Semidehydroascorbic Acid as an Intermediate in Norepinephrine Biosynthesis in Chromaffin Granules*

(Received for publication, February 26, 1991)

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We investigated whether semidehydroascorbic acid was an intermediate in norepinephrine synthesis in chromaffin granules and in electron transfer across the chromaffin granule membrane. Semidehydroascorbic acid was measured in intact granules by electron spin resonance. In the presence of intragranular but not extragranular ascorbic acid, semidehydroascorbic acid was formed within granules in direct relationship to dopamine β-monoxygenase activity. However, semidehydroascorbic acid was not generated when granules were incubated with epinephrine instead of the substrate dopamine, with dopamine β-monoxygenase inhibitors, without oxygen, and when intragranular ascorbic acid was depleted. Experiments using the impermeant paramagnetic broadening agents \[K_2[Cr(C_2O_4)_3]-3H_2O\] and Ni(en)_3(NO_3)_2 provided further evidence that semidehydroascorbic acid was generated only within granules. We also investigated semidehydroascorbic acid formation in the presence of intragranular and extragranular ascorbic acid. Under these conditions, semidehydroascorbic acid was formed on both sides of the granule membrane, and formation was coupled to dopamine β-monoxygenase activity. These data indicate that dopamine β-monoxygenase is reduced by single electron transfer from intragranular ascorbic acid, that transmembrane electron transfer occurs by single electron transfer, and that transmembrane electron transfer is directly coupled to formation of intragranular semidehydroascorbic acid via dopamine β-monoxygenase activity.

Ascorbic acid is required by the enzyme dopamine β-monoxygenase for synthesis of the catecholamine hormone norepinephrine (1, 2). Ascorbic acid's mechanism of action is different for the isolated enzyme as compared to the enzyme in chromaffin secretory granules. For the isolated enzyme there is indirect evidence that ascorbic acid can reduce dopamine β-monoxygenase by two transfers of single electrons (3–5). However, the specific mechanism of reduction is unknown.

For the enzyme in intact secretory granules, the role of ascorbic acid is more complex. The concentration of ascorbic acid is approximately 10 mM in chromaffin granules (6, 7), and dopamine β-monoxygenase is localized exclusively within granules (8–10). Intragranular ascorbic acid is essential for dopamine β-monoxygenase activity (11). If no external ascorbic acid is present, intragranular ascorbic acid is consumed in a 1:1 ratio with norepinephrine biosynthesis. When all the intragranular ascorbic acid has been consumed, norepinephrine biosynthesis stops. In the presence of extragranular ascorbic acid, however, intragranular ascorbic acid is maintained and norepinephrine biosynthesis continues, even though ascorbic acid does not enter granules (11). Based on these data, we and others have postulated that there is a mechanism by which external ascorbic acid transfers electrons across the chromaffin granule membrane to regenerate intragranular ascorbic acid (12–17). This mechanism could involve a protein-mediated electron shuttle across the chromaffin granule membrane.

In intact granules, however, our understanding of transmembrane electron transfer is limited. From experiments on chromaffin granule ghosts and phospholipid vesicles, cytochrome b$_{552}$ has been proposed to be the transmembrane protein which transfers electrons from external ascorbic acid to regenerate the intragranular vitamin (12–15, 18). Based on experiments in chromaffin granule ghosts, intragranular semidehydroascorbic acid was proposed as an electron acceptor (13). Nevertheless, the specific electron acceptor intermediate within intact granules remains unknown. If reduction of this intermediate occurs by single electron transfer, then the intragranular electron acceptor should be semidehydroascorbic acid. If reduction of the intermediate occurs by the simultaneous transfer of 2 electrons, semidehydroascorbic acid should not be formed. In addition, an oxidized intermediate must be generated by external ascorbic acid as it provides electrons for transmembrane electron transfer: the identity of this species is also unknown. If single electron transfer occurs, the extragranular intermediate should be semidehydroascorbic acid, and, if 2 electrons are transferred concurrently, semidehydroascorbic acid should not be detected.

Independent of external ascorbic acid, semidehydroascorbic acid could also be generated during norepinephrine biosynthesis as intragranular ascorbic acid reduces dopamine β-monoxygenase. Within the intact granule it is not known whether ascorbic acid donates one or two electrons at a time to dopamine β-monoxygenase. Again, if the enzyme was reduced in single electron steps, semidehydroascorbic acid should be detected; if the enzyme was reduced by 2 electrons at one time from ascorbic acid, semidehydroascorbic acid should not be present.

