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High-Affinity Sodium-Dependent Uptake of Ascorbic Acid by Rat Osteoblasts

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Summary. Ascorbic acid is essential for the formation of bone by osteoblasts, but the mechanism by which osteoblasts transport ascorbate has not been investigated previously. We examined the uptake of L-[^{14}C]ascorbate by a rat osteoblast-like cell line (ROS 17/2.8) and by primary cultures of rat calvaria cells. In both systems, cells accumulated L-[^{14}C]ascorbate during incubations of 1–30 min at 37°C. Unlike propionic acid, which diffuses across membranes in protonated form, ascorbic acid did not markedly alter cytosolic pH. Initial ascorbate uptake rate saturated with increasing substrate concentration, reflecting a high-affinity interaction that could be described by Michaelis-Menten kinetics (apparent K_m = 30 ± 2 μM and V_{max} = 1460 ± 140 nmol ascorbate/g protein/min in ROS 17/2.8 cells incubated with 138 mM extracellular Na^+). Consistent with a stereoselective carrier-mediated mechanism, unlabeled L-ascorbate was a more potent inhibitor (IC_{50} = 30 ± 5 μM) of L-[^{14}C]ascorbate transport than was D-isoascorbate (IC_{50} = 380 ± 55 μM). Uptake was dependent on both temperature and Na^+, since it was inhibited by cooling to 4°C and by substitution of K^+, Li^+ or N-methyl-D-glucamine for extracellular Na^+. Decreasing the external Na^+ concentration lowered both the affinity of the transporter for ascorbate and the apparent maximum velocity of transport. We conclude that osteoblasts possess a stereoselective, high-affinity, Na^+-dependent transport system for ascorbate. This system may play a role in the regulation of bone formation.

Key Words vitamin C · ascorbic acid · sodium cotransport · osteoblasts

Introduction

Ascorbic acid (vitamin C[1]) plays an important role in bone formation. Vitamin C deficiency (scurvy) is characterized by generalized defects in the extracellular matrices of bone and other connective tissues. In vivo studies have shown that administered [^{14}C]ascorbate accumulates preferentially in sites of active osteogenesis (Hammarström, 1966). In vitro, the formation of bone in organ culture is reduced in the absence of ascorbic acid (Chen & Raisz, 1975) and the vitamin is essential for the formation of a mineralized extracellular matrix by rabbit long bone cells (Anderson et al., 1984) and rat calvaria cells (Bellows et al., 1986).

The organic matrix of bone consists primarily of type I collagen, and ascorbate is required for the synthesis of this and other interstitial collagens (Prockop et al., 1979). To be utilized for osteoid synthesis, ascorbate must first traverse the osteoblast plasma membrane. Some weak organic acids (e.g., propionic acid) can diffuse passively in uncharged protonated form across plasma membranes (Boron, 1983). Ascorbic acid (pK_a's of ~4 and 11), the reversibly reduced form of vitamin C, exists predominantly as a monoanion at physiological pH. It is not clear, however, to what extent uptake of vitamin C can occur by diffusion of protonated ascorbic acid across cell membranes. It has been suggested that ascorbate accumulates in human erythrocytes by an entrapment mechanism (Wagner et al., 1987). Ascorbic acid is thought to be oxidized extracellularly to dehydroascorbic acid, which then diffuses across the erythrocyte plasma membrane. Dehydroascorbic acid is reduced intracellularly, resulting in accumulation of the impermeant ascorbate ion. Alternatively, it has been proposed that uptake of dehydroascorbic acid by erythrocytes (Mann & Newton, 1975), neutrophils, fibroblasts (Bigley et al., 1983), placental cells (Ingermann, Stankova & Bigley, 1986), and leukocytes (Moser, 1987) is mediated by the Na^+-independent glucose transport system. In other tissues, active uptake of ascorbic acid is thought to occur via cotransport with Na^+. Low-affinity (K_m = 2–5 mM ascorbate) Na^+- dependent ascorbate transporters have been reported in epithelium and macrophages (Castronova et al., 1983). Higher affinity (K_m = 10–100 μM ascorbate) systems have been found in tissues with high metabolic requirements for ascorbate (ad-

[1] The term vitamin C is used to refer to the sum of the oxidized and reduced forms of ascorbic acid. However, there is no requirement for dietary vitamin C in rats which, like most animal species, produce ascorbic acid from glucose.

renal medulla and pituitary gland; Diliberto, Heckman & Daniels, 1983; Cullen, May & Eipper, 1986) and in tissues in which vitamin C is transported across epithelial barriers (renal tubule and intestine; Bianchi, Wilson & Rose, 1986; Rose, 1986). Because of the importance of ascorbic acid in bone formation and the possibility that transport of ascorbate may regulate osteogenesis, we examined the mechanism of ascorbate uptake by osteoblasts. This is the first report of a high-affinity, Na^+-dependent ascorbate transport system in bone. A preliminary account has appeared in abstract form (Wilson & Dixon, 1988).

