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# PHOTOOXIDATION OF FERROCYANIDE AND IODIDE IONS AND ASSOCIATED PHOSPHORYLATION IN $NH_2OH$-TREATED CHLOROPLASTS

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## SUMMARY

$NH_2OH$-treated, non-water oxidizing chloroplasts are shown to be capable of oxidizing ferrocyanide and $I^-$ via Photosystem II at appreciable rates ($\geqslant$ 200 $\mu$equiv/h per mg chlorophyll). Using methylviologen as electron acceptor, ferrocyanide oxidation can be measured as $O_2$ uptake, as ferricyanide formation, or as $H^+$ consumption ($2\,Fe^{2+}+2H^++O_2 \rightarrow 2\,Fe^{3+}+H_2O_2$). $I^-$ oxidation can be measured as methylviologen-mediated $O_2$ uptake, or spectrophotometrically, using ferricyanide as electron acceptor. The oxidation product $I_2$ is re-reduced, as it is formed, by unknown reducing substances in the reaction system.

The rate-saturating concentrations of these donors are very high: 30 mM with ferricyanide and 15 mM with $I^-$. Relatively lipophilic Photosystem II donors such as catechol, benzidine and *p*-aminophenol saturate the photooxidation rate at much lower concentrations ($<$ 0.5 mM). It thus seems that the oxidation of hydrophilic reductants such as ferricyanide and $I^-$ is limited by permeability barriers. Very likely the site of Photosystem II oxidation is embedded in the thylakoid membrane or is situated on the inner surface of the membrane.

The efficiency of phosphorylation ($P/e_2$) is 0.5 to 0.6 with ferrocyanide and about 0.5 with $I^-$. In contrast the $P/e_2$ ratio is 1.0 to 1.2 when water, catechol, *p*-aminophenol or benzidine serves as electron donor. These differences imply that only one of two phosphorylation sites operate when ferrocyanide and $I^-$ are oxidized. Ferrocyanide and $I^-$ are also chemically distinct from other Photosystem II donors in that their oxidation does not involve proton release. It is suggested that the mechanism of energy conservation associated with Photosystem II may be only operative when the removal of electrons from the donor results in release of protons (i.e. with water, hydroquinones, phenylamines, etc.).

## INTRODUCTION

Recent investigations on partial pathways of electron transport have established that there are two sites of energy conservation associated with noncyclic electron

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPPS, *N*-2-hydroxylpiperazine-*N'*-2-propanesulfonic acid; HEPES, *N*-2-hydroxylpiperazine-*N'*-2-ethanesulfonic acid.

transport in chloroplasts [1–6]. One of these sites, which we have called Site I, corresponds to the well-known site of phosphorylation between the plastoquinone pool and cytochrome *f* [7, 8]. The newly recognized site, which we have designated Site II, has been placed in the close proximity of Photosystem II [2, 3, 9]. This paper deals with the nature of energy transduction at Site II.

During the past year we have developed a new method of chloroplast treatment ($NH_2OH$ washing) [10] which is highly effective in inactivating the mechanism of water oxidation yet does not impair the coupling mechanism of the chloroplast membrane. The treated chloroplasts are quite active in oxidizing, via Photosystem II, various exogenous reductants such as catechol, *p*-aminophenol, benzidine and dicyanohydroquinone [11]. The transport of electrons from these donors to methylviologen supports phosphorylation with a $P/e_2$ ratio of 1.0 to 1.1. These $P/e_2$ values are virtually the same as the value for noncyclic photophosphorylation involving water oxidation. The $P/e_2$ data for *p*-aminophenol and benzidine also confirm the data which Yamashita and Butler [12] obtained with NADP as acceptor using their Tris-washed chloroplasts. Clearly neither of the sites of phosphorylation is destroyed or even significantly impaired when the mechanism of water oxidation is destroyed. Furthermore, Site II seems to function normally when artificial reductants replace water as the electron source.

Another important clue to the nature of Site II has recently been provided by the experiments of Gould and Izawa [13, 14], who showed that an energy-linked proton translocation is associated with dibromothymoquinone reductions. The reaction is believed to involve only the electron transport span: $H_2O \rightarrow$ Photosystem II $\rightarrow$ plastoquinone pool [9]. They postulated [14], in accordance with the original suggestion of Mitchell [15] (see also Rumberg et al. [16]), that the proton translocation consists of two steps: internal discharge of protons through water oxidation followed by uptake of external protons through reduction of hydrogen carriers (e.g. plastoquinone). According to the chemiosmotic coupling theory [15], it is this Photosystem II-driven proton translocating loop itself that constitutes Site II. Such a loop, if exists, may well operate when artificial hydrogen donors such as hydroquinones and phenylamines replace water, since their oxidation also involves proton release. For technical reasons, however, we have not been able to demonstrate the predicted proton translocation associated with Photosystem II oxidation of artificial hydrogen donors.

Nevertheless, a further important test of the feasibility of this chemiosmotic model of Site II is available. That is to test pure "electron" donors the oxidation of which does not involve proton changes. If indeed the inward discharge of protons from donors (including water) represents the primary step of energy conservation at Photosystem II, then no energy conservation should occur at Photosystem II when it oxidizes non-proton-producing electron donors. Consequently the transport of electrons from such donors to methylviologen, now effectively by-passing Site II, should support phosphorylation only with the efficiency ascribable to Site I alone. The $P/e_2$ expected is approx. 0.5–0.6. These are the values found invariably associated with Photosystem I–donor reactions which may be considered to involve only Site I (e.g. diaminodurene $\rightarrow$ methylviologen) [11].

We have discovered that ferrocyanide and $I^-$ can be oxidized by Photosystem II in $NH_2OH$-washed, non-water-oxidizing chloroplasts. The oxidation of these ions at physiological pH values do not involve proton changes. The transport of

electrons from ferrocyanide and $I^-$ to methylviologen did support phosphorylation with efficiencies near the predicted value. This paper describes these rather unusual chloroplast reactions in some detail.