The simplest explanation to these problems is to couple the process of transmembrane electron transfer to dopamine β-monoxygenase activity in situ. In this scheme (Fig. 1), intragranular semidehydroascorbic acid would be formed by transfer of a single electron from intragranular ascorbic acid to dopamine β-monoxygenase. Semidehydroascorbic acid would then be available to accept an electron transferred across the granule membrane from external ascorbic acid.
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**Fig. 1. A model for transmembrane electron transfer and dopamine β-monoxygenase reduction in chromaffin granules.** Intragranular ascorbic acid reduces dopamine β-monoxygenase by single electron transfer, with formation of semidehydroascorbic acid. In the presence of external ascorbic acid, intragranular semidehydroascorbic acid can be reduced by transmembrane electron transfer. There are 2 steps in the model, indicating that dopamine β-monoxygenase is reduced twice by a single electron. Although oxygen and dopamine are shown associated with the enzyme during its reduction, the precise interaction is unknown. See text for details of charge balance and energy requirements. The abbreviations used are: AA, ascorbic acid; SDA, semidehydroascorbic acid; D/M, dopamine β-monoxygenase.

Dopamine β-monoxygenase would be reduced in two steps of single electron transfer for norepinephrine biosynthesis. The model is consistent with the known stoichiometry of 1 ascorbic acid consumed for 1 norepinephrine formed (11).

To investigate the mechanism proposed in Fig. 1, we measured semidehydroascorbic acid (ascorbate free radical) in intact chromaffin granules by electron spin resonance, a technique which is specific for detecting this radical (19). We investigated semidehydroascorbic acid formation both inside and outside of intact granules as a function of dopamine β-monoxygenase activity, with and without added external ascorbic acid. These experiments provide the first experimental evidence in intact granules that semidehydroascorbic acid may be the electron acceptor for transmembrane electron transfer, that transmembrane electron transfer may be coupled to dopamine β-monoxygenase activity, and that dopamine β-monoxygenase may be reduced by single electron transfer in situ.

**MATERIALS AND METHODS**

**Preparation of Chromaffin Granules**—Chromaffin granules were prepared immediately prior to use from fresh bovine adrenal medulla as described earlier (11, 20). The granules were suspended in 0.3 M sucrose, 55 mM HEPES buffer, pH 6.8, containing 0.033 mg/ml catalase (final concentration). The protein concentration of the granules was approximately 8–10 mg/m1.

**Assays**—[3H]Norepinephrine biosynthesis was measured by a combination of HPLC and scintillation spectrometry (11, 21, 22). Ascorbic acid was measured by HPLC with coulometric electrochemical detection (23). Protein was determined using the Bradford method as described (26), in the concentration range of 2–30 pmol/100 μl.

**Chemicals**—Ascorbic acid, ATP, catalase, dopamine, epinephrine, norepinephrine, dithiothreitol, diethyldithiocarbamic acid, HEPES, and tyramine were purchased from Sigma. [3H]Dopamine was obtained from Du Pont-New England Nuclear. Ni(en)3(NO3)2 was a gift from Dr. Lalage Wakefield (National Cancer Institute). Tris(oxalato) chromate III [K12[Cr(C2O4)3]·3H2O] was prepared as described (27).

**RESULTS**

Semidehydroascorbic Acid Generation within Chromaffin Granules—We investigated formation of semidehydroascorbic acid within intact granules under conditions of norepinephrine biosynthesis in the absence of external ascorbic acid. As shown in Fig. 2A, addition of dopamine and MgATP to the granules gave rise to a two-line ESR spectrum (αH = 1.7 G) typical of the ascorbate free radical. This finding suggests that semidehydroascorbic acid is generated within granules during norepinephrine biosynthesis. The formation of semidehydroascorbic acid was then studied under several control conditions. There was a small signal of semidehydroascorbic acid from the granules alone (Fig. 2B). This signal was...
sistent with the presence of endogenous dopamine, which was ≤10 nmol/mg of protein. The signal was slightly larger when granules were incubated with MgATP (Fig. 2C). This could be accounted for by the effect of MgATP on dopamine β-monoxygenase activity in intact chromaffin granules (22).

