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Ascorbic Acid Transport in Mouse and Rat Astrocytes is Reversibly Inhibited by Furosemide, SITS, and DIDS

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The uptake of L-ascorbic acid (vitamin C) by astrocytes was studied using primary cultures prepared from the neopallium of newborn Swiss CD-1 mice or Sprague-Dawley rats. Initial uptake rates were significantly greater in mouse than in rat astrocytes. Exposure of cultures to 0.25 mM dibutyryl cyclic AMP for 2 weeks changed cell morphology from polygonal to stellate and stimulated ascorbate uptake, with the greatest stimulation occurring in mouse astrocytes. Uptake was specific for the vitamin since it was not diminished by the presence of other organic anions including acetate, formate, lactate, malonate, oxalate, p-aminohippurate, pyruvate and succinate. Ascorbate uptake was Na^+-dependent but did not have a specific requirement for external Cl^- (Cl^-_0). Substitution of Cl^-_0 by Br^- or NO_3^- decreased ascorbate uptake rates by 20-31%; whereas substitution by gluconate or isethionate increased uptake by 20-31%. Ascorbate transport by astroglial cultures from both animal species was rapidly ($\leq$1 min) and reversibly inhibited by the anion transport inhibitors furosemide, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). The rapid and reversible effects of the impermeant inhibitors (SITS and DIDS) are consistent with direct inhibition of ascorbate transporters located in the astroglial plasma membrane.

KEY WORDS: Vitamin C; L-ascorbic acid; astrocytes; furosemide; SITS; DIDS.

INTRODUCTION

Ascorbic acid (vitamin C) is absorbed by the intestine or synthesized by the liver and then delivered through the bloodstream to the brain (1,2). The vitamin is essential for brain function because it is a cofactor in catecholamine biosynthesis (3), facilitiates release of transmitters (4–6), modulates binding of ligands to neural receptors (7–9), and slows rates of transmitter clearance (10–12). There is evidence that ascorbate is actively transported in rat cerebral cortex (13) and that brain cells regulate the extracellular concentration of vitamin C (14). Ascorbate accumulation in cerebral astrocytes is saturable, stereoselective and Na^+-dependent (15). Rose (2) has argued, however, that saturation kinetics may not arise necessarily from uptake of ascorbate by a plasma membrane transport system. Instead, substrate may simply diffuse across the plasma membrane, followed by rate-limiting enzymatic conversion to an impermeant species or carrier-mediated uptake into a subcellular compartment. Thus, it is not yet clear if a saturable ascorbate transport system exists in the plasma membrane of astrocytes.

Over 99% of total ascorbic acid occurs as a monoanion at physiological pH. Anions can enter cells by diffusion, anion-cation cotransport or anion exchange (16). Na^+-dependent ascorbate transport has been observed in several tissues (2), including astrocytes (15), and is thought to represent Na^+-anion cotransport. In bovine pigmented ciliary epithelium the contransporter is electrogenic and appears to carry two Na^+ with each ascorbate molecule

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(17). There also is evidence of Cl^--ascorbate exchange across corneal membranes in an amphibian species (18). In astrocytes it is not known if ascorbate competes with other anions for a common transport site. Direct inhibitors of ascorbate transport, which would be useful for studying the location and mechanism of the vitamin C carrier, have never been identified previously.

Astrocytes cultured from mouse and rat brains possess Cl^--dependent transport systems sensitive to the loop diuretic furosemide and to the stilbene disulfonates, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) (19–22). Activation of these transporters may play a role in the regulation of astroglial cell volume. Clinical administration of anion transport inhibitors has been proposed in the management of traumatic brain injury, to prevent the astroglial swelling that contributes to cytotoxic cerebral edema (23,24). For example, furosemide is reported to exert a beneficial effect on cerebral water and ion contents in cases of peritumoral brain edema (25). It is conceivable, however, that these inhibitors interfere with the metabolism of physiologically important substrates such as vitamin C.

