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Transport mechanisms for vitamin C in the JAR human placental choriocarcinoma cell line

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Abstract

We investigated the transport pathways available for the uptake of vitamin C in the human placental choriocarcinoma cell line, JAR. These cells were found to possess the capacity to accumulate the vitamin when presented either in the oxidized form (dehydroascorbic acid) or in the reduced form (ascorbate). Dithiothreitol and 5,5′-dithiobis(2-nitrobenzoic acid) were used to maintain vitamin C as ascorbate and dehydroascorbic acid, respectively. The uptake of these two forms of vitamin C in JAR cells was found to occur by different mechanisms. The uptake of the dehydroascorbic acid was Na^+-independent and was mediated by facilitative glucose transporters as evidenced from the inhibition of the uptake process by glucose. On the other hand, the uptake of ascorbate was Na^+-dependent and was not sensitive to inhibition by glucose. Substitution of Na^+ with other monovalent cations abolished the uptake of ascorbate completely. The uptake process was, however, not influenced by anions. Kinetic analysis indicated the presence of a single saturable transport system for ascorbate with a Michaelis–Menten constant of $22 \pm 1\ \mu M$. The dependence of the uptake rate of ascorbate on Na^+ concentration exhibited sigmoidal kinetics, suggesting interaction of more than one Na^+ ion with the transporter. The Hill coefficient for the Na^+ interaction was 2, indicating that the Na^+-dependent ascorbate transport is electrogenic. The Na^+-dependent stimulation of ascorbate uptake was primarily due to an increase in the affinity of the transporter for ascorbate in the presence of Na^+. It is concluded that the JAR placental trophoblast cell line expresses two different transport systems for vitamin C: one for the reduced form of the vitamin ascorbate; and the other for the oxidized form of the vitamin dehydroascorbic acid. © 1998 Elsevier Science B.V.

Keywords: Vitamin transport; Ascorbate; Dehydroascorbic acid; JAR choriocarcinoma cell; (Human placenta)

1. Introduction

Vitamin C, one of the water-soluble vitamins, is an important cellular nutrient. It exists in two physiological forms, as L-ascorbic acid (AA), the reversibly reduced form which is anionic at physiological pH, and dehydro-L-ascorbic acid (DHAA), the reversibly oxidized form which is non-ionizable. Here we use vitamin C as a general term to refer to both forms. The major circulating form of the vitamin in blood is AA. The concentration of DHAA in plasma is about $2\ \mu M$, which is very low compared to the concentration of AA ($\sim 60\ \mu M$) [1]. In its reduced form, this

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vitamin is involved in a variety of metabolic reactions, where it serves as a powerful antioxidant and as a cofactor in the synthesis of extracellular matrix proteins [2] and in the modulation of the biosynthesis [3,4], release [5] and clearance [6] of neurotransmitters. It is highly essential for the normal growth and proper bone formation of the fetus. Decreased levels of this vitamin in the mother's plasma has been shown to be the contributory factor in premature separation of the placenta, premature birth and puerperal fever [7]. Though some animals (e.g. rat and rabbit) can synthesize AA from glucose, humans are incapable of endogenous biosynthesis of this vitamin. Therefore, in humans the fetal supply is solely dependent upon proper transfer of this micronutrient across the placenta from maternal to fetal circulation [8–10]. The concentration of AA in fetal circulation is several-fold higher when compared to that in the maternal circulation [8,9,11], indicating that the placenta has a very effective mechanism for active transport of this vitamin from mother to fetus.

The mechanism of transport of vitamin C has been extensively studied in several cell types. In the intestinal mucosal cells [12,13], osteoblasts [14], cerebral astrocytes [15], and alveolar macrophages [16], the concentrative uptake of vitamin C has been shown to occur in the form of AA by a sodium-dependent process. In certain other cell types such as erythrocytes, fibroblasts, and neutrophils, vitamin C entry involves the uptake of DHAA by sodium-independent facilitative glucose transporters and its rapid reduction to AA intracellularly [17]. There have been a few reports on the mechanism of vitamin C transport in the human placenta. Streeter and Rosso [18] have studied the uptake of AA in placental explants and found that, while at very high concentrations the vitamin entry is by simple diffusion, the uptake at lower concentrations is carrier-mediated and is dependent on the presence of sodium ions. Ingermann et al. [19] have suggested that the reversibly oxidized DHAA is the predominant form of vitamin C that enters the syncytiotrophoblast. Their inference was based on their studies of vitamin C uptake into brush border membrane vesicles prepared from the placenta. The uptake of AA into these vesicles was much slower compared to that of DHAA and the uptake of DHAA was inhibitable by 3-O-methyl-D-glucose. More recently, Rybakowski et al. [20], based on perfusion studies in near-term human placentas, have reported that vitamin C enters the trophoblast predominantly as DHAA and that its transfer through the microvillous and basal membranes occurs via facilitative glucose transporters.

In the studies reported here, we investigated the mechanism of vitamin C entry into the trophoblasts using JAR choriocarcinoma cells as the model system. Over the years, this cell line has proven to be a very useful and convenient model for the study of various vitamin transport systems in the human placenta [21–24]. In the present study, the uptake of radioactive vitamin C was measured under conditions in which the vitamin existed either in the oxidized (DHAA) or in the reduced form (AA). Under oxidizing conditions, the uptake of vitamin C was sodium-independent and was inhibited by D-glucose. In contrast, under reducing conditions, the uptake was sodium-dependent, electrogenic and not inhibitable by D-glucose. In light of these findings, we conclude that both AA and DHAA are transported into the JAR cells and that different transport pathways are responsible for the uptake of these two forms of vitamin C. Due to the presence of several-fold higher concentrations of AA compared to DHAA in the maternal blood, we speculate that the sodium-dependent uptake process available for AA is the predominant pathway for the entry of vitamin C into the syncytiotrophoblast from maternal circulation.