Materials and Methods

Materials

Alpha-minimum essential medium (alpha-MEM)[2] and fetal bovine serum were obtained from Gibco Laboratories. L-[1-^{14}C]ascorbic acid (10 mCi/mmol) was purchased from Dupont Canada. L-ascorbate. D-isoascorbate, DL-homocysteine, KCl, LiCl, N-methyl-D-glucamine, ouabain and amiloride were from Sigma. *Bis*(carboxyethyl)-5,6-carboxyfluorescein (BCECF) acetoxymethyl ester was obtained from Molecular Probes. Nigericin was from Behring Diagnostics. All other chemicals were analytical reagent grade.

Cells and Cell Culture

The clonal osteosarcoma cell line ROS 17/2.8 was provided by Dr. G.A. Rodan (Merck, Sharp and Dohme Research Laboratories), grown in alpha-MEM supplemented with 15% heat-inactivated fetal bovine serum and antibiotics, and subcultured twice weekly. Four days prior to uptake measurements, ROS 17/2.8 cells were seeded at a density of 2×10^4 cells/cm^2 into 60- or 100-mm culture dishes.

Bone cells were isolated from fetal rat calvaria by sequential collagenase digestion (Rao et al., 1977; Bellows et al., 1986). Populations harvested at digestion times of 10, 20, 30, 50 and 70 min were designated populations I to V, respectively. Populations IV and V, which are most highly enriched in cells expressing osteoblast markers (Luben, Wong & Cohn, 1976; Rao et al., 1977), were pooled and plated at a density of 1.5×10^4 cells/cm^2 into 60-mm culture dishes containing 3 ml supplemented alpha-MEM. The medium was changed every second or third day. These primary cultures reached confluence after approximately seven days and were used for uptake determinations at 12 days.

[2] The abbreviations used are: Na_o^+, extracellular sodium; NMG, N-methyl-D-glucamine; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; alpha-MEM, alpha-minimum essential medium; pH_i, cytoplasmic pH; HPLC-ED, high performance liquid chromatography with electrochemical detection; BCECF, *bis*(carboxyethyl)-5,6-carboxyfluorescein; IC_{50}, half-maximal inhibitory concentration.

Ascorbic Acid Analysis

The concentration of ascorbic acid in the extracellular medium during 1–30-min incubations with ROS 17/2.8 cell cultures was determined by high performance liquid chromatography with electrochemical detection (HPLC-ED), according to the procedure of Behrens and Madère (1987). At the end of each incubation period, a 900-μl sample of medium was combined with 100 μl of 8.5% metaphosphoric acid and stored at −80°C. 3,4-Dihydroxybenzylamine (1 μM) was included as an internal standard. Immediately upon thawing, the sample was passed through a 45-μm Millex filter and a 20-μl aliquot of the filtrate was injected into the HPLC-ED system. Separation was achieved by reverse-phase HPLC-ED with a Resolve C18 column. The mobile phase was 80 mM sodium acetate buffer containing 1 mM n-octylamine, 15% methanol and 0.015% metaphosphoric acid. The final pH of the mobile phase was 4.6 and the flow rate was 0.9 ml/min. Eluate fractions were collected for scintillation counting and the elution volumes of ascorbic acid and 3,4-dihydroxybenzylamine were determined by electrochemical detection with a Waters M460 amperometric detector. When L-[^{14}C]ascorbic acid standards were analyzed by this system, 95% of the radiolabel co-eluted with the electrochemical peak corresponding to the authentic vitamin. The ascorbic acid concentration in each experimental sample was calculated by interpolation on a standard curve using a computer algorithm.

Measurement of Ascorbate Uptake

To measure cell ascorbate uptake, supplemented alpha-MEM was replaced by serum-free incubation medium containing L-[^{14}C]ascorbate. This 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. Stock solutions of L-[^{14}C]ascorbate, L-ascorbate and D-isoascorbate contained 0.4 mM homocysteine to prevent oxidation. The final concentration of homocysteine in the incubation medium was 4 μM. The pH of the medium was not altered by the presence of these reductants at the concentrations employed. To examine the dependence of ascorbate uptake on external Na^+ (Na_o^+), Na_o^+ was replaced by isosmotic concentrations of N-methyl-D-glucamine, Li^+ or K^+. Osmolality was measured by freezing point depression and Na concentration was measured by flame photometry. In some cases, the effects of ouabain were tested by preincubating ROS 17/2.8 cells with the drug in serum-free medium and comparing the subsequent L-[^{14}C]ascorbate uptake rate to that of preincubated controls. To measure ascorbate uptake rates, cells were incubated with L-[^{14}C]ascorbate for 0–30 min at 4 or 37°C. At the end of this uptake incubation, aliquots of medium were collected for scintillation counting and HPLC-ED analysis. Incubations were terminated by rinsing cultures with ice-cold isosmotic sucrose solution and harvesting the cells. An aliquot of the cell harvest was used for protein measurement (Lowry et al., 1951) and the remainder was combined with scintillation cocktail. The radioactive contents of the cell layer and incubation medium were quantified by liquid scintillation counting. Rate of ascorbate uptake was computed based on the specific activity of L-[^{14}C]ascorbate in the medium and was expressed as nmol ascorbate/g protein/min. Lineweaver-Burk plots were analyzed by linear regression to determine the apparent Michaelis constants (K_m) and maximum rates of ascorbate uptake (V_{max}).

