New Ionic Redox Agents for the Study of Photosynthesis[1]

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Abstract

Numerous ions were tested for their usefulness as electron carriers in the electron transport chain of spinach chloroplasts. It was found that silicomolybdic acid and cobaltinitrite were sufficient electron acceptors for photosystem II while metavanadite and ferrocyanide could be used to donate electrons to photosystem I. Silicomolybdic acid, a large polyanion, proved to be a unique compound accepting electrons before the DCMU block. Cobaltinitrite accepted electrons close to the photophosphorylation site of PS II. Metavanadite donated electrons to PS I in the vicinity of plastocyanin (PC) while ferrocyanide provided a site between plastocyanin and P 700.

Introduction

With the exception of potassium ferri-ferrocyanide (9, 12), few ions have been used as electron carriers in the photosynthetic electron transport chain of chloroplasts. In this study, silicomolybdic acid, a large polyanion (5) and potassium cobaltinitrite are proposed as electron acceptors for photosystem II. The properties of these reactions are compared to the well-known $H_2O \rightarrow$ferricyanide reaction. To act as electron donors to photosystem I, potassium vanadite (vanadium IV), another large polyanion and ferrocyanide (9) were chosen. The oxidation of these compounds is compared to the standard PS I reaction, ascorbate plus TMPD$\rightarrow$methyl viologen (12). By introducing these various ions as electron carriers in photosynthetic electron transport, new approaches are opened up to an understanding of the sequence of the electron transport chain in spinach chloroplasts.

Materials and Methods

Spinach chloroplasts were prepared from market spinach by the method of Jagendorf and Avron (10). Chlorophyll was determined by Arnon's method (1).

The effect of the various anions on photosynthetic electron transport was studied polarographically with a Clark-type electrode. A typical PS II reaction mixture contained in 3 ml total volume: chloroplasts containing 50 μg chlorophyll, 150 μmoles Trizma-Mes, pH 7.0, 30 μmoles $MgCl_2$, 12 μmoles NH_4Cl and 0.2-0.5 mg silicomolybdic acid or 0.2-0.5 mg potassium cobaltinitrite. Silicomolybdic acid reduction was done in the presence of DCMU since this reaction was found to be largely DCMU-insensitive.

PS I reactions contained the following per ml solution: chloroplasts containing 15 μg chlorophyll, sodium ascorbate, 50 μmoles, TMPD 0.2 μmole, methyl viologen, 0.4 μmole, Trizma-Mes, pH 8, 150 μmole, and DCMU 0.03 μmole. The ferrocyanide reaction contained 320 mmoles ferrocyanide in place of TMPD, the metavanadite

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(vanadium IV)—0.4 ml of a saturated solution dissolved in 0.1 N NaOH.

Chelators, such as ortho- or bathophenanthroline were added to chloroplasts in the least possible volume of ethanol—20 λ for silicomolybdic acid reduction, 20-50 λ for cobaltinitrite reduction. PS I reactions were found to be less sensitive to ethanol.

Polylysine, 35,000 M.W., obtained from Sigma, was dissolved in water. A strict order of addition to PS I reactions was observed to get maximum inhibition (4): water, polylysine, and chloroplasts, followed by buffer and all other reaction mixture ingredients.

DBMIB was the gift of Dr. A. Trebst. Silicomolybdic acid was purchased from ICN-K & K Laboratories. It was dissolved in water and filtered before use. In general, the ions tested were dissolved in water if possible; if not, in 1 N NaOH or 1 N HCl. Some gave saturated solutions and had to be filtered.

Results

Optimum concentrations of silicomolybdic acid (0.2 mg), cobaltinitrite (0.5 mg) and metavanadate (1 mmole) per each 2 ml assay are shown in Fig. 1. The pH optimum of the water $\rightarrow$ silicomolybdic acid reaction is from 6-7 (Fig. 2) although all standard PS II reactions in this study were done at pH 7. Since chloroplasts were tightly coupled, the presence of ammonium chloride was necessary. The optimum pH for the $H_2O \rightarrow$metavanadate reaction was 7, as Fig. 2 shows.

