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STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION

IV. THE RELATION OF PROTON FLUXES TO THE ELECTRON TRANSPORT AND ATP FORMATION ASSOCIATED WITH PHOTOSYSTEM II

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SUMMARY

1. By using dibromothymoquinone as the electron acceptor, it is possible to isolate functionally that segment of the chloroplast electron transport chain which includes only Photosystem II and only one of the two energy conservation sites coupled to the complete chain (Coupling Site II, observed $P/e_2 = 0.3$–0.4). A light-dependent, reversible proton translocation reaction is associated with the electron transport pathway: $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone. We have studied the characteristics of this proton uptake reaction and its relationship to the electron transport and ATP formation associated with Coupling Site II.

2. The initial phase of H^+ uptake, analyzed by a flash-yield technique, exhibits linear kinetics (0–3 s) with no sign of transient phenomena such as the very rapid initial uptake ("pH gush") encountered in the overall Hill reaction with methylviologen. Thus the initial rate of H^+ uptake obtained by the flash-yield method is in good agreement with the initial rate estimated from a pH change tracing obtained under continuous illumination.

3. Dibromothymoquinone reduction, observed as O_2 evolution by a similar flash-yield technique, is also linear for at least the first 5 s, the rate of O_2 evolution agreeing well with the steady-state rate observed under continuous illumination.

4. Such measurements of the initial rates of O_2 evolution and H^+ uptake yield an H^+/e^- ratio close to 0.5 for the Photosystem II partial reaction regardless of pH from 6 to 8. (Parallel experiments for the methylviologen Hill reaction yield an H^+/e^- ratio of 1.7 at pH 7.6.)

5. When dibromothymoquinone is being reduced, concurrent phosphorylation (or arsenylation) markedly lowers the extent of H^+ uptake (by 40–60%). These data, unlike earlier data obtained using the overall Hill reaction, lend themselves to

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Abbreviations: P/e_2, ratio of the number of molecules of ATP formed to the number of pairs of electrons transported; H^+/e^-, ratio of the number of hydrogen ions transported to the number of electrons transported; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone).

an unequivocal interpretation since phosphorylation does not alter the rate of electron transport in the Photosystem II partial reaction. ADP, P_i and hexokinase, when added individually, have no effect on proton uptake in this system.

6. The involvement of a proton uptake reaction with an H^+/e^- ratio of 0.5 in the Photosystem II partial reaction $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone strongly suggests that at least 50% of the protons produced by the oxidation of water are released to the inside of the thylakoid, thereby leading to an internal acidification. It is pointed out that the observed efficiencies for ATP formation (P/e_2) and proton uptake (H^+/e^-) associated with Coupling Site II can be most easily explained by the chemiosmotic hypothesis of energy coupling.

INTRODUCTION

The electron transport pathway in isolated chloroplasts can be divided into several segments by the use of appropriate electron donor–acceptor combinations in conjunction with specific inhibitors of certain intermediate electron carriers [1]. When this is done it becomes clear that there are two sites of energy conservation in the phosphorylation-coupled transport of electrons from water to Photosystem I [1–5]. These two coupling sites differ markedly in their characteristics.

Coupling Site I refers to the energy conservation site associated with the transfer of electrons from plastoquinone to cytochrome *f* [6, 7]. This site provides the rate-determining step for the overall Hill reaction [1]. The rate of electron flux through Coupling Site I is greatly increased by ADP plus phosphate under phosphorylating conditions and still more by the presence of phosphorylation uncouplers. The efficiency of phosphorylation (P/e_2) at Site I is dependent on the pH of the medium, with an optimum at pH 8–8.5 where the observed P/e_2 is 0.6–0.7 (ref. 1). ATP formation supported by Coupling Site I is inhibited by the chloroplast energy transfer inhibitor $HgCl_2$ (ref. 8).

Coupling Site II is associated with the transfer of electrons from water to plastoquinone or with the transfer of electrons from water to exogenous lipophilic "Class III" oxidants which intercept electrons before they reach plastoquinone [5, 9]. Coupling Site II differs from Site I in a number of important respects. The rate of electron transport through Site II is independent of phosphorylation and uncoupling [1, 9, 10]. The efficiency of phosphorylation is practically independent of pH (6–9), and the observed P/e_2 ratio is characteristically 0.3–0.4 [1–3, 5, 9, 10]. Moreover, ATP formation supported by Coupling Site II is insensitive to $HgCl_2$ (ref. 8).

These differences suggest that the modes of energy transduction at Sites I and II are somehow different. Therefore we have been prompted to undertake a further investigation into the mechanism by which electron transport may be coupled to phosphorylation at these two sites. This paper deals primarily with further characterizations of the light-induced proton uptake (pH rise) associated with the transfer of electrons from water to dibromothymoquinone via Photosystem II [1]. In previous papers [3, 9, 13] we established that this plastoquinone antagonist [11], which at low concentrations (0.5 μM) blocks the transport of electrons from reduced plastoquinone to cytochrome *f* [12], can act at higher concentrations (25 μM) as an acceptor of electrons from Photosystem II, probably via plastoquinone [9]. In

addition, we have shown that the phosphorylation reaction associated with dibromothymoquinone reduction exhibits all of the characteristics of Coupling Site II outlined above [9].

The use of substrate concentrations of dibromothymoquinone provides a convenient reaction system for studying the nature of Coupling Site II, since the function of the substrate as an inhibitor effectively blocks further electron transport (to Photosystem I) and thereby isolates Site II from Site I. Furthermore, we have recently presented evidence that a reversible "proton pump" is associated with dibromothymoquinone reduction [1]. Since electron transport in this system is unaffected by uncouplers or by phosphorylation, it has been possible to observe directly the effects of ATP formation on the "proton pump" without a complication encountered by previous investigators [14, 15]. Heretofore it has been impossible to study the effect of phosphorylation on proton uptake without increasing the electron transport rate, thereby inadvertently modifying the associated proton uptake. Therefore it has previously not been possible to study the proton pump and phosphorylation as opposing processes in an unambiguous way.

