

# Human HL-60 Myeloid Leukemia Cells Transport Dehydroascorbic Acid Via the Glucose Transporters and Accumulate Reduced Ascorbic Acid

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**The cellular accumulation of vitamin C, a substance critical to human physiology, is mediated by transporters located at the cell membrane, and is regulated in a cell-specific manner. Neoplastic cells may have special needs for vitamin C. Therefore, we investigated the transport of vitamin C in a human myeloid leukemia cell line (HL-60). The HL-60 cells lacked the capacity to transport the reduced form of vitamin C, ascorbic acid, but they showed a remarkable ability to transport the oxidized form of vitamin C, dehydroascorbic acid (DHA). Uptake-accumulation studies indicated that the HL-60 cells accumulated ascorbic acid when provided with DHA. Kinetic analysis showed the presence of two functional ac-**

**tivities involved in the uptake of DHA, one with low affinity and one with high affinity. Cytochalasin B and phloretin, which inhibit the passage of glucose through the facilitative glucose transporters, also inhibited the transport of DHA by HL-60 cells. Transport of DHA was completed by D- but not L-hexoses, and was sensitive to D-hexose-dependent counter transport acceleration. These data support the concept that HL-60 myeloid leukemic cells transport DHA through the facilitative hexose transporters (glucose transporters) and accumulate the reduced form of ascorbic acid.**

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**A** CONSIDERABLE BODY of information exists regarding the role of ascorbic acid (vitamin C) in mammalian physiology. Vitamin C is required for vascular and connective tissue integrity as well as normal hematopoiesis and leukocyte function.<sup>1-3</sup> Humans cannot synthesize vitamin C,<sup>4,5</sup> and therefore, it must be provided exogenously and transported intracellularly, a process that is mediated by specific transporters located at the cell membrane.<sup>6-13</sup> Previous studies indicated the possible existence of several different transport systems in various cells, and even in the same differentiated cell type.<sup>10,14</sup>

Compounds that markedly affect the activity of mammalian glucose transporters also affect the capacity of cells to take up ascorbic acid, suggesting the functional involvement of glucose transporters in this process.<sup>11,15,16</sup> Two different glucose transport systems have been described in mammalian cells: a family of facilitative glucose transporters composed of at least six isoforms expressed in one or more cell types<sup>17</sup> and a sodium-glucose cotransporter expressed in small intestine and kidney.<sup>18</sup> These two families of transporters differ structurally as well as functionally and indirectly, and often contradictory, evidence suggests that both could be involved in the transport of ascorbic acid in mammalian cells.<sup>5,11,13,15,16</sup> The presence of facilitative glucose transporters in every human cell type<sup>17</sup> makes these proteins ideal candidates to fulfill the role of transporters of ascorbic acid. We recently identified the glucose transporters GLUT1, GLUT2, and GLUT4 as

efficient transporters of dehydroascorbic acid (DHA) across cell membranes by expressing these facilitative glucose transporters in *Xenopus* oocytes.<sup>19</sup> We also established that facilitative glucose transporters are involved in the transport and accumulation of vitamin C by normal human neutrophils using specific inhibitors of facilitated glucose uptake.<sup>19</sup> These studies showed that both cell types accumulated the reduced form of ascorbic acid when incubated in the presence of DHA. The identity of the transported form of ascorbic acid has been a controversial and important issue, especially because ascorbic acid is present mainly in its reduced form in blood and in various tissues and cells.<sup>7,9,10,20-23</sup>

Determination of the content of ascorbic acid in cells and tissues has indicated differential accumulation in various normal and neoplastic cell types. Chronic lymphocytic leukemia cells appear to accumulate higher intracellular concentrations of ascorbate than their normal counterparts.<sup>7</sup> However, the molecular mechanisms that regulate the cellular content of ascorbic acid are poorly understood. Although a correlation has been found between the availability of glutathione and changes in cellular ascorbate in whole animal models,<sup>24</sup> *in vitro* experiments have provided contradictory data regarding the enzymatic or nonenzymatic nature of the reduction of ascorbate by glutathione.<sup>25-27</sup> It has also been shown that the maturation of granulocyte-macrophage progenitors *in vitro* is accompanied by a major increase in the cells, ability to take up DHA.<sup>28</sup> Little information is available on the ability of human myeloid leukemic cells to accumulate ascorbic acid and the kinetic aspects of this process.

