Cellular ascorbic acid accumulation occurs in vitro by two distinct mechanisms: transport of ascorbate itself or transport and subsequent intracellular reduction of its oxidized product, dehydroascorbic acid. It is unclear which mechanism predominates in vivo. An easily detectable compound resembling ascorbate but not dehydroascorbic acid could be a powerful tool to distinguish the two transport activities. To identify compounds, 21 ascorbate analogs were tested for inhibition of ascorbate or dehydroascorbic acid transport in human fibroblasts. The most effective analogs, competitive inhibitors of ascorbate transport with \( K_i \) values of 3 \( \mu \)M, were 6-deoxy-6-bromo-, 6-deoxy-6-chloro-, and 6-deoxy-6-iodo-L-ascorbate. No analog inhibited dehydroascorbic acid transport. Using substitution chemistry, \([125I]6\)-deoxy-6-iodo-L-ascorbate (1.4 \( \times \) 10\(^4\) mCi/mmol) was synthesized. HPLC detection methods were developed for radiolabeled and nonradiolabeled compounds, and transport kinetics of both compounds were characterized. Transport was sodium-dependent, inhibited by excess ascorbate, and similar to that of ascorbate. Transport of oxidized ascorbate and oxidized 6-deoxy-6-iodo-L-ascorbate was investigated using \( Xenopus \) laevis oocytes expressing glucose transporter isoform GLUT1 or GLUT3. Oxidation of ascorbate or its analog in media increased uptake of ascorbate in oocytes by 6–13-fold compared with control but not that of 6-deoxy-6-iodo-L-ascorbate. Therefore, 6-deoxy-6-iodo-L-ascorbate, although an effective inhibitor of ascorbate transport, either in its reduced or oxidized form was not a substrate for dehydroascorbic acid transport. Thus, radiolabeled and nonradiolabeled 6-deoxy-6-iodo-L-ascorbate provide a new means for discriminating dehydroascorbic acid and ascorbate transport in ascorbate recycling.

Ascorbic acid (vitamin C, ascorbate) is a required nutrient for humans. Ascorbate is a cofactor in eight intracellular mammalian enzymatic reactions and serves a primary role in the defense against oxidant radicals in vivo (1–3). Loss of two electrons leads to formation of dehydroascorbic acid, which can be reduced back to ascorbate enzymatically or nonenzymatically (4–8).

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Ascorbate accumulation in vivo and in cells occurs by two mechanisms, collectively termed ascorbate recycling (3, 9, 10). Ascorbate is transported as such or is oxidized extracellularly to dehydroascorbic acid, transported by glucose transporters, and reduced internally (3, 11, 12). Under physiologic conditions in the absence of oxidation, ascorbate is the primary, if not the only, substrate available (13, 14). Nevertheless, it remains unknown whether normal intracellular ascorbate accumulation occurs solely due to ascorbate transport itself or whether some fraction of ascorbate is oxidized at the cell membrane to dehydroascorbic acid, transported, and reduced intracellularly. A serious disadvantage in using ascorbate itself to discriminate these mechanisms of transport is that ascorbate can be accumulated by both pathways. To characterize the mechanisms of ascorbate accumulation physiologically, a compound is needed that is similar to ascorbate but is transported by only one mechanism. Such a compound has not been available.

An ideal compound should meet several criteria. It should be similar to ascorbate because this is the substrate found physiologically. The compound should be measurable in small amounts. Its utility would be greatly enhanced if the compound could be detected easily, for example by HPLC or, especially, by radiochemical detection. Current radioactive detection of ascorbate is problematic because of low specific activity of available radiolabeled ascorbate, emphasizing the need for a much higher specific activity radiolabeled analog. Finally, an ideal compound to study transport and accumulation would be specific for mechanisms of ascorbate transport only and would show no activity toward dehydroascorbic acid transport mechanisms.

To address these issues, we synthesized or obtained 21 structural analogs of ascorbate having substitutions or conformational changes at positions 2–6. We studied the ability of each analog to inhibit ascorbate transport in cultured human fibroblasts, cells in which ascorbate transport kinetics have been previously characterized (15). Based on these observations, we synthesized 6-deoxy-6-iodo-l-ascorbate and \([^{125}I]6\)-deoxy-6-iodo-l-ascorbate, a high specific activity ascorbate analog, and we characterized their transport characteristics in fibroblasts and in \( Xenopus \) oocytes expressing glucose transporter isoforms GLUT1 or GLUT3. The results suggested that 6-deoxy-6-iodo-l-ascorbate and \([^{125}I]6\)-deoxy-6-iodo-l-ascorbate may be ideal compounds for quantitation of ascorbate-specific transport.

