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Relationship of Transplasmalemma Redox Activity to Proton and Solute Transport by Roots of *Zea mays*[1]

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ABSTRACT

Transplasmalemma redox activity, monitored in the presence of exogenous ferricyanide stimulates net H^+ excretion and inhibits the uptake of K^+ and α-aminoisobutyric acid by freshly cut or washed, apical and subapical root segments of corn (*Zea mays* L. cv "Seneca Chief"). H^+ excretion is seen only following a lag of about 5 minutes after ferricyanide addition, even though the reduction of ferricyanide occurs before 5 minutes and continues linearly. Once detected, the enhanced rate of H^+ excretion is retarded by the ATPase inhibitors *N,N'*-dicyclohexylcarbodiimide, diethylstilbestrol, and vanadate. A model is presented in which plasmalemma redox activity in the presence of ferricyanide involves the transport only of electrons across the plasmalemma, resulting in a depolarization of the membrane potential and activation of an H^+-ATPase. Such a model implies that this class of redox activity does not provide an additional and independent pathway for H^+ transport, but that the activity may be an important regulator of H^+ excretion. The 90% inhibition of K^+ ($^{86}Rb^+$) uptake within 2 minutes after ferricyanide addition can be contrasted with the 5 to 15% inhibition of uptake of α-aminoisobutyric acid. The possibility exists that a portion of the K^+ and most of the α-aminoisobutyric acid uptake inhibitions are related to the ferricyanide-induced depolarization of the membrane potential, but that the redox state of some component of the K^+ uptake system may also regulate K^+ fluxes.

The presence of redox activity at the surface of plant and animal cells (6, 10) provides an additional mechanism to ATPases for controlling solute fluxes across the plasmalemma. One class of redox activity detected in plants involves the transfer of reducing equivalents across the plasmalemma from a cytosolic donor such as NADH (4) or NADPH (21, 28) to ferricyanide, a nonpermeating oxidant (5, 21). Accompanying this reductase activity is an apparent excretion of H^+, an inhibition of K^+ uptake, and a stimulation of K^+ efflux (5, 14, 17, 27). Because of the central role played by H^+ and K^+ in cell metabolism and solute transport, we have been evaluating various models which attempt to determine the linkage between H^+ and K^+ fluxes and transplasmalemma redox activity.

In one model, the redox reactions occurring in the presence of external ferricyanide are thought to provide a pathway in addition to an H^+-ATPase for H^+ transport (14, 27). Thus, the oxidation of NADPH would result in the transfer of both H^+ and electrons across the plasma membrane, the electrons reducing the ferricyanide and H^+ accumulating in the medium. If the stoichiometry of ferricyanide reduced to H^+ excreted were greater than 1, a depolarization of the membrane potential would result, which in turn could inhibit K^+ uptake and stimulate efflux. Depolarizations during reduction of external iron have already been reported (14, 27).

Redox activity and H^+ transport, according to this model, are stoichiometrically linked, so the ratio of ferricyanide reduced to H^+ exported must be a constant. We will show, however, that this ratio can vary. Furthermore, if redox activity provides a separate and independent pathway for H^+ as the model predicts, ferricyanide-induced H^+ excretion should not be sensitive to inhibitors of ATPases. The data to be presented will indicate that ATPase inhibitors are in fact quite effective.

What is more, we observe a very rapid and marked inhibition of K^+ uptake by ferricyanide, but only a small inhibition of α-aminoisobutyric acid uptake. This may indicate that redox activity interacts with K^+ transport in two different ways, *i.e.* directly with the K^+ transport mechanism as well as indirectly via a depolarization of the Em.[2] In addition, we report the rates of ferricyanide reductase activity of apical *versus* subapical segments and of freshly cut *versus* washed segments, and discuss the relationship between these various rates to the fluxes of H^+, K^+, and AIB.

MATERIALS AND METHODS

Tissue Preparation. Caryopses of *Zea mays* L. cv "Seneca Chief" (Charles Hart Seed Co.) were germinated on tap water-moistened vermiculite. After 2 d in complete darkness, 2 to 5 cm long roots were selected, the vermiculite was removed in aerated, distilled H_2O, and either the apical 5 mm or a subapical segment 5 to 10 mm from the tip was excised. The segments were rinsed for 5 to 15 min in distilled H_2O and then transferred to a buffered preincubation medium. The washing procedure effectively removed any reducing agents leaking from the cells (21). After cutting, and throughout the experiments, the segments were either shaken at 125 rpm on an orbital shaker or bubbled with air. Preliminary experiments determined that aeration by shaking or air bubbling gave equivalent results.

