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Substrate Regulation of Ascorbate Transport Activity in Astrocytes

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Astrocytes possess a concentrative L-ascorbate (vitamin C) uptake mechanism involving a Na^+-dependent L-ascorbate transporter located in the plasma membrane. The present experiments examined the effects of deprivation and supplementation of extracellular L-ascorbate on the activity of this transport system. Initial rates of L-ascorbate uptake were measured by incubating primary cultures of rat astrocytes with L-[^{14}C]ascorbate for 1 min at 37°C. We observed that the apparent maximal rate of uptake (V_{max}) increased rapidly (<1 h) when cultured cells were deprived of L-ascorbate. In contrast, there was no change in the apparent affinity of the transport system for L-[^{14}C]ascorbate. The increase in V_{max} was reversed by addition of L-ascorbate, but not D-isoascorbate, to the medium. The effects of external ascorbate on ascorbate transport activity were specific in that preincubation of cultures with L-ascorbate did not affect uptake of 2-deoxy-D-[^{3}H(G)]glucose. We conclude that the astroglial ascorbate transport system is modulated by changes in substrate availability. Regulation of transport activity may play a role in intracellular ascorbate homeostasis by compensating for regional differences and temporal fluctuations in external ascorbate levels.

KEY WORDS: Ascorbate; astrocytes; autoregulation; rat brain; transport mechanism; vitamin C.

INTRODUCTION

Ascorbate is essential for nervous system function because it is a cofactor in biosynthesis of myelin (1,2) and catecholamines (3), facilitates release of transmitters (4-6), modulates binding of ligands to neural receptors (7-9), and slows rates of transmitter clearance (10-12). Vitamin C homeostasis in the central nervous system is maintained even when plasma ascorbate levels are drastically lowered (13) or elevated (14,15).

Experiments with cortical slices and freshly dissociated tissue have shown that brain cells actively transport ascorbate (16,17,18). Cultured brain cells are usually grown in media that are virtually free of ascorbate. This is true even for serum-supplemented media because the vitamin is not detectable (<0.5 μg/ml) in the commercially available sera commonly used for cell culture (19). Ascorbate is not synthesized by rodent brain cells and is not detectable in either astrocytes or neurons cultured in medium from which the vitamin is absent (20). However, our previous studies showed that type-1 astrocytes of rat and mouse brains possess a specific, high-affinity, Na^+-ascorbate cotransport system in their plasma membrane (21,22). Uptake of L-ascorbate by astrocytes was found to be: i) saturable, stereoselective, temperature- and Na^+-dependent; ii) specific for the vitamin since it was not diminished in the presence of other organic anions including acetate, formate, lactate, malonate, oxalate, p-aminohippurate, pyruvate and succinate; iii) lacking a specific requirement for external Cl^-; and iv) rapidly (≤1 min) and reversibly inhibited by furosemide, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS)

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and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). Rapid and reversible inhibition by the impermeant antagonists, SITS and DIDS, localized the transporter to the plasma membrane. The apparent affinity of the transporter for L-ascorbate was in the micromolar range in astrocytes incubated with a physiological concentration of extracellular Na^+, indicating a high-affinity L-ascorbate transporter. However, the affinity for L-ascorbate was significantly decreased when the extracellular Na^+ concentration was lowered, consistent with Na^+-ascorbate cotransport. Efflux proceeded much more slowly than uptake under our experimental conditions. There is evidence that the transport system is subject to regulation, since prolonged (>1 week) exposure to dibutyryl cyclic AMP increased the apparent maximal transport rate (V_{max}) of L-ascorbate in astrocytes (21,22).

Type-1 astrocytes are an abundant glial cell type comprising approximately 30% of brain volume. It is important to characterize the transport process of astrocytes in order to understand the mechanisms underlying regulation of cerebral ascorbate concentration. The function of vitamin C in astrocytes is not known, but could be investigated by experiments involving depletion and repletion of external ascorbate, as suggested for other cell types (23). The purpose of the present study was to examine the responses of astroglial ascorbate transport activity to changes in extracellular ascorbate concentrations.