2. Materials and methods

2.1. Materials

L-[carboxyl-^{14}C] Ascorbic acid (specific radioactivity 16.7 mCi/mmol) was obtained from Amersham. Ascorbate, dithiothreitol (DTT), and 5,5′-dithio bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma. RPMI-1640 medium was obtained from Mediatech. Penicillin and streptomycin were obtained from Life Technologies. All other chemicals were of analytical grade.

2.2. Cell culture

The JAR human placental choriocarcinoma cell line was purchased from American Type Culture Collection (Rockville, MD) and routinely grown in RPMI-1640 medium, supplemented with 10% fetal

bovine serum, 100 units of penicillin and 100 μg/ml of streptomycin. Confluent cultures were trypsinized and the released cells were seeded in 35 mm petri dishes at a density of 1.5×10^6 cells/dish. A day after seeding, the medium was replaced with fresh medium and the cells were used for uptake measurements the following day.

2.3. Measurement of vitamin C uptake

Uptake measurements were carried out at room temperature. The cell monolayer was washed once with 1.5 ml of the uptake buffer and the uptake measurement was initiated by the addition of 1 ml of uptake buffer containing the [^{14}C] labeled vitamin C. The incubation was continued for a desired length of time following which the uptake was terminated by aspirating the uptake medium and, subsequently, washing the cells thrice with 1.5 ml of ice-cold uptake buffer. The cells were then solubilized with 1 ml of 1% SDS/0.2 N NaOH and transferred to scintillation vials for the determination of the radioactivity associated with the cells. The uptake buffer was prepared fresh each time, the composition of which, in most experiments, was 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl or 140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 mM glucose. In addition, depending on whether the measurement of AA uptake or DHAA uptake was intended, either 1 mM DTT or 1 mM DTNB, respectively, was also included in the buffer. When the effect of D-glucose on the uptake of AA and DHAA was measured, the concentration of NaCl/choline chloride was reduced to 120 mM. In experiments dealing with the effect of various monovalent ions on AA uptake, NaCl was isoosmotically substituted with different salts. When the influence of anions on AA uptake was studied, KCl and $CaCl_2$ were substituted by potassium gluconate and calcium gluconate, respectively, and sodium salts of different anions added. In experiments dealing with the influence of Na^+ on AA uptake, the buffers containing 140 mM NaCl and 140 mM *N*-methyl-D-glucamine chloride were mixed to give uptake buffers of desired Na^+ concentration.

2.4. Protein determination

In each experiment, cells cultured under identical conditions were used to determine the protein content. The medium was removed and the cells were lysed in 1 ml water. The lysate was passed through a 25-gauge needle several times to obtain a homogenate. The protein content of the homogenate was determined by the Lowry method.

2.5. Data analysis

Experiments were made in triplicate and repeated twice. The results are given as means ± S.E. Kinetic analyses were done by non-linear regression and confirmed by linear regression using a commercially available computer program Fig. P, Version 6.0 (Biosoft, Cambridge, UK).

3. Results

3.1. Influence of DTT and DTNB on vitamin C uptake in JAR choriocarcinoma cells

The uptake of vitamin C was first measured in the presence of DTT or DTNB in buffers with, and without Na^+. The results of the experiment are presented in Table 1. In control experiments done in the absence of the modifying agents, significant uptake of the vitamin was seen both, in the presence and absence of Na^+. However, the uptake measured in the presence of Na^+ was two-fold higher, compared

Table 1
Influence of DTT and DTNB on vitamin C uptake in JAR choriocarcinoma cells [a]

	Vitamin C uptake (fmol/mg protein per 30 min)	
	− NaCl	+ NaCl
Control	64.3 ± 0.8	127.0 ± 0.8
DTT	3.0 ± 0.1	104.6 ± 4.8
DTNB	421.8 ± 3.1	362.0 ± 7.4

[a] Uptake of [^{14}C]vitamin C (final concentration, 7.5 nM, 0.13 μCi/dish) was measured in the absence (− NaCl) or presence (+ NaCl) of Na^+. In Na^+-free medium, NaCl was isoosmotically substituted with choline chloride. Cells were washed thrice with 1.5 ml of either Na^+-containing buffer or Na^+-free buffer, prior to initiation of uptake. DTT (1 mM) or DTNB (1 mM) was added along with the radio-labeled vitamin C as indicated. Uptake was measured for 30 min at room temperature. Values are means ± S.E.

to that measured in its absence. Interestingly, when DTT was included in the uptake buffer, there was a drastic decrease in the uptake measured in the absence of Na^+ with practically no change in the uptake observed in the presence of Na^+. The values measured in the presence of Na^+ were about thirty-fold higher, compared to that measured in its absence. In contrast, when DTNB was included in the uptake buffer, increased uptake was seen both, in the absence and presence of Na^+. The values measured were approximately seven-fold higher in the absence of Na^+ and three-fold higher in the presence of Na^+ compared to the control values measured in the absence of the modifying agents. More importantly, the uptake values were comparable in the presence, and in the absence of Na^+ when measured in the presence of DTNB.

