

Inhibition of Ascorbic Acid Transport in Human Neutrophils by Glucose*

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Because of the structural similarity between glucose and ascorbic acid, we investigated the effect of glucose on uptake and accumulation of ascorbic acid in isolated normal human neutrophils. Ascorbic acid accumulation was determined using high-performance liquid chromatography with coulometric electrochemical detection, in conjunction with liquid scintillation spectrometry. Ascorbic acid accumulation in neutrophils is mediated by a high and a low affinity transport activity. In neutrophils from different volunteers, glucose inhibited uptake and accumulation of ascorbic acid by both transport activities 3–9-fold. The mechanism of inhibition was different for each transport activity: inhibition of the high affinity transport activity was noncompetitive, while inhibition of the low affinity activity was competitive. Glucose-induced inhibition of both ascorbic acid transport activities occurred in neutrophils of all donors tested and was fully reversible. Although the mechanism of ascorbic acid accumulation appeared to be different than that for glucose transport, other monosaccharides and glucose transport inhibitors also inhibited ascorbic acid accumulation. These are the first data to suggest that ascorbic acid accumulation in neutrophils can be regulated by compounds of similar structure.

Ascorbic acid is present in normal human neutrophils in millimolar concentrations (1, 2). Ascorbic acid might have several functions in neutrophils including facilitating oxidative destruction of microorganisms, preserving neutrophil integrity, or protecting surrounding tissues from oxidants generated during the respiratory burst (3–9). However, the definitive function of ascorbic acid in neutrophils is unknown. To understand function, one approach is to first regulate intracellular ascorbic acid content. Possible function can then be studied in direct relationship to a wide range of intracellular and extracellular concentrations.

To achieve these goals, we have investigated ascorbic acid transport in human neutrophils. We reported that ascorbic acid is accumulated by the combined actions of a high affinity and a low affinity transport activity (2). Both activities were saturable, temperature-dependent, and accumulated ascorbic acid against a concentration gradient as large as 50-fold.

In this paper, we investigate the regulation of ascorbic acid accumulation into human neutrophils by the action of the

two transport activities. It is possible that substances normally present in blood, or compounds of similar structure to ascorbic acid, can regulate its transport. Glucose has a similar structure, and is the precursor for the vitamin in species which synthesize it (10). Glucose has been proposed to impair ascorbic acid translocation across cell membranes (11). Previous experiments to test this hypothesis have been difficult to interpret since they were performed using erythrocytes and/or dehydroascorbic acid rather than ascorbic acid (12–14). Erythrocytes may not have an active ascorbic acid transport system and do not concentrate ascorbic acid against a gradient (15). Dehydroascorbic acid may not be found at all in normal human plasma or only at very low concentrations, and dehydroascorbic acid is not detectable in human neutrophils (2, 16). Moreover, insensitive assay methods used prevented accurate determination of ascorbic acid *versus* its metabolites or other reducing substances (17, 18). Using a sensitive and specific high-performance liquid chromatographic assay for ascorbic acid, we report here on the effect of glucose on the accumulation of ascorbic acid by each transport activity. The data indicate that glucose has the ability to regulate both transport activities, but by a different mechanism for each.

MATERIALS AND METHODS

Neutrophils were isolated from heparinized whole blood collected from healthy male adult volunteers, receiving no medications or ascorbic acid supplements, as previously described (2). Neutrophils were suspended in Hanks' balanced salt solution without calcium, magnesium, or phenol red (pH 7.4) to a final concentration of 1×10^7 cells/ml. Neutrophils were routinely >95% pure as revealed by Wright's staining and >95% viable by exclusion of trypan blue. To investigate regulation of ascorbic acid transport, freshly isolated neutrophils were plated on 24-well (16-mm diameter) culture plates (Costar, Cambridge, MA), incubated to allow the cells to adhere to the well bottoms, and washed, as previously described (2). Fresh bicarbonate-free suspension buffer containing 1.5 mM Ca^{2+} , 1.3 mM Mg^{2+} , and various concentrations of ascorbic acid and glucose (pH 7.4) was added to each well, final volume 1 ml. Ionic strength was maintained to compensate for changes in glucose concentration. Neutrophils were incubated at 37 °C in a humidified 5% CO_2 atmosphere. At times indicated in the text the buffer was removed, and the cells were washed, extracted, and stored as previously described until analyzed for ascorbic acid and protein (2).

Total intracellular ascorbic acid was determined by high-performance liquid chromatography (HPLC)¹ and [¹⁴C]ascorbic acid was quantitated by a combination of liquid scintillation spectrometry and HPLC, as previously described (2, 17). Protein determinations were performed by the bicinchoninic acid protein assay (19), after solubilizing extracted neutrophils (2). Intracellular total and [¹⁴C]ascorbic acid concentration was determined by conversion of neutrophil protein to a measured intracellular volume, as previously described (2).

Experimental points represent the mean \pm S.D. of at least three

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¹ The abbreviation used is: HPLC, high-performance liquid chromatography.

samples. Error bars have been omitted when the standard deviation was less than the size of the symbol. Statistical analysis of the data was performed using Student's *t* test.

