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SHORT COMMUNICATIONS

Semidehydroascorbate as a Product of the Enzymic Conversion of Dopamine to Norepinephrine

Coupling of Semidehydroascorbate Reductase to Dopamine-β-Hydroxylase

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SUMMARY

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Dopamine-β-hydroxylase, a copper monooxygenase, catalyzes the ascorbate-dependent hydroxylation of dopamine to norepinephrine. In the transfer of two electrons from ascorbate to dopamine-β-hydroxylase, the immediate product of enzyme reduction is not dehydroascorbate, as previously thought, but rather semidehydroascorbate, a free radical. Using the NADH-dependent semidehydroascorbate reductase preparation from rat liver microsomes, generation of semidehydroascorbate during dopamine-β-hydroxylation was observed. In the presence of catalytic amounts of ascorbate, a coupling of dopamine-β-hydroxylase to semidehydroascorbate reductase has been demonstrated: Regeneration of active cofactor is observed by the catalytic oxidation of NADH and formation of β-hydroxylated product; delayed addition of the reductase results in a decrease in the rate of NADH oxidation; and, with further delay, no oxidation of NADH is observed, indicating the lability of the free radical intermediate. At low rates of hydroxylation, a stoichiometric oxidation of NADH was obtained in the presence of the reductase during the formation of octopamine from tyramine. Semidehydroascorbate reductase was found in membrane fractions of the bovine adrenal medulla but not in the soluble fractions. Tyramine-dependent oxidation of NADH, inhibited by fusaric acid and stimulated by fumarate, can be observed using partially purified chromaffin vesicle membranes or large granular fraction membranes. These results are consistent with a role for semidehydroascorbate reductase in dopamine-β-hydroxylation through regeneration of the active cofactor, ascorbate.

INTRODUCTION

Dopamine-β-hydroxylase (EC 1.14.17.1; DBH[1]), a mixed-function oxidase, catalyzes the final step in the biosynthesis of norepinephrine (1). The putative external electron donor for this system is ascorbate. β-Hydroxylation of dopamine was previously described by a reaction mechanism requiring the transfer of two electrons from one molecule of ascorbate to enzyme-bound Cu^{2+} ions with the release of dehydroascorbate; this reduced form of the enzyme was then reoxidized by molecular oxygen and substrate with the formation of the β-hydroxylated product and water (2).

Despite the fact that dehydroascorbate has been accepted as a product of dopamine-β-hydroxylation, there is no evidence for the presence of a system for the reduction of dehydroascorbate to ascorbate in the adrenal medulla or noradrenergic neurons. In fact, the high requirement of dietary ascorbate suggests that mammals may not have a system to regenerate active cofactor from dehydroascorbate.

Recent studies on the mechanism of catechol oxidation by DBH suggested that catalytic oxidation of catechol resulted from the formation of a partially reduced enzyme/catechol semiquinone complex, a species that may undergo autooxidation to regenerate the oxidized form of the enzyme (3). These results and the evidence that several one-electron transferring agents support β-hydroxylation implied that one-electron transfer from reducing agent to enzyme may occur (3).

Studies on the purification and characterization of a

[1] Abbreviations used: DBH, dopamine-β-hydroxylase; SDR, semidehydroascorbate reductase; NADH, nicotinamide adenine dinucleotide, reduced.

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quinone reductase by Nason's laboratory (4) led to the discovery of a rat liver enzyme, semidehydroascorbate reductase (EC 1.6.5.4; SDR[1]), localized on microsomal membranes, which catalyzes the NADH[1]-dependent reduction of semidehydroascorbate to ascorbate (5).

In the present paper we report results which indicate that the product of DBH reduction (first step in the hydroxylation reaction) is not dehydroascorbate, as previously thought, but rather semidehydroascorbate, a free radical. The finding of SDR in membrane fractions prepared from the adrenal medulla suggests a role for this enzyme in dopamine-β-hydroxylation to regenerate the reduced form of the cofactor, ascorbate. A preliminary report of these findings has been presented (6).

