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VANADIUM COMPOUNDS AND FERROCYANIDE AS IONIC REDOX AGENTS IN PHOTOSYNTHESIS

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SUMMARY

The effect of such ionic redox agents as ferrocyanide and several vanadium compounds was determined on photosynthetic reactions of spinach chloroplasts. It was found that:

1. Vanadyl sulfate like ferrocyanide in moderately high concentrations (0.03 M) donates electrons to Photosystem II.

2. Decavanadate in the presence of 2,5-dibromothymoquinone accepts electrons in Photosystem II.

3. In the absence of a block between the two photosystems, decavanadate accepts electrons in Photosystem I in the vicinity of plastocyanin or beyond.

4. Vanadite and ferrocyanide in high concentrations (0.32 M) donate electrons to Photosystem I.

5. On the basis of chelator inhibition and polyoxyethylene sorbitan monolaureate treatment, the vanadite oxidation site is located near plastocyanin while the ferrocyanide site is between plastocyanin and *P*-700.

INTRODUCTION

Few large ions have been used as artificial electron carriers in the electron transport chain of chloroplasts. These include the old standby, potassium ferricyanide [1] as an electron acceptor in Photosystem II and silicomolybdic acid, recently reported by Giaquinta et al. [2] and Barr and Crane [3] to accept electrons before the DCMU block in Photosystem II. Ionic electron donors to Photosystem II include potassium ferrocyanide in relativity low concentrations (0.03 M) and potassium iodide reported by Izawa and Ort [4]. Several other ionic redox agents have been tested in photosynthetic reactions and briefly described by Barr et al. [5]. This is a further exploration of the usefulness of 2 of the most promising ones, potassium ferrocyanide in high concentrations (0.32 M) and vanadite (vanadium IV) as electron donors to Photosystem I.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromothymoquinone; MES, 2 (*n*-morpholino) ethane sulfonic acid; TMPD, *N,N, N', N'*-tetramethyl-*p*-phenylenediamine; Tween 20, polyoxyethylene sorbitan monolaureate.

Vanadium compounds were chosen for this study because they exhibit various valence states (II–V) and can be oxidized easily [6]. The state IV, vanadyl and vanadite ions are quite stable in air. It was assumed that vanadate (vanadium V) could accept electrons somewhere in the chloroplast electron transport chain and vanadite or vanadyl ions (vanadium IV) would donate electrons. This proved to be the case, as data presented in this paper indicate.

MATERIALS AND METHODS

Chloroplasts were prepared from market spinach by the method of Jagendorf and Avron [7]. Chlorophyll was determined by Arnon's method [8].

Oxygen evolution in Photosystem II or uptake with methyl viologen as the acceptor in Photosystem I were measured polarographically with a Clark-type electrode and recorded with a Honeywell recorder. Assay mixtures were those of Brand et al. [9]. In Photosystem I assays containing polylysine, a strict order of addition was observed to get maximum inhibition: water, polylysine and chloroplasts, followed by buffer and other reaction mixture ingredients. Saturating light was used in all experiments.

Tris-treated chloroplasts for studies of vanadyl electron donation to Photosystem II were prepared as previously described [3]. Tween washes of chloroplasts were performed by stirring chloroplasts with a 1 % solution of Tween-20 and recentrifuging at $1200 \times g$ for 10 min to obtain a washed chloroplast pellet. The proportion of chloroplasts to wash solution was 1 mg chlorophyll/50 ml Tween 20. The washed chloroplasts were resuspended in 0.4 M sucrose with 0.05 M NaCl. Plastocyanin to reconstitute Photosystem I activity in Tween-washed chloroplasts was obtained by the procedures of Katoh [10]. The brown protein (rubimedin) used to restore ferrocyanide oxidation was eluted from the same DEAE column used to prepare plastocyanin (Katoh's step 4) with increasing salt concentrations until the brown band came off with 0.4 M NaCl.

Chelators including ortho- or bathophenanthroline, salicylaldoxime and dithizone were added to chloroplasts in the least possible volume of ethanol, 20–50 μl/1.5 ml. Decavanadate was prepared by adding vanadium pentoxide in excess to 0.1 M sodium hydroxide and letting the solution stand overnight at pH 6.2 before filtering off excess oxide. The black vanadite ion was prepared by reducing the decavanadate with ascorbic acid either in the reaction vessel or separately. Vanadyl sulfate was dissolved in water.