It is important to characterize the astroglial transport process because of its potential role in the regulation of cerebral ascorbate concentration. The present study examined the specificity of the astroglial ascorbate transport system for inorganic and organic anions and its susceptibility to pharmacological inhibitors. A preliminary report has appeared in abstract form (26).

EXPERIMENTAL PROCEDURE

Materials. L-[1-^{14}C]ascorbic acid (10 mCi/mmol) was purchased from Dupont Canada. Horse serum was obtained from Gibco Canada. Modified Eagle's minimum essential medium (27) was made using tissue culture-grade chemicals purchased from Sigma. L-ascorbic acid, DL-homocysteine, KCl, LiCl, N-methyl-D-glucamine (NMG), sodium formate, sodium lactate, sodium malonate, sodium oxalate, sodium pyruvate, sodium succinate, sodium p-aminohippurate, furosemide, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and N(6),O(2′)-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP) also were from Sigma. Sodium acetate was purchased from BDH.

Cell Culture. Astroglial cultures were prepared from the neopallium of 1-day-old Swiss CD-1 mice and Sprague-Dawley rats according to the procedures of Hertz et al. (27,28). The cells were plated onto 60 mm petri dishes (Falcon) and grown in modified Eagle's minimum essential medium with 20% horse serum (37°C; 95% air/5% CO_2). The culture medium was changed twice weekly, with the serum concentration reduced to 10% after 1 week. Cultures reached confluency after 2 weeks. They were grown for an additional 2 weeks, in the presence or absence of 0.25 mM dibutyryl cyclic AMP, before being used for uptake experiments. Microscopic examination of cultures stained with sliver showed that neurons were absent. The cultivated cells stained positively for glial fibrillary acidic protein using the procedure of Wilson et al. (29). Treatment with 0.25 mM dibutyryl cyclic AMP transformed the flat polygonal astrocytes to process-bearing stellate astrocytes.

Measurement of Ascorbate Uptake. Cellular uptake of L-[^{14}C]ascorbate was measured at 37°C in serum-free incubation media, essentially as described previously (15). The Na^+- and Cl^--replete medium consisted of (in mM): 134 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 10 glucose and 20 Hepes, adjusted to pH 7.3 with NaOH. The dependence of ascorbate uptake on extracellular Cl^- (Cl^-_o) was studied by isoosmotic replacement of NaCl with the sodium salts of gluconate, isethionate, Br^- or NO_3^-. These low Cl^- media contained 1.8 mM $CaCl_2$ but K^+ gluconate was subtituted for KCl. Osmolality was measured by freezing point depression.

To test the effects of anion transport inhibitors, astrocytes were incubated for 1 min in Na^+- and Cl^--replete medium containing 1 mM furosemide, DIDS or SITS. Acute effects were determined by adding the drugs to cell cultures at the start of the ascorbate uptake incubation. Reversibility was examined by preincubating cell cultures with the inhibitors, subsequently using bovine serum albumin to scavenge reversibly bound inhibitors (30), and then measuring ascorbate uptake during a final 1 min incubation. To remove reversibly bound inhibitors, the cultures were washed once with drug-free medium, then incubated for 1 or 5 min in medium containing 0.5% bovine serum albumin, and finally washed again with drug-free medium (entire procedure at 37°C).

Stock solutions of L-[^{14}C]ascorbate and unlabeled L-ascorbate contained 0.4 mM homocysteine to prevent oxidation. The pH of the medium was not altered by the presence of these reductants at the concentrations employed. Measurement of ascorbic acid by high-performance liquid chromatography with electrochemical detection (31) confirmed that the concentration of the vitamin in the external medium decreased slowly with time, the loss being less than 10% during 30 min incubations with astroglial cultures.

Aliquots of media were collected at the end of each uptake incubation. The incubations were terminated by washing cultures with ice-cold isoosmotic sucrose solution and harvesting the cells. An aliquot of the cell harvest was used for protein measurement (32) and the remainder was combined with scintillation cocktail. The radioactive contents of the media and cells were measured by liquid scintillation counting. Uptake rates were computed based on the specific activity of L-[^{14}C]ascorbate in the media and expressed as nmol ascorbate/g protein/min.

