6-Bromo-6-deoxy-L-ascorbic Acid

AN ASCORBATE ANALOG SPECIFIC FOR Na⁺-DEPENDENT VITAMIN C TRANSPORTER BUT NOT GLUCOSE TRANSPORTER PATHWAYS

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Vitamin C intracellular accumulation is mediated by Na⁺-dependent vitamin C transporters SVCT1 and -2 and dehydroascorbic acid transporters GLUT1 and -3. It is unclear which pathways dominate in vivo. As a new step to resolve this issue, we identified and tested 6-bromo-6-deoxy-L-ascorbic acid as a specific candidate for SVCTs. In high performance liquid chromatography and electron paramagnetic resonance analyses, the reduced compounds ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid were similar. The oxidized products 6-bromo-6-deoxy dehydroascorbic acid (BrDHA) and dehydroascorbic acid (DHA) had comparable stabilities, based on reduction recoveries. Upon expression of GLUT1 or GLUT3 in Xenopus oocytes, BrDHA was neither transported nor bound, in contrast to robust transport of DHA. The findings were not explained by differences in the oocyte reduction of DHA and BrDHA because lysed oocytes reduced both compounds equally. Further, there was no transport of the reduced compound, 6-bromo-6-deoxy-L-ascorbic acid, by GLUT1 or GLUT3. As a prerequisite for investigating 6-bromo-6-deoxy-L-ascorbic acid transported by SVCTs, SVCT2 transport activity in oocytes was enhanced 14-fold by construction and use of a vector that added a fixed poly(A) tail to the 3’ end of cRNA. For SVCT1 and SVCT2 expressed in oocytes, similar \( K_m \) and \( V_{max} \) values were observed for ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid. In human fibroblasts, predicted to have SVCT-mediated ascorbate accumulation, \( K_m \) and \( V_{max} \) values were again comparable for ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid. Using activated human neutrophils, predicted to have ascorbate accumulation mediated predominantly by DHA and GLUT transporters, 6-bromo-6-deoxy-L-ascorbic acid accumulation was <1% of accumulation when compared with ascorbic acid. We conclude that 6-bromo-6-deoxy-L-ascorbic acid is the first transport substrate identified as completely specific for SVCTs, but not GLUTs, and provide a new strategy to determine the contribution of each pathway to ascorbate accumulation.

Vitamin C (ascorbic acid) is accumulated by many tissues, but the transport mechanisms responsible are controversial (1–3). Three possibilities exist: transport and accumulation of ascorbic acid by Na⁺-dependent transporters (4, 5); transport of dehydroascorbic acid (DHA), the first stable product of ascorbate oxidization, by Na⁺-independent facilitative glucose transporters followed by immediate intracellular reduction to ascorbate (6, 7); or a combination of the two (2, 8–10). To distinguish among these possibilities requires the isolated transporters, an appropriate expression system, and a substrate that is specific for the ascorbic acid transporters or DHA transporters but not both.

6-Halo-ascorbates are attractive candidate substrates for two reasons. First, they inhibit ascorbic acid but not DHA transport in cells (11). Second, the products of oxidation of 6-halo-6-deoxy-L-ascorbic acids, the 6-halo-dehydroascorbic acids, cannot cyclize, in contrast to DHA, which exists in solution predominantly as a bicyclic hemiketal (see Fig. 1e) (12). Unfortunately, the properties of the 6-halo-ascorbates themselves have been only partially described (11). Furthermore, it previously has been impossible to perform additional key experiments. Thus, for some time, there has been clear evidence that DHA is transported by some Na⁺-independent glucose transporters (GLUTs) (6, 13). However, distinct Na⁺-independent vitamin C transporters (SVCT1, SVCT2) have been isolated only more recently (14). Based on its distribution, SVCT2 is the best Na⁺-independent transporter candidate to mediate ascorbate tissue accumulation (14). Nonetheless, because the activity of this transporter in expression systems is low (14, 15), the requisite experiments could not be undertaken.

In this study, we describe a means to enhance the activity of SVCT2 ~14-fold in the Xenopus oocyte expression system and characterize the experimental properties of the reduced halo-ascorbate 6-bromo-6-deoxy-L-ascorbic acid and its oxidized product 6-bromo-6-deoxy dehydroascorbic acid (BrDHA). These compounds were tested as substrates for expressed Na⁺-independent vitamin C transporters, SVCT1 and SVCT2, and for expressed DHA transporters, GLUT1 and GLUT3. The results show that 6-bromo-6-deoxy-L-ascorbic acid is completely specific for SVCT1 and -2 and is not transported by GLUT1 and -3, whereas the oxidized form BrDHA is not transported by any of these transporters. These data represent the first full characterization of an ascorbate-specific transporter substrate and indicate that the 6-halo-ascorbate analogs are ideal compounds for resolving outstanding issues in the biology of ascorbate accumulation.