METHODS

Semidehydroascorbate reductase activity was measured spectrophotometrically (Beckman, Acta MVI) by the disappearance of absorbance at 340 nm (7). In this assay, the free radical, semidehydroascorbate, was generated by mixing ascorbate and dehydroascorbate; formation of semidehydroascorbate by this method has been confirmed using electron paramagnetic resonance spectroscopy (8, 9). The reaction mixture contained 0.1 mM NADH, 10 mM ascorbate, 7.5 mM dehydroascorbate (ICN Pharmaceuticals, Inc.), and 100 mM Tris–HCl buffer, pH 7.4, in a final volume of 1 ml. The reaction was carried out at 30°C and was initiated by the addition of the enzyme. Rat liver microsomal SDR was prepared by a modification of the procedure of Schulze and Staudinger (10) and extracted with an acetone/water mixture (9:1) according to the method of Lester and Fleischer (11). The partially inactive SDR of extracted microsomes was reactivated by the addition of 0.5 mM Triton X-45 or Triton X-114 (10). SDR activity was also assayed by a procedure adapted from an observation by Wosilait *et al.* (4). In this procedure, semidehydroascorbate is generated by the ascorbate oxidase-catalyzed oxidation of ascorbate (12). The incubation mixture is identical to that stated previously with the substitution of ascorbate oxidase for dehydroascorbate; the concentration of ascorbate oxidase was adjusted to give the maximal enzyme rate. Ascorbate oxidase was purified from green zucchini squash (*Cucurbita pepomedullosa*) according to the method of Avigliano *et al.* (13).

Dopamine-β-hydroxylase activity was measured spectrophotometrically using pyrocatechol as the external electron donor in place of ascorbate: The *O*-benzoquinone generated in this reaction is reduced nonenzymically by NADH and the oxidation of the latter compound is followed by the decrease in absorbance at 340 nm (14). The reaction mixture contained 20 mM fumarate, 100 mM Tris–HCl buffer, pH 7.0, 0.1 mM NADH, 10 mM pyrocatechol, 5 mM tyramine, 60 μg/ml catalase, and the enzyme preparation to be assayed in a final volume of 1 ml; incubations were carried out at 30°C. DBH was also assayed radiometrically by a modification of the procedure of Friedman and Kaufman (2). The reaction mixture contained the components listed previously except that 10 mM ascorbate and [*side chain*-2-^{14}C]tyramine (Amersham Corp.), 1.0 μCi, were added in place of pyrocatechol.

To measure the coupling of semidehydroascorbate reductase to dopamine-β-hydroxylase, β-hydroxylation was measured as described for DBH activity except that semidehydroascorbate reductase, 10 mM ascorbate, and 0.5 mM Triton X-114 were added to the incubation mixture in place of pyrocatechol. A tyramine-dependent oxidation of NADH was followed by the disappearance of absorbance at 340 nm. Due to the presence of endogenous inhibitors of DBH in the SDR preparation, Cu^{2+} was added to the incubation mixture to give maximal stimulation. Bovine adrenal medullary DBH was prepared by a modification of the method of Ljones *et al.* (15).

Monoamine oxidase activity, catecholamines, and proteins were determined as previously described (16).

For the subcellular distribution studies, the medullae from fresh bovine adrenal glands were dissected and homogenized in 0.25 M sucrose containing 1 mM EDTA and 20 mM Tris–HCl, pH 7.4. Subcellular fractions of the adrenal medulla were prepared by differential and discontinuous sucrose gradient centrifugations as previously described (16).

RESULTS AND DISCUSSION

If semidehydroascorbate is a product of hydroxylation, then SDR could play a role in regeneration of the cofactor for β-hydroxylation. Using the rat liver SDR preparation, formation of the free radical, semidehydroascorbate, was examined. A typical experiment illustrating NADH oxidation with purified DBH and pyrocatechol as the electron donor is shown in Fig. 1A; a DBH- and tyramine-dependent oxidation of NADH is observed. When SDR is incubated in the presence of the ascorbate/dehydroascorbate redox couple, NADH oxidation is observed, demonstrating an enzyme system which catalyzes the electron transfer from NADH to the free radical, semidehydroascorbate (Fig. 1B). In the presence of SDR and ascorbate, a tyramine-dependent oxidation of NADH is observed, suggesting the generation of semidehydroascorbate during dopamine-β-hydroxylation (Fig. 1C); no NADH oxidation is observed in the absence of SDR (see Table 2). Figure 1C also depicts the dependence of NADH oxidation on the concentration of DBH. Indeed, in other experiments, changes in the rate of β-hydroxylation, produced by varying the tyramine concentration, inhibiting DBH with fusaric acid, or activating DBH with the anion, fumarate, caused corresponding changes in the rate of NADH oxidation (Table 1).[2] Furthermore, at low rates of hydroxylation, a stoichiometric oxidation of NADH was obtained in the presence of SDR during the formation of octopamine from tyramine. One such experiment is depicted in Table 1; at 60 μM tyramine the ratio of octopamine formed to NADH oxidized is nearly unity. As the rate of β-hydroxylation is increased, there is an elevation in the ratio of octopamine formed to NADH oxidized. These results suggest a greater loss of semidehydroascorbate through dismutation with increasing rates of free radical generation. In a different experiment, the ratio of the rate of NADH oxidation with 4.0

[2] Diliberto, E. J., Jr., and Allen, P. L., unpublished observations.