Statistics. Results are presented as the mean ± SEM of n experiments, each performed in duplicate or triplicate. Differences between mean values were evaluated using paired or pooled t-tests and a P value of less than 0.05 was considered significant.

RESULTS

Rates of ascorbate uptake were consistently greater in mouse than in rat astroglial cultures. The initial rates of ascorbate uptake by polygonal astrocytes were 124 ± 14 and 68 ± 4 nmol/g/min in mouse (n = 9 experiments) and rat (n = 6) preparations, respectively (P < 0.05). Treatment with dibutyryl cyclic AMP (0.25 mM for 2 weeks) changed astroglial morphology from polygonal to stellate and stimulated ascorbate uptake. As-

corbate uptake rates increased approximately two-fold in rat and three-fold in mouse astrocytes after exposure to dibutyryl cyclic AMP, with the result that the difference between mouse and rat preparations became even larger in stellate than in polygonal cultures. The initial uptake rates in nmol/g/min for stellate astrocytes were: mouse, 428 ± 28 (n = 6); rat, 147 ± 15 (n = 8) ($P < 0.05$).

Astroglial ascorbate uptake was concentrative. Previous authors reported the internal volume of cultured cerebral astrocytes as 2-4 μl/mg protein (33,34). Cell:medium ratios of radiolabeled ascorbate were calculated based on an internal volume accessible to solute of 2 μl/mg protein (33). The mean ratios after 1 min incubation with 5 μM L-[^{14}C]ascorbate were: mouse polygonal, 12 ± 1; mouse stellate, 43 ± 3; rat polygonal, 7 ± 0.4; rat stellate, 15 ± 2.

The substrate specificity of the astroglial ascorbate transporter was investigated by 1 min incubations with the radiolabeled vitamin and the sodium salts of various organic acids. In the presence of 5 μM [^{14}C]ascorbate, 3 mM unlabeled ascorbate inhibited 96% of the uptake of radiolabel into either polygonal or stellate astrocytes. However, the initial rates of [^{14}C]ascorbate uptake were not affected by 3 mM acetate, formate, lactate, malonate, oxalate, p-aminohippurate, pyruvate or succinate (data not shown).

A comparison was made between ascorbate uptake rates in astroglial cultures incubated in either Cl^--replete or nominally Cl^--free media (Figure 1). Removing external Cl^- altered ascorbate uptake by mouse astrocytes, but the nature of the effect depended upon which anions were substituted for Cl^-_o. The rates of ascorbate uptake by both polygonal and stellate astrocytes when Cl^-_o was replaced by various anions increased in the order: NO_3^- < Br^- < Cl^- < isethionate < gluconate (Figure 1).

Ascorbate uptake into astroglial cultures was inhibited within 1 min by the anion transport inhibitors furosemide, SITS and DIDS. Uptake rates for control cultures not treated with inhibitors were measured in each experiment (Figures 2-4). The effect of 1 mM furosemide on rat cultures was to inhibit the initial rate of ascorbate uptake by 72 ± 6 and 78 ± 4 percent in polygonal and stellate astrocytes, respectively (n = 3; Figure 2, top panel). Similarly, in mouse cultures, furosemide inhibited ascorbate uptake by 74 ± 1 percent in polygonal astrocytes and 72 ± 1 percent in stellate cells (n = 4; Figure 2, bottom panel). Removal of furosemide by a 1 min incubation (wash) with a solution containing albumin completely reversed these inhibitory effects in polygonal and stellate astrocytes of both animal species (Figure 2).

