Rate of Transmembrane Electron Transfer in Chromaffin-Vesicle Ghosts†

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ABSTRACT: The chromaffin vesicle of the adrenal medulla contains a transmembrane electron carrier that may provide reducing equivalents for dopamine β-hydroxylase in vivo. This electron-transfer system can be assayed by trapping ascorbic acid inside resealed membrane vesicles (ghosts), adding an external electron acceptor such as ferricytochrome *c* or ferricyanide, and following the reduction of these acceptors spectrophotometrically. Cytochrome *c* reduction is more rapid at high pH and is proportional to the amount of chromaffin-vesicle ghosts, at least at low ghost concentrations. At pH 7.0, ghosts loaded with 100 mM ascorbic acid reduce 60 μM cytochrome *c* at a rate of 0.035 ± 0.010 μequiv min^{-1} (mg of protein)$^{-1}$ and 200 μM ferricyanide at a rate of 2.3 ± 0.3 μequiv min^{-1} (mg of protein)$^{-1}$. The rate of cytochrome *c* reduction is accelerated to 0.105 ± 0.021 μequiv min^{-1} (mg of protein)$^{-1}$ when cytochrome *c* is pretreated with equimolar ferrocyanide. Pretreatment of cytochrome *c* with ferricyanide also causes a rapid rate of reduction, but only after an initial delay. The ferrocyanide-stimulated rate of cytochrome *c* reduction is further accelerated by the protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), probably because FCCP dissipates the membrane potential generated by electron transfer. These rates of electron transfer are sufficient to account for electron transfer to dopamine β-hydroxylase in vivo and are consistent with the mediation of electron transfer by cytochrome *b*-561.

Ascorbic acid is required for the synthesis of two important classes of hormones: catecholamines and peptide hormones. In catecholamine synthesis, dopamine β-hydroxylase needs ascorbic acid to hydroxylate dopamine to norepinephrine. Many peptide hormones are amidated on the carboxy terminus, and the peptide-amidating monooxygenase uses ascorbic acid. Since both of these enzymes are found within the secretory vesicles that store the hormones, these vesicles probably have a mechanism for regenerating internal ascorbic acid.

In the chromaffin vesicles of the adrenal medulla, dopamine β-hydroxylase uses ascorbic acid as a one-electron donor and produces semidehydroascorbate (Diliberto & Allen, 1981; Skotland & Ljones, 1980). We proposed that semidehydroascorbate is reduced back to ascorbate by cytochrome *b*-561 and that cytochrome *b*-561 in turn draws electrons from a cytosolic electron donor (Njus et al., 1983). Thus, cytochrome *b*-561, an integral membrane protein, functions as a transmembrane electron carrier transferring electrons from the cytosol to regenerate internal ascorbic acid.

In neurosecretory vesicles isolated from the posterior pituitary, the peptide-amidating monooxygenase uses ascorbic acid to amidate vasopressin and oxytocin (Bradbury et al., 1982; Eipper et al., 1983a,b). Since the membranes of these vesicles also contain cytochrome *b*-561 (Duong et al., 1984), they may employ the same ascorbate-regenerating mechanism. In fact, cytochrome *b*-561 mediated ascorbate regeneration may be found in a variety of organelles.

To show that the chromaffin-vesicle membrane has a transmembrane electron carrier, we (Njus et al., 1983; Harnadek et al., 1985) measured the membrane potential created when electrons are passed from an internal electron donor (ascorbic acid) to an external electron acceptor (ferricyanide or cytochrome *c*). A more direct and quantitative assay for electron transfer is to follow the reduction of cytochrome *c* or ferricyanide spectrophotometrically. We report here on the characterisitcs of this assay and employ it to measure rates of electron transfer across chromaffin-vesicle membranes. This assay is especially important because it may be used to investigate electron transfer not only in chromaffin vesicles but also in other cytochrome *b*-561 containing vesicles as well.