RESULTS

Transport and Accumulation of Ascorbic Acid in Neutrophils as a Function of External Glucose Concentration—We determined the effect of glucose on transport and accumulation of ascorbic acid in neutrophils by the high affinity and low affinity ascorbic acid transport activities. At 10 μM external ascorbic acid, the high affinity transport activity was responsible for approximately 90% of transport (2). At 200 μM external ascorbic acid, the low affinity transport activity was responsible for 70–80% of transport (Ref. 2; see also Figs. 2A and 2B and "Discussion").² The concentration of plasma glucose in normal humans is approximately 2.5–6.5 mM, while concentrations of 10–20 mM can be found in asymptomatic diabetics (20). Thus, neutrophils were incubated with either 10 or 200 μM [¹⁴C]ascorbic acid and 0.0, 2.5, 5.0, or 10.0 mM glucose for 120 min. For the low affinity transport activity, accumulation of total ascorbic acid as measured by HPLC was linear for 120 min at all concentrations of glucose (Fig. 1A). Ascorbic acid accumulation decreased as extracellular glucose concentrations increased. Endogenous intracellular ascorbic acid concentration at zero time was approximately 1.5 mM. When ascorbic acid transport was quantitated by liquid scintillation spectrometry, endogenous ascorbic acid was not included in the measurement, and the effect of glucose on the low affinity transport activity was more apparent (Fig. 1B). For the high affinity transport activity, ascorbic acid transport measured by liquid scintillation spectrometry was also linear for at least 120 min and was inhibited at increasing glucose concentrations (Fig. 1C). As expected, when glucose regulation of the high affinity transport activity was determined by HPLC, the effect was more difficult to detect due to the small amount of ascorbic acid accumulated at 10 μM external ascorbic acid in comparison to the large concentration of endogenous ascorbic acid in freshly isolated neutrophils (data not shown). For both transport activities, the rate of ascorbic acid transport at the lowest glucose concentration was 3–4-fold greater than at the highest glucose concentration.

To determine whether glucose inhibition of ascorbic acid transport and accumulation was a general phenomenon, neutrophils were obtained from multiple donors and incubated with a wide range of external glucose concentrations. Neutrophils were incubated for 90 min with either 10 or 200 μM [¹⁴C]ascorbic acid and concentrations of extracellular glucose ranging from 0 to 30 mM, depending upon the experiment. The effect of glucose on [¹⁴C]ascorbic acid transport is shown in Figs. 2A and 2B. For both transport activities, glucose inhibited the rate of [¹⁴C]ascorbic acid transport by 3–8-fold. Glucose inhibition of the high and low affinity transport activities was concentration-dependent; inhibition was most pronounced at glucose concentrations between 1 and 4 mM.

By using HPLC to determine total ascorbic acid accumulation, similar effects of glucose were obtained for the low affinity transport activity (Fig. 2C). Since the initial ascorbic acid concentration at isolation varied from 1.0 to 1.6 mM (data not shown), glucose suppressed the rate of ascorbic acid accumulation 4–9-fold (Fig. 2C). HPLC was used to determine the effects of glucose on the high affinity transport activity (data not shown). Regulation of ascorbic acid accumulation also occurred but was more difficult to detect due to the small amount of ascorbic acid transported by the high affinity

