Secretion of Newly Taken-Up Ascorbic Acid by Adrenomedullary Chromaffin Cells

Abstract. *Primary cultures of bovine adrenomedullary cells accumulate carbon-14–labeled ascorbic acid through a saturable and energy-dependent process. The newly taken-up ascorbate is released concomitantly with catecholamines upon stimulation of chromaffin cell secretion. The release of ascorbate is Ca^{2+}-dependent and mediated through activation of nicotinic receptors. These results indicate that exogenous ascorbate taken up into chromaffin cells is incorporated in situ into a secretable compartment, probably the catecholamine-containing chromaffin vesicles.*

In humans the nutritional requirement of ascorbic acid is widely recognized through the role of this vitamin in preventing the pathological state known as scurvy. The lack of ascorbate in the diet of mammals that do not synthesize it results in a rapid preferential depletion of the vitamin in heart, skeletal muscle, and other tissues, but not in brain, where ascorbate content decreases only after prolonged deficiency (*1*). The conservation of ascorbate in brain is indicative of an important role for the vitamin in neural tissue metabolism and suggests the presence of neuronal reutilization (*2*) or accumulation systems for the vitamin (*3*, *4*). Even in mammals that do not depend on dietary ascorbate, an efficient uptake or regeneration system for ascorbic acid must be present in neural tissue since ascorbate is synthesized only in liver (*5*).

The best documented role for ascorbate in nervous tissue is its function as a cofactor for dopamine β-hydroxylase (*6*), an enzyme that is enclosed within the chromaffin vesicles of the adrenal medulla (*7*) and synaptic vesicles of adrenergic neurons (*8*). The enzyme, a mixed-function oxidase, requires an electron donor to catalyze the conversion of dopamine to norepinephrine. Ascorbic acid is the putative electron donor for this reaction (*9*). However, the role of ascorbate in neuronal function does not appear to be restricted to its participation in dopamine β-hydroxylation. Indeed, recent studies have suggested that ascorbate can modulate neurotransmission, that is, ascorbate was shown to inhibit dopamine-sensitive adenylate cyclase in vitro (*10*) and to block amphetamine-induced stereotypy in vivo, an action thought to be mediated by dopaminergic neural systems (*11*). In addition, ascorbate has been shown to inhibit Na^+,K^+-activated adenosine triphosphatase (*12*) and the binding of neurotransmitters to their receptors (*13*).

The physiological role of ascorbate in β-hydroxylation is supported by the high content (~ 20 m*M*) of fully reduced ascorbate found in chromaffin vesicles (*14*). Nevertheless, a vesicular uptake system for ascorbate has not been detected in studies of isolated chromaffin vesicles in vitro (*15*). We now report that primary cultures of bovine adrenomedullary cells accumulate ascorbic acid and that upon stimulation they release the newly taken-up cofactor with catecholamines.

Chromaffin cells from bovine adrenal medulla were isolated and plated in multiwell plates (Falcon) with a density of 1×10^6 cells per well as described by Wilson and Viveros (*16*). Ascorbate uptake and release experiments were performed with the cells in a balanced salt solution containing 150 m*M* NaCl, 4.2 m*M* KCl, 1.0 m*M* NaH_2PO_4, 11.2 m*M* glucose, 0.7 m*M* $MgCl_2$, 2.0 m*M* $CaCl_2$, and 10.0 m*M* Hepes (*N*-2-hydroxyethylpiperazine - *N'* - 2 - ethanesulfonic acid) (*p*H 7.4) (see legends to Table 1 and Fig. 1). Ascorbate analysis of cell extracts was done by a modification of the procedure of Pachla and Kissinger (*17*).