Assay of Ferricyanide Reduction. Fifteen washed 5-mm segments were preincubated at room temperature in 2 ml of 1 mM Tris-Mes (pH 6.1), 1 mM KCl, 1 mM $CaCl_2$, 2.4 mM NaCl, for the time desired. The tissue was transferred to 1.5 ml of fresh medium now also containing 0.8 mM $Na_3Fe(CN)_6$, the $CaCl_2$ was increased to 3 mM to stimulate the reaction rate (11, 25), and the NaCl was eliminated so that the Na^+ concentration remained at 2.4 mM. After the desired time period, the incubation medium was removed, and the quantity of ferrocyanide produced was determined by the colorimetric assay of Avron and

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[2] Abbreviations: Em, membrane potential; AIB, α-aminoisobutyric acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; FC, fusicoccin; p.m.f., protonmotive force; C_o, C_i, external and internal concentration; J_o, J_i, rate of efflux and influx.

Shavit (1).

Medium Acidification. Forty root segments were preincubated in 2 ml of an aerated solution containing the medium detailed above, but without NaCl. After the desired period of washing, the segments were transferred to a glass tube (17 mm i.d. × 40 mm high) containing 3 ml of an aerated solution of 0.5 mM Tris-Mes (pH 6.1), 1 mM KCl, and 3 mM $CaCl_2$ at room temperature. The pH was monitored continuously with a Radiometer combination electrode and a Beckman Expandomatic pH meter connected to an Esterline Angus recorder through a low pass R-C filter (time constant = 0.25 ms) to reduce noise. The pH was adjusted with 5 mM NaOH to 6.3, a baseline was established (usually for about 5–10 min), and $Na_3Fe(CN)_6$ added to a final concentration of 0.8 mM. Concentrations and times of addition of other substances are indicated in the data presented. Additions of only NaCl to give a final concentration of 2.4 mM had no effect on rates of acidification.

For experiments in which ferricyanide reduction was measured along with pH, the acidification medium was removed for assay 20 min after addition of ferricyanide, or, for the experiments presented in Figure 2, after 5 min intervals. The ATPase inhibitors DCCD and DES were added as 100% ethanol solutions to a final ethanol concentration of 1% (v/v); ethanol alone did not inhibit acidification. Sodium orthovanadate was prepared and quantified as reported by O'Neill and Spanswick (20).

Isotope Uptake. Fifteen root segments were washed in water for 5 to 15 min, preincubated in 2 ml of a solution of 10 mM Tris-Mes (pH 6.1), 0.2 mM KCl, 1 mM $CaCl_2$, and 2.4 mM NaCl for the desired length of time, then transferred to 2 ml of an identical solution now containing 3 mM $CaCl_2$ and approximately 1 μM $^{86}Rb^+$ (New England Nuclear, 4–6 × 10^5 cpm/ml) with or without 0.8 mM $Na_3Fe(CN)_6$. The validity of Rb^+ as an analog for K^+ transport in corn roots has been shown previously (16). When ferricyanide was added, the NaCl was eliminated so that all treatments contained equimolar Na^+. The buffer strength was increased to 10 mM compared to the 1 mM used in other experiments to minimize the possibility of indirect changes due to pH. The K^+ concentration chosen was in the range of the saturable component in which ferricyanide has been shown to be most effective (17).

Following the uptake period at room temperature (usually 10 min), the segments were washed by shaking at 4°C in a solution of 1 mM Tris-Mes (pH 6.1), 15 mM KCl, 1 mM $CaCl_2$, and 2.4 mM NaCl for 5 to 10 min to remove most of the counts from the free space. Preliminary experiments showed that the wash treatment in the cold did not appear to cause a loss of accumulated radioactivity from within the cells, a conclusion also reached by others (16). After the wash, the sections were placed in 4.5 ml of Scintisol (Interex Corp.) for extraction and counting.

For uptake of AIB, the preincubation medium contained 10 mM Tris-Mes (pH 6.1), 1 mM $CaCl_2$, 2 mM KCl, and 2.4 mM NaCl. The uptake medium had the identical composition except that the $CaCl_2$ concentration was raised to 3 mM and 1.2 × 10^6 dpm/ml [^{3}H]AIB (10 Ci/mmol, ICN Pharmaceuticals) plus unlabeled AIB were added to make the final amino acid concentration 50 μM. When required, $Na_3Fe(CN)_6$ was added to 0.8 mM. After a 10 to 15 min uptake period, the segments were washed for 10 min in an ice cold solution identical in composition to that used for uptake except that [^{3}H]AIB was omitted. Segments were extracted and counted as above.