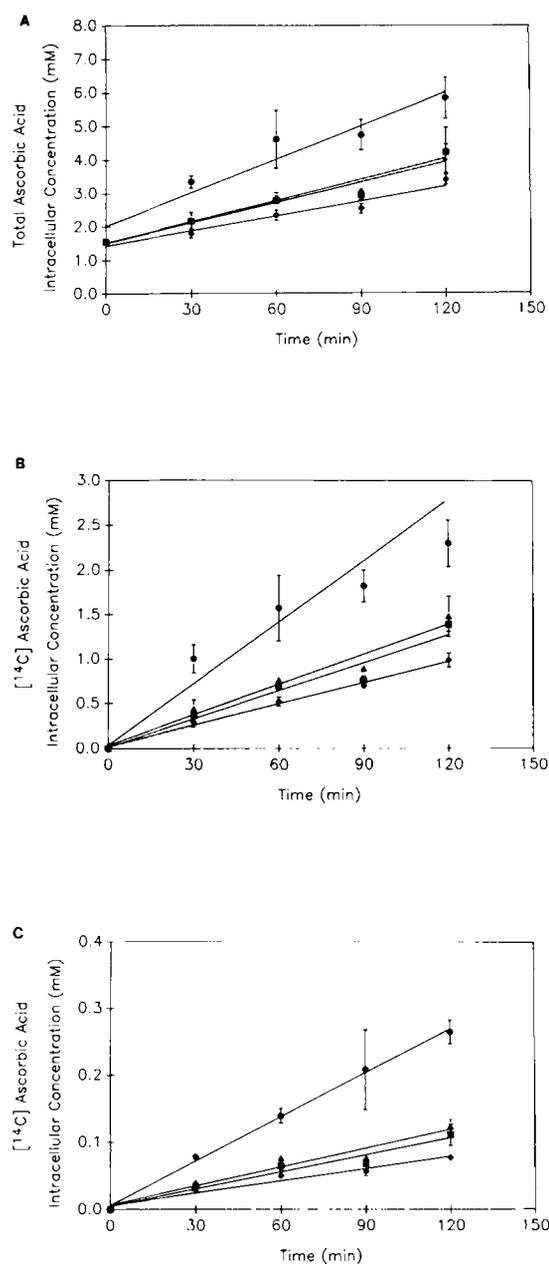


FIG. 1. Effect of glucose on [¹⁴C]ascorbic acid uptake and total ascorbic acid accumulation in neutrophils as a function of time. Neutrophils isolated and plated as described under "Materials and Methods" were incubated in buffer containing either 200 μM (A, B) or 10 μM (C) [¹⁴C]ascorbic acid and either 0 mM (●), 2.5 mM (▲), 5.0 mM (■), or 10 mM (◆) glucose (pH 7.4) for the times indicated: A, accumulation of total ascorbic acid by the low affinity ascorbic acid transport activity; B, uptake of [¹⁴C]ascorbic acid by the low affinity ascorbic acid transport activity; C, uptake of [¹⁴C]ascorbic acid by the high affinity ascorbic acid transport activity. Uptake of [¹⁴C]ascorbic acid was determined by liquid scintillation spectrometry and total ascorbic acid accumulation was determined by HPLC.

transport activity compared to the much larger concentration of endogenous ascorbic acid in neutrophils, as noted above. Ascorbic acid transport at 10 and 200 μM was also studied in the absence of glucose and at three concentrations between 0 and 1 mM glucose from many different volunteers. At 0, 0.25, 0.5, and 0.75 mM glucose, inhibition of ascorbic acid transport was similar to that at 1 mM glucose. Since glucose concentrations less than 1 mM had no additional effect on inhibition, 1 mM glucose was used as the concentration equivalent to no inhib-

² P. Washko and M. Levine, unpublished observations.

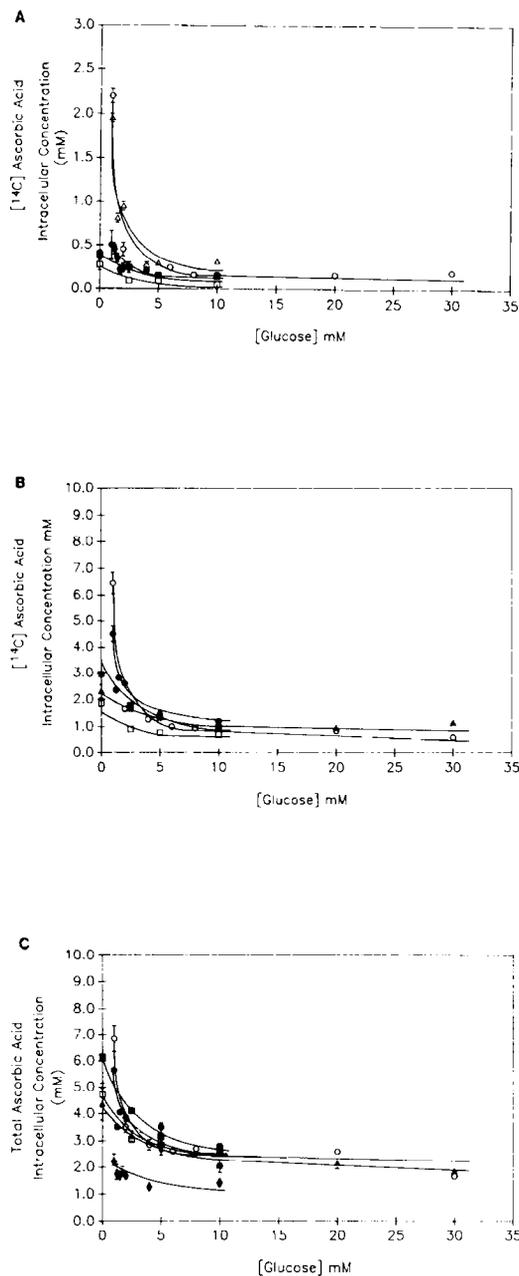


FIG. 2. Effect of glucose concentrations of 0.0–30.0 mM on $[^{14}\text{C}]$ ascorbic acid uptake and total ascorbic acid accumulation in neutrophils from different donors. Plated neutrophils were incubated for 90 min in buffer containing 10 μM (A) or 200 μM (B, C) $[^{14}\text{C}]$ ascorbic acid and the indicated concentrations of glucose at pH 7.4. Each figure includes data from five to six blood donors; each symbol represents a different donor. Data for ascorbic acid uptake were obtained by scintillation spectrometry (A, B); data for total ascorbic acid accumulation were obtained by HPLC (C). To calculate the curves shown, data from each donor were expressed as percent transport inhibited at different concentrations of glucose; dose-effect curves were determined in this way for each donor according to standard pharmacologic principles (21). Each curve represents a dose-effect curve calculated from the data for that patient. A, uptake of $[^{14}\text{C}]$ ascorbic acid by the high affinity ascorbic acid transport activity; B, uptake of $[^{14}\text{C}]$ ascorbic acid by the low affinity ascorbic acid transport activity; C, accumulation of total ascorbic acid by the low affinity ascorbic acid transport activity.

itor in subsequent experiments. Since the hexose monophosphate shunt might be implicated in ascorbic acid transport (22), we included 1 mM glucose in the medium rather than no glucose for kinetic experiments.