Similarly, a small signal was also seen when the granules were incubated with dopamine alone (Fig. 2D). This change in the presence of substrate was also predicted, due to an approximately 10-fold increase in dopamine content in granules incubated under these conditions (data not shown). These data suggest that semidehydroascorbic acid formation is coupled to dopamine β-monoxygenase activity.

If semidehydroascorbic acid is generated within granules during norepinephrine biosynthesis, an ESR signal should be detected under optimum conditions of dopamine β-monoxygenase activity. Likewise, the signal should disappear under conditions when either dopamine β-monoxygenase is inhibited by specific inhibitors or the enzyme cannot function because of the wrong substrate. Results of such experiments are shown in Fig. 3. The experimental conditions for Figs. 3, A and B, are favorable for norepinephrine biosynthesis; in Fig. 3A, granules were incubated with dopamine and MgATP; and, in Fig. 3B, granules were incubated with the artificial substrate tyramine and MgATP. In both cases, semidehydroascorbic acid was generated. In Fig. 3C, granules were incubated with MgATP and epinephrine. Since dopamine β-monoxygenase does not use epinephrine as substrate, there should be little or no norepinephrine biosynthesis under these conditions and, hence, little or no signal for semidehydroascorbic acid. The spectrum in Fig. 3C shows that semidehydroascorbic acid is not formed. We then incubated the granules with inhibitors of dopamine β-monoxygenase (28) and measured formation of semidehydroascorbic acid. The ESR spectra in the presence of dithiothreitol (Fig. 3D) and diethylidithiocarbamate (Fig. 3E) show that the semidehydroascorbic acid signals were decreased. Norepinephrine biosynthesis in the same experiments is shown in Table I. Both inhibitors almost completely inhibited norepinephrine biosynthesis. The finding could not be due to granule lysis or ascorbic acid oxidation, as granules remained intact as indicated by total catecholamines and intragranular ascorbic acid (Table I). These spectra (Fig. 3, A–E) suggest that semidehydroascorbic acid generation is coupled to dopamine β-monoxygenase activity. When the enzyme is inactive, semidehydroascorbic acid is not formed, regardless of intragranular ascorbic acid.

Dopamine β-monoxygenase is a copper containing mixed function oxidase that converts dopamine to norepinephrine only in the presence of oxygen (1, 2, 29, 30). If formation of semidehydroascorbic acid within granules is a function of dopamine β-monoxygenase activity, then without oxygen the signal for semidehydroascorbic acid should disappear. We therefore incubated granules with MgATP and dopamine in gas-permeable Teflon capillary tubing (used in all of these experiments), and semidehydroascorbic acid formation was measured under incubation conditions of room air (Fig. 4A). A second spectrum (Fig. 4B) was obtained while argon was flowed around the same sample. For the third spectrum (Fig. 4C), the argon flow was stopped, and samples were reoxygenated with room air. Semidehydroascorbic acid was generated only when oxygen was present. These data again suggest that the generation of semidehydroascorbic acid within granules is coupled to dopamine β-monoxygenase activity, and, under all conditions when the enzyme cannot catalyze the reaction, semidehydroascorbic acid is not formed.

When chromaffin granules are incubated with MgATP and dopamine, norepinephrine biosynthesis proceeds linearly for 30 min (11, 22). Although intragranular ascorbic acid is consumed, its concentration remains above the in situ value of dopamine β-monoxygenase (11). As intragranular ascorbic acid continues to be consumed and its concentration decreases, norepinephrine biosynthesis decreases. Norepinephrine biosynthesis is no longer detectable as intragranular ascorbic acid falls below 100 μM (11). We therefore incubated granules with MgATP and dopamine and recorded ESR spectra at different time intervals (Fig. 5). We also measured intragranular ascorbic acid under these conditions (Fig. 6). At 90 min, as intragranular ascorbic acid was slightly above the in situ value for ascorbic acid (Fig. 6), the generation of semidehydroascorbic acid began to decrease (Fig. 5B). At 135 min, intragranular ascorbic acid was below in situ level (Fig. 6), and the generation of semidehydroascorbic acid also decreased (Fig. 5C). Beyond 150 min, intragranular ascorbic acid could not be detected, and the semidehydroascorbic acid signal was also not seen (Fig. 5D). This suggests that intragranular semidehydroascorbic acid is not generated when norepinephrine synthesis ceases, in this case due to depletion of intragranular ascorbic acid.

