An Electron Transfer Dependent Membrane Potential in Chromaffin-Vesicle Ghosts†

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ABSTRACT: Adrenal medullary chromaffin-vesicle membranes contain a transmembrane electron carrier that may provide reducing equivalents for intravesicular dopamine β-hydroxylase in vivo. This electron transfer system can generate a membrane potential (inside positive) across resealed chromaffin-vesicle membranes (ghosts) by passing electrons from an internal electron donor to an external electron acceptor. Both ascorbic acid and isoascorbic acid are suitable electron donors. As an electron acceptor, ferricyanide elicits a transient increase in membrane potential at physiological temperatures. A stable membrane potential can be produced by coupling the chromaffin-vesicle electron-transfer system to cytochrome oxidase by using cytochrome *c*. The membrane potential is generated by transferring electrons from the internal electron donor to cytochrome *c*. Cytochrome *c* is then reoxidized by cytochrome oxidase. In this coupled system, the rate of electron transfer can be measured as the rate of oxygen consumption. The chromaffin-vesicle electron-transfer system reduces cytochrome *c* relatively slowly, but the rate is greatly accelerated by low concentrations of ferrocyanide. Accordingly, stable electron transfer dependent membrane potentials require cytochrome *c*, oxygen, and ferrocyanide. They are abolished by the cytochrome oxidase inhibitor cyanide. This membrane potential drives reserpine-sensitive norepinephrine transport, confirming the location of the electron-transfer system in the chromaffin-vesicle membrane. This also demonstrates the potential usefulness of the electron transfer driven membrane potential for studying energy-linked processes in this membrane.

In recent years, the catecholamine-storing chromaffin vesicles of the adrenal medulla have been fertile ground for bioenergetic studies (Carmichael, 1983; Winkler & Westhead, 1980; Ungar & Phillips, 1983). A proton-translocating ATPase[1] pumps H^+ into the vesicle interior and can create a membrane potential (inside positive) or a pH gradient (inside acidic). These gradients, in turn, drive catecholamine transport into the vesicles (Njus et al., 1981a; Knoth et al., 1981b; Johnson et al., 1981). We have recently shown that the chromaffin-vesicle membrane also has the capacity to transfer electrons from internal ascorbate to the external electron acceptors ferricyanide and ferricytochrome *c* (Njus et al., 1983). In vivo, this electron-transfer system may function to import reducing equivalents needed by the intravesicular enzyme dopamine β-hydroxylase. This system may regenerate intravesicular ascorbic acid, the enzyme's immediate source of reducing equivalents, by transferring electrons from an external electron donor to internal semidehydroascorbate. The likely mediator of this electron transfer is cytochrome *b*-561, a major component of the chromaffin-vesicle membrane (Apps et al., 1980, 1984; Duong & Fleming, 1982, 1984).

In our previous studies, we assayed electron transfer by measuring the membrane potential generated by electron transfer from internal ascorbate to an external electron acceptor. We found, using ferricyanide as the electron acceptor, that the membrane potential was stable at 4 °C but short-lived at 37 °C. For that reason, our earlier studies were done at the lower temperature. We report here that stable membrane potentials can be generated at 37 °C by using cytochrome *c*. However, cytochrome oxidase is needed to continually regenerate ferricytochrome *c*, and ferrocyanide is required to facilitate electron transfer. This method of generating membrane potentials at physiological temperatures represents an alternative to using the H^+-translocating ATPase. In particular, the cytochrome *c* dependent membrane potential can be used to drive catecholamine transport into chromaffin-vesicle ghosts.

Materials and Methods

Chromaffin vesicles were prepared from bovine adrenal medulla (Kirshner, 1962). These vesicles were lysed, the membranes allowed to reseal, and the ghosts purified on a Ficoll density gradient (Njus & Radda, 1979). Ascorbate-loaded (or isoascorbate-loaded) ghosts were prepared by lysing in the presence of 0.10 M ascorbate (or isoascorbate)–0.15 M Tris–PO_4, pH 7.0 (Njus et al., 1983). Phosphate-loaded ghosts were lysed in 0.2 M Tris–PO_4, pH 7.0. All experiments were performed within 15 h of the cattle being slaughtered.

Fluorescence of 8-anilinonaphthalene-1-sulfonic acid (ANS) was monitored by using a Perkin-Elmer 204S fluorescence spectrophotometer equipped with a water-jacketed cell holder (Njus et al., 1983). Excitation and emission wavelengths were 380 and 480 nm, respectively. For anoxia experiments (Figure 5), fluorescence was measured by using an Aminco-Bowman fluorescence spectrophotometer. Although this instrument put out a higher noise level, the sample chamber could accommodate a sealed cell. The suspension medium, ANS, and cytochrome *c* or $K_3Fe(CN)_6$ were placed inside a fluorescence cell equipped with a stopcock and a side injection port. This mixture was evacuated and flushed with nitrogen several times.

