THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 21, Issue of May 26, pp. 12584–12592, 1995
*Printed in U.S.A.*

# Accumulation of Vitamin C (Ascorbate) and Its Oxidized Metabolite Dehydroascorbic Acid Occurs by Separate Mechanisms*

(Received for publication, February 1, 1995, and in revised form, March 14, 1995)

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**It is unknown whether ascorbate alone (vitamin C), its oxidized metabolite dehydroascorbic acid alone, or both species are transported into human cells. This problem was addressed using specific assays for each compound, freshly synthesized pure dehydroascorbic acid, the specially synthesized analog 6-chloroascorbate, and a new assay for 6-chloroascorbate. Ascorbate and dehydroascorbic acid were transported and accumulated distinctly; neither competed with the other. Ascorbate was accumulated as ascorbate by sodium-dependent carrier-mediated active transport. Dehydroascorbic acid transport and accumulation as ascorbate was at least 10-fold faster than ascorbate transport and was sodium-independent. Once transported, dehydroascorbic acid was immediately reduced intracellularly to ascorbate. The analog 6-chloroascorbate had no effect on dehydroascorbic acid transport but was a competitive inhibitor of ascorbate transport. The $K_i$ for 6-chloroascorbate (2.9–4.4 μM) was similar to the $K_m$ for ascorbate transport (9.8–12.6 μM). 6-Chloroascorbate was itself transported and accumulated in fibroblasts by a sodium-dependent transporter. These data provide new information that ascorbate and dehydroascorbic acid are transported into human neutrophils and fibroblasts by two distinct mechanisms and that the compound available for intracellular utilization is ascorbate.**

Ascorbate (vitamin C) is accumulated in human tissues as much as 50-fold compared to plasma (1). However, the mechanism of transport is unknown. One possibility is that ascorbate is transported as such. Data supporting this mechanism are that ascorbate transport is concentration dependent, saturable, energy dependent, and sodium-dependent (2–8). Ascorbate but not dehydroascorbic acid is found in plasma from healthy volunteers (9, 10).

Another possibility is that ascorbate is oxidized at or near cell membranes, enters cells as dehydroascorbic acid, and is reduced intracellularly to ascorbate (3, 11–15). Dehydroascorbic acid is transported into neutrophils where it is immediately reduced to ascorbate (11–13). Dehydroascorbic acid was proposed to be transported by a glucose transporter (12–15), which was identified as GLUT I expressed in *Xenopus laevis* oocytes (15). The interpretation of these results was that dehydroascorbic acid was transported via GLUT I and that ascorbate as such was not transported at all (13–15).

There are several explanations for these conflicting conclusions. There have been no direct attempts to distinguish between ascorbate and dehydroascorbic acid as transport substrates. Analog compounds have not been commercially available which could potentially distinguish transported substrates. Interpretation of experiments is also hampered by lack of attention to these issues: substrate purity, specific assays for both substrates, measurement of intracellular mass of both substrates, an assay of analog mass, and the need to account for substrate stability.

We addressed these problems in human cells by directly investigating whether ascorbate transport alone occurs, dehydroascorbic acid transport alone occurs, whether both occur, and whether the carrier mechanisms are similar or different. HPLC[1] electrochemical assays were used for ascorbate and dehydroascorbic acid, substrate purity and stability were accounted for, an ascorbate analog was synthesized, and an assay was developed to measure analog mass. Several distinct experimental techniques provided novel evidence that both ascorbate and dehydroascorbic acid were transported, but by separate mechanisms.

## EXPERIMENTAL PROCEDURES

*Materials*—Ascorbate was purchased from Sigma. [1-$^{14}$C]Ascorbate was purchased from DuPont NEN. Bromine was purchased from Fluka. Anhydrous ether and 2,3-dimercapto-1-propanol were purchased from Aldrich. Dehydroascorbic acid was prepared immediately prior to utilization using the method of bromine oxidation of ascorbic acid as described previously (11). 6-Deoxy-6-chloro-L-ascorbate was synthesized as described previously (16). Identity and purity were confirmed by NMR, thin layer chromatography, mass spectral data, and by comparison of the optical rotation with the literature value (16). All other commercially obtained materials were of the highest grade available.

*Cell Preparation*—Neutrophils were isolated from heparinized whole blood obtained from healthy adult male volunteers and plated as described previously (3). Normal human skin fibroblast strains CRL1497 and CRL1501 were obtained from American Tissue Cell Collection. Fibroblast cultures were maintained as described previously (2). Myelocyte cell lines were obtained from ATCC and were maintained in RPMI 1640 with 10% fetal calf serum and 2 mM glutamine at 37 °C and 5% $CO_2$.

*Methods*—Ascorbate and dehydroascorbic acid transport studies were performed as described previously (2, 11). Experiments with neutrophils and fibroblasts were conducted on plated cells incubated at 37 °C. Culture plates were stationary to avoid cell detachment unless noted, in which case incubation medium was mixed by agitation of plates. Cells were incubated in HEPES/phosphate buffer containing 147 mM NaCl, 5 mM KCl, 1.9 mM $KH_2PO_4$, 1.1 mM $Na_2HPO_4$, 5.5 mM glucose, 0.3 mM $MgSO_4$:$7H_2O$, 1 mM $MgCl_2$:$6H_2O$, 0.3 mM $CaCl_2$:$2H_2O$, and 10 mM HEPES, pH 7.4. For sodium-free buffer, NaCl and $Na_2HPO_4$ were replaced by choline chloride and $K_2HPO_4$. Immediately prior to experiments cells were washed twice in HEPES/phosphate buffer. Where indicated assays were performed in HEPES/phosphate buffer

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol.

with or without sodium. After incubation, plated cells were washed twice with ice-cold phosphate-buffered saline, pH 7.4, which was removed by vacuum suction. The attached plated cells were extracted using 0.5 ml of ice-cold 60% methanol, 1 mM EDTA. After centrifugation at 15,000 × *g* at 4 °C for 10 min, supernatants were frozen at −70 °C until analysis.

For myelocyte tumor cells experiments were performed with suspended cells in test tubes at concentrations of 1–2 × $10^6$/ml in HEPES buffer at 37 °C. Cells were stationary during incubations; settling did not occur. After incubation, cells were washed by centrifugation three times with ice-cold phosphate-buffered saline, pH 7.4. Cell pellets were extracted using 0.5 ml of ice-cold 60% methanol, 1 mM EDTA. After centrifugation at 15,000 × *g* at 4 °C for 10 min, supernatants were frozen at −70 °C until analysis.