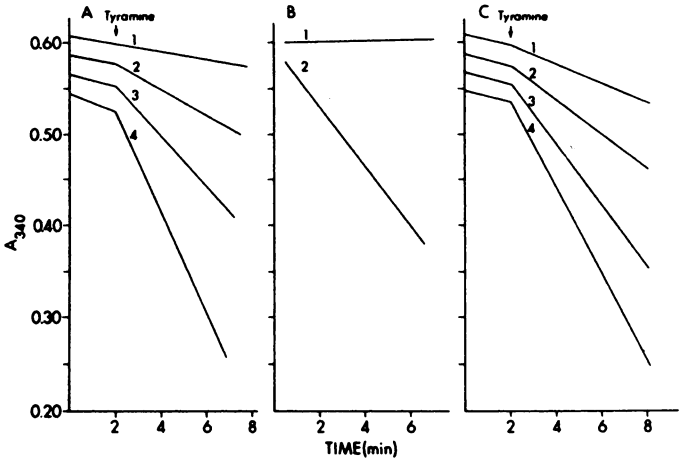

FIG. 1. *The activity of dopamine-β-hydroxylase, semidehydroascorbate reductase, and the dopamine-β-hydroxylase-semidehydroascorbate reductase coupled reaction*

The activity of purified dopamine-β-hydroxylase (DBH) was measured spectrophotometrically by a disappearance in absorbance at 340 nm with pyrocatechol as the electron donor as described in Methods (A). Curves 1, 2, 3, and 4 were generated from 0, 1.6, 4, and 8 μg of purified DBH, respectively. The arrow indicates the addition of tyramine. The activity of the liver microsomal semidehydroascorbate reductase (3 mg wet wt) was followed using the ascorbate/dehydroascorbate redox couple to generate semidehydroascorbate; curves 1 and 2 are in the absence and presence of ascorbate, respectively (B). In C the oxidation of NADH was monitored in the DBH–SDR coupled reaction with ascorbate as the electron donor for DBH. Curves 1, 2, 3, and 4 were generated with different concentrations of DBH: 0, 1.6, 4, and 8 μg, respectively. The arrow indicates the addition of tyramine. NADH oxidation in this reaction occurs through generation of semidehydroascorbate during β-hydroxylation and subsequent reduction of the radical by SDR.

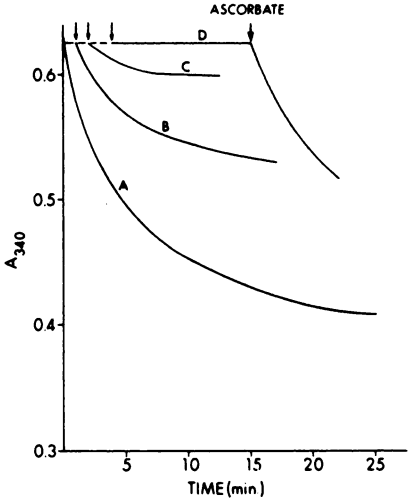

FIG. 2. *NADH oxidation by the dopamine-β-hydroxylase-semidehydroascorbate reductase coupled reaction with catalytic amounts of ascorbate*

The oxidation of NADH by the dopamine-β-hydroxylase/semidehydroascorbate reductase coupled reaction was measured spectrophotometrically by a disappearance in absorbance at 340 nm. The reaction was carried out as described in Methods with dopamine-β-hydroxylase, 18.6 μg, semidehydroascorbate reductase, 6 mg wet wt, and catalytic amounts of ascorbate, 12.5 nmol. Curves A, B, C, and D show the rate of NADH oxidation due to 0, 1, 2, and 4 min-delayed addition of NADH (small arrows). In Curve A the amount of NADH oxidized was threefold greater than the amount of ascorbate added. Delayed addition (Curves B and C) of NADH resulted in a decrease in the rate of NADH oxidation; after a 4-min delay (Curve D), no oxidation of NADH was observed, indicating the labile nature of the free radical semidehydroascorbate. If at this time 12.5 nmol of additional ascorbate was added, a rate of NADH oxidation similar to curve A was observed (Curve D, large arrow).

mM tyramine to the rate with 0.06 mM tyramine was increased with a higher concentration of SDR.