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[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonic acid; ATPase, adenosinetriphosphatase; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; oxonol VI, bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol; Tris, tris(hydroxymethyl)aminomethane.

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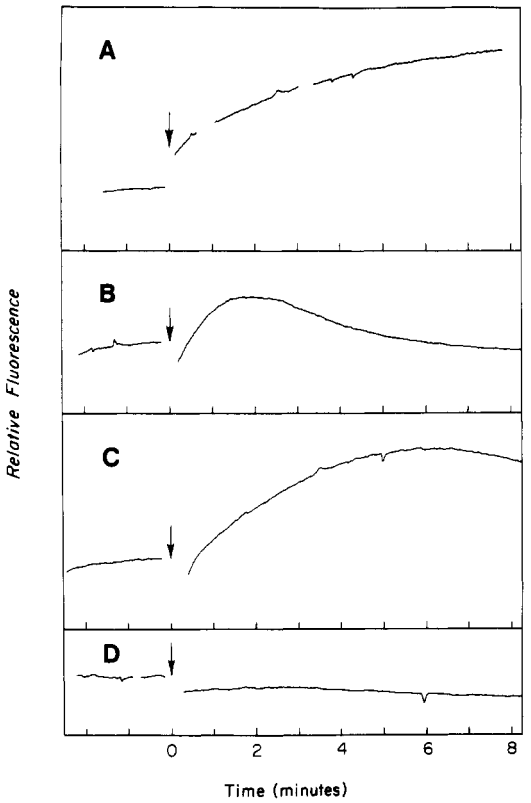

FIGURE 1: Membrane potentials monitored with ANS. For each trace, ascorbate-loaded ghosts (97 μg of protein in 25 μL) were added along with 10 μL of 1 mM ANS to 2.0 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0, at 37 °C. Sample D also received 10 μL of 500 μM FCCP. At $t = 0$, the following were added: (A) 100 μL of 100 mM ATP–100 mM $MgSO_4$, pH 7; (B) 10 μL of 100 mM $K_3Fe(CN)_6$; (C and D) 10 μL of 1.71 mM ferricyanide-treated cytochrome *c*. Fluorescence was monitored as described under Materials and Methods.

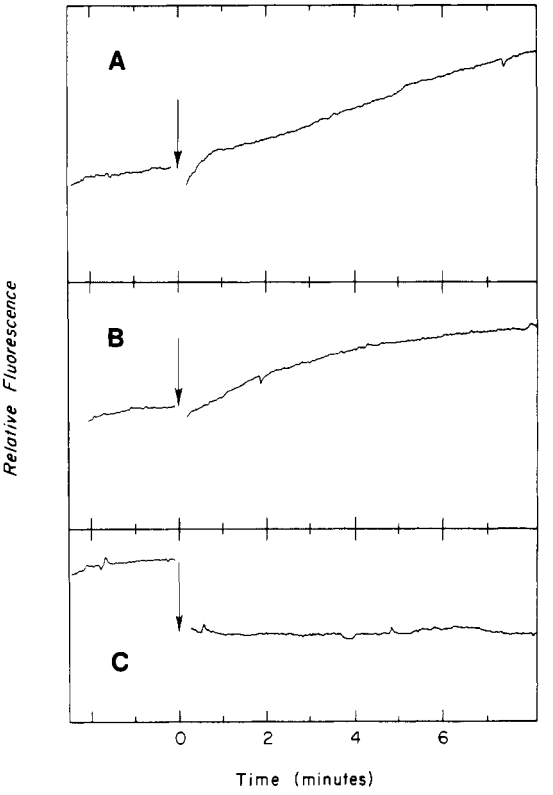

FIGURE 2: Electron transfer dependent membrane potentials generated with different electron donors. For each trace, the appropriate ghost preparation (25 μL) was added along with 10 μL of 1 mM ANS to 2.0 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0, at 4 °C. $K_3Fe(CN)_6$ (10 μL of 100 mM) was added at $t = 0$ and fluorescence monitored as described under Materials and Methods. Ghost samples were (A) ascorbate-loaded ghosts (110 μg of protein), (B) isoascorbate-loaded ghosts (85 μg of protein), and (C) phosphate-loaded ghosts (140 μg of protein).

The chromaffin-vesicle suspension was then injected and the fluorescence followed.

Absorbance of oxonol VI (Figure 3) was measured on an Aminco DW-2 spectrophotometer operated in the dual-wavelength mode (Russell et al., 1985). Absorbance was measured at 625 nm relative to 587 nm. Oxygen consumption (Figure 6) was measured by using a Clark-type oxygen electrode fitted with a Teflon membrane. The sample was placed in a water-jacketed chamber and stirred continuously. Uptake of [^{3}H]norepinephrine (Figures 7 and 8) was assayed by collecting ghosts on cellulose acetate filters (0.45-μm pore size) as described by Knoth et al. (1981a).