In the presence of oxygen at room temperature, AA is rapidly oxidized to DHAA. Addition of DTT not only prevents this oxidation but also reduces the oxidized DHAA back to AA. DTNB, on the other hand, oxidizes AA to DHAA. Thus, vitamin C exists predominantly as AA in the presence of DTT, as DHAA in the presence of DTNB, and a mixture of both in the absence of any modifying agent. Thus, the characteristics of vitamin C uptake observed in the presence of DTT represent the characteristics of AA uptake. Similarly, the characteristics of vitamin C uptake observed in the presence of DTNB represent the characteristics of DHAA uptake. The uptake of AA into JAR cells was almost entirely dependent on the presence of Na^+. The uptake of DHAA into the cell, on the other hand, was independent of Na^+. We then studied the uptake of AA in presence of Na^+ and the uptake of DHAA in the absence of Na^+ at various concentrations of DTT and DTNB, respectively. As can be seen in Fig. 1. the uptake of AA which is higher in the absence of DTT drops down significantly in the presence of 0.5 mM DTT and stays relatively constant at higher concentrations of DTT (Fig. 1(A)). The uptake of DHAA, on the other hand, keeps increasing with increasing concentrations of DTNB (Fig. 1(B)). The higher uptake of vitamin C seen in the absence of DTT may be due to the uptake of the vitamin as DHAA since both, DHAA and AA are present under these conditions. This is supported by the finding that, in the absence of DTNB, the Na^+-independent uptake of the vitamin is considerably high (55 ± 2.5 fmol/mg protein per 30 min). This clearly demonstrates that the observed difference in the uptake of the vitamin under these two conditions was due to the oxido-reduction status of the vitamin. This was confirmed by measuring the uptake of radio-labeled vitamin C in the presence or absence of DTT in cells pretreated for 30 min with DTT as well as in the presence or absence of DTNB in cells pretreated for 30 min with DTNB. The uptakes measured in the absence of DTT and DTNB in cells pretreated with DTT and DTNB, respectively,

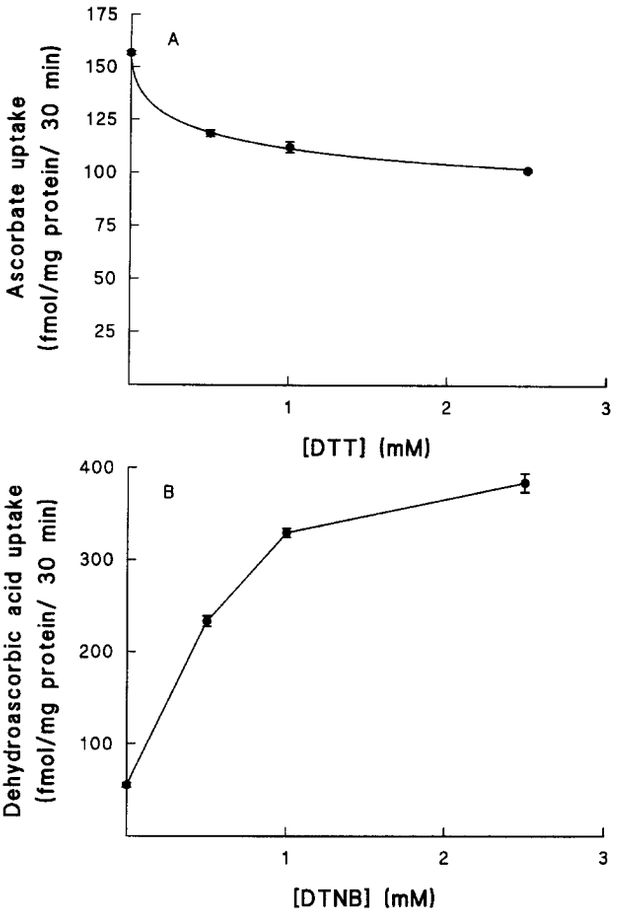

Fig. 1. Dose response of DTT and DTNB on the uptake of (A) ascorbate and (B) dehydroascorbic acid, respectively. The uptake of AA was measured in the presence of NaCl and various concentrations of DTT. The uptake of DHAA was measured in the absence of NaCl and various concentrations of DTNB. Concentration of [^{14}C]vitamin C was 7.5 nM (0.13 μCi/dish). Incubation at room temperature for 30 min. Data given are means ± S.E.

were very close to the values obtained in control cells which were not treated with either of the modifying agents (data not shown). These results indicate that DTT and DTNB do not directly act on the cells to produce the observed effects on the uptake of the vitamin.

Thus, JAR cells can take up vitamin C both as DHAA and as AA. Whereas the uptake as DHAA is sodium-independent, the uptake of the reversibly reduced AA is absolutely dependent on the presence of Na^+ in the extracellular medium. In addition, the uptake as DHAA was three- to four-fold higher than the uptake as AA (351 ± 6 fmol/mg protein per 30 min vs. 118 ± 3 fmol/mg protein per 30 min) when measured in the presence of Na^+.