For measurement of dehydroascorbic acid reducing activity, neutrophil homogenates were prepared. Approximately 1 × $10^9$ purified neutrophils were suspended in 10 ml of ice-cold 10 mM PIPES buffer, pH 7.4, containing 100 mM KCl, 3.5 mM $MgCl_2$, 0.1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Suspended cells were pressurized with $N_2$ for 30 min at 350 pounds/square inch in a nitrogen cavitation bomb. The cavitate was centrifuged at 100,000 × *g* for 60 min, and the supernatant was used as a homogenate. The homogenate was dialyzed for 18 h in 20 mM Tris buffer at 4 °C, pH 7.5. Ascorbate reduced from dehydroascorbic acid by the homogenate was measured using HPLC (17); pure freshly prepared dehydroascorbic acid was the substrate (10). The reaction was performed in 25 μl of Tris buffer, pH 7.5, containing 0.8 mM glutathione, 0.4 mM NADPH, 200 μM dehydroascorbic acid and 10 μl of dialyzed neutrophil homogenate at room temperature. The reaction was performed for 3 min and was terminated by adding 33 μl of ice-cold 90% methanol containing 1 mM EDTA. After centrifugation at 14,000 × *g* for 10 min, 30 μl of supernatant was used immediately for measuring dehydroascorbic acid reduction activity. Concentrated glucose solutions prepared in 20 mM Tris buffer at pH 7.5 were added to assay mixtures to yield the final indicated concentrations.

*Assays*—All measurements of intra- and extracellular ascorbate were performed using high performance liquid chromatography with coulometric electrochemical detection as described previously (17). Dehydroascorbic acid was reduced to ascorbate with 2,3-dimercapto-1-propanol prior to determination by HPLC with coulometric electrochemical detection as described previously (10). [1-$^{14}$C]Ascorbate and [1-$^{14}$C]dehydroascorbic acid were measured using both liquid scintillation spectrometry and HPLC with coulometric electrochemical detection as described previously (11). Purity of radiolabel was determined by HPLC with coulometric electrochemical detection. Intracellular volume was determined as described previously as a function of cell protein, which was measured using bicinchoninic acid (Bio-Rad) (3, 11, 18).

Optimum potentials for 6-deoxy-6-chloro-L-ascorbate detection were determined using the Coulometric Electrochemical Array System (19). The assay was adapted for the HPLC system described for ascorbate quantitation using a mobile phase composed of 55% methanol (v/v), 50 mM $NaH_2PO_4$, 50 μM $NaC_2H_3O_2$, 189 μM dodecyltrimethylammonium chloride, and 36.6 μM tetraoctylammonium bromide at a pH of 4.8 (17). Changes in current were followed on the second electrode. The cell containing the electrochemical detector was reversed so that electrode number two was exposed to the solute first and the electrode was set at a potential of 250 mV.

*Data Analyses*—Kinetics for all substrates were determined when transport was linear; time points for assays were selected as described previously (2, 3, 18). To determine values for the high affinity dehydroascorbic acid transport activity, the low affinity component was subtracted as described previously (2, 3, 18, 20, 21). A line was constructed through the linear portion of the curve from 100–800 μM. The rate for the lower affinity activity was calculated (slope * [external dehydroascorbic acid]) and subtracted from the observed value of $v$. Analysis of glucose inhibition of dehydroascorbic acid transport was performed using the non-linear regression program Enzfitter. Equations for competitive (i) and non-competitive (ii) inhibition were

$$v = V^*S/\left(S + \left(1 + \frac{I}{K_i}\right)K_m\right) \quad \text{(Eq. 1)}$$

$$v = V^*S/\left(S\left(1 + \frac{I}{K_{i1}}\right) + K_m\left(1 + \frac{I}{K_1}\right)\right) \quad \text{(Eq. 2)}$$

$V$ and $K_m$ were determined in the absence of glucose using both Eadie-Hofstee analysis and non-linear regression with the program Enzfitter.

Data points in all figures represent the mean of ≥3 samples ± S.D.; S.D. is not displayed when smaller than point size.

RESULTS

*Competition between Ascorbate and Dehydroascorbic Acid*—To test whether there are separate carriers for ascorbate and dehydroascorbic acid transport, we performed competition experiments using 1-$^{14}$C-labeled substrate with increasing concentrations of unlabeled substrate. Both intracellular isotope amount and mass accumulation were measured. Human neutrophils were incubated with either 50 μM [1-$^{14}$C]ascorbate or [1-$^{14}$C]dehydroascorbic acid and 0–500 μM of either unlabeled ascorbate or dehydroascorbic acid. [1-$^{14}$C]Ascorbate transport was inhibited by extracellular unlabeled ascorbate but was unaffected by dehydroascorbic acid (Fig. 1*A*). Conversely, [1-$^{14}$C]dehydroascorbic acid transport was inhibited by increasing concentrations of extracellular unlabeled dehydroascorbic acid but was unaffected by extracellular ascorbate (Fig. 1*B*).

Mass data for these experiments were always consistent with labeled substrate findings. For example, we predicted that when neutrophils were incubated with [1-$^{14}$C]dehydroascorbic acid and increasing concentrations of the same unlabeled compound, dehydroascorbic acid transport would increase but resulting accumulation would be detected only as ascorbate. These were the observed findings (Fig. 1*C*, data not shown).

From these experiments we could address whether ascorbate transport and accumulation occurred independently of dehydroascorbic acid transport. Neutrophils were incubated with 50 μM [1-$^{14}$C]dehydroascorbic acid and increasing concentrations of external unlabeled ascorbate. Accumulation from ascorbate alone was determined at each point by subtracting [1-$^{14}$C]dehydroascorbic acid transport from total ascorbate mass (Fig. 1*C*, *inset*). The data show that ascorbate transport occurred independently of dehydroascorbic acid and was concentration dependent even in the presence of dehydroascorbic acid. Ascorbate was transported and accumulated at ≥10-fold slower rate compared to dehydroascorbic acid, consistent with other observations (3, 11, 18). Similar findings were observed for competition and mass accumulation in human fibroblasts (data not shown).

