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Characterization of the ascorbic acid transport by 3T6 fibroblasts

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Ascorbic acid transport by 3T6 mouse skin fibroblasts has been characterized using radiometric technique with L-[1-^{14}C]ascorbic acid under the conditions in which oxidation of ascorbic acid was prevented by addition of 1 mM thiourea. The ascorbate transport is temperature-dependent with the energy of activation E and Q_{10} of 13.3 kcal/mol and 2.0, respectively. The transport requires energy and exhibits Michaelis-Menten kinetics with an apparent K_m of 112 μM and V_{max} of 158 pmol/min per mg protein, when the extracellular Na^+ concentration is 150 mM. The ascorbate transport requires presence of extracellular Na^+ and can be inhibited by ouabain treatment. At 40 and 200 μM ascorbate concentrations, respectively, 1.4 and 1.0 moles of Na^+ bound the transporter molecule per each mole of ascorbate transported. Increased Na^+ binding to the transporter at lower ascorbate concentration may signify multiple Na^+-binding sites or ascorbate concentration dependent conformational changes in the transporter molecule. Increasing Na^+ concentration decreases K_m without affecting V_{max}, suggesting that Na^+ increases affinity of ascorbate for the transporter molecule without affecting translocation process. An increase in ascorbate concentration reduces the number of Na^+ bound to the transporter from 1.4 to 1.0. The ascorbate transport is stimulated by Ca^{2+} and other divalent cations. The mechanism of stimulation by Ca^{2+} is not clear. Calcium increases both the K_m and V_{max}. The data presented support the hypothesis that the ascorbate transport by 3T6 fibroblasts is an energy and temperature-dependent active process driven by the Na^+ electrochemical gradient. A potent inhibitor of ascorbate transport is also demonstrated in human serum.

Introduction

Ascorbic acid (vitamin C) is a dietary requirement for man, primates, guinea pigs and flying mammals since they have lost the capacity to synthesize it. Ascorbic acid from diet is absorbed into the circulation and is then transported into cells which utilize it. Similarly, mammals who synthesize ascorbic acid also need to have an ascorbate transport system to satisfy intracellular requirement of ascorbic acid for all tissues except liver, since ascorbic acid is synthesized in the liver of these animals. Indeed, ascorbic acid transport has been demonstrated in a variety of cells.

Although not much is known about the biochemical functions of ascorbic acid, its diverse roles are apparent in a multitude of seemingly unrelated pathological symptoms found in scurvy. Perhaps the clearly understood functions of

Abbreviations: DMEM, Dulbecco-Vogt modification of Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSS, balanced salt solution; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid.

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ascorbic acid are in the hydroxylation of dopamine to norepinephrine [1–3] and in the hydroxylation of proline and lysine residues of nascent collagen [4–7]. Since fibroblasts synthesize collagen, we were interested in studying ascorbic acid transport by fibroblasts.

Ascorbic acid and dehydroascorbic acid are two interconvertible forms of vitamin C and are transported into cells by two different mechanisms (for example, see Ref. 8). Although dehydroascorbic acid is preferentially taken up by neutrophils, erythrocytes and lymphocytes [9,10], ascorbic acid is the prefered form of the vitamin transported by many tissues [11–13]. Ascorbate transport into adrenomedullary chromaffin cells and several other tissues is shown to be by a process which is Na^+-sensitive and energy-dependent [11–15].

While studying ascorbic acid transport by fibroblasts, we observed that bovine serum contains heatlabile factor(s) which when activated by endotoxin, inhibits ascorbic acid transport [16]. In order to study the mechanism of this inhibition by serum factor(s), we undertook detail characterization of the ascorbic acid transport by fibroblasts.