RESULTS

The stimulation of apparent H^+ excretion by ferricyanide (approximately 4.5 μmol g^{-1} fresh weight h^{-1}) is recorded in the upper trace of Figure 1. This change in pH is not due primarily to CO_2 emitted since purging the solution with N_2 after removal of the segments does not substantially change the pH. A titration

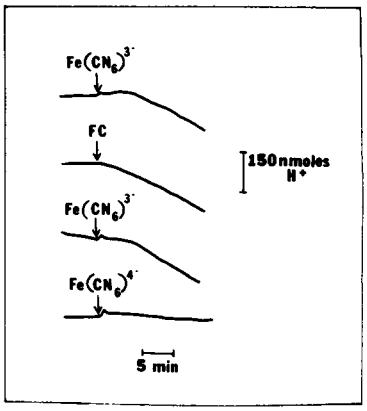

FIG. 1. Acidification by 40, 5 mm apical root segments of an aerated medium. The segments were washed for at least 2 h and solutions added at arrows. Final concentrations were: $Na_3Fe(CN)_6$ and $Na_4Fe(CN)_6$, 0.8 mM; FC, 2 μM. The first, second, and fourth traces were begun at pH 6.3; the third trace was begun at pH 6.7.

curve of this solution from pH 4.0 to 7.0 indicates that the reduction in pH is not due to leakage of organic acid. There is about a 5 min lag before a steady state rate of acidification is observed, however. This lag does not seem to be due to problems related to the detection of H^+ for two reasons. First, a lag of only about 1 min is seen after addition of FC at a concentration which causes an acidification rate similar to that of ferricyanide (Fig. 1, second trace). Second, if the incubation medium is adjusted to pH 6.7 instead of 6.3 (Fig. 1, third trace), a slow rate of acidification occurs, but even under these conditions there is a 5 min or so lag until a new rate of apparent H^+ excretion is established.

Also shown in Figure 1 (fourth trace), is that ferrocyanide has little effect on acidification of the medium. Thus, the process of reduction is required for a stimulation of H^+ excretion, not just the presence of an $Fe(CN)_6$ complex. Furthermore, the inactivity of the Na salt of ferrocyanide illustrates that the action of Na ferricyanide is not related to the presence of Na in combination with an immobile anion. In contrast to previous reports (*e.g.* 17), net H^+ efflux is not detectable until after addition of ferricyanide. This is probably due to our use of 5 mm tips rather than subapical segments, to differences in the cultivar of *Zea mays*, and in the composition of the incubation medium.

A comparison of the rates of ferricyanide reduction and apparent H^+ excretion is shown in Figure 2. The 5 min lag before detection of acidification can be contrasted with the kinetics of redox activity, where reduction of ferricyanide occurs within 5 min (Fig. 2A). Other experiments (not shown) indicate that reduction occurs within 2 min. Thus, the stoichiometry of ferricyanide reduced to H^+ excreted changes markedly over the course of the experiment (Fig. 2B). A steady state ratio usually between 1.3 and 2.1 is attained only after 10 min. This ratio can be compared with the 2.5 reported previously for subapical corn root segments (15) and about 2.0 for iron-depleted bean roots (27). In the latter cases, detailed time-course curves for H^+ transport were not presented. A ratio of 1.0 occurs with *Elodea* leaves (14).

With corn roots, no changes in this ratio at steady state acidification beyond the range just mentioned are seen using freshly cut *versus* washed tissue, or subapical *versus* apical segments. The ratio also remained constant after incubating segments in buffers of various molarities (0.25–1.5 mM), in various ferricyanide concentrations (0.1–0.8 mM), or by changing the tissue to volume ratio; and in all cases a lag period was clearly observed, except for freshly cut segments where acidification began immediately after ferricyanide addition in 3 of 13 trials (data not shown).

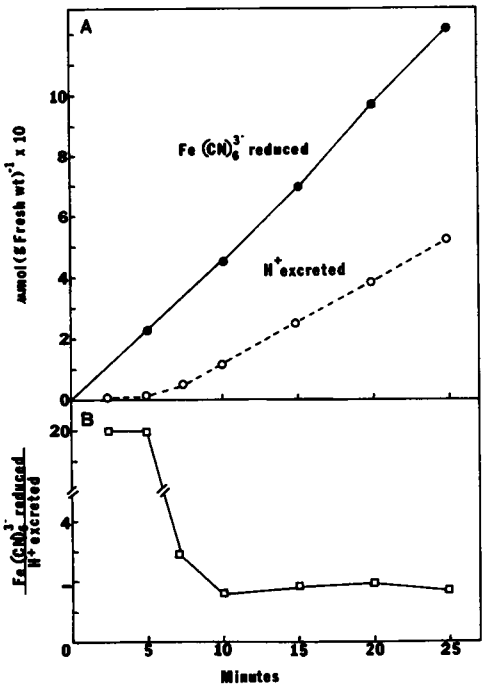

FIG. 2. A, Comparison of ferricyanide reduction and net H^+ excretion by washed, 5 mm apical root segments (initial pH = 6.3). Ferricyanide reduction (initial concentration = 0.8 mM) was determined by removing the medium every 5 min and replacing with fresh medium. H^+ excretion was calculated from a recording similar to that shown by the first trace in Figure 1. B, Ratios of ferricyanide reduced to H^+ excreted were calculated from the data in (A).