*Ascorbate Analogs*—To further distinguish between ascorbate and dehydroascorbic acid transport, the analog 6-deoxy-6-chloro-L-ascorbate (6-chloroascorbate) was synthesized. 6-Chloroascorbate has a chlorine substituted for the hydroxyl on C-6. The remaining structure of the five-member lactone ring and the stereochemistry at carbons 4 and 5 are unchanged (16). A six-position substituted analog was chosen because substitutions on C-5 or C-6 do not appear to change ascorbate function (22, 23). Neutrophils or fibroblasts were incubated with [1-$^{14}$C]ascorbate or [1-$^{14}$C]dehydroascorbic acid and increasing concentrations of 6-chloroascorbate. 6-Chloroascorbate had no effect on dehydroascorbic acid transport but inhibited ascorbate transport 4–10-fold (Fig. 2, *A* and *B*). Inhibition of ascorbate transport by 6-chloroascorbate was competitive with a $K_i$ of 4.4 μM in neutrophils (Fig. 2*A*, *inset*) and 2.9 μM in fibroblasts (Fig. 2*B*, *inset*). Both inhibition constants are similar to the $K_m$ for ascorbate of approximately 10 μM (Fig. 2, *A* and *B*, *insets*) (2, 3, 18). Substitution of other halogens at C-6 had similar effects on ascorbate transport.[2]

*Sodium Dependence of Transport*—While ascorbate transport has been shown to be sodium-dependent in fibroblasts, it is unclear whether sodium is required for dehydroascorbic acid transport (2, 4–8). Fibroblasts were incubated with dehydroascorbic acid in media with and without sodium. Dehydroascorbic acid transport and subsequent reduction to ascorbate were sodium-independent (Fig. 3).

[2] R. W. Welch, A. Crossman, Jr., K. L. Kirk, and M. Levine, manuscript in preparation.

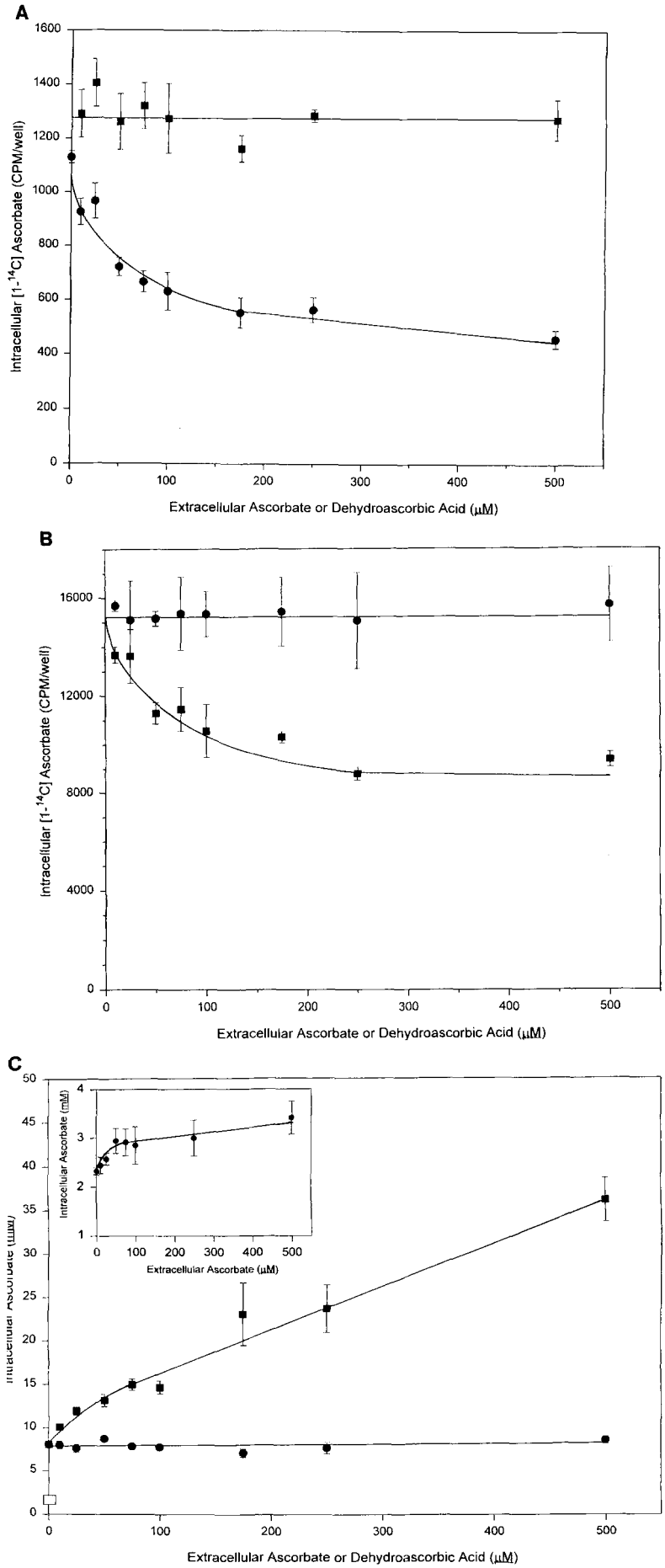


FIG. 1. **Competition between ascorbate and dehydroascorbic acid for transport.** *A*, competition of [1-$^{14}$C]ascorbate by ascorbate and dehydroascorbic acid. Plated human neutrophils were incubated for 30 min at 37 °C with 50 μM [1-$^{14}$C]ascorbate with increasing concentrations of either non-radiolabeled ascorbate ● or freshly prepared non-radiolabeled dehydroascorbic acid (■). *B*, competition of [1-$^{14}$C]dehydroascorbic acid by ascorbate and dehydroascorbic acid. Plated neutrophils were incubated for 30 min at 37 °C with 50 μM [1-$^{14}$C]-dehydroascorbic acid with increasing concentrations of either non-radiolabeled ascorbate ● or non-radiolabeled dehydroascorbic acid (■). *C*, mass accumulation of extracellular ascorbate and dehydroascorbic acid as intracellular ascorbate. Plated neutrophils were incubated for 30 min at 37 °C with 50 μM [1-$^{14}$C]dehydroascorbic acid with increasing concentrations of either non-radiolabeled ascorbate ● or non-radiolabeled dehydroascorbic acid (■). Intracellular ascorbate mass was determined using HPLC with coulometric electrochemical detection. Endogenous ascorbate concentration in the absence of extracellular ascorbate or dehydroascorbic acid was determined by HPLC with coulometric electrochemical detection (□). *Inset*, increase in intracellular ascorbate mass as a function of extracellular ascorbate, in the presence of 50 μM external [1-$^{14}$C]dehydroascorbic acid. Accumulation from ascorbate alone was determined at each point by subtracting accumulation of 50 μM [1-$^{14}$C]dehydroascorbic acid from total intracellular ascorbate mass. Data points in all figures represent the mean of ≥3 samples ± S.D.; S.D. is not displayed when smaller than point size. Assays were as described under "Experimental Procedures."