MATERIALS AND METHODS
Reagents—\([^{14}C]Ascorbic acid (8.0 mCi/mmol) and \([^{125}I]NaI (25 mCi in 71 \mu l of 10^{-3} M NaOH) were purchased from NEN Life Science Products (Boston, MA). Ascorbic acid, 2-deoxyglucose, 3-O-methyl glucose, serine, leucine, threonine, bovine serum albumin, dithiothreitol
Ascorbate Analogs: 6-Deoxy-6-iodo-L-ascorbic Acid

(DTT),1 and EDTA were obtained from Sigma, and methanol was purchased from Baker (Philipsburg, NJ). Other reagents were of the highest available commercial grade.

Ascorbate Analogs—3-O-Benzyl-, 3-O-octadecyl-, 2-O- octadecyl-2-O- myristyl-, 5,6-isopropylidene-, 3-O-methoxymethyl-5,6-isopropylidene-, and 2-O-methyl-3-O-methoxymethyl-L-ascorbic acid were purchased from Institute for Materia Medica (Osaka, Japan). 2-O-sulfato-L-ascorbic acid and L-isoroscorbic acid were obtained from Sigma. L-Ascorbic acid and L-isoroscorbic acid were prepared by Kenner Rice (Laboratory of Medicinal Chemistry, NIDDK, National Institutes of Health). 5-Deoxo-, 6-iodo-5-deoxy-6-phenoxo-5-deoxy- and 6-phenylsulfide-5-deoxynalogs were provided by Dr. C. G. O’ Higgins (Tish International, Peabody, MA). 2-Bromo-6-ido-L-ascorbic acid and 6-O-phenyl-L-ascorbic acid were provided by Tadao Kurata (Ochanomizu University, Tokyo, Japan). The analogs 6-deoxy-, 6-deoxy-6-idoo-, 6-deoxy-6-chloro-, 6-deoxy-6-fluro-, 6-deoxy-6-bromo-, 6-O-phenyl-, 6-O-(3-nitro)-phenyl-, and 6-O-(3-trifluoromethyl)-phenyl-L-ascorbic acid were synthesized as described below.

6-deoxy-L-ascorbic acid and 6-deoxy-6-halo-L-ascorbic acid analogues were prepared using a published procedure with modifications (16). The halogenated analogs were synthesized by halogenation of methyl 2,3-O-isopropylidene-L-gulonate, followed by acid-catalyzed hydrolysis/rearrangement to form the ascorbic acid derivative. This latter step was carried out in methanolic HCl, a modification of the published procedure that gave improved yields of 6-chloro- and 6-bromo-6-ido-L-ascorbic acid analogues. Nucleophilic addition of fluoride and iodide to methyl 2,3-isopropylidene-6-O-trifluoromethylsulfonyl-L-gulonate, followed by methanolic HCl-mediated hydrolysis and rearrangement, produced 6-deoxy-6-fluoro- and 6-deoxy-6-ido-L-ascorbic acids, respectively. In the nucleophilic substitution, use of the trifluoromethanesulfonate ester rather than the p-toluenesulfonate ester (16) resulted in a more rapid reaction and improved yields. 6-Deoxy-L-ascorbic acid was prepared by hydrogenolysis of 6-bromo-L-ascorbic acid over palladium on carbon (16). Melting point and NMR spectral data of these analogues were in agreement with reported values. 6-deoxy-6-iodo-L-ascorbate was measured by HPLC previously (18). 6-Deoxy-6-iodo-L-ascorbate was measured by HPLC with electrochemical detection using the same detection system and settings, but the mobile phase used for ascorbate was modified to contain 55% methanol. [125I]6-Deoxy-6-iodo-L-ascorbic acid was measured using the HPLC separation conditions for unlabeled compound, with the addition of an online scintillation spectrometry detection system after the coulometric detector (12).

Incubation and injection of mRNA coding for the glucose transporter isoforms GLUT1 or GLUT3 as described (12). Three days postinjection, individual oocytes were incubated for 15 min at room temperature with 50 μM of either [125I]ascorbic acid or [125I]6-iodo-6-ido-L-ascorbic acid in the presence or absence of either hydrogen peroxide (H2O2) (8 mM) or xanthine oxidase (0.05 units/ml) (Sigma) and 36 μM DTT. The presence of DTT was due to dilution of DTT present in original stock material. After 15-min incubations, oocytes were washed four times with ice-cold phosphate-buffered saline containing 1 mM phloretin. Individual oocytes were solubilized with 10% SDS, and internalized radioactivity was quantified by scintillation spectrometry (12).