Materials and Methods

Materials

Mouse embryo 3T6 fibroblast cells (ATCC No. CCL 96) were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Dulbecco-Vogt modification of Eagle's medium (DMEM) (GIBCO No. 320-1885), fetal bovine serum (GIBCO No. 200-6140), glutamine, gentamicin, NUNC tissue culture flasks with a surface area of 25 cm^2 (GIBCO No. 163371) were purchased from GIBCO Laboratories, Grand Island, NY, U.S.A. L-[1-^{14}C]Ascorbic acid (specific activity 17 mCi/mmol) was obtained from the Amersham Corporation. Thiourea, ouabain, ascorbic acid, Hepes, and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were purchased from Sigma Chemical Co. Aquasol was obtained from New England Nuclear. All other chemicals were of analytical grade.

Methods

Mouse embryo 3T6 fibroblast cells were grown in NUNC tissue culture flasks with a surface area of 25 cm^2 in DMEM supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine and 100 μg per ml of gentamicin. Cells were grown at 37 °C in an atmosphere of 7.5% CO_2. The growth medium was changed every 48 h until the cells reached near confluency when the flasks were used for experiments. The other details were described elsewhere [17].

Ascorbic acid uptake. Cells near confluency were washed three times with 5 ml of balanced salt solution (BSS; containing 150 mM NaCl, 4.2 mM KCl, 1.0 mM NaH_2PO_4, 11.2 mM D-glucose, 0.7 mM $MgCl_2$, 2.0 mM $CaCl_2$, and 10 mM Hepes, adjusted to pH 7.2 with NaOH). Thiourea (1 mM) was added to the BSS since it prevents oxidation of ascorbic acid. Ascorbic acid (30 μM) was degraded with $t_{0.5}$ of 30 min in phosphate-buffered saline. By adding 1 mM thiourea it was found that no detectable ascorbic acid was degraded in 5 h [18]. The cells were incubated in BSS for 15 min before transport was initiated by addition of L-[1-^{14}C]ascorbic acid. The transport was carried out at 37 °C for 20 min and terminated by removing the radioactive BSS and washing the cell-sheet four times with 5 ml of ice-cold BSS. The washed cell-sheet was digested overnight in 2.5 ml of 0.4 M NaOH. After neutralizing with HCl, 2 ml of the dissolved cells were placed in 10 ml of aquasol in glass scintillation vials and the radioactivity was determined by liquid scintillation spectrometry using a Beckman 150 liquid scintillation counter. An aliquot of the dissolved cells was used for protein determination by the method of Bradford [19]. Intracellular volume was calculated assuming that the cells have a density of 1.2 and the cell-protein constitutes 15% of the wet weight of the cells. An average of triplicate determinations are reported here with each value differing from the average by less than $\pm$ 5%. Results of each experiment reported here have been independently confirmed at least two more times.

Results and Discussion

The liver of most mammals, except humans, guinea pigs and primates, has retained the capacity to synthesize ascorbic acid. Therefore, ascorbic acid level in the cultivated cells, which do not synthesize it, drops steadily in the first few days

[15]. 3T6 fibroblasts which were used in this study and obtained from the American Type Culture Collection, are an established cell-line. Therefore, it is presumed that 3T6 fibroblasts cultivated in the absence of exogenous ascorbic acid would not have any ascorbic acid content of their own. In such cells we studied characteristics of the ascorbic acid transport in vitro by using L-[1-^{14}C]ascorbic acid with a radiometric technique. The transport studies were done in BSS containing 1 mM thiourea used for stabilizing ascorbic acid. Although in our studies, uptake of radioactive ascorbate was followed for a maximum of 30 min, L-[1-^{14}C]ascorbic acid under these conditions was completely stable for at least 5 h [18].