SITS (1 mM) inhibited initial ascorbate uptake rates

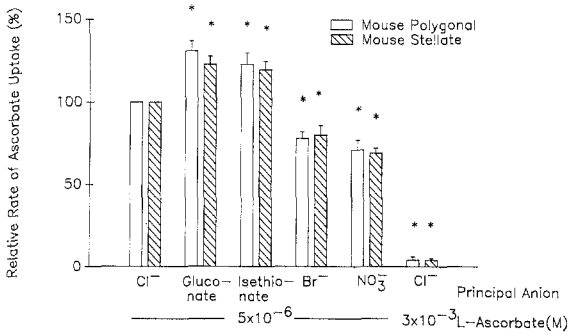


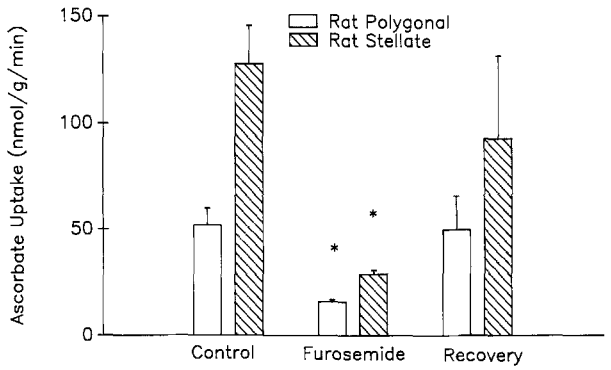
Fig. 1. Anion dependence of ascorbate uptake into mouse astrocytes. Rate of ascorbate uptake was measured using mouse astrocytes incubated for 1 min at 37 °C with 5 μM L-[^{14}C]ascorbate as described under "Experimental Procedure". Gluconate, isethionate, Br^- or NO_3^- were substituted isoosmotically for Cl^- in the external medium. Excess (3 mM) unlabeled L-ascorbate was added to Cl^--containing medium, where indicated, to determine nonspecific uptake. Comparisons are made between polygonal astrocytes that were not treated with dibutyryl cyclic AMP and stellate astrocytes that were treated with 0.25 mM dibuytyryl cyclic AMP for 2 weeks. Data are expressed as percentages of the uptake rates in Cl^--containing medium and are the means ± SE of 5 independent experiments. The uptake rates in Cl^--containing medium were 121 ± 14 and 377 ± 44 nmol ascorbate/g protein/min in polygonal and stellate astrocytes, respectively. *$P < 0.05$ compared to control values.

by 46 ± 11 and 61 ± 5 percent in rat polygonal (n = 4) and stellate (n = 5) astrocytes, respectively (Figure 3, top panel). Ascorbate accumulation was diminished by 74 ± 4 percent in mouse polygonal astrocytes (n = 4) and 69 ± 1 percent in mouse stellate (n = 3) cultures (Figure 3, bottom panel). After removal of SITS by washing with albumin solution, ascorbate uptake rates returned to control levels (Figure 3).

Similarly, 1 mM DIDS slowed initial ascorbate uptake rates in astrocytes (Figure 4). Expressed as percent inhibition of the rates of ascorbate accumulation in control cultures, the results were: rat polygonal, 77 ± 6 (n = 6); rat stellate, 79 ± 6 (n = 5); mouse polygonal, 93 ± 5 (n = 4); mouse stellate, 93 ± 1 (n = 3). These inhibitory effects of DIDS were partially reversed by a 1 min wash with albumin solution (Figure 4). After lengthening the albumin wash period to 5 min, the residual inhibition compared to albumin-washed controls was reduced to the following percentages: rat polygonal, 16 ± 16 (n = 2); rat stellate, 15 ± 8 (n = 3); mouse polygonal, 14 ± 9 (n = 2); mouse stellate, 16 ± 4 (n = 3).

DISCUSSION

Rates of ascorbate uptake were greater in mouse than in rat astrocytes grown under identical conditions,