Ascorbate Analogs: 6-Deoxy-6-iodo-L-ascorbic Acid

Ascorbate Analogs: 6-Deoxy-6-iodo-L-ascorbic Acid

Inhibition of Ascorbate Transport by Analogs—Ascorbic acid analogs with stereochemical inversion or substitutions on carbon 2, 3, 4, 5, and 6 were examined individually for their ability to inhibit ascorbic acid uptake in cultured fibroblasts (Table I). Without analog, ascorbate was accumulated at least 8-fold against a concentration gradient (not shown). Little inhibition of ascorbic acid transport was observed with compounds containing substitutions on carbon 2 or 3. Analogs examined included 2-amino-L-ascorbic acid, 2-sulfato-L-ascorbic acid, 3-O-benzyl-L-ascorbic acid, and 3-O-octadecyl-L-ascorbic acid (Table I). Two other analogs, 2-O-octadecyl-L-ascorbic acid and 2-O-myristyl-L-ascorbic acid, were also tested, but these compounds decreased adherence of fibroblasts to tissue.
culture plates, making interpretation of results difficult. The addition of some of these compounds to human neutrophils did not inhibit ascorbate transport without toxicity as measured by trypan blue dye exclusion (not shown).

\( \delta \)-Ascorbic acid, the enantiomer of \( \lambda \)-ascorbic acid, was a poor inhibitor of ascorbate transport. \( \lambda \)-Ascorbic acid and \( \delta \)-ascorbic acid, with stereochemical inversions at carbons 4 and 5, respectively, were only slightly more effective. 5,6-Isopropylidene-\( \lambda \)-ascorbic acid was a moderate inhibitor, but inhibition was lost with the addition of a methoxymethyl group onto carbon 3. A series of 5-deoxy- and 6-deoxy-\( \lambda \)-ascorbic acid analogs all effectively inhibited ascorbate transport, but carbons 4 and 5, respectively, were more effective. 5,6-Isopropylidene-\( \lambda \)-ascorbic acid was a potent inhibitor, but inhibition was lost with the addition of a methoxymethyl group onto carbon 3. A series of 5-deoxy- and 6-deoxy-\( \lambda \)-ascorbic acid analogs all effectively inhibited ascorbate transport (Table I). This series included 5- and 6-deoxy ascorbic acid, 5-deoxy and 6-deoxy-6-halo-\( \lambda \)-ascorbic acids, and several 6-O-phenyl ethers. The 6-deoxy-6-halo compounds were by far the best inhibitors tested. The chloro, bromo, and iodo analogs were better inhibitors than 5-deoxy-\( \lambda \)-ascorbic acid (Table I).

Based on these data, we examined inhibition kinetics of the 6-halo series in greater detail. All of the 6-deoxy-6-halo-\( \lambda \)-ascorbic acid analogs inhibited ascorbic acid transport competitively (Fig. 1, A–E). For each analog, an inhibition constant \( (K_i) \) was determined by replotting the slopes of the lines against inhibitor concentration (Table II). Without analog, ascorbate was accumulated at least 4.7-fold against a concentration gradient (not shown).

Effects of the 6-halo series on dehydroascorbic acid transport were also examined. Incubations of fibroblasts with \( 40 \mu M \) radiolabeled \( [\text{\textsuperscript{14}C}] \)dehydroascorbic acid in the presence or absence of \( 1 \mu M \) analog showed no difference in dehydroascorbic acid transport or ascorbate accumulation (not shown).

Lack of inhibition of ascorbate transport by analogs with alterations at position 2 or 3 suggests that these positions are needed for functional interaction with the ascorbate transporter. The specificity of the stereochemical configuration at carbons 4 and 5 is indicated by the minimal inhibition observed with the ascorbate stereochemoisomers \( \delta \)-ascorbate, \( \delta \)-isoascorbate, and \( \lambda \)-ascorbate. In contrast, substitutions of \( \lambda \)-ascorbate at carbon 5 or 6 did not appreciably affect the ability of compounds to inhibit ascorbate transport. As noted above, the 6-deoxy-6-halo analogs had lower \( K_i \) values for ascorbic acid transport than ascorbic acid itself. Other structurally related compounds, including 2-deoxyglucose, \( 3 \)-O-methyl glucose, serine, leucine, and threonine, did not inhibit ascorbate transport (not shown).