Net accumulation of L-[^{14}C]ascorbate depends upon the respective rates of uptake and efflux. To assess the importance of

efflux, ROS 17/2.8 cell cultures were incubated with 5 μM L-[^{14}C] ascorbate for 5 min in serum-free medium containing 138 mM Na^+. Cultures were then washed and incubated with ascorbate-free medium for up to 10 min. Apparent efflux rates were calculated by dividing the amount appearing in the medium by the initial L-[^{14}C]ascorbate content of the cultures.

Measurement of Cytosolic pH

Cytosolic pH (pH_i) was measured fluorimetrically using the pH-sensitive dye *bis*(carboxyethyl)-5,6-carboxyfluorescein (BCECF). Monolayers of ROS 17/2.8 cells in supplemented alpha-MEM were loaded at 37°C for 1 hr with 4 μM BCECF acetoxymethyl ester. Cells were then washed, harvested by trypsinization and resuspended at 7×10^5 cells/ml in medium containing (in mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 20 HEPES, adjusted to pH 7.3 with NaOH. BCECF fluorescence was monitored using a fluorescence spectrophotometer (model LS-5, Perkin-Elmer) equipped with a magnetic stirrer and water jacket to maintain cell suspensions at 37°C. Excitation wavelength was set at 495 nm (5-nm slit) and emission at 525 nm (10-nm slit). Calibration of fluorescence *vs.* pH_i was obtained using the K^+-nigericin method (Thomas et al., 1979).

Statistics

Results are presented either as traces representative of at least three experiments or as the mean ± SE of the number of determinations specified. Straight lines were fitted and intercepts calculated by linear regression. IC_{50} values were determined by probit analysis. Differences between mean values were evaluated using the Student's *t* test with *P* values of less than 0.05 considered significant.

Results

Ascorbate Uptake

Accumulation of L-[^{14}C]ascorbate by ROS 17/2.8 cells proceeded linearly with time during incubations of 1 to 30 min (Fig. 1). L-[^{14}C]ascorbate uptake was largely inhibited by lowering the incubation temperature from 37 to 4°C (Fig. 1). Removing Na^+ from the incubation medium by substitution with N-methyl-D-glucamine decrease cellular uptake to the same extent as did low temperature, indicating that virtually all temperature-dependent ascorbate transport was Na^+ dependent (Fig. 1).

The initial rate of uptake was dependent on the concentration of L-[^{14}C]ascorbate and, consistent with Michaelis-Menten kinetics, saturated at high substrate concentrations (Fig. 2). The apparent K_m and V_{max} were found to be 30 ± 2 μM and 1460 ± 140 nmol ascorbate/g protein/min, respectively (n = 3), in 138 mM Na_o^+. Lowering the extracellular Na^+ concentration to 35 mM decreased the apparent affinity and slowed the maximum rate of transport. In

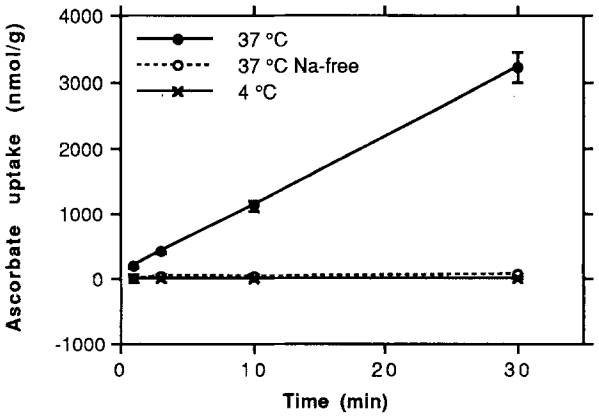

Fig. 1. Time course of ascorbate uptake showing the effects of temperature and external Na^+. Ascorbate uptake was measured using ROS 17/2.8 cells incubated with 5 μM L-[^{14}C]ascorbate as described Materials and Methods. The temperature was 4°C for those incubations so indicated and 37°C for the others. The extracellular Na^+ concentration was 138 mM except for the nominally Na^+-free medium, in which N-methyl-D-glucamine was substituted for Na^+. Ascorbate uptake, expressed in nmol/g protein, is plotted as a function of the incubation time. Data are the means ± SE of triplicate determinations from a single experiment representative of two independent experiments. Error bars were omitted where smaller than the symbol.

35 mM Na_o^+, the apparent K_m and V_{max} were 71 ± 2 μM and 670 ± 100 nmol ascorbate/g protein/min, respectively (n = 3).

The following experiment was performed to distinguish between internalization of the radiolabel and displaceable binding to cell surfaces. After the usual uptake incubation (1 min at 37°C with 5 μM L-[^{14}C]ascorbate), the medium containing the labeled vitamin was replaced for 1 min with medium containing 3 mM unlabeled L-ascorbate. Under these conditions, less than 10% of the L-[^{14}C]ascorbate associated with the cells was displaced by the excess unlabeled L-ascorbate. In contrast, simultaneous incubation of cells with both L-[^{14}C]ascorbate and 3mM unlabeled L-ascorbate decreased uptake by 95% (*see* Figs. 4 and 6). These data indicate that the cell cultures accumulated L-[^{14}C]ascorbate principally through internalization rather than displaceable binding. These data also show that there was little efflux of ascorbate under conditions which in other tissues promote the transactivation of ascorbate efflux (Rose, 1988).