EXPERIMENTAL PROCEDURE

Materials. L-[1-^{14}C]ascorbate (10 mCi/mmol) was purchased from Dupont Canada. 2-Deoxy-D-[^{3}H(G)]glucose (8.3 mCi/mmol) was purchased from New England Nuclear and Amersham Canada. Horse serum was obtained from Gibco Canada. Modified Eagle's minimum essential medium (24) was made using tissue culture-grade chemicals purchased from Sigma. L-ascorbate, D-isoascorbate, DL-homocysteine, and N(6),O(2′)-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP) were also from Sigma.

Cells. Primary cultures of astrocytes were prepared from the neopalium of 1-day-old Sprague-Dawley rats according to the procedure of Hertz et al. (24,25). The cells were plated onto 60 mm petri dishes (Falcon) and grown in modified Eagle's minimum essential medium with 20% horse serum nominally ascorbate-free (37°C; 95% air/5% CO_2). The culture medium was changed twice weekly, with the serum concentration reduced to 10% after 3 days. Cultures reached confluency after 2-3 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 silver showed that neurons were absent. The cultivated cells stained positively for glial fibrillary acidic protein using the procedure of Wilson et al. (26). The morphology of living cell cultures was assessed by phase contrast microscopy. Treatment with 0.25 mM dibutyryl cyclic AMP changed the flat polygonal astrocytes to process-bearing stellate astrocytes.

Preincubation of Astrocytes and Measurement of Transport Kinetics. The initial rate of cellular uptake of L-[^{14}C]ascorbate was measured at 37°C in serum-free incubation medium, essentially as described previously (21). The 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. Aliquots of medium were collected at the end of each uptake incubation. Incubations were terminated by washing cultures with ice-cold, isoosmotic Tris-sucrose solution and harvesting the cells. An aliquot of the cell harvest was used for protein measurement (27) and the remainder was combined with scintillation cocktail. The radioactive contents of the medium and cells were measured by liquid scintillation counting. Ascorbate uptake proceeds linearly with time for at least 3 min (21), so 1 min incubations were used to measure initial uptake rates. Rates were computed based on the specific activity of L-[^{14}C]ascorbate in the medium and expressed as nmol ascorbate/g cell protein/min.

To study the effect of external ascorbate supplementation on transport activity, astrocytes were preincubated for 0-18 h with various concentrations of unlabeled L-ascorbate (physiological substrate) or D-isoascorbate (control compound for assessment of nonspecific effects) prior to measurement of L-[^{14}C]ascorbate uptake. The medium was replenished with L-ascorbate at intervals of 12 h or less. The effect of external ascorbate deprivation was examined by preincubating astrocyte cultures with unlabeled L-ascorbate for 12-18 h (37°C), subsequently washing and incubating (0-6 h) the cultures with ascorbate-free medium, and then measuring L-[^{14}C]ascorbate uptake during a final 1 min incubation.

Stock solutions of L-[^{14}C]ascorbate and unlabeled ascorbate analogs 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.

2-Deoxy-D-[^{3}H(G)]glucose transport studies were carried out as described by Mesmer et al. (28). Cells were grown to confluence in six-well Costar plates (35 × 15 mm). Medium was aspirated and each well washed with 8 ml of phosphate buffered saline (PBS). Nine hundred microlitres of uptake buffer (PBS containing 1 mg/ml bovine serum albumin) were added to each well. Transport studies were carried out at 23°C and were initiated by the addition of 100 μl of radioactive 2-deoxyglucose (0.6 mM). At appropriate times, uptake was terminated by washing the wells rapidly (less than 15 sec) twice with ice cold PBS. In all cases, 1 min uptake assays were performed and cell samples were taken at 15, 30, 45 and 60 sec after the addition of the radioactive substrate. Cells were solubilized with 1 ml of 0.1% Triton X-100; 0.8 ml aliquots were counted in 10 ml of scintillation fluid. Additional wells on each plate were used for protein determinations. Zero-time controls and background counts were subtracted from the raw data.