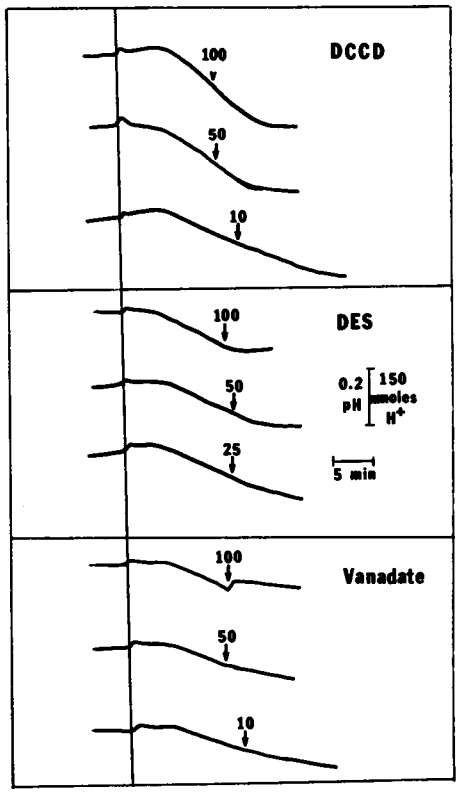

FIG. 3. Effects of DCCD, DES, and sodium orthovanadate on ferricyanide-induced net H^+ excretion. Ferricyanide was added to a concentration of 0.8 mM to washed, 5 mm apical root segments at the vertical line. The inhibitors were added at the arrows; numbers on arrows represent final concentrations in μM. Final ethanol concentration for DCCD and DES was 1% (v/v).

However, the large change in ratio of ferricyanide reduced to H^+ excreted during the 1 to 10 min period after ferricyanide is added (Fig. 2B), leads to the possibility that this class of redox activity involves only electron transport across the plasma membrane and does not provide a unique pathway for H^+ excretion. To determine the extent to which H^+ excretion during redox activity is dependent on a H^+-ATPase, various concentrations of the ATPase inhibitors, DCCD, DES, and vanadate, were added during ferricyanide-induced H^+ excretion (Fig. 3). DCCD inhibits this acidification completely after 5 min at both 100 and 50 μM, while 10 μM DCCD inhibits 40% after a 10 min lag. A lag of only about 1 min is seen before inhibitions by DES; acidification is inhibited 100% after 5 min with 100 μM DES, 80% by 50 μM DES, and 50% by 25 μM. The final ethanol concentration with either DCCD or DES was 1% (v/v), which by itself did not inhibit net H^+ excretion.

Vanadate inhibits acidification of the medium after only a very short lag period (Fig. 3), although the inhibitions are less complete than with the other drugs—75% by 100 μM vanadate, 50% by 50 μM vanadate, and 25% by 10 μM. Little or no net H^+ excretion is observed if the inhibitors (at 100 μM) are added at the same time as the ferricyanide (data not shown).

To examine the possibility that the inhibitors were preventing ferricyanide-induced acidification merely by interfering with the reduction of ferricyanide, the latter parameter was measured using identical inhibitor concentrations (Fig. 4). DES is without detectable effects on ferricyanide reduction at the concentrations and time periods used. DCCD at 50 and 100 μM inhibits about 50% of the redox activity by 15 min and the effects of vanadate are most striking at 100 μM when an inhibition of 40% occurs after 5 min. It seems clear, however, that at these concentrations and time periods, the inhibitors are much less effective at inhibiting ferricyanide reduction than they are at preventing the apparent excretion of H^+. For example, 5 to 10 min after addition of the inhibitors at 50 μM, ratios of ferricyanide reduced to H^+ excreted are 17, 103, and 4 for DCCD, DES, and vanadate, respectively.

The transport of electrons unaccompanied by H^+ which appears to occur during the first 5 min after addition of ferricyanide, could lead to conditions which directly or indirectly affect solute fluxes. Kochian and Lucas (17) report that ferricyanide markedly inhibits K^+ uptake after 15 min, and we show here (Fig. 5) that an inhibition of 90% is seen within 5 min; uptake rates remain inhibited to that degree as long as the ferricyanide is present, and in other experiments (not shown) inhibitions up to 80% occur within 1 min. When ferricyanide is removed, rates of K^+ uptake recover to 50% of the rate of the controls by 2 min and are 80% of controls by 10 min. Extrapolation to 0-time of the lines connecting the points in Figure 5 suggests that not all of the apoplastic counts are removed by the washing procedure. Thus, the degree of inhibition of uptake by ferricyanide may be even greater.