Molecular Identity of the Transported Substrate

Ascorbic acid spontaneously oxidizes to dehydroascorbic acid in aqueous solutions at physiological pH and temperature. However, the reduced

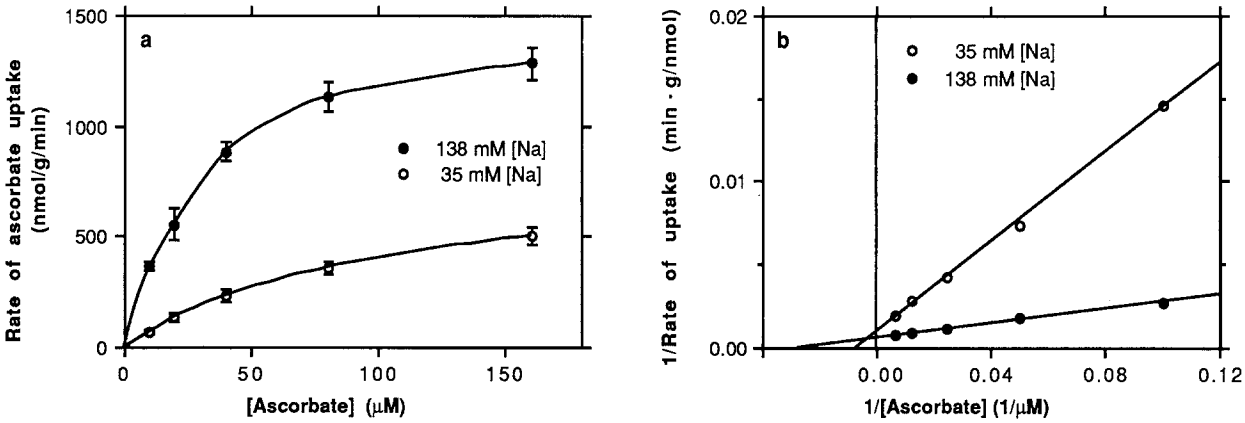

Fig. 2. Saturable uptake of L-[^{14}C]ascorbate by ROS 17/2.8 cells as a function of the extracellular concentrations of L-[^{14}C]ascorbate and Na^+. ROS 17/2.8 cells were incubated in media containing either 35 or 138 mM Na^+ for 1 min at 37°C with the indicated concentration of L-[^{14}C]ascorbate. N-methyl-D-glucamine was substituted for Na^+ in the low Na^+ medium. L-[^{14}C]ascorbate uptake in the presence of excess (3 mM) unlabeled L-ascorbate was found to be less than 5% of the total uptake and was subtracted from the total uptake to obtain the saturable uptake. (*a*) Rate of saturable uptake is plotted as a function of ascorbate concentration. Data are the means ± SE of three independent experiments each with triplicate determinations. (*b*) Lineweaver-Burk plots of the data illustrated in panel *a*. Lines were fitted by least squares and had correlation coefficients >0.99

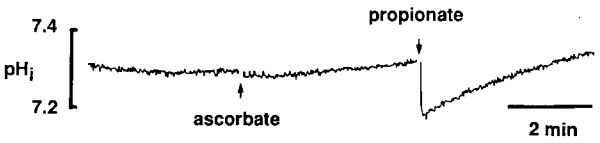

Fig. 3. Acute effects of L-ascorbate and propionate on cytosolic pH. The pH_i of ROS 17/2.8 cells loaded with BCECF was monitored fluorimetrically as described in Materials and Methods. Small volumes of concentrated sodium ascorbate and sodium propionate (5 mM final concentrations) were applied, where indicated, to cells suspended in Na^+-containing buffer, pH 7.3, 37°C. The tracing is representative of six tracings from three independent experiments

Table 1. Concentration of ascorbic acid in external medium during incubation with ROS 17/2.8 cell cultures[a]

Incubation period (min)	[Ascorbic acid] (μM)
1	5.3 ± 0.2
3	5.4 ± 0.1
10	5.3 ± 0.1
30	4.6 ± 0.1

[a] Cultures were incubated for the indicated periods at 37°C with incubation medium containing homocysteine (4 μM) and L-[^{14}C]ascorbic acid (nominally 5 μM, 10 mCi/mmol). Aliquots of media were collected at the end of each incubation period and assayed for ascorbic acid by HPLC-ED as described in Materials and Methods. Data are means ± SE of triplicate determinations from a single experiment representative of two independent experiments.

vitamin can be protected by the antioxidant homocysteine. We assessed the stability of ascorbic acid in the incubation medium under the experimental conditions used in this study. The concentration of authentic ascorbic acid in the incubation medium was analyzed by HPLC-ED and was found to decline only slightly during prolonged incubations (Table 1). These data suggest that the principal moiety transported was ascorbate, not dehydroascorbic acid.