All experiments were carried out in cells maintained for 3 days in culture when endogenous ascorbate levels had decreased to about 12 percent of the original content (*18*). Ascorbic acid was effectively taken up by isolated chromaffin cells. We characterized the uptake and found that it is temperature-dependent, is linear for up to 30 minutes, and follows Michaelis-Menten kinetics with an apparent Michaelis constant (K_m) of 30 μ*M* (*4*). Table 1 shows some of the characteristics of this transport system. After 30 minutes of incubation with 200 μ*M* ascorbate, we obtained an accumulation of 1.13 ± 0.11 nmole per 10^6 cells which, in addition to the endogenous content, is equivalent to a concentration of about 1.0 m*M* if we assume that the cofactor is evenly distributed throughout the intracellular water. This indicates a fivefold concentration ratio with respect to the medium. At lower levels of ascorbate (10 μ*M*), a concentration ratio as high as 50-fold was attained (*18*). Replacement of Na^+, by either sucrose or Li^+, as well as addition of ouabain to the medium, strongly inhibited ascorbate uptake, suggesting a dependency on a Na^+ gradient. Accordingly, 2,4-dinitrophenol and low temperature blocked the accumulation of the cofactor. High-performance liquid chromatography analysis of the cell extracts after incubation with L-[1-^{14}C]ascorbate revealed that greater

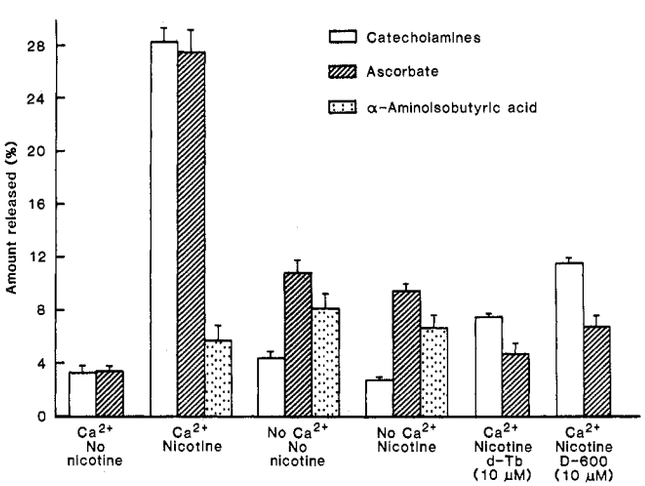

Fig. 1. Effect of nicotine on [^{14}C]ascorbate release from chromaffin cells in primary culture. Cells (1×10^6 cells per well) were first incubated with 0.5 ml of balanced salt solution containing 200 μ*M* [^{14}C]ascorbate and 0.5 μ*M* [^{3}H]AIB (where indicated) for 30 minutes at 37°C and then washed at room temperature by replacing the medium at intervals of 5, 5, and 10 minutes, with 0.5 ml of balanced salt solution. Cells were then stimulated for 10 minutes at room temperature by the addition of 0.45 ml of balanced salt solution containing 10 μ*M* nicotine and 10 μ*M* ouabain to prevent ascorbate reuptake. This concentration of ouabain was found not to modify the basal or nicotine-induced release of catecholamines. D-600 and *d*-tubocurarine were present during the last 10-minute wash and during the stimulation period. After stimulation, the medium and cell extracts were analyzed for [^{14}C]ascorbate and catecholamines (*27*). Results are expressed as the mean ± standard deviation ($N = 3$) of a typical experiment repeated at least four times with similar results.

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than 90 percent of the newly taken-up ascorbate was maintained reduced, a form required for the hydroxylation of dopamine. Since β-hydroxylation of dopamine occurs inside the chromaffin vesicle, we attempted to obtain evidence for accumulation of ascorbate inside these vesicles.