Glucose-induced Inhibition Kinetics of Ascorbic Acid Trans-

port: **High Affinity Transport Activity**—Since the uptake of 10 and 200 μM ascorbic acid was linear for at least 120 min at glucose concentrations as high as 10 mM, 90-min time points were suitable for determining concentration-dependent kinetics of the high affinity transport activity. Experiments were performed using $[^{14}\text{C}]$ ascorbic acid, and uptake was measured using liquid scintillation spectroscopy. Neutrophils were incubated with six different concentrations of $[^{14}\text{C}]$ ascorbic acid and five different concentrations of glucose. The concentrations of ascorbic acid used were those which would be transported predominantly by the high affinity transport activity (2). As shown in Fig. 3 glucose inhibition of $[^{14}\text{C}]$ ascorbic acid uptake by the high affinity transport activity was concentration-dependent. Lineweaver-Burk analysis revealed that this apparent inhibition was predominantly noncompetitive, with an apparent K_i of approximately 2.0 mM (inset, Fig. 3). The apparent K_m for ascorbic acid uptake at 5 mM extracellular glucose was 3.6 μM , in agreement with the apparent K_m reported for the high affinity transport activity (2).

Glucose-induced Inhibition Kinetics of Ascorbic Acid Transport: Low Affinity Transport Activity—We determined the linear phase of ascorbic acid accumulation by the low affinity transport activity under conditions permitting maximal transport without toxicity. Based on the results in Figs. 2 and 3, 1 mM extracellular glucose was used. Since the low affinity transport activity saturates at millimolar concentrations of extracellular ascorbic acid (2), 5 and 10 mM ascorbic acid concentrations were selected; ascorbic acid accumulation was measured by HPLC. Ascorbic acid transport was linear for 60 min, and accumulation was ≥ 2 -fold for each concentration (data not shown). Based on these data, concentration-dependent kinetics were determined at 30 min.

Neutrophils were incubated with five different concentrations of glucose and six concentrations of ascorbic acid. Although concentrations of ascorbic acid used were those which would be transported predominantly by the low affinity transport activity (2), the data still had to include transport from the high affinity activity, especially at the ascorbic acid concentration of 200 μM . The contribution of the high affinity activity was determined by calculating high affinity V_{max} at different glucose concentrations (Fig. 3; data not shown for 1.75 mM glucose). The high affinity component was subtracted from all data points in Fig. 4. As seen in Fig. 4A, glucose inhibited ascorbic acid uptake by the low affinity transport activity in a concentration-dependent fashion. The effect of ascorbic acid concentrations from 0.2 to 2.0 mM can be seen

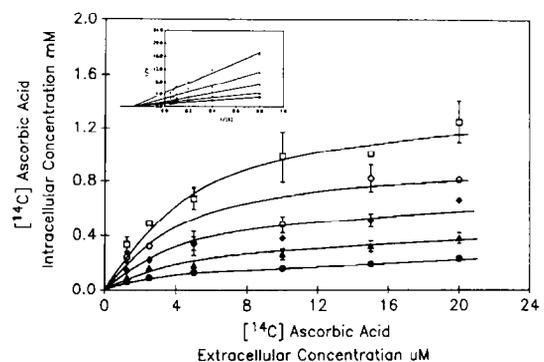


FIG. 3. Kinetics of glucose-induced inhibition of ascorbic acid uptake by the high affinity ascorbic acid transport activity. Plated neutrophils were incubated for 90 min in buffer containing either 10 mM (●), 2 mM (▲), 1.5 mM (◆), 1.25 mM (○), or 1 mM (□) glucose and the concentrations of $[^{14}\text{C}]$ ascorbic acid indicated in the figure (pH 7.4). Uptake of $[^{14}\text{C}]$ ascorbic acid was determined by liquid scintillation spectrometry (inset, Lineweaver-Burk analysis).