Cytochrome oxidase was assayed at 25 °C by the method of Cooperstein & Lazarow (1951). Activities are expressed as second-order rate constants. Succinic dehydrogenase activity at 25 °C was determined by using 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) as the electron acceptor (Pennington, 1961). Since ascorbate-loaded ghosts contain some intrinsic INT-reducing activity, samples were referenced against controls in which succinate was replaced by an equal concentration of malonate. Protein was assayed by using biuret reagent (Casey et al., 1976).

Equine cytochrome *c* was either used directly or oxidized by treating with $K_3Fe(CN)_6$ (Njus et al., 1983). Cytochrome *c*, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, reserpine, Hepes, FCCP, and ATP were obtained from Sigma Chemical Co. [^{3}H]Norepinephrine was purchased from New England Nuclear, D-isoascorbic acid from ICN K & K Laboratories, and ANS (Mg^{2+} salt) from Eastman Organic Chemicals. Oxonol VI was kindly donated by Dr. James Russell of the National Institutes of Health.

RESULTS

The fluorescent probe ANS is a convenient indicator of the membrane potential in chromaffin-vesicle ghosts (Bashford et al., 1975; Drake et al., 1979; Njus & Radda, 1979; Njus et al., 1983). An increased membrane potential (inside positive) is reflected in an enhanced ANS fluorescence. When ATP is added to a chromaffin-vesicle ghost suspension at 37 °C, the inwardly directed H^+-translocating ATPase generates a membrane potential and increases ANS fluorescence (Figure 1A). A similar fluorescence enhancement is elicited by adding either ferricyanide or ferricyanide-treated cytochrome *c* to a suspension of ascorbate-loaded ghosts (parts B and C of Figure 1). That this fluorescence enhancement is a response to membrane potential is indicated by the fact that the response is inhibited by the protonophore FCCP (Figure 1D).

The simplest explanation for this membrane potential is that it is generated by the movement of electrons from the internal donor to the external acceptor. Phosphate-loaded ghosts do not produce an ANS response (Figure 2C), confirming the requirement for an internal electron donor. This also shows that chromaffin-vesicle ghosts do not contain an endogenous electron donor such as residual catecholamine Either ascorbate or isoascorbate can serve as the internal electron donor (parts A and B of Figure 2), so the electron-transfer pathway is apparently not stereospecific.

Although either ferricyanide or ferricyanide-treated cytochrome *c* can be used as the electron acceptor, the response to ferricyanide is transient at 37 °C while the response to cytochrome *c* is stable (Figure 1). To show that this reflects a difference in the membrane potentials and is not an artifact

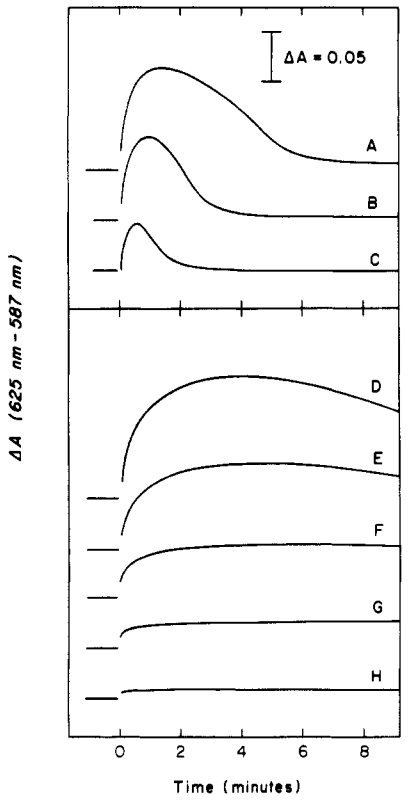

FIGURE 3: Membrane potentials monitored with oxonol VI. For each trace, ascorbate-loaded ghosts (342 μg of protein in 20 μL), ascorbate oxidase (40 μL of 1 mg/mL), and oxonol VI (14 nmol in 7 μL of ethanol) were added to 3.0 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0, at 25 °C. At $t = 0$, $K_3Fe(CN)_6$ was added as follows: (A) 100 nmol, (B) 50 nmol, and (C) 25 nmol. Cytochrome *c* was added in portions of (D) 15 nmol, (E) 7.5 nmol, (F) 3.8 nmol, and (G) 1.9 nmol, along with 30 nmol of potassium ferrocyanide. In (H), cytochrome *c* (15 nmol) was added with no ferrocyanide.

of the ANS probe, we monitored membrane potentials using oxonol VI (Figure 3). This voltage-sensitive dye has been used to measure membrane potentials in other systems (Bashford & Thayer, 1977; Smith & Chance, 1979; Russell et al., 1985). Although oxonol VI responds more quickly than ANS to changes in membrane potential, the two probes yield similar results. Figure 3 also shows the dependence of the membrane potential on the concentration of the electron acceptor. The maximum response requires approximately 30 μM ferricyanide but only ~5 μM cytochrome *c*.