In Figure 6 it can be seen that the retardation of K^+ uptake is dependent on the concentration of ferricyanide in the medium; a 50% inhibition is seen at about 80 μM ferricyanide. The shape of the concentration curve and the point of half-maximal activity is similar to that obtained when the rate of ferricyanide reduction is measured (21). Also shown in Figure 6 is that the inhibition of K^+ uptake is related to ferricyanide-induced transmembrane redox activity, since additions of ferrocyanide are ineffective. The small inhibition of K^+ uptake seen at 0.8 mM ferrocyanide is probably due to redox activity catalyzed by ferricyanide which was produced from ferrocyanide by an oxidase at the cell surface (21). No inhibitions of K^+ uptake are seen if 250 μg/ml oxidized Cyt *c* is added instead of ferricyanide; no Cyt *c* reduction could

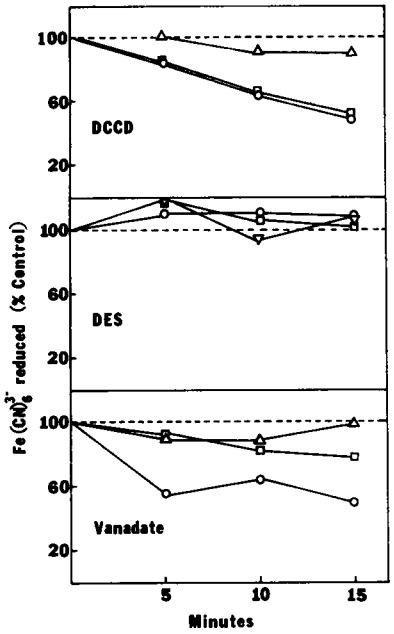

FIG. 4. Effects of DCCD, DES, and sodium orthovanadate on ferricyanide reduction. Washed, 5 mm apical root segments were incubated in a medium containing 0.8 mM ferricyanide with or without various concentrations of the inhibitors. (○), 100 μM; (□), 50 μM; (▽), 25 μM; (△), 10 μM. The medium was removed every 5 min for determination of ferrocyanide produced and was replaced with fresh medium. Average values for controls without inhibitors was 4.75 μmol ferricyanide reduced/g fresh weight·h. Replicates of the mean of 3 or more experiments are presented. SE was usually 10% or less of the mean.

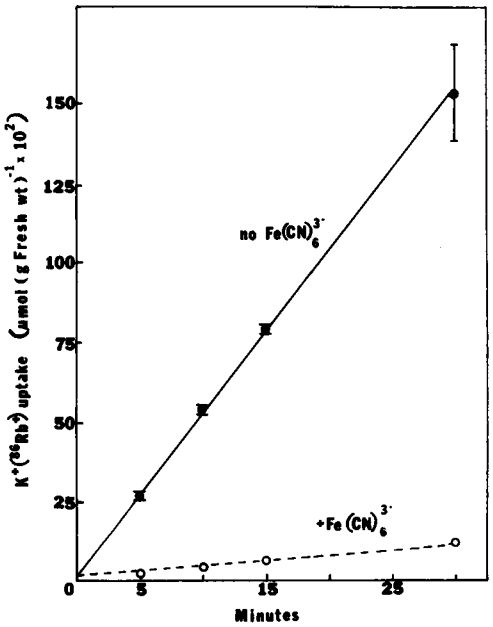

FIG. 5. Time course of K^+ ($^{86}Rb^+$) uptake into 5 mm apical root segments washed for 1 h in the presence or absence of 0.8 mM ferricyanide. All treatments contained 0.2 mM KCl and 2.4 mM Na^+. Bars represent SE of the mean; where not shown, bars did not extend beyond the symbol.

be detected with corn root segments (21).

The inhibitory effects of ferricyanide on K^+ uptake were further investigated using root tissues with different capabilities for ferricyanide reductase activity and for solute uptake. The

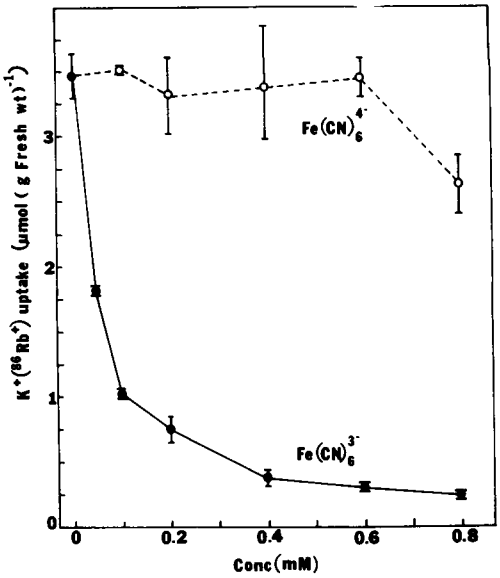

FIG. 6. Comparison of $Na_3Fe(CN)_6$ and $Na_4Fe(CN)_6$ on K^+ ($^{86}Rb^+$) uptake into 5 mm apical root segments washed for 1 h. The uptake media were adjusted with NaCl so that all solutions contained 3.2 mM Na^+. Final K^+ concentration was 0.2 mM. Bars represent SE of the mean.