In addition, the radioactivity from the cells, which had just transported ascorbic acid, was extracted in metaphosphoric acid and analyzed by thin-layer chromatography as described earlier [18]. The results indicated that more than 95% of the radioactivity comigrated with ascorbic acid [16], suggesting that it is the transport of ascorbic acid and not its degradation product which is being studied under our experimental conditions. The time course for L-[1-^{14}C]ascorbic acid transport into 3T6 fibroblasts was studied. The ascorbate transport was linear for 30 min when the ascorbate concentration was 2, 20 or 100 μM (data not shown). In subsequent studies the initial uptake between 0 and 20 min of incubation was used to determine the initial transport velocity. 3T6 fibroblasts concentrated ascorbic acid against the concentration gradient. At an ascorbate concentration of 2.4 μM for 30 min, a 25-fold higher concentration of ascorbate was found within the cells as compared to the extracellular fluid. At higher ascorbate concentrations, the ratio of intracellular to extracellular ascorbic acid was relatively lower despite the higher rate of ascorbate transport. For example, at 200 μM ascorbate concentration, the intracellular to extracellular ratio of ascorbate was about three.

The ascorbate transport by 3T6 fibroblasts exhibited saturation kinetics suggesting a carrier mediated process (Fig. 1). The transport process follows a Michaelis-Menten relationship with an apparent K_m of 112 μM and V_{max} of 158 pmol/min per mg protein (Fig. 1) when the normal BSS was used containing 150 mM Na^+. The K_m for

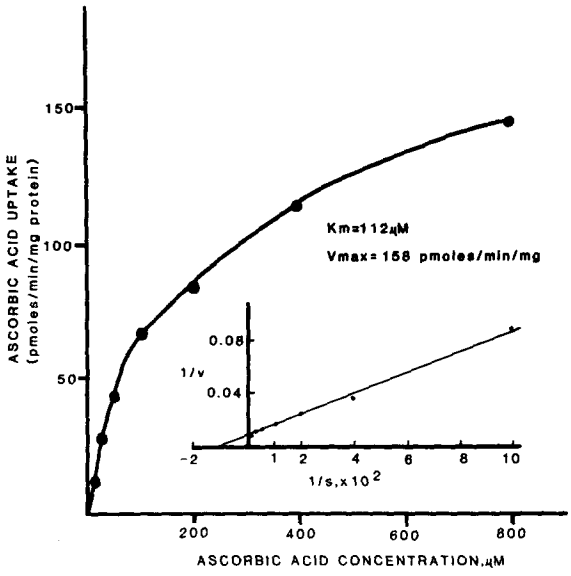

Fig. 1. Ascorbate transport as a function of ascorbate concentration. When the grown cells were near confluency, they were rinsed three times with BSS and incubated for 10 min in BSS containing 1 mM thiourea. The varied concentrations of ascorbate along with radioactive ascorbate, were added to different flasks and the transport was terminated after 20 min. Other experimental details are given in Materials and Methods. The inset is a Lineweaver-Burk plot of the reciprocal of the rate of ascorbate transport as a function of the reciprocal of ascorbic acid concentration.

ascorbate transport by 3T6 fibroblasts is higher than the K_m of 16.6 μM and 29 μM observed for the adrenal cortical [20] and chromaffin cells [15], respectively. The ascorbate transport by 3T6 fibroblasts is a temperature dependent process (Fig. 2). The Arrhenius plot follows a straight line showing the energy of activation (E) of 13.3 kcal/mol. Between 20 °C and 40 °C a Q_{10} value of 2 is observed indicating doubling of the rate of transport when the temperature is increased by 10 °C. Moser and Weber [21] found E of 25 kcal/mol for ascorbate transport in the granulocytes. Diliberto et al. [15] observed two values of E, 3.5 and 22 kcal/mol, for adrenal chromaffin cells. To examine if the energy production is required for ascorbate transport, the cells were pretreated with 2,4-dinitrophenol or iodoacetate for 30 min at 37 °C. The rate of ascorbate transport by the treated cells was at 30% of the control. The results indicate that the continuous generation of energy by aerobic metabolism is required to meet the energy expenditure for the ascorbate transport