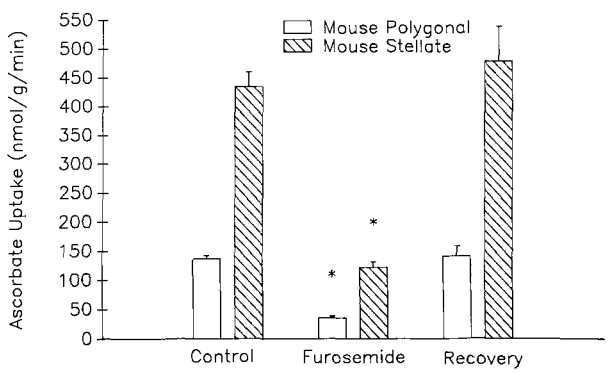
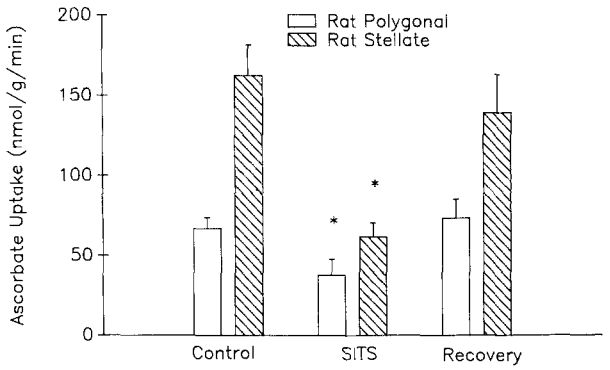


Fig. 2. Effect of furosemide on ascorbate uptake by primary cultures of rodent astrocytes. Rates of uptake were measured using mouse and rat astrocytes incubated for 1 min at 37°C with 5 μM L-[^{14}C]ascorbate as described under "Experimental Procedure". The acute effect of 1 mM furosemide was determined by adding it to the cell cultures at the beginning of the ascorbate uptake incubation. Reversibility was examined by preincubating cell cultures with the inhibitor, removing the inhibitor, and then measuring ascorbate uptake during a subsequent 1 min incubation. To remove furosemide, the cultures were washed once with drug-free medium, then incubated for 1 min in medium containing 0.5% bovine serum albumin, and finally washed again with drug-free medium (entire procedure at 37°C). Comparisons are made between polygonal astrocytes that were not treated with dibutyryl cyclic AMP and stellate astrocytes that were treated with 0.25 mM dibutyryl cyclic AMP for 2 weeks. Uptake rates are expressed as nmol ascorbate/g protein/min. Data are means ± SE of 3-4 independent experiments performed in triplicate. *$P < 0.05$ compared to control values.



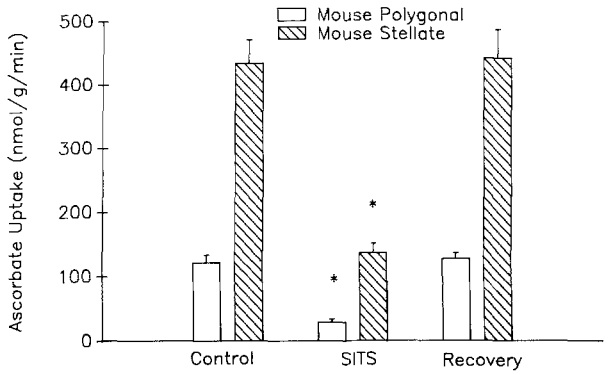


Fig. 3. Effect of SITS on ascorbate uptake by primary cultures of rodent astrocytes. Rates of uptake were measured using mouse and rat astrocytes incubated for 1 min at 37°C with 5 μM L-[^{14}C]ascorbate as described under "Experimental Procedure". The acute effect of 1 mM SITS was determined by adding it to the cell cultures at the beginning of the ascorbate uptake incubation. Reversibility was examined by preincubating cell cultures with the inhibitor, removing the inhibitor, and then measuring ascorbate uptake during a subsequent 1 min incubation. To remove SITS, the cultures were washed once with drug-free medium, then incubated for 1 min in medium containing 0.5% bovine serum albumin, and finally washed again with drug-free medium (entire procedure at 37°C). Comparisons are made between polygonal astrocytes that were not treated with dibutyryl cyclic AMP and stellate astrocytes that were treated with 0.25 mM dibutyryl cyclic AMP for 2 weeks. Uptake rates are expressed as nmol ascorbate/g protein/min. Data are means ± SE of 3-5 independent experiments performed in triplicate. *$P < 0.05$ compared to controls values.

with the greatest discrepancy evident after prolonged exposure to dibutyryl cyclic AMP. This interspecific difference is not generalized to all transport processes since there is no difference in the rate of creatine uptake by mouse and rat astroglial cultures (35). K^+ influx velocity is, however, 10-fold greater in mouse (1000 - 2000 nmol/mg/min) than in rat (100-200 nmol/mg/min) astrocytes (36).