The results presented in this paper show that there is a quantitative relationship between the electron transport-dependent accumulation of hydrogen ions and ATP formation at Coupling Site II. The results also suggest that Coupling Site II in chloroplasts may be identified with the water-oxidation reaction, which seems to release protons to the inside of the chloroplast lamellar membrane.

MATERIALS AND METHODS

Chloroplasts (intact, naked lamellae) were isolated in the cold from leaves of fresh market spinach (*Spinacia oleracea* L.) as described in a previous paper in this series [1]. For some experiments the HEPES–NaOH buffer in the suspension medium was replaced with either phosphate or arsenate and the bovine serum albumin was omitted. The concentration of chloroplasts in the final stock suspension was adjusted so that the amount of buffer carried over into the reaction mixture gave a final concentration of 0.5 or 1.0 mM.

Changes in hydrogen ion concentration in the reaction mixture were detected with a Corning semi-micro ("Tri-purpose") combination pH electrode connected to a fast responding Heath/Schlumberger EU200-30 pH electrometer module equipped with a Heath/Schlumberger EU200-02 DC offset module to facilitate scale expansion. The output from the electrometer was recorded on an Esterline-Angus strip chart recorder. The response half-time for the pH measuring system was in the order of 0.5–1 s. Changes in pH were normally monitored with a scale expansion of 0.1 pH unit full scale (10 inches) on the recorder. The noise level at this amplification was less than 0.002 pH unit.

In routine pH experiments, reactions were run in a final volume of 2.0 ml in thermostated vessels at 18 °C with continuous stirring. Prior to illumination the reaction mixture was adjusted to the desired initial pH with small volumes of dilute NaOH or HCl. Actinic illumination was supplied by a 500-W slide projector. The beam was passed through a 500-ml round-bottomed flask containing a dilute $CuSO_4$ solution (which served both as an infrared filter and as a condensing lens) and through an orange glass filter (transmission > 600 nm) plus a Corning I-69 heat filter. The

light intensity was approximately 700 kergs $\cdot s^{-1} \cdot cm^{-2}$ (600–700 nm). At the end of each experiment the pH changes registered on the chart paper were translated into H^+ equivalents by titrating the reaction mixture in the light with a known amount of 0.001 M HCl.

Electron transport was measured as oxygen evolution (dibromothymoquinone as acceptor) or oxygen uptake (methylviologen as electron acceptor) [16] using a Clark-type membrane-covered oxygen electrode. For these experiments a 3.0-ml reaction mixture was used. When both electron transport and proton uptake were determined, the pH rise was measured in an identical reaction mixture in the same apparatus, substituting the pH electrode for the oxygen electrode.

RESULTS

Light-induced pH rise associated with dibromothymoquinone reduction

We have previously presented evidence that the electron transport pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone, which includes Coupling Site II but not Coupling Site I, is associated with a light-dependent, reversible proton uptake [1]. These results have been confirmed and extended (Fig. 1).

Fig. 1 depicts the general pattern of the light-induced pH rise in the medium. Above pH 8.1, where reduced dibromothymoquinone is rapidly reoxidized by molecular oxygen [9] (and therefore no exhaustion of the electron acceptor occurs), the pH rise can be observed many times using repeated light cycles (trace A). Below pH 8, where the reoxidation rate is very slow, the pH shift can be maintained only as long as the reduction of dibromothymoquinone continues. As the reduction approaches completion and the electron transport slows down, a gradual reversal of the pH change is observed even in the light, and eventually the pH returns to the original level (trace B). Subsequent illuminations do not restore the pH rise. If the light is turned off before the complete exhaustion of the electron acceptor, a second light cycle does induce a small pH rise, the extent of which depends upon the amount of oxidized dibromothymoquinone remaining. The uncoupler gramicidin D (4 μg/ml) completely abolishes the light-induced pH response (data not shown).

If ferricyanide is added to the reaction mixture so that the photoreduced dibromothymoquinone is continually reoxidized by the excess ferricyanide, a reversible pH rise superimposed on an irreversible pH drop is observed (Fig. 1 trace C). This is repeatable even below pH 8 since the reoxidation of reduced dibromothymoquinone by ferricyanide is very rapid at any pH above 6. The irreversible pH drop is due to the protons produced by the oxidation of water according to the equation:

$$1/2\ H_2O + Fe^{3+} \rightarrow 1/4\ O_2 + Fe^{2+} + H^+.$$

Thus the rate of proton production can also be used as a means for determining the rate of electron transport. As trace D shows, both the transient pH rise and the lag in the light-induced pH drop due to the superimposed proton uptake are eliminated by the uncoupler gramicidin, although the rate of electron transport (measured as proton production) is scarcely affected. This confirms our previous observations that the rate of electron transport associated with dibromothymoquinone reduction is not increased by uncouplers [9].