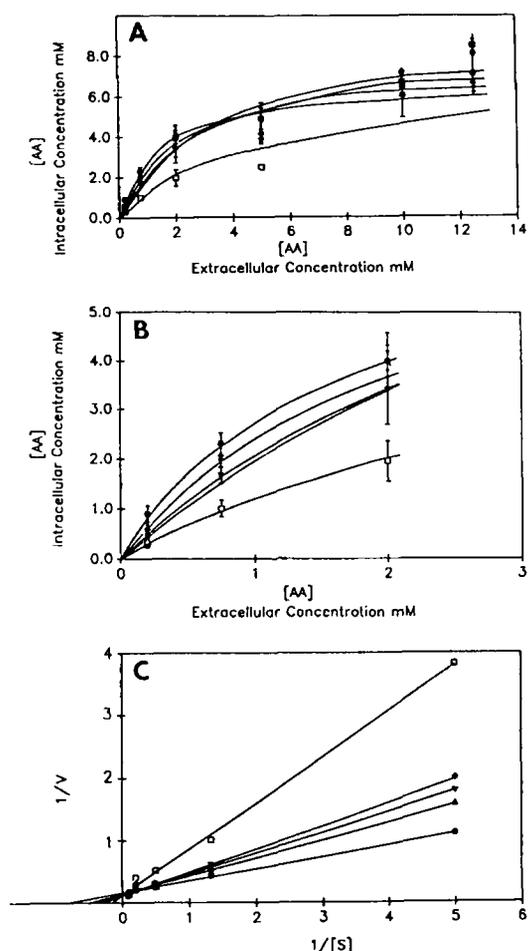


FIG. 4. Kinetics of glucose-induced inhibition of total ascorbic acid accumulation by the low affinity ascorbic acid transport activity. Plated neutrophils were incubated for 30 min in buffer containing either 1 mM (●), 1.25 mM (▲), 1.75 mM (▼), 2 mM (◆), or 10 mM (□) glucose and the concentrations of ascorbic acid indicated in the figure (pH 7.4): A, accumulation of total intracellular ascorbic acid as a function of extracellular ascorbic acid and glucose concentrations; B, detail of the data presented in A between 0 and 2 mM extracellular ascorbic acid; C, Lineweaver-Burk analysis of the data presented in A. Total ascorbic acid accumulation was determined by HPLC.

more clearly in Fig. 4B. Lineweaver-Burk analysis revealed that the apparent inhibition was predominantly competitive (Fig. 4C), with an apparent K_i of approximately 1.8 mM. The apparent K_m for ascorbic acid uptake at 5 mM extracellular glucose was calculated to be 3.8 mM, also in agreement with previous findings (2).

Reversibility of Glucose-induced Inhibition—If the glucose-induced inhibition of ascorbic acid transport by the high and low affinity transport activities was truly noncompetitive and competitive, respectively, both types of inhibition should be reversible upon removal of inhibitor. As shown in Figs. 5A and 5B neutrophils incubated with 200 μ M [14 C]ascorbic acid and 10 mM glucose transported less ascorbic acid than neutrophils incubated with the same concentration of ascorbic acid and 1 mM glucose, as expected. However, if cells initially preincubated for 60 min in 10 mM glucose were then incubated in 1 mM glucose and 200 μ M [14 C]ascorbic acid, uptake of ascorbic acid was equal to the uptake of ascorbic acid by neutrophils incubated in 1 mM glucose without preincubation (Figs. 5A and 5B). Thus, the inhibitory effect of glucose on the low affinity ascorbic acid transport activity was reversible. Similar results were obtained for the high affinity transport

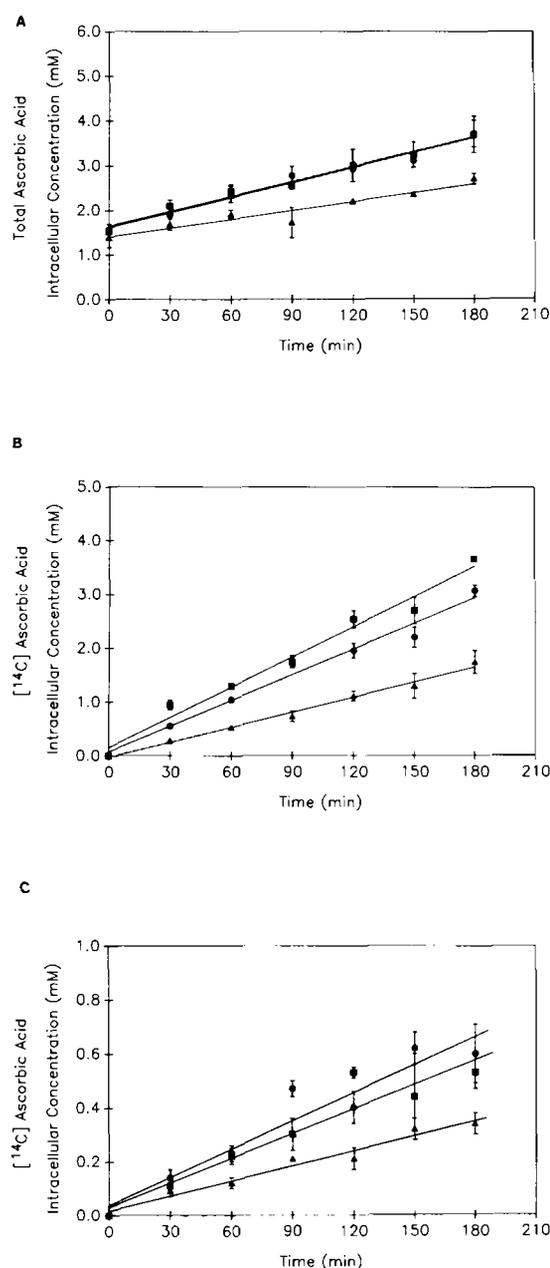


FIG. 5. Reversibility of glucose-induced inhibition of [14 C] ascorbic acid uptake and total ascorbic acid accumulation in neutrophils. In A and B, plated neutrophils were incubated with 200 μ M [14 C]ascorbic acid and either 1 mM (●) or 10 mM (▲) glucose for the indicated times. Neutrophils indicated by (■) were first preincubated with 10 mM glucose for 60 min, washed, and then immediately incubated in 1 mM glucose and 200 μ M [14 C]ascorbic acid for the indicated times. A, accumulation of total ascorbic acid by the low affinity ascorbic acid transport activity as determined by HPLC. B, uptake of [14 C]ascorbic acid by the low affinity ascorbic acid transport activity as determined by liquid scintillation spectrometry. In C, plated neutrophils were incubated with 10 μ M [14 C]ascorbic acid and either 1 mM (●) or 10 mM (▲) glucose for the indicated times. Neutrophils indicated by (■) were first preincubated in 10 mM glucose for 60 min, washed, and then immediately incubated in 1 mM glucose and 10 μ M [14 C]ascorbic acid for the indicated times. Uptake of [14 C] ascorbic acid was determined by liquid scintillation spectrometry.

activity when ascorbic acid transport was measured by liquid scintillation spectroscopy (Fig. 5C).