well-documented stimulation of K^+ influx after washing can be readily observed (Table I, column 2). Freshly cut apical segments show higher rates of K^+ uptake than freshly cut subapical segments, but the per cent increase by washing is much greater for subapical segments. These data are qualitatively similar to those for phosphate uptake into segments from different locations of roots of a different variety of *Zea mays* (18). Ferricyanide inhibits all treatments to about the same degree except the fresh, subapical segments, but since the counts associated with these segments in ferricyanide are so low, it is likely that a large percentage of the radioactivity represents residual isotope which was not washed from the free space.

The very striking effects of ferricyanide on K^+ uptake are not reflected in the uptake of AIB (Table II). Uptake by control, apical root segments is stimulated as the pH is reduced, evidence consistent with the H^+ co-transport model (22). An inhibition of AIB uptake by ferricyanide is observed mainly at pH 5.0, and the effect is quite small compared to that seen for K^+. In other experiments performed at pH 5.0 (Table III), inhibitions of 10 to 15% by ferricyanide are observed regardless of whether the tip segments are freshly cut or are washed for 2.5 h, although the rate of uptake into washed segments is 50% greater than into the fresh. Ferricyanide effects on fresh or washed subapical segments was identical to that seen for apical tissue (data not shown). Redox activity induced by the electron donor NADH, on the other hand, inhibits amino acid uptake into sugar cane cells more completely (30), a result not observed in corn root segments (data not shown).

DISCUSSION

The reduction of ferricyanide, a reaction occurring at the cell surface (4, 21), results in the apparent excretion of H^+ across the plasma membrane, but not until after a lag period of about 5 min (Figs. 1–4). Thus, as stated earlier concerning the localization of the site of auxin action (24), one can not assume that a compound has an intercellular site of action just because a lag period exists before its effects on the plasma membrane become evident. This lag in response to ferricyanide may be peculiar to corn roots, however, since acidification occurs immediately after additions of ferricyanide to cultured carrot cells (4) and to oat

Table I. *Ferricyanide Reduction and Effects of Ferricyanide on Uptake of K^+ ($^{86}Rb^+$) into Freshly Cut or Washed Apical or Subapical Root Segments*

Apical segments (0–5 mm from the tip) or subapical segments (5–10 mm from the tip) were used 10 min after cutting or after a 4-h wash. Ferricyanide reduction was determined after 20 min. K^+ uptake was determined by a 10 min incubation in 0.2 mM KCl containing $^{86}Rb^+$. When added, the final concentration of ferricyanide was 0.8 mM. Data are the mean ± SE.

	Ferricyanide Reduced	K^+ Uptake		
		−Ferricyanide	+Ferricyanide	% of control
	μmol g^{-1} fresh wt h^{-1}			
Apical segments				
Fresh	4.73 ± 0.25	1.89 ± 0.15	0.19 ± 0.03	10
Washed	3.07 ± 0.60	4.59 ± 0.37	0.43 ± 0.02	9
Subapical segments				
Fresh	2.98 ± 0.33	0.41 ± 0.04	0.09 ± 0.01	22
Washed	3.68 ± 0.34	3.05 ± 0.21	0.34 ± 0.01	11

Table II. *Effects of pH and Ferricyanide on AIB Uptake into Washed, Apical Root Segments*

Following a wash period of 2.5 h, root segments (0–5 mm from the tip) were pretreated for 12 min with or without 0.8 mM ferricyanide. [^{3}H] AIB was then added to a final concentration of 50 μM. After 12 min more, the tissue was washed and counted. Data are the mean ± SE.

pH	Ferricyanide		% of Control
	−	+	
	μmol AIB g^{-1} fresh wt h^{-1} × 10^2		
7.0	21.6 ± 0.8	21.4 ± 0.8	99
6.0	36.6 ± 1.5	33.2 ± 0.3	91
5.0	52.8 ± 0.5	44.0 ± 1.0	83

Table III. *Effects of Ferricyanide on Uptake of AIB into Freshly Cut or Washed Apical Root Segments*

Apical segments (0–5 mm from the tip) were incubated in 50 μM [^{3}H] AIB at pH 5.0 with or without 0.8 mM ferricyanide 10 min after cutting (fresh) or 150 min after cutting (washed). Data are the mean ± SE.