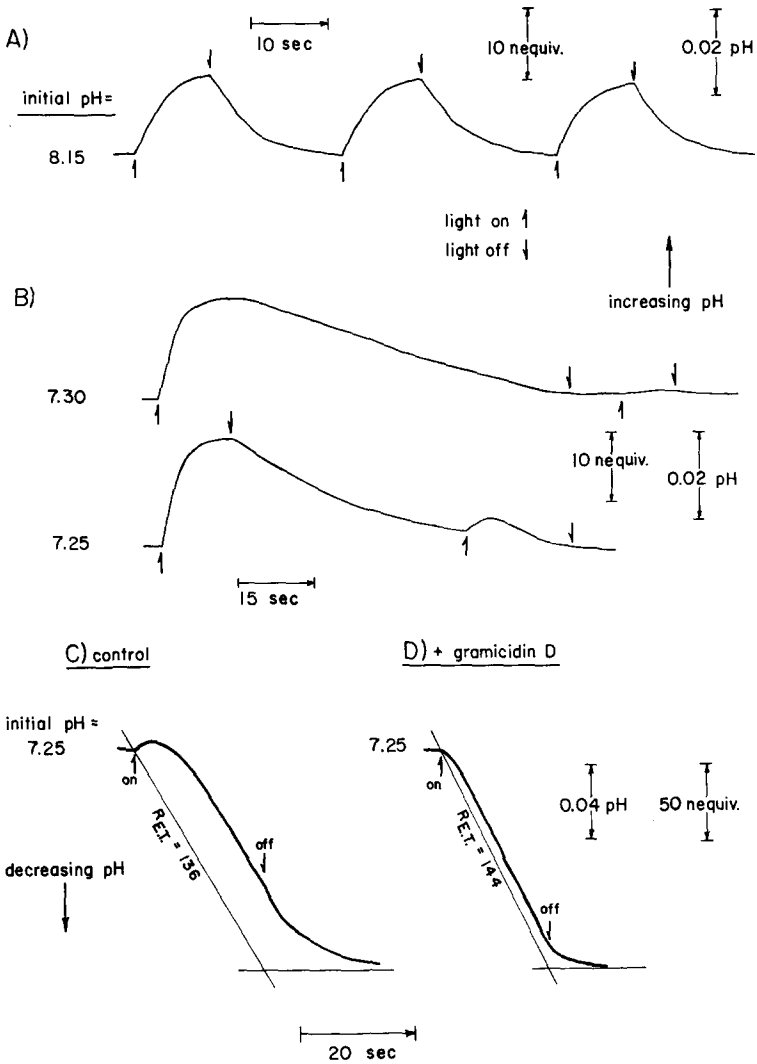

Fig. 1. Light-induced pH changes associated with the partial electron transport pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone in isolated chloroplasts. The 2.0-ml reaction mixture contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM NaCl, 0.5 mM HEPES–NaOH buffer, 20 μM dibromothymoquinone and chloroplasts containing 100 μg chlorophyll. In the experiments presented in traces C and D, 0.5 mM potassium ferricyanide was added to the reaction to continually reoxidize the photoreduced dibromothymoquinone. Note that the rate of electron transport ($R_{E.T.}$; in μequiv $\cdot$ h^{-1} $\cdot$ mg chlorophyll^{-1}) is not increased by the uncoupler gramicidin D. For further explanation see the text.

Stoichiometry between electron transport and proton uptake (H^+/e^-)

The data presented in Fig. 1 are most easily interpreted in terms of a transmembrane H^+ gradient associated with the Photosystem II partial reaction $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone. However, the significance of this proton gradient and its relation to the coupling mechanism cannot be evaluated critically

until the efficiency of proton uptake (H^+/e^-) is determined. Moreover, determination of H^+/e^- in this case is of special interest, since the dibromothymoquinone partial reaction includes only one (Site II) of the two coupling sites associated with the complete Hill reaction [3, 9]. Thus the observed efficiency of ATP formation (P/e_2) supported by this partial electron transport pathway is lower than the efficiency of the complete chain where both coupling sites are operating ($P/e_2 = 0.3$–0.4 versus 1.1–1.2, respectively) [1]. Therefore one might reasonably expect the efficiency of proton uptake associated with Site II to be correspondingly low. However, the determination of an H^+/e^- ratio involves a variety of difficulties, and, in fact, no truly unequivocal method of measuring it has as yet been developed. For a more complete discussion of these difficulties, see review articles by Jagendorf [17], Walker and Crofts [18] and Schwartz [19]. In the experiments outlined below we have observed the initial kinetics of proton uptake and electron transport by two different methods in an attempt to circumvent these difficulties and to accurately measure the H^+/e^- in the dibromothymoquinone partial reaction.

The response time ($t_{\frac{1}{2}} \leqslant 1$ s) of the pH assay system used in these experiments (see Methods) was considerably faster than the apparent kinetics of the pH rise ($t_{\frac{1}{2}} \simeq 4$–5 s) and therefore it seemed possible that the relatively linear initial phase

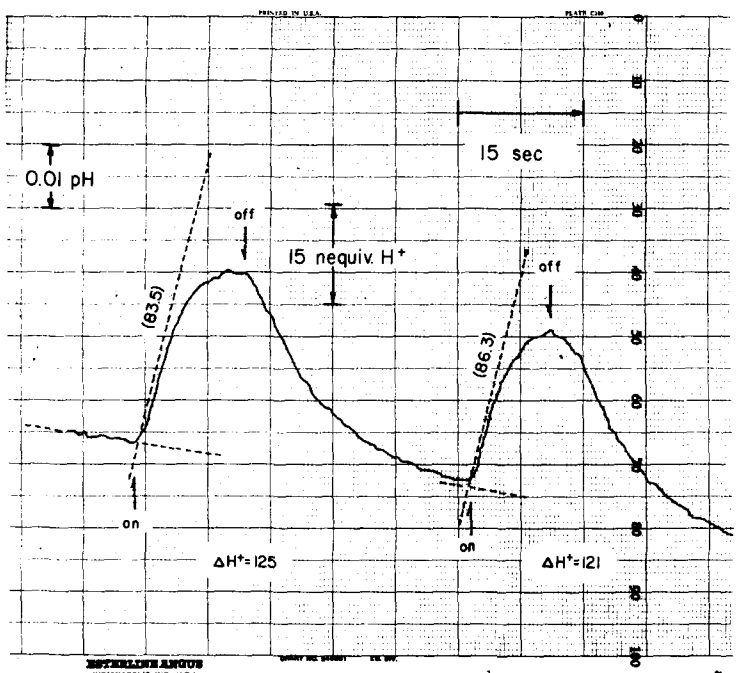

Fig. 2. Recorder tracing of the apparent kinetics of the reversible pH rise associated with Photosystem II electron transport from water to dibromothymoquinone. The reaction mixture was as in Fig. 1, trace A, except the HEPES buffer was replaced by 2.25 mM Na_2HPO_4 and contained chloroplasts equivalent to 145 μg chlorophyll. The initial pH was 8.16. Numbers in parentheses are the apparent initial rates of proton uptake in μequiv $H^+ \cdot h^{-1} \cdot$ mg chlorophyll^{-1}. The steady-state extent of the pH rise (ΔH^+; in nequiv · mg chlorophyll^{-1}) was determined by titrating the reaction mixture in the light with known volumes of 0.001 M HCl (not shown).