All of these experiments suggest that semidehydroascorbic acid was generated only within chromaffin granules rather than outside granules. Ascorbic acid was present at a high concentration in granules under a variety of conditions, yet semidehydroascorbic acid was generated only when dopamine β-monoxygenase was active. Since the enzyme is localized exclusively to the chromaffin granule interior, no external ascorbic acid was present, and the granules did not lyse, semidehydroascorbic acid had to be formed inside the granules. In addition, the rate of efflux of ascorbic acid under these incubation conditions is very low, so that ascorbic acid would not be available outside granules for formation of semidehydroascorbic acid (11). Nevertheless, additional experiments were performed to confirm that semidehydroascor-
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TABLE I
Effect of dithiothreitol (DTT) and diethyldithiocarbamate (DDC) on [3H]norepinephrine biosynthesis, intragranular ascorbic acid, and total catecholamines

Chromaffin granules were incubated for 30 min with 1 mM MgATP, 10 mM [3H]dopamine, and 2 mM concentration of listed inhibitors. Ascorbic acid, total catecholamines, and [3H]norepinephrine biosynthesis were measured as described under "Materials and Methods."

<table>
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<tr>
<th>Compound</th>
<th>[3H]Norepinephrine biosynthesis (nmol/30 min/mg protein)</th>
<th>Ascorbic acid (mM)</th>
<th>Total catecholamines (nmol/mg protein)</th>
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<tr>
<td>None</td>
<td>25.0 ± 1.5</td>
<td>6.4 ± 0.5</td>
<td>1374 ± 61</td>
</tr>
<tr>
<td>DTT</td>
<td>2.4 ± 0.5</td>
<td>10.1 ± 0.4</td>
<td>1292 ± 52</td>
</tr>
<tr>
<td>DDC</td>
<td>5.0 ± 0.6</td>
<td>10.0 ± 0.2</td>
<td>1305 ± 48</td>
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Fig. 4. Generation of semidehydroascorbic acid in the presence and absence of room air. Granules were mixed with 1 mM MgATP and 10 mM dopamine, and the spectrum was recorded in the presence of room air (A). Argon was then flowed around the sample, and the spectrum was recorded (B). The argon was then withdrawn, and the spectrum (C) was recorded in the presence of room air. The amount of semidehydroascorbic acid formed was (picomoles/mg of protein): A, 1.88; B, <0.10; C, 2.15.

Fig. 5. Disappearance of semidehydroascorbic acid signal with time. Chromaffin granules were mixed with 1 mM MgATP and 10 mM dopamine and incubated at 37 °C. At different times, a sample was taken for recording the ESR spectrum. A, 0 min; B, 90 min; C, 135 min; D, 200 min. The amount of semidehydroascorbic acid formed was (picomoles/mg of protein): A, 2.98; B, 1.60; C, 1.04; D, <0.10.

Fig. 6. Depletion of intragranular ascorbic acid. Chromaffin granules in buffer were incubated at 37 °C with 1 mM MgATP and 10 mM dopamine. Samples were withdrawn at indicated times for ascorbic acid determination.

Fig. 7. Generation of semidehydroascorbic acid in the presence of [K₃[Cr(C₂O₄)₃]·3H₂O] and Ni(en)₃(NO₃)₂. The amount of semidehydroascorbic acid formed was (picomoles/mg of protein): A, 2.16; B, 1.88; C, 2.43. A, granules + 1 mM MgATP + 10 mM dopamine; B, granules + 1 mM MgATP + 10 mM dopamine + 2.5 mM [K₃[Cr(C₂O₄)₃]·3H₂O]; C, granules + 1 mM MgATP + 10 mM dopamine + 3.0 mM Ni(en)₃(NO₃)₂.