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The abbreviations used are: DHA, dehydroascorbic acid; BrDHA, 6-bromo-6-deoxy DHA; GLUT, facilitative glucose transporters; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; RT, reverse transcriptase; SVCT, Na⁺-dependent ascorbic acid transporters.
**Materials and Methods**

**Reagents**—[14C]Ascorbic acid (4–8 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (PerkinElmer Life Sciences). Tris(2-carboxyethyl)-phosphine hydrochloride, superoxide dismutase, catalase, glutathione, diethiothreitol, EDTA, phosphate buffers, and bromoform were purchased from Sigma. Media was purchased from Hyclone (Logan, UT). Histopaque-1083, HEPES, porcine-12-myristate-13-acetate, N-formyl-methionyl-leucyl-phenylalanine, and dimethyl sulfoxide were obtained from Sigma. (3-3-Cholamidopropyl)dimethylammonio-1-propanesulfonate was purchased from J. T. Baker. Dextran T-500 was from Amersham Biosciences. Dulbecco’s modified minimal essential medium was from BIOSOURCE Inc. All other reagents were of the highest available grade.

**Synthesis of Ascorbate Analogues**—6-Deoxy-6-halo-L-ascorbic acids were prepared using a previously published procedure (16) with modifications (11).

**HPLC Analysis**—HPLC mass was measured by reverse phase HPLC using a 4.6 mm 250 × 4.6 mm C-18 column (Columbia, Phenomenex, Torrence, CA) with coulometric electrochemical detection as described previously (17). 6-Deoxy-6-bromo, -iodo, and -chloro-L-ascorbic acid mass were also measured by HPLC, using the same detection system and settings but with a modification of the mobile phase methanol:water ratio to 47.5:52.5%. Bromine was used as described previously (13) to oxidize ascorbic acid and 6-halo ascorbate analogs to dehydroascorbic acid and 6-deoxy-6-bromo, -iodo, and -chloro dehydroascorbic acid, respectively. To quantitate the capacity of oocyte lysates to oxidize ascorbic acid and 6-halo ascorbate analogs to dehydroascorbic acid, respectively. To quantitate the capacity of oocyte lysates to reduce DHA to ascorbic acid, lysates were incubated with [14C]DHA for 10 min. The resulting DHA was then used to separate [14C]ascorbic acid from [14C]dehydroascorbic acid. To separate [14C]ascorbic acid from endogenous ascorbic acid, eluted fractions were collected after HPLC separation at 10-s intervals and analyzed by scintillation spectrometry.

**Electron Paramagnetic Resonance**—Ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid were adjusted to a final concentration of 250 μM in the presence of 50 mM phosphate-buffered saline (pH 7.4) and 50 μM EDTA. The free radical reaction was initiated by adding ferric sulfate (Fe(II)SO₄) at a final concentration of 5 mM. For electron paramagnetic resonance measurements of the ascorbic acid free radical and 6-deoxy-6-bromo ascorbyl acid free radical, samples from the reaction mixture were drawn into a gas-permeable Teflon capillary tube (Zeus Industries) of 0.81-mm inner diameter, 0.38-mm wall thickness, and 15-cm length. Each capillary was folded four times, inserted into a narrow quartz tube open at both ends (2.9-mm diameter), and then placed into the cavity of a Varian E-109 X-band spectrometer. Electron paramagnetic resonance settings were as follows: 1.25-G modulation amplitude, 100-kHz modulation frequency, and 10-milliwatt microwave power.

**Spectrophotometry**—Spectrophotometric measurements were performed using an 8453A Hewlett Packard diode array spectrophotometer set at absorbance 260 nm. The kinetics of ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid oxidized equivalents were studied following the addition of reducing agents, 10 mM Tris(2-carboxyethyl)-phosphate hydrochloride or 0.04 mM MgCl₂ glutaredoxin (18) and 10 mM glutathione. Visualization of recovery was achieved using UV-visible Chemstation software. Each data point represents one sample reaction repeated three times.

**Fluorometric and Construct**—Rat GLUT1 and human GLUT3, SVCT1, and SVCT2 cDNA were prepared by cutting plasmid vectors with the appropriate restriction enzymes followed by an *in vitro* transcription reaction using the SP6 or T7 Message mMachine kit (Ambion). Poly(A) tailing of SVCT1 and -2 cDNA was undertaken using a poly(A) tailing kit (Ambion). The integrity of newly synthesized cRNA was assessed using a 1% agarose-6% formaldehyde gel. cRNA concentration was determined using an 8453A Hewlett Packard diode array spectrophotometer (Cary 1E).

**Construction of Human SVCT2p(A) 18 pCR2.1**—Human skin fibroblasts were cultured as described (5). Strain CRL-1497 was obtained from the American Type Culture Collection and grown in Dulbecco’s modified minimal essential medium with 4.5 g glucose/liter and without glutamine (BIOSOURCE) supplemented with 0.1 mM minimum essential medium non-essential amino acid solution, 2 mM L-glutamine, 10% fetal calf serum. Ascorbic acid or 6-bromo-6-deoxy-L-ascorbic acid, freshly prepared [14C]DHA, or BrDHA at the indicated concentrations for the times specified. After incubation at room temperature, oocytes were washed four times with ice-cold phosphate-buffered saline. Individual oocytes per replicate were solubilized with 10% SDS, and internalized radioactivity was quantified by scintillation spectrometry as pmol/oocyte. Each data point represents the mean value of 10–20 oocytes ± S.D. Each experiment was repeated a minimum of three times with similar results.