We show here that human myeloid HL-60 cells possess a remarkable capacity to transport the oxidized form of vitamin C, DHA, and accumulate reduced ascorbic acid. Kinetic analysis and competition studies indicated that the transporters of DHA have glucose transporter-like properties. Our data indicate that HL-60 cells transport DHA, but accumulate the reduced form of ascorbic acid, and point to an important role for facilitative glucose transporters in the transport of DHA in human myeloid leukemic cells.

## MATERIALS AND METHODS

Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. Two hours before the experiment, cells were suspended in incubation buffer (15 mmol/L HEPES

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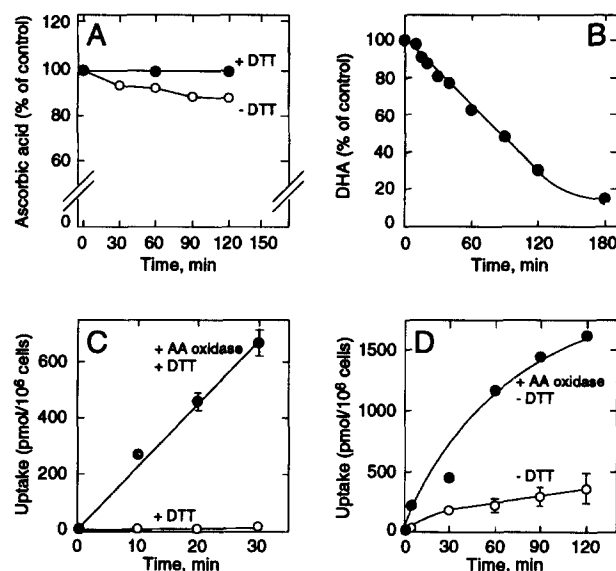
pH 7.6, 135 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L CaCl<sub>2</sub>, 0.8 mmol/L MgCl<sub>2</sub>, 0.1 mmol/L dithiothreitol [DTT]) at a final concentration of  $2 \times 10^6$  cells/mL. Cell viability was greater than 95% as determined by trypan blue exclusion.

For uptake assays, 0.5 mL of incubation buffer containing  $2 \times 10^6$  cells/mL was added to 0.5 mL of incubation buffer containing 0.1 mmol/L DTT and the appropriate concentrations of ascorbic acid in the absence (for studies using the reduced form) or in the presence (for studies using the oxidized form) of 0.1 to 20 U ascorbate oxidase.<sup>19</sup> Solutions of ascorbic acid were prepared daily and were tested for the presence of the oxidized or the reduced form by spectrophotometry and/or high performance liquid chromatography (HPLC). DTT at 0.1 mmol/L was added to the solutions to prevent the oxidation of ascorbic acid to DHA in the absence of ascorbate oxidase.<sup>19</sup> The uptake assay mixture also contained 0.1 to 0.5  $\mu$ Ci of L-[<sup>14</sup>C]-ascorbic acid (specific activity 4.74 mCi/mmol, New England Nuclear (NEN)-DuPont, Wilmington, DE). In the standard assay, the mixture was incubated for 10 minutes at room temperature and the cells were collected by centrifugation and washed twice by centrifugation in cold (4°C) stopping solution (phosphate-buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup>, and containing 100  $\mu$ mol/L phloretin and 20  $\mu$ mol/L cytochalasin B) before solubilization in 0.2 mL of 50 mmol/L TRIS-HCl pH 7.8 containing 0.2% sodium dodecyl sulfate. The cell-associated radioactivity was determined by scintillation spectrometry. A sample in which the cells were immediately centrifuged in the presence of cold stopping solution was used as a control for nonspecifically trapped radioactivity. Hexose uptake assays were performed as previously described<sup>19</sup> using 2-[1,2-<sup>3</sup>H(N)]-deoxy-D-glucose (specific activity 26.2 Ci/mmol, NEN-DuPont, Wilmington, DE). When appropriate, competitors and inhibitors were added to the uptake assays at the concentrations indicated in the respective figures, and/or the cells were preincubated in their presence. For determination of internal cell volume, HL-60 cells were suspended in medium supplemented with 0.1 mmol/L 3-O-methyl-D-glucose and traces of <sup>3</sup>H-3-O-[methyl-<sup>3</sup>H]-D-glucose, incubated for 0 and 4 hours at room temperature and processed as above. This procedure allowed us to estimate an internal volume of 0.3  $\mu$ L per 10<sup>6</sup> cells.

