Vol. 56, No. 3, 1974 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

PHOTOSYNTHETIC CONTROL AND PHOTOPHOSPHORYLATION IN PHOTOSYSTEM II OF ISOLATED SPINACH CHLOROPLASTS

P. Heathcote and D.O. Hall, Dept. Plant Sciences
King's College, 68 Half Moon Lane, London SE24 9JF, England.

Received December 10,1973

Two cycles of photosynthetic control have been observed in isolated spinach chloroplasts in the presence of lipophilic class III electron acceptors, which may accept electrons at PS II. ADP/O ratios of 0.8 to 0.9 were recorded; rates of oxygen evolution were stimulated by phosphorylating reagents and uncouplers. Addition of the plastoquinone antagonist DBMIB decreased photosynthetic control, oxygen evolution and photophosphorylation. We believe that there is a coupling site associated with PSII which can be rate limiting. Comparison of the P/2e ratios observed with class I and class III electron acceptors leads us to propose that more than 0.6 and possibly approaching one molecule of ATP can be formed for every pair of electrons transported from water to PSII acceptors.

Winget et al[1] proposed that more than one molecule of ATP was formed per pair of electrons transferred through the non-cyclic electron transport chain. This work was extended[2], while others demonstrated photosynthetic control and ADP/O's of above one[3-7]. Such observations suggested that there were two ATP coupling sites in non-cyclic electron transport. Neumann et al[8] suggested from work with sub-chloroplast photosystem I (PSI) particles that both of these were situated on the intermediary electron transport chain and associated with PSI. Saha et al[9] surveyed electron acceptors and found that non-lipid soluble electron acceptors that did not uncouple (called class I) accepted electrons after both coupling sites. Lipid soluble acceptors (class III) accepted electrons after one coupling site, and the photophosphorylation associated with the photoreduction of class III acceptors had a P/2e half that associated with class I acceptors. (P/2e = ATP/2e = ADP/O; see ref.7).

Trebst et al[10] recently introduced the electron transport inhibitor dibromothymoquinone (DBMIB) as a plastoquinone antagonist, which enabled both Trebst and Reimer[11] and Izawa et al[12] to demonstrate the proportion of photophosphorylation associated with the photoreduction of class III acceptors which could be solely due to a PSII associated coupling site. Trebst and Reimer[11] reported that the photoreduction of p-benzoquinone and 2,6-dimethylbenzoquinone (2,6 DBQ) in the presence of DBMIB were coupled with a P/2e of 0.3; electron transport was stimulated by uncouplers. Ferricyanide (FeCy) photoreduction in the presence of DBMIB had a P/2e ratio of only 0.16, and was not stimulated by uncouplers. Izawa *et al*[12] reported that the photoreduction of class III acceptors in the absence of DBMIB was coupled with a P/2e approaching half of the over all P/2e of 1.1 to 1.3 observed with class I acceptors. Uncouplers and subtrates of photophosphorylation did not affect the high rates of electron transport in the presence of class III acceptors. The photoreduction of FeCy and class III acceptors in the presence of DBMIB (and of DBMIB alone[13]) only had a P/2e of 0.3 to 0.4. These results were confirmed by use of pre-incubation of the chloroplasts with the plastocyanin antagonists, KCN and poly-lysine[14-16] Izawa[12,14] thus proposed that class III acceptors could accept a percentage of electrons at a site subsequent to plastocyanin, and after the coupling site associated with PSI. Further work[17] confirmed that there was a coupling site associated with PSII, with a P/2e of 0.3 to 0.4. This, if added to the P/2e of 0.6 observed for the PSI coupling site, closely approached the overall P/2e of about 1.1 for PSI + PSII. Rates of O_2 evolution in the PSI system showed stimulation by phosphorylating reagents and sensitivity to uncouplers[17]. Strotmann and Von Gosslen[18] reported similar effects but they computed a PSI P/2e of 1.0. Although electron transport in the presence of class I acceptors showed stimulation when phosphate was added in the presence of ADP, DBMIB or KCN removed this effect[17]. Work by Gimmler[19,20] on whole cells of *Dunaliella* has demonstrated photophosphorylation associated with DBMIB - insensitive light-induced conformational changes, and that fluorescence of PSII is under the energy control of its coupling site.

