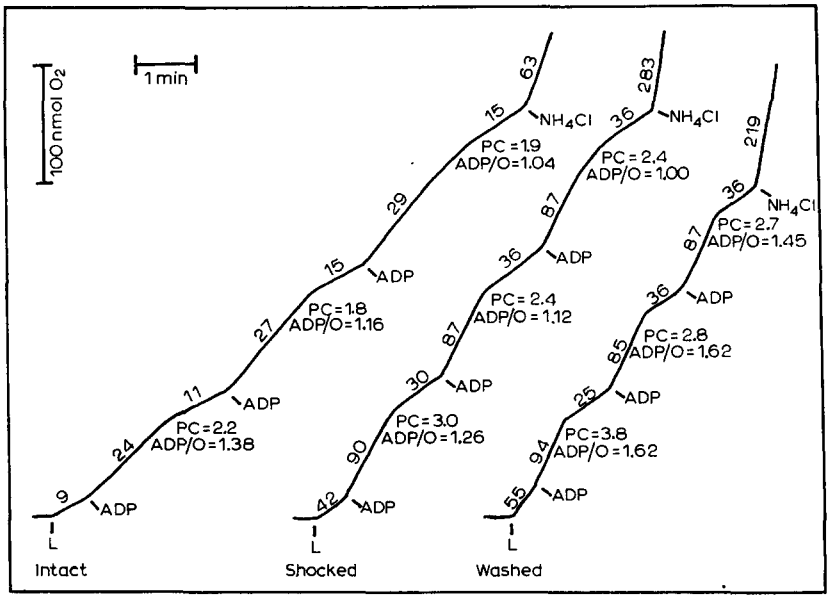

Fig. 5. Successive cycles of photosynthetic control by pea chloroplasts with ferricyanide as electron acceptor. Oxygen evolution was measured as described in Fig. 2. Each addition of ADP contained 186 nmol and NH_4Cl was added to a final concentration of 5 mM. Numbers along the traces indicate μmol O_2 · mg chlorophyll^{-1} · h^{-1}. PC = photosynthetic control ratio.

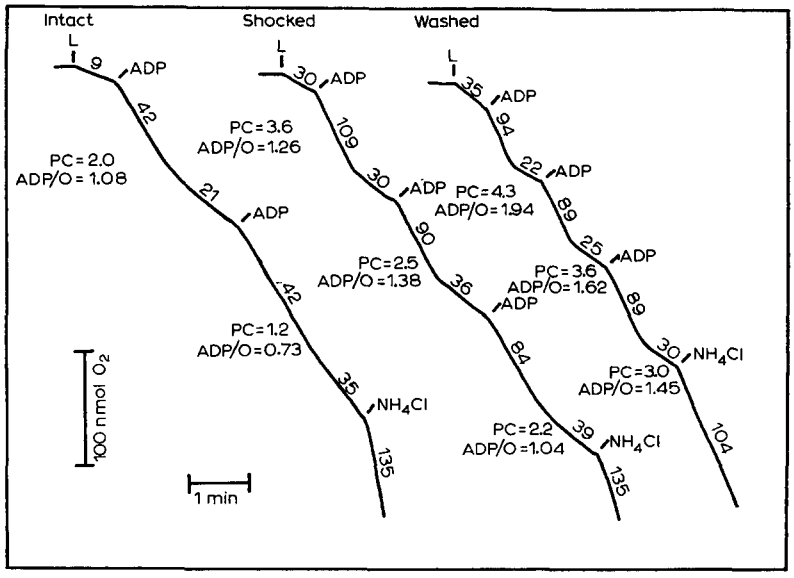

Fig. 6. Successive cycles of photosynthetic control by pea chloroplasts with methyl viologen as electron acceptor. Oxygen uptake was measured as described in Fig. 3. Each addition of ADP contained 186 nmol and NH_4Cl was added to a final concentration of 5 mM. Numbers along the traces indicate μmol O_2 · mg chlorophyll^{-1} · h^{-1}. PC = photosynthetic control ratio.