	Ferricyanide		% of Control
	−	+	
	μmol AIB g^{-1} fresh wt h^{-1} × 10^2		
Fresh	40.2 ± 1.2	34.0 ± 0.1	85
Washed	61.9 ± 2.4	55.6 ± 0.7	90

coleoptile segments (B Rubinstein, AI Stern, unpublished results).

Assuming that the drop in pH in response to ferricyanide represents excretion of H^+, it becomes important to determine if redox activity provides a pathway for H^+ across the plasma membrane in addition to, and independent from, that usually associated with a H^+-ATPase. If a separate pathway exists, one might expect a fixed stoichiometry of ferricyanide reduced to H^+ excreted as electrons transported across the plasma membrane reduce ferricyanide and the accompanying H^+ accumulate in the medium. But for the first 5 min after its addition to root segments, ferricyanide is reduced, but little, if any, acidification of the medium is detected (Fig. 2). Furthermore, a comparison of Figures 3 and 4 shows that additions of DCCD, DES, or vanadate produce variable ratios of ferricyanide reduced to H^+ excreted (actual values can range from less than 4 to over 100) even though the drugs are added after the acidification has come to steady state.

Because different ratios of ferricyanide reduced to H^+ excreted can be obtained, it is possible that redox activity, as measured here, involves transport only of electrons across the plasma membrane. The acidification that occurs 5 min after the addition of ferricyanide may be due to activation of an ATPase, since this net H^+ excretion is inhibited by the various ATPase inhibitors (Fig. 3). Although the effects of ATPase inhibitors on ferricyanide-stimulated H^+ transport have not been reported previously, it should be noted that the elevated H^+ export of Fe-deficient plants (which also have high cell surface redox activity) is inhibited by 5 μM DCCD and 50 μM DES (23). In corn root protoplasts, the acceleration of net H^+ efflux by exogenous NADH, a different type of redox activity than that measured here, is, nevertheless, inhibited by DCCD, DES, and vanadate (19); Lin (19) has concluded that the H^+ efflux driven by NADH oxidation has properties similar to those of an H^+-ATPase.

A revised model to explain the action of ferricyanide on H^+ excretion is shown in Figure 7. The presence of ferricyanide results in the oxidation of a cytosolic donor (indicated as NADPH) with the electrogenic transport of electrons across the plasma membrane. For at least 5 min after ferricyanide is added, H^+ could accumulate in the cytosol. The aggregate effect of this redox activity would be a depolarization of the membrane potential and possibly even an acidification of the cytosol until H^+ excretion finally occurs via the H^+-ATPase. A depolarization of the Em and acidosis of the cytosol have been implicated in activating the H^+-ATPase (8, 12).

The evidence just presented supports this scheme as do reports of a depolarization of the Em after ferricyanide is added to *Elodea* leaves (14), to Fe-starved bean roots (27), and a depolarization of 15 to 20 mV after barley roots were bathed in 0.5 mM ferricyanide (B Etherton, unpublished results). A model of a

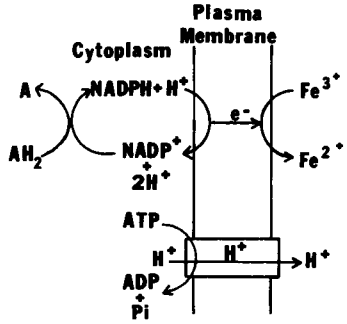

FIG. 7. Hypothetical model for the stimulation of net H^+ excretion by transplasmalemma redox activity. The reduction of ferricyanide at the outer surface of the cell ($Fe^{3+} \rightarrow Fe^{2+}$) is accomplished by electron transport across the plasma membrane from a cytosolic donor such as NADPH. The NADP is reduced by reactions in the cytosol ($AH_2 \rightarrow A$). Electron flow leads to a depolarization of the Em, perhaps to an acidosis of the cytosol as well, and a stimulation of the activity of the H^+-transporting ATPase (lower portion of the diagram).

different sort, but still involving an interaction between transplasma membrane redox activity and H^+-ATPase, was proposed by Sachs *et al.* (26) for gastric parietal cells.

Before adopting the model outlined in Figure 7, other points must be considered. First, the acidification being measured is only a net excretion of H^+, with H^+ uptake assumed to be constant, especially during the first 5 min after addition of ferricyanide or in the presence of ATPase inhibitors. If H^+ uptake were changing markedly during these conditions, the calculations of ferricyanide reduced to H^+ excreted presented here would be invalid.

Second, our evidence for the involvement of an ATPase during electron transport across the plasma membrane depends on the specificity of the inhibitors used. In this regard, these compounds do not inhibit ferricyanide reduction (Fig. 4) to the same extent as net H^+ export (Fig. 3), and their effects on the latter process are rapid and occur at rather dilute concentrations. Furthermore, it has been shown that with proper precautions DCCD (9, 23), DES (2), and vanadate (15) do not have major effects on intracellular metabolism. It is thus likely that under our conditions, too, the agents are acting primarily as inhibitors of plasma membrane ATPases.

