Biochimica et Biophysica Acta, 552 (1979) 129—142

BBA 78301

Na^+-DEPENDENT, ELECTRONEUTRAL L-ASCORBATE TRANSPORT ACROSS BRUSH BORDER MEMBRANE VESICLES FROM GUINEA PIG SMALL INTESTINE

LUCA SILIPRANDI, PAOLO VANNI, MARKUS KESSLER and GIORGIO SEMENZA

Laboratorium für Biochemie der E.T.H., Universitätstrasse 16, CH-8092 Zürich (Switzerland)

(Received August 22nd, 1978)

Key words: L-Ascorbate transport; Heterologous inhibition; Electroneutral transport; (Brush border vesicle, Guinea pig)

Summary

In brush border vesicles from guinea pig small intestine L-ascorbate transport is Na^+-dependent and electroneutral (in the presence of Na^+, as shown by its lack of response to either positive or negative $\Delta\psi$ across the membrane).

L-Ascorbate transporter has the kinetic characteristics of a mobile carrier (K_m for L-ascorbate, 0.3 mM). D-Isoascorbate (erythorbate) seems to be another, but poorer, substrate of the same transporter.

L-Ascorbate transport is subjected to heterologous inhibition by D-glucose.

Introduction

Whereas in the rat [1—3] and hamster (Caspary, W., unpublished observations quoted in ref. 4) the intestinal absorption of L-ascorbate is minimal and takes place via 'diffusion', it is significant and probably mediated by a carrier in both guinea pig and man [4—6], two species in which L-ascorbate is a vitamin. Using preparations of surviving intact small intestine, ascorbate absorption was shown to be Na^+ dependent in vitro in both latter species [4,5].

In recent years, preparations of vesicles from intestinal brush border membranes were reported (e.g. refs. 7 and 8). They have proved of great value in differentiating between 'primary' and 'secondary' active transport mechanisms (e.g. refs. 7 and 8) and in demonstrating the rheogenic nature of some Na^+-dependent intestinal transport systems [9,10].

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

In the present study we have characterized the transport of L-ascorbate across the brush border membrane by using what are perhaps the most efficient brush border membrane vesicles from the small intestine [11,8]. We decided to re-investigate this problem and to extend previous studies which utilized whole tissue preparations for various reasons: (i) When surviving tissue preparations are used to study L-ascorbate uptake, partially contradictory requirements have to be met, i.e. that of having a good oxygenation of the tissue, but at the same time that of avoiding extensive oxidation of L-ascorbate itself. (ii) Membrane vesicles are the preparation of choice in the elucidation of some aspects of transport mechanisms, e.g. whether a given transport is rheogenic or not. L-Ascorbate absorption is reported to be Na^+ dependent. Since L-ascorbic acid has a pK'_1 of 4.17 (from ref. 12), if the transport of 1 Na^+ is associated with that of 1 ascorbate$^-$, this Na^+-dependent system should be electrically neutral. (iii) Counterflow experiments can be carried out in a more straight forward way on membrane vesicles than in whole tissue preparations. (iv) D-Isoascorbate (erythorbate), the epimer of L-ascorbate in C_5, may interfere with the absorption of the latter. Although D-isoascorbate has only negligible vitamin C activity, it is admitted in some countries as a technological antioxidant additive to foodstuffs.

We present here evidence that ascorbic acid is transported into brush border membrane vesicles from guinea pig small intestine apparently by a single, saturable carrier with an apparent K_m value, under the conditions used, of approx. 0.3 mM. The system is activated by Na^+; in the presence of this cation transport of ascorbic acid is electrically neutral. L-Ascorbate uptake is subjected to heterologous inhibition by D-glucose, in all likelihood via increased intravesicular Na^+ concentration. (Interactions occurring between different transport systems (e.g. between D-glucose transporter and neutral amino acid transporter) are referred to as 'heterologous').

D-Isoascorbate is a fully-competititve inhibitor ($K_i \simeq 20$ mM) and an efficient elicitor of L-ascorbate transport.

Materials and Methods

Chemicals. All reagents were of highest purity available. L-[1-^{14}C]Ascorbic acid was purchased from New England Nuclear, Boston, MA. D-[1-^{3}H]Glucose and L-[1-^{14}C]glucose from Radiochemical Centre Amersham, England. If open once, the vials of radioactive L-ascorbic acid were resealed under nitrogen and stored at −20°C. As judged from the uptake, storage for some days under these conditions did not alter ascorbic acid.

All chemical used were reagent grade and were obtained from Merck, Darmstadt (F.R.G.), Sigma, St. Louis MO (U.S.A.) or Fluka, Buchs (Switzerland), except for the preparation of monactin plus nonactin (approx. 1 : 3, w/w), which was a generous gift from Professor W. Simon, ETH Zürich.

Vesicle preparation. The vesicles of small intestinal brush border membranes of guinea pig were prepared from fresh intestine, as described by Kessler et al. [8]. The animals were killed by a blow on the neck, the small intestine was quickly removed, rinsed with cold saline and everted. The mucosa was scraped off with a glass slide. The scrapings from 3 to 6 guinea pigs were suspended in

60—120 ml 300 mM mannitol, 10 mM Hepes/Tris (pH 7.5) and processed as described in ref. 8. For the experiments with rabbit small intestine, we used frozen material. The experimental procedure remained the same [8].

Transport measurements. The uptake of L-ascorbate, D- and L-glucose by brush border membrane vesicles was determined at 20°C. The incubation medium contained, unless stated otherwise: 300 mM D-mannitol, 10 mM Hepes/Tris (pH 7.5), 100 mM NaSCN, varying concentrations of L-ascorbate and of dithiothreitol (approximately one half of those of L-ascorbate) with approx. 20 mg membrane protein/ml (i.e. with many more vesicles than in refs. 7—10). The high concentration of D-mannitol both inside (from last step in vesicle preparation) and outside the vesicle minimized the effects of osmotic gradients across the membrane.