Effect of Ascorbate on Cytoplasmic pH (pH_i)

In other cells, it has been shown that uptake of weak organic acids (e.g., propionic acid) can occur by rapid diffusion of the uncharged protonated form of the acid across the plasma membrane (Boron, 1983). Once within the cell, dissociation of the acid results in cytoplasmic acidification. To examine whether appreciable uptake of ascorbic acid might occur by such a mechanism, we compared in ROS 17/2.8 the acute effects on pH_i of ascorbate and propionate (Fig. 3). Whereas the addition of 5 mM sodium ascorbate had little effect on pH_i, the same concentration of sodium propionate caused rapid acidification of the cytosol.

In another experiment, we tested if the marked dependence of ascorbate uptake on Na_o^+ arises from indirect coupling of Na^+/H^+ antiport to the uptake of protonated ascorbic acid. The uptake of L-[^{14}C]ascorbate by ROS 17/2.8 cells was measured in the presence or absence of amiloride, an inhibitor of Na^+/H^+ exchange (Kleyman & Cragoe, 1988). Al-

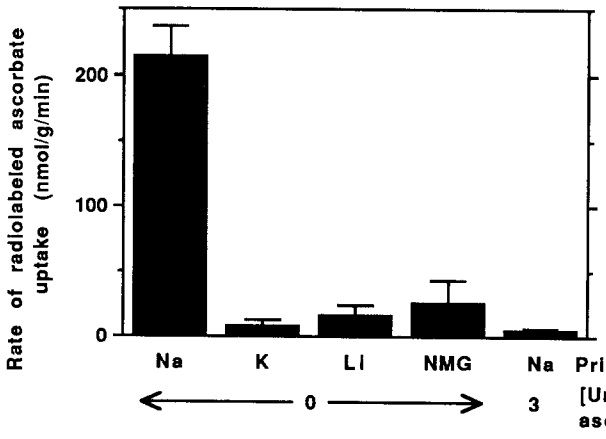

Fig. 4. Cation dependence of the rate of radiolabeled ascorbate uptake. Rate of L-[^{14}C]ascorbate uptake was measured as described in Materials and Methods, using ROS 17/2.8 cells incubated for 1 min at 37°C with 5 μM L-[^{14}C]ascorbate in medium containing 138 mM Na^+. Where indicated, K^+, Li^+ or N-methyl-D-glucamine (NMG) were substituted isosmotically for Na^+ in the external medium. Excess (3 mM) unlabeled L-ascorbate was added to Na^+-containing medium where indicated, in order to determine nonspecific uptake. Data are the means ± SE from three independent experiments each with triplicate determinations

though 1 mM amiloride virtually abolishes Na^+/H^+ exchange activity in ROS 17/2.8 cells (Dixon & Kassim, 1988), it had no effect on ascorbate uptake. Rate of L-[^{14}C]ascorbate uptake during 1-min incubation with 1 mM amiloride was 100 ± 3% of control values in three independent experiments with triplicate replications. These results suggest that there is little passive diffusion of the protonated form of ascorbic acid across the plasma membrane and that uptake is not coupled to Na^+/H^+ antiport.

Na^+ Dependence of Ascorbate Transport

Uptake of L-[^{14}C]ascorbate by ROS 17/2.8 cells was strongly dependent on the presence of external Na^+ (Figs. 1 and 2). Substitution of K^+, Li^+ or N-methyl-D-glucamine for extracellular Na^+ failed to support uptake of the vitamin (Fig. 4). The effect of Na_o^+ on ascorbate uptake was concentration dependent (Fig. 5), but was not consistent with Michaelis-Menten kinetics.

The following experiments were carried out to assess the possibility that the effects of temperature and Na_o^+ on ascorbate uptake (Figs. 1 and 2) arose from changes in the rate of L-[^{14}C]ascorbate efflux. After an uptake incubation of 5 min at 37°C with 5 μM L-[^{14}C]ascorbate, the medium containing the labeled vitamin was replaced by an ascorbate-free medium. The percentages of initial cell L-[^{14}C]ascorbate content that appeared in the medium following 10-min incubations were 14 ± 1 at 4°C in 138 mM Na_o^+, 16 ± 3 at 37°C in 138 mM Na_o^+, and 16 ± 2 at 37°C in nominally Na^+-free medium in which N-methyl-D-glucamine was substituted for Na^+ (mean ± range of duplicate determinations). This insensitivity of the apparent efflux to temperature and Na_o^+ indicates that loss of radiolabel by the cultures likely arose from reversible extracellular binding instead of carrier-mediated efflux of ascorbate.