3.2. Time course of vitamin C uptake into JAR cells in the presence of DTT or DTNB

Time course of the uptake of 7.5 nM ^{14}C-labeled vitamin C into JAR cells was first measured in the presence of DTT in choline chloride or sodium chloride-containing buffer. There was no significant uptake observed in choline chloride buffer. However, rapid uptake of the radiolabel was observed in Na^+-containing medium and the uptake was linear for at least 45 min (Fig. 2(A)). It was clearly evident that the presence of Na^+ in the extracellular medium markedly stimulated AA uptake. The uptake measured in sodium chloride-containing buffer was appoximately twenty-seven-fold greater than that measured in choline chloride-containing buffer at 30 min incubation. In contrast, when the experiment was done in the presence of DTNB instead of DTT, rapid accumulation of vitamin C was observed in the presence as well as in the absence of Na^+ (Fig. 2(B)). The level of intracellular accumulation of the vitamin was similar in the presence and in the absence of extracellular Na^+, at all time points studied. This uptake rate was three to four-fold higher than the Na^+-dependent uptake rate of AA measured in the presence of DTT. These results confirm that JAR cells accumulate vitamin C as AA by a sodium-dependent transport system and as DHAA by a sodium-independent system, and that the uptake of the vitamin as DHAA is three to four times more rapid compared to the uptake as AA.

3.3. Effect of D-glucose on the uptake of vitamin C in the presence of DTT or DTNB

The influence of D-glucose on the uptake of the vitamin as AA or DHAA was examined by measuring the uptake in the presence of either DTT (AA uptake) or DTNB (DHAA uptake) and in the presence of increasing concentrations of glucose. The osmolality of the uptake buffer was maintained con-

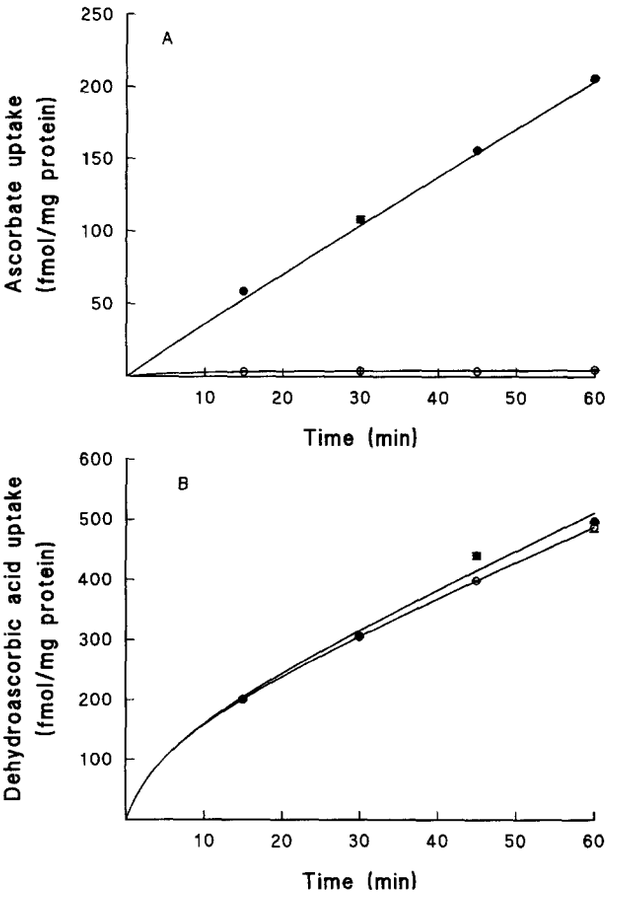

Fig. 2. Time course of uptake for (A) ascorbate and (B) dehydroascorbic acid in JAR cells. (A) DTT (1 mM) was added to the uptake buffer to maintain vitamin C in the reduced ascorbate form, and (B) DTNB (1 mM) was added to the uptake buffer to maintain vitamin C in the oxidized dehydroascorbic acid form. The composition of the uptake buffer was 25 mM Hepes/Tris (pH 7.5), 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and either 140 mM NaCl or 140 mM choline chloride. Uptake was measured in both cases in the (○) absence or (●) presence of Na^+. Concentration of [^{14}C]vitamin C was 7.5 nM (0.13 μCi/dish). Values are means ± S.E.