of the pH tracings (Fig. 2) might provide a reasonably accurate estimate of the initial rate of proton uptake. This assumption would be invalid, of course, if the pH rise involved any transient kinetics faster than the instrument response time, such as the initial "pH gush" ($t_{\frac{1}{2}} \simeq 0.10$ s) described earlier by Izawa and Hind [20]. In order to detect the possible involvement of such transient kinetics, we have examined pH changes induced by a series of brief illuminations (flash duration 1–3 s) with intervening dark periods to allow for the lag due to instrument response time. That is, a sufficient dark period was introduced between successive flashes to allow the instrument to record the entire pH change which had been completed during the flash. This dark period was not long enough to allow any significant decay of the pH change, however. Somewhat surprisingly, we could detect no evidence for any significant "gush" phenomena at all associated with dibromothymoquinone reduction (Fig. 3, trace b), the rate of proton uptake determined for the first flash being essentially the same as the rate determined for a subsequent flash. Plot c of Fig. 3 represents the early phase of proton uptake reconstructed by summing the flash yields and illumination times of trace b. A very good agreement was found between the initial slope of the linear phase of the reconstructed time-course curve (< 4 s; trace c) and the initial slope of the continuous recorder tracing (trace a). This is not surprising since, as trace b shows, there was no significant overshoot of the pH response after a flash except for the small amount attributable to the instrumental time lag. Nor was there a rapid enough dark decay to obscure the flash yield. In other words, each flash yield determination did seem to accurately represent the extent of the pH rise which had been essentially completed during the flash. Thus the initial slope of a pH rise curve obtained under continuous light must in fact be a fairly reliable measure of the actual initial rate of proton influx.

The rate of electron transport in the same time range (0–5 s after light on) was measured as O_2 evolution using a duplicate reaction mixture in the same apparatus (Fig. 3, traces d–f). Flash experiments were even more important in this case, because of the very slow response time ($t_{\frac{1}{2}} \simeq 2$ s) of the membrane-covered oxygen electrode used. The flash experiments again detected no sign of transient phenomena (Fig. 3, trace e) and again the reconstructed slope agreed rather precisely with the steady-state slope determined under continuous light (traces d, f). (Transient kinetics such as those discovered by Joliot et al. [21] are far beyond the resolution of our instrument.) The ratio H^+/e^- determined from the data presented in this set of experiments (Fig. 3) was 0.51.

The use of intermittent brief periods of illumination described above makes transient differences between initial rates and steady state rates quite obvious when these differences do exist. Thus the initial "pH gush" known to be associated with the reduction of methylviologen by chloroplasts [20] is clearly revealed by the flash technique (Fig. 4b) even though the pH changes recorded during continuous illumination conceal the transient (Fig. 4a). However, if the tracing for the continuous illumination is corrected for the instrument response time according to the method of Izawa and Hind [20], there is good agreement between the kinetics of the pH rise as determined by the flash yield technique and the corrected kinetics for continuous illumination. The "pH gush" amounted to one mole of H^+ for each 20–30 moles chlorophyll in this experiment. However, no corresponding initial fast phase in oxygen production could be detected (Fig. 4b), indicating that the "pH-gush" observed under some