Materials and Methods

Chromaffin vesicles were prepared from bovine adrenal medulla as described by Kirshner (1962). These were lysed and the ghost membranes resealed and purified on a Ficoll density gradient (Njus & Radda, 1979). Ascorbate-loaded ghosts were prepared by lysing in the presence of 0.10 M ascorbate and 0.15 M Tris[1]/PO_4, pH 7.0 (Njus et al., 1983). Ascorbate-free ghosts were prepared by lysing in the presence of 0.2 M Tris/PO_4, pH 7.0 (Njus & Radda, 1979). All experiments were performed within 15 h of the cattle being slaughtered.

Absorbance of cytochrome *c* was recorded either with a Cary 118C spectrophotometer set at 550 nm (Figures 2 and 3) or with an Aminco DW-2 spectrophotometer operated in the dual-wavelength mode (Figures 1 and 4–6). In the latter case, absorbance was measured at 550 nm relative to 587 nm. To calculate rates of electron transfer, molar extinction coefficient differences (reduced minus oxidized) were taken as 2.0×10^4 at 550 nm and as -2.0×10^3 at 587 nm.

Absorbance of ferricyanide (Figure 7) was measured with the Aminco DW-2 spectrophotometer operated in the dual-wavelength mode. Absorbance was measured at 480 nm relative to 410 nm. The molar extinction coefficient difference (reduced minus oxidized) was taken as 1.0×10^3.

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[1] Abbreviations: *A*, absorbance; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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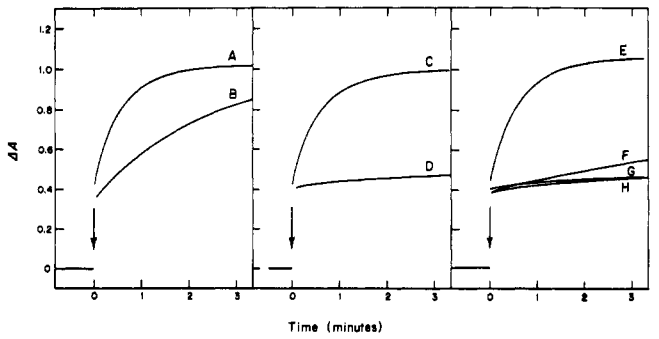

FIGURE 1: Electron-transfer-dependent cytochrome *c* reduction. Samples were incubated in 1 mL of 0.4 M sucrose, 10 mM Hepes, and 250 μM KCN, pH 7.0, at 22 °C for 2 min. Then, at $t = 0$, 20 μL of 3 mM cytochrome *c* and 3 mM ferrocyanide was added and the absorbance recorded. Samples contained (A) ascorbate-loaded ghosts, (B) ascorbate-loaded ghosts with 20 μg of ascorbate oxidase, (C) 50 nmol of ascorbate, (D) 50 nmol of ascorbate with 20 μg of ascorbate oxidase, (E) ascorbate-free ghosts with 50 nmol of ascorbate added externally, (F) ascorbate-free ghosts with 20 μg of ascorbate oxidase and 50 nmol of ascorbate added externally, (G) ascorbate-free ghosts with 20 μg of ascorbate oxidase, or (H) ascorbate-free ghosts alone. Ascorbate-loaded ghosts were added to a final concentration of 82.7 μg of protein/mL while ascorbate-free ghosts were added to a final concentration of 94.0 μg/mL.

Unless specified otherwise, ascorbate oxidase and cyanide were included in the medium in all experiments. Ascorbate oxidase (20 μg/mL) was added to destroy free ascorbic acid external to the vesicles. Cyanide (250 μM) was added to prevent reoxidation of cytochrome *c* by mitochondrial cytochrome oxidase contaminating the chromaffin-vesicle ghost preparation.

Protein was assayed with biuret reagent (Casey et al., 1976). Equine cytochrome *c* (type III), ascorbate oxidase, Hepes, Mes, and Tris were obtained from Sigma Chemical Co., St. Louis, MO. FCCP was purchased from Chemical Dynamics Corp., South Plainfield, NJ.