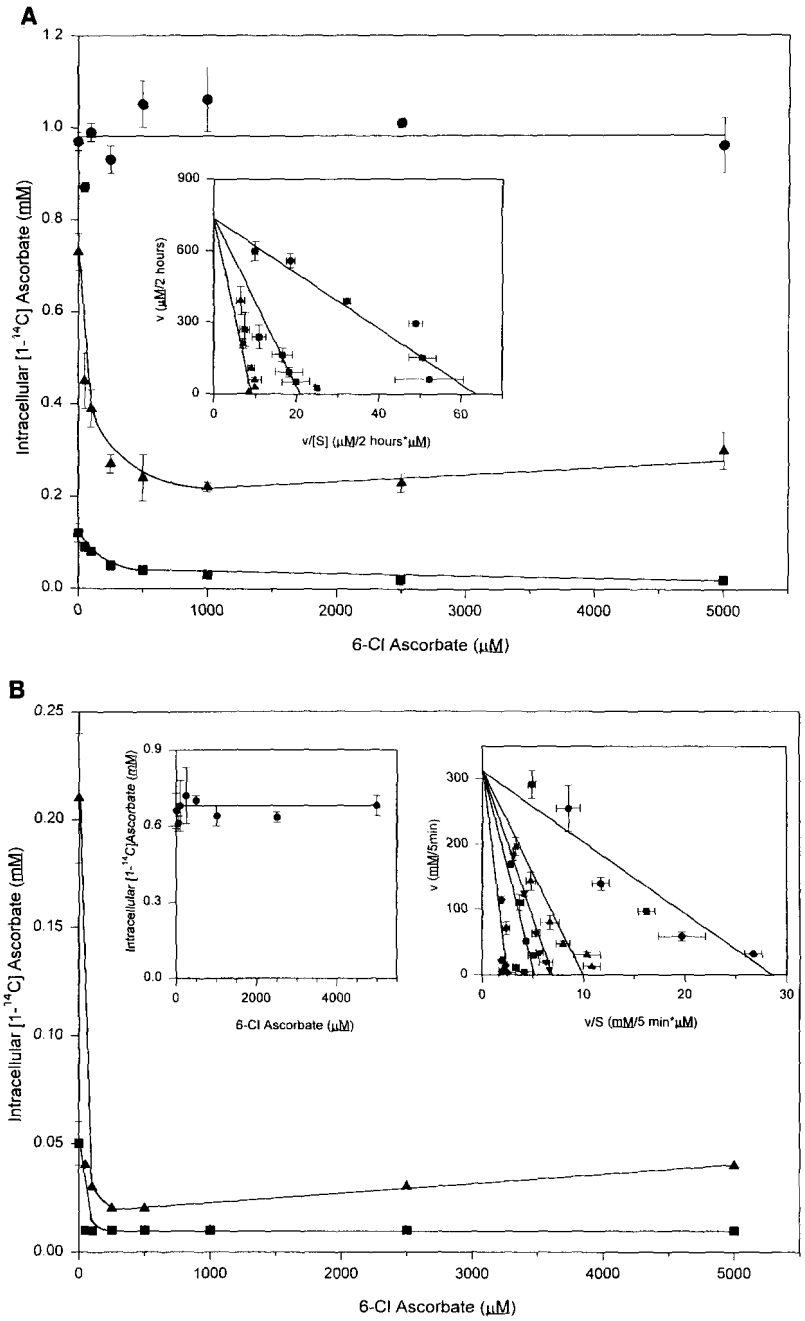


FIG. 2. **Discrimination between ascorbate and dehydroascorbic acid transport by 6-deoxy-6-chloroascorbate.** *A*, neutrophils were incubated with 50 μM [1-$^{14}$C]dehydroascorbic acid for 10 min (●), or 50 μM [1-$^{14}$C]ascorbate for 10 (■) or 60 (▲) min at 37 °C, with increasing concentrations of 6-chloroascorbate. *Inset*, kinetics of transport inhibition by 6-chloroascorbate. Neutrophils were incubated with [1-$^{14}$C]ascorbate (1.2–60 μM) and 6-chloroascorbate for 90 min at 37 °C. Data are displayed in the Eadie-Hofstee format. 6-Chloroascorbate concentrations (μM) were 0 ●, 12 (■), 30 (▲). Assays were performed as described under "Experimental Procedures." *B*, human fibroblasts were incubated with 50 μM [1-$^{14}$C]ascorbate for 30 (■) or 90 (▲) min, or 50 μM [1-$^{14}$C]dehydroascorbic acid for 30 min at 37 °C (*left inset*), with increasing concentrations of 6-chloroascorbate. *Right inset*, kinetics of transport inhibition by 6-chloroascorbate. Fibroblasts were incubated for 2 h at 37 °C with [1-$^{14}$C]ascorbate (1.2–60 μM) and 6-chloroascorbate. 6-Chloroascorbate concentrations (μM) were 0 ●, 3 (▲), 6 (▼), 12 (■), 30 (◆). Assays were performed as described under "Materials and Methods."

Sodium dependence of transport was investigated in neutrophils. Because sodium-free buffers activated normal neutrophils but not myelocyte tumor cells (data not shown), four cell lines were studied. Cells were incubated with [1-$^{14}$C]ascorbate in the presence or absence of sodium. Measurement of both mass and radiolabel indicated ascorbate transport was sodium-dependent (Table I). Cells were also incubated with [1-$^{14}$C]ascorbate, sodium, and unlabeled dehydroascorbic acid. Dehydroascorbic acid had virtually no effect on transport of [1-$^{14}$C]ascorbate. Nevertheless, dehydroascorbic acid produced a >15-fold increase in intracellular ascorbate mass above that expected from [1-$^{14}$C]ascorbate alone (Table I). These data provide additional evidence that ascorbate and dehydroascorbic acid transport occur by distinct mechanisms and that ascorbate transport is sodium-dependent. In contrast to ascorbate, dehydroascorbic acid transport and intracellular reduction in neutrophils is sodium-independent (11). Taken together, the data indicate that the requirement for sodium is another means to distinguish ascorbate from dehydroascorbic acid transport.