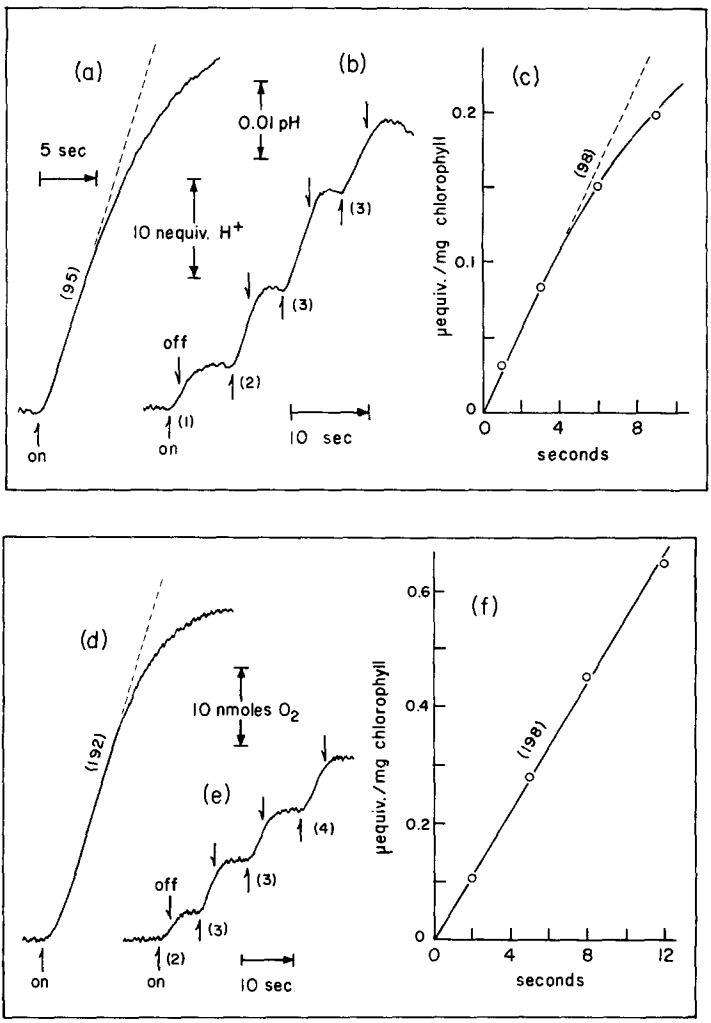

Fig. 3. Initial kinetics of the light induced pH rise and electron transport measured under both flash and continuous illumination with dibromothymoquinone as the electron acceptor. The reaction mixture (3.0 ml) was as in Fig. 1, trace a, except that the concentration of dibromothymoquinone was 50 μM and the reaction contained chloroplasts equivalent to 150 μg chlorophyll. The initial pH was 7.4. Trace a: recorder tracing of the apparent initial kinetics of the pH rise observed under continuous illumination. The rate of H^+ uptake in μequiv $H^+ \cdot h^{-1} \cdot$ mg chlorophyll^{-1} was determined from the essentially linear initial portion of the tracing and is shown in parentheses. Trace b: recorder tracing of the pH rise induced by flash illumination. Numbers in parentheses represent the flash duration (in s). Trace c: reconstructed time course of the initial portion of the pH rise from flash yield determinations (trace b). Note that the rate of proton uptake measured by the flash technique agrees very well with the rate determined under continuous illumination (trace a). Trace d: recorder tracing of the kinetics of O_2 evolution under continuous illumination. The rate of electron transport (in μequiv $\cdot h^{-1}$ $\cdot$ mg chlorophyll^{-1}) is shown in parentheses. Trace e: recorder tracing of O_2 evolution measured by the flash yield technique. Numbers in parentheses represent flash duration (in seconds). Trace f: reconstructed time-course for O_2 evolution determined from the flash experiment (trace e). Note that the rate determined by this technique agrees very well with the steady-state rate determined under continuous illumination (trace d).

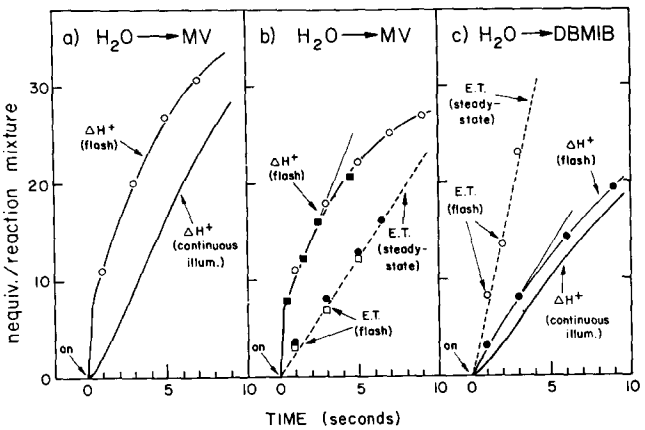

Fig. 4. Comparison between the initial rates of proton uptake and electron transport for the complete electron transport chain ($H_2O \rightarrow$ methylviologen) and the Photosystem II partial reaction $H_2O \rightarrow$ dibromothymoquinone. Reaction conditions were as in Fig. 3 with the following exceptions. In the experiments presented above in a and b, dibromothymoquinone (DBMIB) was omitted and 100 μM methylviologen (MV) was added. When dibromothymoquinone was the electron acceptor, electron transport (E.T.) was observed as O_2 evolution. When methylviologen was the acceptor, electron transport was followed as O_2 uptake [16]. The chlorophyll concentration was 50 μg/ml. (a) Comparison between the observed pH change (ΔH^+) under continuous illumination and by a flash yield technique. Note that the large "pH gush", which is clearly evident in the kinetics obtained by the flash technique, is masked in the continuous tracing due to the instrumental response lag. Nevertheless the slope of the relatively linear portion of the pH rise immediately following the "gush" is in good agreement with the slope obtained with continuous illumination. The initial pH for this experiment was 6.8. (b) Comparison between the kinetics of H^+ uptake (ΔH^+) and electron transport (E.T.) when methylviologen is the electron acceptor. The data shown is from two identical experiments done at pH 7.6. Note that the rate of electron transport determined under continuous illumination (dashed line) agrees very well with the electron transport rate determined by the flash technique (points), and that there is apparently no initial burst of electron transport corresponding to the initial "pH gush". The H^+/e^- ratio determined for this experiment (see text) was 1.7. (c) An experiment similar to b above except the electron transport pathway included only Photosystem II and only Coupling Site II. Dibromothymoquinone served as the electron acceptor. Note that the initial "pH gush" is absent in this system. The H^+/e^- ratio calculated for this experiment (pH 7.2) was 0.45 (See also Fig. 3).

conditions cannot be simply explained in terms of an initial rapid rate of electron transport associated with plastoquinone reduction. The ratio H^+/e^- observed during methylviologen reduction (calculated from the relatively linear portion of the early phase of the pH rise after the initial gush) was 1.7. Note again (Fig. 4c) that there was no significant initial rapid phase of proton uptake when dibromothymoquinone was being reduced and that the ratio H^+/e^- was much lower, about 0.5. Thus the proton pump associated with dibromothymoquinone reduction (involving only Coupling Site II) is distinguished from the proton pump associated with methylviologen reduction (involving both Sites I and II) in two ways: the reaction involving only Site II lacks an intitial rapid phase and is less than half as efficient as the combined sites in transporting H^+ across the lamellar membranes.

Table I summarizes the results of three independent series of experiments performed with different chloroplast preparations. While the ratio H^+/e^- for the dibromothymoquinone reducing system is relatively constant (0.35–0.5) over a wide

TABLE I

STOICHIOMETRY BETWEEN PROTON UPTAKE AND ELECTRON TRANSPORT (H^+/e^-) WITH DIBROMOTHYMOQUINONE OR METHYLVIOLOGEN AS ELECTRON ACCEPTOR

Reactions were run as described in Figs 3 and 4. The basic reaction mixture was as in Fig. 5 except in Expt 1, where the phosphate buffer was replaced by 0.5 mM HEPES–NaOH. The chlorophyll concentration was 100 μg/2ml. Note that when dibromothymoquinone served as the electron acceptor so that only Coupling Site II was being utilized, the ratio H^+/e^- was relatively constant (0.35–0.51) over a wide pH range. When methylviologen served as the electron acceptor (utilizing Coupling Sites I and II), the H^+/e^- ratio was much higher.

Expt No.	Electron acceptor	Initial pH	Initial rate (μequiv · h^{-1} · mg chlorophyll^{-1})		H^+/e^-
			H^+ uptake	Electron transport	
1	Dibromothymo-quinone	6.22	93**	218**	0.42
		6.60	102	225	0.45
		7.00	89	234	0.38
		7.48	81	212	0.38
2*	Dibromothymo-quinone	7.20	100**	198**	0.51
		7.60	63	146	0.43
		8.00	48	125	0.38
3*	Dibromothymo-quinone	7.30	69**	172**	0.40
		8.13	60**	168**	0.36
		8.15	53	153	0.35
	Methylviologen	7.40	103**	59**	1.74
		7.75	108**	63**	1.71

* Phosphate buffer (2.5 mM).