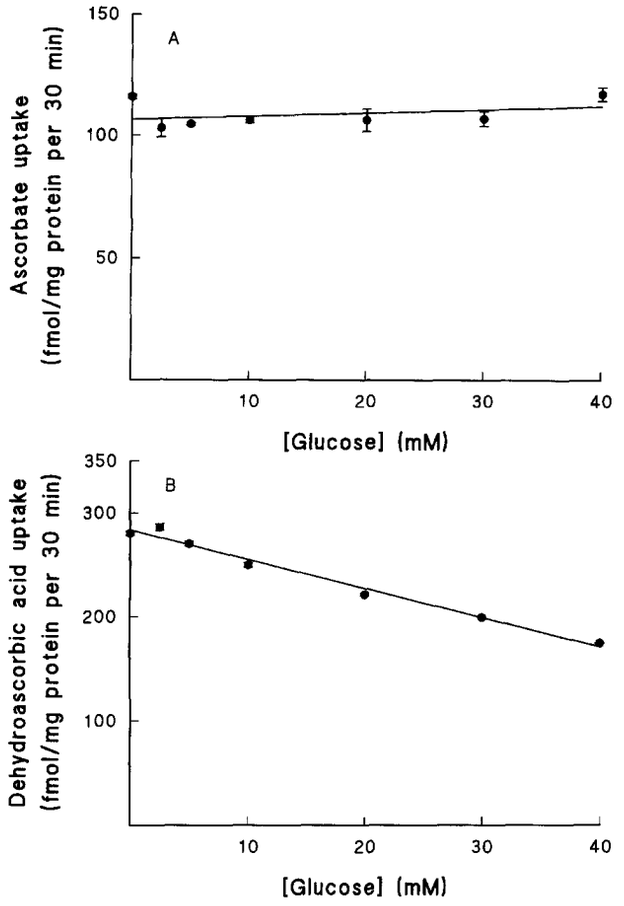

Fig. 3. Effect of D-glucose on the uptake of (A) ascorbate and (B) dehydroascorbic acid in JAR cells. (A) DTT (1 mM) was added to the uptake buffer to maintain vitamin C as ascorbate, and (B) DTNB (1 mM) was added to the uptake buffer to maintain vitamin C as dehydroascorbic acid. Uptake of ascorbate was measured in the presence of NaCl, whereas uptake of dehydroascorbic acid was measured in the absence of NaCl. Concentration of [^{14}C]vitamin C was 5 nM (0.08 μCi/dish). Concentration of D-glucose in the uptake buffer was varied between 0 and 40 mM. Osmolality of the buffer was maintained by adding appropriate concentrations of mannitol. Values are means ± S.E.

stant by adding appropriate amounts of mannitol. The uptake as AA was measured in the presence of 120 mM NaCl, and the uptake as DHAA was measured in the presence of 120 mM choline chloride. The results are presented in Fig. 3. D-glucose failed to inhibit the uptake of AA even at the highest concentration used (40 mM) (Fig. 3(A)). In contrast, the uptake of DHAA was significantly inhibited by glucose (Fig. 3(B)). At a concentration of 40 mM, glucose caused 40% inhibition of DHAA uptake.

These results clearly demonstrate that the uptake of vitamin C in JAR cells takes place by two independent pathways. The uptake of AA occurs by an Na^+-dependent ascorbate transporter which is insensitive to D-glucose. The uptake of DHAA, on the other hand, occurs by a Na^+-independent transport system which is inhibitable by glucose.

3.4. Influence of monovalent cations and anions on the uptake of AA

The role of different monovalent cations and anions in the transport of vitamin C as AA was then studied at two different concentrations of ascorbate (7.5 nM and 10 μM) (Table 2). DTT (1 mM) was added to the uptake medium to maintain vitamin C as AA. The influence of cations on the uptake was measured in the presence of various Cl^- salts containing different cations. The uptake of AA was drastically reduced when Na^+ was replaced with Li^+, K^+, or choline, regardless of the concentration of AA used. That the uptake was negligible in the absence of Na^+ shows that Na^+ is obligatory for the transport process at both concentrations of AA used. The influence of anions on the uptake was measured in

Table 2
Ionic dependence of ascorbate uptake in JAR cells[a]

	Ascorbate uptake	
	7.5 nM (fmol/mg protein per 30 min)	10 μM (pmol/mg protein per 30 min)
NaCl (control)	198.1 ± 0.4 (100)	142.6 ± 8.6 (100)
NaF	167.5 ± 4.3 (85)	141.6 ± 2.4 (99)
Na gluconate	172.3 ± 2.6 (87)	108.8 ± 2.0 (76)
LiCl	3.6 ± 0.1 (2)	5.8 ± 0.2 (4)
KCl	3.9 ± 0.1 (2)	12.6 ± 1.2 (9)
Choline Cl	2.8 ± 0.3 (1)	7.4 ± 1.0 (5)

[a] Confluent cells were incubated with 7.5 nM [^{14}C]ascorbate (0.13 μCi/dish) in the absence or presence of 10 μM ascorbate for 30 min either in the control buffer (25 mM Hepes/Tris (pH 7.5), 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM glucose and 140 mM NaCl) or in buffer in which NaCl was replaced with 140 mM of various inorganic salts. When influence of anions was studied, KCl and $CaCl_2$ in the buffer were replaced with potassium gluconate and calcium gluconate, respectively. DTT (1 mM) was added to the uptake buffer to keep ascorbate in reduced form. After incubation for 30 min, the cells were washed with the respective buffer (ice-cold) and the radioactivity associated with the cells was quantified. Values are means ± S.E.

the presence of Na^+ salts containing different anions. Substitution of Cl^- with F^- or gluconate decreased the uptake only slightly. Thus, Cl^- is not mandatory for the uptake of AA by the Na^+-dependent ascorbate transporter.

3.5. Kinetics of AA uptake by the Na^+-dependent ascorbate transporter in JAR cells

The kinetics of AA uptake in the presence of 1 mM DTT and NaCl was measured over a 2.5–50 μM concentration range. The incubation time was 30 min. The concentration of [^{14}C]AA was kept constant at 7.5 nM. The non-specific diffusional component was determined by the uptake observed in the presence of 5 mM unlabeled AA. This was subtracted from the total transport to calculate the carrier-mediated component. The uptake rate of the carrier-mediated component was found to be hyperbolically related to AA concentration, indicating saturability of the uptake process (Fig. 4). Kinetic parameters of the uptake process were then calculated by non-linear regression analysis and confirmed by linear regression. The values for the Michaelis–Menten constant (K_t) and maximal velocity (V_{max}) were found to be 22.4 ± 0.8 μM and 483 ± 7 pmol/mg protein per 30 min, respectively.