**TABLE I**

| Modification | Ascorbate analog                  | Ascorbate transport (percentage of transport with no inhibitor) |
|--------------|-----------------------------------|---------------------------------------------------------------|
|              |                                   | 0 \( \mu M \) | 10 \( \mu M \) | 100 \( \mu M \) | 1000 \( \mu M \) |
| None         | \( \lambda \)-Ascorbic acid       | 100 ± 4.6    | 64 ± 0.8    | 19 ± 0.6     | 6 ± 0.3  |
| Carbon 2     | 2-Amino-                          | 100 ± 6.4    | 100 ± 4.3   | 89 ± 2.1     | 70 ± 4.9  |
| Carbon 2     | 2-Sulfate-                        | 100 ± 11     | 89 ± 5.0    | 102 ± 8.0    | 82 ± 2.0  |
| Carbon 3     | 3-O-Benzyl-                       | 100 ± 22     | 76 ± 4.9    | 95 ± 9.8     | 71 ± 4.9  |
| Carbon 3     | 3-O-Octadeyl-                     | 100 ± 5.3    | 102 ± 3.5   | 107 ± 5.3    | 109 ± 12  |
| Carbon 4 and 5| \( \delta \)-                     | 100 ± 5.0    | 100 ± 5.0   | 100 ± 0.0    | 79 ± 0.0  |
| Carbon 4     | \( \lambda \)-Iso-               | 100 ± 5.0    | 84 ± 5.0    | 84 ± 5.0     | 53 ± 0.0  |
| Carbon 5     | \( \delta \)-Iso-                | 100 ± 5.0    | 90 ± 0.0    | 70 ± 0.0     | 40 ± 10   |
| Carbons 5 and 6 | 5,6-Isopropylidene-             | 100 ± 0.2    | 74 ± 0.0    | 68 ± 0.0     | 26 ± 0.0  |
| Carbons 3, 5, and 6 | 3-O-Methoxymethyl, 5,6-isopropylidene- | 100 ± 4.5 | 100 ± 0.0 | 91 ± 0.0 | 82 ± 9.1 |
| Carbon 6     | 6-Deoxy-                          | 100 ± 12     | 75 ± 4.0    | 51 ± 9.0     | 11 ± 0.0  |
| Carbon 6     | 6-Deoxyfluoro-                    | 100 ± 19     | 79 ± 8.0    | 30 ± 7.0     | 17 ± 3.0  |
| Carbon 6     | 6-Deoxychloro-                   | 100 ± 10     | 33 ± 5.0    | 50 ± 0.0     | 5.0 ± 0.0 |
| Carbon 6     | 6-Deoxybromo-                    | 100 ± 10     | 33 ± 0.0    | 6.0 ± 0.0    | 6.0 ± 0.0 |
| Carbon 6     | 6-Deoxyiodo-                     | 100 ± 12     | 35 ± 6.0    | 6.0 ± 0.0    | 6.0 ± 0.0 |
| Carbon 6     | 6-Deoxyphenyl-                   | 100 ± 12     | 74 ± 0.0    | 68 ± 0.0     | 26 ± 0.0  |
| Carbon 6     | 6-Deoxynitrophenyl-              | 100 ± 16     | 74 ± 3.0    | 58 ± 4.0     | 13 ± 1.0  |
| Carbon 6     | 6-Deoxytrifluorophenyl           | 100 ± 15     | 70 ± 3.0    | 45 ± 5.0     | 10 ± 0.0  |
| Carbon 5     | 5-Deoxy-                          | 100 ± 9.3    | 74 ± 1.5    | 30 ± 1.8     | 8 ± 1.0   |
| Carbons 5 and 6 | 6-Ido-5-deoxy-                  | 100 ± 6.8    | 20 ± 1.5    | 4 ± 3.3      | 4 ± 0.5   |
| Carbons 5 and 6 | 6-Phenoxy-5-deoxy-              | 100 ± 4.6    | 66 ± 1.7    | 76 ± 3.2     | 23 ± 1.7  |
| Carbons 5 and 6 | 6-Phenylsulfide-5-deoxy-        | 100 ± 8.0    | 73 ± 2.9    | 20 ± 1.1     | 12 ± 1.2  |
available $^{14}$C-labeled ascorbate, resulting in a marked increase in measurement sensitivity. This is illustrated in Fig. 2C, where the HPLC profile of 35 pmol of $[^{125}$I]6-deoxy-L-ascorbic acid is compared with that of 1000 pmol of $^{14}$C-ascorbate.