## MATERIALS AND METHODS

### *Isolation and $NH_2OH$-treatment of chloroplasts*

Chloroplasts (unfragmented naked lamellae) were isolated from commercial spinach (*Spinacia oleracea* L.) as described elsewhere [11], and suspended in a medium containing 0.2 M sucrose, 5 mM *N*-2-hydroxylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaOH buffer (pH 7.5) and 2 mM $MgCl_2$. The chlorophyll concentration of this stock suspension was approx. 2 mg/ml. Hydroxylamine treatment of chloroplasts [10] was performed as follows: 1 vol. of the chloroplast stock suspension was added to 10 vol. of a freshly prepared medium containing sucrose, buffer and $MgCl_2$ as above and in addition 5 mM $NH_2OH$ plus 1 mM EDTA. The mixture was allowed to stand at room temperature (21 °C) for 20 min, then diluted with cold, $NH_2OH$-free suspending medium, and centrifuged at $4000 \times g$ for 5 min at 0 °C. The sedimented chloroplasts were washed twice by centrifugation with a large volume of the suspending medium to remove $NH_2OH$ and EDTA, and finally suspended in the same medium.

### *Reagents*

$K_4Fe(CN)_6$ was recrystallized from a warm saturated aqueous solution by slowly cooling it to −10 °C. Colorless crystalls of catechol, *p*-aminophenol hydrochloride and benzidine dihydrochloride were obtained from charcoal-treated aqueous solutions by cooling (catechol) or by adding excess HCl at 0 °C (*p*-aminophenol and benzidine). Lyophilized bovine blood superoxide dismutase (specific activity, 3000 units/mg) was purchased from Truett Laboratories (Dallas, Texas). The enzyme was dissolved in 10 mM HEPES (pH 7.5), dialyzed overnight against the same medium, then stored at −20 °C.

### *Assays*

Electron transport from water or artificial electron donors to methylviologen was assayed routinely as the $O_2$ uptake resulting from the reoxidation of reduced methylviologen. A membrane-covered (Clark-type) oxygen electrode was used for these $O_2$ assays. Optical monitoring of reactions (e.g. ferrocyanide oxidation) was performed using a monochromator from a Beckman DU spectrophotometer with Gilford electronics. The monochromator was modified to permit illumination of the reaction cuvette (light path 1 cm) in a thermostated holder. Absorption spectrum determinations were conducted using a Cary 15 recording spectrophotometer. Changes in the pH of the reaction mixture were measured using a Corning semi-micro combination glass electrode connected to a Heath-Schlumberger EU200-300 electrometer equipped with an EU 200-02 offset module. The actinic light used was a rate-saturating orange light (600–700 nm; approx. 700 kergs $\cdot$ $cm^{-2} \cdot s^{-1}$). The reaction temperature was 19 °C. Phosphorylation was measured as [$^{32}$P]ATP formation by the method detailed elsewhere [17].

RESULTS

*Ferrocyanide-supported electron transport and phosphorylation*

All of the experiments described in this paper (except those of Fig. 1B) were carried out using $NH_2OH$-washed, non-$O_2$-producing chloroplasts. The electron acceptor employed was usually methylviologen. The chloroplast preparations used were free of catalase activity, and this facilitated the measuring of electron transport as the aerobic reoxidation of reduced methylviologen.

The top trace of Fig. 1A confirms that the $NH_2OH$-washed chloroplasts used were practically totally unable to reduce methylviologen with electrons from water. However, when high concentrations of ferrocyanide (> 10 mM) were added, a rapid uptake of $O_2$ was observed. The fact that exactly half of the $O_2$ taken up in the light was released on addition of catalase indicates that all of the $O_2$ taken up was reduced to the level of $H_2O_2$. The reaction is highly sensitive to 3-(3,4-dichlorophenyl)-1,1dimethylurea (DCMU) (bottom trace) indicating an obligatory involvement of Photosystem II.

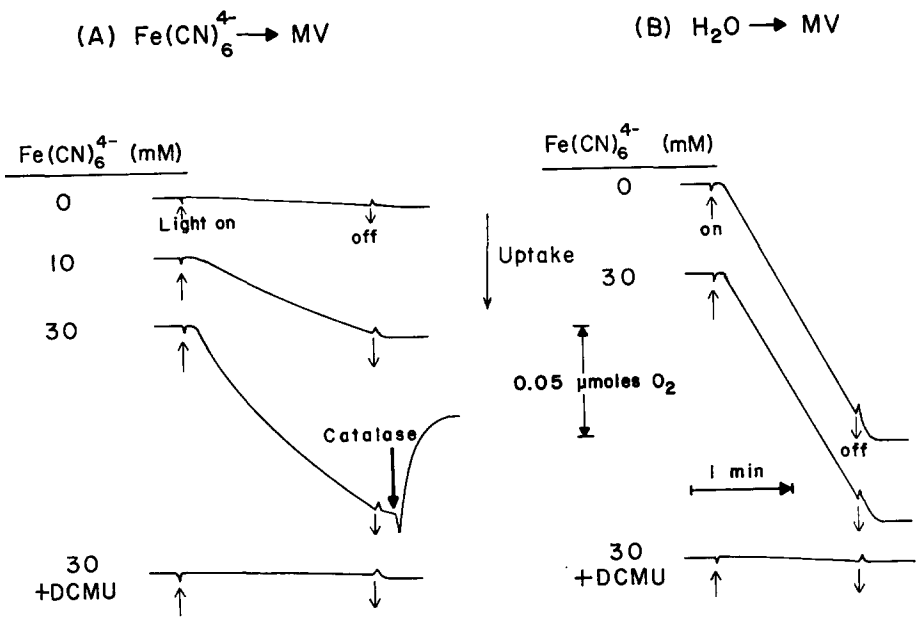


Fig. 1. A. Electron transport from ferrocyanide to methylviologen (MV) in $NH_2OH$-washed chloroplasts as observed by $O_2$ uptake. The reaction mixture (2 ml) contained 0.1 M sucrose, 50 mM HEPPS (*N*-2-hydroxylpiperazine-*N'*-2-propane sulfonic acid)–NaOH buffer (pH 8.0), 2 mM $MgCl_2$, 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$, 0.5 mM methylviologen, $NH_2OH$-washed chloroplasts equivalent to 40 μg chlorophyll, and the indicated concentrations of $K_4Fe(CN)_6$. When used, DCMU was 2 μM. B. Lack of effect of ferrocyanide addition on methylviologen Hill reaction in normal chloroplast. The conditions were as in A except normal, $O_2$-producing chloroplasts were used.