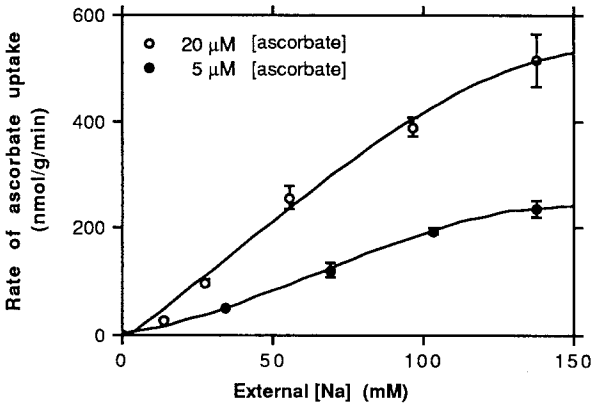

Fig. 5. Effect of external Na^+ concentration on the rate of saturable ascorbate uptake by ROS 17/2.8 cells. ROS 17/2.8 cells were incubated with 5 or 20 μM L-[^{14}C]ascorbate for 1 min at 37°C. Extracellular Na^+ concentration was varied by substitution with N-methyl-D-glucamine. Plotted are the initial rates of saturable ascorbate uptake as a function of external Na^+ concentration. Nonspecific uptake was defined as the uptake of L-[^{14}C]ascorbate in the presence of excess (3 mM) unlabeled L-ascorbate and was subtracted from the total uptake to determine saturable uptake. Data are the means ± SE of a single experiment representative of four independent experiments each performed in triplicate

The Na^+/K^+-ATPase inhibitor ouabain was used to increase intracellular Na^+ levels. Incubation of ROS 17/2.8 cells in 138 mM Na_o^+ medium containing ouabain (0.5 mM, 2 hr, 37°C) increased intracellular Na content five-fold (Table 2). Following Na-loading by ouabain, the L-[^{14}C]ascorbate uptake rate decreased by 64% (Table 2).

Specificity of Ascorbate Uptake

Competition experiments indicated that ascorbate uptake was stereoselective (Fig. 6). Unlabeled L-ascorbate inhibited L-[^{14}C]ascorbate uptake with an

Table 2. Effects of ouabain on cellular Na content and rate of ascorbate uptake[a]

	Cellular Na (μmol/culture dish)	Rate of ascorbate uptake (nmol/g/min)
Control	203 ± 14	138 ± 4
Ouabain	1008 ± 54[b]	49 ± 7[b]

[a] ROS 17/2.8 cell cultures were washed three times with serum-free alpha-MEM, then preincubated for 2 hr at 37°C in serum-free alpha-MEM with or without 0.5 mM ouabain. Subsequently, cellular Na content and L-[^{14}C]ascorbate uptake were measured in parallel but separate experiments. To measure cellular Na, the cultures (8×10^6 cells/culture dish) were washed three times with 150 mM LiCl solution and harvested in 3 mM LiCl solution. The cell harvest was sonicated, sedimented and the Na in the supernatant measured by emission flame photometry. Uptake of L-[^{14}C]ascorbate was determined by 1-min incubations at 37°C in medium containing 138 mM Na^+ and 5 μM L-[^{14}C]ascorbate as described in Materials and Methods. Data are the means ± SE of three independent experiments performed in duplicate (cellular Na content) or triplicate (L-[^{14}C]ascorbate uptake).
[b] $P < 0.05$ compared to control value.

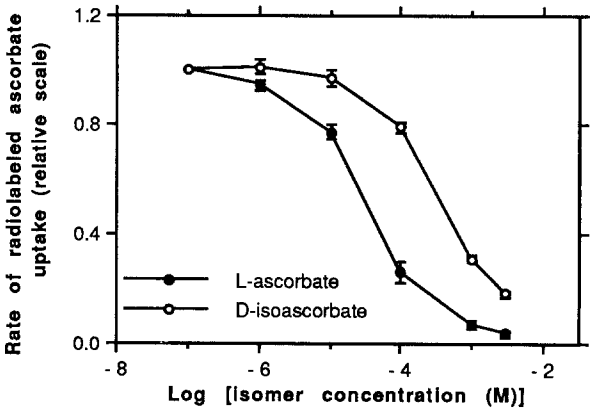

Fig. 6. Inhibition of radiolabeled ascorbate uptake by unlabeled L-ascorbate and its isomer, D-isoascorbate. Uptake was measured using ROS 17/2.8 cells incubated for 1 min at 37°C in medium containing 138 mM Na^+, 5 μM L-[^{14}C]ascorbate and the indicated concentration of unlabeled L-ascorbate or D-isoascorbate. Results are expressed as relative rates of L-[^{14}C]ascorbate uptake. Uptake in the presence of 10^{-7} M unlabeled L-ascorbate or D-isoascorbate was normalized to one. Data are the means ± SE of three independent experiments each performed in triplicate

IC_{50} of 30 ± 5 μM ($n = 3$), whereas D-isoascorbate, an epimer of L-ascorbate, inhibited L-[^{14}C]ascorbate uptake with an IC_{50} of 380 ± 55 μM ($n = 3$). Thus, L-ascorbate was 12-fold more potent as a competitor for the uptake site. Omission of glucose from the medium during 1-min incubations did not affect L-[^{14}C]ascorbate uptake (*data not shown*). The lack of an inhibitory effect of 10 mM glucose in medium containing 5 μM L-[^{14}C]ascorbate suggests that uptake of ascorbate by osteoblasts is not mediated by a glucose transport system.