RESULTS

Electron transfer from ascorbate trapped inside chromaffin-vesicle ghosts to external cytochrome *c* can be observed by monitoring the membrane potential (inside positive) created by charge movement (Njus et al., 1983; Harnadek et al., 1985). A more direct method, however, is to measure the reduction of cytochrome *c* spectrophotometrically. As shown in Figure 1, cytochrome *c* is reduced when it is added to a suspension of ascorbate-loaded chromaffin-vesicle ghosts. That the ascorbate is sequestered inside the ghosts is demonstrated by the fact that it is insensitive to ascorbate oxidase (Figure 1A,B). The amount of ascorbate oxidase added should have been sufficient to destroy all of the ascorbate in the ascorbate-loaded ghosts. The ghosts contained less than 50 nmol of ascorbate since they would not bleach 50 nmol of 2,6-dichlorophenolindophenol. Fifty nanomoles of pure ascorbate was completely destroyed by ascorbate oxidase (Figure 1C,D) as was 50 nmol of ascorbate added externally to an ascorbate-free ghost suspension (Figure 1E,F). The failure of ascorbate-free ghosts to reduce cytochrome *c* (Figure 1G,H) also demonstrates that the internal reductant is ascorbate rather than an endogenous electron donor such as residual catecholamine.

As one might expect, increasing the concentration of ascorbate-loaded ghosts increases the rate of cytochrome *c* reduction (Figure 2). The rate is approximately proportional to ghost concentration when small amounts of ghosts are added but saturation occurs at higher concentrations. The rate of cytochrome *c* reduction also increases as the pH is raised

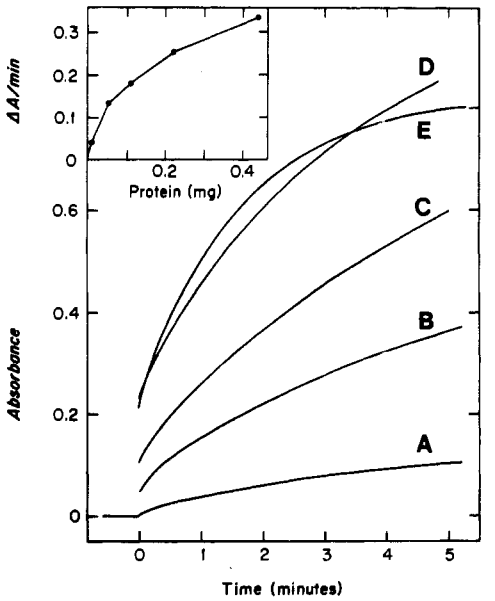

FIGURE 2: Dependence of the rate of electron transfer on ghost concentration. Alliquots of an ascorbate-loaded ghost suspension were added to 1 mL of 0.4 M sucrose, 10 mM Hepes, and 250 μM KCN, pH 7.0. Ascorbate oxidase (2 μg/μL of ghost suspension) was added to each sample and allowed to incubate at 22 °C for 5 min. Then, at $t = 0$, 10 μL of 6 mM cytochrome *c* was added and the absorbance at 550 nm monitored. Samples received the following volumes of ghost suspension (11.1 mg of protein/mL): (A) 1, (B) 5, (C) 10, (D) 20, and (E) 40 μL. The inset shows the slopes measured in the first 30 s plotted as a function of the ghost concentration.

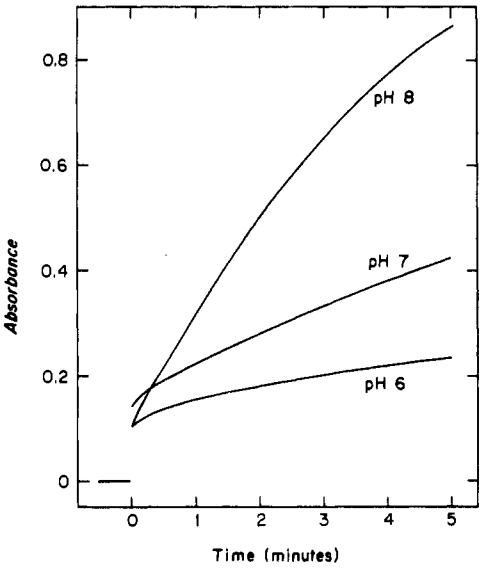

FIGURE 3: Dependence of the rate of electron transfer on pH. Ascorbate-loaded ghosts (195 μg of protein) were added to 1 mL of 0.4 M sucrose, 10 mM Hepes, 10 mM Mes, and 250 μM KCN at the indicated pH. Ascorbate oxidase (20 μg) was added to all samples and allowed to incubate at 22 °C for 5 min. Then, at $t = 0$, 10 μL of 6 mM cytochrome *c* was added and the absorbance at 550 nm monitored.