Our results confirm the observations[7] of photosynthetic control (a state II to state III to state IV cycle[4]) and photophosphorylation associated with the photoreduction of class III acceptors. In the present work ADP/O and ATP^{32}/O ratios between 0.8 and 0.9 were observed with class III acceptors, which were usually half the values observed with class I acceptors. 1.0μM DBMIB decreased rates of oxygen evolution, photosynthetic control, stimulation by uncouplers, and the ADP/O. Although the fraction by which photophosphorylation was decreased agreed with Izawa[12], the P/2e's with different acceptors were not all decreased to the same value and some acceptors exhibited DBMIB-resistant P/2e's above 0.6.

It is believed that the results presented demonstrate that the coupling site associated with PSII has a rate-limiting step. DBMIB-insensitive class III acceptor photophosphorylation has produced up to 0.65 molecules of ATP^{32} for every pair of electrons transported. With class III acceptors in the absence of DBMIB, or with 1.0 μM DBMIB as an electron acceptor, a P/2e approaching one is observed.

METHODS. Chloroplasts (unbroken, type A[21]) were isolated from commercially purchased Spinacea oleracea and assayed as ref. 7 except that ascorbate was omitted from the grinding medium. ADP/O values were calculated by the method of Hall et al[5] and ATP^{32} formation assayed by the method of Hagihara and Lardy[22]. DBMIB was the gift of Prof. A.V. **Trebst,** DAD was the gift of Dr. N.E. Good and was also purchased from Eastman Kodak together with phenylenediamene di HCl (PD) and 2,5 dimethylbenzoquinone (2,5 DBQ). 2,6 DBQ was obtained from the Aldrich Chemical Co. DAD and PD were used as stock 10 mM solutions in water; 2,5 - and 2,6 DBQ and DBMIB were dissolved in ethanol at 50 mM and 2 mM concentrations, respectively. All class III acceptors were used in the presence of 2 mM FeCy to keep them oxidised and to prevent donation of electrons to PSI. Variation in experimental results was due to the variability of spinach used during the summer months in which the experiments were performed.

RESULTS and DISCUSSION Photosynthetic control, and ADP/O ratios approaching one, can be observed with optimum concentrations of the class III acceptors DAD_{ox} (Fig.1) and 2,6 DBQ (Fig.2). Oxygen electrode experiments showed that two cycles of photophosphorylation could be observed at these optimum concentrations. Addition of DBMIB decreased photosynthetic control and ADP/O's and uncouplers

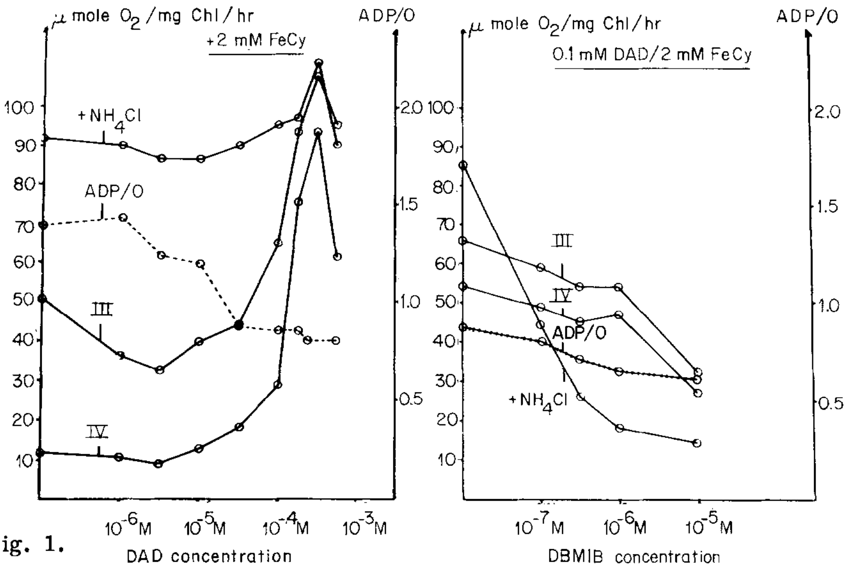

Fig. 1.