An important consequence of these findings is the possible role of SDR in dopamine-β-hydroxylation in the adrenal medulla. If this is the case, DBH activity should be dependent on the continuous regeneration of the cofactor, ascorbate, by SDR. With catalytic amounts of ascorbate, a coupling of DBH to SDR was observed, i.e., oxidation of NADH in excess of the ascorbate added (Fig. 2). The four curves in Fig. 2 show the rate of NADH oxidation using catalytic amounts of ascorbate. In curve A, NADH was added at time zero; after 29 min of incubation, the amount of NADH oxidized was threefold greater than the amount of ascorbate added. Delayed addition of NADH resulted in a decrease in the rate of NADH oxidation (Fig. 2, curves B and C). With further delay, no oxidation of NADH was observed, indicating the labile nature of the free radical, semidehydroascorbate; if at this time additional ascorbate was added, a rate of NADH oxidation similar to curve A was observed (Fig. 2, curve D). DBH to SDR coupling was confirmed in stoichiometry experiments, where concomitant oxidation of NADH and formation of β-hydroxylated product were obtained in the presence of catalytic amounts of ascorbate.[2]

The possibility that NADH oxidation may be a consequence of superoxide anion generation during β-hydroxylation was considered (17, 18).[3] It has been shown that a NADH binding protein, such as lactate dehydrogenase (LDH), can catalyze the oxidation of NADH by superoxide anion, apparently through activation of the NADH molecule (19). Thus, the question of whether

TABLE 1

Stoichiometry of the DBH–SDR coupled reaction as a function of tyramine concentration

Semidehydroascorbate reductase (3 mg wet wt), prepared from rat liver microsomes, was coupled to purified DBH (4 μg) and the tyramine-dependent oxidation of NADH was followed by the disappearance of absorbance at 340 nm as described in Methods. After 10 min of incubation, aliquots were removed to measure the formation of octopamine radiometrically using [*side chain*-2-^{14}C]tyramine. Note that at low rates of hydroxylation (0.06 mM tyramine), there is nearly stoichiometric oxidation of NADH during the formation of octopamine from tyramine.

Tyramine	NADH oxidation	Octopamine formation	Ratio, octopamine/NADH
mM	*nmol/10 min*	*nmol/10 min*	
0.06	7.07	8.42	1.19
0.25	17.20	27.32	1.59
1.00	31.83	57.70	1.81
4.00	40.03	90.51	2.26

[3] Rajagopalan, K. V., personal communication.

SDR is functioning nonspecifically as a NADH binding protein was examined. The addition of superoxide dismutase (0.5–10 μg/ml) had no effect on the rate of NADH oxidation by the DBH–SDR coupled reaction; the lowest concentration of superoxide dismutase inhibited cytochrome *c* reduction by 91% using the xanthine oxidase system for generating superoxide anion (Table 2). In the DBH–SDR coupled reaction, LDH (38–150 μg/ml) was without effect. Of greater significance was the fact that LDH could not substitute for SDR, indicating that no superoxide anion is generated in the DBH system (Table 2). These results confirm our previous observations showing a lack of inhibition of DBH by superoxide dismutase (17). Thus, it appears unlikely that superoxide anion is involved in the oxidation of NADH by the DBH–SDR coupled reaction.

The present results indicate that the product of DBH reduction is not dehydroascorbate, but rather semidehydroascorbate. In the absence of SDR, the amounts of dehydroascorbate and β-hydroxylated product formed are equivalent, i.e., ascorbate is oxidized stoichiometrically during the hydroxylation reaction (2).[4] Since two electrons are required for each hydroxylation cycle, two molecules of ascorbate should be oxidized to semidehydroascorbate for each molecule of β-hydroxylated product formed. The mechanism for dopamine-β-hydroxylation then appears to involve multiple steps such that reduction of the enzyme occurs through a series of one electron transfers. Since the site of oxidation–reduction in DBH appears to be enzyme-bound copper (2), one possible explanation of the present results is that only one Cu^{2+} is exposed at the enzyme active site for interaction with ascorbate. In this mechanism, reduction of the first Cu^{2+} is followed by release of semidehydroascorbate and transfer of an electron from the first to the second Cu^{2+}; complete reduction of DBH involves transfer of an electron from a second ascorbate molecule to the first Cu^{2+} and release of another semidehydroascorbate molecule. This fully reduced DBH (with two electrons) can interact with substrate and oxygen to complete the hydroxylation cycle, reoxidizing the enzyme and releasing β-hydroxylated product and water. Intramolecular electron transfer from one copper ion to another has been demonstrated for the enzyme, laccase (20, 21). Alternatively, the two enzyme-bound coppers may be spaced far enough apart so as to require reduction by different ascorbate molecules. Finally, the oxidation–reduction potential of the semidehydroascorbate/ascorbate couple as opposed to the dehydroascorbate/ascorbate couple may determine one electron transfer from ascorbate to enzyme-bound Cu^{2+}.