The incubation was stopped by addition of ice-cold 250 mM NaCl, the suspension filtered through a Sartorius filter (0.6 μm pore size) under suction, and washed once with the same ice-cold stop solution.

Radioactivity was measured in a scintillation counter; protein was determined according to Lowry et al. [13].

Results

All experiments were repeated 3—8 times with consistent results. The individual experiments reproduced are representative. The points in the figures are averages of 3—5 individual determinations.

Binding vs. transport across the brush border membrane

By far most of the L-ascorbate taken up by guinea pig brush border vesicles is transported into their osmotic space (i.e. across the membrane), rather than bound to the membrane itself. This conclusion is based on the following observations: (i) the ratio medium [L-ascorbate] to the amount of L-ascorbate associated with the vesicles at equilibrium is not statistically different at the various concentrations of L-ascorbate investigated. For example, this ratio is 1360 ± 120 · ml^{-1} · mg protein at 0.3 mM L-ascorbate and is 1110 ± 90 · ml^{-1} · mg protein at 0.1 mM L-ascorbate in the experiment of Fig. 1. In addition, this ratio is independent of the substrate considered: in Fig. 6 (see below) the ratio is 780 · ml^{-1} · mg protein for L-ascorbate (Fig. 6A) and 770 · ml^{-1} · mg protein for D-glucose (Fig. 6B). Similar conclusions were arrived at when comparing with the uptake of L-glucose (data not shown). (ii) The amount of L-ascorbate present in the osmotic space of the vesicles at equilibrium must be equal to zero at zero osmotic space. As shown in Fig. 2B this is actually the case, no ascorbate being associated with the brush border membrane vesicles at infinite medium osmolarity, i.e. at zero vesicular osmotic space. (iii) The presence of another anion of similar structure in large excess (D-isoascorbate) does not reduce the amount of L-ascorbate associated with the vesicles at equilibrium (see Fig. 3B). Thus, the amount of L-ascorbate bound to the membrane as counter ion (or in some other way) must be negligibly small or nil.

This is contrary to what is observed with cations: the amounts of, e.g. choline, Na^+, Ca^{2+} bound by these membranes are quite large [8]. This difference in binding capacity for cations and (an) anion(s) is not unexpected, in

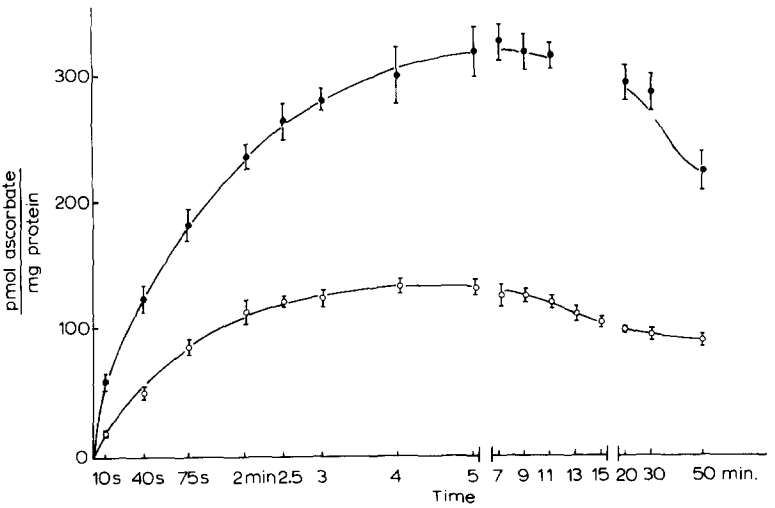


Fig. 1. Time course of L-ascorbate uptake in membrane vesicles from guinea pig small intestine. The concentration of L-ascorbate was 0.3 mM (upper curve) or 0.1 mM (lower curve), respectively. The vesicles, which were preequilibrated in 300 mM D-mannitol and 10 mM Hepes/Tris (pH 7.5), were incubated at 20°C in a medium of the following composition: 100 mM NaSCN, 300 mM D-mannitol, 10 mM Hepes/Tris (pH 7.5), 0.3 or 0.1 mM sodium ascorbate and dithiothreitol at concentrations approximately equal to one half of those of L-ascorbate. The bars indicate the S.E.

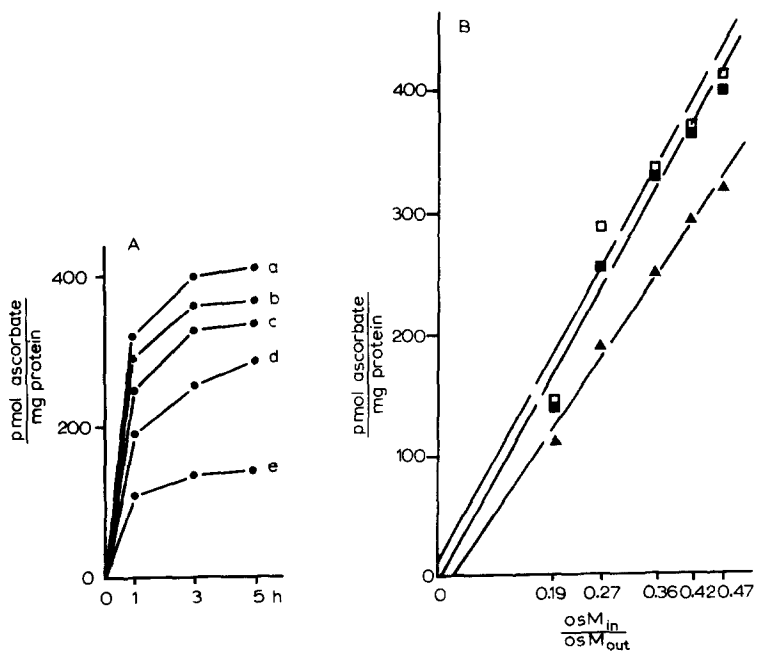


Fig. 2. L-Ascorbate uptake as a function of the osmolarity of the incubation medium. The vesicles were prepared in 100 mM D-mannitol, 10 mM Hepes/Tris (pH 7.5) and were incubated in 0.13 mM L-[^{14}C]-ascorbate, 50 mM NaSCN, 8 mM Hepes/Tris (pH 7.5) and varying concentrations of D-mannitol. (A) Time course of L-ascorbate uptake. D-Mannitol concentrations were: 100 mM (a), 125 mM (b), 165 mM (c), 250 mM (d) or 400 mM (e). (B) L-Ascorbate uptake at 1 (▲), 3 (■) or 5 (□) h vs. the ratio between the osmolarity values of the inner and outer media.