Membrane-permeant, they can be used to eliminate the extragranular semidehydroascorbic acid. Thus, intragranular and extragranular semidehydroascorbic acid signals can be distinguished. [K₃[Cr(C₂O₄)₃]·3H₂O], 2.5 mM, or Ni(en)₃(NO₃)₂, 3.0 mM, eliminated the ascorbate free radical signal when the ascorbate free radical was generated without granules, using ascorbic acid/Fe³⁺/Cu²⁺ (data not shown). We then incubated granules with MgATP, dopamine, and either [K₃[Cr(C₂O₄)₃]·3H₂O] (Fig. 7B), Ni(en)₃(NO₃)₂ (Fig. 7C), or no line-broadening agent (Fig. 7A). The ascorbate free radical signal was virtually the same under all conditions. These experiments indicate that semidehydroascorbic acid is generated within chromaffin granules. As a control for granule integrity, we measured norepinephrine biosynthesis, total catecholamines,
and intragranular ascorbic acid in the presence of \([K_3\text{Cr(C}_2\text{O}_4)\text{]}_3 \cdot 3\text{H}_2\text{O}\). \([K_3\text{Cr(C}_2\text{O}_4)\text{]}_3 \cdot 3\text{H}_2\text{O}\) had no effect on any of these indices, indicating that the granules were intact and functional (Table II).

**Semidehydroascorbic Acid Generation Outside Chromaffin Granules**—Extragranular ascorbic acid transfers either 1 or 2 electrons across the chromaffin granule membrane to regenerate intragranular ascorbic acid, which is consumed during norepinephrine biosynthesis. If single electrons from external ascorbic acid undergo transmembrane transfer, semidehydroascorbic acid should be formed outside granules. To investigate this possibility, chromaffin granules were first incubated with MgATP and dopamine, and the spectrum was recorded (Fig. 8A). The signal represents semidehydroascorbic acid within granules, as described above. Granules were then incubated with MgATP, dopamine, and external ascorbic acid. The semidehydroascorbic acid signal obtained in this experiment was larger (Fig. 8D) than the signal without added ascorbic acid (Fig. 8A). The increased signal can be explained by extragranular semidehydroascorbic acid formation from the added ascorbic acid. To verify this, granules were incubated with MgATP, dopamine, ascorbic acid, and \([K_3\text{Cr(C}_2\text{O}_4)\text{]}_3 \cdot 3\text{H}_2\text{O}\). \([K_3\text{Cr(C}_2\text{O}_4)\text{]}_3 \cdot 3\text{H}_2\text{O}\) was used to selectively eliminate the extragranular semidehydroascorbic acid signal, without affecting the intragranular signal (see Fig. 7B above). In the presence of \([K_3\text{Cr(C}_2\text{O}_4)\text{]}_3 \cdot 3\text{H}_2\text{O}\) (Fig. 8C), the semidehydroascorbic acid signal was indistinguishable from the condition of no external ascorbic acid (Fig. 8A). These data indicate that semidehydroascorbic acid is also formed outside of chromaffin granules in the presence of extragranular ascorbic acid.

It remained possible that the increased semidehydroascorbic acid signal in the presence of external ascorbic acid was due to simple air oxidation of added vitamin. To investigate this possibility, granules were incubated with MgATP and ascorbic acid without dopamine, and the spectrum was recorded. The semidehydroascorbic acid signal obtained (Fig. 8D) was similar to that obtained when granules were incubated with MgATP in the absence of ascorbic acid (see Fig. 2C). This suggests that under our experimental conditions the semidehydroascorbic acid signal is not due to air oxidation of external ascorbic acid.

**Semidehydroascorbic Acid Generation Outside and Inside Chromaffin Granules Is Coupled to Dopamine β-Monoxygenase Activity**—The formation of semidehydroascorbic acid outside chromaffin granules in the presence of external ascorbic acid could be coupled to electron transfer to intragranular semidehydroascorbic acid. If this is true, and, since formation of intragranular semidehydroascorbic acid is coupled to dopamine β-monoxygenase activity, then in the presence of external ascorbic acid semidehydroascorbic acid formation on both sides of the chromaffin granule membrane should be coupled to dopamine β-monoxygenase activity. Thus, if dopamine β-monoxygenase is inhibited, there should be no semidehydroascorbic acid signal either inside or outside chromaffin granules. To investigate this, chromaffin granules were first incubated with MgATP, dopamine, and ascorbic acid. The signal shown in Fig. 9A is the combined signal of semidehydroascorbic acid generated outside and inside the granules. The next spectrum (Fig. 9B) was obtained in the absence of oxygen, by flowing argon around the sample. Argon completely eliminated the semidehydroascorbic acid signal within 2 min. When the same sample was reoxygenated, the semidehydroascorbic acid signal reappeared (Fig. 9C) within 2 min. The chromaffin granules were then incubated with specific inhibitors of dopamine β-monoxygenase in the presence of MgATP, dopamine, and ascorbic acid. Both dithiothreitol and diethylthiobarbiturate completely eliminated the semidehydroascorbic acid signal (Fig. 9, D and E). These experiments show that when dopamine β-monoxygenase was inhibited there was no semidehydroascorbic acid formation on either side of the chromaffin granule membrane. The experiments with diethylthiobarbiturate provide additional evidence that semidehydroascorbic acid signal is not due to air oxidation of external ascorbic acid. Therefore, formation of both extragranular and intragranular semidehydroascorbic acid is coupled to dopamine β-monoxygenase activity.