For experiments measuring intracellular concentrations of ascorbic acid and DHA, cells were washed three times with incubation buffer at 4°C before lysis using 60% methanol containing 1 mmol/L EDTA. Lysates were stored at -70°C until analysis and analyzed by HPLC.<sup>20</sup> Samples were separated on a Whatman strong anion exchange Partisil 10 SAX (4.6 × 25-cm) column (Whatman, Hillsboro, OR). A Whatman-type WCS solvent-conditioning column was used and the eluates monitored with a Beckman System Gold liquid chromatograph (Beckman Instruments, Irvine, CA) with a diode array detector and radioisotope detector arranged in series. Ascorbic acid was monitored by absorbance at 265 nm and by radioactivity. DHA shows no absorption at 265 nm and was monitored by radioactivity. HPLC analysis showed that ~95% to 98% of the radioactivity present in the initial DTT-treated samples migrated in the position corresponding to reduced ascorbic acid. Monitoring absorbance at 265 nm showed a small difference in the retention times of the absorbance peak of reduced ascorbic acid (retention time = 11.66 minutes) as compared with the elution of the radioactive peak (retention time = 11.80 minutes), caused by the experimental arrangement consisting of a diode array detector and radioactivity detector connected in series. The remaining radioactive material eluted in a position coincident with the DHA generated by treating the samples with ascorbate oxidase before the chromatographic separation. Treatment with ascorbate oxidase caused the disappearance of the peaks of absorbance and radioactivity eluting at 11.66 minutes and 11.80 minutes, with the generation of a new radioactive peak (containing 100% of the radioactivity) eluting at 3.44 minutes.

## RESULTS

**Stability of ascorbic acid and DHA.** In the presence of oxygen, ascorbic acid is rapidly oxidized to dehydroascorbic acid and then to further hydrolysis products.<sup>29,30</sup> Samples of ascorbic acid incubated in buffers lacking DTT underwent oxidation as indicated by a decrease in the absorbance at 265 nm (data not shown). This oxidation process was confirmed by the HPLC analysis that showed a time-dependent decrease in the radioactive peak corresponding to ascorbic acid (Fig 1A), with a concomitant increase in the DHA peak. This analysis also indicated that DTT inhibited the oxidation of ascorbic acid (Fig 1A). We generated DHA by adding ascorbate oxidase to samples of ascorbic acid containing 0.1 mmol/L DTT. The oxidation of ascorbic acid was followed

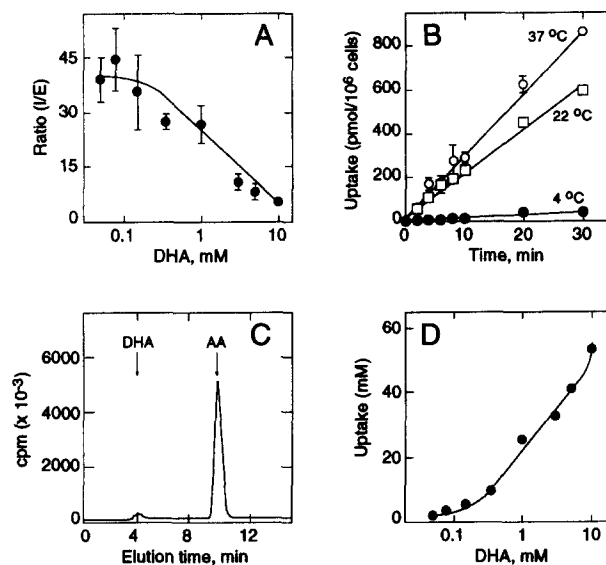


**Fig 1. Stability and transport of the reduced and the oxidized forms of ascorbic acid by HL-60 cells.** (A) Stability of ascorbic acid in solution as assessed by HPLC. Time course of the oxidation of ascorbic acid dissolved in buffer with (●; +DTT) or without (○; -DTT) 0.1 mmol/L DTT. Samples of ascorbic acid were incubated at room temperature for up to 120 minutes before analysis by HPLC. The radioactivity associated with the ascorbic acid peak at each time was quantitated and expressed as percent of the radioactivity at zero time. (B) Stability of DHA in solution. Samples of ascorbic acid (0.1 mmol/L in buffer containing 0.1 mmol/L DTT) were oxidized to DHA by treatment with 0.1 U ascorbate oxidase for 1 minute. Afterwards, samples were incubated at room temperature for the different times indicated in the figure before increasing the concentration of DTT to 3 mmol/L. The generation of the reduced form of ascorbic acid was followed spectrophotometrically immediately after adding DTT by measuring relative to a control sample treated with ascorbate oxidase at zero time and containing 3 mmol/L DTT to inhibit the oxidation of DHA (○). (C) Uptake of reduced and oxidized forms of ascorbic acid in the presence of DTT. Cells were incubated in medium containing 0.1 mmol/L DTT and 50  $\mu$ mol/L ascorbic acid untreated (○; +DTT) or treated with ascorbate oxidase (●; +AA oxidase). (D) Uptake of reduced and oxidized forms of ascorbic acid in the absence of DTT. Cells were incubated in medium lacking DTT and containing 50  $\mu$ mol/L ascorbic acid untreated (○; -DTT) or treated with ascorbate oxidase (●; +AA oxidase). Results correspond to the mean  $\pm$  SD of four samples.