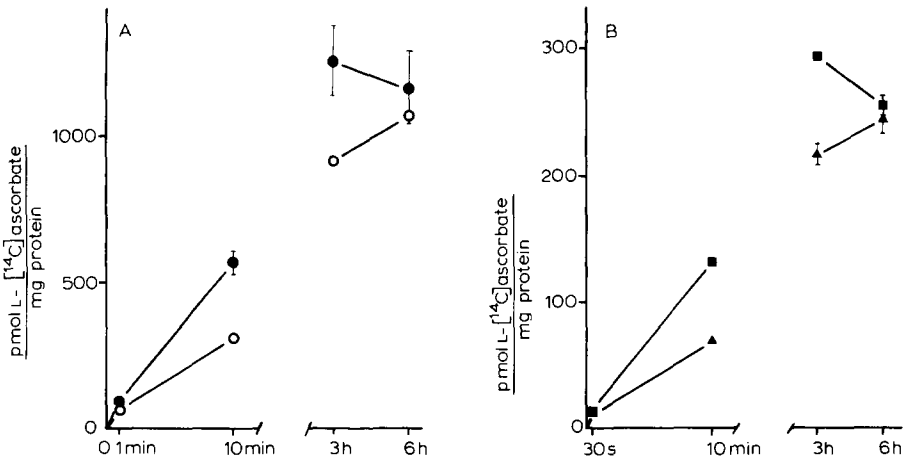


Fig. 3. Counterflow of L-[^{14}C]ascorbate elicited by unlabeled L-ascorbate (A) or by unlabeled D-isoascorbate (B). The vesicles were preincubated in 300 mM D-mannitol, 10 mM Hepes/Tris, (pH 7.5), 100 mM NaSCN, 10 μg monactin/nonactin mixture per mg protein and the unlabeled elicitor (20 mM L-ascorbate in A, 150 mM D-isoascorbate in B) for 3—4 h at 20°C. Incubation proper was also carried out at 20°C by diluting the preincubated suspension 20-fold. The incubation medium had the following final composition: 300 mM D-mannitol, 10 mM Hepes/Tris (pH 7.5), 100 mM NaSCN and L-[^{14}C]ascorbate (1.3 mM in A, 0.3 mM in B). Symbols in A: ●, samples preincubated in unlabeled L-ascorbate; ○, samples preincubated in the absence of elicitor. Symbols in B: ■, samples preincubated in unlabeled D-isoascorbate; ▲, samples preincubated in the absence of elicitor. The bars indicate the S.E. When not given, the S.E. were smaller than the symbols used.

view of the negative ζ-potential of these vesicles (Hauser, H., unpublished observations, 1978).

Time course and overall kinetic parameters of L-ascorbate uptake

L-Ascorbate uptake deviates from linearity even before 10 s, which is the shortest incubation time examined (Fig. 1). Elsewhere [14] we have pointed out the importance of using initial velocities for the calculation of both K_m and J_{max} values (the flux equations of co-transport for Na^+_{in}, $substrate_{in} \neq 0$ are prohibitively clumsy unless highly restrictive, and thus dangerous, assumptions are made). For accurate kinetic analysis the use of the initial linear range of uptake is thus imperative.

In the case of L-ascorbate uptake, however, this very range of the time course of uptake is not accessible to accurate measurements, due to the low transport capacity of the vesicles for this substrate (approx. 10 times smaller than that for D-glucose, under optimal conditions). This obstacle could not be compensated for by the moderately high specific radioactivity of the L-ascorbate used or by the rather large amounts of vesicles used in these studies (compared to those used earlier for glucose or amino acid transport, e.g. refs. 7—10).

The next best solution for obtaining reliable overall kinetic parameters from close-to-initial uptake data is to measure for various times of incubation both the substrate uptake values at infinitely large medium concentrations and the substrate concentrations yielding half maximum uptake values. If these values do not change drastically with the time of incubation, their extrapolation to zero time should provide reliable K_m and J_{max} values. Such a set of experiments

TABLE I

DEPENDENCE OF L-ASCORBATE UPTAKE ON THE TIME OF INCUBATION

For experimental conditions, see Materials and Methods. Results are expressed as mean ± S.E. The number of experiments are in parentheses.

Incubation time	L-Ascorbate concentration (mM) yielding half-maximum uptake	L-Ascorbate uptake at infinitely large L-ascorbate concentration (pmol · mg^{-1} protein)
10 s	0.296 ± 0.009 (3)	153 ± 7.4 (3)
50 s	0.293 ± 0.017 (3)	414 ± 26 (3)
90 s	0.315 ± 0.018 (3)	578 ± 31 (3)
2.5 min	0.392 ± 0.018 (3)	743 ± 89 (3)
4 min	0.437 ± 0.018 (3)	838 ± 76 (3)
150 min	1.773 ± 0.219 (4)	2069 ± 168 (4)

is shown in Table I. Clearly, the K_m values obtained at 10, 50 and 90 s are not significantly different. An initial K_m of approx. 0.3 mM under these experimental conditions should thus be close to the correct value. For J_{max} however, a similarly accurate extrapolation is not possible, although a value of 20—25 pmol · s^{-1} · mg^{-1} protein is likely to be an acceptable estimate. The K_m value obtained here with brush border vesicles is about one third as large as the value reported in the literature for intact surviving small intestine [5]. A similar discrepancy was observed earlier in the K_m values for D-glucose intestinal uptake [14,15,18] and is probably due to the different media used and/or to the effect of unstirred layers on the kinetic parameters of transport in intact small intestine.