Factors affecting silicomolybdic acid reduction by chloroplast photosystem II are shown in Table I. Inhibition of oxygen evolution rates is given by ethanol, sodium bicarbonate, and potassium permanganate. Chelators, such as bathophenanthroline also inhibited the water$\rightarrow$ silicomolybdic acid pathway by 10% or more, depending on the concentration but orthophenanthroline gave little inhibition of this reaction. Both chelators strongly inhibited the $H_2O \rightarrow$ferricyanide reaction. Another difference between the reduction of silicomolybdic acid and ferricyanide in chloroplasts was that potassium permanganate showed no inhibition of the $H_2O \rightarrow$ferricyanide pathway indicating that all components of the water$\rightarrow$silicomolybdic acid pathway are not the same in the 2 reactions.

Table 2 describes other chloroplast photosystem I and II acceptors in the following reactions: $H_2O \rightarrow$cobaltinitrite, $H_2O \rightarrow$metavanadate (vanadium V), $H_2O \rightarrow$ruthenium red, and $H_2O \rightarrow$nitroprusside blue. The reduction of these compounds by chloroplasts is also inhibited by DCMU, DBMIB, Tween-20, and polylysine from 45-100%. Since water$\rightarrow$ cobaltinitrite is completely inhibited by DCMU, it is clearly a PS II reaction while the water$\rightarrow$metavanadate passway encompasses both photosystems. The other 2 reactions described, $H_2O \rightarrow$rutherium red and $H_2O \rightarrow$nitroprusside blue, are also mixed reactions. They have not been studied in detail and are presented here only to show that rutherium red and nitroprusside blue are able to accept electrons from the chloroplast electron transport chain.

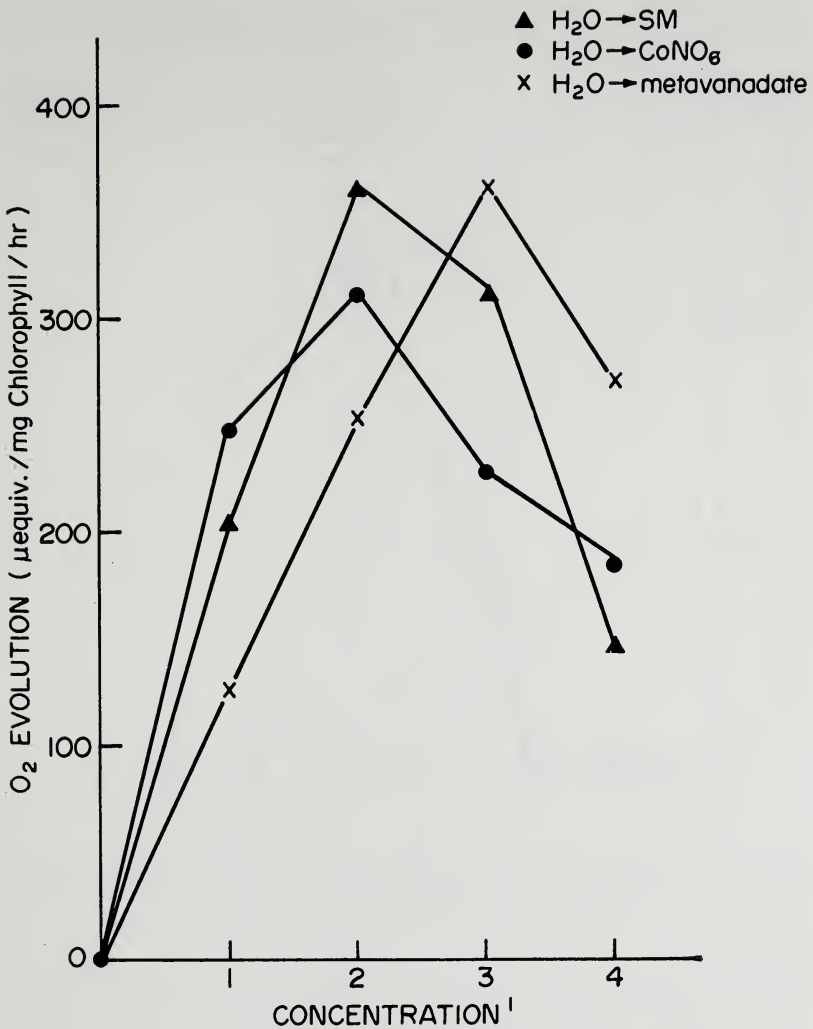

FIGURE 1. *The effect of silicomolybdic acid, cobaltinitrite, and metavanadate concentration on oxygen evolution rates in photosystem II of spinach chloroplasts.*

Vanadyl sulfate and ferrocyanide in low concentrations (0.032 M) were found to donate electrons to PS II (Table 3). The vanadyl sulfate reaction was completely sensitive to DCMU, ferrocyanide 70%.