Permeant analogs of cyclic AMP enhance phosphorylation of astroglial proteins and transform flat polygonal astrocytes to process-bearing, stellate astrocytes in vitro (37, 38). In situ, cerebral cyclic AMP concentration increases following ischemia (39) and astroglial stellation resembles the induction of reactive astrocytes that follows brain trauma (40). We observed that prolonged exposure to 0.25 mM dibutyryl cyclic AMP caused stellation and increased the rates of ascorbate uptake in rat and, especially, mouse astrocytes. Dibutyryl cyclic AMP also stimulates glutamate and GABA uptake by primary cultures of mouse cerebral astrocytes (41,42).

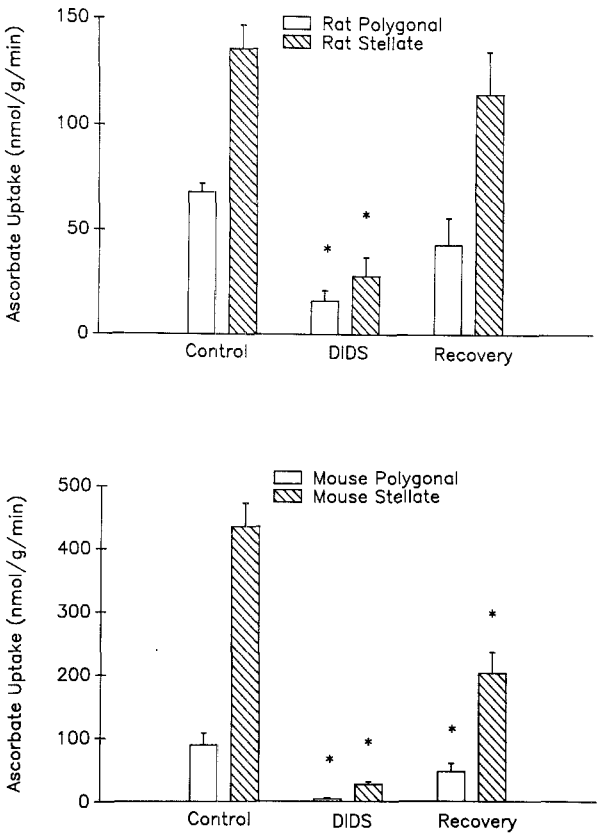


Fig. 4. Effect of DIDS on ascorbate uptake by primary cultures of rodent astrocytes. Rates of uptake were measured using mouse and rat astrocytes incubated for 1 min at 37°C with 5 μM L-[^{14}C]ascorbate as described under "Experimental Procedure". The acute effect of 1 mM DIDS was determined by adding it to the cell cultures at the beginning of the ascorbate uptake incubation. Reversibility was examined by preincubating cell cultures with the inhibitor, washing, and then measuring ascorbate uptake during a subsequent 1 min incubation. Cultures were washed once with drug-free medium, then incubated for 1 min in medium containing 0.5% bovine serum albumin, and finally washed again with drug-free medium (entire procedure at 37°C). Comparisons are made between polygonal astrocytes that were not treated with dibutyryl cyclic AMP and stellate astrocytes that were treated with 0.25 mM dibutyryl cyclic AMP for 2 weeks. Uptake rates are expressed as nmol ascorbate/g protein/min. Data are mean ± SE of 3-6 experiments performed in triplicate. *$P < 0.05$ compared to control values.

However, not all transport systems are similarly regulated. Studies with primary cultures of mouse astrocytes failed to detect any effect of dibutyryl cyclic AMP-induced stellation on deoxyglucose uptake (43). Moreover, dibutyryl cyclic AMP-induced stellation has no effect on the initial rate of Na^+ uptake in primary cultures of rat astrocytes (44).