Because 6-chloroascorbate was a competitive inhibitor of ascorbate transport, we investigated whether 6-chloroascorbate could utilize the ascorbate transport mechanism for accumulation against a concentration gradient with a sodium requirement. Fibroblasts were incubated with 50 μM 6-chloroascorbate in the presence or absence of sodium for varying times (Fig. 3, *inset*). 6-Chloroascorbate was accumulated against a concentration gradient in the presence of sodium but was not transported at all without sodium. These data suggest that 6-chloroascorbate is accumulated by the same mechanism as ascorbate.

*Effects of Glucose on Transport and Reduction*—D-Glucose is a non-competitive inhibitor of ascorbate transport in neutrophils (18), and dehydroascorbic acid is transported via the glucose transporter GLUT I (15). Therefore, we investigated whether glucose could be used to differentiate between ascor-

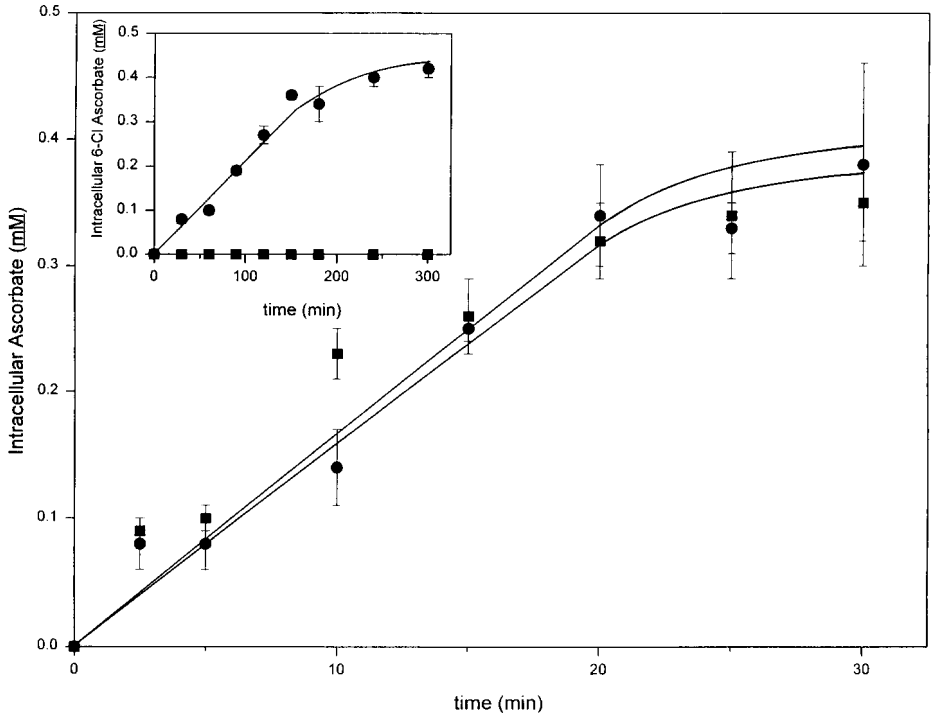


FIG. 3. **Sodium requirement for transport.** Dehydroascorbic acid transport with and without sodium. Plated fibroblasts were incubated with 100 μM dehydroascorbic acid with ● or without (■) sodium, for 0–30 min at 37 °C. Intracellular ascorbate was analyzed by HPLC with coulometric electrochemical detection as described under "Materials and Methods." *Inset*, fibroblasts were incubated with 50 μM 6-chloroascorbate ● or without (■) sodium for 0–300 min at 37 °C. 6-Chloroascorbate was determined by a new coulometric electrochemical HPLC assay as described under "Materials and Methods."

TABLE I
*Ascorbate and dehydroascorbic acid transport in myelocytes*

Four different myelocyte cell lines were incubated for 30 min at 37 °C with 100 μM [1-$^{14}$C]ascorbate with sodium, 100 μM [1-$^{14}$C]ascorbate without sodium, or 100 μM [1-$^{14}$C]ascorbate plus 200 μM dehydroascorbic acid with sodium in HEPES/phosphate buffer, pH 7.4. Dehydroascorbic acid was freshly prepared to purity. Cell concentration was $1 \times 10^6$ cells/assay. [l-$^{14}$C]Ascorbate uptake was determined using scintillation spectroscopy (Scint. Spec.) and HPLC with coulometric electrochemical detection (Mass) as described under "Materials and Methods."

| | THP-1 | | U937 | |
|---|---|---|---|---|
| | Ascorbate pmol/1 × $10^6$ cells | | Ascorbate pmol/1 × $10^6$ cells | |
| | Mass | Scint. Spec. | Mass | Scint. Spec. |
| 100 μM [1-$^{14}$C]ascorbate (+$Na^+$) | 38.3 ± 7.7 | 45.9 ± 4.7 | 47.0 ± 13.5 | 62.3 ± 13.6 |
| 100 μM [1-$^{14}$C]ascorbate (−$Na^+$) | 3.5 ± 0.2 | 13.5 ± 0.1 | 11.8 ± 1.9 | 24.8 ± 2.5 |
| 100 μM [1-$^{14}$C]ascorbate (200 μM dehydroascorbic acid, +$Na^+$) | 733.2 ± 27.9 | 47.1 ± 0.5 | 999.9 ± 50.8 | 57.4 ± 1.1 |
| | PLB | | HL60 | |
| | Ascorbate pmol/1 × $10^6$ cells | | Ascorbate pmol/1 × $10^6$ cells | |
| | Mass | Scint. Spec. | Mass | Scint. Spec. |
| 100 μM [1-$^{14}$C]ascorbate (+$Na^+$) | 45.4 ± 7.3 | 58.5 ± 7.9 | 98.1 ± 3.4 | 94.9 ± 2.2 |
| 100 μM [1-$^{14}$C]ascorbate (−$Na^+$) | 10.5 ± 0.5 | 20.0 ± 3.2 | 18.5 ± 13.5 | 28.3 ± 1.3 |
| 100 μM [1-$^{14}$C]ascorbate (200 μM dehydroascorbic acid, +$Na^+$) | 1372.9 ± 37.8 | 65.3 ± 3.8 | 1583.4 ± 3.8 | 94.9 ± 2.0 |