Counterflow experiments

Preloading of the vesicles with either L-ascorbate (Fig. 3A) or D-isoascorbate (Fig. 3B) followed by dilution produced an increase in L-[^{14}C]ascorbate uptake (and, with D-isoascorbate as the elicitor, a transient accumulation of L-[^{14}C]-ascorbate also), in spite of the absence of any Na^+ gradient across the membrane. The experiments were carried out in the presence of both Na^+ and monactin (plus nonactin), i.e. the membrane was electrically short-circuited and changes in intravesicular Na^+ concentrations (which the efflux of the elicitor may otherwise have induced) were prevented by the ionophore. Thus, the accelerated influx and the transient accumulation of L-ascorbate, respectively, cannot be ascribed to any indirect coupling (e.g. via movements of elicitors and/or Na^+ by way of transporters other than that of L-ascorbate) and are most likely to be attributed to a counterflow mechanism.

Cation and anion dependence

The experiment in Fig. 4 clearly shows that external Na^+ accelerates L-ascorbate uptake, an observation agreeing with data of the literature on whole tissue preparations in vitro [4,5]. Neither choline nor K^+ can substitute for Na^+. Differences in cation permeabilities can be ruled out as the possible mechanism for this Na^+ effect, since these vesicles have similar permeabilities for Na^+ and K^+ [15]. The presence of Na^+ is also necessary for the transient, albeit small, L-ascorbate overshoot (Fig. 1).

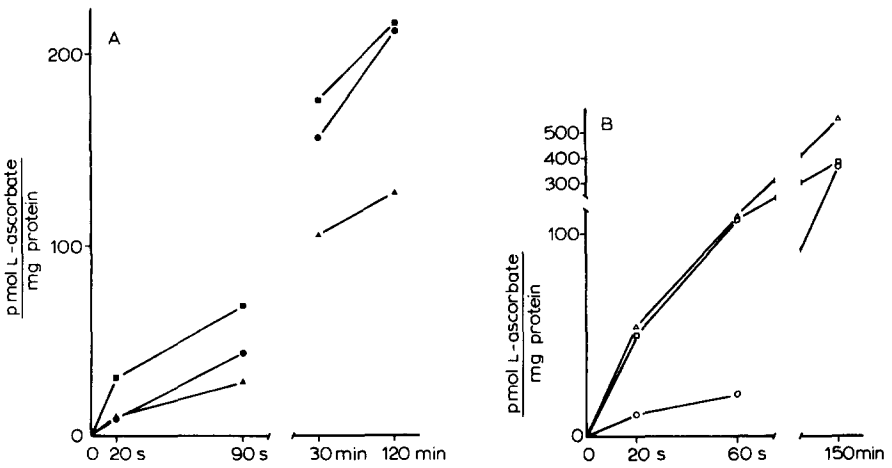
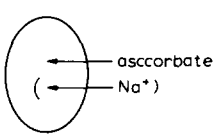
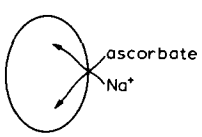
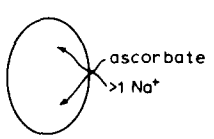


Fig. 4. Uptake of L-ascorbate into brush border membrane vesicles from guinea pig small intestine. At the start of the incubations, the concentration of D-mannitol was 300 mM and that of Hepes/Tris, pH 7.5, was 10 mM at both sides of the membrane. The following components were present only in the outer medium: 0.3 mM L-[^{14}C]ascorbate and these salts: in A, 100 mM NaSCN (■), KSCN (●) or choline SCN (▲). In B, 50 mM Na_2SO_4 (△), 100 mM NaSCN (□) or KSCN (○).

TABLE II

POSSIBLE MECHANISMS OF FLUX COUPLING BETWEEN L-ASCORBATE AND Na^+ MOVEMENTS ACROSS BRUSH BORDER MEMBRANES FROM GUINEA PIG SMALL INTESTINE

Indirect (electrical) coupling of fluxes	Direct coupling of fluxes (co-transport)	
	Flux ratio 1 : 1	Flux ratio 1 : >1
Driving force: membrane $\Delta\Psi$ (positive inside) ("rheogenic", "electrogenic")	Driving force: $\Delta\mu$ of Na^+. electrically neutral (no $\Delta\Psi$ dependence)	Driving force: $\Delta\bar{\mu}$ of Na^+. $\Delta\Psi$-stimulated (negative inside) ("rheogenic", "electrogenic")
		
Physiologically unlikely	Physiologically sensible	Physiologically sensible
Unlikely because of lack of inhibition by $SCN^-_0 >> SCN^-_I$ (i.e. by $\Delta\Psi$, negative inside) (Fig. 4b)	Likely because of lack of any effect of $SCN^-_0 >> SCN^-_I$ (i.e. of $\Delta\Psi$, negative inside) (Fig. 4b)	Ruled out because of lack of ascorbate uptake by $SCN^-_0 >> SCN^-_I$ (i.e. by $\Delta\Psi$, negative inside), in the presence of Na^+ (Fig. 4b)
Ruled out because ascorbate uptake is not accelerated by K^+, a cation for which the membrane has about the same permeability as for Na^+ (Fig. 4b)	$\Delta\Psi$ (positive inside) should (and does) accelerate ascorbate uptake in the absence of Na^+_0. It should have no effect (and does not have any) on ascorbate uptake in the presence of Na^+_0 (Table III)	Ruled out because a $\Delta\Psi$ (positive inside) should inhibit L-ascorbate uptake. It does not (Table III)
Ruled out because $\Delta\Psi$ (positive inside) should accelerate ascorbate uptake. It does not (Table III)		

In principle (Table II), this synport acceleration by Na^+ could be due to (i) indirect (electrical) coupling: the entry of the L-ascorbate anion would be braked by the negative membrane potential which it builds up. The entry of Na^+ would collapse this $-\Delta\psi$. Alternatively, Na^+ for which the membrane has a larger permeability than for L-ascorbate, would produce a $\Delta\psi$, positive inside, which would 'suck in' L-ascorbate. (ii) The acceleration by Na^+ could be due to direct coupling of fluxes, i.e. both Na^+ and L-ascorbate would be transported by the same agency with an ascorbate : Na^+ flux ratio of 1 : 1 or of 1 : >1.