The addition of superoxide dismutase had no inhibitory effect on the ferrocyanide-supported $O_2$ uptake even at an enzyme activity of 300 units/ml reaction mixture. This amount of superoxide dismutase is 3- to 10-fold greater than the amount required to completely eliminate the oxidation of ascorbate or of ascorbate–

TABLE I

ELECTRON TRANSPORT FROM VARIOUS ARTIFICIAL DONORS TO METHYLVIOLOGEN VIA PHOTOSYSTEM II AND PHOTOSYSTEM I AND THE ASSOCIATED PHOSPHORYLATION IN $NH_2OH$-WASHED CHLOROPLASTS

Basic reaction conditions were as in Fig. 1A except for the variations in the electron donor used. When catechol and *p*-aminophenol were used the reaction mixture contained 0.5 mM ascorbate as electron reservoir. When used, superoxide dismutase (SOD) was 300 units/ml reaction mixture for the first three donors and 100 units/ml for the rest; DCMU, 2 $\mu$M. $I^-$ was added as KI. $Mn(oxine)_2$ was a 1 : 2 mixture (mole/mole) of $MnCl_2$ and 8-hydroxyquinoline. For details of the reactions involving catechol, *p*-aminophenol and benzidine, see ref. 11. Reaction time, 30 s.

| Donor | Concn (mM) | $O_2$ uptake ($\mu$moles/h per mg chlorophyll) | | | ATP formation ($\mu$moles/h per mg chlorophyll) | | | $P/e_2$ (= $P/O_2$ with SOD) |
|---|---|---|---|---|---|---|---|---|
| | | No addition | +SOD | +DCMU | No addition | +SOD | +DCMU | |
| $Fe(CN)_6^{4-}$ | 30 | 44* | 45* | 5 | 24* | 25* | 0 | 0.56 |
| $I^-$ | 20 | 102 | 100 | 8 | 47 | 46 | < 2 | 0.46** |
| $Mn(oxine)_2$ | 0.5 | 75 | 72 | 5 | 43 | 44 | 0 | 0.61 |
| Catechol | 0.5 | 182 | 102 | 10 | 116 | 112 | < 2 | 1.10 |
| *p*-Aminophenol | 0.5 | 152 | 91 | 10 | 95 | 93 | < 2 | 1.02 |
| Benzidine | 0.5 | 92 | 88 | 5 | 94 | 93 | 0 | 1.06 |

* Time-averaged values (0–30 s) of rapidly diminishing rates. Initial rates at $t = 0$ were presumably 2- to 3-fold faster (cf. Fig. 3).

** At $t = 0$ the ratio probably exceeded 0.5 (cf. Fig. 7).

donor couples by superoxide radical anion, the precursor of $H_2O_2$ (see Table I; see also refs. 11, 18, 19). Clearly there was no radical oxidation of ferrocyanide to contribute to $O_2$ consumption. We may thus safely conclude that the pair of electrons utilized for the reduction of $O_2$ to $H_2O_2$ originates almost exclusively from the light-dependent biological oxidation of ferrocyanide. Uptake of one molecule of $O_2$ therefore corresponds to transport of a pair of electrons from ferrocyanide to methylviologen (hence $P/O_2 = P/e_2$; see below).

The parallel experiment shown in Fig. 1B was performed with normal, water-oxidizing chloroplasts. The active reduction of methylviologen with electrons from water was virtually unaffected by the presence of 30 mM ferrocyanide. Apparently ferrocyanide cannot replace water as the electron donor as long as the water-oxidizing mechanism is intact.

The spectrophotometric data of Fig. 2A, B demonstrate that ferricyanide is formed in approximately the amount predicted from the amount of ferrocyanide-supported $O_2$ uptake. Illumination of reaction mixtures containing ferrocyanide caused irreversible absorbance changes in the 400–450-nm region, and the wavelength dependence of these changes agreed well with the absorption spectrum of ferricyanide. In Fig. 2B the reversible change at 480 nm and the reversible portion of the change at 420 nm represent light-scattering changes of chloroplasts. The absorbance changes plotted in Fig. 2A have been corrected for these light-scattering changes. In this experiment the absorbance change at 420 nm caused by 60 s illumination was 0.028, which corresponds to a formation of 54 nmoles ferricyanide in the 2-ml reaction mix-

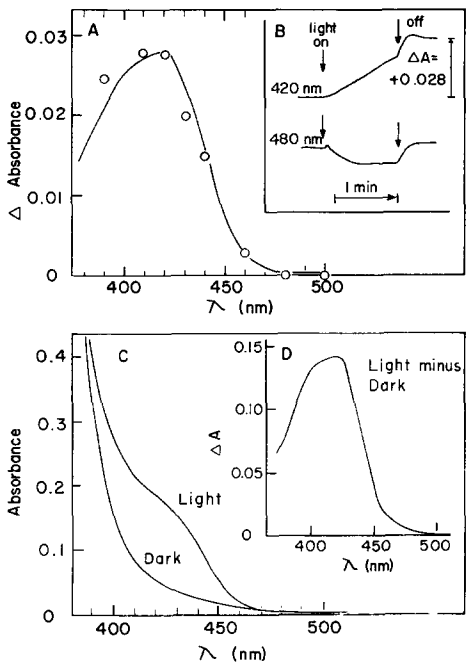


Fig. 2. Electron transport from ferrocyanide to methylviologen (MV) in $NH_2OH$-washed chloroplasts as observed by spectrophotometric determination of ferricyanide formation. In A, the curve represents the absorption spectrum of 27 $\mu$M ferricyanide; the circles represent light-induced absorbance changes of the reaction mixture at different wavelengths corrected for light-scattering changes as in B. Conditions were as in Fig. 1A with 30 mM ferrocyanide and 20 $\mu$g chlorophyll per ml. In C, the curve designated "light" indicates the absorption spectrum of a filtrate of the reaction mixture exposed to light for 60 s; "dark", a dark control. D. The light minus dark difference spectrum. In this filtration experiment, the reaction mixture (8 ml) contained 0.1 M sucrose, 50 mM HEPPS–NaOH buffer (pH 8.0), 2 mM $MgCl_2$, 0.5 mM methylviologen, 30 mM $K_4Fe(CN)_6$ and $NH_2OH$-washed chloroplasts equivalent to 100 $\mu$g chlorophyll per ml.

ture. A parallel experiment with a duplicate reaction mixture showed that 24 nmoles of $O_2$ (48 nequiv) were consumed during the same period of illumination. This is in good agreement with the optical data. The "Millipore"-filtration experiment of Fig. 2C, D unequivocally demonstrated the light-dependent formation of ferricyanide (for details, see legend for Fig. 2C, D). As indicated by the experiment of Fig. 2A, B, the oxidation of ferrocyanide could be followed spectrophotometrically. However, the high sensitivity of our optical system to light-scattering changes made this alternative method rather impractical.