by measuring the decrease in absorbance at 265 nm and was confirmed by quantitative HPLC analysis. As assessed by HPLC, about 0.01% of the radioactivity (26 cpm) eluted in the position corresponding to ascorbic acid, with the bulk (214,240 cpm, >99%) eluting in the position of DHA. Thus, a sample of 50  $\mu\text{mol/L}$  ascorbic acid contained less than 5 nmol/L ascorbic acid after treatment with ascorbic acid oxidase. DHA was reduced back to ascorbic acid with recovery of the absorbance at 265 nm by adding 3 mmol/L DTT to samples still containing ascorbate oxidase, a procedure that we used as a simple assay to estimate the stability of DHA in solution. This assay was possible because of the irreversible nature of the hydrolysis of DHA, a process that cannot be reversed by reducing agents.<sup>9,30</sup> The capacity of DHA to be reduced decreased steadily in a time-dependent fashion. Only about 50% of the original amount of ascorbic acid was recovered by adding 3 mmol/L DTT to a sample of DHA maintained for 90 minutes at room temperature in the presence of 0.1 mmol/L DTT (Fig 1B), a result consistent with previous determinations of the stability of DHA in solution using HPLC to directly quantitate DHA.<sup>30</sup> HPLC analysis confirmed the presence of ascorbic acid in samples of DHA treated with 3 mmol/L DTT. Overall, these data indicate that it is possible to study the transport of the reduced or the oxidized form of ascorbic acid under carefully controlled experimental conditions.

**Selective transport of DHA.** The HL-60 cells had a remarkable capacity to transport DHA (Fig 1C), and uptake was linear for the duration of the experiments. The cells could not transport the reduced form of ascorbic acid (Fig 1C). To eliminate the possibility that the  $\text{H}_2\text{O}_2$  generated during the treatment with ascorbate oxidase could influence the cellular uptake of DHA,  $\text{H}_2\text{O}_2$  was added to a preparation of ascorbic acid containing DTT. No cellular accumulation of ascorbic acid was observed when this sample was used in the uptake assay. Consistent with this result, absorption spectrometry showed that no dehydroascorbic acid was generated under these conditions. Additional control experiments indicated that ascorbic acid incubated for long periods of time in the presence of DTT was not taken up by the cells. The cells did accumulate radioactive material when presented with ascorbic acid in the absence of DTT (Fig 1D), an experimental condition that leads to the oxidation of ascorbic acid with generation of DHA. However, the cellular uptake of ascorbate was only a fraction (<20%) of the uptake observed when the cells were provided with DHA (Fig 1D). This result is consistent with the observations showing the oxidation of ~15% of the ascorbic acid to DHA in a 2-hour incubation period in buffers lacking DTT (Fig 1A). Overall, these experiments indicate that oxidation of ascorbic acid to DHA is essential for uptake of the vitamin by the HL-60 cells. Oxidation can be induced by simply incubating ascorbic acid in solution under aerobic conditions, and treatment with ascorbate oxidase is only required to quantitatively generate DHA.

**Cellular accumulation of ascorbic acid.** An estimated intracellular volume of 0.3  $\mu\text{L}$  per  $10^6$  HL-60 cells was used to express the measured values of transported radioactive