** Measured by flash yield determinations. Other values are from continuous tracings.

range of pH values (6.2–8.2), there is some tendency for the lower H^+/e^- values to be associated with the higher pH values. Several considerations lead us to believe that the true efficiency of proton translocation in this system is even more pH-independent than actually observed, however, and that the true value for the ratio H^+/e^- is most likely about 0.5, the highest value actually encountered at the lower pH regimes (pH 6–7). At higher pH's, especially above pH 8, the outward diffusion of protons in the light is probably much faster and therefore competes more efficiently with proton accumulation (uptake). This was apparent in the experiments, since the dark decay process observed after turning off the light was approximately twice as fast at pH 8 as at pH 7 (decay half-time = 7 s at pH 8; 15 s at pH 7). It was also apparent in the flash experiments that the initial linear phase of the pH rise at pH 8 was somewhat shorter than at pH 7.3 (see Fig. 3, trace c).

Effect of phosphorylation and arsenylation on proton uptake

Both the chemiosmotic and the chemical coupling hypotheses predict that concomitant phosphorylation or arsenylation should decrease the steady-state extent of the proton uptake [22]. Several workers have indeed observed a decrease in the pH rise by concomitant phosphorylation or arsenylation [15, 23], but the opposite effect — a stimulation of proton uptake by arsenylation — has also been reported [14]. As has been pointed out by Dilley and Shavit [15], the acceleration of electron transport in ordinary noncyclic electron transport systems by phospho-

rylation (or in some cases by the effect of ions in the phosphorylation medium, e.g. Mg^{2+}), and resultant increases in the rate of proton flux could easily mask the true effect of phosphorylation on proton gradients. Moreover, the addition of ADP (or ATP) itself has also been shown to cause marked increases in proton uptake [23]. However, by using the Photosystem II-dependent dibromothymoquinone reduction one avoids these complications since the electron transport in this system is not stimulated by phosphorylation [9], nor is proton uptake enhanced by the addition of ADP (cf. Table II). This reaction therefore provides a convenient system in which to test the effect of phosphorylation on proton uptake.

TABLE II

EFFECT OF PHOSPHORYLATION AND ARSENYLATION ON THE EXTENT OF THE LIGHT-INDUCED PROTON UPTAKE ASSOCIATED WITH THE ELECTRON TRANSPORT PATHWAY $H_2O \rightarrow$ PHOTOSYSTEM II $\rightarrow$ DIBROMOTHYMOQUINONE

Reactions were run as in Figs 5 and 6. Final concentrations of the additions were: P_i, 2.5 mM; ADP, 75 μM; hexokinase, 1 μg/2ml (plus 10 mM glucose); $HAsO_4^{2-}$, 2.5 mM. The extent of the proton uptake was determined by titration in the light as described in Methods. Note that the presence of a complete phosphorylation or arsenylation system significantly lowers the steady-state extent of the proton uptake, although the individual phosphorylating agents have no effect by themselves (Expt 1).

Expt No.	Initial pH	Additions	ΔH^+ extent (nequiv/mg chlorophyll)	(%)
1	8.14	None	42	(100)
		ADP	42	(100)
		Hexokinase	40	(98)
		ADP, P_i, hexokinase	26	(62)
2	8.15	P_i	56	(100)
		ADP, P_i, hexokinase	22	(40)
3	8.22	P_i	70	(100)
		ADP, P_i, hexokinase	39	(56)
4	8.27	$HAsO_4^{2-}$	55	(100)
		$HAsO_4^{2-}$, ADP	44	(80)
5	8.25	$HAsO_4^{2-}$	73	(100)
		$HAsO_4^{2-}$, ADP	59	(81)

In Fig. 5, trace A demonstrates the reproducibility of the pH rise in a phosphate-containing suspension over repeated light cycles. This reproducibility greatly facilitated the experiments since the effect of an additive (e.g. ADP) could be examined without preparing a new reaction mixture. Trace B shows that in the absence of hexokinase, the addition of ADP initiates ATP formation which can be followed as the irreversible consumption of protons according to the equation:

$$ADP^{3-} + HPO_4^{2-} + H^+ \rightarrow ATP^{4-} + H_2O \qquad \text{(pH 8)}$$

Clearly the extent of the reversible proton uptake (seen as a proton efflux after turning off the light) is smaller under phosphorylating conditions (+ADP) than under non-phosphorylating conditions (−ADP). This is demonstrated in a different way in trace C, where the hexokinase+glucose system is used to consume the ATP