Polylysine, mol. wt 35 000 was obtained from Sigma, DBMIB was a gift from Dr A. Trebst. Vanadium pentoxide and vanadyl sulfate were purchased from K & K Laboratories.

RESULTS

Vanadyl sulfate is remarkably selective as a donor to Photosystem II as shown by complete inhibition of the vanadyl → methyl viologen reaction by DCMU. This reaction is also almost completely inhibited by DBMIB (Table I). This reaction has a pH optimum at pH 6.5.

The vanadyl reaction is similar to the oxidation of moderately low concentra-

TABLE I

VANADIUM COMPOUNDS AND FERROCYANIDE AS ELECTRON DONORS IN SPINACH CHLOROPLASTS

The vanadyl→methyl viologen reaction contained (2 ml): chloroplasts (50 μg chlorophyll), 100 μmol tris (hydroxymethyl)aminomethane-MES, pH 7, 20 μmol $MgCl_2$, 8 μmol NH_4Cl, 1.6 μmol methyl viologen and 40 mmol vanadyl sulfate. The ferrocyanide→methyl viologen reaction contained all of the reactants above except 0.032 M ferrocyanide in place of vanadyl.

Reaction	Additions	Acceptor reduced (μequiv/mg chlorophyll/h)	Inhibition (%)
H_2O→methyl viologen	None	558	0
in 0.8 M Tris-treated chloroplasts	None	0	100
Vanadyl→methyl viologen			
in Tris-treated chloroplasts	None	248	–
	DCMU (3μg/ml)	0	100
	DBMIB (0.04 μg/ml)	248	0
	Polylysine (35 000 mol.wt) (0.2 mg/ml)	181	27
Ferrocyanide (0.032 M)→methyl			
viologen in Tris-treated chloroplasts	None	428	–
	DCMU (3μg/ml)	248	42
	DBMIB (0.04 μg/ml)	338	21
	Polylysine (0.2 mg/ml)	558	0

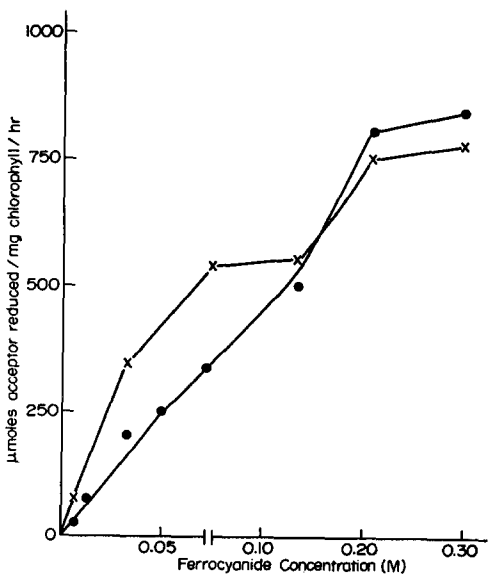

Fig. 1. The effect of various concentrations of ferrocyanide on the ferrocyanide oxidation assay by Photosystem I in presence or absence of DCMU. The reaction mixture contained (4 ml vol.): chloroplasts with 100 μg chlorophyll, 200 μmol sodium ascorbate, 1.6 μmol methyl viologen, 600 μmol tris(hydroxylmethyl)amino methane-MES, pH 8, 1.2 μmol DCMU, and varying concentrations of ferrocyanide. ×, ferrocyanide; ●, ferrocyanide with DCMU.

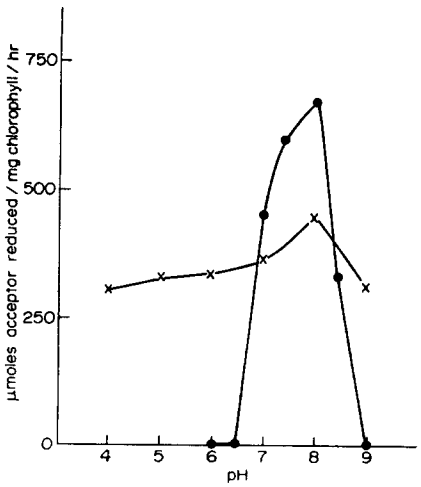

Fig. 2. The effect of pH on the ascorbate plus decavanadate or ferrocyanide→methyl viologen reaction. Reaction conditions as in Fig. 1 except pH of buffer varied. 0.3 M ferrocyanide and 0.04 M vanadite were used. ●, vanadite; ×, ferrocyanide.

tions of ferrocyanide (0.03 M) which also donates electrons to Photosystem II as previously described [4] except that vanadyl appears to be a more specific donor for Photosystem II on the basis of relative DCMU inhibition.