The antiradiation agent, 2-mercaptoethylguanidine, inhibited norepinephrine synthesis in intact chromaffin vesicles without inhibiting dopamine uptake. Subsequently, this compound was shown to exert its effect by inhibition of DBH (22). The mechanism of inhibition of DBH by 2-mercaptoethylguanidine was found to involve the interaction of DBH with two inhibitor molecules. Since the site of inhibition appeared to be through chelation of enzymic Cu^{2+}, these results suggested that two

[4] Diliberto, E. J., Jr., and Kaufman, S., unpublished observations.

TABLE 2

Effects of superoxide dismutase and lactate dehydrogenase on NADH oxidation of the DBH–SDR coupled reaction

Semidehydroascorbate reductase (3 mg wet wt) was coupled to purified DBH (4 μg) as described in Methods. The tyramine-dependent oxidation of NADH was followed by the disappearance of absorbance at 340 nm. Activity of the DBH–SDR coupled reaction is expressed in nanomoles NADH oxidized per minute. Incubations were carried out in the presence or absence of superoxide dismutase or lactate dehydrogenase; in experiment III lactate dehydrogenase was substituted for semidehydroascorbate reductase. The lowest superoxide dismutase concentration produced 91% inhibition of cytochrome *c* reduction by the xanthine oxidase system.

Experiment	Additions[a]	NADH oxidation
		nmol/min
I	None	4.94
	SOD, 0.5 μg/ml	4.26
	SOD, 1 μg/ml	5.01
	SOD, 5 μg/ml	5.25
	SOD, 10 μg/ml	5.45
II	None	4.90
	LDH, 38 μg/ml	4.94
	LDH, 150 μg/ml	4.50
III	None	4.18
	LDH, 150 μg/ml, -SDR	0

[a] SOD, superoxide dismutase; LDH, lactate dehydrogenase; SDR, semidehydroascorbate reductase.

Cu^{2+} were exposed at the enzyme active site (23). The previous proposal that only one Cu^{2+} is exposed at the enzyme active site may indicate that the attachment of the first inhibitor molecule induces a change in the conformation, enabling a greater accessibility of the enzymic copper for binding with the second molecule of inhibitor.

These proposals, describing the mechanism of one-electron transfers for dopamine-β-hydroxylation, were based on the enzyme mechanism studies by Friedman and Kaufman (2) and kinetic studies by Goldstein *et al.* (24) which support a model where each active site accepts two electrons from the electron donor before binding with O_2 and the assignment of a redox role to the copper of DBH. In contrast to this ping-pong mechanism obtained with ascorbate as the electron donor, Ljones and Flatmark (25) reported a sequential mechanism for DBH with potassium ferrocyanide as the electron donor. From these kinetic studies and electron paramagnetic resonance spectroscopy data (26), Ljones *et al.* suggested the involvement of only one copper atom per enzyme active site in the hydroxylation cycle and binding of O_2 before complete reduction of the enzyme. If this is the case, then reduction of the Cu^{2+} is followed by transfer of the electron to bound O_2 and release of semidehydroascorbate; complete reduction of DBH involves transfer of an electron from a second ascorbate molecule to the Cu^{2+} and release of another semidehydroascorbate molecule. However, this type of mechanism is not consistent with the data of Friedman and Kaufman (2), where evidence was presented showing complete reduction of the enzyme anaerobically.