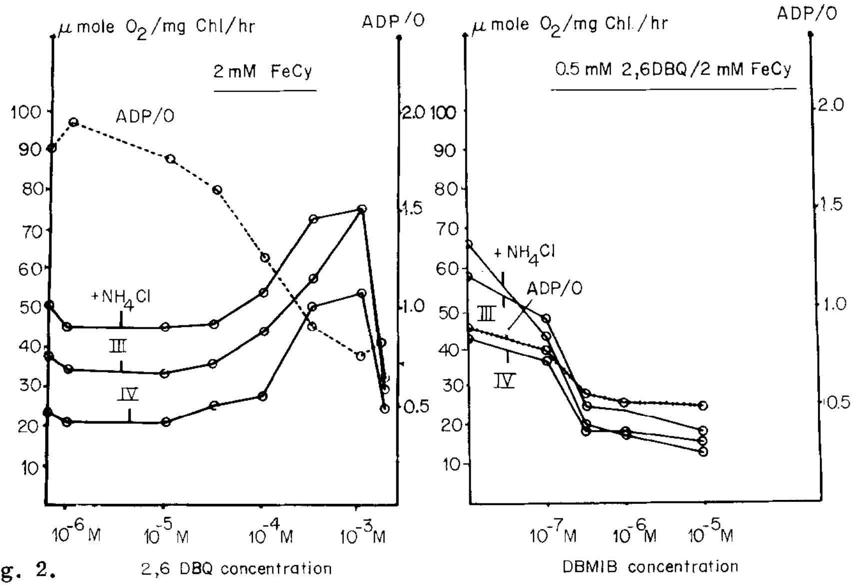

Fig. 2.

(NH_4Cl and CCCP) inhibited rather than stimulated O_2 evolution. From Fig. 3 it would appear that 1.0 μM DBMIB effectively blocks electron transport to PSI, since when added to a $H_2O \longrightarrow$ FeCy system this concentration gave maximum inhibition (90%) of phosphorylating rates of O_2 evolution. Above this

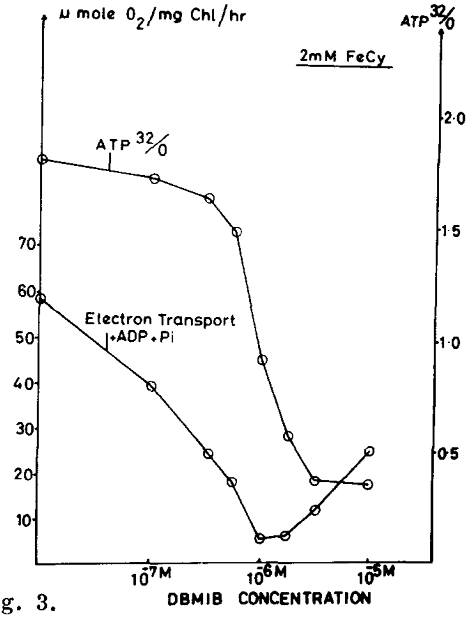

Fig. 3.

concentration rates of oxygen evolution start to increase since DBMIB can act as a PSII acceptor[12,13]. Table I summarises photosynthetic control and ADP/O's with four class III acceptors ± DBMIB, and confirms these results with ATP^{32} measurements. As the photosynthetic control is quite low (1.2-1.6), measurements of ADP/O's will be prone to inaccuracy, and ATP^{32}/O's were used for comparing P/2e ratios. With class III acceptors (also 1.0 μM DBMIB) the stoichiometry of photophosphorylation (0.8-0.9) is approximately half that observed with the class I acceptor FeCy (1.9). 1.0 μM DBMIB decreases the ATP^{32}/O ratios by 30-40% (from 0.9 to 0.6) with DAD$_{ox}$ and PD$_{ox}$, and by 40-50% with the DBMIB analogues (substituted quinones) 2,5 and 2,6 DBQ (from 0.9 to 0.5). It could be assumed that if the P/2e for the PSII site is subtracted from the P/2e observed with FeCy, the P/2e for the PSI coupling site should result. As Table I demonstrates, if only the DBMIB-insensitive PSII photophosphorylation is subtracted P/2e's of 1.2 to 1.4 result. Photophosphorylation in the presence of class I acceptors (e.g. FeCy, methyl viologen (MV)) could include a contribution by cyclic or pseudo-cyclic electron transport. However there is no evidence to support this view[2,4,7].