The data shown in Figure 3 also demonstrate that cytochrome *c* will elicit an increase in membrane potential only if ferrocyanide is present. As discussed below, ferrocyanide apparently accelerates the rate of cytochrome *c* reduction by ascorbate-loaded ghosts. Ferricyanide, which will be reduced to ferrocyanide by the ascorbate-loaded ghosts, causes a similar stimulation. The cytochrome *c* used in Figures 1, 4, 5, and 8 and in our previous work (Njus et al., 1983) was oxidized by pretreatment with ferricyanide. Because cytochrome *c* binds anions, including ferri- and ferrocyanide (Stellwagen & Cass, 1975), the ferricyanide-treated cytochrome *c* preparation contains enough of these anions to stimulate electron transfer.

The stability of the membrane potential elicited by cytochrome *c* relative to the potential induced by ferricyanide (Figure 3) suggests that, while ferricyanide acts as a terminal electron acceptor, cytochrome *c* may act as an intermediate and be reoxidized by cytochrome oxidase present in mitochondrial membranes contaminating the chromaffin-vesicle ghost preparation. If this is the case, then the response to cytochrome *c* should be inhibited by cyanide, a cytochrome

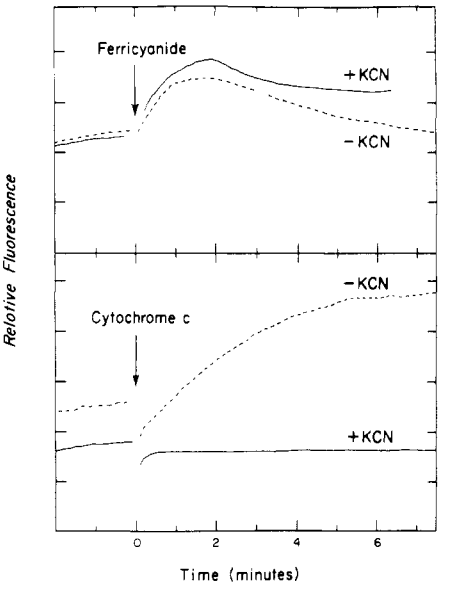

FIGURE 4: Effect of cyanide on electron transfer dependent membrane potentials. For each trace, ascorbate-loaded ghosts (710 μg of protein in 150 μL) were added to 2.0 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0, along with 10 μL of 1 mM ANS. KCN (10 μL of 50 mM) was added as indicated. At $t = 0$, ferricyanide-treated cytochrome *c* (10 μL of 1.44 mM) or $K_3Fe(CN)_6$ (10 μL of 100 mM) was added and fluorescence monitored at 37 °C.

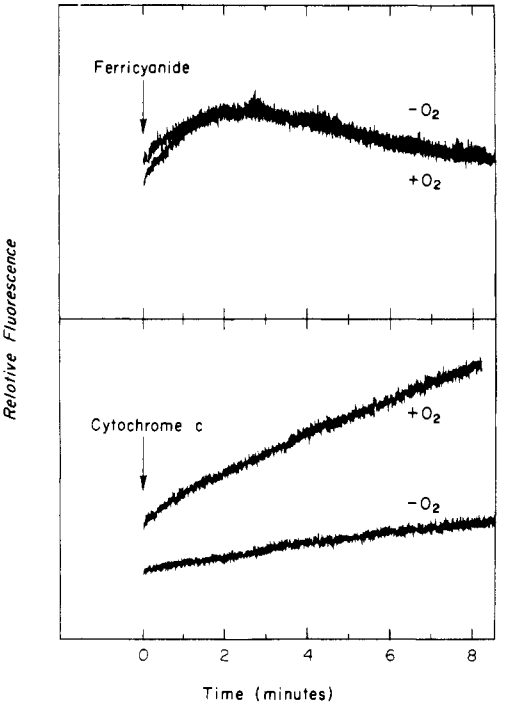

FIGURE 5: Effect of anoxia on electron transfer dependent membrane potentials. For each trace, 10 μL of 1 mM ANS was added to 2.0 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0. Ferricyanide-treated cytochrome *c* (10 μL of 1.37 mM) or $K_3Fe(CN)_6$ (10 μL of 100 mM) was also added, and the mixture was evacuated and flushed with nitrogen in a sealed cuvette. At $t = 0$, 25 μL of ascorbate-loaded ghosts (97 μg of protein) was added by injection through a serum stopper and fluorescence was monitored at 23 °C. Traces with O_2 were obtained by readmitting air to the cuvette after the evacuation procedure.

oxidase inhibitor, and should require O_2. In accordance with these expectations, KCN abolishes the cytochrome *c* dependent membrane potential but has no effect on the ferricyanide-dependent potential (Figure 4). Moreover, anoxia abolishes the response to cytochrome *c* but not to ferricyanide (Figure 5).