(Figure 3). Rates at pH 7 and pH 8 are about 30% and 130% faster than the rate at pH 6.

We have observed that electron transfer from internal ascorbate to external cytochrome *c* will generate a membrane potential only if ferrocyanide or ferricyanide is present (Harnadek et al., 1985). We suggested that these anions stimulate the rate of electron transfer. As shown in Figure 4, the rate of cytochrome *c* reduction is greatly accelerated by ferrocyanide. Data collected from many experiments using different ghost preparations yielded the following average rates:

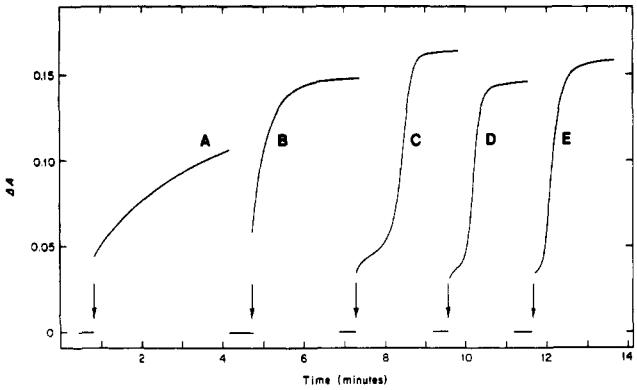

FIGURE 4: Effect of ferrocyanide and ferricyanide on electron-transfer-dependent cytochrome *c* reduction. Ascorbate-loaded ghosts (193 μg of protein) were added to 1 mL of 0.4 M sucrose, 10 mM Hepes, and 250 μM KCN, pH 7.0. Ascorbate oxidase (20 μg) was added and the sample allowed to incubate at 22 °C for 5 min. Cytochrome *c* (10 nmol premixed with the additions indicated below) was added at the arrow and absorbance recorded. Samples received (A) cytochrome c alone, (B) cytochrome *c* + 10 nmol of ferrocyanide, (C) cytochrome *c* + 10 nmol of ferricyanide, (D) cytochrome *c* + 5 nmol of ferricyanide, or (E) cytochrome *c* + 2.5 nmol of ferricyanide.

Untreated cytochrome *c* (60 μM) is reduced at a rate of 0.035 ± 0.010 μequiv min^{-1} (mg of protein)$^{-1}$ (12 determinations using ghosts from five different preparations). Cytochrome *c* treated with equimolar ferrocyanide is reduced at a rate of 0.105 ± 0.021 μequiv min^{-1} (mg of protein)$^{-1}$ (15 determinations from seven preparations). Ferrocyanide does not affect the rate at which cytochrome *c* is reduced by ascorbate in the absence of chromaffin-vesicle membranes (G. J. Harnadek, unpublished observation).

When cytochrome *c* is pretreated with equimolar ferricyanide, it is reduced at an accelerated rate (Figure 4C), but only after an initial lag. Presumably, the ferricyanide must first be reduced to ferrocyanide; then, cytochrome *c* reduction begins at the ferrocyanide-treated rate. Consistent with this idea is the fact that lower concentrations of ferricyanide produce shorter lag times (Figure 4D,E). These delay times imply a rate of ferricyanide reduction of 0.06 μequiv min^{-1} (mg of protein)$^{-1}$. The lower ferricyanide concentrations also produce accelerated rates of cytochrome *c* reduction, indicating that the effective concentration is quite low. The rate of cytochrome *c* reduction is shown as a function of ferrocyanide concentration in Figure 5. Reduction of 60 μM cytochrome *c* is stimulated half-maximally by about 10 μM and maximally by about 100 μM ferrocyanide. At the lower cytochrome *c* concentrations used in Figure 4, maximal rates seem to be obtained with less ferrocyanide.