TABLE II

INHIBITION OF STATE 4 ELECTRON TRANSPORT BY PHLORIZIN IN PEA CHLOROPLASTS

Oxygen evolution or uptake was measured as described in Figs. 2 and 3. ADP production was measured as described in Methods. Phlorizin was added to a final concentration of 1 mM. FeCN, ferricyanide; MV, methyl viologen.

Acceptor	Chloroplasts	Oxygen evolution or uptake (μmol O_2 · mg chlorophyll^{-1} · h^{-1})			% Inhibition	ADP/O	ADP production (μmol · mg chlorophyll^{-1}·h^{-1})
		State 4	+Phlorizin	+NH_4Cl			
FeCN	Intact	10	8	71	20	1.09	14.8
FeCN	Shocked	34	28	164	18	1.17	24.6
FeCN	Washed	20	20	163	0	1.58	4.6
MV	Intact	25	21	176	16	0.97	14.8
MV	Shocked	46	34	169	26	1.48	24.6
MV	Washed	22	22	162	0	1.95	4.6

Successive additions of small amounts of ADP resulted in a continual decrease of both photosynthetic control ratio and ADP/O ratio with ferricyanide (Fig. 5), or methyl viologen (Fig. 6). The decrease in ADP/O ratio suggests the presence in the chloroplast suspension of an ADP regenerating reaction which increased as the total ATP concentration increased.

The State 4 rate of electron transport would be increased by the continual turnover of ADP. Energy transfer inhibitors such as phlorizin [14] would inhibit the phosphorylation of ADP and thus inhibit electron transport. Table II shows that 1 mM phlorizin inhibited State 4 electron transport by 16–26 % in intact and shocked chloroplast suspensions with either ferricyanide or methyl viologen as the electron acceptor. Phlorizin also inhibited the rate of oxaloacetate-dependent oxygen evolution by 25–35 %, whereas the uncoupler-stimulated rate was inhibited by less than 5 % (Fig. 4). Heber and Kirk [13] reported 15 % inhibition of oxaloacetate-dependent oxygen evolution by phlorizin in intact spinach chloroplasts.

Osmotic shock of the chloroplasts followed by a single wash through 250 mM sucrose resulted in increased ADP/O and photosynthetic control ratios (Table II, Figs. 5 and 6). After washing, ADP/O ratios of 1.7–2.0 were obtained with methyl viologen. The ADP/O ratios with ferricyanide were consistently lower than with methyl viologen, ranging from 1.4 to 1.6 after washing. The decrease in ADP/O ratio with successive additions of ADP was greatly reduced in washed chloroplasts (Figs. 5 and 6). The State 4 rate of electron transport in washed chloroplasts was insensitive to phlorizin (Table II).

The increase in ADP/O ratio and decrease in phlorizin inhibition after washing suggests that the ATP-consuming reaction was soluble and remained in the supernatant. Rates of ATP hydrolysis by intact chloroplasts in the dark ranged from 11 to 22 μmol · mg chlorophyll^{-1} · h^{-1} and after osmotic shock this increased to 18–47 μmol · mg chlorophyll^{-1} · h^{-1}. In washed chloroplasts the rate was much lower, ranging from 3 to 9 μmol · mg chlorophyll^{-1} · h^{-1}. Table II shows typical values for phlorizin inhibition, ADP/O ratio and rate of ADP production in the three classes of pea chloroplasts used.

In contrast to pea chloroplasts, preparations of isolated spinach chloroplasts did not show any significant increase in ADP/O ratio after washing. Both shocked and washed spinach chloroplasts gave high ADP/O ratios of 1.5–1.7 and 1.8–2.0 with ferricyanide and methyl viologen, respectively (Table III). These data suggested that the ATP-consuming reactions present in pea chloroplasts were absent or had low activity in spinach chloroplasts. The low rate of ATP hydrolysis by spinach chloroplasts (5–10 μmol · mg chlorophyll^{-1} · h^{-1}) was not significantly decreased after washing (Table III).