bate and dehydroascorbic acid transport. To do so it was necessary to characterize dehydroascorbic acid transport kinetics. Dehydroascorbic acid uptake (2–800 μM) demonstrated low and high affinity components (Fig. 4*A*). Kinetics of the low affinity component were indeterminate due to two factors: appearance of intracellular dehydroascorbic acid and dehydroascorbic acid toxicity (Fig. 4*B*). At external dehydroascorbic acid concentrations >800 μM intracellular radiolabel exceeded intracellular ascorbate mass. Intracellular dehydroascorbic acid which was not reduced to ascorbate accounted for the difference. At extracellular dehydroascorbic acid concentrations >2000 μM cell death occurred. Subsequent kinetic measurements were performed at extracellular dehydroascorbic acid concentrations ≤800 μM so that dehydroascorbic acid transport and not reduction was measured. For the high affinity transport component $K_m$ = 35.5 ± 9 μM and $V_{max}$ = 0.75 ± 0.4 mM/min (Fig. 4*C*). Contrary to previous reports (15), glucose inhibition was noncompetitive with inhibition constants of $K_i$ = 2.37 ± 0.79 mM and $K_{ii}$ = 2.66 ± 0.38 mM (20, 21). Graphic analysis of observed and calculated uptake rates were compared and supported these conclusions, as did algebraic analysis of the Eadie-Hofstee plot (Fig. 4*C*, *inset*) (20, 21). These data indicate that glucose is a non-competitive inhibitor of dehydroascorbic acid transport and that glucose cannot be used to distinguish ascorbate and dehydroascorbic acid transport activities in neutrophils. External dehydroascorbic acid concentrations were stable for the time course of the experiments (11) (data not shown).

For interpretations of dehydroascorbic acid transport kinetics to be accurate, transport and not reduction must be the rate-limiting step. Mass transport data (Fig. 4*B*) imply that transport is the rate-limiting step for external dehydroascorbic acid concentrations ≤800 μM. To address the issue further, we investigated whether glucose inhibited dehydroascorbic acid reduction. For these experiments it was necessary to partially characterize the dehydroascorbic acid reduction activity of neutrophils. Dehydroascorbic acid reduction in neutrophil homogenates was similar to that expected from the same number of whole cells, was localized to cytosol, was dependent on cell

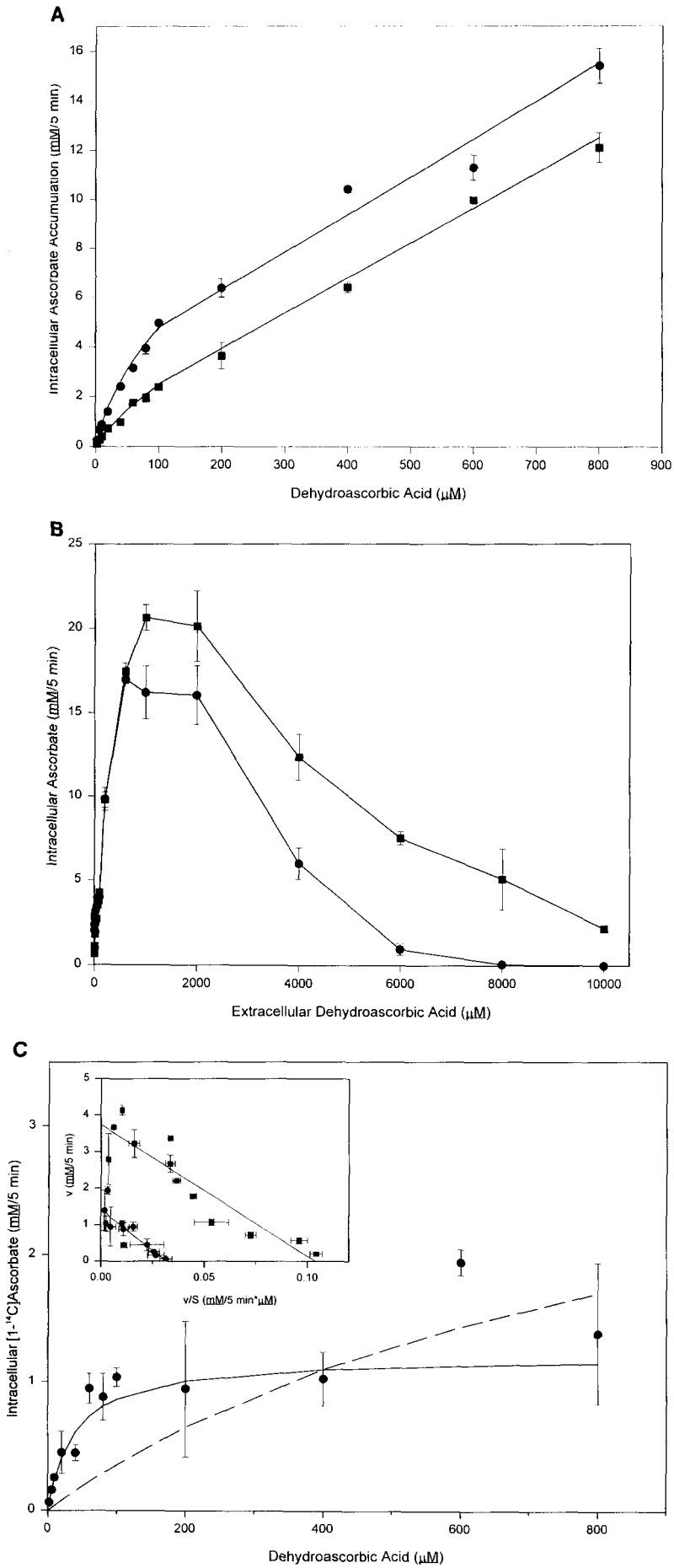


FIG. 4. **Dehydroascorbic acid transport kinetics and inhibition by glucose.** *A*, neutrophils were incubated for 5 min at 37 °C with freshly prepared [1-$^{14}$C]dehydroascorbic acid (2–800 μM) with (■) and without ● 5.5 mM glucose. Ascorbate was determined by scintillation spectroscopy as described under "Materials and Methods." *B*, neutrophils were incubated for 5 min at 37 °C with freshly prepared [1-$^{14}$C]dehydroascorbic acid (2–10,000 μM) without glucose. Intracellular measurements represent radiolabel (■) and mass ●. Ascorbate was determined by scintillation spectroscopy and HPLC with coulometric electrochemical detection. *C*, inhibition equations were tested using the non-linear regression analysis program Enzfitter (—, non-competitive inhibition; - - - -, competitive inhibition). Data points represent the high affinity activity in the presence of 5.5 mM glucose. Activity from the low affinity transport activity was subtracted and the analysis was performed as described under "Materials and Methods." *Inset*, Eadie-Hofstee analysis of the substrate velocity plot with ● and without (■) glucose. Ascorbate was determined by scintillation spectroscopy; all intracellular label was ascorbate. Data for the low affinity component were subtracted as described under "Materials and Methods" and replotted for the high affinity component using the weighted means of the corrected rates.