The transport of electrons from ferrocyanide to methylviologen supports phosphorylation. In Fig. 3, the time course curves for $O_2$ uptake and ATP formation were obtained by yield determinations, using a series of identical reaction mixtures illuminated for different periods of time. Both processes exhibited similar, almost biphasic kinetics. The plots revealed an unexpectedly fast initial phase of $O_2$ uptake which continuous $O_2$ tracing failed to detect (cf. Fig. 1A) obviously because of the slow response of the conventional membrane-covered $O_2$ electrode used. In this experiment the rate of electron transport computed on the basis of the total $O_2$ uptake

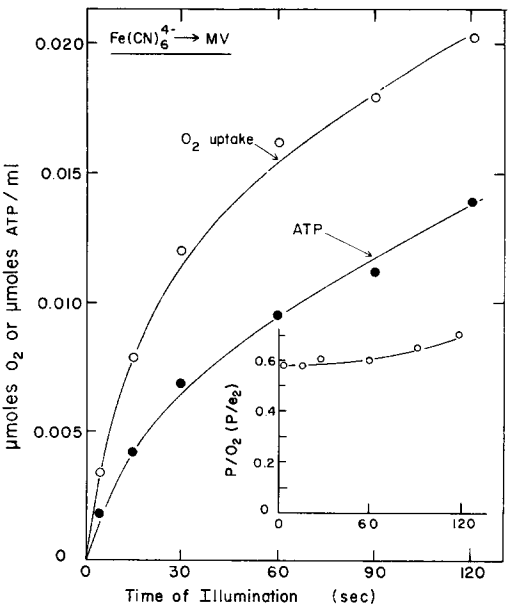


Fig. 3. Time courses of electron transport from ferrocyanide to methylviologen (MV) and of associated phosphorylation in $NH_2OH$-washed chloroplasts. The time courses were determined using a series of identical reaction mixtures illuminated for indicated periods of time. Conditions were as in Fig. 1A with 30 mM ferrocyanide and 40 μg chlorophyll per ml.

induced by 5 s illumination was 220 μequiv (110 μmoles $O_2$) per h per mg chlorophyll. The true initial rate at $t = 0$ presumably exceeded 300 in the μequiv units. These rates are comparable to the rate of the normal Hill reaction. The cause of the biphasic kinetics will be discussed in a later section.

Although the rates of electron transport and phosphorylation decreased quickly with time, their ratio ($P/O_2$ or $P/e_2$; actually the ratio of yields) held level at 0.6 for about 60 s, then gradually rose (Fig. 3, inset). This phenomenon can be explained if one assumes that the $P/e_2$ ratio intrinsic to the ferrocyanide oxidation indeed approximates the predicted value of 0.6 (see Introduction). As the reaction proceeds, however, the oxidation product ferricyanide accumulates and begins to replace methylviologen as the electron acceptor, thereby introducing an unmeasurable electron flow from ferrocyanide to ferricyanide. This unmeasurable (cyclic) electron flow still supports phosphorylation at an unchanged rate. Consequently the apparent $P/e_2$ is inflated. The experiment of Fig. 4 suggests that this interpretation is probably correct. Low concentrations of exogenously added ferricyanide readily replaced methylviologen as the electron acceptor, and thereby converted the measurable electron transport ferrocyanide → methylviologen to undetectable electron transport ferrocyanide → ferricyanide without changing the phosphorylation rate. Close examination of data will show that the apparent rise in the $P/e_2$ depicted in Fig. 3 is about the extent one would expect from the data of Fig. 4.

Very high concentrations of ferrocyanide ($\geqq$ 30 mM) are required to saturate electron transport and phosphorylation (Fig. 5). However, the efficiency with which the electron transport is coupled to phosphorylation ($P/e_2 = 0.53$ in this experiment)

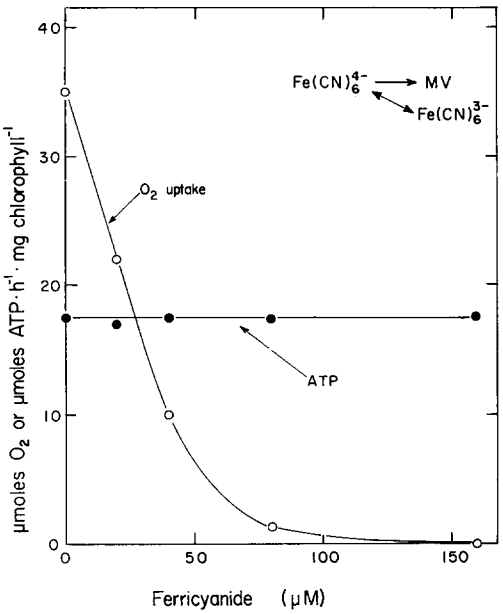


Fig. 4. Effect of increasing concentrations of exogenous ferricyanide on the ferrocyanide → methyl viologen (MV) reaction and associated phosphorylation in $NH_2OH$-washed chloroplasts. The composition of the reaction mixture was as in Fig. 1A with 30 mM ferrocyanide, 30 μg chlorophyll per ml and the indicated concentration of ferricyanide added. The reaction time was 30 s. Note that the phosphorylation remains unchanged as the $O_2$ uptake is suppressed by ferricyanide addition, suggesting the conversion of noncyclic photophosphorylation to cyclic photophosphorylation mediated by ferro-/ferri-cyanide turn over.

remains constant over a wide range of ferrocyanide concentrations. This latter fact speaks strongly against the possibility that the low $P/e_2$ values (0.5–0.6) result from an uncoupling effect of ferrocyanide. In fact, we found no evidence that ferrocyanide could act as an uncoupler in the concentration range tested. The presence of 30 mM ferrocyanide had virtually no effect on the phosphorylation coupled to the Hill reaction in normal chloroplasts (data not shown).