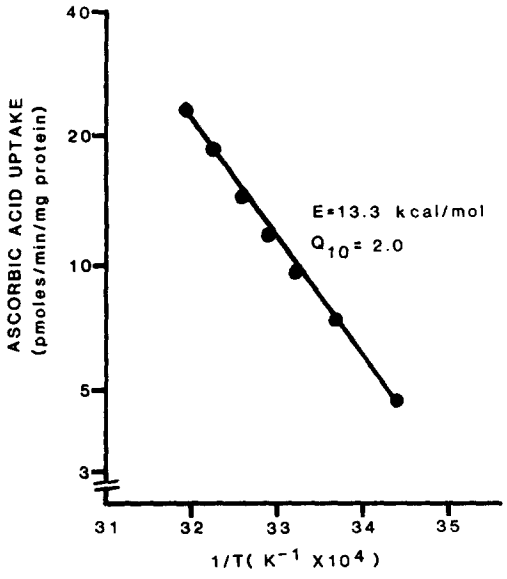

Fig. 2. Arrhenius plot of the effect of temperature on ascorbate transport. Cells when near confluency were rinsed three times with BSS and incubated in BSS containing 1 mM thiourea for 10 min at the respective temperatures. Radioactive ascorbic acid was then added at 2.75 μM (spec. act. 7.6 mCi/mmol) and incubated for 20 min at the respective temperatures. Other experimental details were as given in Materials and Methods. $(-E/2.3\ R) = (\log v_1 - \log v_2)/(T_1 - T_2)$ is the slope of the line on Arrhenius plot where E is the energy of activation, v_1 and v_2 are velocities at the absolute temperatures T_1 and T_2, respectively, and R is the gas constant. Q_{10} is v at T/v at $(T - 10)$.

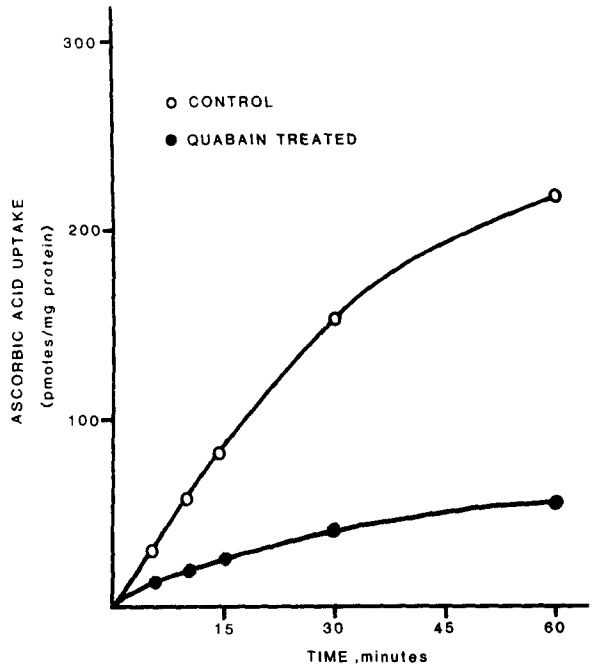

Fig. 3. Effect of ouabain treatment on ascorbate transport. Cells when near confluency were rinsed with BSS and incubated further for 2 h with BSS (control) or with BSS containing 100 μM of ouabain. Radioactivity ascorbate was then added at 2.75 μM (spec. act. 7.6 mCi/mmol) and ascorbate transport was carried out for indicated time. Other details are as given in Materials and Methods.

process. The observation was confirmed using carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone in which case the cells treated with 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone for 10 min at 37°C showed 65% inhibition. The results indicate that the ascorbate transport by 3T6 fibroblasts is an energy- and temperature-dependent process.