Mechanism i (indirect, electric coupling) and mechanism ii with a flux ratio >1 can be ruled out (Table II) because: (a) a diffusion potential (negative inside) produced by a NaSCN gradient (out > in) neither accelerates nor inhibits L-ascorbate uptake (Fig. 4B: the uptake of L-ascorbate is the same in the presence of a NaSCN or Na_2SO_4 gradient. Since the permeability of these vesicles for SO_4^{2-} is much smaller than for SCN^- [15], the former anion must produce a smaller $\Delta\psi$, negative inside). (b) A diffusion potential (positive inside, this time produced by an in > out SCN^- gradient, Table III) neither accelerates nor inhibits L-ascorbate uptake in the presence of Na^+. Similar, although less clear-cut results were obtained with a $\Delta\psi$ (positive inside) produced by valinomycin and K^+ (out > in). (c) Finally, indirect, electrical coupling, if operative, should also be possible with K^+ in addition of Na^+, because the permeabilities of this membrane for these two cations are similar [15]. However, K^+ does not accelerate L-ascorbate uptake (Fig. 4B).

The conclusion seems thus inescapable that Na^+-dependent L-ascorbate transport is electrically neutral, rather than rheogenic (Table II, direct coupling with flux ratio 1 : 1), and that Na^+ is co-substrate of L-ascorbate transport. This conclusion is strengthened further by the following experiment. If the L-ascorbate carrier can operate, albeit more slowly, in the absence of Na^+, L-ascorbate transport should be rheogenic in the absence of this cation, with a $\Delta\psi$ (positive inside) accelerating L-ascorbate uptake. This is actually the case (see Table III,

TABLE III

EFFECT OF $\Delta\Psi$ (POSITIVE INSIDE) ON L-ASCORBATE UPTAKE ± Na^+

Expt. No. Conditions expected to preveal at the start of incubation	Vesicles preincubated in SCN^- or in SO_4^{2-} Incubations in 0.3 mM L-ascorbate ± Na^+			
	Major components	Concentrations (mM) at time zero		pmol L-ascorbate / mg protein · 15 s
		In	Out	
1. $\Delta\Psi$ (positive inside) and $Na^+_{out} > Na^+_{in}$	choline thiocyanate Na_2SO_4	200 —	10 20	25.0 ± 3 (3)
2. No (or little) $\Delta\Psi$ and $Na^+_{out} > Na^+_{in}$	choline sulfate Na_2SO_4	100 —	5 20	24.4 ± 4.7 (3)
3. $\Delta\Psi$ (positive inside) and no Na^+	choline thiocyanate choline sulfate	200 —	10 20	16.35 ± 2.23 (3)
4. No (or little) $\Delta\Psi$ and no Na^+	choline sulfate	100	25	1.08 ± 1.03 (3)

Expt. 3). The $\Delta\psi$ (positive inside) was generated by a choline thiocyanate gradient (in > out), because the permeability of these membranes for SCN^- is much larger than that for choline [15]. An alternative explanation, i.e. that intravesicular SCN^- is exchanged against ascorbate via 'exchange diffusion', i.e.