Using HPLC, we examined the stability of both radiolabeled and nonradiolabeled 6-deoxy-6-iodo-L-ascorbate in water; 60% methanol, 1 mM EDTA; and BSS. Stability was maintained for 6 h at 4 °C, and <5% loss occurred after 2 h at 37 °C (not shown). 0.1 mM DTT prevented loss at 37 °C and was added in experiments examining kinetics of cellular uptake. Although degradation was minimal in 60% methanol, 1 mM EDTA at 4 °C alone, some degradation occurred during cell extraction (not shown).

**Fig. 1. Inhibition of ascorbate transport by 6-deoxy-6-halo-L-ascorbate series.** Cultured human fibroblasts were incubated for 2 h with $[^{14}$C]ascorbic acid in the presence of 6-deoxy-L-ascorbic acid (A), 6-deoxy-6-fluoro-L-ascorbic acid (B), 6-deoxy-6-chloro-L-ascorbic acid (C), 6-deoxy-6-bromo-L-ascorbic acid (D), and 6-deoxy-6-iodo-L-ascorbic acid (E). $[^{14}$C]ascorbate concentrations were 1.2–60 μM. Analog concentrations were 0 (●), 120 (■), 300 (▲), and 600 μM (▼) for 6-deoxy-L-ascorbic acid and 0 (●), 37.5 (■), 75 (▲), 150 (▼), and 300 μM (▲) for all other compounds. Double reciprocal plots of triplicate determinations ± S.D. are shown.
shown). Therefore, 1 mM DTT was added during cell extraction with 60% methanol/EDTA to prevent analog loss.

**Transport of [125I]6-Deoxy-6-iodo-L-ascorbic Acid**—Because HPLC accurately measured nonradiolabeled 6-deoxy-6-iodo-L-ascorbate, its transport properties and kinetics were examined in cultured human skin fibroblasts. Accumulation of 50 μM analog was linear for 2 h, occurred 4-fold against a concentration gradient, and was inhibited nearly 80% by 1 mM L-ascorbate (not shown).

We determined whether transport and accumulation of 6-deoxy-6-iodo-L-ascorbate are sensitive to the presence of sodium, as is ascorbate transport. Fibroblasts were incubated with analog 5–130 μM in balanced salt solution with or without sodium, which was replaced by equimolar amounts of choline and potassium. Without sodium, transport and accumulation of the ascorbate analog were inhibited as much as 95% (Fig. 3).

Transport kinetics of 6-deoxy-6-iodo-L-ascorbate were determined by incubating fibroblasts with analog 3.75–900 μM for 1 h at 37 °C (Fig. 4A). Data analysis demonstrated two component transport, an initial high affinity component and a secondary linear component, within the range of analog concentrations used. The high affinity component was calculated by subtracting a line extrapolated from the linear portion of the curve (15). As calculated by Eadie-Hofstee transformation (inset) of these data, the apparent Km was 6.7 μM and Vmax of 51.5 μM/h.

**Table II**

Inhibition constants (K_i) of 6-halo analogs for ascorbate transport

| Compound                           | K_i (μM) |
|------------------------------------|----------|
| 6-Deoxy-L-ascorbic acid            | 140      |
| 6-Deoxy-6-fluoro-L-ascorbic acid   | 32       |
| 6-Deoxy-6-chloro-L-ascorbic acid   | 2.9      |
| 6-Deoxy-6-bromo-L-ascorbic acid    | 2.8      |
| 6-Deoxy-6-iodo-L-ascorbic acid     | 3.2      |
| L-Ascorbic acid                    | 16       |

**FIG. 2.** HPLC analysis of 6-deoxy-6-iodo-L-ascorbate and [125I]6-deoxy-6-iodo-L-ascorbate. Reverse-phase HPLC chromatograms are shown of 6-deoxy-6-iodo-L-ascorbate, 50 pmol injected, measured using electrochemical detection (A), and [125I]6-deoxy-6-iodo-L-ascorbate (specific activity 1.4 × 10^4 mCi/mmol), 35 pmol injected, from a 1:1000 dilution of original product, measured with either electrochemical detection (B) or radiometric detection (C). Both samples contained 1 mM DTT. Inset to C, [14C]ascorbic acid, 1000 pmol injected measured with radiometric detection, shown for comparison (specific activity 8.0 mCi/mmol).