It has been shown that ascorbate transport by ileal mucosa [12,22] and chromaffin cells [15] is inhibited by ouabain, a natural glycoside. Since ouabain is an inhibitor of $(Na^+ + K^+)$-ATPase, it is possible that the resultant elimination of Na^+-gradient is responsible for the inhibited ascorbate transport. We examined the effects of ouabain and extracellular Na^+ on the ascorbate transport. The grown cells were incubated for 2 h in BSS either with or without ouabain, after which ascorbate transport was studied as shown in Fig. 3. The ouabain-treated cells showed nearly 70% inhibition in the rate of ascorbate transport as compared to the controls. To examine if the ascorbate transport is dependent on extracellular Na^+ concentration, the cells were exposed to BSS containing various concentrations of Na^+ (Li^+ was added to make total $Na^+ + Li^+$ concentration of 150 mM) for 10 min, after which the rate of ascorbate transport was studied. As shown in Fig. 4, the rate of ascorbate transport was dependent on the extracellular Na^+ concentration at both high (200 μM) and low (40 μM) ascorbate concentrations. The results were confirmed in the experiment in which isomolar sucrose was used instead of Li^+ to make isotonic BSS. These results show that extracellular Na^+ is required for the ascorbate transport by 3T6 fibroblasts and that ouabain inhibits ascorbate transport probably by inhibiting $(Na^+ + K^+)$-ATPase and thereby eliminating Na^+ gradient. The data from Fig. 4 when plotted according to the Hill's equation, indicated the n_H, the number of Na^+ molecules that bind to the

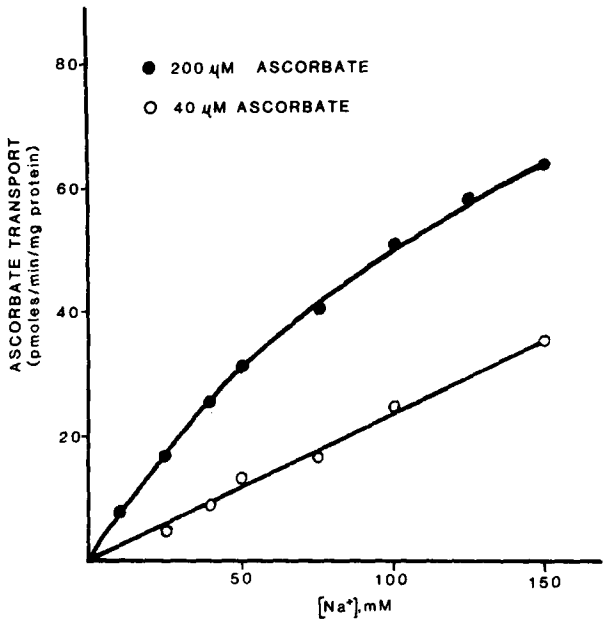

Fig. 4. Effect of extracellular Na^+ concentration on ascorbate transport. Experimental details are given in Materials and Methods except that BSS had varied concentration of Na^+. Li^+ was added so that total ($Na^+ + Li^+$) was 150 mM. Radioactive ascorbic acid (spec. act. 0.2 mCi/mmol) was added at either 40 or 200 μM concentration and ascorbate transport was terminated after 20 min as described in Materials and Methods. The uptake in the absence of Na^+ was about 10–15% of the maximum and has been subtracted.