Optimum concentrations of metavanadite (0.4 ml) and ferrocyanide (20 mg) are given in Fig. 3. In this case total volume of the reaction mixture was 5 ml. Optimum pH for these 2 PS I reactions is 8 (Fig. 4). Fig. 5 shows factors affecting ferrocyanide oxidation in PS I. It can

[1] *silicomolybdic acid concentration was (1, 2, 3, 4) 0.1, 0.2, 03, 0.4 mg/assay; cobaltinitrite concentration—0.25, 0.5, 1, and 2.5 mg; metavanadate concentration—0.5, 1, 2, and 4 mmoles.*

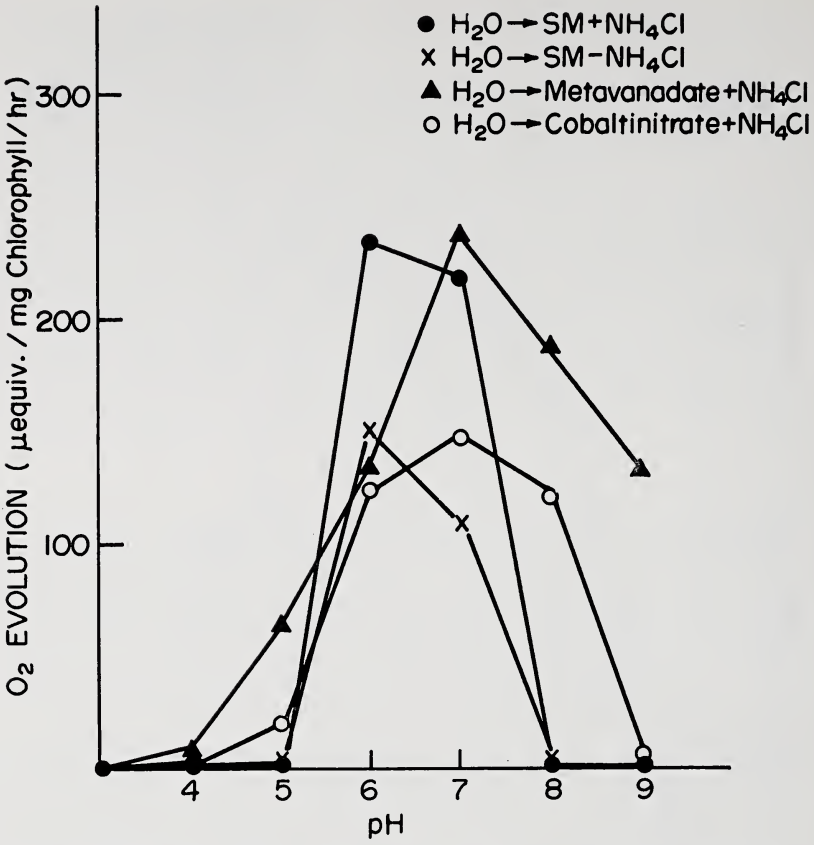

FIGURE 2. *The effect of pH on silicomolybdic acid, metavanadate, anl cobaltinitrite reduction in chloroplasts. NH_4Cl was present in all assays except where silicomolybdic acid reduction was compared to rates in its absence.*

be seen that polylysine and ethanol stimulate this reaction while such chaotropic agents as potassium iodide and sodium thiocyanate inhibit it.

In Table 4 factors affecting vanadite (vanadium IV) oxidation by photosystem I are described. Tween-20 gave a complete inhibition of the rate which could be restored by exogenous plastocyanin. Polylysine also inhibited vanadite oxidation by PS I. Rates were affected to varying degrees by the chelators, ortho- or bathophenanthroline, salicylaldoxime and dithizone.