**Fibroblast Culture**—Human skin fibroblasts were cultured as described (5). Strain CRL-1497 was obtained from the American Type Culture Collection and grown in Dulbecco’s modified minimal essential medium with 4.5 g glucose/liter and without glutamine (BIOSOURCE) supplemented with 0.1 mM minimum essential medium non-essential amino acid solution, 2 mM L-glutamine, 10% fetal calf serum. Ascorbic acid or 6-bromo-6-deoxy-L-ascorbic acid, freshly prepared [14C]DHA, or BrDHA at the indicated concentrations for the times specified. After incubation at room temperature, oocytes were washed four times with ice-cold phosphate-buffered saline. Individual oocytes per replicate were solubilized with 10% SDS, and internalized radioactivity was quantified by scintillation spectrometry as pmol/oocyte. Each data point represents the mean value of 10–20 oocytes ± S.D. Each experiment was repeated a minimum of three times with similar results.

**Neutrophil Isolation**—Neutrophils were isolated from heparinized whole blood collected from healthy male volunteers (6). Neutrophils were separated from other cellular components by Histopaque-1083 density centrifugation followed by dextran sedimentation and osmotic lysis of residual erythrocytes with hypotonic saline. Neutrophils were suspended in Hanks’ balanced salt solution without calcium, magnesium, or phenol red (pH 7.4) to a concentration of 1 × 10⁶ cells/ml using particle count and size analyzer (Z2, Coulter Corp.). For ascorbic acid transport experiments, neutrophils were plated on 24-well culture plates (Corning) to a final concentration of 1 × 10⁶ cells/ml in 10 mM HEPES-P0 buffer (pH 7.4) containing 147 mM NaCl, 5 mM KCl, 1.9 mM KH₂PO₄, 1.1 mM NaHPO₄, 5 mM glucose, 0.3 mM MgSO₄·7H₂O, 1.0 mM MgCl₂·6H₂O, 1.5 mM CaCl₂·2H₂O. Neutrophils were incubated to allow the cells to attach to the wells for 1 h and then washed with HEPES buffer.

**Fibroblast and Neutrophil Transport Assays**—Fibroblasts were plated on 6-well culture plates (3506, Costar) and grown for ~7 days until confluent. 30 min before experiments, culture medium was replaced with Dulbecco’s modified minimum essential medium without fetal bovine serum. Ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid were added to plated fibroblasts at the indicated concentration, with superoxide dismutase (11.3 μg/ml), catalase (450 units/ml), and glutathione (100 μM) added as protective oxidation of ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid. After a 2-h incubation, the medium was transferred for external ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid measurement. Fibroblasts were washed two times with ice-cold phosphate-buffered saline (pH 7.4), and intracellular ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid were extracted with 75% methanol, 1 mM EDTA for 5 min. Cellular ascorbic acid was assayed with the 6-bromoascorbic acid protein assay kit (Pierce) (20) after solubilization with 0.1 mM NaOH, 1% 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate. Neutrophils were activated with phorbol 12-myristate 13-acetate (55 ng/ml) and N-formyl-methionyl-leucyl-phenylalanine (5 μM). Neutrophils were incubated in HEPES containing superoxide dismutase (11.3 μg/ml), catalase (450 units/ml), and diethiothreitol (100 μM), and 50 μM 6-bromo-6-deoxy-L-ascorbic acid or 14Cascorbic acid in 24-well culture plates for 60 min. After incubation, the extracellular buffers were removed, and external substrate concentrations were determined by HPLC and scintillation spectrometry. Neutrophils were washed with cold HEPES buffer twice and extracted with 60% methanol, 1 mM EDTA for 10 min on ice. Intracellular substrate concentrations were then determined as described above.

Each fibroblast and neutrophil experiment was repeated three times with similar results. Transport results were calculated as concentrations based on intracellular volumes (5, 19), and fibroblast kinetics were expressed as Eadie-Hofstee plots. Error bars were omitted when S.D. was less than symbol size.