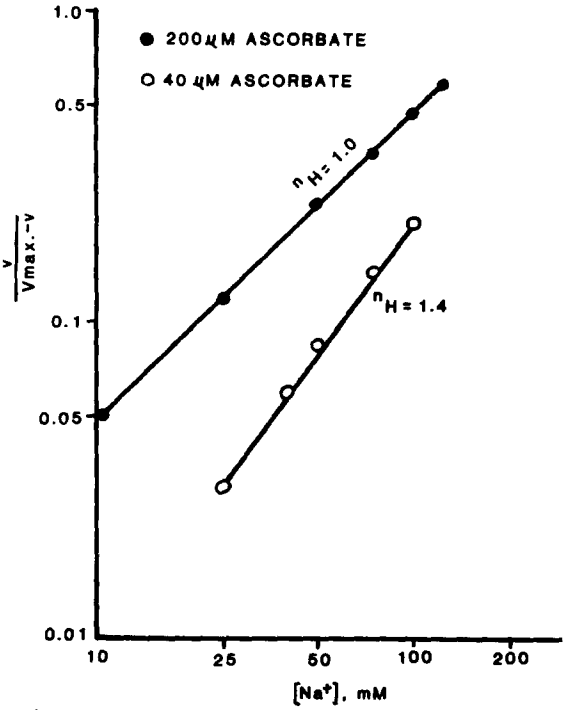

Fig. 5. Transport of ascorbic acid as a function of external Na^+ concentration: Hill's plot. The data from Fig. 4 were replotted according to the Hill's equation. Slope of the line, n_H approximates the number of Na^+ that bind to the carrier for each mole of ascorbate transported.

transporter for each molecule of ascorbate transported (Fig. 5). The values for each n_H obtained from the slopes of the curves suggest that at relatively lower ascorbate concentration (40 μM) more Na^+ molecules bind per molecule of ascorbate transported. The numbers of Na^+ molecules bound per molecule of ascorbate transported are 1.0 and 1.4 at 200 μM and 40 μM ascorbate concentrations, respectively. This suggests that at lower ascorbate concentrations, binding of Na^+ is enhanced. The concentration of Na^+ required to produce one-half the maximal rate of uptake, $[Na^+]_{0.5}$, is obtained from the abscissa for each curve corresponding to the ordinate value of 1.0. The values for $[Na^+]_{0.5}$ obtained from Fig. 5 are 200 mM and 300 mM Na^+ at 200 μM and 40 μM ascorbate concentration, respectively. The effect of extracellular Na^+ on K_m and V_{max} of the ascorbate transport was also examined. The ascorbate transport was carried out at various ascorbate concentrations when the extracellular Na^+ concentration was either 20, 60 or 150 mM in the BSS. The results as shown in Fig. 6 clearly suggest that the extracellular Na^+ has profound effect on the K_m of the transport and has no effect on V_{max}. Increasing the extracellular Na^+ from 20 mM to 150 mM reduces the K_m from 365 to 129 μM. In other words, Na^+ has a significant effect on the affinity between ascorbate and the transporter. Increasing Na^+ concentration increases the affinity between ascorbate and the putative carrier molecule but has no effect on the translocation process.

The ascorbate transport system also requires a divalent cation, in addition to its requirement for monovalent Na^+. As shown in Fig. 7, the rate of ascorbate transport increases as a function of Ca^{2+} concentration. In the absence of Ca^{2+}, the rate of ascorbate transport was about 15% of the maximum. The concentration of Ca^{2+} required for one-half the maximum stimulation of ascorbate transport is approx. 25 μM. The precise function