The rate of ferrocyanide oxidation peaks at pH 7.7 but exhibits a wide skirt on the acid side (Fig. 6). The associated phosphorylation and its efficiency ($P/e_2$) peak at pH 8 and quickly approach zero below pH 7. It should be noted, however, that the rates presented in Fig. 6 (also Figs 4 and 5) were computed on the basis of the total $O_2$ uptake yield and the ATP yield resulting from 30 s illumination. Since the kinetics of ferrocyanide oxidation are non-linear (Fig. 3), these are time-averaged values of quickly diminishing rates. As a result, it is quite possible that the pH profiles for the rates of electron and ATP formation are somewhat distorted. However, the pH profile for the essentially time-independent term $P/e_2$ is presumably free from such distortion.

*$I^-$-supported electron transport and phosphorylation*

$I^-$ serves as an efficient donor of electrons to Photosystem II in $NH_2OH$-treated chloroplasts. Electron transport and phosphorylation both proceed more

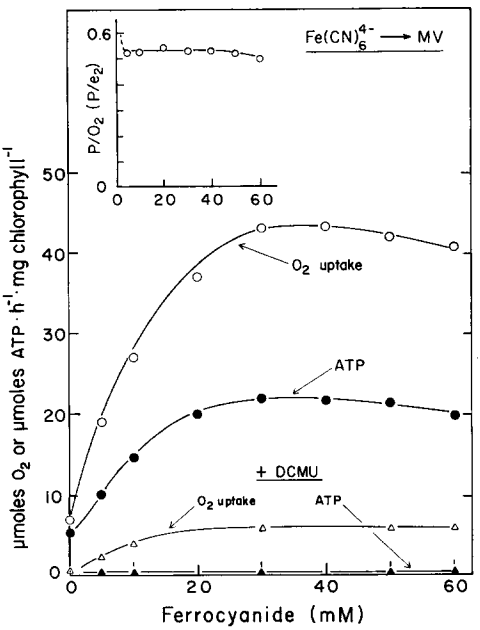


Fig. 5. Electron transport from ferrocyanide to methylviologen (MV) and associated phosphorylation in $NH_2OH$-washed chloroplasts as a function of the ferrocyanide concentration. The composition of the reaction mixture was as in Fig. 1A with 40 $\mu$g chlorophyll per ml and the indicated concentrations of ferrocyanide. The reaction time was 30 s.

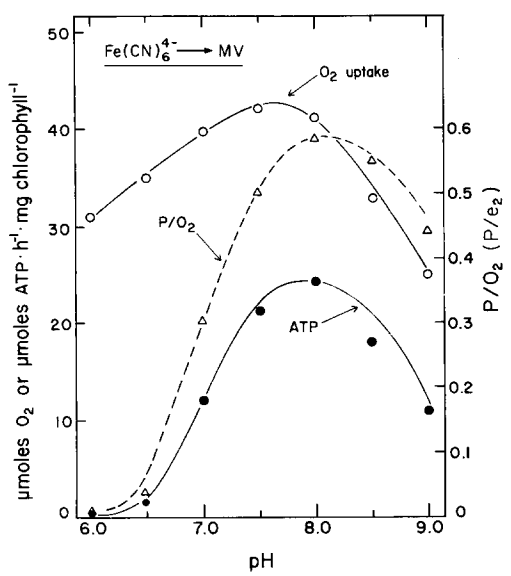


Fig. 6. Electron transport from ferrocyanide to methylviologen (MV) and associated phosphorylation in $NH_2OH$-washed chloroplasts as a function of pH. The composition of the reaction mixture was as in Fig. 1A with 30 mM ferrocyanide and 40 $\mu$g chlorophyll per ml. The buffers used (all 50 mM) were: MES (2-(*N*-morpholino)ethanesulfonic acid)–NaOH (pH 6.0 and 6.5), HEPES–NaOH (pH 7.0 and 7.5) and HEPPS–NaOH buffer (pH 8.0–9.0). The reaction time was 30 s.

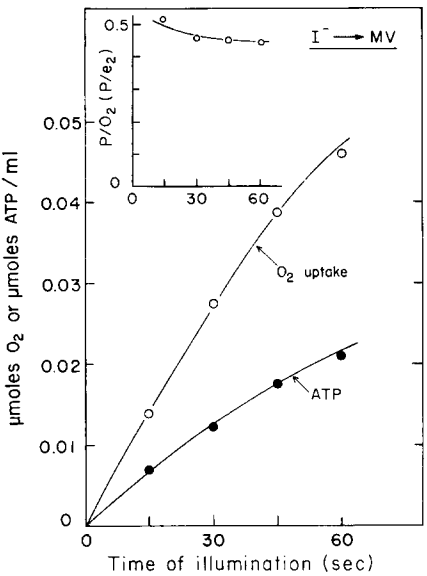


Fig. 7. Time courses of electron transport from $I^-$ to methylviologen (MV) and associated phosphorylation in $NH_2OH$-washed chloroplasts. The basic reaction conditions were as in Fig. 1A except that $K_4Fe(CN)_6$ was replaced by 20 mM KI. Chlorophyll, 40 $\mu$g/ml. For procedures, see Fig. 3.

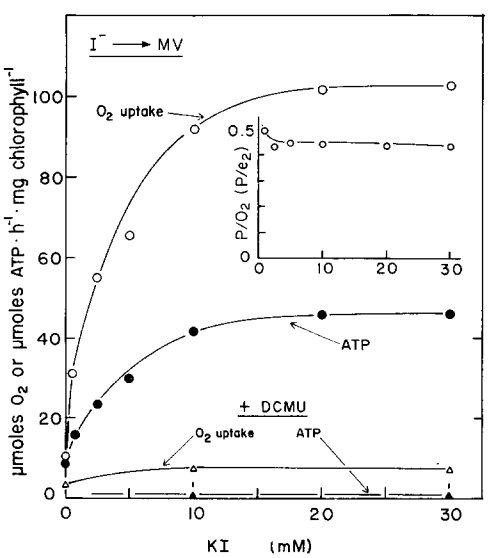


Fig. 8. Electron transport from $I^-$ to methylviologen (MV) and associated phosphorylation as a function of the KI concentration. The basic conditions were as in Fig. 1A except that ferrocyanide was replaced by indicated concentrations of KI. When used, DCMU was 1.5 $\mu$M. Chlorophyll, 40 $\mu$g/ml. The reaction time, 30 s.

linearly than in the ferrocyanide-oxidizing system. The $P/e_2$ ratio exceeds 0.5 initially ($< 15$ s) but falls slightly with the reaction time (Fig. 7). $I^-$ photooxidation and associated phosphorylation become saturated at a KI concentration of about 15 mM (Fig. 8). The $P/e_2$ ratio (0.45 as measured after 30 s illumination) was almost completely independent of the concentration of KI. Again this constancy of $P/e_2$ values speaks strongly against the possibility of the low $P/e_2$ resulting from uncoupling by $I^-$. Concentrations of KI up to 30 mM had essentially no uncoupling effect when tested on the phosphorylation associated with the methylviologen Hill reaction in normal chloroplasts. ($I^-$ does not easily replace water as the donor in normal chloroplasts.) Electron transport and phosphorylation supported by iodide oxidation share a common pH optimum at 8 (Fig. 9). As in the ferrocyanide-oxidizing system, the phosphorylation, and therefore the $P/e_2$ ratio, steeply approach zero below pH 7. A marked stimulation of electron transport by concomitant phosphorylation was also noted.