**DISCUSSION**

We describe here semidehydroascorbic acid formation in intact chromaffin granules. In the absence of extragranular vitamin, semidehydroascorbic acid was generated only inside granules. Although ascorbic acid was present in millimolar concentrations within granules, semidehydroascorbic acid was formed only when intragranular dopamine β-monoxygenase was active. In the presence of external vitamin, semidehydroascorbic acid was generated inside and outside of chromaffin granules; generation of the radical on both sides of the granule membrane was again coupled to dopamine β-monoxygenase activity. Furthermore, generation of semidehydroascorbic acid outside granules was dependent on generation of the free radical within granules. We take these data to indicate that dopamine β-monoxygenase within chromaffin granules is reduced by single electrons from intragranular ascorbic acid. The intragranular semidehydroascorbic acid that is formed is then available for reduction by single electron transfer from external ascorbic acid, resulting in generation of semidehydroascorbic acid outside the chromaffin granule (see Fig. 1).

The data also indicate that reduction of intragranular dopamine β-monoxygenase is independent of external vitamin. If external vitamin is unavailable, semidehydroascorbic acid will still be formed as long as internal ascorbic acid is present to reduce dopamine β-monoxygenase. Two semidehydro-
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The amount of semidehydroascorbic acid formed was (picomoles/mg of protein): A, 2.43; B, 4.65; C, 2.43; D, 0.12. A, granules + 1 mM MgATP + 10 mM dopamine; B, granules + 1 mM MgATP + 10 mM dopamine + 1 mM ascorbic acid; C, granules + 1 mM MgATP + 10 mM dopamine + 1 mM ascorbic acid + 2.5 mM $\text{K}_2\text{[Cr(C_2O_4)S}_3\text{H}_2\text{O}]$; D, granules + 1 mM MgATP + 1 mM ascorbic acid.

corbid acid molecules can then disproportionate, and the net result is that intragranular ascorbic acid may be oxidized. If external vitamin is present, intragranular semidehydroascorbic acid may be reduced to ascorbic acid by transmembrane electron transfer.

These data confirm two independent hypotheses concerning semidehydroascorbic acid in chromaffin granules. One hypothesis involves reduction of dopamine $\beta$-monooxygenase. The enzyme was originally believed to be reduced by 2 electrons simultaneously, with formation of dehydroascorbic acid (30). Alternatively, since ascorbate oxidase oxidizes ascorbic acid to semidehydroascorbic acid (32), dopamine $\beta$-monooxygenase was proposed by others to oxidize ascorbic acid in an analogous manner (3, 29). Although indirect evidence using isolated enzyme preparations supported this concept (3-5), the data in this paper provide the first evidence for the reduction of dopamine $\beta$-monooxygenase in situ.

The second hypothesis addressed in this paper concerns the mechanism of transmembrane electron transfer in chromaffin granules. Although transmembrane electron transfer must occur, the mechanism is dependent on the number of electrons transferred. Using standard redox potentials, 2-electron transfer is more favorable, with formation of dehydroascorbic acid (33). Experiments using chromaffin granule ghosts suggest single electron transfer, with formation of semidehydroascorbic acid (14). However, there are disadvantages in using membrane ghosts, which can be avoided by using intact granules. Intact granules are a normal component of chromaffin cells, and the endogenous granule contents are not eliminated as in ghosts. Since the granules are intact and have not been disrupted and resealed, problems with incomplete resealing and sidedness of resealing are avoided. By using intact granules, endogenous dopamine $\beta$-monooxygenase can be the driving force for electron transfer, without complications of the line-broadening agent itself inducing formation of semidehydroascorbic acid (14). The experiments in this paper using intact granules indicate that semidehydroascorbic acid is the intermediate in transmembrane electron transfer in situ.