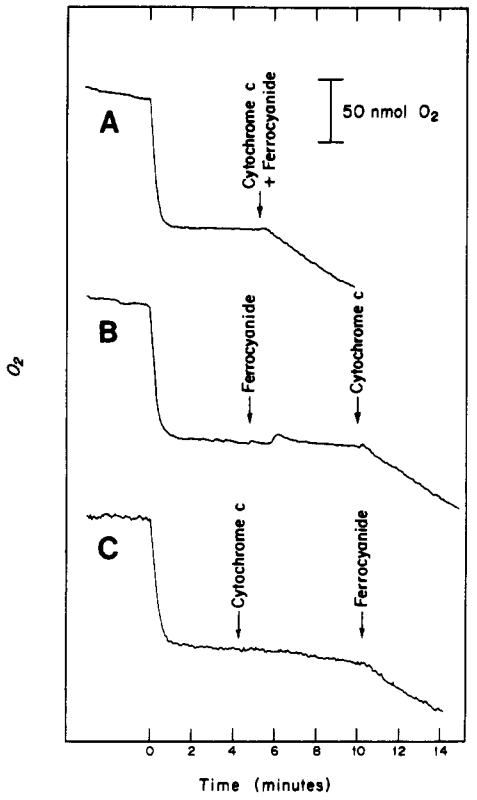

FIGURE 6: Oxygen consumption dependent on electron transfer. For each trace, the oxygen electrode chamber was filled with 1.24 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0, and 50 μL of ascorbate-loaded ghosts (413 μg of protein) at 26.5 °C. At $t = 0$, ascorbate oxidase (25 μL of 1 mg/mL) was added. Cytochrome c (30 μL of 6 mM) and ferrocyanide (30 μL of 6 mM) were added at the times indicated.

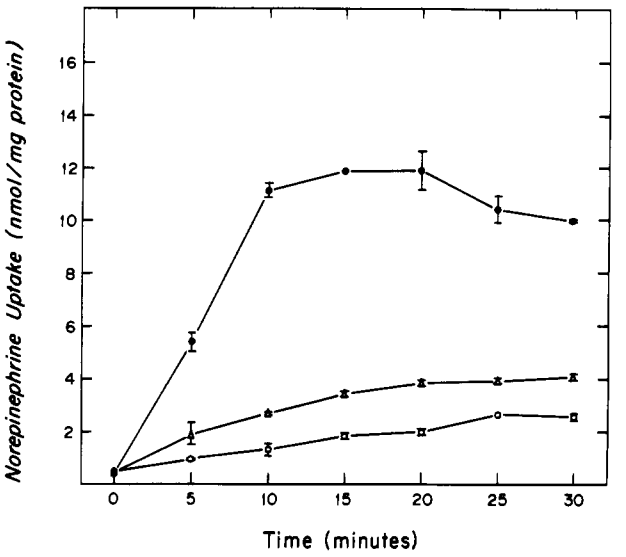

FIGURE 7: Norepinephrine transport driven by the electron transfer dependent membrane potential. Ascorbate-loaded ghosts (3.13 mg of protein in 200 μL) were added to 8.0 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0, along with 20 μL of 6 mM cytochrome c (△), 6 mM ferrocyanide (○), or both (●). At $t = 0$, 50 μL of 5 mM [^{3}H]norepinephrine (2.5 μCi) was added. The suspension was incubated at 37 °C, and 1-mL samples were collected by filtration at the times indicated. Each point is the average (±SD) of values obtained in two separate experiments.

Since cytochrome oxidase is coupled to the electron-transfer system, we should be able to measure oxygen consumption accompanying electron transfer. As shown in Figure 6, cytochrome c addition initiates oxygen consumption at a rate of about 12 nmol/min. This rate is constant for several minutes, indicating that the redox state of cytochrome c quickly reaches a steady state. Oxygen consumption occurs slowly in the absence and rapidly in the presence of ferrocyanide. Ferrocyanide does not stimulate cytochrome oxidase activity (data not shown), so it must accelerate cytochrome c reduction by ascorbate-loaded ghosts. This is consistent with the stimulatory effect of ferrocyanide on the cytochrome c dependent membrane potential. Ferrocyanide is not acting as an electron donor for oxygen consumption: The amount of ferrocyanide added is insufficient to reduce the amount of O_2 taken up, and further addition of ferrocyanide after oxygen consumption has slowed does not stimulate further O_2 uptake (data not shown).

Chromaffin-vesicle ghost preparations contain some external ascorbate because the 100 mM ascorbate in which they are prepared is not completely removed by density gradient centrifugation. The amount of O_2 taken up following ascorbate oxidase addition (Figure 6) indicates that the ghost preparation contains about 4 mM external ascorbate or 4% of the original concentration. In Figure 3, ascorbate oxidase was added to the suspension medium to ensure that ferricyanide and cytochrome c would be reduced only by electron transfer from intravesicular ascorbate. There should be ~80 nmol of external ascorbate in this experiment. In the absence of ascorbate oxidase, this should reduce added ferricyanide, increasing the amount of ferricyanide required to elicit the maximum membrane potential. External ascorbate should have less effect on the cytochrome c dependent membrane potential since cytochrome c is reoxidized by cytochrome oxidase. These differences are observed when ascorbate oxidase is omitted (data not shown).