**RT-PCR Analysis**—Glut1, Glut3, SVCT1, and SVCT2 Expression—RNA from human fibroblasts and fibroblasts was extracted using QIAamp RNA blood and RNeasy kits (Qiagen), respectively. RT-PCR were performed using Superscript One-Step RT-PCR kit with Platinum Taq (Invitrogen) and primers at a final concentration of 1 μM. Human primers are as follows: GLUT1 forward primer, 5’-AATGAGACAGGGTGCTGGACGTCG-3’; GLUT1 reverse primer, 5’-ATCCTGATGCTAAGCCGCGTGGAG-3’; GLUT3 forward primer, 5’-TCCCTTACCCCTCCCTCCGCCATGGCGA-3’; GLUT3 reverse primer, 5’-CTTCCTACCCCTCCCTCCGCCATGGCGA-3’.
GLUT3 reverse primer, 5'-CTTTCTCTCTCTATATCCCTAGTGCGGC-3'; SVCT1 forward primer, 5'-ATGAGGGCCCCGAGGAGCCTCG-3'; SVCT1 reverse primer, 5'-TGGAACAGGGCGACCCGAGAT-3'; SVCT2 forward primer, 5'-CTGACCTCAGTGGCGATCTAC-3'; SVCT2 reverse primer, 5'-CATGTCAGGTAGTGCTGTAGCCCA-3'. Human kidney and whole brain total RNA (1 µg/µl) were used as controls for gene expression (Clontech), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (Clontech) were used to determine PCR efficiency: G3PDH forward primer, 5'-TGAAGGCGGGCTACCGATG-3'; G3PDH reverse primer, 5'-CATGTGGGCGCATGCTCCACAC-3'. For cDNA synthesis and predenaturation, 1 cycle of 50 °C for 20 min and 94 °C for 2 min was performed on total RNA. For PCR amplification, 40 cycles of 94 °C for a 15-s denaturation, 55 °C for 30 s of annealing, 68 °C for a 50-s extension, and 1 cycle of 72 °C for a 5-min final extension were performed using Peltier thermal cycler (MJ Research).

**RESULTS**

Characterization of 6-Halo-ascorbic Acid Analogs and Halodehydroascorbic Acid Analogs—To dissect vitamin C transport pathways, an ideal substrate would be transported by SVCTs but not GLUTs (or vice versa). We chose to investigate 6-halo ascorbic acid analogs because the reduced forms of these analogs are similar to ascorbic acid in structure (Fig. 1) and thus should be transported by SVCT1 and SVCT2. On the other hand, the oxidized form of ascorbic acid (DHA) has been shown to exist in solution as hydrates and can also form a cyclic hemiketal (Fig. 1f) (12). This latter structure cannot be formed in the absence of the 6-hydroxyl group. If this planar cyclic hemiketal is important for transport, the 6-halo ascorbic acid analogs should behave quite differently in the oxidized form and should not be transported by GLUT1 and GLUT3.

A sensitive and accurate platform for analog detection must be available to study transport specificities. Fig. 2A is a representative HPLC chromatogram of 100 pmol of ascorbic acid and 6-deoxy-6-bromo, -iodo, and -chloro-L-ascorbic acid and shows the reduced forms of the analogs to be pure with no detectable secondary or interfering peaks. The quantification of analog transport can therefore be undertaken by HPLC analysis.

In the use of analogs to dissect vitamin C transport pathways, analogs must be completely oxidized, with oxidation similar to that of ascorbic acid. Fig. 2B is a representative chromatogram of 100 pmol of DHA and 6-deoxy-6-bromo, -iodo, and -chloro dehydroascorbic acid. The figure shows that 6-halo dehydroascorbic acid analogs are undetectable by HPLC analysis and are thus completely oxidized, similar to DHA. Because of the ease of synthesis and purification of 6-bromo-6-deoxy-L-ascorbic acid and its similarity to other 6-halo-ascorbates, subsequent studies focused on this compound.

As a further test of oxidation profiles, the formation of ascorbyl radical intermediates was measured from ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid (Fig. 2C). Ferric(II) sulfate, which auto-oxidizes to ferrous(III) sulfate under neutral pH and aerobic conditions (21), was used to drive the formation of the free radical species. In the absence of Fe(II), no free radical species were observed, indicating that ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid were stable in solution under the test conditions. An electron paramagnetic resonance signal was detected only in the presence of Fe(II), indicating the production of ascorbyl and 6-deoxy-6-bromo ascorbyl free radical adducts. The areas under the curve for ascorbyl and 6-deoxy-6-bromo ascorbyl free radicals were similar, indicating similar degrees of oxidation.

To validate transport studies, it is necessary to show that the oxidized analog, BrDHA, is stable enough to be available for transport, similar to DHA. The analysis of stability could not be assessed directly by HPLC because oxidized analogs and DHA were undetectable (Fig. 2B). Instead, stability was measured by determining the recovery (reduction) of oxidized analog at various time points after oxidation, using spectrophotometry for detection. Fig. 3, A and B, demonstrate that DHA and BrDHA were 80–100% recovered 0–20 min after oxidation. At 40 min after oxidation, DHA and BrDHA were 100 and 60% recovered, respectively. As a further test of recovery, glutathione and glutathione were used as a reducing system after
oxidation (18). DHA and BrDHA were 100% recovered (100% reduced) up to 20 min after oxidation (data not shown). Subsequent experiments with oxidized analog were undertaken within 20–40 min of oxidation.