In Table 5, 2 photosystem I reactions—ascorbate plus TMPD→ methyl viologen and ascorbate plus ferrocyanide→methyl viologen—are compared after various chloroplast treatments. Washing chloroplasts with 1% Tween-20 solution almost abolished TMPD oxidation and decreased ferrocyanide oxidation by half. TMPD oxidation could be completely restored by addition of plastocyanin, but plastocyanin had no effect on the ferrocyanide rate. Ferrocyanide oxidation in Tween-washed chloroplasts could only be restored by the addition of the 0.4 M NaCl

TABLE 1. *Factors Affecting Silicomolybdic Acid Reduction by Chloroplast Photosystem II.*

Reaction	Additions	O_2 evolution (μequiv./mg chlorophyll/hr)	Inhibition (%)
H_2O➤SM	None	312	—
	DCMU	288	8
	Ethanol (20 λ)	228	27
	Ethanol (50 λ)	150	52
	Bathophenanthroline (0.05 mg)	246	21
	Orthophenanthroline (0.05 mg)	310	1
	Sodium bicarbonate (0.1 mmole)	86	72
	Potassium permanganate (10 μmoles)	182	42
	Potassium permanganate (25 μmoles)	78	75
H_2O➤ferricyanide	None	306	—
	DCMU	0	100
	Ethanol (20 λ)	272	11
	Ethanol (50 λ)	248	19
	Bathophenanthroline (0.05 mg)	116	62
	Orthophenanthroline (0.05 mg)	72	76
	Sodium bicarbonate (0.1 mmole)	276	10
	Potassium permanganate (10 μmoles)	312	0
	Potassium permanganate (50 μmoles)	186	39

fraction from a DEAE column containing an unknown factor. The addition of polylysine to chloroplasts decreased TMPD oxidation but stimulated ferrocyanide oxidation. Incubation of chloroplasts with bathocuproine, a lipophilic chelator with an affinity for copper, for 1 hr decreased the TMPD and ferrocyanide rates, but the water-soluble bathophenanthroline sulfonate had no effect. A 5-min. incubation with dithizone, another copper chelator, showed some inhibition of the TMPD rate, but longer periods of incubation resulted in stimulation. Dithizone had little effect on ferrocyanide oxidation, as did salicylaldoxime which again inhibited TMPD oxidation.

In Table 6, less successful ionic electron donors or acceptors for both photosystems in spinach chloroplasts are described. The wide variety of unsuccessful compounds which are known to undergo oxidation-reduction reactions implies specificity of the successful ones.

Discussion

Various ions which exhibit several possible redox states, such as vanadium compounds II-V, appear to be likely candidates as electron carriers in the electron transport chain of chloroplasts. However, only ferri-ferrocyanide (9, 12) has been used for such a purpose previously.

TABLE 2. *Factors Affecting the Reduction of Other Chloroplast Photosystem I and II Acceptors.*

Reaction	Additions	O_2 evolution (μequiv./mg chlorophyll/hr)	Inhibition (%)
H_2O➤cobaltinitrite	None	338	—
	DCMU	0	100
	DBMIB	124	63
	Orthophenanthroline (0.05 mg)	189	44
	Bathophenanthroline (0.05 mg)	169	50
H_2O➤metavanadate	None	362	—
	DCMU	40	89
	DBMIB	136	62
	Orthophenanthroline (0.05 mg)	68	81
	Bathophenanthroline (0.05 mg)	182	50
	Tween-20 (2.5×10^{-3}%)	96	73
	Tween-20 (5×10^{-3}%)	36	90
	Polylysine (0.1 mg)	0	100
H_2O➤ruthenium red	None	143	—
	DCMU	78	45
	DBMIB	39	73
H_2O➤nitroprusside	None	142	—
	DCMU	68	52
	DBMIB	42	70

In this study, it was chosen to test several other ions to serve as artificial electron acceptors in photosystem II or as electron donors to photosystem I. Of numerous compounds tested, silicomolybdic acid and cobaltinitrite, metavanadate and ferrocyanide were found to function best in chloroplasts.