With increasing concentrations of ferrocyanide there is an increasing tendency

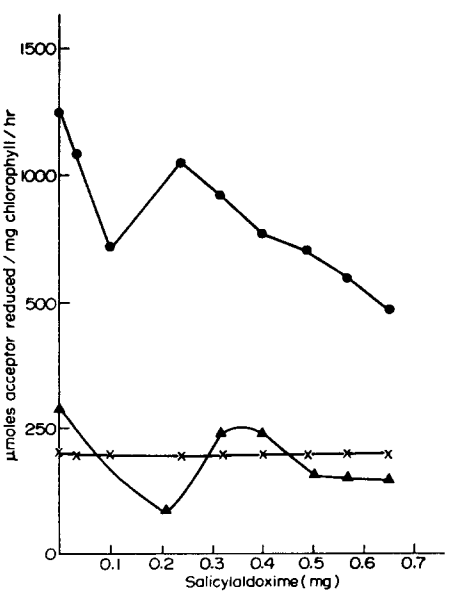

Fig. 3. The effect of salicylaldoxime on TMPD, ferrocyanide or vanadite oxidation by Photosystem I. Reaction conditions as in Fig. 1; salicylaldoxime added in concentrations indicated. For the TMPD reaction, 0.1 mg TMPD was added; ferrocyanide and vanadite concentrations were 0.3 M and 0.04 M respectively. ●, TMPD; ×, ferrocyanide; ▲, vanadite.

TABLE II

FACTORS AFFECTING VANADITE AND FERROCYANIDE OXIDATION BY PHOTOSYSTEM I IN SPINACH CHLOROPLASTS

The ascorbate+decavanadate→methyl viologen reaction contained (4 ml): chloroplasts with 100 μg chlorophyll, 200 μmol sodium ascorbate, 1.6 μmol methyl viologen, 600 μmol tris (hydroxymethyl) aminomethane-MES, pH 8, 1.2 μmol DCMU and 0.04 M decavanadate. The ascorbate+ferrocyanide reaction contained all reactants as above except 0.32 M ferrocyanide in place of decavanadate. The ascorbate+TMPD reaction contained all reactants as above except 0.1 mg TMPD in place of decavanadate or ferrocyanide. Saturating levels of plastocyanin were added as indicated.

Reaction	Additions*	O_2 uptake (μequiv/mg chlorophyll/h)	Inhibition (%)
Ascorbate+decavanadate →methyl viologen	None	255	0
	DBMIB (0.04 μg/ml)	265	0
	After extraction with 1 % Tween 20	0	100
	Tween 20 and plastocyanin	255	0
	Polylysine (M.W. 35 000) (0.1 mg/ml)	113	56
	Polylysine (0.2 mg/ml)	56	76
	Orthophenanthroline (0.05 mg)	204	20
	Bathophenanthroline (0.05 mg)	56	78
	After 5 min. incubation with dithizone (0.2 mg/mg chlorophyll)	306	0
	Salicylaldoxime (0.2 mg)	85	67
Ascorbate+Ferrocyanide (0.32 M)→methyl viologen	None	819	0
	DMBIB (0.04 μg/ml)	1326	0
	After extraction with 1 % Tween 20	480	41
	After extraction with 1 % Tween 20 plus plastocyanin	480	41
	After extraction with 1 % Tween 20 plus 0.4 M NaCl fr. from DEAE	987	0
	Polylysine (0.1 mg/ml)	1410	0
	After 5 min. incubation with dithizone (0.2 mg/mg chlorophyll)	789	4
Ascorbate+TMPD→methyl viologen	None	1578	0
	DBMIB (0.04 μg/ml)	2115	0
Ascorbate+TMPD →methyl viologen	After extraction with 1 % Tween 20	168	89
	After extraction with 1 % Tween 20 plus saturating plastocyanin	1518	0
	Polylysine (0.2 mg/ml)	282	88
	After 5 min incubation with dithizone (0.2 mg/mg chlorophyll)	1665	0
	Orthophenanthroline (0.05 mg)	1692	0
	Bathophenanthroline (0.05 mg)	1974	0
	Salicylaldoxime (0.2 mg)	1665	0