**Fig 2. Accumulation of ascorbic acid in HL-60 cells incubated in the presence of DHA.** (A) Uptake of DHA. HL-60 cells were incubated for 30 minutes in incubation medium containing different concentrations of radioactive DHA. The accumulated radioactivity was expressed on a volume basis using an intracellular volume of 0.3  $\mu\text{L}$  per  $10^6$  cells. Data are presented as the ratio of the radioactivity accumulated in the HL-60 cells to that present in the extracellular medium (I/E). (B) Effect of temperature on the uptake of DHA. Uptake of DHA was measured at the temperatures indicated in the figure. Under the conditions of the experiment ( $10^6$  cells per mL, 50  $\mu\text{mol/L}$  DHA), an uptake of 15 pmol per  $10^6$  cells corresponds to an intracellular concentration in equilibrium with the extracellular medium. (C) Identification of the reduced form of ascorbic acid in HL-60 cells by HPLC. HL-60 cells were incubated for 30 minutes in medium containing radioactive DHA, extracted with methanol-EDTA, and the extract analyzed by HPLC. The arrows show the expected elution times of ascorbic acid (AA) and DHA. Greater than 90% of the intracellularly trapped radioactivity eluted in the position corresponding to ascorbic acid. (D) Intracellular accumulation of ascorbic acid. Using an internal volume of 0.3  $\mu\text{L}$  per  $10^6$  cells, the data from (A) were expressed as intracellular concentrations of ascorbic acid.

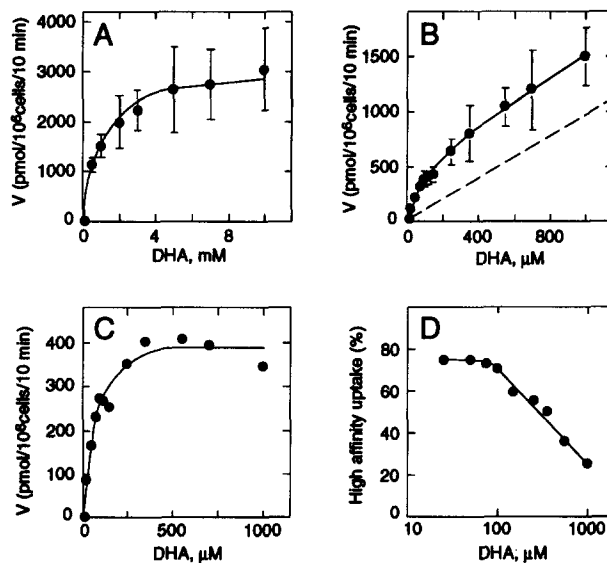
ascorbic acid on a concentration basis. At the end of the 30-minute incubation period in the presence of 50  $\mu\text{mol/L}$  DHA, the HL-60 cells accumulated ~30 times the amount of radioactive material expected assuming simple facilitated uptake (Fig 2A). This effect was dependent on the concentration of DHA during the assay, with greater ratios of internal to external concentrations observed at the lower concentrations of DHA. The cellular uptake of DHA was highly dependent on the incubation temperature, with the cells at 32°C accumulating ~70% of the radioactivity accumulated at 37°C (Fig 2B). Cells incubated at 4°C were still able to take up DHA; however, no cellular accumulation of radioactive material above the amount expected for simple equilibrium was observed (Fig 2B).

The question of the form of vitamin C accumulated in HL-60 cells is important because it has been determined that only reduced ascorbic acid is present in mammalian cells studied *in vivo* and *in vitro*.<sup>7,9,10,20-23</sup> HPLC analysis indicated that greater than 95% of the radioactivity taken up by the

cells incubated in the presence of DHA eluted in the position corresponding to reduced ascorbic acid (Fig 2C). This material eluted in the position corresponding to DHA when the cellular extracts were treated with ascorbate oxidase before the HPLC separation, confirming the identity of the accumulated vitamin C as reduced ascorbic acid. This information was used to calculate the concentration of reduced ascorbic acid accumulated in cells incubated in the presence of different concentrations of DHA (Fig 2D). The analysis showed that the HL-60 cells were able to accumulate ascorbic acid at concentrations as high as 50 mmol/L when incubated in the presence of millimolar concentrations of DHA (Fig 2D). These results indicate that the transport and reduction of DHA back to ascorbic acid are tightly coupled. This conclusion is supported by the fact that it took less than 0.5 minutes to stop the uptake reaction before preparing the cellular extracts for HPLC analysis, and in all cases studied, at least 95% of the intracellularly trapped ascorbate after an uptake period of 30 minutes was present in the reduced form. The analysis also provided further evidence for the selective transport of DHA by the HL-60 cells. When incubated in the presence of 50  $\mu\text{mol/L}$  DHA, the HL-60 cells accumulated an intracellular concentration of ascorbic acid of 1 mmol/L (Fig 2D). Considering an intracellular volume of 0.3  $\mu\text{L}$  per  $1 \times 10^6$  cells, it follows that  $2 \times 10^6$  cells accumulated 0.6 nmoles of reduced ascorbic acid. However, the HPLC analysis indicated that no more than 0.005 nmoles of reduced ascorbic acid were present in the initial sample of DHA. Thus, even if all the reduced ascorbic acid present in the incubation medium was preferentially taken up by the cells, it could account for less than 1% of the ascorbic acid accumulated intracellularly.