Silicomolybdic acid was found by Giaquinta *et al.* (5) to accept electrons in PS II before the DCMU block, presumably at Q. This reaction did not support photophosphorylation. Since silicomolybdic acid is a large polyanion, it is assumed that it does not penetrate the thylakoid membrane. As such it is a useful tool in studying membrane sidedness. The fact that the $H_2O \rightarrow$silicomolybdic acid pathway is affected by low concentrations of ethanol and is strongly inhibited by

TABLE 3. *Ionic Donors to Chloroplast Photosystem II.*

Reaction	Additions	O_2 uptake (μequiv./mg chl/hr)	Inhibition (%)
Vanadyl sulfate	None	344	0
	DCMU	0	100
	DBMIB	56	84
Ferrocyanide	None	84	0
	DCMU	25	70

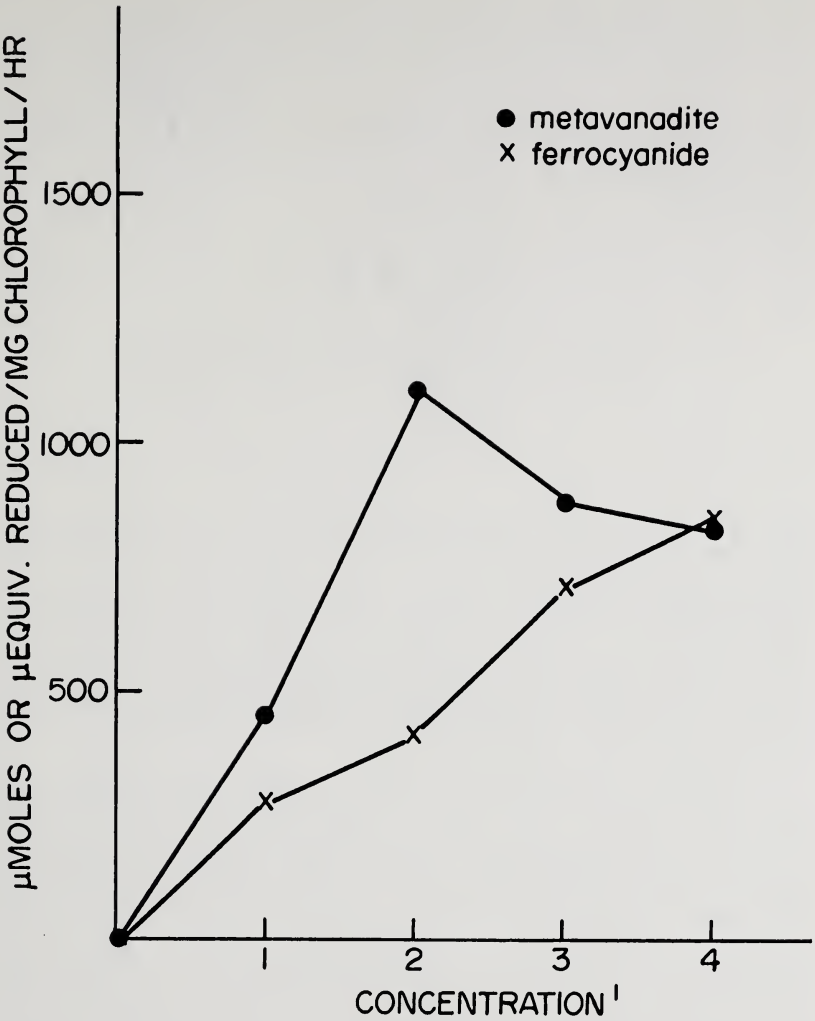

FIGURE 3. *The effect of metavanadite and ferrocyanide concentration on oxygen production by photosystem I in spinach chloroplasts.*

sodium bicarbonate and potassium permanganate whereas the water→ ferricyanide reaction keeps going under these conditions (Table 1), points to silicomolybdic acid accepting electrons on a sidepath branching off from Q, the initial electron acceptor from the PS II reaction center chlorophyll of the traditional Z-scheme of electron transport chain in chloroplasts.