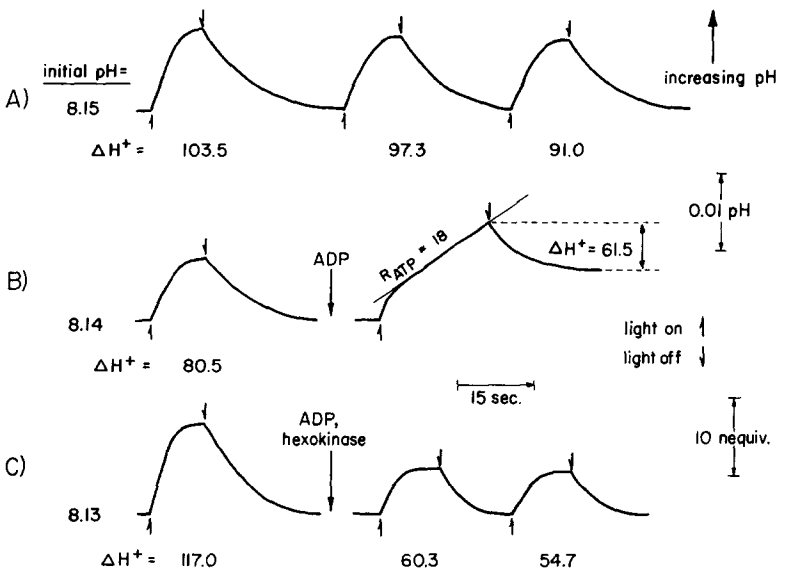

Fig. 5. Effect of phosphorylation on the extent of the light-induced proton uptake associated with electron transport from water to dibromothymoquinone. Reaction conditions are as in Fig. 1, trace A, except that the HEPES buffer was replaced by 2.5 mM Na_2HPO_4. The extent of the proton uptake (ΔH^+) was determined as described in Methods. Trace A illustrates the repeatability of the pH rise over repeated light–dark cycles. Trace B shows that the addition of ADP (final concentration 75 μM) causes an irreversible proton consumption in the light (due to ATP formation) and results in a decrease in the extent of the reversible proton uptake (seen as a dark efflux after turning off the light). This irreversible proton consumption was eliminated in trace C by the addition of hexokinase (1 μg/2ml) and glucose (10 mM) with the ADP (75 μM). Again a significant decrease in the extent of the proton uptake is observed. (The additives themselves had only a negligible effect on the buffering capacity of the reaction mixture.)

as it is produced and thus eliminate the irreversible proton consumption due to ATP formation. Hexokinase alone has no effect on the extent of the pH rise (cf. Table II).

If arsenate replaces phosphate (with or without hexokinase) a situation similar to the ADP+P_i+hexokinase system should be observed, since the unstable arsenylated ADP does not accumulate. Fig. 6 shows that indeed this is the case. A marked lowering in the extent of proton uptake is observed upon addition of ADP to an arsenate containing medium (in this case without hexokinase).

Table II summarizes the effect of various additions on the extent of the proton uptake associated with dibromothymoquinone reduction. It can be seen that in this system ADP, hexokinase or phosphate alone have no effect on the extent of proton uptake. The lack of effect by ADP in this system is in contrast with the observation of McCarty et al. [23]. Using the methylviologen system, they too noted that hexokinase had no effect on proton uptake, but they did observe a large stimulation by low concentrations of ADP. We have confirmed their observation using methylviologen as electron acceptor, although in our chloroplast preparations the maximum stimulation obtained was only 30–40%. The complete lack of ADP effect on proton uptake in the partial reaction involving only Coupling Site II suggests that the stimu-

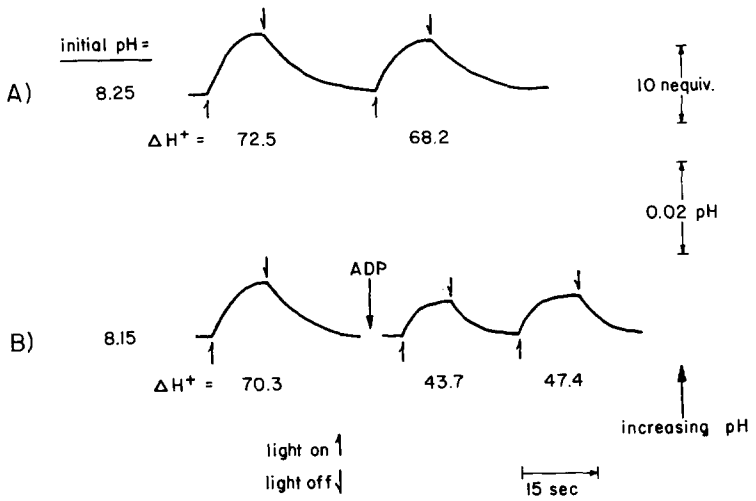

Fig. 6. Effect of arsenylation on the extent of the light induced proton uptake associated with electron transport to dibromothymoquinone. Reaction conditions were the same as in Fig. 5. except the Na_2HPO_4 buffer was replaced by 2.5 mM Na_2HAsO_4 and no hexokinase plus glucose was added. Note that the extent of the H^+ uptake was significantly smaller when ADP was added to the reaction mixture. The arsenylation of ADP does not result in an irreversible proton consumption, however, since the arsenylated nucleotide hydrolyzes rapidly.

latory effect of ADP may be expressed only when the electron transport system includes Coupling Site I.

DISCUSSION

The characteristics of the proton pump associated with the electron pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ dibromothymoquinone, revealed in this study, may be summarized as follows: (i) approximately one proton is taken up for every pair of electrons transported (or H^+/e^- ratio = 0.5; observed range, 0.4–0.5), (ii) the efficiency of proton uptake is essentially pH-independent (between 6.2 and 8.2), and (iii) the kinetics of proton uptake, as observed by flash-yield determinations, show no sign of burst phenomena (such as the "pH-gush" which is clearly seen in the methylviologen reducing system). It should be stressed here that the light-induced pH rise in the medium (proton uptake) described in this paper has no direct bearing on the chemistry of the oxidation or reduction of dibromothymoquinone per se, as demonstrated by the fact that gramicidin abolishes the reversible pH rise but has no effect on dibromothymoquinone reduction [1, 9] (see also Fig. 1, traces C and D).

In other words, the reduction of dibromothymoquinone consumes the same number of H^+ as are generated in the oxidation of water and therefore any pH changes observed in the medium must represent the formation of proton gradients.

Mechanism of proton translocation associated with Photosystem II electron transport

The mechanism of the Photosystem II "proton pump" described above and its efficiency ($H^+/e^- = 0.5$) can be explained most easily if we assume that the mem-

brane-bound electron transport enzymes are arranged within the membrane in such a manner as to favor the vectoral movement of protons directly involved in electron transport. Such an anisotropy in the electron transport chain could result in a proton uptake with the observed stoichiometry (H^+/e^- ratio) according to two models:

$$H_2O \rightarrow 1/2\ O_2 + 2e^- + 2H^+_{(inside)}$$
$$DBMIB + 2e^- + H^+_{(inside)} + H^+_{(outside)} \rightarrow DBMIBH_2$$
$$H_2O + DBMIB + H^+_{(outside)} \rightarrow 1/2\ O_2 + DBMIBH_2 + H^+_{(inside)}$$

or

$$H_2O \rightarrow 1/2\ O_2 + 2e^- + H^+_{(inside)} + H^+_{(outside)}$$
$$DBMIB + 2e^- + 2H^+_{(outside)} \rightarrow DBMIBH_2$$
$$H_2O + DBMIB + H^+_{(outside)} \rightarrow 1/2\ O_2 + DBMIBH_2 + H^+_{(inside)}$$

where DBMIB and $DBMIBH_2$ represent the oxidized and reduced forms of dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) respectively. The stoichiometric formation of the fully reduced dibromothymoquinone below pH 8 has in fact been confirmed (see Fig. 3). It should be noted concerning the above models that, in theory, any intermediate situation between these two extremes is possible. One thing is clearly indicated by these formulations, however. That is, in any situation, a large portion (50–100%) of the protons produced in the oxidation of water must be released to the inside of the thylakoid, and a similar and complementary portion of the protons needed for the hydrogenation of dibromothymoquinone must come from the outside (i.e. 50–100% inversely related to the percentage of protons from water released inside).