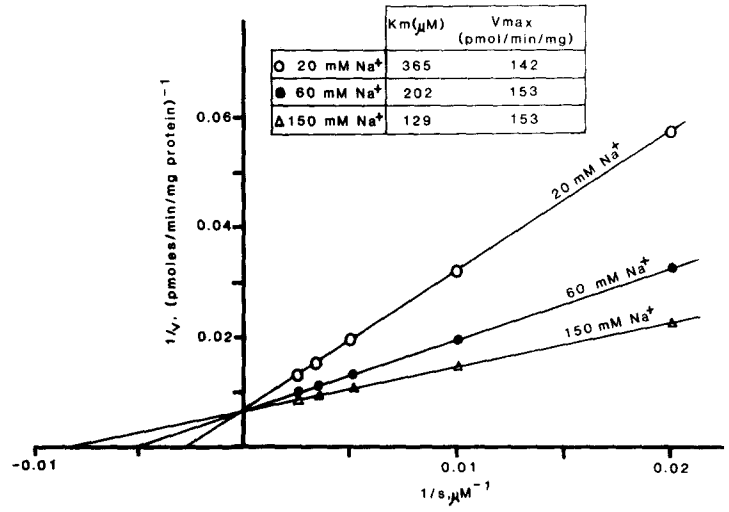

Fig. 6. Effect of external Na^+ concentration on kinetics of ascorbate transport. Details are as in Fig. 1 except that Na^+ concentration in BSS was either 20, 60 or 150 mM and that Li^+ was added to make total ($Na^+ + Li^+$) concentration of 150 mM. The data presented are in the form of Lineweaver-Burk plot of the reciprocal of v, the rate of ascorbate transport, and s, the ascorbate concentration. The inset is a tabulation of the values of K_m and V_{max} at different extracellular Na^+ concentrations. While the V_{max} remained unchanged, the K_m decreases as the Na^+ concentration in the BSS increases.

for Ca^{2+} in the ascorbate transport is not clear, since Ca^{2+} seems to affect both the K_m and V_{max}. As shown in Fig. 8, increasing Ca^{2+} concentration from 20 μM to 2 mM increases K_m as well as V_{max}. A number of other divalent cations share with calcium the ability to stimulate ascorbate transport. Mn^{2+}, Mg^{2+}, Co^{2+} or Ni^{2+} stimulated ascorbate transport either equal to or slightly more than the stimulation obtained by calcium, when each was added at a 25 μM concentration. The Ca^{2+}-dependent stimulation of ascorbate transport was also observed in adrenal cortical [20] and chromaffin cells [15].

Finally, we examined if human serum contained any regulatory factor(s) for the ascorbate transport. As shown in Table I, normal human serum contained a heat-labile factor which inhibited ascorbic acid transport by 3T6 fibroblasts. At 4% concentration, human serum caused 76% inhibition of the transport. We have confirmed this observation with three other samples

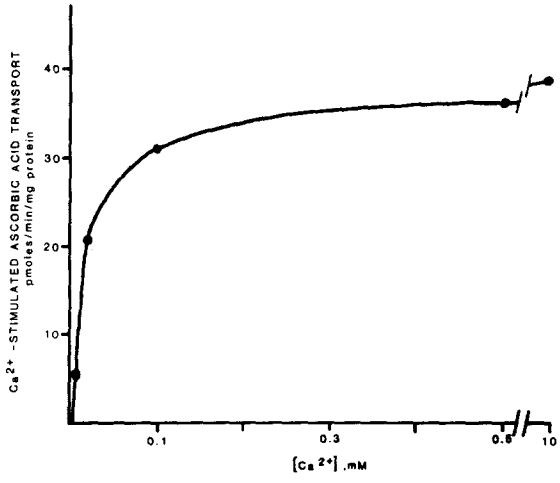

Fig. 7. Effect of Ca^{2+} on the ascorbate transport. Ascorbate transport was studied as described in Materials and Methods except that BSS was prepared without addition of Ca^{2+} or Mg^{2+}. Indicated amount of Ca^{2+} was then added. The ascorbate transport was carried out for 20 min when the ascorbate concentration was 100 μM (0.2 mCi/mmol). The ascorbate uptake in the absence of added Ca^{2+} was about 10% of the maximum and was subtracted from each point. Addition of 10 μM EDTA or EGTA completely inhibited the ascorbate transport, however, cells started detaching from the flasks.

TABLE I

EFFECT OF HUMAN SERUM ON ASCORBATE TRANSPORT

The values are means ± S.D. of four determinations. The ascorbate transport was studied in a total volume of 2.5 ml, the details of which are given in the Materials and Methods. The ascorbate concentration was 2.75 μM.