Since the reduction of cobaltinitrite was found to be entirely DCMU-sensitive and also, ortho- and bathophenanthroline-sensitive (Table 2),

[1] *metavanadite concentration was (1, 2, 3, 4) 0.2, 0.4, 0.6 and 0.8 ml/assay; ferrocyanide concentration—2, 5, 10 and 20 mg.*

TABLE 4. *Factors Affecting Photosystem I Vanadite Oxidation.*

Reaction	Additions	PS I Rate (μmoles acceptor reduced/mg chlorophyll/hr)	Inhibition (%)
Ascorbate plus metavanadite→ methyl viologen	None	255	—
	Tween-20 (0.1%)	0	100
	" plus PC	255	0
	Polylysine (0.1 mg)	113	56
	Polylysine (0.2 mg)	56	78
	Bathophenanthroline (0.05 mg)	56	78
	Orthophenanthroline (0.05 mg)	204	20
	Salicylaldoxime (0.2 mg)	85	67
	Dithizone (0.5 mmole)	306	0

cobaltinitrite is assumed to accept electrons between Q and immediately after the DCMU block of PS II. This is Gould and Izawa's (7) proposed photophosphorylation site for PS II which they assay by the $H_2O \rightarrow$dimethylbenzoquinone reaction. Further support for cobaltinitrite accepting electrons at this site comes from Barr and Crane's data on the behavior of uncoupler protection against chelator inhibition at phosphorylation sites in the electron transport chain (3). Cobaltinitrite reduction in PS II is chelator-sensitive and can be protected by such uncouplers as CCCP (unpublished data).

Ascorbate plus metavanadite→methyl viologen and ascorbate plus ferrocyanide→methyl viologen are photosystem I reactions. It is well known that ascorbate plus TMPD→methyl viologen donate electrons to PS I before plastocyanin because Tween washes or sonication which remove plastocyanin stop this reaction. Activity can be restored by exogenous plastocyanin. A comparison of metavanadite and TMPD oxidation in Tween-washed chloroplasts shows that both reactions are inhibited when plastocyanin is removed (Tables 4, 5) and both can be restored by exogenous plastocyanin. This data places the metavanadite oxidation site before or close to plastocyanin. However, the metavanadite site appears to be different from the TMPD site on the basis of chelator inhibition: TMPD oxidation is frequently stimulated by bathophenanthroline whereas metavanadite can be inhibited by bathophenanthroline.

The third PS I electron donor site studied involves ferrocyanide in high concentrations (0.32 M). A comparison of this reaction in Tween-washed chloroplasts with TMPD oxidation (Table 5) shows that it loses about a half of its activity and that the loss cannot be restored by plastocyanin. Restoration of the normal rate is possible only by the addition of an unknown component, a protein which can be eluted from a DEAE column by the 0.4 M NaCl gradient fraction after dialysis. Since ferrocyanide oxidation appears to be independent of plastocyanin, ferrocyanide must donate electrons to PS I after the plastocyanin site, or somewhere between plastocyanin and P 700, the

TABLE 5. *Treatments Affecting Chloroplast Photosystem I Reactions.*

Treatment	Additions	TMPD (μmoles acceptor reduced/mg chlorophyll/hr)	Ferrocyanide (μmoles acceptor reduced/mg chlorophyll/hr)
Before Tween-20	None	1692	819
After extraction with 1% Tween-20	None	84	480
	PC	1596	480
	0.4 M NaCl from DEAE column	732	987
After water wash	None	1128	810
Untreated	None	1974	846
	PL	300	1410
Untreated	None	1974	846
After 1 hr. incubation with bathocuproine (5 mg in 0.2 ml ETOH/mg chlorophyll)	None	366	282
After 1 hr. incubation with bathocuproine sulfonate (5 mg/mg chlorophyll)	None	1833	789
Untreated	None	2000	780
After 5 min. incubation with dithizone (0.2 mg/mg chlorophyll)	None	1437	732
After 30 min. incubation with dithizone	None	2358	705
Untreated	None	1538	658
Untreated	Salicyladoxime (0.1 mg)	750	658