In an attempt to explain the consistent reports of ADP/O ratios below 1.3 [4, 5, 12], the effect of various assay conditions on ADP/O ratio was investigated. As shown in Fig. 7 the ADP/O ratio was virtually pH-independent in the pH range 7–9 for both ferricyanide and methyl viologen. In contrast, the photosynthetic control ratio showed a well defined optimum at pH 7.5. The decrease in photosynthetic control ratio at higher pH values was due to faster rates of State 4 electron transport. According to Mitchell's chemiosmotic hypothesis [23] coupling between electron transport and phosphorylation is by means of an electrochemical gradient, and control of electron transport is achieved by a back pressure caused by a high pH or electrical

TABLE III

ELECTRON TRANSPORT AND ADP/O RATIOS FOR INTACT, SHOCKED AND WASHED SPINACH CHLOROPLASTS

Oxygen evolution or uptake was measured as described in Figs. 2 and 3. The rate of ADP production was measured as described in Methods. FeCN, ferricyanide; MV, methyl viologen; PC, photosynthetic control ratio.

Acceptor	Chloroplasts	μmol O_2 · mg chlorophyll^{-1} · h^{-1}				PC	ADP/O	ADP production
		State 2	State 3	State 4	NH_4Cl			(μmol · mg chlorophyll^{-1}·h^{-1})
FeCN	Intact	3	27	9	73	3.0	1.68	5.1
FeCN	Shocked	20	56	18	215	3.1	1.68	8.6
FeCN	Washed	30	71	18	252	3.9	1.68	6.6
MV	Intact	12	27	17	127	1.6	1.56	5.1
MV	Shocked	20	50	20	131	2.5	1.95	8.6
MV	Washed	11	46	17	138	2.7	1.98	6.6

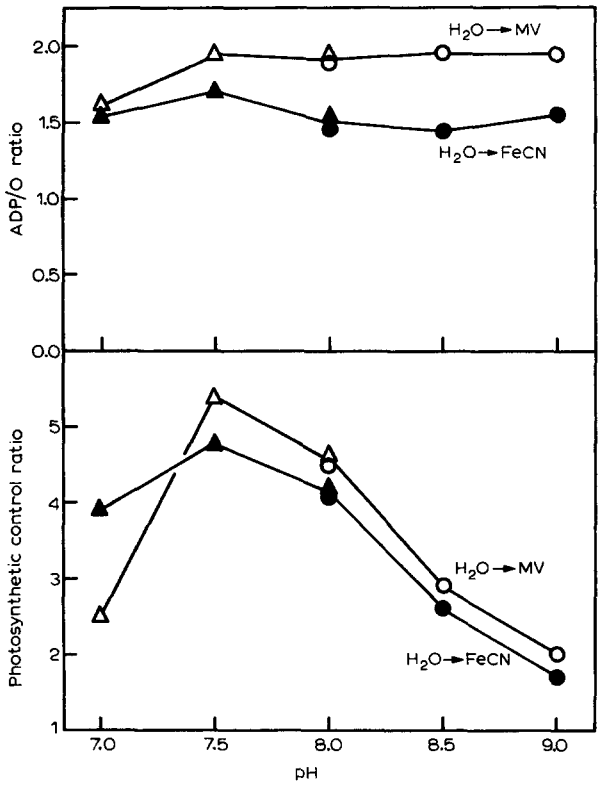

Fig. 7. Effect of pH on ADP/O and photosynthetic control ratios of washed pea chloroplasts. Oxygen evolution with ferricyanide (solid symbols) or oxygen uptake with methyl viologen (open symbols) was measured as described in Figs. 2 and 3. The buffers employed were 50 mM HEPES (△, ▲) or 50 mM Tricine (○, ●). ADP was added to a final concentration of 0.18 mM. The sucrose concentration was 100 mM. FeCN, ferricyanide; MV, methyl viologen.

gradient across the membrane. In terms of Mitchell's hypothesis, the increase in State 4 rates at pH values above 7.5 suggests a higher rate of leakage of protons out of the chloroplasts grana.