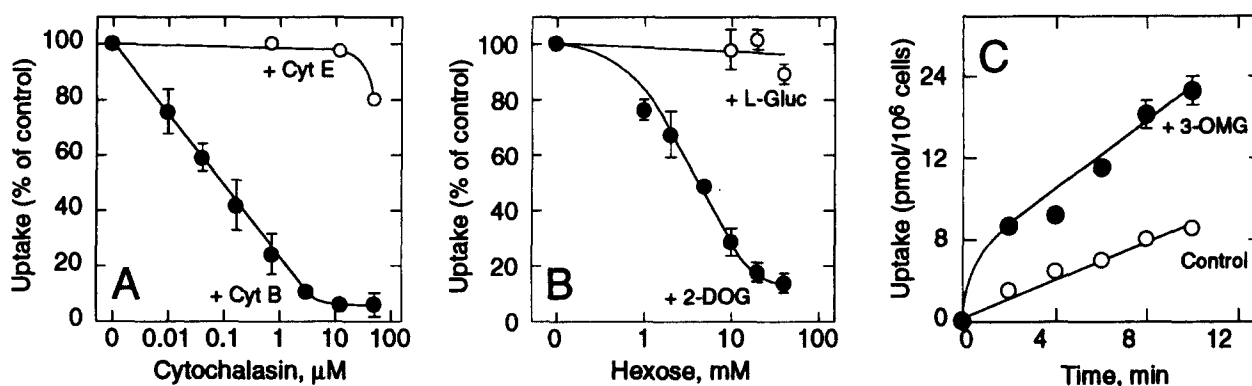
**Kinetics of DHA uptake.** Kinetic studies indicated that the uptake of DHA occurred in a concentration-dependent fashion and showed saturation at less than 10 mmol/L DHA (Fig 3A). Lineweaver-Burk analysis showed an apparent Michaeli's constant ( $K_m$ ) of 2.8 mmol/L, and a maximal velocity ( $V_{max}$ ) of 2 pmol per  $10^6$  cells per minute for the uptake of DHA by the HL-60 cells. Further analysis indicated the presence of a second, high-affinity functional component involved in the uptake of DHA (Fig 3B). After correcting for the contribution of the low-affinity component (Fig 3, B and C), an apparent  $K_m$  of 45  $\mu\text{mol/L}$  and a  $V_{max}$  of 0.3 pmol per  $10^6$  cells per minute were estimated for the high-affinity component. Thus, similar to human neutrophils, and to *Xenopus laevis* oocytes expressing mammalian glucose transporters,<sup>19</sup> promyelocytic HL-60 cells showed the presence of two functional activities involved in the uptake of DHA, one high-capacity low-affinity component and one low-capacity high-affinity component. In terms of the relative contribution of each functional component to the cellular uptake of dehydroascorbic acid, our data allowed us to estimate that at physiologic concentrations of ascorbate, most of the uptake involves high-affinity component (Fig 3D).

**Hexose transporter-like properties of the DHA transporter.** The transport of DHA in HL-60 cells was completely inhibited by cytochalasin B (Fig 4A), a specific inhibitor of facilitated hexose uptake.<sup>17</sup> Cytochalasin B caused



**Fig 3.** Kinetic analysis of the uptake of DHA by HL-60 cells. (A) Saturation curve for the low-affinity uptake of DHA. Uptake was measured in the presence of millimolar concentrations of DHA. (B) Evidence for high-affinity uptake of DHA. Uptake was measured in the presence of concentrations of DHA ranging from 20  $\mu\text{mol/L}$  to 1 mmol/L. The dotted line was used to obtain the values presented in C. (C) Saturation curve for the high-affinity uptake of DHA. Data were obtained from B subtracting single points on the dotted line from the corresponding values on the curve of uptake versus concentration of DHA. (D) Importance of the high-affinity uptake of DHA. Data in B and C were used to estimate the relative contribution of the high-affinity uptake to the total uptake of DHA by HL-60 cells. Where appropriate, data represent the mean  $\pm$  SD of at least four samples.