As mentioned before, electron transfer from internal ascorbate to external cytochrome *c* generates a membrane potential in the presence of ferrocyanide (Harnadek et al., 1985). This membrane potential (positive inside) should slow the rate of electron transfer. The uncoupler FCCP will dissipate the membrane potential. FCCP, therefore, should accelerate the rate of electron transfer to ferrocyanide-treated cytochrome *c*. This effect is shown in Figure 6. FCCP does not increase the rate in the absence of ferrocyanide (data not shown) consistent with the absence of an electron-transfer-generated membrane potential under these conditions.

Electron-transfer-generated membrane potentials can be elicited by ferricyanide as well as by cytochrome *c* (Njus et al., 1983). Therefore, ferricyanide should also be reduced by ascorbate-loaded ghosts. This is confirmed by Figure 7. It is more difficult to follow ferricyanide reduction because the extinction coefficient change (reduced minus oxidized) is

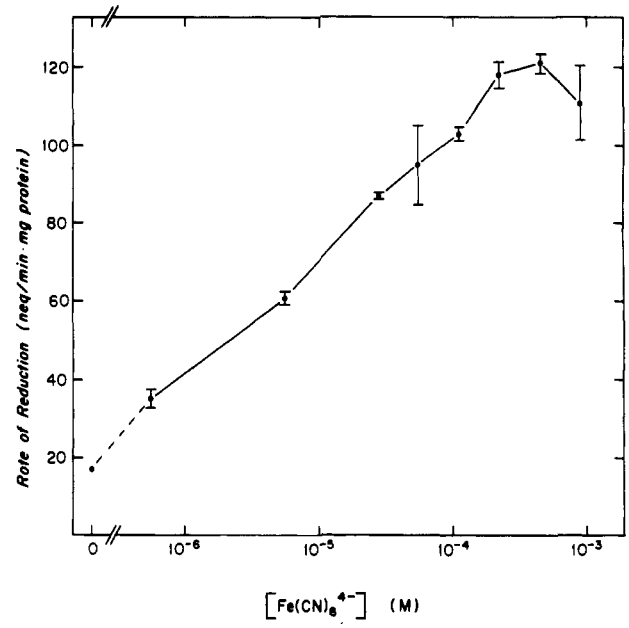

FIGURE 5: Dependence of the rate of electron transfer on ferrocyanide concentration. Each point is the average of three replicate samples. For each sample, ascorbate-loaded ghosts (176 μg of protein) were added to 1 mL of 0.4 M sucrose, 10 mM Hepes, and 250 μM KCN, pH 7.0, containing 20 μg of ascorbate oxidase and allowed to incubate at 22 °C for 5 min. Cytochrome *c* (60 nmol) mixed with various amounts of ferrocyanide was added and the absorbance monitored. The rate of cytochrome *c* reduction was calculated from the rate of absorbance change as described in the text.