One factor which had a pronounced effect on ADP/O ratios was the phosphate concentration. Fig. 8 shows the effect of phosphate concentration on ADP/O ratio at pH 7.5 and pH 8.5 with washed pea chloroplasts. In the presence of 10 mM K_2HPO_4 this preparation gave an ADP/O ratio of 1.55 with ferricyanide at pH 7.5. Decreasing the concentration of K_2HPO_4 to 5 mM (as used by Good's group [4, 5]) decreased the ADP/O ratio to 1.37. At 3.3 mM K_2HPO_4 (as used by Trebst [11]) the ADP/O ratio was further decreased to 1.26. The decrease in ADP/O ratio with concentrations of K_2HPO_4 below 10 mM was repeatedly observed with chloroplasts that showed high ADP/O ratios. In some preparations where the ADP/O ratio was lower than 1.45 under standard conditions (10 mM P_i), decreasing the phosphate concentration had a less pronounced effect than that shown in Fig. 8, suggesting that some other factor (such as intactness of the grana lamellae) was limiting the efficiency of phosphorylation. The decrease in ADP/O ratio with decreasing K_2HPO_4 concentrations was not

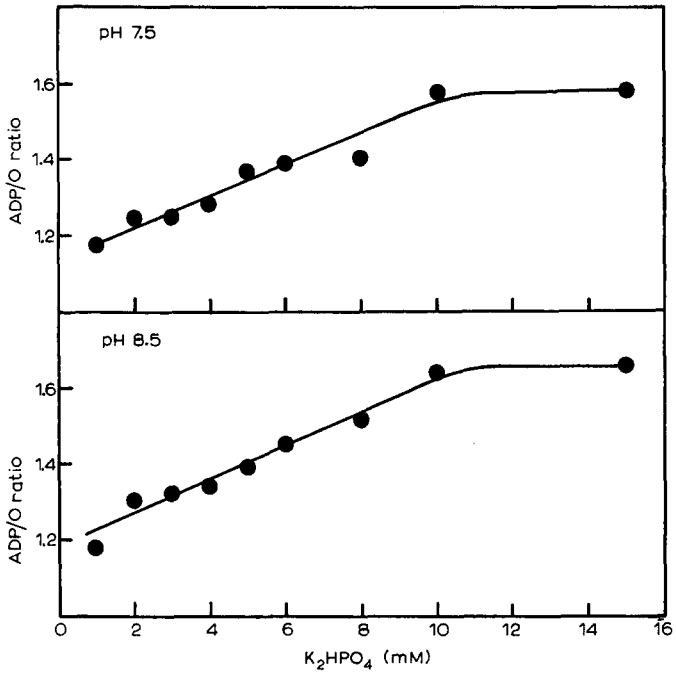

Fig. 8. Effect of phosphate concentration on ADP/O ratio of washed pea chloroplasts. The ADP/O ratio is the average of two cycles of photosynthetic control using ferricyanide. The medium contained 100 mM sucrose, 5 mM $MgCl_2$, 1.3 mM ferricyanide, 50 mM HEPES (pH 7.5) or 50 mM Tricine (pH 8.5) and variable concentrations of K_2HPO_4.

due to K^+ effects as lower ratios were observed with 5 mM K_2HPO_4 in the presence or absence of 10 mM KCl.

Decreasing the Mg^{2+} concentration to 2 mM [4, 5] did not alter the ADP/O ratio or the photosynthetic control ratio. Hall's group [3, 24] suggested that EDTA is required for optimum photosynthetic control and include 2 mM EDTA in their reaction media. The addition of 2 mM EDTA was not required for optimal photosynthetic control and ADP/O ratios in our preparations.

DISCUSSION

West and Wiskich [25] demonstrated photosynthetic control with chloroplasts (Class I) that were intact as determined by phase contrast microscopy and suggested that intact chloroplasts might be required to show tightly coupled electron transport. From the data of Figs. 2–4 it is obvious that chloroplasts with functionally intact outer membranes (i.e. chloroplasts capable of high rates of CO_2 fixation) are impermeable to ferricyanide and to substrate amounts of ADP, and conversely that intact chloroplasts are not required for measurement of photosynthetic control. The Class I chloroplasts of West and Wiskich [25] were not capable of CO_2-dependent oxygen evolution. Either their Class I chloroplasts had damaged or swollen outer membranes or the electron transport measured was only that carried out by broken chloroplasts

in the preparation. The decrease in photosynthetic control and ADP/O ratio after sonication reported by West and Wiskich [25] was probably the result of disruption of the grana lamellae, rather than rupture of the outer membrane. Similar studies in this laboratory suggest that coupled electron transport requires grana lamellae that are not swollen or separated.