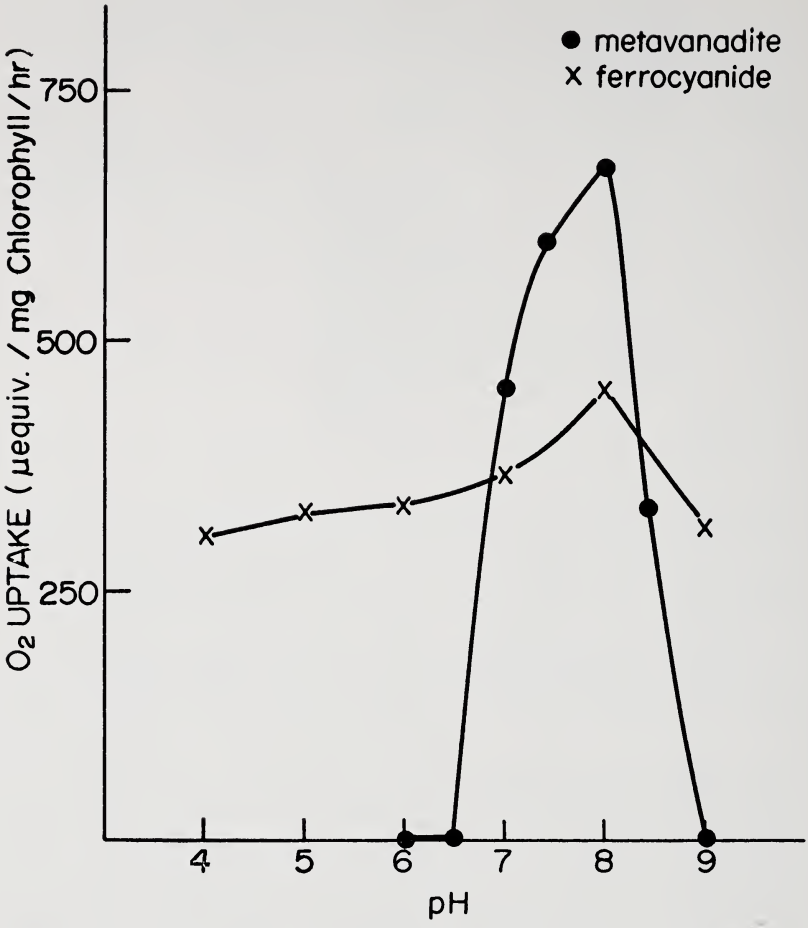

FIGURE 4. *The effect of pH on metavanadite and ascorbate oxidation by photosystem I of spinach chloroplasts.*

TABLE 6. *Less Successful Ionic Electron Donors or Acceptors for Photosynthetic Reactions.*

Reaction	Oxygen evolution or uptake (μequiv./mg chlorophyll/hr)
H_2O➤rhodium chloride	0
H_2O➤perrhenic acid	0
Perrhenic acid➤MV	17
H_2O➤sodium tantalate	0
H_2O➤sodium ruthenate	39
H_2O➤manganicyanide (dark)	52
H_2O➤manganicyanide (light-dark)	65
H_2O➤manganicyanide (without chloroplasts)	72
H_2O➤ruthernium potassium cyanate	33