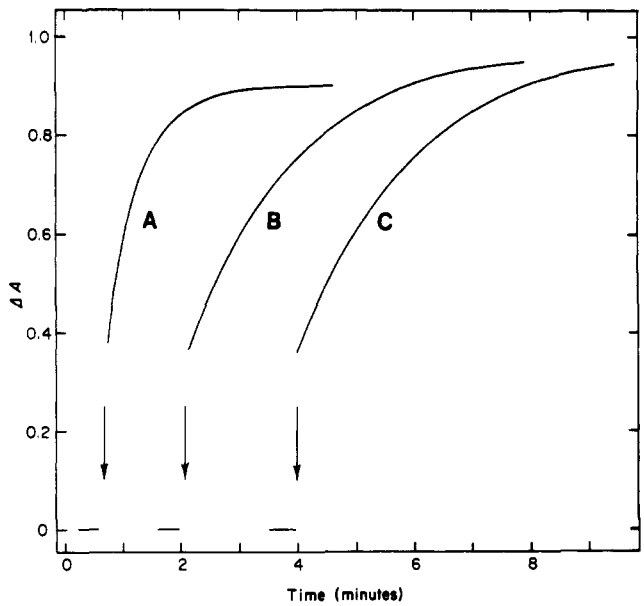

FIGURE 6: Effect of FCCP on electron-transfer-dependent cytochrome *c* reduction. Ascorbate oxidase (20 μg) was added to ascorbate-loaded ghosts (178 μg of protein) in 1 mL of 0.4 M sucrose, 10 mM Hepes, and 250 μM KCN, pH 7.0, and incubated for 5 min at 22 °C. FCCP or ethanol was added after 2 min of incubation. Then, 20 μL of 3 mM cytochrome *c* and 3 mM ferrocyanide was added at the arrow and absorbance monitored. Samples received (A) 10 μL of 250 μM FCCP (in ethanol), (B) 10 μL of ethanol, or (C) no additions.

smaller by a factor of 20. Fortunately, this difference is more than offset by the fact that ascorbate-loaded ghosts reduce ferricyanide much more rapidly than they reduce cytochrome *c*. The rate of reduction of 200 μM ferricyanide appears to be about 2.3 ± 0.3 μequiv min^{-1} (mg of protein)$^{-1}$ (three determinations from two ghost preparations) or about 70 times the rate for untreated 60 μM cytochrome *c*. This rate is also

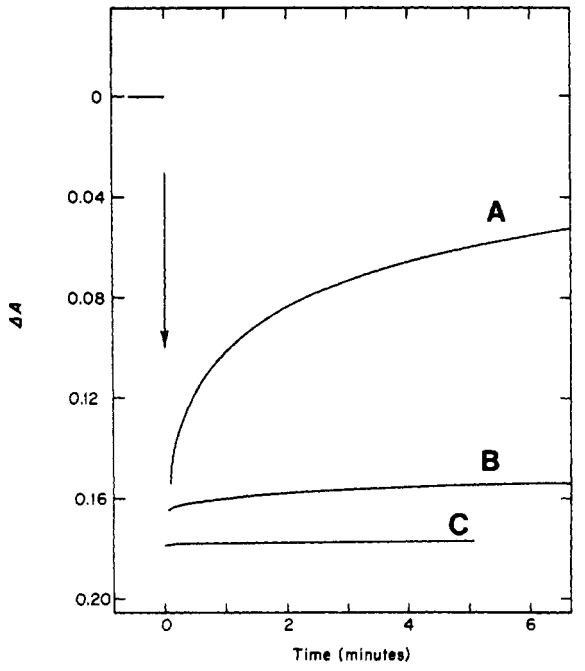

FIGURE 7: Electron-transfer-dependent ferricyanide reduction. Ghost samples were added to 1 mL of 0.4 M sucrose, 10 mM Hepes, and 250 μM KCN, pH 7.0, along with ascorbate oxidase (20 μg). After a 5-min incubation at 22 °C, 20 μL of 10 mM ferricyanide was added (at $t = 0$) and the absorbance recorded. Samples received (A) ascorbate-loaded ghosts (91 μg of protein), (B) ascorbate-free ghosts (87 μg of protein), or (C) no ghosts.

about 40 times the rate of reduction of 2.5–10 μM ferricyanide calculated from the lag times in Figure 4. This suggests that the rate of reduction is proportional to ferricyanide concentration. There is a possibility that ferricyanide will be reduced by catecholamines contaminating the ghost preparation. That this is not a problem is indicated by the fact that ascorbate-free ghosts do not reduce ferricyanide (Figure 7B).