To assess mitochondrial contamination in the chromaffin-vesicle ghost preparation, we assayed cytochrome oxidase and succinic dehydrogenase at 25 °C. Cytochrome oxidase activity in three preparations averaged 16 ± 3 mL (mg of protein)$^{-1}$, while succinic dehydrogenase activity averaged 31 ± 4 nmol min^{-1} (mg of protein)$^{-1}$.

The presence of mitochondrial membranes in the chromaffin-vesicle ghost preparation raises the possibility that the electron transfer dependent membrane potential is generated across a membrane other than the chromaffin-vesicle membrane. To show that electron transfer is truly across the chromaffin-vesicle membrane, we tested the ability of the membrane potential to drive norepinephrine transport. When cytochrome c is added externally, ascorbate-loaded chromaffin-vesicle ghosts take up [^{3}H]norepinephrine if ferrocyanide is present (Figure 7). Since the internal volume of the ghosts is about 3 μL/mg of protein (Knoth et al., 1981b), the norepinephrine concentration gradient reaches a value of ~130 following the addition of cytochrome c and ferrocyanide. This uptake is inhibited by reserpine (Figure 8). Since reserpine-sensitive norepinephrine transport is a marker for chromaffin-vesicle membranes, electron transfer must be creating a potential difference across this membrane.

Discussion

A number of investigators (Njus et al., 1981b; Wakefield et al., 1982; Grouselle & Phillips, 1982) have suggested that the chromaffin vesicle may have a transmembrane electron-transfer system to import reducing equivalents. These reducing equivalents are needed by the intravesicular enzyme dopamine β-hydroxylase, a mixed-function oxidase that catalyzes the hydroxylation of dopamine to norepinephrine. In this reaction, one atom from molecular oxygen is inserted into dopamine and the other is reduced to H_2O. Although intravesicular ascorbic acid may serve as the immediate electron donor for

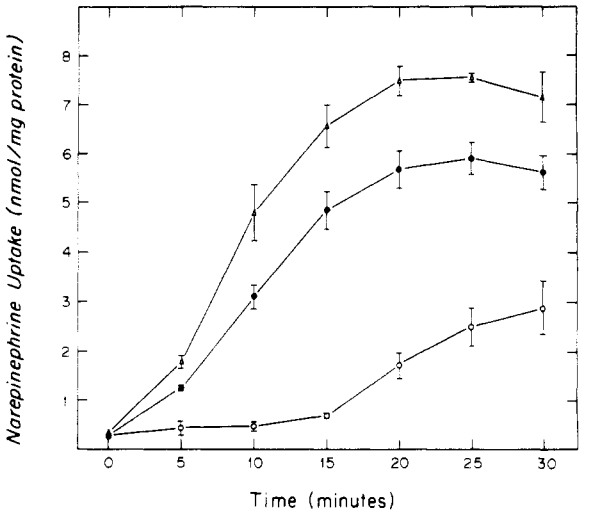

FIGURE 8: Inhibition of norepinephrine uptake by reserpine. Ascorbate-loaded ghosts (1.27 mg of protein in 200 μL) were added to 8.0 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0, along with 20 μL of 6.85 mM ferricyanide-treated cytochrome *c* and additions as specified below. At t = 0, 50 μL of 10 mM [^{3}H]norepinephrine (2.5 μCi) was added. The suspension was incubated at 37 °C, and 1-mL samples were collected by filtration at the times indicated. Each point is the average (±SD) of values obtained in two separate experiments. (△) No additions; (○) +200 nmol of reserpine in 20 μL of 60% acetone–40% ethanol (v/v); (●) +20 μL of solvent.

this reaction, the chromaffin vesicle must take up reducing equivalents to regenerate the ascorbate. A likely candidate for a transmembrane electron carrier is cytochrome *b*-561. This cytochrome is the second most abundant protein in the chromaffin-vesicle membrane and appears to span the membrane (Konig et al., 1976; Abbs & Phillips, 1980; Apps et al., 1980; Duong & Fleming, 1984). Moreover, cytochrome *b*-561 has a midpoint reduction potential (+100 to +140 mV) suitable for this function (Flatmark & Terland, 1971; Apps et al., 1984).

To test for an electron-transfer pathway across the chromaffin-vesicle membrane, we looked for the membrane potential that should be creatd by electron transfer from an internal electron donor to an external electron acceptor (Njus et al., 1983). Although the direction of electron transfer in this assay is opposite that presumed to occur in vivo, it is technically easier to trap a reductant inside chromaffin-vesicle ghosts than to trap an oxidant. We showed that an electron transfer dependent membrane potential could be generated by electron transfer from internal ascorbate to either external ferricyanide or external ferricytochrome *c*. At 4 °C, the membrane potential is stable and can be assayed either by measuring the distribution of the permeant SCN^- ion or by following the enhancement of ANS fluorescence (Njus et al., 1983). The fluorescent probe, although it is a qualitative indicator, can be used to follow the transient responses observed at higher temperatures. For that reason, we have used it in the work described here.