The data in this paper also confirm the stoichiometry in situ of the rates of norepinephrine formation and intragranular ascorbic acid consumption. Using intact chromaffin granules without external ascorbic acid, we have shown that norepinephrine biosynthesis matches intragranular ascorbic acid consumption in a 1:1 ratio (11). These findings are substantiated here. Norepinephrine biosynthesis in chromaffin granules incubated without external ascorbic acid was 25-26 nmol/mg of protein/30 min (Tables I and II). Ascorbic acid consumption over 30 min under identical conditions was approximately 5-6 mM (Fig. 6, Table II), corresponding to 22-26 nmol/mg of protein. Both norepinephrine formation and ascorbic acid consumption are linear for 30 min under these conditions (11). These data verify that intragranular ascorbic acid is consumed 1:1 as norepinephrine is formed in intact chromaffin granules.
For these stoichiometric calculations to be valid, there should be no replacement of the substrate as it is consumed, and no metabolism of semidehydroascorbic acid. For ascorbic acid consumption and norepinephrine formation, these conditions are satisfied, since without external ascorbic acid intragranular vitamin is not replaced as it is consumed, and norepinephrine is not further metabolized in isolated granules over 30 min. By contrast, similar calculations cannot be made to relate an amount of semidehydroascorbic acid to a rate of ascorbic acid consumption, and a rate of norepinephrine formation. Although amounts of semidehydroascorbic acid in granules have been determined, semidehydroascorbic acid amount is not equivalent to the rate of semidehydroascorbic acid formation nor the rate of its disappearance, since semidehydroascorbic acid as an intermediate is both formed and consumed. Thus, it is not possible to correlate the amount of semidehydroascorbic acid to the rate of norepinephrine formation, nor to calculate a stoichiometric relationship between semidehydroascorbic acid and norepinephrine formation.

Since semidehydroascorbic acid formation is minimal in the presence of external ascorbic acid without dopamine (Fig. 8D), it is unlikely that the radical is formed by comproportionation. Also, reaction conditions for formation of semidehydroascorbic acid by comproportionation are unfavorable (34). Under the conditions used in these experiments, the concentration of semidehydroascorbic acid formed by comproportionation would be well below the detection limit for semidehydroascorbic acid.

The experiments here support the mechanism of transmembrane electron transfer in Fig. 1. However, there are several issues which remain to be addressed. One point concerns charge balance across the chromaffin granule membrane. We have recently shown that regeneration of intragranular ascorbic acid is independent of MgATP and the membrane potential across the chromaffin granule membrane (35). Nevertheless, when transmembrane electron transfer occurs, a positive charge is required for charge balance, but the mechanism of charge balance is not known. The driving force for reduction of intragranular semidehydroascorbic acid also remains to be determined. An additional issue not addressed by the model is the fate of extragranular semidehydroascorbic acid. Although there are proposals that semidehydroascorbic acid dismutates, with further rapid metabolism of dehydroascorbic acid. An additional issue not addressed by the model is the fate of extragranular, semidehydroascorbic acid. Although there are proposals that semidehydroascorbic acid dismutates, with further rapid metabolism of dehydroascorbic acid. Another possibility is that extragranular semidehydroascorbic acid formation, nor the rate of semidehydroascorbic acid consumption and norepinephrine formation, these conditions are satisfied, since without external ascorbic acid intragranular vitamin is not replaced as it is consumed, and norepinephrine is not further metabolized in isolated granules over 30 min. By contrast, similar calculations cannot be made to relate an amount of semidehydroascorbic acid to a rate of ascorbic acid consumption, and a rate of norepinephrine formation. Although amounts of semidehydroascorbic acid in granules have been determined, semidehydroascorbic acid amount is not equivalent to the rate of semidehydroascorbic acid formation nor the rate of its disappearance, since semidehydroascorbic acid as an intermediate is both formed and consumed. Thus, it is not possible to correlate the amount of semidehydroascorbic acid to the rate of norepinephrine formation, nor to calculate a stoichiometric relationship between semidehydroascorbic acid and norepinephrine formation.

Acknowledgments—We thank Dr. Murli Krishna Cherukuri for his helpful suggestions and Dr. Peter Riesz for the use of his ESR facilities.

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