The saturable component of [^{14}C]ascorbate uptake into astroglial cultures is mediated by a Na^+-dependent transport mechanism that is specific for ascorbate. All of the saturable ascorbate uptake into rat astrocytes incubated at 37°C with 5 μM L-[^{14}C]ascorbate was prevented by the omission of Na^+ from the incubation medium, since Na^+-free media decreased uptake to the same extent as did excess unlabeled L-ascorbate (15). Nominally Na^+-free media also decreased the initial ascorbate uptake rates by mouse astrocytes to 15 ± 5 percent of control values measured in Na^+-replete medium ($n = 4$; unpublished observations). Acetate, formate, lactate, malonate, oxalate, para-aminohippurate, pyruvate or succinate each failed to affect the initial rate of saturable ascorbate uptake. Therefore, the transport process for ascorbate is distinct from the mechanisms that translocate unsubstituted monocarboxylic acids (formic and acetic), hydroxymonocarboxylic acids (lactic), ketomonocarboxylic acids (pyruvic), dicarboxylic acids (oxalic, malonic and succinic), and the acturic acid derivative p-aminohippuric acid.

Cl^-_0 substitution had only small effects on the initial rate of ascorbate uptake in astrocytes (Figure 1). Furthermore, these effects varied depending upon which anion was substituted for Cl^-_0. These data indicate that the Na^+-dependent ascorbate transport mechanism does not have a specific requirement for external Cl^-. The large effects of furosemide, SITS and DIDS contrast with the relative insensitivity of astroglial ascorbate uptake to Cl^- and other external anions. This contrast indicates that the effects of these inhibitors are not secondary to alterations in Cl^- gradients.

[^{14}C]Ascorbate accumulation in astrocytes was blocked by unlabeled ascorbate (Figure 1), consistent with a saturable uptake mechanism. Rose (2) has argued that saturation may not arise from a plasma membrane transporter since it could result from transmembrane diffusion followed by intracellular metabolism or compartmentation. However, the rapid and reversible effects of furosemide, SITS and DIDS suggest that these inhibitors interact directly with the astroglial ascorbate transporter. Furthermore, since SITS and DIDS are impermeant, the plasma membrane must be the location of the ascorbate transporter in these cells.

Astroglial ascorbate uptake (present experiments) and $Na^+/K^+/Cl^-$cotransport (21) are both rapidly inhibited by 1 mM furosemide. However, ascorbate uptake is also sensitive to SITS (present experiments) while the $Na^+/K^+/Cl^-$ cotransporter is not (21). DIDS blocks ascorbate efflux from bovine adrenomedullary cells that possess a Na^+-ascorbate cotransporter similar to the astroglial mechanism (45), but does not affect Na^+-independent ascorbate uptake into human red cells (46). Thus, DIDS inhibition of vitamin C translocation may be specific for Na^+-ascorbate cotransport systems.

Stimulation of astroglial anion transport systems

causes cerebrocortical swelling under certain pathological conditions (19,47). Both cerebrocortical swelling and associated uptake of ions are prevented by furosemide or SITS (48). Furosemide has been observed to have a beneficial effect on the water and electrolyte content of cerebral tissue in cases of peritumoral brain edema (25). Therefore, inhibitors of anion transport systems may be useful clinically for preventing the cytotoxic cerebral edema associated with traumatic brain injury (23,24). The present experiments found that the astroglial ascorbate transporter is susceptible to inhibition by furosemide and stilbene disulfonates. Thus, anion transport inhibitors administered clinically to prevent cytotoxic edema may interfere with the regulation of cerebral vitamin C levels.

In conclusion, the present study provides evidence that the saturable, Na^+-dependent uptake of ascorbate by astrocytes (15) is mediated by a specific carrier located in the plasma membrane. Furthermore, the transport system may be regulated physiologically since the rate of uptake increases following chronic exposure to a permeant analog of cyclic AMP in vitro, particularly in the mouse astroglial model.

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