TABLE II

*Effect of glucose on dehydroascorbic acid reduction by neutrophil homogenates*

Homogenate preparation, incubation conditions, and ascorbate measurements are described under "Materials and Methods." Dehydroascorbic acid concentration was 200 μM, glutathione concentration was 0.8 mM, and NADPH concentration was 0.4 mM. The experiment was performed using at least three different homogenates with similar results.

| Glucose (mM) | Ascorbate (pmol/3 min) | | | | |
|---|---|---|---|---|---|
| | 0 | 2 | 5 | 10 | 20 |
| Dialysate + dehydroascorbic acid + glutathione + NADPH | 658.3 | 550.1 | 649.4 | 662.5 | 705.1 |
| Dialysate + dehydroascorbic acid | 53.0 | 58.9 | 60.1 | 56.8 | 57.5 |
| Dialysate alone | <10 | <10 | <10 | <10 | <10 |
| Glutathione + NADPH + dehydroascorbic acid (no dialysate) | <10 | <10 | <10 | <10 | <10 |

number, was non-dialyzable, and was found in the retentate using centrifugal ultrafiltration (data not shown). Dehydroascorbic acid reduction required reduced glutathione and NADPH for maximal activity (Table II). Activity could not be accounted for by simple chemical reduction of dehydroascorbic acid and was protein-mediated (24–27). Glucose was tested as an inhibitor of dehydroascorbic acid reduction and had no effect (Table II). These data together with those in Fig. 4*B* indicate that for dehydroascorbic acid concentrations ≤800 μM, reduction is not a rate-limiting step for transport, and the assumptions for determining transport kinetics are correct.

*Substrate Stability*—It remained possible that inadvertent ascorbate oxidation could influence some experimental results. External ascorbate was not oxidized under the experimental conditions used here (Fig. 1, Table I, data not shown) (3, 11, 18). Nevertheless, additional confirmatory experiments were performed. Thiol reagents such as DTT can prevent ascorbate oxidation and reduce dehydroascorbic acid to ascorbate (9, 10). We investigated the effects of DTT on ascorbate and dehydroascorbic acid transport. If no oxidation of external ascorbate occurred, DTT should not influence ascorbate transport. By contrast, DTT will reduce dehydroascorbic acid to ascorbate. Since the rate of ascorbate transport is >10-fold slower than that of dehydroascorbic acid, its transport should be decreased by DTT as reduction occurs. Neutrophils were incubated with either 200 μm dehydroascorbic acid or [1-$^{14}$C]ascorbate and increasing concentrations of DTT. Both [1-$^{14}$C]ascorbate uptake and mass accumulation were measured. As expected, DTT decreased dehydroascorbic acid transport as measured by intracellular ascorbate accumulation (Fig. 5*A*). DTT had no effect on [1-$^{14}$C]ascorbate transport and ascorbate accumulation at extracellular DTT concentrations up to 100 μM (Fig. 5, *B* and *C*). These data provide additional evidence that inadvertent oxidation of ascorbate did not occur. Ascorbate transport in the presence of DTT was not observed in neutrophils by others (15) probably because the incubation time was too short.

## DISCUSSION

We report here new evidence that both ascorbate and dehydroascorbic acid are transported into human cells, but by separate mechanisms. A number of experimental criteria distinguished the two transport systems. Competition experiments showed that ascorbate but not dehydroascorbic acid competed with [1-$^{14}$C]ascorbate for transport. Conversely, dehydroascorbic acid but not ascorbate competed with [1-$^{14}$C]dehydroascorbic acid for transport. Both ascorbate transport and accumulation occurred in the presence of dehydroascorbic acid and were independent of it. Likewise, dehydroascorbic acid was transported independently of ascorbate. The specially synthesized analog 6-chloroascorbate had no effect whatsoever on dehydroascorbic acid transport. By contrast, this analog competitively inhibited ascorbate transport with a $K_i$ similar to the $K_m$ for ascorbate transport. Ascorbate transport was sodium-dependent, while dehydroascorbic acid transport was sodium-independent. Finally, 6-chloroascorbate was transported and accumulated against its concentration gradient only in the presence of sodium.

It is theoretically possible that a single transport protein has two independent properties, such that ascorbate and dehydroascorbic acid transport could be mediated by the same protein. Several lines of evidence suggest this is not likely. GLUT I expressed in oocytes transports glucose and dehydroascorbic acid, but not ascorbate (15). Despite use of insensitive assay techniques, the data suggest that one single transport protein (GLUT I) does not have independent transport properties for ascorbate and dehydroascorbic acid. Glucose transporters I-V are well characterized (28, 29). They do not appear to be sodium-dependent nor to have sites indicative of sodium dependence. As shown here, such sodium dependence is required for ascorbate but not dehydroascorbic acid transport. Recently, ascorbate transport activity was expressed in oocytes using mRNA from rabbit kidney (30). Activity was sodium-dependent and uptake of radiolabeled ascorbate was inhibited by excess unlabeled ascorbate but not glucose, implying that the putative ascorbate transporter is mediated by a different protein than that responsible for dehydroascorbic acid transport. It is unclear whether one or several mRNAs are responsible for ascorbate transport activity and there are no clones for the transporter. These problems must be solved before the transporter is expressed definitively. Until this can be accomplished it is necessary to demonstrate functionally that there are two distinct transport activities. The data in this paper are the first to suggest that this is the case.