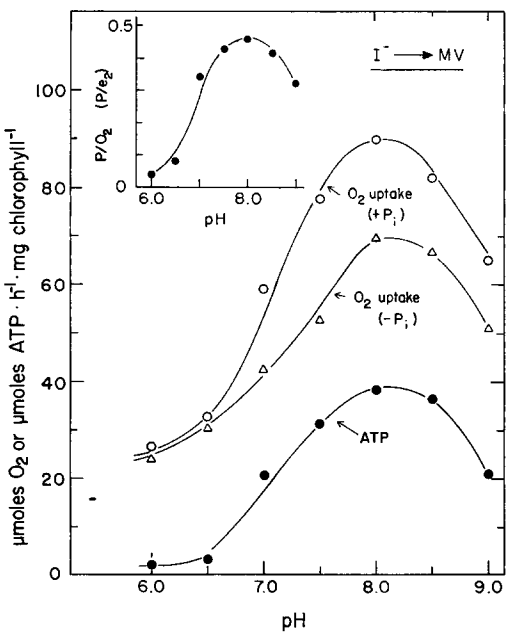


Fig. 9. Electron transport from $I^-$ to methylviologen (MV) and associated phosphorylation in $NH_2OH$-washed chloroplasts as a function of pH. The basic conditions were as in Fig. 1A except that ferrocyanide was replaced by 20 mM KI and that the buffers used were as in Fig. 6. Chlorophyll, 40 $\mu$g/ml. The reaction time, 30 s.

Attempts to detect the oxidation product of $I^-$, free $I_2$ (or $I_3^-$), were unsuccessful. This is not surprising in view of the high reactivity of $I_2$. The amount of $I_2$ formed by the end of 30 s illumination (the routine reaction time) would have been of the order of 50 nmoles in the 2-ml reaction mixture, an amount which could have easily been (and indeed was) consumed even by minute impurities of the chemicals present in the mixture such as sucrose and buffer. A test with chloroplasts exhaustively washed and suspended in phosphate buffer showed, however, that

chloroplasts themselves can readily consume an amount of exogenous $I_2$ which is nearly equivalent to the amount of chlorophyll present. Since the $I_2$ produced (which would have been able to oxidize ferrocyanide) actually disappeared quickly, it was possible to use ferricyanide as the electron acceptor, replacing methylviologen, and observe the reaction photometrically as ferricyanide reduction. In this study, however, we have not used this optical method for quantitative experiments for the technical reason described for ferrocyanide oxidation.

In the methylviologen-reducing system there was no indication of $H_2O_2$ formed being consumed by a reaction with $I^-$ within the reaction time of 30 s. Not surprisingly, neither of the higher potential halogen ions $Cl^-$ and $Br^-$ was found able to substitute for $I^-$ as the electron donor for Photosystem II.

The electron transport and phosphorylation data pertaining to the ferrocyanide- and $I^-$-oxidizing systems are summarized in Table I, and contrasted with the data for several other Photosystem II donor reactions which give $P/e_2$ of 1.0 to 1.1. For the details of the latter reactions, see ref. 11. Also included are preliminary data for the photooxidation of another metal complex, $Mn^{2+}$ (8-hydroxyquinoline)$_2$, which we found to serve as a good electron donor for Photosystem II. Again a $P/e_2$ value of approx. 0.6 was found. Characterization of this new donor reaction has not been completed yet.

*Changes in the pH of the medium associated with photooxidation of ferrocyanide and $I^-$*

The methylviologen-mediated aerobic photooxidation of ferrocyanide should consume protons according to the formula:

$$2\,Fe(CN)_6^{4-} + 2\,H^+ + O_2 \xrightarrow[\text{methylviologen}]{2e^- \text{ transport}} 2\,Fe(CN)_6^{3-} + H_2O_2 \qquad (1)$$

Consequently the pH of a weakly buffered suspending medium should rise irreversibly as the reaction proceeds. One might also expect this process to support an energy-linked proton translocation since the process is coupled (at Site I, according to our model). Traces *a* and *b* of Fig. 10 verify these predictions. Proton uptake did occur upon illumination, and it did comprise two components: a gramicidin-insensitive, irreversible component (aerobic ferrocyanide oxidation or Eqn 1) and a gramicidin-sensitive, reversible component (energy-linked proton uptake). The initial slope (0–20 s) of $H^+$ consumption in trace *a* is approximately 230 $\mu$equiv/h per mg chlorophyll, which is diminished to 110 in the presence of gramicidin (trace *b*). The latter rate, which should represent the electron transport rate (Eqn 1), is in fair agreement with the rate measured as $O_2$ uptake under comparable conditions (130 $\mu$equiv or 65 $\mu$moles $O_2$ per h per mg chlorophyll).