None of the effects of glucose on ascorbic acid accumulation could be explained by oxidation of extracellular ascorbic acid, since extracellular ascorbic acid concentrations did not

change during these experiments (2). The findings here using scintillation spectroscopy also could not be accounted for by oxidation of [^{14}C]ascorbic acid, since extracellular and intracellular radiolabeled ascorbic acid was approximately 95% pure (23).

The kinetic analyses shown are from individual donors. We confirmed kinetic analyses in different donors (data not shown).

Effect of Glucose Transport Inhibitors on Ascorbic Acid Transport—Since competitive inhibition of glucose transport occurs among structurally related sugars (24, 25), glucose and ascorbic acid might share a common transport system. However, this possibility is unlikely for several reasons. Glucose transport in neutrophils occurs by facilitated diffusion, which is cation-independent (14, 24, 26). By contrast, ascorbic acid is accumulated in neutrophils against a concentration gradient as shown here and elsewhere, and ascorbic acid transport is cation and temperature-dependent (2, 23). To further distinguish whether a common transporter was possible, and since exchange of extracellular and intracellular glucose occurs in neutrophils (25), we studied whether glucose could exchange with endogenous ascorbic acid in neutrophils. When neutrophils containing 1.5 mM ascorbic acid were incubated in buffer containing from 0 to 30 mM glucose for 150 min, there was no change in intracellular ascorbic acid (data not shown). Also, when neutrophils containing 1.5 mM ascorbic acid were incubated in medium without vitamin, there was no loss of intracellular ascorbic acid for as long as 5 h (data not shown). If glucose and ascorbic acid shared a common transporter, intracellular ascorbic acid should have equilibrated with extracellular media, and intracellular ascorbic acid should have exchanged with extracellular glucose. These observations suggest that the mechanism of ascorbic acid transport is different than that for glucose entry.

Nevertheless, due to the structural similarity of ascorbic acid and glucose, and the effects of glucose on ascorbic acid transport, we predicted that some inhibitors of glucose transport would effect ascorbic acid translocation. We investigated the effects of hexoses and pentoses on high affinity and low affinity ascorbic acid transport. For the high affinity activity, neutrophils were incubated with 10 μM ascorbic acid and the hexoses/pentoses shown in Table I. All sugars at 10 mM compared to 1 mM inhibited ascorbic acid accumulation. By contrast, ascorbic acid accumulation at 200 μM was unaffected by any of these sugars except 2-deoxy-D-glucose and, as ex-

TABLE I
Effect of monosaccharides on ascorbic acid transport:
high affinity activity

Isolated, plated neutrophils were incubated for 60 min in buffer containing 10 μM [^{14}C]ascorbic acid and either 1 mM or 10 mM of the monosaccharides listed in the table (pH 7.4). [^{14}C]Ascorbic acid was determined by liquid scintillation spectrometry in conjunction with high-performance liquid chromatography. Values are the mean \pm S.D. of three samples.

Monosaccharide	Ascorbic acid transport	
	1 mM monosaccharide	10 mM monosaccharide
D-Glucose	0.99 \pm 0.17	0.17 \pm 0.01 ^a
L-Glucose	1.13 \pm 0.10	0.32 \pm 0.02 ^b
D-Mannose	0.90 \pm 0.04	0.22 \pm 0.01 ^b
D-Galactose	1.10 \pm 0.03	0.26 \pm 0.03 ^c
D-Xylose	1.21 \pm 0.07	0.31 \pm 0.01 ^b
D-Ribose	1.13 \pm 0.09	0.46 \pm 0.03 ^b
Methyl-D-glucoside	1.17 \pm 0.05	0.37 \pm 0.03 ^c
2-Deoxy-D-glucose	0.78 \pm 0.01	0.09 \pm 0.01 ^c

Significantly different from corresponding 1 mM value: ^a $p < 0.02$; ^b $p < 0.01$; ^c $p < 0.001$.

pected, D-glucose (data not shown). These data indicate the ascorbic acid transport activities can be distinguished by the inhibitory action of monosaccharides. The inhibition of ascorbic acid transport by these compounds differs from their effects on glucose transport (24, 27, 28), again suggesting that ascorbic acid and glucose are transported by separate mechanisms in neutrophils.

We studied the effects of glucose transport inhibitors on high affinity and low affinity ascorbic acid transport. Neutrophils were incubated with 10 μM [^{14}C]ascorbic acid and the inhibitors in Table II. The data indicate that most inhibited ascorbic acid transport. Cytochalasin B was a more effective inhibitor than other cytochalasins, similar to the effects of cytochalasins on glucose transport (29). Neutrophils were also incubated with 200 μM ascorbic acid and inhibitors. To determine whether any inhibitors were toxic, ascorbic acid accumulation by HPLC was measured. As shown in Table III, ascorbic acid accumulation was inhibited in a similar pattern compared to the high affinity transport activity. Ascorbic acid accumulation was prevented but endogenous ascorbic acid was maintained in the presence of cytochalasin B or phloretin, suggesting that these inhibitors were not toxic to neutrophils (see Table III).