Generation of reactive oxygen intermediates is a normal process in aerobic organisms. However, these oxidants do not oxidize ascorbate outside resting (unactivated) neutrophils or normal fibroblasts. The evidence is that external ascorbate was not oxidized by resting neutrophils, and dehydroascorbic acid was detected externally only when cells were activated (3, 11, 18). Additional data are seen in Fig. 1 and Table I. If oxidants from resting neutrophils or fibroblasts oxidized external [1-$^{14}$C]ascorbate, extracellular dehydroascorbic acid would have inhibited accumulation of newly formed [1-$^{14}$C]dehydroascorbic acid. Also, if oxidation of external [1-$^{14}$C]ascorbate occurred outside resting cells, extracellular dehydroascorbic acid could have changed the rate or amount of [1-$^{14}$C]ascorbate accumulation. None of these findings were observed. Possibilities to explain why aerobic metabolites did not oxidize ascorbate outside resting neutrophils and fibroblasts include: compartmentalization or unavailability of oxidants to external ascorbate because of the site of oxidant production, a slow rate

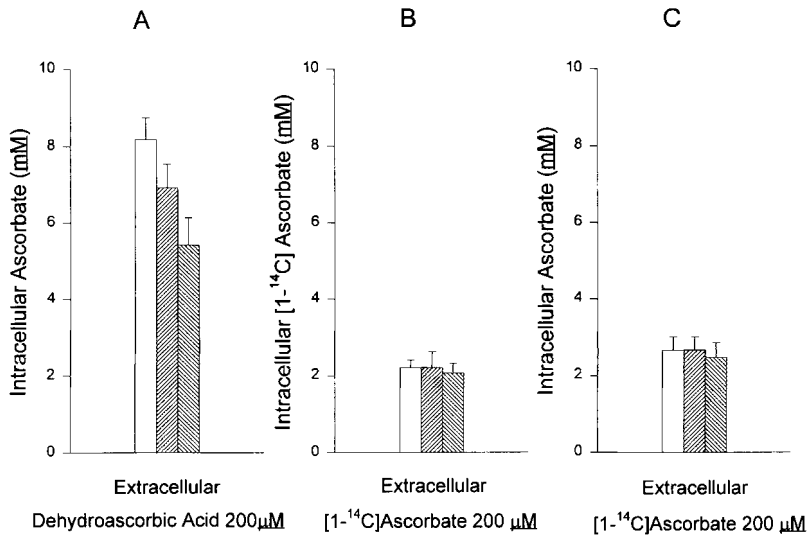


FIG. 5. **Effects of DTT on ascorbate and dehydroascorbic acid transport.** Human neutrophils were incubated with 200 μM freshly prepared dehydroascorbic acid for 10 min (*A*) or 200 μM [1-$^{14}$C]ascorbate for 60 min (*B* and *C*) at 37 °C with increasing concentrations of DTT. DTT concentrations were 0 (□), 50 (▨), or 100 (▩) μM. Ascorbate was determined by HPLC (*A* and *C*) or scintillation spectroscopy (*B*). Assays were as described under "Materials and Methods."

of oxidant production, and quenching of generated oxidants before they are available to external ascorbate.

We and others proposed that intracellular ascorbate is utilized for protection against permeant oxidants made by activated neutrophils (11, 31, 32). Oxidants from activated neutrophils oxidize extracellular ascorbate to dehydroascorbic acid. Dehydroascorbic acid is more rapidly transported than ascorbate and is immediately reduced intracellularly to ascorbate for potential oxidant protection. High concentrations of intracellular ascorbate would be available at the same time they would be needed to quench newly formed oxidants generated by activated neutrophils. Oxidant generation by neutrophils can be as high as 200 nmol oxidant/$10^6$ cells·h (33, 34). It was predicted that for ascorbate to be important for oxidant quenching, rapid intracellular reduction of oxidized ascorbate would have to occur (34). The data here and elsewhere suggest that ascorbate recycling, or reduction within cells of newly formed extracellular dehydroascorbic acid, occurs in neutrophils. In addition, reutilization of extracellular dehydroascorbic acid occurs when neutrophils are exposed to bacteria and ascorbate but not to ascorbate alone.[3]

There may be other explanations why ascorbate recycling occurs. In theory, it is possible that dehydroascorbic acid is reduced in cells so that ascorbate can be continuously exported to replenish extracellular ascorbate consumed by oxidants. However, ascorbate efflux does not occur from plated fibroblasts or neutrophils (2, 18, data not shown). The results were unaffected by mixing the incubation medium: efflux still did not occur (data not shown). The best current explanation for ascorbate recycling is that it rapidly provides high concentrations of ascorbate for intracellular oxidant quenching at the time oxidants are generated by activated neutrophils.

We found here that glucose was a non-competitive inhibitor of dehydroascorbic acid transport, in contrast to findings by others (15). There are several reasons for the discrepancy. Incubation conditions used by others contained ascorbate, DTT, and ascorbate oxidase to form dehydroascorbic acid (15). True substrate concentrations were not measured directly. Under these conditions external dehydroascorbic acid concentrations would not be constant, making kinetic measurements unreliable. Other analyses of substrate-velocity curves were performed improperly (15). The two components of biphasic kinetics can be separated by extrapolating the linear portion of the substrate-velocity curve at higher substrate concentrations to the *y* axis. The rate contribution represented by this line is then subtracted from the original data (2, 3, 18, 20, 21). Another suitable method is to utilize non-linear regression programs, as used in this paper. However, the method used by others (15) of drawing a line from the origin to a single point is invalid for determining kinetics for a biphasic system. Other problems were that no mass measurements were reported for kinetic analyses, and the few mass measurements performed for other experiments utilized the insensitive and nonspecific method of thin layer chromatography. Differences between kinetic results here and elsewhere (15) cannot be accounted for by diffusion limitations in the extracellular buffer. This is because dehydroascorbic acid uptake and its inhibition by glucose were identical when buffers were either mixed or stationary (data not shown).

From findings by others (15), it was not clear whether dehydroascorbic acid transport or its reduction was rate limiting in the presence of glucose. For kinetic interpretations to be correct, transport would have to be the rate-limiting step, and glucose itself should not interfere with intracellular reduction. Using mass measurements, we show here that transport was the rate-limiting step only at dehydroascorbic acid concentrations ≤800 μM. Dehydroascorbic acid reduction was incomplete at extracellular concentrations above 800 μM. We also demonstrated directly that glucose itself did not interfere with reduction in neutrophil homogenates. Only with this information is it possible to perform correct kinetic calculations for high affinity dehydroascorbic acid transport. Despite previous claims (15), calculations for low affinity transport were not valid because these issues were not accounted for.

The mechanism of dehydroascorbic acid reduction in cells is unknown. Although the isolated proteins protein disulfide isomerase and glutaredoxin have dehydroascorbic acid reducing activity (25), it is unclear whether these or any other proteins actually mediate reduction in cells (27). Substantial evidence suggests that dehydroascorbic acid reduction occurs only chemically with glutathione as the reductant (24, 26, 27, 35–37). The data in this paper provide new evidence that dehydroascorbic acid reduction in neutrophils is protein mediated and cannot be accounted for via chemical reduction by glutathione. Definitive information on the identity of the protein(s) involved will be best provided using activity-based purification; this work is in progress.

[3] Y. Wang, T. Russo, S. Chanock, and M. Levine, manuscript in preparation.

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