DCCD and vanadate do inhibit ferricyanide reduction somewhat (Fig. 4), perhaps by affecting the availability of the electron donor or by some other nonspecific action. DCCD, for example, may be binding to a protein involved with electron transfer as well as by binding to protein associated with an ATPase (13). Vanadate interferes with phosphorylation (7), so an effect on ferricyanide reduction may indicate that a phosphorylated intermediate is involved in electron transfer across the plasma membrane as it is in electron transfer in the chloroplast (3).

Finally, the applicability of this model (Fig. 7) to the regulation of H^+ transport *in vivo* should be considered. It is possible, for example, that ferricyanide is intercepting electrons at a distance from the *in vivo* (apoplastic) acceptor, thereby short-circuiting electron flow; this might cause H^+ excretion by a series of events which may never occur under more normal conditions. Alternately, the ferricyanide may only be substituting for the *in vivo* (apoplastic) acceptor, which could be an oxidized component of the soil or of the plasma membrane. In this case, the ferricyanide may mimic the usual events leading to H^+ excretion, but perhaps change the rates at which they occur.

We also attempted to determine if the effects of ferricyanide on solute fluxes are due to a direct action on the transport mechanism or are indirect, perhaps by a depolarization of the Em. The uptake of AIB is inhibited only slightly by additions of ferricyanide (Tables II and III). Assuming that the rate of uptake is directly proportional to p.m.f., the sum of the membrane potential and the pH gradient across the plasma membrane, one can estimate the effect on uptake of the 20 mV depolarization, which occurs just after ferricyanide addition to unstressed root tissue (27; B Etherton, unpublished data). The calculations show that the inhibitions seen at pH 5 and 6 can be accounted for by the depolarization of the Em. However, there is no effect of ferricyanide at pH 7 (Table II), even though reduction occurs rapidly at this pH (22). Perhaps at the higher external pH, some factor other than the p.m.f. becomes limiting for AIB uptake.

The inhibition of K^+ uptake, in contrast to that of AIB, is inhibited rapidly and markedly by the redox process occurring in the presence of ferricyanide (Figs. 5 and 6). To see if this inhibition is due entirely to the depolarization of the Em induced by ferricyanide, it is possible to use a form of the Ussing-Teorell flux ratio equation ($Em = 59 \log (C_o \cdot J_o / C_i \cdot J_i)$ assuming that K^+ fluxes are largely passive (29). With values of 0.2 mM for C_o, and reasonable values of 50 to 200 mM for C_i, and 2.5 μmol/h·g fresh weight for J_o, the J_i at an Em of -130 mV would be inhibited 55% compared to the J_i at -150 mV, an amount considerably less than the 90% inhibition actually observed. It is possible, therefore, that the very marked inhibition of K^+ uptake seen in the presence of ferricyanide is not due entirely to the depolarization of the Em, but may be due, in part, to oxidation of some component of the uptake mechanism.

Regarding the relationship between the capacity for redox activity and the rate of K^+ uptake, there seems to be little correlation between the two. For example, when apical root segments are washed for 4 h, their ability to reduce ferricyanide decreases by 35% (Table I), and their ability to take up K^+ increases by 140% (Table I). However, when subapical segments are washed for 4 h, reductase activity now increases by about 25% and the rate of K^+ uptake increases 640% (Table I). These data imply that there is no close link between the ability of the cells to take up K^+ and the capacity for transplasmalemma membrane redox activity, at least for the activity measured when ferricyanide is added. But, one cannot eliminate the possibility that metabolic or environmental factors may regulate K^+ uptake by interacting with the redox system much as ferricyanide does.

We also considered the possibility that redox activity interacts with the recovery of K^+ uptake which occurs during washing. After observing the action of exogenous NADH, Kochian and Lucas (17) concluded that this class of redox activity causes a wound response similar to that of cutting. In Table I, it can be seen that ferricyanide does in fact reduce the rate of K^+ uptake in washed, subapical segments to the level of that in the freshly cut tissue without ferricyanide; but the result may be fortuitous for this class of redox activity, since marked inhibitions are also seen in freshly cut apical and subapical segments, and, as mentioned in "Results," a recovery to 80% of controls is seen in only 10 min. Furthermore, ferricyanide does not reduce the rate of AIB uptake into washed segments to that of the freshly cut segments (Table III). It appears that added ferricyanide is not acting largely by mimicking processes associated with the shock of cutting the tissue; a similar conclusion was drawn by Kochian and Lucas (17). The increase in redox activity by subapical segments seen after washing (Table I) may represent an inducible redox activity in these tissues, and may be comparable qualitatively to that occurring in dicots when iron is limiting (6).

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