Table I ADP/O and ATP^{32}/O with class I and III acceptors

	O_2 electrode expts.			ATP^{32} formation expt.		
	State III	+ NH_4	ADP/O	O_2 evolution (+ ADP + Pi)	overall ATP^{32}/O	computed PSI ATP^{32}/O
(1) FeCy (2mM)	43	82	1.77	70	1.90	-
" + DBMIB	6	6	-	7	0.94	-
(2) DAD$_{ox}$ (0.1mM)	66	86	0.87	93	0.96	1.28
" + DBMIB	54	18	0.65	63	0.62	
(3) 2,6 DBQ (0.5mM)	58	66	0.87	74	0.96	1.42
" + DBMIB	23	17	0.51	50	0.48	
(4) PD$_{ox}$ (0.1mM)	112	114	0.87	128	0.94	1.25
" + DBMIB	90	42	0.63	95	0.65	
(5) 2,5 DBQ (1mM)	41	50	0.91	88	0.92	1.34
" + DBMIB	23	22	0.57	54	0.56	

O_2 evolution in µmoles/mg. chlorophyll/hr. All class III acceptors contained 2mM FeCy in the reaction mixture. DBMIB was 1µM. Computed PSI ATP^{32}/O obtained by subtracting the DBMIB-insensitive component of electron transport from the overall ATP^{32}/O. ATP^{32} formation and ATP^{32}/O ratios were linear with time over 5 min. Dark controls were about 1% of FeCy rate.

Table II ADP/O and ATP^{32}/O with PS I + II and PS II systems

	O_2 electrode expt.		ATP^{32} formation expt.	
	State III	ADP/O	O_2 exchange	ATP^{32}/O
$H_2O \longrightarrow$ FeCy	+ 51	1.60	+ 65	1.68
$H_2O \longrightarrow$ FeCy/DBMIB	+ 5	-	+ 6	0.69
$H_2O \longrightarrow$ DAD$_{ox}$	+ 76	0.77	+ 92	0.85
$H_2O \longrightarrow$ DAD$_{ox}$/DBMIB	+ 51	0.60	+ 52	0.54
$H_2O \longrightarrow$ MV	- 41	1.64	- 51	1.74
$H_2O \longrightarrow$ MV/DBMIB	- 1	-	- 2	0.33
$H_2O \longrightarrow$ DAD $\longrightarrow$ MV	- 24	1.64	- 52	1.77
$H_2O \longrightarrow$ DAD/DBMIB $\longrightarrow$ MV	- 18	1.44	- 18	1.57
$H_2O \longrightarrow$ PD/DBMIB $\longrightarrow$ MV	- 3	-	*	*

Conditions as in Table I MV = 50µM. * = not measured.

Confirmation of the overall P/2e in the presence of a class III acceptor (DAD) and the class I acceptor(MV) is provided by table II. Normally the addition of 1.0 µM DBMIB will block electron transport from H_2O to MV (Izawa[12]).

However, the addition of 0.1 mM DAD provides a substitute for plastoquinone which is blocked by DBMIB, and DAD thus carries a proton and an electron across the membrane. As Table II shows nearly a doubling of the P/2e ratios is observed when a H_2O —> DAD/DBMIB —> MV system is substituted for an H_2O —> DAD/FeCy system (i.e. PSII + I is substituted for PSII). This experiment provides additional evidence that DAD is a coupled PSII electron acceptor and that the P/2e for the PSII coupling site could approach one. If only the DBMIB-insensitive portion of the P/2e observed with DAD as an acceptor is deducted from the overall P/2e of the H_2O —> DAD/DBMIB —> MV system, again a calculated P/2e of above one results. DAD is participating in the H_2O —> DAD/DBMIB —> MV system, since PD does not appear to be able to reverse the DBMIB inhibition possibly because it cannot transfer protons across the membrane[23]. There is no evidence to suggest that DBMIB is a PSII photophosphorylation inhibitor, although concentrations above saturating (1 μM) further inhibit P/2e in an H_2O —> DBMIB/FeCy system (Fig.3).