3.6. Influence of Na^+ on AA uptake by the Na^+-dependent ascorbate transporter

Since the transport of AA exhibits an obligatory dependence on Na^+, we investigated the influence of Na^+ on the kinetics of AA transport. Since this involves varying the concentration of Na^+ in the uptake buffer by isoosmotic replacement by *N*-methyl-D-glucamine, we first tested the effect of *N*-methyl-D-glucamine chloride on AA uptake. This was done by measuring the uptake of AA in the presence of 90 mM NaCl and, either 50 mM of the chloride salt of either lithium, choline or *N*-methyl-D-glucamine or 100 mM mannitol. As seen in Table 3, the isoosmotic replacement of *N*-methyl-D-glucamine chloride with either LiCl, choline chloride, or mannitol did not significantly alter the uptake of AA. This proves that *N*-methyl-D-glucamine has no effect on AA uptake and, hence, was used to substitute for Na^+ to investigate the influence of Na^+ on AA transport. To

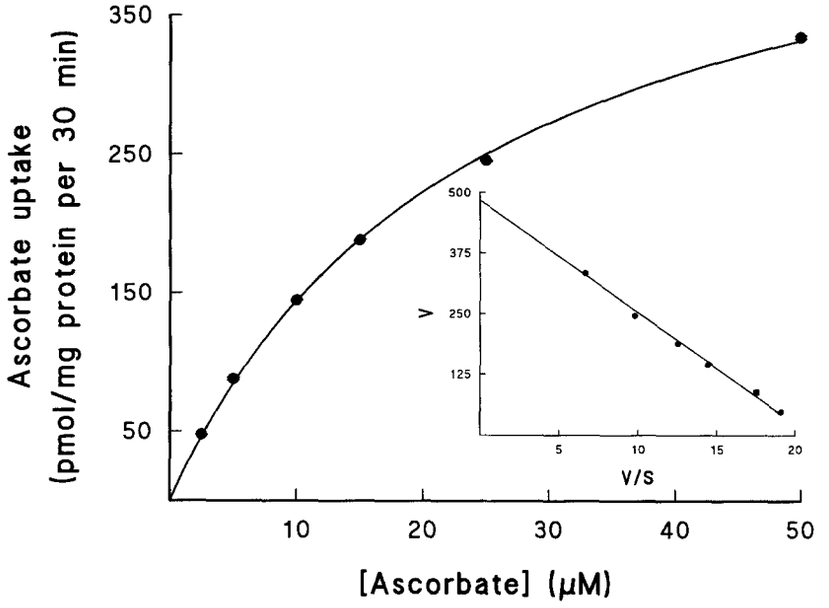

Fig. 4. Kinetics of ascorbate uptake in JAR cells. Uptake of ascorbate was measured in JAR cells with a 30 min incubation in the presence of NaCl. DTT (1 mM) was added to the uptake medium to maintain vitamin C as ascorbate. Concentration of ascorbate was varied from 2.5 μM to 50 μM. The concentration of [^{14}C]ascorbate was kept constant at 7.5 nM (0.13 μCi/dish). The amount of radio-label uptake in the presence of excess (5 mM) unlabeled ascorbate was used to calculate the diffusional component. Data given are means ± S.E. and represent only the saturable component. Inset: Eadie–Hofstee transformation of the same data.

Table 3
Influence of *N*-methyl-D-glucamine chloride on AA uptake[a]

	Ascorbate uptake (fmol/mg protein per 30 min)
N-methyl-D-glucamine chloride	109.21 ± 1.23
Lithium chloride	97.38 ± 3.04
Choline chloride	112.06 ± 1.06
Mannitol	116.62 ± 2.74

[a] Uptake of 7.5 nM [^{14}C]ascorbate (0.13 μCi/dish) was studied in confluent monolayers of JAR cells in the presence of 90 mM NaCl and 50 mM of the indicated inorganic salt or 100 mM of mannitol in the extracellular medium. The uptake buffer was supplemented with 1 mM DTT to maintain the ascorbate in the reduced state. Following 30 min incubation at room temperature, the cells were washed thrice with ice-cold uptake buffer and the radioactivity associated with the cells quantified. Results are means ± S.E.

analyze kinetically the dependence of AA uptake on the concentration of Na^+, uptake of AA was measured at increasing concentrations of Na^+ in the uptake buffer (range, 0–140 mM). The measurement was made in the presence of DTT to keep the vitamin in the reduced state as AA. The relationship between Na^+-dependent AA uptake (i.e. uptake in the presence of Na^+ minus the uptake in the absence of Na^+) and Na^+ concentration was found to be sigmoidal, suggesting an Na^+/AA coupling ratio of more than one (Fig. 5). To calculate the number of Na^+ ions interacting with the carrier, the data were analyzed using the Hill-type equation

$$v = \frac{V_m[Na^+]^n}{K_{0.5}^n + [Na^+]^n}$$

where v is Na^+-dependent AA uptake, V_m the AA uptake at infinite concentration of Na^+, $K_{0.5}$ the concentration of Na^+ necessary to cause AA uptake equal to 50% of V_m, and n the number of Na^+ ions interacting with the carrier per transport cycle. This analysis gave a $K_{0.5}$ value of 45 ± 2 mM, a V_m value of 189 ± 4 fmol/mg protein per 30 min and an n value of 2.1 ± 0.1. The value for n was confirmed from the slope of the Hill plot (2.0 ± 0.04) (Fig. 5, inset). An n value of 2.0 indicates that, for every AA molecule transported, two molecules of Na^+ ions are cotransported into the cell. At physiological pH, AA is a carboxylate anion with a single negative charge. Since the AA : Na^+ stoichiometry is 1 : 2, AA uptake