Characteristics of Ascorbate Uptake by Primary Cultures of Calvaria Cells

Fetal rat calvaria cells require ascorbate to form mineralized bone nodules in vitro (Bellows et al., 1986). As observed for ROS 17/2.8 cells, the accumulation of L-[^{14}C]ascorbate by fetal rat calvaria cells was sensitive to temperature, dependent on Na_o^+ and inhibited by excess unlabeled L-ascorbate (Fig. 7). However, expressed on a per gram protein basis, the rate of saturable uptake in primary cultures (Fig. 7) was slower than in ROS 17/2.8 (Fig. 4). This difference likely arises from the more extensive extracellular matrix secreted by these primary cultures. Nonspecific uptake (per gram protein) was similar in both systems. These observations suggest that a saturable, Na^+-dependent ascorbate uptake mechanism exists in both the transformed osteoblast-like cell line ROS 17/2.8 and primary cultures of bone cells enriched in osteoblasts.

Discussion

Ascorbic acid is essential for the formation of bone by osteoblasts (Anderson et al., 1984; Bellows et al., 1986). In this study, we have examined and characterized the mechanism by which osteoblasts transport ascorbate. Two cell systems were used as in vitro osteoblast models. The first was a clonal osteoblast-like cell line, ROS 17/2.8, which displays many of the phenotypic characteristics of osteoblasts. These characteristics include responsiveness to parathyroid hormone, high alkaline phosphatase activity, synthesis of osteocalcin and formation of a mineralized extracellular matrix in vivo (Majeska & Rodan, 1985; Shteyer et al., 1986). The second model was primary cultures of rat calvaria cells enriched in osteoblasts. These primary cultures form mineralized, bone-like nodules by a process that has been shown to be dependent on vitamin C (Bellows et al., 1986).

Using L-[^{14}C]ascorbate, it was demonstrated that both ROS 17/2.8 (Fig. 1) and primary bone cell cultures (Fig. 7) can accumulate vitamin C. In other cells types, both carrier-mediated and nonsaturable diffusion pathways have been proposed as the mechanisms underlying transmembrane movement of ascorbic acid and dehydroascorbic acid (Rose, 1988). Our observations weigh against the possibil-

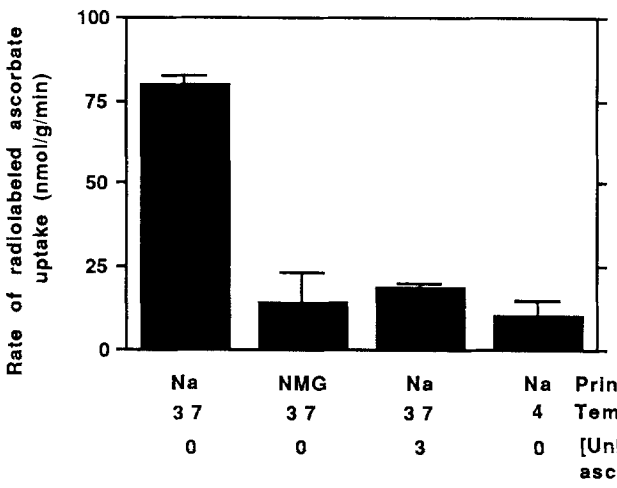

Fig. 7. Rate of radiolabeled ascorbate uptake by primary cultures of rat calvaria cells: effects of temperature, Na_o^+ and excess unlabeled L-ascorbate. Primary osteoblast-enriched cultures of rat calvaria cells were incubated with 5 μM L-[^{14}C]ascorbate for 1 min as described in Materials and Methods. The temperature was 4°C for the indicated incubations and 37°C for the others. N-methyl-D-glucamine was substituted for 138 mM Na^+ in the incubation medium where indicated. Excess (3 mM) unlabeled L-ascorbate was added to the 138 mM Na^+ medium to determine nonspecific uptake. Plotted are the initial rates of uptake under each condition. Data are the means ± SE of six determinations from two independent experiments

ity that, in osteoblasts, vitamin C diffuses passively across the plasma membrane. First, under the experimental conditions employed, there was only a slight decrease in the concentration of extracellular ascorbic acid (Table 1), consistent with minimal oxidation of the ascorbate anion to uncharged dehydroascorbic acid, which may be capable of diffusing across biological membranes (Wagner et al., 1987). Secondly, in contrast to propionate, ascorbate had a negligible effect on cytosolic pH (Fig. 3), consistent with minimal accumulation of ascorbate by nonionic diffusion. Lastly, the rate of uptake of ascorbate by osteoblasts saturated at high concentrations of ascorbate (Figs. 2, 4, 6, and 7), consistent with a carrier-mediated process.