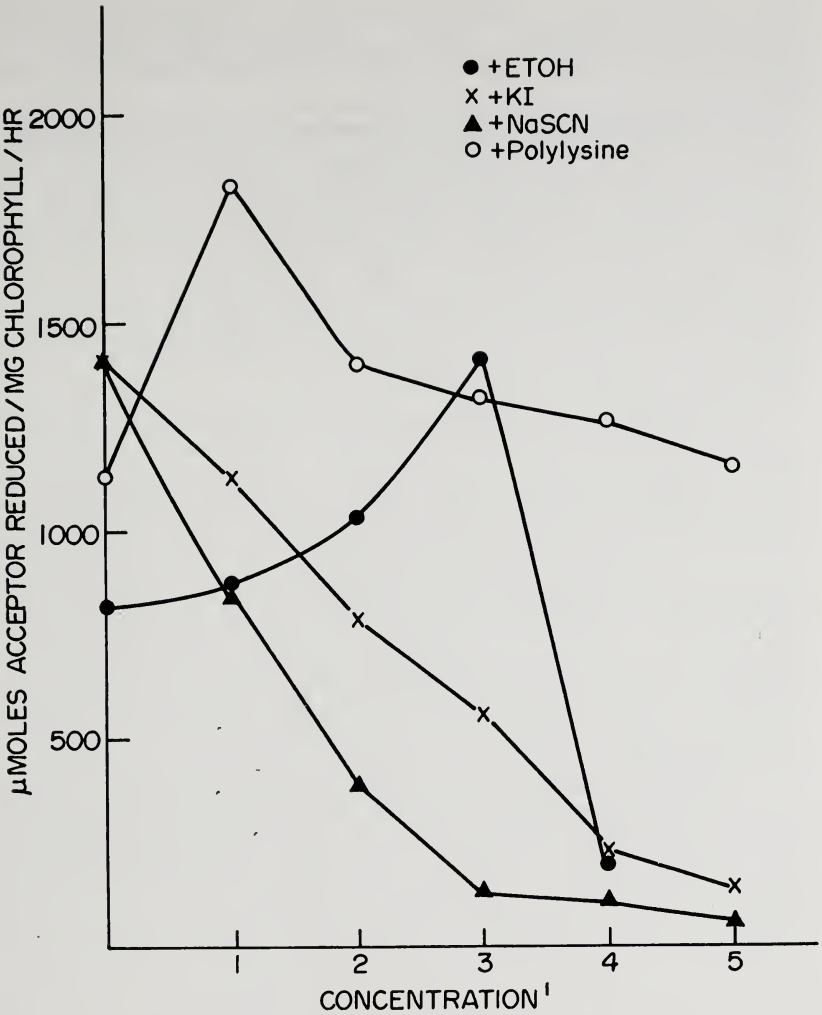

FIGURE 5. *Factors affecting ferrocyanide oxidation by photosystem I in spinach chloroplasts.*

reaction center chlorophyll in PS I. An examination of ferrocyanide reaction sites after incubation with various chelators, such as bathocuproine, salicylaldoxime or dithizone, discloses varied results: bathocuprione inhibits ferrocyanide oxidation while dithizone or salicylaldoxime does not effect it. The three chelators also have different effects on TMPD oxidation: bathocuproine and salicylaldoxime which most likely affect the copper in plastocyanin are strong inhibitors; dithizone

[1] *ethanol concentration was (1, 2, 3, 4)—0.1, 0.2, 0.5 and 1 ml; KI concentration—300, 600, 1200, and 3000 mmoles; NaSCN concentration—300, 600, 1200, 1500 and 3000 mmoles; polylysine concentration—0.1, 0.2, 0.3, 0.4 and 0.5 mg.*

inhibits during short incubation periods but stimulates the reaction over a 30 min. incubation period. A chelator-stimulated site in the vicinity of plastocyanin has been reported previously by Barr and Crane (3).

The metavanadite and ferrocyanide data can also be interpreted according to the branch pathway scheme of Haehnel (8) who put plastocyanin and cytochrome f on separate pathways to P 700. In this case vanadite would donate electrons primarily to the plastocyanin branch, ferrocyanide to the other branch, and TMPD to both pathways.

The apparent donation of electrons from metavanadite to plastocyanin would indicate that plastocyanin or another component in that pathway is on the outer surface of the chloroplast membrane since metavanadite is not soluble in lipids.

In contrast to the negatively charged metavanadite, the positive vanadyl ion donates electrons to methyl viologen mostly before the DCMU site and, therefore, reacts before photosystem II. This site may be similar to the site where low concentrations of ferrocyanide donate electrons to PS II (Table 3), as described by Izawa and Ort (9). The ability of both positive and negative ions to act as electron donors before PS II indicates that a portion of the PS II system may lie exposed on the exterior of the membrane. Although Arntzen *et al.* (2) showed by freeze-etch studies of chloroplasts that large PS II particles were on the inside of the membrane, other studies using non-penetrating DABS isotope labelling (6) and immunological studies (11) have shown that a portion of PS II may be accessible from the outside of the membrane.

In summary, the use of ionic electron carriers has been investigated in photosynthetic electron transport. Two new electron acceptors for PS II and two electron donors for PS I have been described. Silicomolybdic acid was found to be unique in accepting electrons before the DCMU block, cobaltinitrite immediately after it in PS II. Metavanadite and ferrocyanide, electron donors to PS I, were useful in studying the region between plastocyanin and P 700.

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