strong inhibition of the transport of DHA with an inhibitory concentration ( $IC_{50}$ ) of  $\sim 0.6 \mu\text{mol/L}$ , but no major effect of cytochalasin E (a noninhibitory analog of cytochalasin B) was observed (Fig 4A). The effect of cytochalasin B on the uptake of DHA paralleled its effect on the uptake of 2-deoxy-D-glucose, both in the degree of inhibition and the dose dependence (data not shown). In related experiments, 2-deoxy-D-glucose, but not L-glucose, inhibited the uptake of DHA by the HL-60 cells (Fig 4B) with an  $IC_{50}$  of 4 mmol/L. Two-deoxy-D-glucose inhibited the uptake of DHA mediated by both the high-affinity (measured using 10  $\mu\text{mol/L}$  DHA) and low-affinity components (measured using 1 mmol/L DHA). We repeated the kinetic experiments using an incubation medium containing 10 mmol/L glucose, an experimental protocol widely used to measure uptake of ascorbic acid by mammalian cells.<sup>6,8-10,14,15</sup> Under these conditions, we were still able to detect the presence of two functional activities involved in the uptake of DHA by the HL-60 cells. By Lineweaver-Burk analysis, the low-affinity component showed an apparent  $K_m$  of 6.5 mmol/L, and a  $V_{max}$  of 5 pmol per  $10^6$  cells per minute. When corrected for the contribution of the low-affinity component, the high-affinity component had an apparent  $K_m$  of 200  $\mu\text{mol/L}$ , and a  $V_{max}$  of 0.2 pmol per  $10^6$  cells per minute. These values are in agreement with previously published values obtained using similar glucose-containing buffers for the uptake of



**Fig 4.** Glucose transporter-like properties of the transporter of DHA present in HL-60 cells. (A) Effect of cytochalasins on transport of DHA. Cells were incubated for 5 minutes in the presence of different concentrations of cytochalasin B (●; +Cyt B) or cytochalasin E (○; +Cyt E) and uptake was then assessed. (B) Effect of hexoses on transport of DHA. The uptake assay was performed in the presence of different concentrations of 2-deoxy-D-glucose (●; +2-DOG) or L-glucose (○; +L-Gluc). (C) Counter-transport acceleration of the uptake of DHA by HL-60 cells. Cells were incubated for 60 minutes in medium alone (○; control) or in medium containing 5 mmol/L 3-O-methyl-D-glucose (●; +3-OMG), washed, and tested for their ability to take up 5  $\mu\text{mol/L}$  DHA. Data represent the mean  $\pm$  SD of four samples.

ascorbic acid by mammalian cells.<sup>6,8-10,14,15</sup> These results were confirmed in experiments in which uptake of low micromolar concentrations of DHA was measured in the presence of different concentrations of 2-deoxy-D-glucose (data not shown). Two-deoxy-D-glucose inhibited the uptake of DHA with an inhibition constant ( $K_i$ ) of about 1.3 mmol/L.

To further analyze the properties of the ascorbate transporters present in HL-60 cells, we studied their sensitivity to counter-transport acceleration. This phenomenon is observed when a transported molecule is present on both sides of the plasma membrane (intracellular and extracellular compartments), and is a typical characteristic of the mammalian facilitative glucose transporters.<sup>17</sup> The transport of DHA was subjected to counter transport acceleration in HL-60 cells pre-equilibrated with 3-O-methyl-D-glucose, a nonmetabolizable hexose that is reversibly transported in and out of the cells (Fig 4C). However, no effect was observed in cells preincubated with L-glucose, a nontransported hexose (data not shown). Taken together, the above results indicate that facilitative hexose transporters participate in the transport of DHA in HL-60 cells.

#### DISCUSSION

We found that HL-60 cells transported only the oxidized form of vitamin C, DHA, and that they were unable to transport the reduced form, ascorbic acid. Our data also indicated that the reduced form, ascorbic acid, was the form of the vitamin accumulated in cells exposed to DHA. These findings are consistent with the concept that the transport of DHA is coupled to the reduction of the transported molecule in the interior of the cell, a mechanism that allows for the observed accumulation of ascorbic acid. The reduced form of ascorbic acid is the main form of the vitamin present in blood and available for cellular uptake, and only low concentrations of DHA have been detected in serum.<sup>7,9,10,20-23</sup> We propose that the accumulation of cellular ascorbic acid involves at least three steps: extracellular oxidation of

ascorbic acid to DHA, facilitated transport of DHA, and intracellular reduction of DHA to ascorbic acid.