The methylviologen-mediated photooxidation of $I^-$ probably would have exhibited very similar proton changes, if the $I_2$ produced had remained in the medium as did ferricyanide. However, as indicated in the preceding section, the $I_2$ produced is quickly reduced again by ambient oxidizable substances. These chemical reactions apparently involve stoichiometric production of acids (e.g. $AH_2 + I_2 \rightarrow A + 2\,H^+ + 2\,I^-$) to compensate for the proton consumption due to electron transport ($2\,I^- + 2\,H^+ + O_2 \rightarrow I_2 + 2\,H_2O_2$). Consequently the only detectable proton change is a totally gramicidin-sensitive reversible proton translocation (traces *c* and *d*). Trace *e* confirms that $NH_2OH$-washed chloroplasts used in this experiment were totally

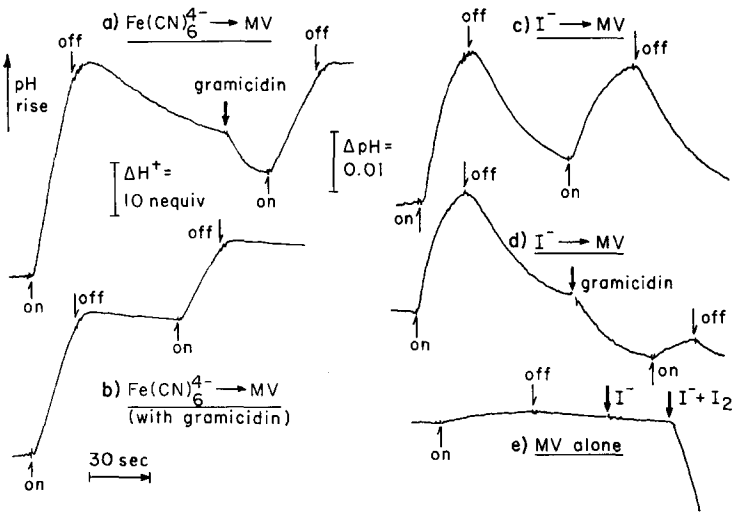


Fig. 10. Changes in the pH of the medium associated with the transport of electrons from ferrocyanide (traces *a* and *b*), $I^-$ (traces *c* and *d*) and water (trace *e*) to methylviologen (MV) in $NH_2OH$-washed chloroplasts. The basic constituents of the reaction mixture (2 ml) were 0.1 M sucrose, 0.5 mM HEPPS–NaOH buffer (pH 7.6), 2 mM $MgCl_2$, 0.1 mM methylviologen and chloroplasts equivalent to 25 $\mu$g chlorophyll per ml. When used, potassium ferrocyanide was 30 mM. KI, used alone, was 20 mM. Gramicidin, when added, was 2 $\mu$g/ml. In trace *e*, $I_2$ was added in the form of $I_3^-$ (final concentrations, 0.5 mM $I_2$ plus 2 mM $I^-$). Trace *e* shows the lack of light-induced pH rise in the absence of artificial electron donors added and also an acid production induced by the addition of $I_2$. For explanations, see text.

inactive in the absence of artificial reductants added. The trace also demonstrates an acid production induced by exogenously added $I_2$ (as $I_3^-$).

## DISCUSSION

Ferrocyanide ($E_0' = 0.43$ V) and $I^-$ ($E_0' = 0.53$ V) can be ranked with benzidine ($E_0' = 0.65$ V at pH 5) [20] and dicyanohydroquinone ($E_0 = 0.97$ V; $E_0'$ unknown) [21] among the weakest artificial reductants oxidized by illuminated chloroplasts. Predictably the oxidation of these substances requires Photosystem II, as indicated by its DCMU sensitivity. However, ferrocyanide and $I^-$ are unique in that the transport of electrons from these two reductants to methylviologen through the two photosystems supports phosphorylation only with $P/e_2$ values of 0.5 to 0.6. The same pathway mediates phosphorylation with $P/e_2$ values of 1.0 to 1.2 when the electron donor is water, catechol, benzidine or *p*-aminophenol (Table I; see also ref. 11). The low efficiencies of phosphorylation obtained with ferrocyanide and $I^-$ cannot be explained as being due to uncoupling by the donors themselves, since the $P/e_2$ values are independent of donor concentrations over very wide ranges (Figs 5 and 8). Nor is it likely that any kind of product inhibition is largely responsible for the low phosphorylation efficiencies, since the $P/e_2$ value is essentially independent of the reaction time (< 60 s) when ferrocyanide is the donor (Fig. 3). The $P/e_2$ does decrease with the reaction time in $I^-$ oxidation, but does so only slightly (0.5–0.4 in 60 s; Fig. 7).

We are thus led to the only remaining simple interpretation: one of the two sites of phosphorylation is inoperative when ferrocyanide or $I^-$ serves as electron donor to Photosystem II. It seems logical to deduce further that the inoperative site is one which is proximal to the point of entry of electrons from the donors, i.e. Site II. Indeed, the characteristics of phosphorylation associated with the oxidation of ferrocyanide and $I^-$ are quite close to what one would expect from the involvement of Site I alone. Gould and Izawa [13] have presented strong evidence that the pathway of electrons from reduced 2,6-dichlorophenolindophenol to methylviologen via Photosystem I includes only Site I. Phosphorylation and its efficiency associated with this pathway peak at pH 8 or slightly above (where $P/e_2$ is 0.65 to 0.68 [11]) and sharply decline toward zero between pH 6.5 and 7. Unpublished data of J. M. Gould for another Photosystem I donor reaction, diaminodurene → methylviologen, also show an almost identical pH profile of phosphorylation with a maximal $P/e_2$ of 0.6. Similar maximal $P/e_2$ values of 0.57 to 0.62 have also been found with diaminotoluene and *p*-phenylenediamine as donors [11]. These properties of Photosystem I-mediated phosphorylation, which presumably originate from the mechanism of coupling at Site I, are unmistakably shared in common by the phosphorylation supported by Photosystem II-initiated transport of electrons from ferrocyanide and iodide to methylviologen (Figs 6 and 9). The only perceptible (and unexplained) difference is the slightly higher $P/e_2$ values found for the pathway reduced 2,6-dichlorophenolindophenol → methylviologen (see above).

It should be added here that the characteristics of phosphorylation attributable to Site II, so observed in the partial electron transport systems involving only Photosystem II ($H_2O$ → dibromothymoquinone [9] and $H_2O$ → 2,5-dimethylquinone [13]), are quite different. Both electron transport and phosphorylation peak around pH 7.6 and their ratio ($P/e_2 = 0.3$ to 0.4) is remarkably independent of pH between 6 and 9*.