	Ascorbate transport (pmol/min per mg protein)	Percentage of control
1. BSS	6.12 ± 0.15	100
2. BSS + human serum (1%)	5.02 ± 0.11	82
3. BSS + human serum (2%)	4.56 ± 0.12	75
4. BSS + human serum (4%)	1.47 ± 0.10	24
5. BSS + heat-inactivated human serum (4%)	5.88 ± 0.14	96

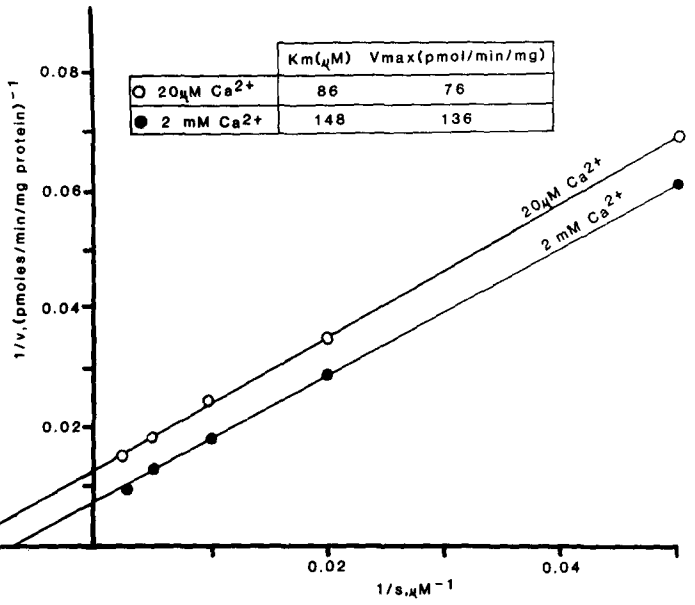

Fig. 8. Effect of calcium on kinetics of ascorbate transport. Details are same as in Fig. 1 except that Mg^{2+} was deleted from the BSS and Ca^{2+} was added at either 20 μM or 2 mM concentration. The data are presented in the Lineweaver-Burk plot of the reciprocal of v, the rate of ascorbate transport, and s, the ascorbate concentration. The inset is a tabulation of the values of K_m and V_{max} at two calcium concentrations in the BSS.

of human sera from normal healthy volunteers and with human serum purchased commercially from Cooper Biomedicals, Malvern, PA. Serum in the presence of 1 mM thiourea has no effect on the stability of ascorbic acid (Ref. 18, and unpublished data). We have earlier [16] demonstrated that fetal bovine serum contained a heat-labile factor which inhibited ascorbate transport only after its interaction with bacterial endotoxin. In this respect the inhibitor in human serum is different from that in bovine serum. It is possible that the inhibitors in both the sera are similar except that human serum contained it in an 'active' form. Our more recent preliminary data suggest that the inhibitor is derived from the activation of complement system in serum [23]. Further characterization of the inhibitor and mode of inhibition is underway. It is conceivable that the inhibitor could provide meaningful insight into the transport process and could help characterize the transporter molecule. The physiological significance of the inhibitor and its role in vitamin C nutrition remains unknown.

The data presented here reveal the characteristics of ascorbate transport by 3T6 fibroblasts. The transport process is a carrier-mediated saturable active process which is temperature- and energy-dependent. It exhibited normal Michaelis-Menten kinetics with K_m of 112 μM and V_{max} of 158 pmol/min per mg protein. The transport process requires extracellular Na^+ which reduces the K_m but does not affect V_{max} of the process. Calcium and other divalent ions stimulate the rate of transport and the mechanism and significance of such stimulation is not fully understood.

With few differences, the basic characteristics of ascorbate transport by 3T6 fibroblasts are similar to the transport systems described for other cells [13–15,20]. The high affinity Na^+-dependent cotransport system seems to be so widespread, it is a testimony to the importance of ascorbic acid in cellular metabolism. However, our understanding of the biochemical functions of ascorbic acid is limited to the hydroxylation of proline and lysine in nascent collagen [4–7] and the hydroxylation of dopamine to norepinephrine [1–3]. Our ignorance about yet unknown biochemical functions of ascorbic acid is evident in many seemingly unrelated pathological lesions found in scurvy. Therefore our search must focus on the possibility of yet unknown biochemical functions of this important vitamin.

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