To quantify oocyte transport of oxidized analog by HPLC, oocytes must be able to internally reduce any oxidized analog if it is transported. To this end, internal oocyte reduction capacity was assessed using mechanically lysed oocytes incubated for 10 min with 10–320 μM [14C]DHA or BrDHA (Fig. 4). The reduction of BrDHA was measured directly by HPLC. [14C]DHA reduction to [14C]ascorbic acid was analyzed using HPLC to separate the two compounds, with the quantification of each peak by scintillation spectrometry as described under “Materials and Methods.” [14C]DHA was used because oocytes contain ascorbic acid, and endogenous ascorbic acid created an interfering high background for the assessment of DHA reduction capability. Fig. 4 displays the scintillation and HPLC analysis of the concentration/recovery curve. These data show that the oocyte reductions of [14C]DHA and BrDHA were equivalent over the selected concentration range. Thus, if BrDHA is transported into oocytes, the analog will be reduced and readily measured.

Analyses of BrDHA Transport by GLUTs—Having confirmed the stability and reduction properties of BrDHA, as described above, transport of this analog was studied in GLUT1- and GLUT3-injected oocytes, with DHA transport as controls (Fig. 5). Intracellular accumulation of 300 μM [14C]DHA and BrDHA was assayed by scintillation spectrometry and HPLC, respec-

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**Fig. 2.** HPLC and electron paramagnetic resonance analysis of reduced and oxidized forms of ascorbic acid and 6-halo ascorbate analogs. **A,** 100 pmol of ascorbic acid (i) and 100 pmol of 6-deoxy-6-chloro (ii), -bromo (iii), and -iodo-L-ascorbic acid (iv) were injected and measured by electrochemical detection. Reverse phase chromatograms are shown. **B,** 10 mM ascorbic acid and 6-halo ascorbate analogs were oxidized using bromine, N2 gas (see “Materials and Methods”), diluted 1:1000, and 100 pmol of DHA, 6-deoxy-6-chloro, -bromo, and -iodo dehydroascorbic acid analogs were injected and measured by electrochemical detection. Reverse phase chromatograms are shown. **C,** electron paramagnetic resonance analysis of ascorbyl and 6-bromo ascorbyl free radical intermediates. Measurement conditions were phosphate-buffered saline (pH 7.4) with 50 μM EDTA. i, 5 μM Fe(II) S04; ii, 250 μM ascorbic acid; iii, 250 μM 6-bromo-6-deoxy-L-ascorbic acid; iv, 5 μM Fe(II) S04 plus 250 μM ascorbic acid; v, 5 μM Fe(II) S04 plus 250 μM 6-bromo-6-deoxy-L-ascorbic acid.
tively. $^{14}$C]DHA transport via GLUT1 and -3 was linear for 40 min, whereas BrDHA transport and reduction were undetectable by HPLC analysis. These data indicate that BrDHA is not transported at all by GLUT1 and GLUT3.

The possibility was tested that BrDHA binds to, but is not transported by, GLUT1 and GLUT3. $^{14}$C]DHA uptake by GLUT1 and GLUT3 was measured when unlabeled DHA or BrDHA concentrations were varied (Fig. 6). Unlabeled DHA competed with $^{14}$C]DHA transport mediated by GLUT1 and GLUT3, whereas unlabeled BrDHA had no effect. These data indicate that oxidized analog does not interact with the substrate binding sites of either GLUT1 or GLUT3, whereas DHA interacts as expected.

**Analyses of 6-Bromo-6-deoxy-L-Ascorbic Acid Transport**—Previous studies using *Xenopus* oocytes have shown SVCT2 transporter activity to be almost 300 times below that of SVCT1 (14, 15). Therefore, before the accurate quantification of 6-bromo-6-deoxy-L-ascorbic acid transport using SVCT2-injected oocytes could be undertaken, an enhanced expression system needed to be obtained. Polyadenylation was a potential method to enhance translation (22). Fig. 7 shows the effects of poly(A) tailing on SVCT1 and -2 ascorbic acid transport in oocytes. As expected, $^{14}$C]ascorbic acid transport mediated by SVCT1 was easily detectable by scintillation spectrometry, whereas SVCT2 ascorbic acid transport was near the lower limit of detection. The addition of a 150–200-base poly(A) tail to the 3’ end of SVCT1 cRNA had little effect on transporter activity, whereas poly(A) tailing enhanced SVCT2 transporter activity in oocytes ~10-fold. In support, when SVCT2 cRNA with a fixed poly(A)$_{18}$ tail was injected into oocytes, ascorbic acid transport activity was enhanced ~14-fold when compared with non-polyadenylated SVCT2 cRNA. All subsequent transport experiments were carried out using human SVCT2p(A)$_{18}$ cRNA derived from the human SVCT2p(A)$_{18}$ pCR2.1 clone. This method was selected because SVCT2 transport activity was highest, and the length of the poly(A) tail was not subject to random variation.