* Additions/ml of reaction mixture where indicated; otherwise total amount reactant added.

to donate electrons to Photosystem I as evidenced by decreasing DCMU inhibition (Fig. 1). Maximum rates are obtained at the highest concentrations of ferrocyanide which are limited to 0.32 M by solubility of ferrocyanide in water. This reaction has a pH optimum at 8.0 (Fig. 2). High concentrations of vanadyl (0.1 M) do not show a DCMU-insensitive reaction because of vanadium toxicity.

This oxidation of ferrocyanide through Photosystem I is unusual in that it is not inhibited by inhibitors of plastocyanin function and is restored by a soluble protein but not by plastocyanin after plastocyanin extraction. Polylysine, at concentrations which completely inhibit the ascorbate plus TMPD → methyl viologen reaction, stimulates the Photosystem I ferrocyanide oxidation (Table II). Dithizone which gives 30 % inhibition [5] and salicylaldoxime which causes up to 60 % inhibition of the ascorbate plus TMPD → methyl viologen reaction have very little effect on the oxidation of ferrocyanide through Photosystem I (Fig. 3).

With short-term incubation (up to 3–5 min), low levels of bathophenanthroline (1–2 μg/ml) stimulate both ferrocyanide and ascorbate-TMPD oxidation through Photosystem I (Fig. 4). After longer incubation (more than 15 min) ascorbate-TMPD oxidation is inhibited whereas ferrocyanide oxidation retains the stimulation. The inhibition of ascorbate-TMPD oxidation is reversed by added plastocyanin [3]. Higher concentrations of bathophenanthroline (1 mg/ml) inhibit both reactions with 3–5 min incubation.

Extraction of chloroplasts with 1 % Tween-20 causes complete loss of ascorbate-TMPD → methyl viologen and about a 50 % loss of ferrocyanide → methyl viologen activity (Table II). The ascorbate-TMPD reaction is completely restored by plastocyanin whereas addition of plastocyanin has no effect on the ferrocyanide

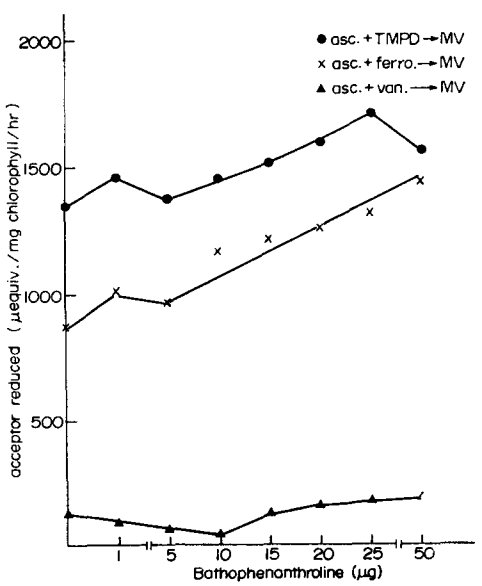

Fig. 4. The effect of bathophenanthroline on TMPD, ferrocyanide or vanadite oxidation by Photosystem I. Reaction conditions as in Fig. 1; bathophenanthroline added in concentrations indicated. For the TMPD reaction 0.1 mg TMPD was added to the reaction mixture. Ferrocyanide concentration was 0.3 M; vanadite, 0.04 M. ●, TMPD; ×, ferrocyanide; ▲, vanadite.