Secretion of catecholamines occurs by exocytosis, with the all-or-none release of the whole content of the chromaffin vesicle directly to the extracellular space (*19*). Thus, if the [^{14}C]ascorbate is accumulated in chromaffin vesicles, it should be cosecreted with catecholamines upon stimulation of the chromaffin cells. Figure 1 shows that newly taken-up [^{14}C]ascorbate is indeed released concomitantly with catecholamines upon stimulation with nicotine (10 μ*M*). Nicotine-induced release of ascorbate is Ca^{2+}-dependent, since it is significantly decreased in the absence of the metal ion. Furthermore, the release is strongly inhibited by the action of D-600, a specific blocking agent of Ca^{2+} channels (*20*). The omission of Ca^{2+} from the medium increased by threefold the basal release of [^{14}C]ascorbate, but not that of catecholamines. This increased release in the absence of Ca^{2+} is probably from a cytosolic compartment (*21*). To rule out the possibility that the nicotine-induced release of [^{14}C]ascorbate was of cytoplasmic origin, we incubated chromaffin cells simultaneously with [^{14}C]ascorbic acid and α-[*methyl*-^{3}H]aminoisobutyric acid ([^{3}H]AIB), an amino acid that is actively transported but not metabolized (*22*). As shown in Fig. 1, the unstimulated release of [^{3}H]AIB (in Ca^{2+}-free medium) was intermediate between the percentage release of endogenous catecholamines and [^{14}C]ascorbate. When ascorbate and catecholamine release was stimulated by nicotine, there was no further increase of [^{3}H]AIB in the medium. Furthermore, *d*-tubocurarine, which blocks the nicotinic receptor (*23*), prevented the release of [^{14}C]ascorbic acid, indicating that the process is mediated through the activation of the chromaffin cell nicotinic receptor.

Ascorbate transport into cerebrospinal fluid is mediated by an active uptake system in the choroid plexus (*3*). Uptake of ascorbate from the extracellular space into brain cells also appears to involve an active transport system (*3*). The adrenal medulla is derived from the neural crest and has been repeatedly used as a model system to study the biology of catecholaminergic neurons (*19*, *24*). Our results, in a homogeneous cell preparation, confirm the presence of an active transport system for ascorbic acid into neural cells. Furthermore, the Ca^{2+}-dependent, nicotine-induced secretion of newly taken-up ascorbate strongly suggests that the chromaffin cell has a mechanism to accumulate the cofactor into the catecholamine storage vesicle, the site of intracellular dopamine β-hydroxylation. Whether the primary source of ascorbate is through cellular uptake from the blood plasma or through the putative cytoplasmic regeneration system (*2*), a vesicular uptake system would be essential to maintain the supply of the β-hydroxylating cofactor and therefore keep pace with the demand for catecholamine biosynthesis. At this point, however, we cannot disregard the possibility that the cofactor is first concentrated by another subcellular compartment from which it is subsequently transferred to the chromaffin vesicle.

Table 1. Uptake of ascorbate by chromaffin cells in primary culture. Plated chromaffin cells (1×10^6 cells per well) were washed three times with 0.5 ml of balanced salt solution. Uptake studies were initiated by addition of 0.5 ml of balanced salt solution containing 200 μ*M* [^{14}C]ascorbate and 1 m*M* thiourea (*26*) to each well; the cells were then incubated for 30 minutes at 37°C. At the end of the incubation period, the cells were placed on ice and washed three times with ice-cold balanced salt solution (0.5 ml per well), and ascorbate uptake was determined by the amount of radioactivity remaining in the cells (*17*). Where the effects of ouabain and 2,4-dinitrophenol were tested, the reagents were added directly into the balanced salt solution and the cells were incubated for 30 minutes at 37°C before addition of ascorbate. No Na^+ in the incubation medium was achieved by substitution with equimolar amounts of Li^+ or sucrose. The effect of low temperature was studied by incubating the cells in an ice bath. The amount of [^{14}C]ascorbate uptake in the control was 1.13 ± 0.11 nmole per 1×10^6 cells ($N = 9$).