Izawa and coworkers suggest[17] that the P/2e for the PSI coupling site is greater than that for the PSII coupling site (0.6 as opposed to 0.4). In the light of the chemiosmotic theory for coupling of electron transport to photophosphorylation a coupling site with energy control could simply be envisaged as that area of the electron transport chain capable of translocating a proton across the thylakoid membrane. In fact if one considers the scheme based on the experiments by Junge and coworkers[24-26] the water-splitting mechanism situated on the inside of the thylakoid membrane could release one proton. A second proton is released on the inside of the membrane by plastoquinone which has accepted an electron and bound a proton on the outside of the membrane and thus carries a hydrogen atom across the membrane. Class III acceptors could accept electrons after PSII on the outside of the membrane and in the conditions of our experiments pass them immediately to saturating concentrations of FeCy and not carry a proton back across to the inside of the membrane. Thus class III acceptors would intercept electrons between two coupling sites. If a coupling site is simply the translocation of a proton each site should show the same P/2e since the H+/ATP should be constant, assuming the lower "driving force" (proton gradient) generated by only one PS is converted to ATP with the same efficiency when two PS operate with a higher "driving force". As yet there is no evidence that a constant leakage rate of protons (or membrane potential decay) should affect PSII more than PSI. The results presented again support a P/2e of approaching two for non-cyclic electron transport. Accepting Junge's scheme of 4 H+ translocated per two electrons, this implies an actual H+/ATP of 2, but Rumberg *et al*[28,29] report values of 3 or 4.

Acknowledgements P.H. is the recipient of a Science Research Council Studentship. We thank Dr. M.C.W. Evans for discussions.

1. Winget, G.D., Izawa, S. and Good, N.E. (1965). Biochem. Biophys. Res. Commun. **21**, 438-443.
2. Izawa, S. and Good, N.E. Biochim. Biophys. Acta. (1968). **162**, 380-391.
3. Horton, A.A. and Hall, D.O. (1968). Nature, **218**, 286-388.
4. West, K.R. and Wiskich, J.T. (1968). Biochem. J. **109**, 527-532.
5. Hall, D.O., Reeves, S.G. and Baltscheffsky, H. (1971). Biochem. Biophys. Res. Commun. **43**, 359-366.
6. West, K.R., Wiskich, J.T. (1973). Biochim. Biophys. Acta, **292**, 197-205.
7. Reeves, S.G. and Hall, D.O. (1973). Biochim. Biophys. Acta, **314**, 66-78.
8. Neumann, J., Arntzen, C.J. and Dilley, R.A. (1971). Biochem. **10**, 866-873
9. Saha, S., Ouitrakul, R., Izawa, S. and Good, N.E. (1971). J. Biol. Chem. **246**, 3204-3209.
10. Trebst, A., Harth, E. and Draber, W. (1970). Z. Naturforsch. **25b**. 1157-9.
11. Trebst, A. and Reimer, S. (1973). Biochim. Biophys. Acta, **305**, 129-139.
12. Izawa, S., Gould, J.M., Ort, D.R., Felker, P. and Good, N.E. (1973). Biochim. Biophys. Acta, **305**, 119-128.
13. Gould, J.M. and Izawa, S. (1973). Eur. J. Biochem. **37**, 185-192.
14. Ouitrakul, R. and Izawa, S. (1973). Biochim. Biophys. Acta, **305**, 105-118.
15. Izawa, S., Kraayenhof, R., Ruuge, E.K. and DeVault, D. (1973). Biochim. Biophys. Acta, **314**, 328-339.
16. Ort, D.R., Izawa, S., Good, N.E. and Krogmann, D.W. (1973). FEBS lett. **31**, 119-122.
17. Gould, J.M. and Izawa, S. (1973). Biochim. Biophys. Acta, **314**, 211-223.
18. Strotmann, H. and Von Gosslen, C. (1973). Z. Natursforsch. **27b**, 445-455.
19. Gimmler, H. (1973). Z. Pflanzenphysiol. **68**, 289-307.
20. Gimmler, H. (1973). Z. Pflanzenphysiol. **68**, 385-390.
21. Hall, D.O. (1972). Nature New. Biol. **235**, 12.
22. Hagihara, B. and Lardy, H.A. (1959). J. Biol. Chem. **235**, 889-894.
23. Hauska, G., Trebst, A. and Draber, W. (1973). Biochim. Biophys. Acta. **305**, 632-641.
24. Junge, W., Rumberg, B. and Schröder, H. (1970). Eur. J. Biochem. **14**, 575-581.
25. Junge, W. (1972). in 'Excited states in photosynthesis and vision'. British Photobiology Society, Chapter V. In press.
26. Junge, W. (1972). Abstr. VI Int. Photobiol. Congr., Bochum. p. 34.
27. Schröder, H., Muhle, H. and Rumberg, B. (1971). Proc. 2nd. Int. Congr. Photosynth. Res. Stresa, **2**, 919-930.
28. Rumberg, B. (1972). Abstr. VI. Int. Photobiol. Congr., Bochum. p. 36