HPLC analysis was used to determine initial uptake rates of 300 $\mu$M 6-bromo-6-deoxy-L-ascorbic acid into SVCT1-, SVCT2p(A)$_{18}$-, GLUT1-, and GLUT3-injected oocytes. Uptake...
of 6-bromo-6-deoxy-L-ascorbic acid via SVCT1 was linear for at least 10 min. The rate of SVCT2-mediated 6-bromo-6-deoxy-L-ascorbic acid uptake was lower than SVCT1 but was linear for 60 min (Fig. 8). 6-Bromo-6-deoxy-L-ascorbic acid transport via GLUT1- and GLUT3-expressing oocytes was not detected by HPLC analysis for as long as 60 min. These findings were consistent with data showing that [14C]ascorbic acid was not transported by GLUTs (data not shown) and indicate that analogs are specific for SVCT1 and SVCT2 and are not transported by GLUT1 or GLUT3. As an additional measure of substrate specificity, BrDHA was tested as a substrate for SVCT1 and SVCT2 and was not transported by either transporter (data not shown).

To determine kinetics, SVCT1- and SVCT2p(A)18 cRNA-injected oocytes were incubated with 10–300 μM [14C]DHA or BrDHA at room temperature. Oocytes were washed, and intracellular uptake was quantified by scintillation spectrometry or HPLC analysis for as long as 60 min. These findings were consistent with data showing that [14C]ascorbic acid was not transported by GLUTs (data not shown) and indicate that analogs are specific for SVCT1 and SVCT2 and are not transported by GLUT1 or GLUT3. As an additional measure of substrate specificity, BrDHA was tested as a substrate for SVCT1 and SVCT2 and was not transported by either transporter (data not shown).

To determine kinetics, SVCT1- and SVCT2p(A)18 cRNA-injected oocytes were incubated with 10–300 μM [14C]ascorbic acid or 6-bromo-6-deoxy-l-ascorbic acid for 10 and 60 min, respectively. Intracellular substrate uptake was quantified by scintillation spectrometry or HPLC, and apparent transport kinetics constants were determined by Eadie-Hofstee analysis. For SVCT1-mediated ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid transport, an apparent \( K_m \) of 170 and 33 μM and apparent \( V_{\text{max}} \) of 8 and 12.9 pmol/min/oocyte were obtained, respectively (Fig. 9). For SVCT2-mediated ascorbic acid and 6-bromo-6-deoxy-l-ascorbic acid transport, an apparent \( K_m \) of 21 and 15.8 μM and apparent \( V_{\text{max}} \) of 0.6 and 0.8 pmol/min/oocyte were obtained, respectively. These data show that transport kinetics of ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid were similar and that 6-bromo-6-deoxy-L-ascorbic acid actually has a higher affinity than ascorbate for SVCT1.

Analog Transport in Human Fibroblasts and Neutrophils—To validate the above oocyte data, the transport properties of ascorbic acid, 6-bromo-6-deoxy-l-ascorbic acid, and their oxidized products were next studied in cells. Fibroblasts and neutrophils were chosen because in these cells, the dominant mechanisms responsible for regulating internal vitamin C concentrations are the Na\(^+-\)dependent (SVCT) pathway (5, 11) and the GLUT pathway (8–10; 13), respectively. Before transport studies were initiated, RT-PCR analyses of fibroblast and neutrophils were undertaken to confirm the presence of candidate transport pathways. Fibroblast and neutrophils express SVCT2, GLUT1, and GLUT3 (Fig. 10 A) and thus have the
potential to transport ascorbic acid, DHA, and 6-bromo-6-deoxy-L-ascorbic acid.

Intracellular accumulation of ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid was readily detectable in cultured fibroblasts (Fig. 10B) and showed comparable transport kinetics. For ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid, respectively, apparent $K_m$ values were 27.0 and 4.1 $\mu$M, and apparent $V_{\text{max}}$ values were 245.2 $\mu$M/2 h and 157.3 $\mu$M/2 h. Based on these data and those above for oocytes, we conclude that ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid are accumulated by the SVCT pathway and have very similar transport properties.

Activated neutrophils accumulate ascorbic acid predominantly by the GLUT pathway, and unactivated neutrophils also transport ascorbic acid, although at a substantially diminished rate (8–10). Before investigating analog transport in activated neutrophils, it was necessary to study unactivated neutrophils for comparison purposes. Unactivated neutrophils were incubated with [14C]ascorbic acid or 6-bromo-6-deoxy-L-ascorbic acid for varying times. [14C]Ascorbic acid was used because endogenous ascorbic acid concentrations, 1.5 mM in unactivated neutrophils, would produce a high background with HPLC analysis. Superoxide dismutase, catalase, and dithiothreitol were also added to prevent external substrate oxidation by neutrophil derived oxidants. As expected, the data show that [14C]ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid uptake was comparable and low (Fig. 10C).