The identity of the molecular components and the functional characteristics of the intracellular mechanisms involved in the reduction of the newly transported DHA to ascorbic acid is a matter of controversy. No activity of dehydroascorbate reductase has been consistently shown in cells such as human neutrophils that accumulate millimolar concentrations of ascorbic acid.<sup>25</sup> It has been proposed that reduction of DHA could be nonenzymatic and dependent only on the intracellular content of reduced glutathione.<sup>26</sup> However, no clear relationship has been found between the differential capability of cells to accumulate ascorbic acid and their respective intracellular concentrations of glutathione.<sup>27</sup> On the other hand, a mutual dependency for the steady-state intracellular concentrations of ascorbic acid and glutathione has been clearly shown in whole animal systems.<sup>24</sup> In this regard, thioredoxin and protein disulfide isomerase are suggested to have the activity of a dehydroascorbate reductase,<sup>31</sup> opening the possibility that they could be involved in the intracellular reduction of DHA.

A plausible explanation for the existence of a cycle of oxidation-transport reduction may be related to the differences in stability of both forms of ascorbic acid in solution. The oxidation of ascorbic acid in solution is reversible and easily prevented by low concentrations of a reducing agent such as DTT. However, DHA is unstable in solution, undergoing hydrolysis in a reaction that is essentially irreversible and is not prevented by reducing agents.<sup>29,30</sup> These considerations may explain the prevalence of the reduced form of ascorbic acid in human blood, but the physiologic mechanisms that maintain ascorbic acid in a reduced state in blood are unknown. In vitro, there is no need to invoke any special mechanism, apart from the presence of oxygen, for the initial oxidation step to DHA. Others investigators have also observed accumulation of ascorbic acid in cells incubated in the presence of ascorbic acid in solutions lacking DTT,<sup>10</sup> an

observation consistent with the oxidation of ascorbic acid to DHA in solution. Likewise, anaerobic conditions have been reported to inhibit the accumulation of ascorbic acid by mammalian cells.<sup>32</sup> There is no information available on the physiologic mechanisms active *in vivo* responsible for the oxidation of ascorbic acid. Nonetheless, our data indicate that a physiologic process leading to the oxidation of ascorbic acid with the concomitant production of DHA will increase the cellular accumulation of ascorbic acid. This concept is especially relevant to those cells of the host-defense system that, when activated, generate superoxide intracellularly and extracellularly.<sup>12,33</sup>

We recently showed that mammalian glucose transporters are efficient transporters of DHA using the *Xenopus laevis* oocyte expression system.<sup>19</sup> We also established that facilitative glucose transporters are involved in the transport of DHA by human neutrophils using kinetic analysis and specific inhibitors of facilitated hexose uptake.<sup>19</sup> Our present data indicate that glucose transporters mediate the transport of DHA by human myeloid leukemic HL-60 cells. Inhibition and competition studies indicated a high degree of functional similarity between the transporters of DHA and the mammalian hexose transporters. The counter-transport acceleration induced by the intracellular presence of 3-O-methyl-D-glucose lends further support to this conclusion. Kinetic studies showed the presence of two functional activities, one with high affinity and one with low affinity for the uptake of DHA. The apparent  $K_m$ 's of the high- and the low-affinity functional activities identified in HL-60 cells were equivalent to those measured in *Xenopus* oocytes expressing mammalian glucose transporters and in human neutrophils. Based on similar results, it was concluded in previous studies that human neutrophils probably possess two different transport systems involved in the transport and accumulation of ascorbic acid.<sup>10</sup> There is also a report suggesting that human erythrocytes express a high-affinity pathway for the transport of ascorbic acid that is not related to the transport of hexoses based on the results of kinetic studies.<sup>14</sup> However, this functional activity appears to correspond to the high-affinity component described by us in *Xenopus* oocytes expressing mammalian hexose transporters, in human neutrophils and now in HL-60 cells. Indeed, the apparent  $K_m$  estimated for the pathway in erythrocytes using an uptake assay in the presence of glucose, is similar to the apparent  $K_m$  estimated for the high-affinity component evident in HL-60 cells when the uptake assays were also performed in the presence of glucose. We propose that HL-60 cells have only one transport system involved in the uptake of DHA, and that this system corresponds molecularly to the facilitative glucose transporter. The cellular accumulation of reduced ascorbic acid is accomplished by the transport of DHA by a facilitated mechanism coupled to its intracellular reduction to ascorbic acid.

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