In a number of cell types, uptake of dehydroascorbic acid is mediated by the Na^+-independent glucose transporter (Mann & Newton, 1975; Bigley et al., 1983; Ingermann et al., 1986; Moser, 1987; however, *see* Bianchi & Rose, 1986). This system cannot account for the transport of vitamin C reported here, as is evident from the following observations. First, external glucose did not inhibit uptake. Second, little dehydroascorbic acid was formed under the conditions employed in these studies (Table 1). Third, uptake of ascorbate by bone cells was dependent on external Na^+ (Figs. 1, 2, 4, 5 and 7). Last, the affinity of the transporter for ascorbate was much higher (apparent K_m of 30 μM; Figs. 2 and 6) than that reported for the interaction of dehydroascorbic acid with the glucose transporter (apparent K_m of 2–3 mM; Bigley et al., 1983).

Taken together, our results suggest that uptake of ascorbate into osteoblasts is mediated by a high-affinity carrier, most likely a Na^+-ascorbate cotransporter. The dependence of the rate of ascorbate uptake on external ascorbate concentration was consistent with Michaelis-Menten kinetics (Fig. 2) yielding an apparent K_m of 30 μM in 138 mM Na_o^+. As expected, this apparent K_m was identical to the IC_{50} obtained for inhibition of L-[^{14}C]ascorbate uptake by unlabeled ascorbate (Fig. 6). Also consistent with a carrier-mediated mechanism, uptake was temperature-sensitive (Fig. 1) and stereoselective (Fig. 6). A similar mechanism for uptake of ascorbate has been described in bovine adrenomedullary chromaffin cells (Diliberto et al., 1983) and murine AtT20 pituitary corticotropic tumor cells (Shields, Gibson & Glembotski, 1986). In these earlier studies, saturable Na^+-dependent uptake of ascorbate was observed with apparent K_m of 29 and 31.5 μM, respectively.

Transport of ascorbate by both ROS 17/2.8 cells and primary osteoblast cultures was markedly dependent on external Na^+ (Figs. 1, 2, 4, 5 and 7). This dependence was not, however, consistent with Michaelis-Menten kinetics (Fig. 5). The sigmoidal shape of these curves (Fig. 5) suggests a stoichiometry of more than one Na^+ for every ascorbate ion cotransported across the plasma membrane and has been observed for other organic ions cotransported with Na^+ (Wheeler, 1979; Larsson, Hertz & Schousboe, 1986). In ROS 17/2.8 cells, the dependence of the rate of ascorbate uptake on the concentration of external ascorbate was measured at both 35 and 138 mM Na_o^+ (Fig. 2*a*). Lineweaver-Burk plots of these data (Fig. 2*b*) indicate that increasing Na_o^+ concentration gave rise to significant increases in both the affinity of the transporter for ascorbate (apparent K_m decreased from 71 ± 2 to 30 ± 2 μM) and the apparent V_{max} (from 670 ± 100 to 1460 ± 140 nmol/g/min). These data are in marked contrast to the effects of Na_o^+ on the kinetics of ascorbate uptake by bovine adrenomedullary (Diliberto et al., 1983) and mouse pituitary tumor (Shields et al., 1986) cells. In these systems, stimulation of in-

creased ascorbate uptake by Na_o^+ was consistent with first order kinetics; moreover, increasing Na_o^+ increased the affinity of the transporter for ascorbate without affecting the apparent V_{max} (Diliberto et al., 1983; Shields et al., 1986). We conclude that rat osteoblast-like cells possess a Na_o^+-dependent ascorbate transport system with a similar affinity for ascorbate as the systems found in bovine adrenomedullary (Diliberto et al., 1983) and mouse pituitary tumor (Shields et al., 1986) cells, but differing significantly in the nature of its interaction with external Na^+.

The effect of intracellular Na^+ on ascorbate uptake was assessed with ouabain. Incubations of ROS 17/2.8 cells with ouabain both increased internal Na content and slowed the initial rate of ascorbate uptake (Table 2). A possible explanation for the effect of ouabain on uptake is that this agent reduces the electrical potential difference across the membrane which, if the stoichiometry is two or more Na^+ per ascorbate, is likely to strongly influence influx. It is also possible that intracellular Na^+ exerts a transinhibitory effect on ascorbate uptake, as has been reported for certain Na^+-amino acid cotransport systems (Schultz & Curran, 1970).

Little is known about the intracellular distribution of ascorbate. ROS 17/2.8 cells take up L-[^{14}C]ascorbate at a constant rate for at least 30 min (Fig. 1) and minimal efflux is observed either in the presence or absence of extracellular ascorbate. These observations suggest that once internalized, ascorbate is rapidly compartmentalized, bound or metabolized to a nonpermeant species. Compartmentation of ascorbate in the lumen of the rough endoplasmic reticulum would be consistent with its putative role as a cofactor in the post-translational modification of procollagen (Prockop et al., 1979). Indeed, it has been observed that microsomes prepared from chick embryo limb bone cells possess a low-affinity, iron-dependent ascorbate transport mechanism (Peterkofsky, Tschank & Luedke, 1987). This microsomal system appears distinct from the higher affinity, Na^+-dependent plasma membrane transporter described here.

In conclusion, our data are consistent with the presence of a Na^+-ascorbate cotransporter in the plasma membrane of osteoblasts. Since ascorbic acid is required for osteogenesis, the possibility exists that regulation of osteoblast ascorbate transport plays a role in the physiological control of bone formation.

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