As the outer envelope of chloroplasts is impermeable to substrate amounts of ADP, the problem appears to be to rupture the outer membrane of intact chloroplasts without damaging the grana lamellae to any significant extent. We have found that this is best achieved by decreasing the osmolarity of the reaction medium or by very brief exposure to water followed by addition of an osmoticum such as sucrose. If the chloroplasts were assayed in a medium containing less than 100 mM sucrose or were exposed to water for more than 30–40 s the photosynthetic control and ADP/O ratios were decreased, indicating damage to the grana lamellae. Any treatment which damages the lamellae resulted in a decrease in ADP/O ratio from 1.6–2.0 to below 1.3. Similarly, chloroplasts suspended in media lacking bovine serum albumin showed a decrease in ADP/O ratio to less than 1.3 within 30 min at 0 °C. The exact nature of stabilization of chloroplasts by serum albumin is not known, although it may act by binding fatty acids, which uncouple chloroplasts [26]. Galactolipase from bean leaf chloroplasts releases fatty acids from subchloroplast particles, but the fatty acids can be bound by serum albumin to prevent damage to the subchloroplast particles [27].

The decrease in ADP/O ratio with successive cycles of ADP (Figs. 5 and 6), the inhibition of State 4 by phlorizin (Table II) and the increase in ADP/O after washing (Figs. 5 and 6 and Table II) were all consistent with the presence of an ATP consuming reaction in the chloroplast preparation. Although the rate of ADP production was not high (Table II) in comparison with rates of electron transport, it was sufficient to lower ADP/O ratios significantly. Over longer periods of incubation the decrease in ADP/O ratio would be more apparent as higher concentrations of ATP would accumulate. The nature of the ATP-consuming reaction is not known. It is apparent, however, that the reaction must have a relatively high affinity for ATP, as the amount of ADP added for measurement of ADP/O ratios was low (0.1–0.2 mM). The reaction was probably associated with a soluble enzyme of the stroma or one bound to the outer membrane, as it was readily removed into the supernatant after washing. This suggests that the reaction does not involve the coupling factor of photophosphorylation. The inhibition of oxaloacetate-dependent oxygen evolution by phlorizin (Fig. 4) suggests that the ATP-consuming reaction occurred in intact chloroplasts, resulting in continual recycling of adenine nucleotides and increased electron transport. Phlorizin would inhibit the phosphorylation of ADP, although it may not have any effect on the ATP consuming reaction. Phlorizin inhibition of State 4 rates of electron transport or of oxaloacetate-dependent oxygen evolution varied between 15 and 60 %, compared to 15 % inhibition reported by Heber and Kirk [13] for spinach chloroplasts. From the data of Table II, a chloroplast preparation which showed 20–26 % inhibition by phlorizin and a rate of ATP hydrolysis of 25 μmol · mg chlorophyll^{-1} · h^{-1} gave ADP/O ratios of 1.2–1.5 prior to washing and 1.6–2.0 after washing. It is evident that even a low rate of ADP production (which results in low phlorizin inhibition) is sufficient to lower the ADP/O ratio significantly. This may explain the ADP/O ratios of 1.2–1.3 calculated by Heber and Kirk [13].

Kraayenhof et al. [28] have reported the presence of an ATP-consuming re-

action in Class I chloroplasts with rates similar to those reported here. They suggested that the reaction reflected the reversibility of the coupling factor ATPase. The reversibility of the coupling factor ATPase, whilst decreasing control ratios as a result of increased State 4 rates, would not affect ADP/O ratios. Whilst chloroplasts are illuminated, electron transport would force the equilibrium $ADP+P_i \rightleftharpoons ATP$ to the right and ATP hydrolysis by the coupling factor could occur only in the absence of ADP and P_i or in the presence of an uncoupler. Thus, the ADP/O ratio can be decreased only by a separate ATP-consuming reaction that occurs simultaneously with phosphorylation. For this reason it is unlikely that the ATPase reported by Kraayenhof et al. [28] was due to the chloroplast coupling factor.