**FIG. 3.** Sodium dependence of 6-deoxy-6-iodo-L-ascorbate uptake in human fibroblasts. Fibroblasts were incubated with increasing concentrations of analog at 37 °C for 1 h in the presence (●) or absence (□) of sodium ions. After incubations, cells were washed, and intracellular 6-deoxy-6-iodo-L-ascorbate was extracted using 60% methanol 1 mM EDTA as described under “Materials and Methods.” Data are mean ± S.D. expressed as μM accumulation based on previously determined intracellular fibroblast volume (15).

**FIG. 4.** Apparent transport kinetics of 6-deoxy-6-iodo-L-ascorbate in human fibroblasts. A, fibroblasts were incubated with analog for 1 h at 37 °C in BSS medium, and intracellular accumulation was measured as described under “Materials and Methods.” B, data plotted were derived from A by extrapolation of a line from the linear portion of the dose-response curve in A and subtraction from each data point. Eadie-Hofstee transformation (inset) of these data is also shown. Apparent transport kinetics derived in this manner were Km of 6.7 μM and Vmax of 51.5 μM/h.

Ascorbate Analogs: 6-Deoxy-6-iodo-L-ascorbic Acid

**FIG. 2.** HPLC analysis of 6-deoxy-6-iodo-L-ascorbate and [125I]6-deoxy-6-iodo-L-ascorbate. Reverse-phase HPLC chromatograms are shown of 6-deoxy-6-iodo-L-ascorbate, 50 pmol injected, measured using electrochemical detection (A), and [125I]6-deoxy-6-iodo-L-ascorbate (specific activity 1.4 × 10^4 mCi/mmol), 35 pmol injected, from a 1:1000 dilution of original product, measured with either electrochemical detection (B) or radiometric detection (C). Both samples contained 1 mM DTT. Inset to C, [14C]ascorbic acid, 1000 pmol injected measured with radiometric detection, shown for comparison (specific activity 8.0 mCi/mmol).
Transport and accumulation of $^{[125]}$I-6-deoxy-6-iodo-L-ascorbate (10 or 20 mM) in cultured fibroblasts were inhibited by L-ascorbate in a concentration-dependent fashion (Fig. 5A). Maximal inhibition of 87 and 81% for 10 and 20 mM, respectively, occurred with an external concentration of 1 mM ascorbate. Transport was examined with and without sodium (Fig. 5B). Without sodium, $^{[125]}$I-6-deoxy-6-iodo-L-ascorbate transport was inhibited as much as 93%.

Apparent transport kinetics of $^{[125]}$I-6-deoxy-6-iodo-L-ascorbate were studied by incubating cultured fibroblasts with analog 3.75–800 μM for 1 h at 37°C (Fig. 6). Similar to results with nonradiolabeled compound, data analysis demonstrated a two component transport process: an initial high affinity component and a later nonsaturating linear component. The slope of the linear portion was identical to that seen with transport without sodium (not shown). Derivation of the data, by subtracting a line extrapolated from the linear portion of the curve followed by Eadie-Hofstee transformation, resulted in an apparent $K_m$ of 5.2 μM and an apparent $V_{max}$ of 17.2 μM/h. Nonlinear curve fitting yielded similar results (not shown). High affinity sodium-dependent and low affinity sodium-independent $^{[125]}$I-6-deoxy-6-iodo-L-ascorbate transport are consistent with ascorbate transport data in fibroblasts (15).

Dehydroascorbic Acid Transporters and $^{[125]}$I-6-Deoxy-6-iodo-L-ascorbic Acid—Interpretations of experiments examining transport of ascorbic acid and dehydroascorbic acid are complicated by the reversible nature of substrate oxidation and reduction. The ability to distinguish ascorbate transport from dehydroascorbic acid transport with a specific analog would be a useful tool. As described above, the 6-haloascorbate analogs did not inhibit dehydroascorbic acid transport in fibroblasts. Unlike ascorbic acid, the 6-deoxy-6-haloascorbate analogs when oxidized cannot form a stable cyclic hemiketal structure in solution because of the absence of a free 6-OH group. If, as seems possible, it is this stabilized cyclic form of dehydroascorbic acid that is transported, oxidized 6-halo analogs, including $^{[125]}$I-6-deoxy-6-iodo-L-ascorbate, should not be transported by mechanisms that transport dehydroascorbic acid.