Statistics. Results are presented as the mean ± SEM of n experiments each with triplicate replications. In figures, error bars were omitted when the standard error was less than the size of the symbol. To determine the apparent Michaelis constant (K_m) and maximum rate of uptake (V_{max}) from Lineweaver-Burk and Eadie-Hofstee plots, straight lines were fitted and intercepts calculated by linear regression. Comparisons between mean values based on a single level of treatment (e.g. V_{max} for cells preincubated 6 h with or without 100 μM L-ascorbate) were evaluated using paired *t*-tests. For simultaneous comparisons of two or more treatments, differences between means were evaluated using repeated measures analysis of variance and the Tukey-

Kramer test (29). For all statistical tests, a P value of <0.05 was considered significant.

RESULTS

External L-ascorbate deprivation increased astroglial L-[^{14}C]ascorbate transport activity (Figure 1). Transferring astrocytes from medium containing ascorbate (100 μM nominal) to ascorbate-free medium increased the L-[^{14}C]ascorbate uptake rate ($P < 0.05$). In contrast, adding L-ascorbate to the medium of previously ascorbate-free cultures led to decreased initial rates of L-[^{14}C]ascorbate uptake ($P < 0.05$, Figure 2). The effects of both substrate deprivation and supplementation were rapid. Most of the change in astroglial L-ascorbate transport rate was achieved within 1 h of raising or lowering the concentration of the vitamin in the medium (Figures 1 and 2).

These changes were specific effects on the L-ascorbate transport system, since the presence or absence of 100 μM external L-ascorbate during the preincubation period did not change either the cellular morphology or protein content of the astroglial cultures (data not shown). Furthermore, preincubation of astrocytes with ascorbate did not inhibit 2-deoxy-D-[^{3}H(G)]glucose uptake. In stellate astrocytes the initial rates of 2-deoxy-D-[^{3}H(G)]glucose uptake were 1878 ± 506 nmol/g protein/min under control (ascorbate-free) conditions and 2092 ± 488 nmol/g protein/min after 6 h preincubation with 100 μM external L-ascorbate (mean ± SEM from three independent experiments).

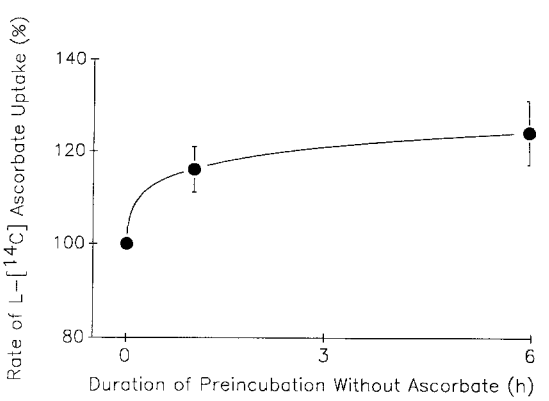

Fig. 1. Time course of the effect of substrate deprivation on astroglial L-ascorbate uptake. Stellate astrocytes were maintained in medium containing 100 μM nominal concentration of unlabeled L-ascorbate for 12-18 h and then transferred to ascorbate-free medium for the indicated times. Subsequently, L-[^{14}C]ascorbate uptake was measured during 1 min incubations with 5 μM of the radiolabeled vitamin (10 mCi/mmol). Results are expressed as percentages of the uptake rates by astrocytes preincubated 18 h with L-ascorbate (100% = 91 ± 11 nmol/g protein/min, $n = 4$). Plotted are the means ± SEM of four independent experiments each performed in triplicate.