As we have already pointed out, the oxidation of ferrocyanide to ferricyanide is a simple valence change at physiological pH's, because of the very high stability of both forms of the complex. No detectable $H^+$ or $OH^-$ change occurs as indicated by the pH-independent $E_0'$ value. Neither does the oxidation of $I^-$ to $I_2$ induce $H^+$ changes perhaps except above pH 9 where IOH formation begins. In contrast, all of those Photosystem II donors including water which gave higher $P/e_2$ values (1.0–1.2), i.e. catechol, benzidine, *p*-aminophenol and dicyanohydroquinone, are hydrogen donors, the oxidation of which involves, or presumably involves, nearly stoichiometric release of protons at physiological pH values ($\Delta E_0'/\Delta pH \approx -0.06$ V or $\Delta H^+/\Delta e^- \approx 1$; no data for dicyanohydroquinone [21] or for benzidine above pH 6 [20]). Thus, if one is allowed to ignore the question regarding the fate of the oxidation product of iodide (see below), our results would seem to provide a strong experimental basis for

* It should also be noted that our arguments involving the $P/e_2$ ratios of individual coupling sites are all based on experiments with conventional Class II chloroplasts (envelope- and stroma-free, largely unfragmented chloroplast lamellae) which give an overall $P/e_2$ of 1.0–1.2 in standard noncyclic photophosphorylation. After the completion of this manuscript a paper by Heathcote, P. and Hall, D. O. ((1974) Biochem. Biophys. Res. Commun. 56, 767–774) has been published in which evidence is presented that in their particular type of chloroplasts giving an overall $P/e_2$ of 1.7, the $P/e_2$ ratio of Site II (Photosystem II coupling site) may be about 0.6, instead of 0.3–0.4 in our chloroplasts. Heathcote and Hall suggest that the $P/e_2$ ratio of Site I (Photosystem I coupling site) in their chloroplasts preparation may be as high as 1.0, rather than 0.6 in our preparation.

the chemiosmotic model of energy conservation at Photosystem II [16, 22] or, in our terminology, at Site II, with a strong emphasis on proton relationships rather than on membrane potentials.

Our results also offer a possible explanation of the intermediate efficiency of phosphorylation ($P/e_2 = 0.8$–$0.9$) found associated with the Photosystem II-dependent oxidation of ascorbate [11]. That is, the oxidation of ascorbic acid anion (i.e. above pH 4.5) to the neutral molecule dehydroascorbate, the demonstrated end product [23], liberates only one proton for a pair of electrons removed, thus: $AH^- \rightarrow A + H^+ + 2e^-$. It may be that Site II is only half-operative with ascorbate as the electron source. We consider here the nominal distinction between one-electron donors (e.g. ferrocyanide) and two-electron donors (e.g. catechol) as probably unimportant here. In fact the flash experiments of Bennoun and Joliot [24] and of Babcock and Sauer [25] indicated that the oxidation of $NH_2OH$, hydroquinone and *p*-phenylenediamine by Photosystem II is via a one-quantum (hence presumably via a one-electron) process requiring no charge accumulation.

The chemiosmotic model of energy conservation at Photosystem II requires, though perhaps not absolutely, that artificial electron donors as well as water be oxidized at or near the inner surface of the thylakoid membrane. There are several lines of circumstantial evidence for this. The concentrations of ferrocyanide and $I^-$ required to saturate oxidation rates are extremely high: 30 and 15 mM, respectively. These values are in striking contrast with the rate-saturating concentrations ($< 0.5$ mM) of other Photosystem II donors such as catechol, benzidine, *p*-aminophenol, etc. (Table I; see also refs 10 and 11). Ascorbate, when acts as the direct electron donor for Photosystem II, saturates the oxidation rate at an intermediate but still relatively high concentration of 5 mM [10]. It thus appears that the concentration requirement is primarily a function of the liposolubility of the donor; nonionic, nonpolar (lipophilic) donors having the highest accessibility to the oxidation site while ionic, highly polar (hydrophilic) donors the lowest. The implication is clearly that the reductants must penetrate the lipid membrane to reach the oxidation site. Such a location of the oxidation site could also explain the biphasic kinetics of oxidation of ferrocyanide, the least permeant reductant (Fig. 3). The initial rapid phase where the oxidation rate approaches the rate of the normal Hill reaction, may represent the oxidation of that portion of ferrocyanide which has already permeated the membrane (possibly into the internal space of the thylakoid) while the subsequent much slower phase may represent a diffusion-limited process. The existence of a strong permeability barrier limiting ferrocyanide oxidation is also suggested by the preliminary experiment of D. R. Ort (unpublished) which indicated that the rate of oxidation of ferrocyanide can be markedly increased by the addition of sub-uncoupling concentrations of digitonin.

$I^-$ seems to permeate the thylakoid membrane somewhat more easily than does ferrocyanide, as suggested by the slightly lower concentration requirement and the more linear kinetics of oxidation. However, the $I^-$ oxidizing system is not nearly as easily characterized as is the ferrocyanide-oxidizing system, because the unstable oxidation product $I_2$ (or possibly $I^\cdot$) is quickly reduced again by unknown reducing substances in the reaction mixture. (Ferricyanide does not react with these substances.) Moreover, this re-reduction of $I_2$ is accompanied by an acid production. Thus, $I^-$ is indirectly a proton-producing electron donor. Nevertheless, the $P/e_2$ data suggest that Site II cannot operate when $I^-$ serves as electron donor. If the chemiosmotic

model of Site II is valid, then the implication is that the protons released by the reduction of $I_2$ are not available for phosphorylation. According to the model, this could only mean that the chemical reduction of $I_2$ and the associated proton production take place predominantly on the outside or the outer surface of the thylakoid membrane. Such a situation does not seem impossible, since $I_2$ is a highly diffusable substance, and since it is conceivable that the region of the membrane where the strong oxidant of Photosystem II is produced may be so deficient in oxidizable substances as to allow $I_2$ a brief life-time to diffuse away. The re-reduction of $I_2$ could then take place preferentially on the outer surface of the membrane where abundant oxidizable substances are available as impurities of the medium. This interpretation is at least consistent with the fact that no important membrane constituents seem to be destroyed by the $I_2$ produced. As shown in Fig. 7 $I^-$ oxidation and associated phosphorylation proceed almost linearly for at least 60 s, during which time the $I_2$ produced and consumed reaches the amount equivalent to chlorophyll present.

Admittedly the interpretation given above for the fate of $I_2$ still leaves ample room for debate. In fact, we feel that the apparent total "uselessness" of protons produced by the nonbiological reduction of $I_2$ may far better be reconciled with the modified chemiosmotic theory of Williams [26]. The theory assigns the key role to localized proton concentrations induced within the hydrophobic membrane by the oxidation of hydrogen carriers, rather than to proton gradients subsequently formed across the membrane. Whichever the model, however, our observations described in this paper strongly point to the role of protons in the main sequence of energy transduction at Photosystem II.

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