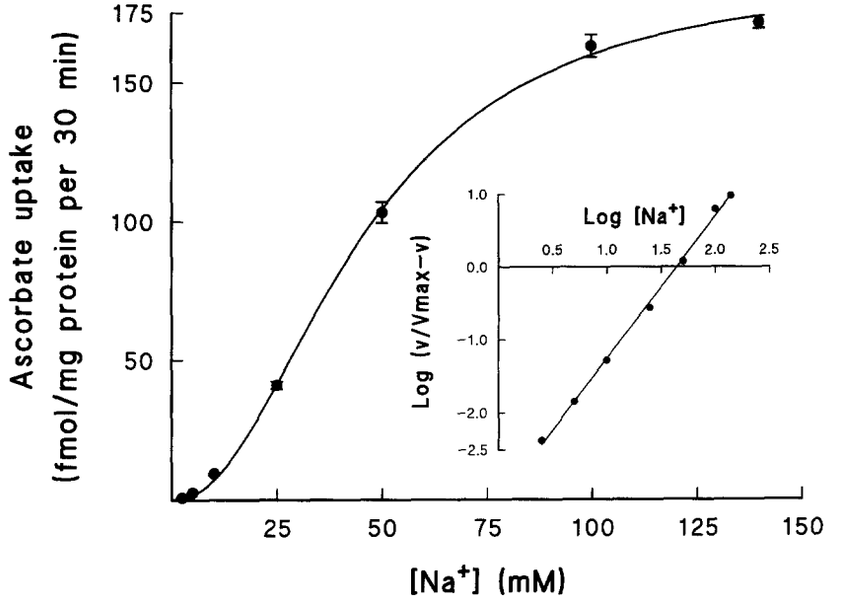

Fig. 5. Effect of Na^+ on the uptake of ascorbate in JAR cells. Ascorbate uptake was studied in JAR cells with a 30 min incubation in the presence of 1 mM DTT and increasing concentrations of Na^+ (0–140 mM). The concentration of Cl^- was kept constant at 140 mM. The osmolality of the medium was kept constant by replacing Na^+ with appropriate concentrations of *N*-methyl-D-glucamine. Inset: Hill plot of the same data. Values are means ± S.E.

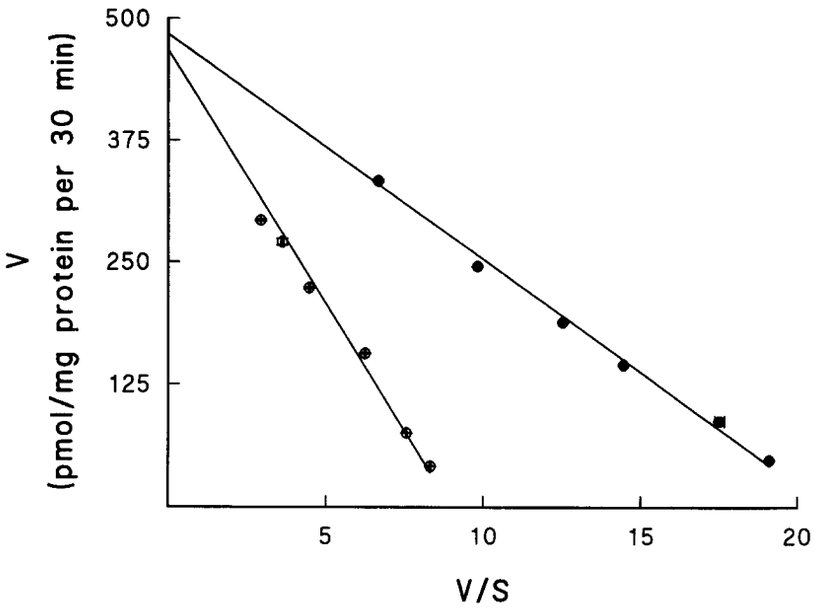

Fig. 6. Effect of Na^+ on the saturation kinetics of ascorbate uptake. Uptake of ascorbate was measured in JAR cells with a 30 min incubation in the presence of 1 mM DTT and either (○) 40 mM or (●) 140 mM Na^+. *N*-methyl-D-glucamine was used to adjust osmolality. The concentration of ascorbate was varied, in the 5–100 μM range, in presence of 40 mM Na^+ and in the 2.5–50 μM range, in the presence of 140 mM Na^+. The concentration of the labeled ascorbate was kept constant at 7.5 nM (0.13 μCi/dish). The amount of radiolabel taken up in the presence of 5 mM unlabeled ascorbate was used to calculate the diffusional component. Results are given as Eadie–Hofstee plots for the saturable component. Values are means ± S.E.

is expected to result in the transfer of a net positive charge across the membrane. Thus, the Na^+-dependent AA uptake should be electrogenic and potential-sensitive.