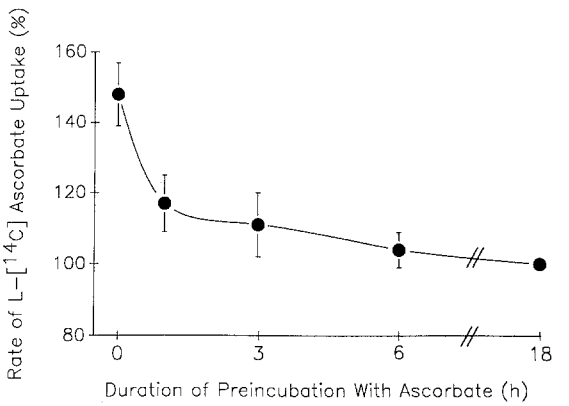

Fig. 2. Time course of the effect of substrate supplementation on astroglial L-ascorbate uptake. Stellate astrocytes were cultured in nominally ascorbate-free incubation medium. On the day of the experiment, the cells were preincubated in medium containing 100 μM nominal concentration of unlabeled L-ascorbate for the indicated times. To measure the initial rate of L-[^{14}C]ascorbate uptake after preincubation, astrocytes were incubated for 1 min with 5 μM of the radiolabeled vitamin (10 mCi/mmol). Results are expressed as percentages of the uptake rates by glial cultures preincubated 18 h with L-ascorbate (100% = 91 ± 11 nmol/g protein/min, $n = 4$). Plotted are the means ± SEM of four independent experiments each performed in triplicate.

Ascorbate transport activities in both polygonal and stellate astrocytes were decreased by preincubation with high concentrations of external L-ascorbate (Figure 3). When compared to L-[^{14}C]ascorbate uptake under control conditions (i.e. after preincubation in nominally ascorbate-free medium), uptake rates after 6 h preincubation with 300 μM L-ascorbate were inhibited by 38 ± 2% in polygonal astrocytes and 42 ± 3% in stellate astrocytes. A significant ($P < 0.05$) decrease was achieved by 6 h preincubation with external L-ascorbate concentrations as low as 30 μM in stellate cells and 100 μM in polygonal cells (Figure 3).

Decreased L-ascorbate transport activity was observed following preincubation with L-ascorbate but not D-isoascorbate (Figure 3). Even 300 μM external D-isoascorbate, which was the highest concentration tested, did not have the effect induced by 30 μM L-ascorbate during the 6 h preincubation period (Figure 3).

The effects of external ascorbate deprivation on the kinetics of L-[^{14}C]ascorbate transport were investigated. Astroglial cultures preincubated either with or without extracellular L-ascorbate demonstrated initial velocities of L-[^{14}C]ascorbate uptake that were concentration-de-

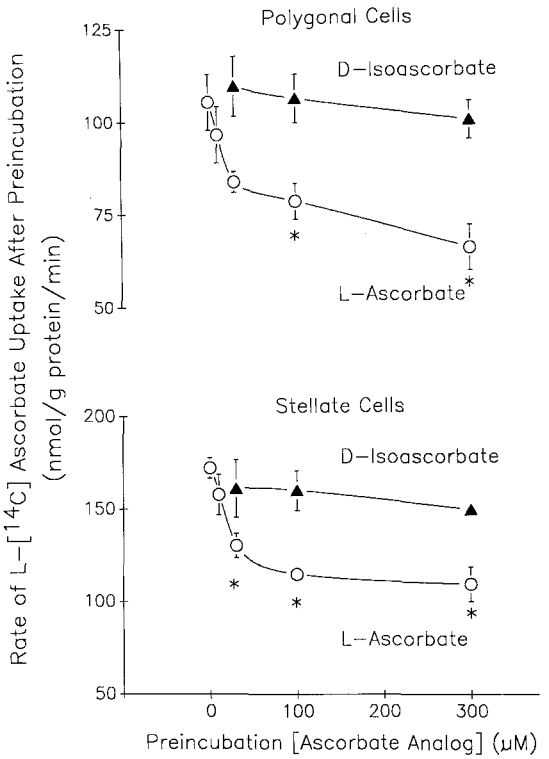

Fig. 3. Effects of changes in extracellular concentration of unlabeled L-ascorbate or its epimer, D-isoascorbate, during 6 h preincubations, on the subsequent rate of L-[^{14}C]ascorbate uptake. To measure the initial rate of uptake after preincubation, astrocytes were incubated for 1 min with 5 μM L-[^{14}C]ascorbate. The **top panel** shows results obtained with polygonal astrocytes (cultures that did not receive dibutyryl cyclic AMP). The **bottom panel** shows results obtained with stellate astrocytes (cultures that were treated with 0.25 mM dibutyryl cyclic AMP for 2 weeks). Each point represents the mean ± SEM of three independent experiments performed in triplicate. * $P < 0.05$ compared to control cells preincubated without L-ascorbate.