Neutrophils were activated by the addition of phorbol 12-myristate 13-acetate (55 ng/ml) or N-formyl-methionyl-leucyl-phenylalanine (5 $\mu$M), and activation resulted in a substantial increase in internal ascorbic acid concentrations (Fig. 10C). Increased internal ascorbic acid concentrations are due to ascorbate recycling, whereby activated neutrophils oxidize external ascorbic acid to DHA, which is then transported into the intracellular compartment of the neutrophil, via GLUT1 and GLUT3, and then reduced to ascorbic acid (8–10; 13). Based on the above oocyte data, activated neutrophils were predicted to be unable to transport BrDHA via GLUT1 or GLUT3. This prediction proved correct: intracellular 6-bromo-6-deoxy-l-ascorbic acid concentrations were similar in activated and unactivated neutrophils (Fig. 10C). These data indicate that 6-bromo-6-deoxy-l-ascorbic acid and its oxidized product BrDHA are not substrates for GLUT1 and GLUT3 in cells. Transport properties in fibroblasts and neutrophils thus are consistent with the above oocyte data for all compounds studied: ascorbic acid, 6-bromo-6-deoxy-l-ascorbic acid, DHA, and BrDHA.

**DISCUSSION**

Ascorbic acid and its oxidized product, dehydroascorbic acid, have distinct transport pathways, mediated by SVCTs and
GLUTs, respectively. Both pathways produce intracellular accumulation of ascorbic acid. The purpose of the present study was to identify and characterize the properties of an analog substrate that would be specific for only one of these pathways. This is a necessary first step toward the long range goal of using substrate analogs to discriminate vitamin C accumulation mechanisms and functions in vivo. The data in this study show that the synthetic analog 6-bromo-6-deoxy-L-ascorbic acid is completely specific for the SVCT pathway. 6-Bromo-6-deoxy-L-ascorbic acid was transported by SVCTs nearly identically to ascorbic acid, and chemical and stability properties were similar for both compounds. Like ascorbic acid, 6-bromo-6-deoxy-L-ascorbic acid was not transported by GLUTs. In contrast, whereas the ascorbate oxidation product DHA was transported by GLUT1 and -3, the oxidized product BrDHA was not transported or bound by the same GLUTs, nor was it transported by SVCTs. These data demonstrate that 6-bromo-6-deoxy-L-ascorbic acid is specific for SVCTs and that this analog will be a useful agent to discriminate mechanisms of vitamin C accumulation.

Despite many studies, the dominant mechanism responsible for intracellular accumulation of ascorbic acid has remained unclear. Explanations for these discrepancies include substantial variations in assay sensitivity, different cell lines, use of non-physiologic concentrations of transport substrates, variable transport conditions, and inadvertent or intentional substrate oxidation (2). To discriminate between the relevant transport pathways, there are two general strategies: development of a tissue transporter knock-out animal or development of a chemical knock-out substrate. A homozygous SVCT2 knock-out mouse has already been developed (23). Many fetal tissues of the knock-out animal were deficient in vitamin C, suggesting that SVCT2 plays an important role in vivo. Because the knock-out animals died within minutes after birth, conclusions about the role of SVCT2 are most relevant to development in utero and parturition (23). The alternative strategy is that described in this study, development of a chemical knock-out substrate. The ideal compound would be structurally similar to vitamin C, demonstrate similar redox and stability characteristics, and use exclusively either the SVCT or the GLUT transport pathway but not both. We tested 6-bromo-6-deoxy-L-ascorbic acid as a candidate compound and showed its utility as a chemical DHA transport knock-out.

Our rationale for using 6-halo-ascorbic acid analogs was based in part on their structures obtained from 13C NMR (12). The reduced forms of the 6-halo ascorbic acid analogs are structurally similar to ascorbic acid, and we predicted that they would be transported by SVCT1 and SVCT2. Oxidized ascorbic acid, or DHA, theoretically can have multiple forms in aqueous solution, including the 2,3-diketolactone structure (Fig. 1c), the hydrated form of this (Fig. 1d), and the hydrated bicyclic
hemiketal (Fig. 1e). The only form detected by $^{13}$C NMR spectroscopy in aqueous solution is the hydrated bicyclic hemiketal (Fig. 1e). The formation of this requires the presence of the C6 hydroxyl group (12). The overall geometry of this molecule and the presence of multiple hydroxyl groups has suggested a similarity of this structure to that of glucose (24). This structural similarity in part led to the hypothesis nearly 30 years ago that DHA and glucose would share the same transport mechanism (25), a hypothesis that was subsequently verified for several facilitated glucose transporters (6, 13, 26). For 6-halo ascorbates such as 6-bromo-6-deoxy-l-ascorbic acid, the C6 hydroxyl group is replaced with halogen. When 6-bromo-6-deoxy-l-ascorbic acid is oxidized, the hydrated bicyclic hemiketal cannot form because of the C6 halogen. BrDHA, represented by the 2,3-diketolactone (Fig. 1h) or the hydrated form (Fig. 1i), lacks structural similarity to glucose, and we predicted that it would not be transported or bound by GLUTs. We have confirmed our hypothesis in this report.