Discussion

It has often been suggested that the chromaffin vesicle has a transmembrane electron transfer system to import reducing equivalents needed by the intravesicular enzyme dopamine β-hydroxylase (Njus et al., 1981, 1983; Wakefield et al., 1982; Grouselle & Phillips, 1982). The electron-transfer system is thought to regenerate intravesicular ascorbic acid, which is the enzyme's preferred reducing agent. A likely candidate for a transmembrane electron carrier is cytochrome *b*-561 as it spans the chromaffin-vesicle membrane (Apps et al., 1980; Duong & Fleming, 1984), is the second most abundant protein in that membrane, and has a midpoint reduction potential (100–140 mV) appropriate for this function (Flatmark & Terland, 1971; Apps et al., 1984). It now appears that neurosecretory vesicles from the neurohypophysis have a similar electron-transfer system to import reducing equivalents for a different intravesicular enzyme, peptide-amidating monooxygenase. These vesicles, too, have membrane-bound cytochrome *b*-561 (Duong et al., 1984) and contain ascorbic acid (Russell et al., 1985).

We have been studying electron transfer across the chromaffin-vesicle membrane by trapping ascorbate inside the vesicles and adding an electron acceptor externally. In previous studies (Njus et al., 1983; Harnadek et al., 1985), we observed the membrane potential generated by electron transfer. Here, we have followed electron transfer more directly by monitoring spectrophotometrically the reduction of the electron acceptor. This outward electron transfer is in the direction opposite that thought to occur in vivo. However, since the electron-transfer system presumably functions to maintain internal ascorbate stores, any vesicle having this system should contain ascorbic acid and be assayable by this method. Because the assay may be applicable to electron-transfer studies in many kinds of secretory and synaptic vesicles, it should be carefully characterized.

In using this assay, we have taken two precautions. First, ascorbate oxidase is included to eliminate any external ascorbate, thus ensuring that reduction is caused by sequestered ascorbate. Second, cyanide is included to prevent reoxidation of cytochrome *c* by cytochrome oxidase. In many vesicle preparations, contamination by mitochondrial membranes can be a problem. When these precautions are taken, it is apparent that internal ascorbate reduces either ferricytochrome *c* (Figure 1) or ferricyanide (Figure 7) added externally. Moreover, at low membrane concentrations, the rate of cytochrome *c* reduction is linearly dependent on the ghost concentration (Figure 2). Therefore, the rate can be used as a quantitative assay for electron-transfer activity.

We suggested earlier that cytochrome *b*-561 is the transmembrane electron carrier. Further evidence for this is the correlation between the presence of cytochrome *b*-561 and the occurrence of transmembrane electron transfer. The membranes of neurohypophyseal secretory vesicles contain cytochrome *b*-561 (Duong et al., 1984) and also exhibit transmembrane electron transfer (Russell et al., 1985). Moreover, purified cytochrome *b*-561 reconstituted into phospholipid vesicles seems to catalyze electron transfer (Srivastava et al., 1984). It is instructive to compare the rates of electron transfer catalyzed by each of these preparations. We found that chromaffin-vesicle ghosts containing 100 mM ascorbate reduce cytochrome *c* at a rate of 35 nequiv min^{-1} (mg of membrane protein)$^{-1}$ and ferricyanide at a rate of 2.3 μequiv min^{-1} mg^{-1}. Russell et al. (1985) observed that neurohypophyseal secretory vesicles reduce cytochrome *c* at a rate of 1.1–1.6 nequiv min^{-1} (mg of protein)$^{-1}$ and ferricyanide at a rate of 0.1 μequiv min^{-1} mg^{-1}. Therefore, both vesicles reduce ferricyanide about 70 times faster than they reduce cytochrome *c*. These rates also correlate with the relative amounts of cytochrome *b*-561 in the two membranes. Chromaffin-vesicle ghosts reduce cytochrome *c* at a rate of 5 min^{-1} per cytochrome *b*-561 while the value in neurosecretory vesicles is 3.5 min^{-1}. Reconstituted cytochrome *b*-561 reduces cytochrome *c* with a turnover number of 570 min^{-1} (Srivastava et al., 1984), so the reconstituted system exhibits an anomalously high rate of electron transfer.

Our assay measures electron transfer in the reverse direction and uses nonphysiological electron acceptors. Nevertheless, if electron uptake in vivo occurs at a rate within 1 order of magnitude of the rates observed here, it will be sufficient to provide electrons for dopamine β-hydroxylase. The V_{max} for dopamine transport into chromaffin-vesicle ghosts is about 1 nmol min^{-1} (mg of protein)$^{-1}$ (Knoth et al., 1981), far less than the rate of electron transfer to cytochrome *c*.