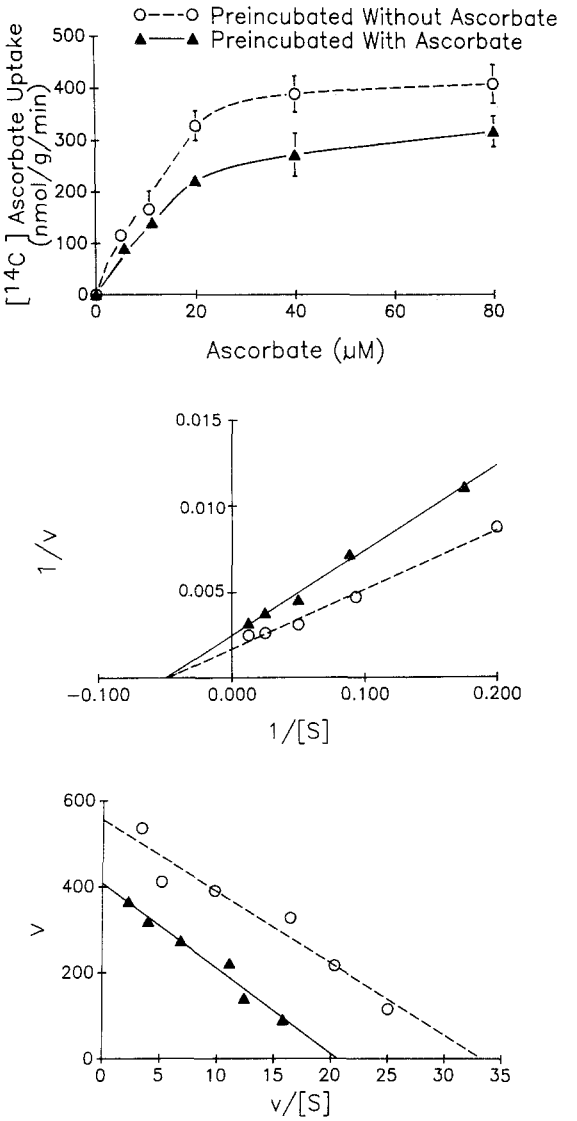

Fig 4. Kinetics of L-[^{14}C]ascorbate uptake by stellate astrocytes after 6 h preincubation with or without 100 μM unlabeled L-ascorbate. To measure the initial rate of uptake after preincubation, astrocytes were incubated for 1 min with the indicated concentrations of L-[^{14}C]ascorbate. The nonspecific uptake, measured in the presence of 3 mM unlabeled L-ascorbate, has been subtracted to calculate saturable uptake. The **top panel** shows the rates of saturable L-[^{14}C]ascorbate uptake. Points represent the mean ± SEM of three independent experiments each performed in triplicate. The **middle panel** is a Lineweaver-Burk plot of the data illustrated in the top panel. [S] = external concentration of L-[^{14}C]ascorbate (μM); v = initial rate of L-[^{14}C]ascorbate uptake (nmol/g protein/min). Lines were fitted by linear regression and had correlation coefficients >0.99. The **bottom panel** is an Eadie-Hofstee plot of the same data. Correlation coefficients were 0.98 for stellate astrocytes preincubated without L-ascorbate and 0.98 for stellate astrocytes preincubated with 100 μM L-ascorbate for 6 h.