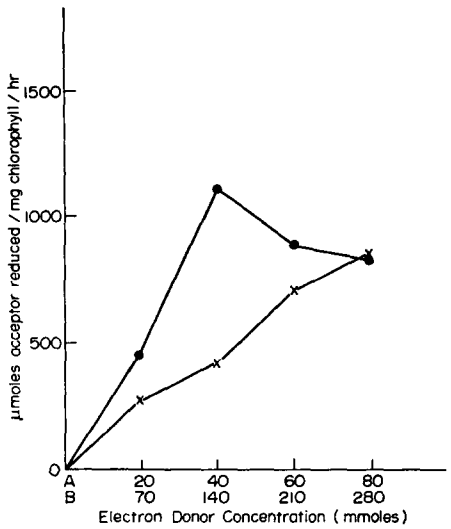

Fig. 5. The effect of various concentrations of vanadite and ferrocyanide as electron donors to Photosystem I. Reaction mixture as in Fig. 1. Scale A, mmol vanadite added; Scale B, mmol ferrocyanide added. ●, vanadite; ×, ferrocyanide.

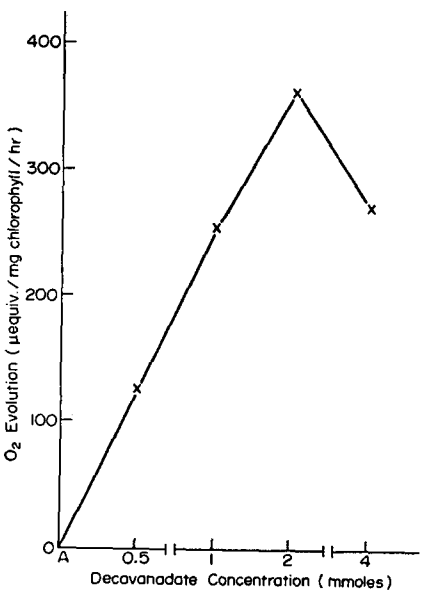

Fig. 6. The effect of various concentrations of decavanadate on oxygen evolution by spinach chloroplasts. The reaction mixture contained (2 ml volume); chloroplasts with 50 μg chlorophyll, 100 μmol tris(hydroxymethyl)aminomethane-MES, pH 7, 20 μmol $MgCl_2$, 8 μmol NH_4Cl and varying amounts of decavanadate. ×, $H_2O \rightarrow$decanavadate.

oxidation. A brown protein which is eluted from a DEAE-cellulose column after plastocyanin (0.4 M NaCl) restores the ferrocyanide oxidation activity but does not restore ascorbate-TMPD activity. Ferredoxin does not restore the ferrocyanide oxidation.

When decavanadate is reduced by ascorbate or dithiothreitol to vanadite, it acts as an electron donor to Photosystem I (Fig. 5). The oxidation through methyl viologen is not inhibited by DCMU or DBMIB. The pH optimum for this reaction is at 8.0, and only a narrow range of pH is tolerated in contrast to ferrocyanide oxidation (Fig. 2).

The response of vanadite oxidation to inhibition by salicylaldoxime (Fig. 3) or polylysine and to plastocyanin extraction is similar to the response of the ascorbate plus TMPD → methyl viologen reaction (Table II). Activity is completely lost after

TABLE III

A COMPARISON OF THE EFFECT OF VARIOUS INHIBITORS ON DECAVANADATE, FERRICYANIDE AND METHYL VIOLOGEN REDUCTION IN SPINACH CHLOROPLASTS

The H_2O→decavanadate reaction contained (2 ml): chloroplasts (50 μg chlorophyll), 100 μmol tris(hydroxymethyl)aminomethane-MES pH 7, 20 μmol $MgCl_2$, 8 μmol NH_4Cl and 1–2 mmol decavanadate. The H_2O→ferricyanide reaction contained 2.5 mmol ferricyanide in place of decavanadate, and the H_2O→methyl viologen reaction 1.6 μmol methyl viologen. Saturating amounts of plastocyanin, added where indicated, usually contained 0.5 μmol phosphatidylcholine.