By its action on glucose metabolism, fluoride inhibits glucose transport (30). If ascorbic acid and glucose shared the same carrier, we predicted that fluoride would either inhibit or have no effect on ascorbic acid transport. For the low and high affinity transport activities, the data show that fluoride stimulates, suggesting again that the mechanism of ascorbic acid entry is different than that of glucose.

DISCUSSION

We report here that both ascorbic acid transport activities in human neutrophils are reversibly inhibited by glucose, but by different mechanisms. The high affinity transport activity was inhibited noncompetitively, while the low affinity transport activity was inhibited competitively. In both cases there was marked decrease in the uptake and accumulation of

TABLE II

Effect of inhibitors on ascorbic acid uptake: high affinity activity

Isolated, plated neutrophils were incubated for 120 min in buffer (pH 7.4) containing 10 μM [^{14}C]ascorbic acid and the following concentrations of inhibitors: cytochalasins B, H, J, phloretin, and phlorizin 100 μM in 1% ethanol (final concentration); cytochalasin D 100 μM in 1% Me_2SO (final concentration); forskolin 10 μM in 1% ethanol (final concentration); potassium fluoride 10 mM in buffer alone. The control value was for neutrophils incubated in buffer containing 1% Me_2SO . Similar values were obtained for neutrophils incubated in 1% ethanol or buffer alone (data not shown). [^{14}C]Ascorbic acid uptake was determined by liquid scintillation spectrometry in conjunction with high-performance liquid chromatography. Values shown are the mean \pm S.D. of three samples.

Ascorbic acid transport	
mM	
Control	0.37 \pm 0.04
Cytochalasin	
B	0.09 \pm 0.01 ^c
D	0.24 \pm 0.01 ^a
H	0.24 \pm 0.01 ^a
J	0.22 \pm 0.02 ^c
Phloretin	0.01 \pm 0.00 ^c
Phlorizin	0.31 \pm 0.03 ^b
Forskolin	0.16 \pm 0.01 ^c
Potassium fluoride	1.51 \pm 0.17 ^c

^a Significantly different from control value: ^a $p < 0.05$; ^b $p < 0.02$; ^c $p < 0.01$.

TABLE III

Effect of inhibitors on ascorbic acid accumulation: low affinity activity

Isolated, plated neutrophils were incubated in buffer (pH 7.4) containing 200 μM ascorbic acid and the following concentrations of inhibitors: cytochalasin B, phloretin, and phlorizin 100 μM in 1% ethanol (final concentration); forskolin 10 μM in 1% ethanol (final concentration); potassium fluoride 10 mM in buffer alone. Control was for neutrophils incubated in 1% Me_2SO . Similar results were obtained for neutrophils incubated in 1% ethanol or buffer alone (data not shown). Total ascorbic acid accumulation was determined by high-performance liquid chromatography. Values are the mean \pm S.D. of three samples. T_0 indicates initial ascorbic acid concentration and T_{120} indicates ascorbic acid concentration after incubation for 120 min.

	Intracellular ascorbic acid concentration	
	T_0	T_{120}
	<i>mM</i>	
Control	1.75 \pm 0.11	2.61 \pm 0.05
Cytochalasin B	1.91 \pm 0.15	1.56 \pm 0.17 ^b
Phloretin	1.81 \pm 0.05	1.78 \pm 0.03 ^c
Phlorizin	1.77 \pm 0.06	2.81 \pm 0.09
Forskolin		2.12 \pm 0.10 ^a
Potassium fluoride	1.79 \pm 0.01	4.93 \pm 0.12 ^c

Significantly different from T_{120} control value: ^a $p < 0.05$; ^b $p < 0.02$; ^c $p < 0.01$. T_0 values for cytochalasin B and phloretin were not significantly different from the corresponding T_{120} values.

ascorbic acid in neutrophils. Inhibition of ascorbic acid transport by glucose did not require glucose preincubation, suggesting that glucose is acting on the ascorbic acid transport activities directly. The apparent K_m calculated for each transport activity at 5 mM glucose was similar to our previously reported values (2), confirming our observations about the kinetic properties of the two transport activities. Although the magnitude of the glucose effect varied in neutrophils from different donors, glucose inhibited ascorbic acid accumulation in all neutrophils.