From prior investigations, 6-halo ascorbic acid analogs showed promise to be specific for the Na$^+$-dependent ascorbic acid transport pathway (4). $^{125}$I[6-Deoxy-6-iodo-l-ascorbic acid was transported into fibroblasts by a saturable Na$^+$-dependent process, whereas $^{125}$I[6-deoxy-6-iodo-l-ascorbic acid and $^{125}$I[6-deoxy-6-ido dehydroascorbic acid were not transported into GLUT1- and GLUT3-expressing oocytes. Based on these data, it was proposed that the Na$^+$-dependent pathway was responsible for intracellular accumulation of ascorbic acid in vitro. However, this conclusion was premature for several reasons. First, it was not possible to study the Na$^+$-dependent transport mechanism in isolation at that time because SVCT1 and SVCT2 had not been identified. Second, because the stability of the oxidized analog was not determined, it was uncertain whether oxidized 6-halo ascorbic acids either were not transported or were simply unstable and had hydrolyzed before they could be transported. Third, it could not be determined whether or not oxidized analog was transported because it had not been demonstrated that oocytes were capable of reducing the oxidized analog. Fourth, analog binding without transport was not fully addressed. In this report, we have resolved all of these issues.

In Xenopus oocytes, SVCT2 transport activity was previously reported to be low (14, 15). It was therefore necessary to enhance expression before analog transport specificities could be evaluated. Several general modifications have been tried to enhance the expression of transport proteins in Xenopus oocytes. For example, enhanced expression has been demonstrated when cRNA is either flanked with the 5′- and 3′-untranslated region of the Xenopus laevis β-globin gene (27) or polyadenylated at the 3′-untranslated region (22). We accomplished polyadenylation of SVCT2 cRNA at the 3′ end either by incubating with Escherichia coli poly(A) polymerase or by in vitro transcription of an SVCT2 poly(A)$_{18}$ cDNA expression vector. Both methods enhanced SVCT2 transport activity, presumably by stabilizing the cRNA, with subsequent enhanced levels of protein expression targeted to the oocyte plasma membrane. To this end, we were able to improve SVCT2 transport activity 10–14-fold, with slightly but reproducibly better activity with the human SVCT2p(A)$_{18}$ pCR2.1 expression vector. This expression vector was chosen because it provided fixed polyadenylation, as opposed to variable polyadenylation with the E. coli poly(A) polymerase method.

Previous studies using ascorbic acid or DHA as substrate, although useful, have not been able to determine which substrate is being transported at the cell surface. This experimental problem, we believe, has been circumvented using 6-halo ascorbic acid analogs. Kinetics analyses of 6-bromo-6-deoxy-l-
ascorbic acid using oocytes injected with SVCT1 and SVCT2 indicate that analog transport parameters are similar to those of ascorbic acid. This was confirmed in experiments with cultured human fibroblasts. The data indicate that accumulation of ascorbic acid and 6-bromo-6-deoxy-L-ascorbic acid are very similar in human cells in which the SVCT pathway is expected to dominate. Further confirmation of 6-bromo-6-deoxy-L-ascorbic acid specificity for the SVCT pathway was also obtained in experiments with activated human neutrophils. These cells are predicted to accumulate ascorbic acid by external oxidation to DHA, transport of DHA by GLUTs, and immediate intracellular reduction to ascorbate (ascorbate recycling) (8–10). When ascorbic acid was the substrate, accumulation increased nearly 2 orders of magnitude in activated neutrophils when compared with unactivated controls. This increase was absent when 6-bromo-6-deoxy-L-ascorbic acid was the substrate, providing further cellular evidence that the analog is specific for the SVCT pathway. When considered together, the in vitro data suggest that of the three potential mechanisms for intracellular vitamin C accumulation, the best supported hypothesis is that the SVCT pathway maintains internal levels of ascorbic acid under normal physiologic non-oxidative conditions, whereas the GLUT-mediated DHA pathway is most likely to be a local phenomenon, restricted to specialized oxidizing conditions, such as those found in the surrounding milieu of the activated neutrophil.

Cell experiments, although useful, may not ultimately resolve the long range issues regarding which substrates are actually transported across the membrane in vivo. In human plasma, ascorbic acid is readily detected, and in healthy adults, concentrations are between 50 and 75 μM (28, 29). In contrast, DHA cannot be readily detected and is estimated to be between 0 and 2% of the ascorbic acid concentration found in plasma (30). Although DHA concentrations are at the limits of detection by current means, it is conceivable that low concentrations of DHA are present in human plasma in vivo. Despite the overwhelming presence of ascorbic acid when compared with DHA, it remains possible that DHA is the dominant substrate transported. An example of a comparable situation is that of thyroid hormone in human plasma, in its T3 and T4 forms. Although T4 predominates, as ~99% of detected total thyroid hormone, the active form at the nuclear receptor is actually T3, and T4 is essentially inactive unless modified (31). Similarly, for ascorbic acid and DHA, there is a potential discrepancy between the dominant substrate found and the dominant substrate transported in vivo (2). Whether ascorbate accumulation is mediated by DHA or ascorbic acid has been a central issue in ascorbate biology for more than 50 years (32, 33). Based on the results in the present report, ascorbate analogs offer a novel means to resolve this issue and to test the contribution of each transport mechanism toward tissue accumulation of vitamin C in vivo.

REFERENCES