Reaction	Additions	O_2 evolution or uptake (μequiv/mg chlorophyll/h)	Inhibition (%)
H_2O→decavanadate	None	530	0
	DCMU (3 μg/ml)	0	100
	DBMIB (0.04 μg/ml)	45	91
	DBMIB (4 μg/ml)	59	89
	After extraction with 1 % Tween 20	11	98
	After extraction with 1 % Tween 20 plus saturating plastocyanin	418	20
	Polylysine (mol.wt 35 000) (0.1 mg/ml)	0	100
H_2O→ferricyanide	None	310	0
	DCMU (3 μg/ml)	0	100
	DBMIB (0.04 μg/ml)	113	64
	After extraction with 1 % Tween 20	212	32
	After extraction with 1 % Tween 20 plus saturating plastocyanin	332	0
	Polylysine (0.1 mg/ml)	400	0
H_2O→methyl viologen	None	750	0
	DCMU (3 μg/ml)	45	94
	DBMIB (0.04 μg/ml)	192	75
	DBMIB (4 μg/ml)	79	90
	After extraction with 1 % Tween 20	11	99
	After extraction with 1 % Tween 20 plus saturating plastocyanin	322	57
	Polylysine (0.1 mg/ml)	34	95

Tween extraction and is restored by plastocyanin. The response of vanadite oxidation to dithizone differs from TMPD in that no inhibition is observed (Table II). The response to bathophenanthroline differs in that vanadite oxidation is inhibited at low concentrations (3–5 min incubation) whereas both TMPD and ferrocyanide oxidations are stimulated under these conditions (Fig. 4).

Overall, it would appear that TMPD and vanadite oxidation by Photosystem I requires plastocyanin but that ferrocyanide oxidation does not.

Ferricyanide and methyl viologen are well known acceptors for water oxidation through Photosystems I and II. Decavanadate (vanadium V) also serves as an acceptor in this type of reaction (Fig. 6). The reduction of decavanadate is 90–100 % inhibited by DCMU. The small DCMU-insensitive reaction may indicate reduction in Photosystem II similar to that seen with other large anions such as the heteropoly molybdates and tungstates [3]. The inhibition of decavanadate reduction by DBMIB (60–90 %) is similar in extent to inhibition of ferricyanide reduction.

The reduction of decavanadate and ferricyanide differ in the response to Tween extraction and polylysine inhibition (Table III). Tween extraction completely inhibits decavanadate reduction; activity is not restored by addition of plastocyanin. The reaction $H_2O \rightarrow$ methyl viologen shows a similar Tween effect. On the other hand, ferricyanide reduction is not inhibited by Tween extraction and shows little increase in activity when plastocyanin is added. Polylysine gives a strong inhibition of both decavanadate and methyl viologen reduction but causes no inhibition of ferricyanide reduction. The strong inhibition of decavanadate reduction by agents which affect plastocyanin indicates that this reagent reacts at or after the site of plastocyanin.

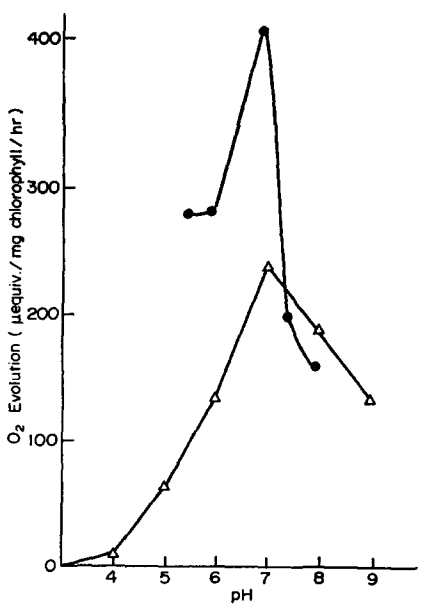

Fig. 7. The effect of pH on the $H_2O \rightarrow$decavanadate (△) and $H_2O \rightarrow$ferricyanide reaction (●). Reaction conditions as in Fig. 5 except pH of the buffer varied. 2 mM decavanadate and 0.032 M ferrocyanide were used.

Both ferricyanide and decavanadate reduction show a pH optimum at 7 (Fig. 7).

DISCUSSION

It is apparent that ions of vanadium IV and V can be as useful and as specific as ferricyanide in the study of photosynthetic electron transport. We feel that ionic reagents of this type will be important in determining the exposure and orientation of redox carriers in the thylakoid membrane.