pendent and saturable following Michaelis-Menten kinetics (Figure 4). For stellate astrocytes preincubated in medium containing L-ascorbate (100 μM for 6 h) or maintained in ascorbate-free medium, Lineweaver-Burk plots of the data indicated the apparent V_{max} were 407 ± 29 and 605 ± 54 nmol ascorbate/g protein/min ($P < 0.05$), respectively, and the apparent K_m were 20 ± 1 and 21 ± 2 μM ascorbate, respectively ($n = 3$; Figure 4). Eadie-Hofstee plots of the data for stellate astrocytes preincubated in medium containing L-ascorbate (100 μM for 6 h) or maintained in ascorbate-free medium, indicated the apparent V_{max} were 390 ± 50 and 554 ± 95 nmol ascorbate/g protein/min ($P < 0.05$), respectively, and the apparent K_m were 17 ± 1 and 17 ± 2 μM ascorbate, respectively ($n = 3$; Figure 4). Therefore, both analyses showed that the increase in transport activity associated

with substrate deprivation involved a significant increase in the apparent maximal uptake rate with no change in the affinity of the astroglial transport system for external L-ascorbate.

DISCUSSION

Altered metabolic states and levels of secretory activity can result in gross changes in the availability of L-ascorbate to various cells. Regulation of ascorbate transport rates is a plausible mechanism for controlling intracellular ascorbate concentrations in the face of a constantly changing extracellular supply. The results of previous in vivo studies are consistent with regulation of vitamin C transport by external ascorbate levels. *i*) In guinea pigs, which cannot synthesize ascorbate, ascorbate-deficient diets lead to a prolonged half-life of vitamin C in brain (13). *ii*) Ingestion of excess vitamin C by guinea pigs slowly changes intestinal ascorbate transport with the result that initial uptake rates are decreased (30,31). For example, the apparent V_{max} of Na^+-dependent ascorbate uptake in ileum was 32% lower in animals fed an ascorbate-enriched ration for 14 days, compared with controls fed a maintenance ration (31). These data from long-term whole-animal experiments suggested that autoregulation of ascorbate transport may occur in cerebral and intestinal tissue, but did not provide information about cellular mechanisms. For example, apparent downregulation might have resulted from altered transport rates but also from altered catabolism of ascorbate, systemic compensations or nonspecific toxic effects. In particular, in vivo experiments did not determine if high-affinity, Na^+-dependent ascorbate transport could be altered by changes in substrate availability.

There does not appear to be any previous evidence for ascorbate-dependent regulation of ascorbate uptake in cell systems. Pheochromocytoma cells express a high-affinity ascorbate transport system, yet preincubation with millimolar concentrations of the vitamin in the culture medium did not alter subsequent uptake of L-[^{14}C]ascorbate (32).

Accumulation of ascorbate by brain cells is especially interesting because the half-life of the vitamin is several-fold longer in the brain than in any other organ studied (liver, heart, kidneys, adrenals, spleen; 13) and this half-life is influenced by cellular transport (33). The present experiments used primary cultures of cerebral astrocytes that are useful brain cell models (34). Although some cell types possess both high- and low-affinity ascorbate transporters (e.g. human neutrophils - 23), virtually all ascorbate uptake by astrocytes appears to be through a high-affinity, Na^+-dependent system (21).

The apparent affinity of the astroglial ascorbate transporter is an order of magnitude greater for L-ascorbate than for D-isoascorbate (21), a stereoisomer that does not naturally occur in rodent brain (35) and has only 1/20 the antiscorbutic activity of vitamin C (13,36). The decrease in astroglial vitamin C transport activity induced by external ascorbate is also stereoselective. Whereas 30 μM L-ascorbate and 300 μM D-isoascorbate were equally effective in competitively blocking L-[^{14}C]ascorbate uptake when either of the unlabeled analogs was added to the medium at the same time as the radiolabeled vitamin (21), the present experiments found that preincubation with 30 μM L-ascorbate was significantly more effective than 300 μM D-isoascorbate in reducing subsequent L-[^{14}C]ascorbate uptake. These data suggest that interaction of ligand with the external face of the ascorbate transporter is not, of itself, sufficient to modulate transport activity. More importantly, the data show that this effect does not arise from nonspecific damage by extracellular reductant. Furthermore, the effects of external ascorbate on L-ascorbate transport activity were apparently specific in that astroglial morphology, protein content and 2-deoxy-D-[^{3}H(G)]glucose uptake were unaffected.