Conditions	Cell uptake (%)
Control	100.0
4°C	2.0
10 μ*M* ouabain	25.9
No Na^+	10.5
1 m*M* 2,4-dinitrophenol	23.3

While the present studies were in progress, depolarization-induced release of ascorbate was demonstrated in rat brain synaptosomes in vitro as well as by electrical stimulation of the thalamocortical pathway in situ (*25*). Although these results are consistent with the uptake and the release of ascorbate from neurons, ascorbate efflux from synaptosomes was Ca^{2+}-independent, indicative of a nonexocytotic process (*25*). Our results show that the release of newly taken up ascorbate from chromaffin cells in culture is proportional to the release of endogenous catecholamines, is Ca^{2+}-dependent, and is nicotine receptor–mediated. These observations are characteristic of exocytotic secretion (*19*, *24*). The evidence supporting exocytotic secretion of ascorbate in this report complements the recent data on ascorbate effects on neurotransmitter receptors and Na^+,K^+-activated adenosine triphosphatase (*10–13*), suggesting a novel neuromodulatory role for this vitamin.

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18. Ascorbic acid levels decrease very rapidly after the cells are plated and by the third day their content (0.8 nmole per 10^6 cells) is close to one-tenth of that on the plating day (E. J. Diliberto, Jr., G. Dean, S. P. Wilson, unpublished observations).
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22. α-Aminoisobutyric acid and norepinephrine are actively transported into cells of the central nervous system by a Na^+-dependent process but the amino acid is not taken up by the storage vesicles and is not coreleased with norepinephrine upon electrical stimulation [F. Orrego, J. Jankelevich, L. Ceruti, E. Ferrera, *Nature (London)* **251**, 55 (1974); O. Vargas and F. Orrego, *J. Neurochem.* **26**, 31 (1976); F. Orrego and R. Miranda, *ibid.*, p. 1033]. Therefore, α-[*methyl*-^{3}H]aminoisobutyric acid (New England Nuclear) was used as a model for an extravesicular compartment marker.
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The Human Genes for *S*-Adenosylhomocysteine Hydrolase and Adenosine Deaminase Are Syntenic on Chromosome 20

Abstract. *Human–Chinese hamster cell hybrids and a monoclonal antibody to human* S-*adenosylhomocysteine hydrolase were used to identify chromosome 20 as the location of the human gene for this enzyme. The gene for adenosine deaminase had previously been mapped to this chromosome. The activity of* S-*adenosylhomocysteine hydrolase is dependent in vivo on that of adenosine deaminase, since the substrates for the deaminase, adenosine and deoxyadenosine, respectively, inhibit and inactivate* S-*adenosylhomocysteine hydrolase in genetic or drug-induced adenosine deaminase deficiency. This functional dependence and the likelihood that* S-*adenosylhomocysteine hydrolase, a eukaryotic enzyme, arose later than adenosine deaminase, which occurs in prokaryotes as well as eukaryotes, suggest that the occurrence of their genes on the same chromosome may have evolutionary significance. In addition, the unusual capacity of* S-*adenosylhomocysteine hydrolase to form stable complexes with adenosine and its cofactor, nicotinamide adenine dinucleotide, suggest that evolution of its gene may have involved recombination of a portion of the adenosine deaminase gene with an adenine nucleotide domain–coding sequence of another preexisting gene.*

In genetic deficiency of adenosine deaminase (ADA; E.C. 3.5.4.4) (*1*), accumulation of adenosine and 2′-deoxyadenosine results in profound depletion of lymphoid tissues and severe combined immunodeficiency disease. Investigation into the biochemical effects of these purine nucleosides has led to recognition that the activity of a second enzyme involved in the metabolism of adenosine, *S*-adenosylhomocysteine (AdoHcy) hydrolase (E.C. 3.3.1.1), is dependent in vivo on that of ADA. *S*-Adenosylhomocysteine hydrolase catalyzes the reversible hydrolysis of AdoHcy to adenosine and L-homocysteine (*2*). This reaction is important because AdoHcy is a potent inhibitor, as well as a product, of *S*-adenosylmethionine–dependent transmethylation, a step that occurs in the biosynthesis, modification, or regulation of the function of nucleic acids, proteins, phospholipids, and biogenic amines (*3*) (see Fig. 1). Cleavage of AdoHcy is the only source of homocysteine, which is used in the synthesis of the essential amino acid methionine in a reaction that also produces tetrahydrofolate, a cofactor for several enzymes participating in the synthesis of nucleic acid precursors.