The pH of the medium affects the rate of cytochrome *c* reduction (Figure 3) but not the midpoint reduction potential of either cytochrome *c* or cytochrome *b*-561 (Apps et al., 1984). The pH may affect the interaction between the two cytochromes through an electrostatic effect: raising the pH will increase negative surface charge. This may facilitate interaction between cytochrome *b*-561 and the cationic cytochrome *c*.

As shown in Figure 6, dissipating the membrane potential with FCCP increases the rate of electron transfer to cyto-

chrome *c* in the presence of ferrocyanide. Since this electron transfer is not rapid enough to generate a membrane potential in the absence of ferrocyanide (Harnadek et al., 1985), FCCP should affect only the ferrocyanide-stimulated rate. This is in fact observed. ATP addition slows electron transfer, but the reason for this effect is unclear. Since ATP fuels an H^+-translocating ATPase (Njus et al., 1981), the ATP-dependent membrane potential (inside positive) could be slowing electron transfer. However, ATP might also be competing with ferrocyanide for anion-binding sites on cytochrome *c* or cytochrome *b*-561. Because of this ambiguity, we have not pursued the ATP effect.

It is not surprising that chromaffin vesicles reduce ferricyanide faster than they reduce cytochrome *c*. Ferricyanide ($E_0' = 420$ mV) has a higher midpoint reduction potential and will have a higher affinity for electrons than cytochrome *c* ($E_0' = 255$ mV). The acceleration of cytochrome *c* reduction caused by ferrocyanide is not so easily rationalized, however. The simplest explanation is that the vesicles reduce ferricyanide to maintain a very high ratio of free ferrocyanide to free ferricyanide. If the ferrocyanide/ferricyanide ratio were greater than 100, then the reduction potential would be less than 300 mV and might be low enough to reduce cytochrome *c*. If this were the case, however, the small fraction of ferricyanide would not be sufficient to mediate electron flow at the observed rate. In the presence of 60 μM ferrocyanide, cytochrome *c* reduction occurs at a rate of 0.105 μequiv min^{-1} (mg of protein)$^{-1}$. Therefore, 0.6 μM ferricyanide (or less) must be capable of accepting electrons from cytochrome *b*-561 at this rate. We found, however, that 200 μM ferricyanide is reduced at a rate of 2.3 μequiv min^{-1} mg^{-1} and 2.5–10 μM ferricyanide is reduced at a rate of 0.06 μequiv min^{-1} mg^{-1} (Figure 4). Consequently, it is doubtful that 0.6 μM ferricyanide would accept electrons at a rate of 0.105 μequiv min^{-1} mg^{-1}. For that reason, we suspect that electron flow from cytochrome *b*-561 to cytochrome *c* is mediated by bound rather than free ferrocyanide.

It is unlikely that ferrocyanide binds to cytochrome *c* since maximal stimulation of electron transfer occurs at ferrocyanide concentrations that are less than equimolar with cytochrome *c*. Moreover, although cytochrome *c* has ferrocyanide binding sites, these have binding constants of 1 mM and 30 mM (Stellwagen & Cass, 1975), concentrations far above the concentrations of ferrocyanide needed to stimulate electron transfer (Figure 5). Instead, we suggest that ferrocyanide binds to a cationic site on cytochrome *b*-561. This cationic character would also cause cytochrome *b*-561 to react more slowly with the cationic cytochrome *c* molecule, especially at lower pH. Moreover, a positively charged site would allow cytochrome *b*-561 to interact with cytosolic ascorbic acid, which may be the natural electron donor in vivo (Njus et al., 1981, 1983, Wakefield et al., 1982).

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Registry No. FCCP, 370-86-5; cytochrome *c*, 9007-43-6; ferrocyanide, 13408-63-4; ferricyanide, 13408-62-3; ascorbic acid, 50-81-7.

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