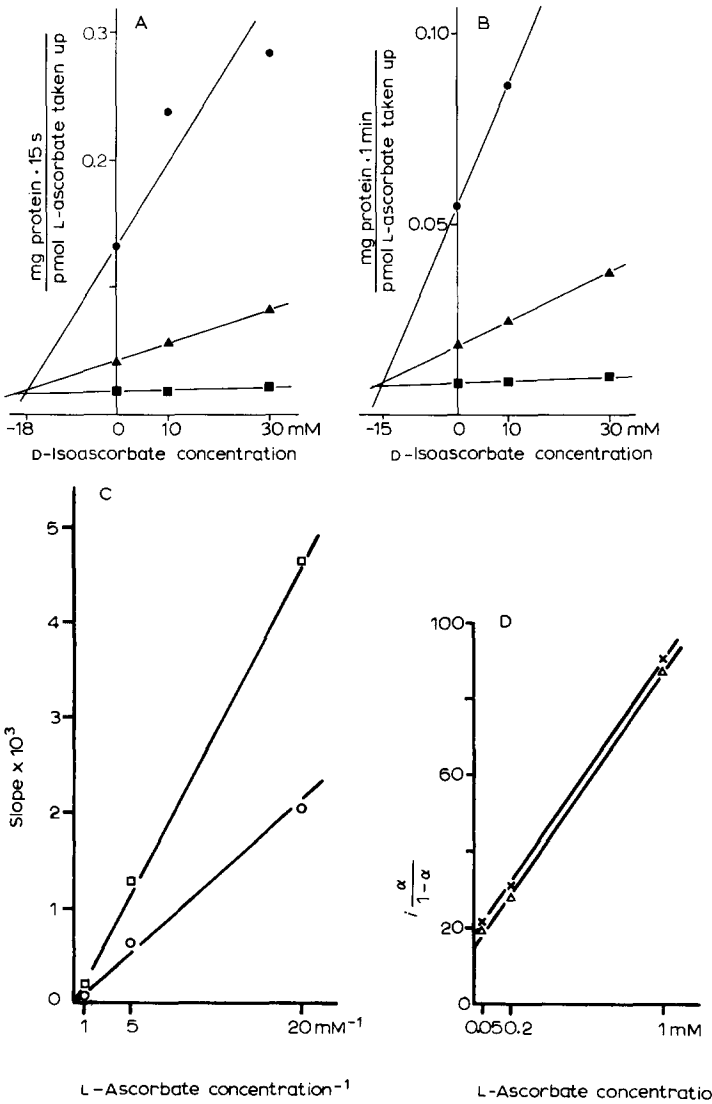


Fig. 5. Competitive inhibition of L-ascorbate uptake into brush border membrane vesicles from guinea pig small intestine, by D-isoascorbate. Standard incubation conditions. (A and B) Dixon plots from the uptake values at 15-s (A) and 1-min (B) incubations times, respectively. L-Ascorbate concentrations: 1 mM (■), 0.2 mM (▲) or 0.05 mM (●). (C) Secondary plots from the Dixon plots of A and B; the slopes are plotted vs. the reciprocal of L-ascorbate concentrations. The lines extrapolate through the origin, both at 15 s (□) and 1 min (○) uptake, which indicates the inhibition to be fully-competitive. (D) Hunter-Downs plot of $i \cdot \alpha/(1-\alpha)$ vs. L-ascorbate concentrations, where i is the concentration of D-isoascorbate and α is the ratio between the velocity of uptake in the presence of inhibitor and the velocity of uptake without inhibitor. The slopes are >0, which indicates again a fully-competitive inhibition. The intercept on the yaxis corresponds to the K_i. Symbols: X,1 min uptake; △, 15 s uptake.

as substrates of the same carrier, is unlikely, because of the different chemical properties of these two anions, and because in the presence of Na^+ the gradient of SCN^- (in > out) does not accelerate L-ascorbate uptake (Table III, Expts. 1 and 2).

Inhibition by D-isoascorbate

An isomer of L-ascorbate, D-isoascorbate, inhibits L-ascorbate uptake (Figs. 5A—5D), in agreement with a previous report in the literature on intact surviving tissue in vitro [5]. As pointed out in a previous section, kinetic analysis of L-ascorbate uptake in these vesicles cannot be based on truly initial velocities. In examining the kinetic nature of D-ascorbate uptake we resorted, therefore, to determine the overall kinetic parameters of at least two as short as possible times of incubations (i.e. 15 s and 1 min). Figs. 5A—5D show the result in Dixon plots (primary and secondary), as well as Hunter and Downs plot. The inhibition is clearly fully competitive with a K_i value of approx. 19.7 mM. Since D-isoascorbate (Fig. 3B) acts as elicitor, it is nearly certain that this compound is a substrate, rather than an inhibitor of the L-ascorbate transport system. We have no radioactive D-isoascorbate at our disposal to carry out uptake experiments with this compound.

Inhibition of L-ascorbate uptake by D-glucose

Figs. 6A and 6B show that D-glucose inhibits L-ascorbate uptake, but not vice

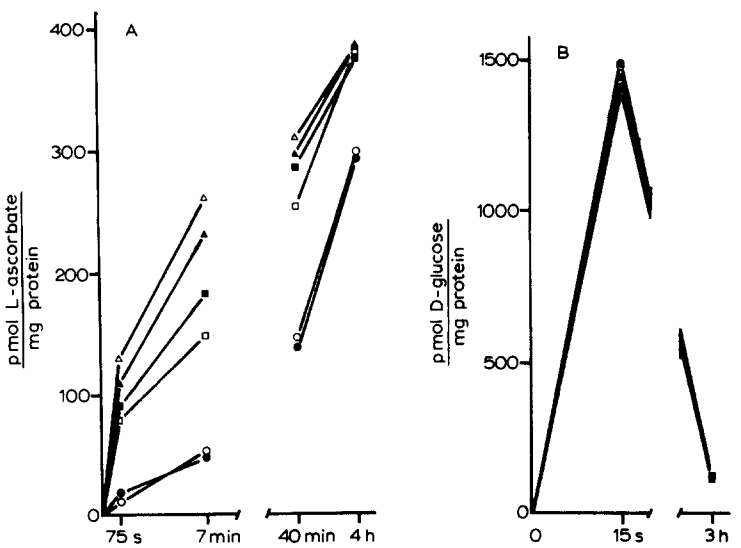


Fig. 6, Heterologous inhibition of L-ascorbate uptake by D-glucose and lack of inhibition of D-glucose uptake by L-ascorbate, in brush border membrane vesicles from guinea pig small intestine. (A) Inhibition of L-ascorbate uptake by D-glucose. Upper four lines, standard incubation conditions (i.e. at the start, 100 mM NaSCN, 0.3 mM L-ascorbate). In addition, △, no D-glucose; ▲, 0.1 mM; ■, 1 mM and, □, 10 mM D-glucose added together with the substrate and NaSCN. Lower two lines: standard conditions, but with 100 mM KSCN instead of NaSCN. ●, no D-glucose; ○, with 10 mM D-glucose added at the start. (B) Lack of inhibition of D-glucose uptake by L-ascorbate. At the start of the incubation, the inner medium of the vesicles was 300 mM D-mannitol plus 10 mM Hepes/Tris, pH 7.5. The outer medium was: 300 mM D-mannitol, 10 mM Hepes/Tris, pH 7.5, 100 mM NaSCN, 0.1 mM D-[^{3}H]glucose and 0, 0.3, 1 or 8 mM L-ascorbate. The points are so close that they are not resolved by the drawing.

TABLE IV

RELEASE OF D-GLUCOSE INHIBITION OF L-ASCORBATE UPTAKE BY PHLORIZIN IN BRUSH BORDER MEMBRANE VESICLES FROM GUINEA PIG SMALL INTESTINE

Medium concentrations at time zero: 0.3 mM L-[^{14}C]ascorbate, 100 mM NaSCN, ± 10 mM D-glucose, ± 0.25 mM phlorizin. Present at both sides of the membrane at time zero: 300 mM D-mannitol, 10 mM Hepes/Tris, pH 7.5. Results are expressed as the mean ± S.E. The number of experiments are in parentheses.