Dehydroascorbic acid is transported by glucose transport isoforms GLUT1 or GLUT3 expressed in *Xenopus laevis* oocytes (12). To examine transport of $^{[125]}$I-6-deoxy-6-iodo-L-ascorbate, oocytes expressing either GLUT1 or GLUT3 were incubated with [14C]ascorbate or $^{[125]}$I-6-deoxy-6-iodo-L-ascorbic acid in the presence or absence of the oxidizing agents hydrogen peroxide or xanthine/xanthine oxidase (Fig. 7A and B). Consistent with previously reported data demonstrating dehydroascorbic acid transport by GLUT1 and GLUT3 (12, 19), oxidation of [14C]ascorbate by hydrogen peroxide or xanthine/xanthine oxidase resulted in a 6- or 13-fold increase in oocyte radiolabel uptake (Fig. 7A). In the absence of oxidation, transport of ascorbate was not different from that in control oocytes not
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DISCUSSION

Goals of these experiments were to identify an ascorbate analog that is transported exclusively by ascorbate transporters, with no activity toward dehydroascorbic acid transport mechanisms. As a first step, the ability of ascorbate analogs to inhibit ascorbate transport was examined. These results provided insights into structure/activity relationships of transport inhibition. Inhibition of ascorbate transport required a C-4S absolute configuration in a five-member reduced ring with no substitutions on carbon 2 or 3. Alterations at positions 2–4 effectively eliminated or greatly reduced the ability of these compounds to interact with the ascorbate transport processes. In contrast, several compounds with substitutions on carbons 5 and 6 were inhibitors of ascorbate transport. Even analogs with comparatively large substituents, including 6-O-aryl ethers, were effective inhibitors. Of all analogs tested, 6-deoxy-6-halo-L-ascorbic acids were the best inhibitors. None of the 6-halo analogs inhibited dehydroascorbic acid transport.

These initial data suggested that 6-deoxy-6-iodo-L-ascorbic acid would be a promising candidate for further development. To that end it was necessary to develop detection systems for this analog, characterize its transport characteristics as an ascorbate-like compound, and examine its selectivity toward ascorbate and dehydroascorbic acid transporters. We established a new method to quantitate 6-deoxy-6-iodo-L-ascorbic acid by HPLC with electrochemical detection. Sensitivity and specificity of quantitative detection of the analog by this method were comparable with the results obtained for ascorbic acid. This method was used to show that 6-deoxy-6-iodo-L-ascorbic acid was accumulated in fibroblasts with transport kinetics comparable with those previously described for ascorbate (15). Ascorbate transport in fibroblasts has a Na +–dependent high affinity component (Km of 6 μM) (15), and 6-deoxy-6-iodo-L-ascorbic acid showed a Na +–dependent high affinity component with similar kinetics (Km of 5.2 μM).

The possibility of incorporating radiiodine into 6-deoxy-6-iodo-L-ascorbic acid sparked our interest in developing this analog as a new tool to study ascorbate transport and function. Synthesis was performed by incorporating [125I]iodide into methyl 2,3-isopropylidene-6-deoxy-6-iodo-L-gulonate, followed by acid-catalyzed rearrangement to the radiolabeled derivative. The specific activity of the final compound was approximately 1000-fold greater than that of commercially available [14C]ascorbate. Radiolabeled and nonradioabeled analogs had identical HPLC elution times. Cellular transport of the radiolabeled compound was sodium-dependent, was inhibited by ascorbate, and had an apparent transport affinity similar to that of ascorbate.

Although stability of both radiolabeled and nonradioabeled compounds was less than that of ascorbic acid, this was addressed by utilizing either low temperatures (≤ 4 °C during HPLC analysis) or by the addition of 0.1–1 mM DTT. Greater instability of these compounds may be related to their inability to form stable hemiketal structures after oxidation as discussed below.

Data from several laboratories (9, 10, 20–22) strongly support the existence in cells of separate transport processes for ascorbate and dehydroascorbic acid. Because of the dual nature of ascorbate accumulation and because ascorbate can be oxidized to dehydroascorbic acid, experiments examining transport and accumulation of these compounds can be difficult to perform. Specific inhibitors of either transport activity would be very useful in the analysis of the relative roles of ascorbate and dehydroascorbic acid in cellular accumulation. Although glucose and similar molecules inhibit dehydroascorbic acid transport (23), in some cells glucose also inhibits ascorbate transport (24). In the current studies, the 6-halo analogs were expressing glucose transport proteins. Transport of [125I]6-deoxy-6-iodo-L-ascorbic acid was also not different from control un.injected oocytes. In contrast to the results with [14C]ascorbate, oxidation of [125I]6-deoxy-6-iodo-L-ascorbic acid resulted in no increased uptake (Fig. 7B).