We show here that the membrane potential requires an internal electron donor since phosphate-loaded ghosts do not produce a response (Figure 2). Equally important is the demonstration that chromaffin-vesicle ghosts do not contain an endogenous electron donor. Although ghost membranes retain only about 5% of the 550 mM catecholamine present in intact chromaffin vesicles, this still represents a significant concentration of internal reducing equivalents. Catecholamines do not reduce the chromaffin-vesicle cytochrome *b*-561 because their midpoint reduction potential (+380 mV) is above that of the cytochrome (+100 to +140 mV). Therefore, the failure of residual catecholamine to serve as an internal electron donor is consistent with the involvement of cytochrome *b*-561 in electron transfer.

We reported before (Njus et al., 1983) that both ferricyanide and ferricytochrome *c* are suitable external electron acceptors. It is apparent, however, that cytochrome *c* does not function as a simple electron acceptor. First, cytochrome *c* acts as an intermediate electron carrier and is reoxidized by cytochrome oxidase. For that reason, cytochrome *c* is effective at lower concentrations and for longer times than ferricyanide. The inhibition of the cytochrome *c* dependent potential by cyanide (Figure 4) and by anoxia (Figure 5) confirms the involvement of cytochrome oxidase. Second, the rate at which cytochrome *c* accepts electrons is greatly accelerated by ferrocyanide. This is seen in the ferrocyanide requirement exhibited by the oxonol VI response (Figure 3), O_2 consumption (Figure 6), and norepinephrine uptake (Figure 7).

Ferrocyanide stimulates the rate of electron transfer from the chromaffin-vesicle membrane (presumably cytochrome *b*-561) to cytochrome *c*. Although free ferrocyanide has a midpoint reduction potential (E_0' = +420 mV) that is unfavorable for mediating electron flow from cytochrome *b*-561 (E_0' = +140 mV) to cytochrome *c* (E_0' = +255 mV), ferricytochrome *c* and ferrocyanide form a stable complex that enhances the rate of electron exchange between them (Stellwagen & Cass, 1975). This does not completely explain our ferrocyanide effect, however, since ferrocyanide produces maximal stimulation at concentrations less than equimolar with cytochrome *c*. Moreover, these ferrocyanide concentrations are on the order of 10 μM (Figures 3 and 7); this is far too low to give significant binding to the two ferrocyanide sites on cytochrome *c* that have association constants of 960 M^{-1} and 310 M^{-1} (Stellwagen & Cass, 1975). Instead, ferrocyanide may form a complex with cytochrome *b*-561, and this complex may interact with cytochrome *c*. We suggest that the external face of cytochrome *b*-561 interacts more favorably with small anions like $Fe(CN)_6^{4-}$ than with large cationic structures such as cytochrome *c*. This would be consistent with the hypothesis that cytochrome *b*-561 functions in vivo to accept electrons from cytosolic ascorbic acid.

The rate of electron transfer can be determined from the rate of oxygen consumption (Figure 6). In the presence of ferrocyanide, O_2 consumption was 12 nmol/min or 30 nmol min^{-1} (mg of protein)$^{-1}$. Each oxygen molecule accepts four electrons, so the electron-transfer rate is approximately 120 nequiv min^{-1} (mg of protein)$^{-1}$. This is comparable to the rate of H^+ translocation catalyzed by the chromaffin-vesicle ATPase, which has an activity of about 30 nmol min^{-1} (mg of protein)$^{-1}$ and a stoichiometry of two H^+ per ATP (Njus et al., 1978). Since the ATPase and electron transfer create comparable membrane potentials (Figure 1; Njus et al., 1983), the rate of electron transfer and the rate of H^+ translocation should be similar. This correspondence suggests further that the cytochrome *c* dependent membrane potential can be accounted for strictly in terms of transmembrane electron flow. Nevertheless, the possibility that some of the charge movement is mediated by a redox-driven ion pump, analogous to cytochrome oxidase (Krab & Wikstrom, 1978), cannot yet be excluded.

The cytochrome oxidase activity present in the chromaffin-vesicle ghost preparation is sufficient to mediate the observed electron flow. Given a cytochrome oxidase activity of 16 mL min^{-1} mg^{-1} and the cytochrome *c* concentrations used here (7 μM), cytochrome oxidase would oxidize 110 nmol of cytochrome *c* min^{-1} (mg of protein)$^{-1}$. Since both succinic

dehydrogenase activity and cytochrome oxidase activity are found in the chromaffin-vesicle ghost preparation, it is likely that the cytochrome oxidase activity is located on mitochondrial membranes contaminating the preparation.

The presence of mitochondrial membranes in the ghost preparation raises the possibility that the electron transfer dependent membrane potential is being generated across a membrane other than the chromaffin-vesicle membrane. The observation of electron transfer dependent norepinephrine transport (Figures 7 and 8) is a good indication that this is not the case, however, since reserpine-sensitive norepinephrine transport is a definitive marker for chromaffin-vesicle membranes.