The exact point at which the protons eventually used in the reduction of dibromothymoquinone enter into the electron transport chain is not known. However, there is kinetic evidence suggesting that dibromothymoquinone is primarily reduced via the plastoquinone pool [9], in line with the fact that its inhibitory site for electron transport is on the Photosystem I side of plastoquinone [24]. It seems reasonable to assume, therefore, that the protons which eventually are used in the reduction of dibromothymoquinone actually enter the electron transport chain at the level of one of the plastoquinones, or even at the level of the primary electron acceptor for Photosystem II, which may also be a quinone-type substance [25].

However, we hasten to point out that it is still premature to assign the H^+/e^- ratio of 0.5, observed for this "isolated" Photosystem II reaction, to the proton translocation efficiency intrinsic to the electron transport pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ plastoquinone since, for example, we have no clear idea of how the membrane permeating electron/hydrogen carrier dibromothymoquinone might affect the anisotropy of that region of the membrane surrounding the plastoquinones. Some indication that the intrinsic value might be higher than 0.5 has been offered by Reinwald et al. [26], who showed a kinetic correspondence between the initial fast proton uptake ("pH gush") and plastoquinone reduction. They calculated the $\Delta H^+/\frac{1}{2}\Delta$ plastoquinone ratio ($= H^+/e^-$) to be exactly 1. On the other hand their interpretation of the "pH gush" may be questioned. As we have shown in Fig. 3e and f the "gush" as observed in this laboratory is not associated with an increased rate of elec-

tron transport. Furthermore there are also reports describing anomalously high $H^+/h\nu$ ratios (e.g. 5) [27, 28] at the onset of illumination. This too suggests a different mechanism of pH change rather than an increased rate of electron transport. Nevertheless, it seems quite possible that in the normal, complete electron transport system the efficiency of proton translocation (H^+/e^-) attributable to the $H_2O \rightarrow$ Photosystem II $\rightarrow$ plastoquinone portion of the chain may be somewhat higher than the values approaching 0.5 observed when this system is isolated from the complete chain.

The relation between the accumulation of H^+ and the mechanism of phosphorylation at Coupling Site II

As shown in Table I, the efficiency of proton uptake (H^+/e^-) associated with dibromothymoquinone reduction (i.e. associated with Coupling Site II) is 0.4–0.5 over a wide pH range (6.2–8.2). A striking parallel is found in the efficiency of phosphorylation, $P/e_2 = 0.3$–0.4 (ref. 9) which is also essentially pH-independent over the same pH range. Furthermore, we have demonstrated unequivocally that concurrent phosphorylation markedly depresses the steady-state extent of proton uptake in this system (see Fig. 5 and Table II). These observations tempt us to postulate a coupling mechanism for Site II which involves the obligatory participation of H^+. Indeed, the H^+/e^- ratio is so close to the P/e_2 ratio that one is inclined to give considerable credence to the "chemiosmotic" coupling hypothesis of Mitchell [29].

The chemiosmotic hypothesis, as applied to chloroplast photophosphorylation, postulates that the efflux of 2 protons through the membrane-bound ATP synthesizing enzyme produces 1 ATP ($H^+/ATP = 2$). Various attempts have been made in recent years to determine this H^+/ATP ratio experimentally. Junge et al. [30] obtained a value of 3 from their studies of flash-induced proton uptake and 515 nm absorbance changes. The same value was also obtained by Schröder et al. [31] who studied the kinetics of the dark proton efflux after the steady state. (A prototype of this latter experiment by Schwartz [32] gave a value of 2). Using somewhat more direct measurements based on post-illumination phosphorylation (X_E) experiments Izawa [33] found a value of 2.4. Thus, the experimentally determined H^+/ATP ratios range between 2 and 3, which should be regarded as in good agreement with the hypothetical value of 2.

In the Photosystem II reaction dealt with here, an approximation of the H^+/ATP ratio may be made from the H^+/e^- and P/e_2 values by assuming (a) that the H^+/e^- ratio determined from initial rates applies to the steady state, and (b) that all protons translocated are available for ATP formation. If we take the Site II ratio of H^+/e^- as 0.5 (see Results) and the Site II P/e_2 as approximately 0.35 (see refs 3, 9), it follows that the requirement for protons in ATP formation (H^+/ATP) is approximately 2.9. While this is in good agreement with Mitchell's hypothetical value of 2 (ref. 29), it most likely represents an overestimation of the true H^+/ATP ratio since there is probably a non-coupled, unspecific outward diffusion of protons competing with the ATP synthesizing pathway. Based on the data presented in refs 31 and 33, a rough estimation of the amount of this unspecific proton efflux may be made. Summarizing those published data, it can be reasonably concluded that the non-phosphorylating efflux constitutes 20–40% of the total proton efflux (pH 8). The H^+/ATP ratios corrected for this component would now fall very near 2.

These considerations strongly implicate proton gradient formation as the mechanism of energy conservation at Coupling Site II. In addition, it seems likely that Site II can be identified as the water-oxidation reaction: the protons lost from water being discharged to the inside of the thylakoid. If this is so, the insensitivity of the electron transport rate to uncouplers of phosphorylation is easily understood [10]; the electron transport results from an essentially irreversible photochemical reaction which is, as a consequence of its irreversibility, insensitive to concentrations of its proton product.

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