To verify our interpretation of these results, oxidation of [14C]ascorbic acid and [125I]6-deoxy-6-iodo-L-ascorbic acid during incubations was measured by HPLC. Because DTT was present in the [125I]6-deoxy-6-iodo-L-ascorbic acid stock solution, all incubations were adjusted to contain similar amounts of DTT (37 μM), and oxidants were adjusted accordingly to assure adequate substrate oxidation. Initial concentrations of [14C]ascorbic acid and [125I]6-deoxy-6-iodo-L-ascorbic acid were 50 μM. Fifteen minutes after the addition of xanthine/xanthine oxidase, [14C]ascorbic acid concentration decreased 60%, and [125I]6-deoxy-6-iodo-L-ascorbic acid concentration decreased 55% (not shown). After the addition of H2O2, [14C]ascorbic acid decreased 85%, and [125I]6-deoxy-6-iodo-L-ascorbic acid decreased 90% (not shown). No oxidation occurred in control samples (not shown).

Therefore, [125I]6-deoxy-6-iodo-L-ascorbate and its oxidized product are not transported by either GLUT1 or GLUT3-mediated mechanisms of dehydroascorbic acid transport, irrespective of the oxidation state of the analog.

Fig. 7. Uptake of oxidized [14C]ascorbate or [125I]6-deoxy-6-iodo-L-ascorbate by Xenopus oocytes expressing glucose transport proteins. Xenopus oocytes expressing GLUT1 (open bars) or GLUT3 (hatched bars) were incubated in BSS medium for 15 min at room temperature with 100 μM [14C]ascorbate (A) or [125I]6-deoxy-6-iodo-L-ascorbate (B) in the presence or absence of either hydrogen peroxide (H2O2) (8 mM) or xanthine (50 μM)xanthine oxidase (0.05 units/ml). Oocytes were then washed with phosphate-buffered saline and solubilized with 10% SDS, and internalized radioactivity was quantified. Filled bars represent control, uninjected oocytes. [125I]6-Deoxy-6-iodo-L-ascorbate was used in tracer amounts; the addition of nonradioabeled analog resulted in a final specific activity of 207 mCi/mmol. Data are means ± S.D. of 10–20 oocytes.
very effective inhibitors of ascorbate transport but did not affect dehydroascorbic acid transport.

Dehydroascorbic acid exists in solution predominantly as a hydrated hemiketal (4), which has a half-life of approximately 6 min at pH 7.4 at 37 °C (25). The 6-halo analogs do not have the necessary OH group required for cyclization to form this structure. Based on this consideration, we reasoned that oxidation of 6-deoxy-6-iodo-L-ascorbic acid would lead to compounds that could not utilize transport mechanisms known to transport dehydroascorbic acid. To test this hypothesis, we expressed in Xenopus oocytes GLUT1 and GLUT3, glucose transport proteins that efficiently transport dehydroascorbic acid (12). Oxidation of 6-deoxy-6-iodo-L-ascorbic acid by either hydrogen peroxide or xanthine/xanthine oxidase failed to induce transport in this system. However, when ascorbate was oxidized similarly, transport of newly formed dehydroascorbic acid resulted in a 6–13-fold increase in ascorbic acid accumulation. Thus, although 6-deoxy-6-iodo-L-ascorbic acid was a potent competitive inhibitor of ascorbate transport, its oxidation did not lead to its transport and accumulation via pathways similar to those of dehydroascorbic acid. This observation demonstrates that 6-deoxy-6-iodo-L-ascorbic acid can be used to characterize the specific contribution of ascorbate transport pathways to total intracellular ascorbic acid accumulation.

In addition to sodium-dependent transport, ascorbate is transported in fibroblasts by a low affinity sodium-independent temperature-sensitive component (15). The low affinity transport activity could not be accounted for by diffusion and appeared to be carrier-mediated (15). In this paper, we found that transport activity could not be accounted for by diffusion and appears to be temperature-sensitive component (15). The low affinity trans-transported in fibroblasts by a low affinity sodium-independent transport activity include secondary transport processes mediated by the sodium-dependent transport protein or transport mediated by a distinct protein. Experiments to test these possibilities may be feasible using 6-deoxy-6-iodo-L-ascorbic acid together with a functionally expressed ascorbate transporter, which has now become available (26).

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