	L-Ascorbate taken up in 2 min (pmol/mg protein)
Control	123.7 ± 5.5 (4)
+ 10 mM D-glucose	82.9 ± 1.5 (4)
+ 0.25 mM phlorizin	107.2 ± 2.4 (4)
+ 10 mM D-glucose + 0.25 mM phlorizin	110.3 ± 2.8 (3)

versa. As phlorizin, a well known fully-competitive inhibitor of D-glucose transport across the small intestinal brush border membrane (e.g. refs. 15—18), does not inhibit significantly L-ascorbate uptake (Table IV), it can be ruled out that L-ascorbate is taken up by the brush border vesicles by the same carrier(s) as D-glucose.

Does D-glucose interact with L-ascorbate carrier directly? This possibility also can be ruled out, because phlorizin, although not interacting with L-ascorbate carrier does release the inhibition of L-ascorbate uptake by D-glucose (Table IV). Thus this inhibition depends on the operation of the D-glucose carrier. It also depends on the presence of external Na^+ (Fig. 6A).

Summing up, the inhibition of L-ascorbate uptake by D-glucose is heterologous (the two compounds do not share the same carrier) and depends on the presence of Na^+. Heterologous interactions (including non-mutual inhibition of unidirectional fluxes, as in this case) are known to occur among several Na^+-dependent systems of the small intestine [19—22].

Discussion

The transport mechanism of L-ascorbate across the brush border vesicles from guinea pig small intestine

L-Ascorbate is transported by a 'mobile carrier' mechanism, as shown by the following criteria: (i) saturation (Table I); (ii) counterflow by unlabeled substrate and by an analogue, D-isoascorbate (Fig. 3); (iii) competitive inhibition by the same analogue, (presumably a substrate of the same transport system (Fig. 5); (iv) lack of this transport system in other species in which L-ascorbate is not a vitamin (e.g. in the rat [1,2]; in the hamster, Caspary, W., unpublished observations quoted in ref. 4; in the rabbit, our own unpublished observations). In these latter species L-ascorbate uptake, if any, does not exceed that of markers of so-called 'passive diffusion', such as L-glucose or D-mannitol.

In agreement with current ideas on the 'carrier' mechanism we do not imply with the above statement that we expect a 'rotating' or 'diffusing' carrier to be operative in L-ascorbate transport, but rather that the kinetic criteria (particularly criterion (ii)) for a mobile (part of the) transporter are fullfilled.

Electroneutrality of L-ascorbate-Na^+ co-transport

As summarized in Table II and as reported in detail under Results, L-ascorbate uptake is activated by Na^+ (Fig. 4) and, in the presence of this cation, is electrically neutral. This can only indicate a 1 : 1 co-transport of L-ascorbate and Na^+ by the same carrier, the driving force of the system being the sum of the gradients in the chemical (rather than electrochemical) gradients of L-ascorbate and Na^+.

It is not surprising, of course, that the co-transport of a monovalent anion and Na^+ is electroneutral. This, however, is only the second time that such an electroneutral Na^+ co-transport is demonstrated. The other example is, to our knowledge, the transport of phosphate across the same membrane [26].

Heterologous inhibition of L-ascorbate uptake by D-glucose (Fig. 6 and Table IV)

The inhibition of L-ascorbate by D-glucose is heterologous in nature (i.e. it occurs between substrates not sharing the same carrier, as shown by the lack of significant effect of phlorizin on L-ascorbate uptake, Table IV), depends on the operation of the D-glucose carrier (it is released by phlorizin) and on the presence of Na^+ (Fig. 6A). It has the characteristics, therefore, of similar Na^+-dependent heterologous interactions between transport systems of the small intestine [19—22].

Although the heterologous inhibition of L-ascorbate uptake by D-glucose may not have important physiological significance, it is of considerable theoretical interest. In fact, although heterologous interactions of this kind were originally suggested by Alvarado [19] to indicate the existence and operation of polyfunctional carriers, they can be explained without difficulty within the framework of Crane's co-transport mechanism [21,22], via a collapse of the driving forces related to the Na^+ gradient. Originally [21] emphasis was put to the increased trans (i.e. cellular) concentration of Na^+ which is produced by the operation of a Na^+-dependent system: the Na^+ entered by this route would 'brake' from the inside the operation of a neighbouring, equally Na^+-coupled transport system ('trans-Na^+-hypothesis'). Later, as the Na^+-dependent transport of neutral substrates was recognized to be rheogenic, i.e. driven by $\Delta\tilde{\mu}_{Na^+}$ across the membrane [9,10], it was suggested, and rightly so, that the collapse of $\Delta\psi$ would be (mainly) responsible for heterologous interactions [22]. Murer et al.'s experiments [22] were designed more to emphasize the importance of $\Delta\psi$ than to negate that of the Na^+ concentration gradient. It is now interesting to find, to our knowledge for the first time, a system in which the heterologous inhibition cannot be due to electric coupling (i.e. via collapse of $\Delta\psi$). In fact, L-ascorbate uptake is unaffected by either positive or negative $\Delta\psi$ (Fig. 4B and Table III), although it is accelerated by Na^+_{out} (Fig. 4) and is inhibited by D-glucose in the presence of Na^+ only (Fig. 6). Thus in this heterologous interaction only changes in intravesicular Na^+ and/or glucose concentrations can play a role. There are, therefore, at least two straight-forward explanations for the heterologous inhibition of L-ascorbate uptake by D-glucose: (i) D-glucose may interact with the ascorbate carrier at its intravesicular side (the substrate-inhibitor specificity of this carrier may be different at the two sides of the membrane, as it has been shown for the monosaccharide carrier of the erythro-

cyte membrane); (ii) the increased intravesicular Na^+ (entered by way of the D-glucose carrier along with glucose) can produce a trans inhibition of L-ascorbate unidirectional influx, or a Na^+, ascorbate efflux, as suggested in the 'trans-Na^+-hypothesis' [21]. The latter mechanism, if operative, would place this heterologous interaction into the framework of the other, Na^+-dependent interactions among small intestinal transport systems, the common mechanism being, in all cases, a decrease in $\Delta\tilde{\mu}_{Na^+}$: in some systems and experimental conditions the overriding factor would be the electric component [22], in others the Na^+ concentration component.