The residual ATP-consuming reaction in washed pea chloroplasts and in spinach chloroplasts did not appear to affect ADP/O ratios (Tables II and III, Figs. 5 and 6). This may be because the reaction was too slow to significantly affect the ADP/O ratio. Alternatively the ATP-consuming reaction in washed chloroplasts may have a low affinity for ATP and does not operate under the conditions used for measurement of ADP/O ratios. Preliminary experiments suggest that there may be two ATP-consuming reactions in our chloroplast preparations; one with a high affinity for ATP, which is removed into the supernatant, and another that is weakly bound to the chloroplast lamellae but has a low affinity for ATP. The experiments with spinach chloroplasts (Table III) show that washing as such does not alter ADP/O ratios.

In spinach chloroplasts or washed pea chloroplasts the ADP/O ratio with ferricyanide was consistently lower than with methyl viologen (Figs. 5 and 6, Tables II and III). The plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) completely inhibits electron transport from water to methyl viologen, whilst electron transport from water to ferricyanide is inhibited by only 60 % [29]. The DBMIB-insensitive pathway of ferricyanide reduction is coupled to phosphorylation giving an ATP/O ratio of 0.7 [30]. If the DBMIB-insensitive pathway of ferricyanide reduction occurs in the absence of DBMIB, the ADP/O ratio for ferricyanide will vary between 0.7 and 2.0, depending on the percentage of electron transport passing through the DBMIB-insensitive pathway. The ADP/O ratio normally measured for ferrycianide (1.5–1.6) suggests that 30–40 % of the electron transfer is through the DBMIB-insensitive pathway in the absence of DBMIB.

Analysis of the ADP/O ratio with varying assay conditions showed that a minimal phosphate concentration of 9–10 mM was required for maximum ADP/O ratio at either pH 7.5 or pH 8.5. Santarius and Heber [31] reported phosphate concentrations between 4 and 25 mM in nonaqueously isolated chloroplasts. Thus inclusion of 10 mM phosphate in the reaction medium is not greatly different from the normal conditions in intact chloroplasts. The reason for decrease in ADP/O with decreasing phosphate concentrations below 10 mM (Fig. 8) is not clear.

In any discussion of the efficiency or stoichiometry of photophosphorylation the most important value is the maximum ADP/O ratio that can be measured. The ADP/O ratio can be significantly decreased by damage or degradation of the chloroplast membranes, by the presence of any ATP-consuming reactions or by assay conditions that are not optimal. It is the maximum ADP/O ratio measured which is relevant, as this reflects the limit set by the true stoichiometry of phosphorylation. From a large number of experiments in this laboratory these maxima are 1.7 for ferricyanide and 2.0 for methyl viologen. This suggests that chloroplasts can achieve

ADP/O ratios above 1.5, which provides sufficient ATP not only for CO_2 fixation but for other energy-requiring synthetic and maintenance functions that occur within the chloroplast. It should be noted that ATP and NADPH are not used solely for CO_2 fixation in chloroplasts and a strict stoichiometry of 1.5 ATP per NADPH is not required, particularly if any pseudocyclic or cyclic electron transport occurs. If a pseudocyclic pathway of electron transport were present in chloroplasts to produce additional ATP, a fine control mechanism would have to exist to regulate between the noncyclic electron transport (producing NADPH and ATP) and the pseudocyclic pathway (producing only ATP) in order to achieve the correct balance between NADPH and ATP required for CO_2 fixation. At present, no evidence exists for a pseudocyclic pathway or for any mechanism to regulate such a pathway.

ACKNOWLEDGEMENTS

This work was supported by the Australian Research Grants Committee and by a Commonwealth Post-Graduate Award (S.P.R.).

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