We have shown that a stable membrane potential can be generated by electron transfer if ferrocyanide is present to catalyze electron transfer to cytochrome *c* and if cytochrome oxidase is allowed to reoxidize cytochrome *c*. The ferrocyanide dependence provides a good control for showing that cytochrome *c* dependent phenomena (e.g., norepinephrine uptake and O_2 consumption) involve electron transfer across the chromaffin-vesicle membrane. Coupling electron transfer to cytochrome oxidase introduces a way of measuring the rate of electron transfer. Finally, the observation of electron transfer dependent norepinephrine uptake proves that the chromaffin-vesicle membrane is the site of electron transfer and that the electron transfer dependent membrane potential can be used to drive energy-linked processes in these vesicles.

Registry No. Ascorbic acid, 50-81-7; isoascorbic acid, 89-65-6; ferricyanide, 13408-62-3; ferrocyanide, 13408-63-4; cytochrome *c*, 9007-43-6; oxygen, 7782-44-7; cyanide, 57-12-5.

REFERENCES

Abbs, M. T., & Phillips, J. H. (1980) *Biochim. Biophys. Acta 595*, 200–221.

Apps, D. K., Pryde, J. G., & Phillips, J. H. (1980) *Neuroscience 5*, 2279–2287.

Apps, D. K., Boisclair, M. D., Gavine, F. S., & Pettigrew, G. W. (1984) *Biochim. Biophys. Acta 764*, 8–16.

Bashford, C. L., & Thayer, W. S. (1977) *J. Biol. Chem. 252*, 8459–8463.

Bashford, C. L., Radda, G. K., & Ritchie, G. A. (1975) *FEBS Lett. 50*, 21–24.

Carmichael, S. W. (1983) *The Adrenal Medulla*, Vol. 3, Eden Press, Westmount, Quebec, Canada.

Casey, R. P., Njus, D., Radda, G. K., & Sehr, P. A. (1976) *Biochem. J. 158*, 583–588.

Cooperstein, S. J., & Lazarow, A. (1951) *J. Biol. Chem. 189*, 665–670.

Drake, R. A. L., Harvey, S. A. K., Njus, D., & Radda, G. K. (1979) *Neuroscience 4*, 853–861.

Duong, L. T., & Fleming, P. J. (1982) *J. Biol. Chem. 257*, 8561–8564.

Duong, L. T., & Fleming, P. J. (1984) *Arch. Biochem. Biophys. 228*, 332–341.

Flatmark, T., & Terland, O. (1971) *Biochim. Biophys. Acta 253*, 487–491.

Grouselle, M., & Phillips, J. H. (1982) *Biochem. J. 202*, 759–770.

Johnson, R. G., Carty, S. E., & Scarpa, A. (1981) *J. Biol. Chem. 256*, 5773–5780.

Kirshner, N. (1962) *J. Biol. Chem. 237*, 2311–2317.

Knoth, J., Isaacs, J. M., & Njus, D. (1981a) *J. Biol. Chem. 256*, 6541–6543.

Knoth, J., Zallakian, M., & Njus, D. (1981b) *Biochemistry 20*, 6625–6629.

Konig, P., Hortnagl, H., Kostron, H., Sapinsky, H., & Winkler, H. (1976) *J. Neurochem. 27*, 1539–1554.

Krab, K., & Wikstrom, M. K. (1978) *Biochim. Biophys. Acta 504*, 200–214.

Njus, D., & Radda, G. K. (1979) *Biochem. J. 180*, 579–585.

Njus, D., Sehr, P. A., Radda, G. K., Ritchie, G. A., & Seeley, P. J. (1978) *Biochemistry 17*, 4337–4343.

Njus, D., Knoth, J., & Zallakian, M. (1981a) *Curr. Top. Bioenerg. 11*, 107–147.

Njus, D., Zallakian, M., & Knoth, J. (1981b) in *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P., & Hinkle, P. C., Eds.) pp 365–374, Addison-Wesley, Reading, MA.

Njus, D., Knoth, J., Cook, C., & Kelley, P. M. (1983) *J. Biol. Chem. 258*, 27–30.

Pennington, R. J. (1961) *Biochem. J. 80*, 649–654.

Russell, J. T., Levine, M., & Njus, D. (1985) *J. Biol. Chem.* (in press).

Smith, J. C., & Chance, B. (1979) *J. Membr. Biol. 46*, 255–282.

Stellwagen, E., & Cass, R. D. (1975) *J. Biol. Chem. 250*, 2095–2098.

Ungar, A., & Phillips, J. H. (1983) *Physiol. Rev. 63*, 787–843.

Wakefield, L. M., Cass, A. E., & Radda, G. K. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 893.

Winkler, H., & Westhead, E. (1980) *Neuroscience 5*, 1803–1823.