D-Isoascorbate (erythorbate), a likely substrate of L-ascorbate transport in guinea pig small intestine

The epimer in C_5 of L-ascorbate, D-isoascorbate, was tested both as an inhibitor (Fig. 5) and as an elicitor (Fig. 3B). The behavior was that expected for a substrate of the same transport system: D-isoascorbate is a fully-competitive inhibitor (K_i, approx. 20 mM) and an efficient elicitor. Thus, the conclusion seems warranted that this compound is a substrate of the L-ascorbate transport system. We could not test this directly since D-isoascorbate is not available in radioactive form. D-Isoascorbate is easily absorbed from the small intestine in vivo [23].

The competition of D-isoascorbate for the same transport system as L-ascorbate may make it a potential antivitamin C. Although the inhibition of intestinal absorption may be rather small, D-isoascorbate does reduce the uptake of L-ascorbate into various tissues of the guinea pig in vivo [24]. These actions of D-isoascorbate should be carefully considered, particularly in view of the fact that some population groups derive all or most of their intake of vitamin C from the L-ascorbate which is added as antioxidative agent to their industrially processed foodstuff [25].

The difference between the K_m of L-ascorbate and the K_i of D-isoascorbate would correspond, if these magnitudes were both 'pure' dissociation constants, to a difference in binding energy of approx. 2 kcal/mol. This difference would be compatible with a hydrogen bond less being involved in the binding of the carrier with D-isoascorbate as compared with its binding with L-ascorbate.

L-Ascorbate uptake in small intestinal brush border membrane vesicles from rabbit

In vesicles from rabbit small intestine L-ascorbate uptake is not larger than that of L-glucose (e.g. in 15-s incubations under standard conditions the uptake values were per mg membrane protein, 15 and 25 pmol, respectively. The difference was not statistically significant. As a comparison, vesicles from guinea pig small intestine took up under identical conditions approx. twice as much L-ascorbate as L-glucose). This observation adds one more species to the list of those (see Introduction) which apparently lack a carrier for intestinal L-ascorbate uptake, and in which this compound is not a vitamin.

Acknowledgements

This work was partially supported by the S.N.S.F., Berne, to whom sincere thanks are addressed. We also thank Professor W. Simon, E.T.H., Zürich, for

the gift of monactin plus nonactin and Professor D.F. Diedrich, Lexington, KY, (U.S.A.) for critically reading the manuscript.

References

1 Hornig, D., Weber, F. and Wiss, O. (1973) Biochem. Biophys. Res. Commun. 52, 168—172
2 Davis, M.G., Murphy, P.R., Crawshaw, S.J. and McGladrie, K. (1977) Nutr. Metab. 21, 266—268 (Suppl. 1)
3 Spencer, R.P., Purdy, S., Hoeldtke, R., Bow, T.M. and Markulis, M.A. (1963) Gastroenterology 44, 768—773
4 Stevenson, N.R. and Brush, M.K. (1969) Am. J. Clin. Nutr. 22, 318—326
5 Mellors, A.J., Nahrwold, D.L. and Rose, R.C. (1977) Am. J. Physiol. 233, E374—E379
6 Nelson, E.W., Lane, H., Fabri, P.J. and Scott, B. (1978) J. Clin. Pharmacol. 18, 325—335
7 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K. (1973) J. Biol. Chem. 248, 25—32
8 Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136—154
9 Murer, H. and Hopfer, U. (1974) Proc. Natl. Acad. Sci. U.S. 71, 484—488
10 Sigrist-Nelson, K., Murer, H. and Hopfer, U. (1975) J. Biol. Chem. 250, 5674—5680
11 Schmitz, J., Preiser, H., Maestracci, D., Gosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98—112
12 The Merck Index (1976) (Windholz, M., Budavari, S., Stroumtsos, L.Y. and Fertig, M.N., eds.), 9th edn., Merck, Rahway, NJ
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Toggenburger, G., Kessler, M., Rothstein, A., Semenza, G. and Tannenbaum, C. (1978) J. Membrane Biol. 40, 269—290
15 Kessler, M. (1978) Dissertation an der ETH, Zürich
16 Alvarado, F. and Crane, R.K. (1962) Biochim. Biophys. Acta 56, 170—172
17 Diedrich, D.F. (1966) Arch. Biochem. Biophys. 117, 248—256
18 Tannenbaum, C., Toggenburger, G., Kessler, M., Rothstein, A. and Semenza, G. (1977) J. Supramol. Struct. 6, 519—533
19 Alvarado, F. (1966) Science 151, 1010—1013
20 Schultz, S.G. and Curran, P.F. (1970) Physiol. Rev. 50, 637—718
21 Semenza, G. (1971) Biochim. Biophys. Acta 241, 637—649
22 Murer, H., Sigrist-Nelson, K. and Hopfer, U. (1975) J. Biol. Chem. 250, 7392—7396
23 Hornig, D. (1977) Acta Vitaminol. Enzymol. (Milano) 31, 9—14
24 Hornig, D., Weber, F. and Wiss, O. (1974) Experientia 30, 173—174
25 Schlettwein-Gsell, D. (1972) In. Z. Vitaminforsch. 42, 3—20
26 Berner, W., Kinne, R. and Murer, H. (1976) Biochem. J. 160, 467—474