To test whether glucose affected each transport activity, we initially chose external ascorbic acid concentrations of 10 and 200 μM . At 10 μM , 90% of transport is mediated by the high affinity activity (2). At 200 μM , the high affinity component is saturated, and transport occurs by both activities. The contribution of the low affinity activity to total accumulation is 70–80% (2).² This conclusion is also justified from data in this paper. In Figs. 2A and 2B transport of 10 and 200 μM ascorbic acid at 5 mM glucose can be compared. As noted in the figure legend, a distinct symbol was used for each volunteer. At 5 mM glucose, neutrophils from three volunteers were studied at both 10 and 200 μM ascorbic acid. Neutrophils from another volunteer were studied at 4 and 6 mM glucose, using 10 and 200 μM ascorbic acid. The concentration of 10 μM is above K_m for the high affinity activity (2; Fig. 3), so that V_{max} of high affinity activity is less than 2-fold transport at 10 μM . For simplicity, high affinity V_{max} can be overestimated by calculating it as $2 \times$ transport at 10 μM . For each volunteer this number can be compared to measured transport at 200 μM ascorbic acid, and the contribution of high affinity transport to transport at 200 μM is obtained. At 5 mM glucose the contribution of high affinity activity is 24, 22, and 29%; at 4 and 6 mM glucose the contribution is 39 and 48%, respectively. To reiterate, these calculations overestimate the contribution of high affinity transport to transport at 200 μM , since high affinity V_{max} is actually less than $2 \times$ transport at 10 μM . Evidence that 10 μM is above K_m for the high affinity activity is seen from Fig. 3, in which K_m was 3.6 μM , in agreement with other measurements (2). In summary, the data indicate

that at 200 μM ascorbic acid, 70–80% of transport occurs by the low affinity activity.

Likewise, the contribution of the high affinity transport activity to transport at 200 μM ascorbic acid for different glucose concentrations other than 5 mM can be determined by comparing Figs. 2A and 2B. For each volunteer, the data show that at different glucose concentrations the contribution of high affinity transport to transport at 200 μM ascorbic acid is similar, in the range of 20–30%. In summary, the data in Figs. 1 and 2 for 200 μM ascorbic acid transport include both the low and high affinity activities. Nevertheless, when the contribution of the high affinity activity is accounted for, there is still a definitive effect of glucose on ascorbic acid transport by the low affinity activity.

From the data shown, we also calculated how the high affinity activity contributed to low affinity transport with different glucose concentrations. From Fig. 3, V_{max} of the high affinity transport activity at each glucose concentration was determined. Each V_{max} was compared to transport at ascorbic acid concentrations from 200 μM to 12 mM (see Fig. 4). At 200 μM external ascorbic acid, high affinity transport contributed 20–30% of ascorbic acid accumulation at the different glucose concentrations. At higher ascorbic acid concentrations, the contribution by the high affinity activity was minimal.

Nevertheless, all data shown in Fig. 4 for low affinity activity have the high affinity transport component subtracted.

Neutrophils from some volunteers were more active than in others in accumulation of ascorbic acid and inhibition by glucose. This variation in ascorbic acid transport is not surprising and is consistent with differences in ascorbic acid absorption in normal humans. In volunteers on metabolic wards given the same amount of ascorbic acid, plasma concentrations vary as much as 4-fold (31, 32). This variation is due in part to differences in ascorbic acid intestinal transport, which would be expected to differ among volunteers due to genetic variations in the transporter activities. Such variations are consistent with normal variation in physiologic processes.

Despite the variation in ascorbic acid transport when different individuals are compared, for each volunteer there was always inhibition of ascorbic acid transport by glucose. Inhibition induced by glucose was at least 3-fold, and for some volunteers was 9-fold. This can be seen in Fig. 2A for the high affinity activity by comparing intracellular [¹⁴C]ascorbic acid concentration as a function of external glucose for each volunteer. Similar inhibition occurs when the external [¹⁴C] ascorbic acid concentration is 200 μM , in Fig. 2B. The inhibition induced by glucose when total intracellular ascorbic acid is measured by HPLC (Fig. 2C) is again similar. For this inhibition to be apparent, endogenous ascorbic acid at zero time must be subtracted. Total intracellular ascorbic acid includes endogenous ascorbic acid, which was present when the neutrophils were isolated. This concentration varied for the different volunteers from 1.0 to 1.6 mM ascorbic acid. When endogenous ascorbic acid from each volunteer was subtracted, glucose inhibited ascorbic acid accumulation at 4–9-fold.

Despite the variations in ascorbic acid transport in different volunteers exposed to the same external ascorbic acid concentration, the K_m s for ascorbic acid transport by the high and low affinity ascorbic acid transport activities reported here (3.6 μM and 3.8 mM) are very similar to the values found in other volunteers (2). Given this similarity, variations in ascorbic acid transport in different volunteers could be due to

amount of transporter protein present. To learn more about the variation of ascorbic acid transport in different volunteers, it will be necessary to isolate the ascorbic acid transport protein(s).

It is possible that glucose analogs may provide a means to isolate the ascorbic acid transporter(s). These tools are needed since there are few available ascorbic acid analogs. The experiments with monosaccharides and inhibitors show that compounds structurally similar to glucose are useful in characterizing the two ascorbic acid transport activities. On the basis of the data here, ascorbic acid analogs are also being synthesized as new tools for characterizing ascorbic acid transport. By studying the effects of glucose and structurally related compounds, we hope to obtain agents to isolate the transport protein(s).

Because it is an inhibitor of glucose metabolism and transport, the effects of fluoride on ascorbic acid transport were investigated. The stimulation of ascorbic acid accumulation by fluoride may be due to its other actions on the respiratory burst in neutrophils (33, 34). Consistent with this possibility, the effects of fluoride were concentration-dependent and were independent of Na^+ or K^+ as the cation (data not shown). Thus, experiments investigating ascorbic acid accumulation in activated neutrophils are also in progress.

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