Substrate-dependent regulation of membrane transport has been extensively documented for the Na^+-independent glucose transport system. Maintenance of various cell types in the absence of D-glucose has been shown to increase the apparent V_{max} for hexose transport with no change in the apparent K_m (37). Glucose deprivation of primary rat brain astroglial cells for 2-12 h gives rise to an increase in glucose transport activity as well as in the amount of glucose transporter protein and mRNA (38). Half-maximal induction of glucose transport activity occurs by 2-3 h and the maximal increase in the glucose transport activity is observed within 12 h of transferring glucose-replete astroglial cells to a nominally glucose-free medium (38). Longer periods of starvation may be less effective, since Hara et al. (39) reported that glucose starvation for 40-65 h does not change subsequent 2-deoxy-D-glucose uptake activity in cultured rat astrocytes. Dissociated cells from rat brain show accelerative exchange of 2-deoxy-D-glucose (i.e. transstimulation of transport). When these cells are preincubated for 20 min in the presence of various concentrations of unlabeled 2-deoxyglucose or 3-*O*-methylglucose, so as to increase intracellular sugar concentrations, the subsequent rate of 2-deoxy-D-[^{3}H(G)]glucose uptake is enhanced (40). No evidence for accelerative exchange

was found in our experiments with astroglial ascorbate transport. Na^+-dependent ascorbate transport has a much higher affinity (apparent K_m = 17-21 μM ascorbate; present experiments) than does Na^+-independent hexose transport (apparent K_m = 360 μM for 2-deoxyglucose; 41) by similar rat astroglial cultures.

Na^+-dependent plasma membrane transport systems which are modulated by external substrate deprivation include: i) Na^+-glucose cotransport activity in enterocytes which increases during glucose deprivation (42); ii) Na^+-amino acid cotransport in fibroblasts, hepatocytes and C6 glioma cells which increases during amino acid deprivation (43–48); iii) Na^+-phosphate cotransport in several cell types which increases during phosphate deprivation (49). In these three cases, Na^+-dependent substrate uptake responds to substrate deprivation (i.e. exposure to substrate-free medium) with an increase in V_{max} but without change in K_m. In some cases, stimulation of transport requires gene transcription and protein synthesis (48,49). However, amino acid deprivation of <1 h can increase cellular amino acid transport activity through kinetic regulation (release from transinhibition) that does not require protein synthesis (44–47). The mechanism underlying the stimulation of astroglial ascorbate V_{max} by vitamin C deprivation has not yet been determined, but may involve: *i*) changed kinetic properties of transporters, affecting V_{max} but not K_m (e.g. release from transinhibition), or *ii*) an increased number of functional transporters due to slowed degradation of existing transporters, activation of nonfunctional (cryptic) transporters, redistribution of transporters from an intracellular compartment to the plasma membrane, or synthesis of transporters de novo. However, synthesis of new transporters seems unlikely in view of the rapidity of the change in ascorbate transport rate following a change in external ascorbate concentration. Under physiological conditions, ascorbate is an important reducing agent (50). Therefore, "adaptation" to vitamin C deprivation may involve an alteration in intracellular redox state (51) with subsequent effects on the transport process.

The concentration of ascorbate in brain extracellular fluid continually fluctuates. For example, extracellular ascorbate concentration in rat striatum varies according to a circadian pattern, with a rise during the nocturnal increase in motor activity (52). Additionally, more rapid changes in rat striatal ascorbate can be observed following tail-pinch (53). Extracellular ascorbate levels also differ between brain regions. For example, extracellular ascorbate concentrations in the white matter of the corpus callosum are higher than in adjacent areas of grey matter (striatum and cortex; 54). Additionally, amphetamine-induced increases in extracellular ascorbate concentration are greater in the caudate than in the nucleus accumbens of rat brain (55). These reports indicate large temporal and regional variations in extracellular ascorbate concentration. Our data are consistent with a transport system that plays a role in regulating intracellular ascorbate levels, because changes in transport activity may compensate for fluctuations in extracellular ascorbate concentration.

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