Two mechanisms account for the dependence of the activity of AdoHcy hydrolase on that of ADA. First, because the equilibrium constant for AdoHcy hydrolysis is highly unfavorable (*2*), further metabolism of adenosine or homocysteine is required to promote AdoHcy cleavage and prevent its accumulation. In cultured lymphoid cells treated with ADA inhibitors, AdoHcy accumulation, causing inhibition of transmethylation reactions, is largely responsible for the toxic effects of adenosine in the concentration range 5 to 50 μ*M* (*4*, *5*). In addition to the inhibitory effect of adenosine, we have shown that deoxyadenosine is a "suicide-like" substrate analog that causes the inactivation of purified AdoHcy hydrolase (*6*), and of AdoHcy hydrolase in ADA-inhibited cultured cells (*7*). This effect of 2′-deoxyadenosine results in severe deficiency of AdoHcy hydrolase activity in the erythrocytes of ADA-deficient children (*8*), and in the erythrocytes and lymphoblasts of patients with lymphocytic leukemias when they are treated with the drug 2′-deoxycoformycin, a potent inhibitor of ADA that causes deoxyadenosine accumulation in vivo (*9*, *10*).

We now report that the genes for ADA and AdoHcy hydrolase are located on the same human chromosome. We mapped the location of AdoHcy hydrolase by using a mouse monoclonal antibody directed against human placental AdoHcy hydrolase (*11*) to develop an immunoprecipitation method for detecting human AdoHcy hydrolase in human-hamster hybrid cell lines that contain different subsets of human chromosomes. Somatic cell hybrids and monoclonal antibodies have recently been used to map the genes for human liver and muscle isozymes of 6-phosphofructokinase (*12*).

The monoclonal antibody designated

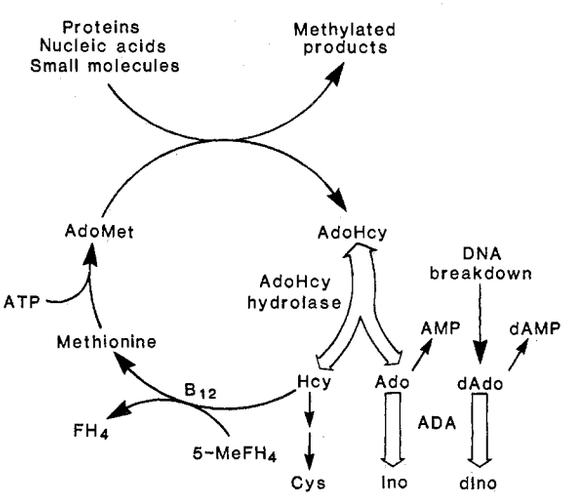

Fig. 1. Metabolic functions of AdoHcy hydrolase and ADA. Abbreviations: *AdoMet*, *S*-adenosylmethionine; FH_4, tetrahydrofolate; $5\text{-}MeFH_4$, 5-methyltetrahydrofolate; B_{12}, vitamin B_{12}; *Hcy*, homocysteine; *Cys*, cysteine; *Ado*, adenosine; *Ino*, inosine; *dAdo*, 2′-deoxyadenosine; *dIno*, 2′-deoxyinosine; *AMP*, adenosine monophosphate; *dAMP*, 2′-deoxyadenosine monophosphate; *ADA*, adenosine deaminase; and *AdoHcy*, *S*-adenosylhomocysteine.

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