As shown by Izawa and Ort [4], moderately low concentrations of ferrocyanide (0.03 M) donate electrons to Photosystem II. Vanadyl ions also show a rather specific donation at this region. In contrast to ferrocyanide, the oxidation of vanadyl leads to the generation of a large amount of protons.

$$VO^{2+} + 2H_2O \rightarrow VO_3^{-1} + 4H^+ + e^-$$

This reaction should, therefore, be of interest in the study of photophorphorylation. Since the vanadyl ion is relatively large, positively charged, insoluble in butanol and is not oxidized in mitochondria without addition of detergent [11] it is reasonable to propose that it reacts with an exposed site on the oxidizing side of Photosystem II. Exposure of a portion of Photosystem II on the outer surface of the thalakoid membrane has been shown by labeling with impermeable probes [12, 13].

The oxidation of vanadite through Photosystem I resembles the oxidation of TMPD in most respects. Polylysine [9] which appears to inhibit plastocyanin inhibits each of these reactions. Salicylaldoxime, which Trebst [14] has proposed to inhibit plastocyanin function, also inhibits these reactions with a characteristic concentration dependence that appears to involve a stimulation effect superimposed on an inhibitory effect. Salicylaldoxime has also been shown to inhibit on the oxidizing side of Photosystem II [15–18]. We have recently observed inhibition of the DCMU-insensitive water → silicomolybdate reaction by salicylaldoxime [19]. The lack of DCMU inhibition of vanadite oxidation would appear to exclude the Photosystem II site as the basis for salicylaldoxime inhibition in sucrose-sodium chloride chloroplasts.

Elstner et al. [20] have shown that detergents can be used to selectively extract plastocyanin. After Tween extraction we find that both vanadite and methyl viologen oxidation by Photosystem I is inhibited and that the activity can be restored by plastocyanin. Vanadite oxidation, therefore, takes place after plastoquinone and before or at plastocyanin. The inhibition of vanadite oxidation by very low concentrations of bathophenanthroline contrasts with the consistent stimulation of TMPD oxidation. This suggests that there is a site which is not involved in TMPD oxidation but is involved in vanadite oxidation. We have previously observed a site of this type in the oxidation of diaminodurene by Photosystem I [19]. Since vanadite ions are not soluble in butanol and require detergent to be oxidized in mitochondria it is again logical to suggest that the site of vanadite oxidation should be on the exterior of the thalakoid.

The oxidation of ferrocyanide at high concentrations through Photosystem I shows remarkably different features to vanadite or TMPD oxidation. The inhibitor and Tween extraction studies indicate no requirement for plastocyanin. It is evident that a part of the oxidation after Tween extraction may proceed by direct interaction

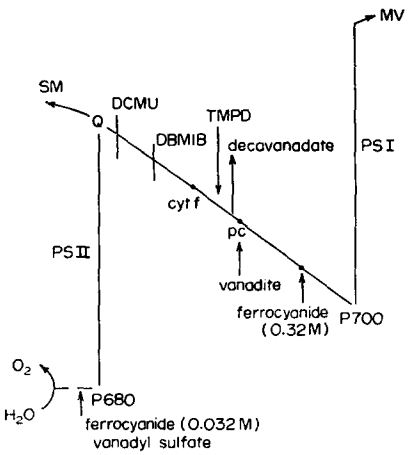

Fig. 8. The Z-scheme of photosynthetic electron transport with possible ferrocyanide and vanadium reaction sites indicated.

with *P*-700. The restoration of additional activity by the brown protein fraction indicates that an additional component is required for ferrocyanide oxidation which does not enhance the oxidation of the other donors in Tween-treated chloroplasts. The active component resembles a compound which we have previously described as rubimedin [21]. Recently, Malkin and Aparicio [22] have reported the presence of a high potential iron sulfur protein in chloroplasts. Haehnel [23] has proposed a branched pathway to *P*-700 through plastocyanin and cytochrome *f*. It is possible that our results could be explained by oxidation of vanadite through the plastocyanin pathway and ferrocyanide through the cytochrome *f* pathway.

Differences are also seen in the reduction of vanadate and ferricyanide by the photosynthetic electron transport chain. Both of these ions accept electrons primarily after the DBMIB inhibition site although decavanadate shows somewhat stronger DBMIB inhibition. The Tween extraction studies and polylysine inhibition indicates that ferricyanide reduction occurs before the plastocyanin site whereas decavanadate is reduced at or after plastocyanin (Fig. 8). Reduction of ferricyanide at cytochrome *f* would be consistent with the spectrophotometric studies of Horton and Cramer [24].

ACKNOWLEDGEMENTS

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