The subcellular localization of SDR in the adrenal medulla has been examined using appropriate enzyme

TABLE 3

Subcellular distribution of semidehydroascorbate reductase activity in the bovine adrenal medulla

Bovine adrenal medullae were homogenized 1:10 (w/v) in 0.25 M sucrose containing 1 mM EDTA and 20 mM Tris–HCl, pH. 7.4. Subcellular fractions of the homogenate were prepared by differential and discontinuous sucrose gradient centrifugations as previously described (16). Since semidehydroascorbate reductase (SDR) was found to be unstable and thought to be inactivated by peroxides (11), catalase (crystalline suspension, Boehringer–Mannheim), 30 μg/ml, was added to all sucrose solutions. Membranes of the large granular fraction were prepared by osmotic lysis of the particles in 10 mM Tris–HCl buffer, pH 7.4, containing catalase, 30 μg/ml; after centrifugation at 30,000*g* for 30 min, the membranes were resuspended 1:10 (w/v) in the Tris–HCl buffer. Semidehydroascorbate reductase activity was measured spectrophotometrically using the ascorbate oxidase/ascorbate system (AO/ascorbate) or the dehydroascorbate/ascorbate redox couple (DHA/ascorbate) to generate semidehydroascorbate as described in Methods. The higher SDR activity with the AO/ascorbate system is due to the higher concentration of free radical generated by that system as compared to the DHA/ascorbate redox couple. Aliquots of each fraction were taken for assay of catecholamines, proteins, monoamine oxidase (MAO), and dopamine-β-hydroxylase (DBH). The values in parentheses represent the percentages of the large granular fraction.

Fractions	Total protein	Total SDR activity		Total MAO	Total DBH	Total catecholamines
		AO/ascorbate	DHA/ascorbate			
	mg	*nmol/min*		*nmol/min*	*nmol/min*	*μmol*
		Differential centrifugation				
Homogenate	153.90	1627	—	58.02	6341	90.19
Large granular (P_2)	55.02	1082	674	32.52	3956	35.43
(% in membranes [P_2M])	(67.3)	(60.8)	—	(92.5)	(43.2)	—
(% in soluble [P_2S])	(23.4)	(0.3)	—	—	—	(97.4)
Microsomal (P_3)	7.76	194	121	6.42	596	1.72
Postmicrosomal supernatant (S_3)	39.03	0	0	—	285	20.45
		Discontinuous sucrose gradient centrifugation of P_2				
Mitochondria (0.8/1.3 M sucrose)	3.38	252	157	9.81	153	0.43
1.3/1.7 M Sucrose interface	7.78	264	164	10.45	495	2.98
Chromaffin vesicles	23.16	54	37	4.01	1106	14.96
Remaining gradient	18.75	264	—	12.92	1693	17.78

markers for the various subcellular fractions (Table 3). Using two different assays for measuring SDR activity, the enzyme was found to be localized on several membrane fractions but not in the soluble fractions. Differential centrifugation revealed that most of the SDR activity was in the microsomal and large granular fractions. Separation of the latter fraction by sucrose density gradient centrifugation showed that the highest specific activity of SDR was on the mitochondrial membrane. Furthermore, in gradients designed to give the least mitochondrial contamination of the chromaffin vesicle fraction, the SDR activity of the vesicle fraction can be attributed to the presence of small amounts of mitochondria. Thus, SDR does not appear to be located on the chromaffin vesicle membrane. On the other hand, DBH is a chromaffin vesicle component with intravesicular active sites. For these results to be consistent with a physiological role for SDR in β-hydroxylation through regeneration of active cofactor, either mitochondria and chromaffin vesicles have permanent or transient connections to allow for direct coupling of these enzymes or the rate of transfer of the free radical from one particle to another is faster than the rate of dismutation. Membranes prepared from an adrenal medullary large granular fraction (containing chromaffin vesicles, lysosomes, and mitochondria) (16) showed a tyramine-dependent oxidation of NADH which was inhibited by fusaric acid and stimulated by fumarate.[2] Thus, *in vitro* coupling of the two membrane-bound enzymes, DBH and SDR, can be demonstrated. Since ascorbate is the putative cofactor for DBH and the intravesicular concentration of ascorbate was estimated to be 13 mM (27), a system for transfer of ascorbate in the reduced form should be present in the chromaffin vesicle.

Recently, Tirrell and Westhead studied the uptake of ascorbate and dehydroascorbate into chromaffin vesicles and found that dehydroascorbate but not ascorbate may enter these particles by diffusion (28). If this is the case, then vesicular ascorbate may require a system similar to that suggested for ATP, i.e., the direct transfer of ATP from the mitochondrial compartment into the vesicles (29). This type of mechanism would be supported by the high specific activity of SDR on the mitochondrial membrane. Currently, these two mechanisms are being considered in our investigation of the role of SDR in dopamine-β-hydroxylation.

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