3.7. Influence of Na^+ on the kinetics of AA uptake

Since the uptake of AA is an Na^+-dependent process, it was of interest to determine the influence of Na^+ on the kinetic parameters of AA uptake. The uptake was measured in the presence of DTT at two different concentrations of NaCl, 40 mM and 140 mM (Fig. 6). The osmolality was maintained constant using *N*-methyl-D-glucamine chloride. The concentration of AA was varied from 5 μM to 100 μM in the case of 40 mM NaCl, and from 2.5 μM to 50 μM in the case of 140 mM NaCl. The concentration of labeled AA was kept constant at 7.5 nM in all cases. The non-specific diffusional component was determined by measuring the uptake in the presence of 5 mM AA. The incubation was carried out for 30 min at room temperature and the data obtained were analyzed by both non-linear and linear regression methods. At a Na^+ concentration of 140 mM, K_t for AA uptake was 22 ± 1 μM and V_{max} was 484 ± 7 pmol/mg protein per 30 min. When the concentration of Na^+ was lowered to 40 mM, the value for K_t increased more than two-fold to 47 ± 2 μM with no significant change in V_{max} (468 ± 13 pmol/mg protein per 30 min). These results show that Na^+ activates AA uptake primarily by enhancing the affinity of the transporter for the substrate.

4. Discussion

The results of the present investigation show that the JAR cells take up vitamin C as both, AA and DHAA by two different mechanisms. Similar conclusion has been drawn to explain vitamin C uptake in neutrophils [25]. The uptake of DHAA is sodium-independent, and inhibitable by glucose. In contrast, the uptake of AA was sodium-dependent, electrogenic and was not inhibited by glucose. Such an electrogenic Na^+-ascorbate cotransport has been de-

scribed in cultured bovine pigmented ciliary epithelial cells [26] and in rat astroglia [27]. There are several reports favoring the hypothesis that the principal pathway of vitamin C uptake into the placenta is as DHAA [19,20]. The transported DHAA is reduced back to AA inside the cell, providing a mechanism of its concentrative accumulation inside the cell. Various tissues of the body, including the placenta, are thought to contain an enzyme which can reduce the transported DHAA back to AA [28]. A glutathione-dependent dehydroascorbic acid reducing activity has recently been purified and cloned from human neutrophils [29]. The protein, glutaredoxin, is a 11 kDa protein with a K_m for dehydroascorbic acid of 250 μM. The uptake of DHAA has been shown to be catalyzed by the sodium-independent facilitative glucose transporters. Of the several known isoforms of sodium-independent glucose transporters, GLUT1, GLUT2 and GLUT4 have been shown to be efficient transporters of DHAA [30]. The inhibition of the sodium-independent DHAA uptake by glucose observed in JAR cells suggests that the facilitative glucose transporters may be involved in this transport process. Of the four known isoforms, placenta and JAR cells have been shown to abundantly express GLUT1 and GLUT3 [31–33]. Supporting this mechanism of vitamin C entry is the report by Norkus et al. [10] wherein perfusion studies with guinea pig placenta simulating maternal hyperglycemia showed inhibition of ascorbic acid uptake. Though similar studies have not been done in human placenta, Ingermann et al. [34], studying the effect of glucose on DHAA uptake in isolated placental microvillous membrane vesicles, have suggested that severe hyperglycemia in maternal blood decreases the transplacental transfer of this vitamin. Though unusual, this facilitative transport system has a higher transport rate in comparison with its active counterpart and can build up the intracellular concentration of vitamin C significantly. This is probably because of the very high levels of expression of the facilitative glucose transporters on the cell membrane coupled to the effective intracellular reduction of the transported DHAA to AA. However, the major drawback for this mechanism to operate in vivo is the low concentration of DHAA in maternal blood which is only ca. 2 μM [1]. Since the affinity of GLUTs for DHAA is in the millimolar range in the absence of any competing monosaccharide [34], the importance of this mechanism in placental vitamin C uptake remains questionable. For this mechanism to operate under in vivo conditions, AA has to be oxidized to DHAA at an extracellular site before being transported into the syncytiotrophoblast. Though activated human neutrophils have been shown to do so [35], no such activity has been reported in the placenta. In addition, DHAA has been shown to be toxic to several cells, including the placental trophoblast cells [36].

The second pathway of vitamin C uptake is by the sodium-dependent ascorbate transporter. The substrate for this transporter is the reversibly reduced AA. Though previous studies have suggested the presence of this transport pathway in the placenta [18,20], no detailed kinetic analysis of the Na^+-dependent AA transport in trophoblasts has been done to date. Our studies in JAR cells have shown that the Na^+/ascorbate cotransporter is of a high affinity type with a K_t of 22 μM for ascorbate. The transport process is electrogenic and obligatorily dependent on Na^+. This suggests that the driving force for the transporter is the transmembrane electrochemical Na^+ gradient. Similar sodium-dependent ascorbate transport systems with comparable affinity for AA have been described in osteoblasts [14,37], neutrophils [35], and several other cell types [12,13,26,27]. Recently, a sodium-dependent ascorbate transport activity was expressed in oocytes using mRNA from rabbit kidney [38].

In conclusion, the data presented clearly demonstrate that vitamin C is accumulated in JAR cells both as DHAA and AA by separate mechanisms. We speculate that similar mechanisms are operative in the human placenta. Since the concentration of AA in the blood is at least an order of magnitude higher than that of DHAA (60 μM vs. 2 μM), the uptake of ascorbate into the syncytiotrophoblast via the Na^+/ascorbate cotransporter is most likely to be the major pathway responsible for the entry of vitamin C from the maternal circulation into the placenta.

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