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Na^+-DEPENDENT, POTENTIAL-SENSITIVE L-ASCORBATE TRANSPORT ACROSS BRUSH BORDER MEMBRANE VESICLES FROM KIDNEY CORTEX

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L-Ascorbate is taken up into brush border vesicles from kidney cortex of rat, rabbit and guinea pig by an efficient, Na^+-dependent and potential-sensitive transport process. This uptake shows saturation (K_m: 0.1–0.3 mM) and is strongly stimulated by low concentrations of NO_3^-. Erythorbate (D-isoascorbate) seems to be another, but poorer, substrate of the same transporter.

Among the mammals so far investigated, only guinea pigs and man, for whom L-ascorbate is a vitamin, have in the small-intestinal brush border an Na^+-dependent, saturable transport system for this compound [1–8]. Using vesicles from intestinal brush border membranes, we have previously shown that this system in the guinea pig [7] and in man [8] has the kinetic characteristics of a 'mobile carrier', is electroneutral (probably due to a 1 : 1 L-ascorbate : Na^+ flux ratio) and is inhibited fully-competitively by D-erythorbate (D-isoascorbate), which is presumably another substrate of the system (it elicits counterflow).

We report in the following the characterization of the transport system for L-ascorbate in kidney proximal tubuli, as studied in brush border membrane vesicles from the cortex. This system, in analogy with that of the small intestine has the kinetic characteristics of a 'mobile carrier' (saturation, fully-competitive inhibition, counterflow) depends on the presence of Na^+ in the outer medium and shows heterologous inhibition by D-glucose. In other respects, however, the renal L-ascorbate transport system differs from that of the small intestine: e.g., it responds to membrane potential and is present, contrary to the intestinal system, also in species for which ascorbate is not a vitamin (e.g., rat, rabbit).

Materials and Methods

1. Preparation of kidney brush border vesicles

Kidney brush border vesicles were prepared by a slightly modified procedure as described in [9,10]. For each experiment, five rats were killed, the kidneys excised and the cortices dissected from the medulla and collected in 300 ml of ice-cold 10 mM mannitol, 2 mM Tris-HCl, pH 7.1. The cortex pieces were homogenized in a mixer of the waring blender type at maximum speed for 2 min. In order to precipitate non-brush border material solid $MgCl_2$ was added to the homogenate to a final concentration of 10 mM. After standing in the cold for 15 min the homogenate was spun down at 3 000 × *g* for 15 min. The supernatant was decanted and centrifuged at 27 000 × *g*

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Abbreviations: DTT, dithiothreitol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Mes, 2-(*N*-morpholino)propanesulphonic acid.

for 30 min. The pellet was resuspended in 75 ml of 10 mM mannitol, 2 mM Tris-HCl, pH 7.1 and dispersed with a Teflon Potter-Elvehjem homogenizer. The precipitation procedure was repeated by adding again solid $MgCl_2$ to a final concentration of 10 mM. After standing 10 min in the cold, the suspension was centrifuged at $3\,000 \times g$ for 15 min. The supernatant was centrifuged once again at $27\,000 \times g$ for 30 min. The pellet, which contained purified brush border vesicles was normally resuspended in ice-cold 300 mM mannitol, 0.02% KN_3 and 10 mM Tris-Hepes, pH 7.5. As documented by appropriate marker enzymes the procedure adopted yielded a satisfactory membrane preparation. In fact, a specific marker of these membranes, i.e., aminopeptidase M, showed a 12.5-fold increase in specific activity as compared to the homogenate (data not shown); other enzymes known not to be associated with the brush border membrane were not enriched. Therefore, the procedure worked out in Refs. 9 and 10 proved satisfactory in our hands also and with the minor modifications mentioned above.

2. Transport measurements

The compositions of the incubation media are given in the legends. All incubation and preincubation media contained (also if not explicitely stated) 300 mM mannitol and 10 mM Tris-Hepes, pH 6.8.

As in previous studies [7,8], DTT was always present at concentrations approximately one half of those of L-ascorbate. Uptake experiments were performed at room temperature according to Kessler et al. [11] *, usually as follows: 10 μl vesicles (20 mg protein/ml) were placed on the bottom of a clear polystyrene test tube fitted into a vibration device controlled by an electric timer. 10 μl of the incubation medium containing the radioactively labelled substrate were placed approx. 1 mm apart from the vesicles. At the start of the timer, the shaking of the vibrator rapidly mixed the two drops together (within less than 80 ms). At the chosen time (usually, 5 s) 2 ml of cold isoosmolar NaCl ('stop solution') were automatically injected into the incubation test tube, which strongly slowed down the uptake. The sample was then quickly filtered through a wet cellulose nitrate microfilter (diameter 0.6 μm) and washed with another 5 ml of the cold stop solution. The time required for the washing was approx. 10 s. The radioactivity retained by the filter was determined by liquid scintillation counting.

In order to avoid oxidation of L-ascorbate by oxygen, incubations lasting longer than 2 min were performed under nitrogen.

3. Chemical

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 from Radiochemical Centre Amersham, England. Once opened, the stock solution of L-[^{14}C]ascorbic acid was resealed under nitrogen and kept at –20°C. The stock solution was stored for not longer than three weeks. Dehydroascorbate was prepared from L-ascorbate by oxidation with benzoquinone [12]. Choline sulphate was prepared by ion-exchange chromatography as described previously [13]. Microfilters (Cellpore, CP-060, diameter 0.6 μm) were purchased from Inchema SA., CH 1249 Avully, Switzerland. Protein was determined according to Bradford [14], with the reagent kit from Bio-Rad; bovine serum albumin was used as standard.

Results

1. Time course of L-ascorbate uptake; K_m and V values

As shown in Fig. 1 the uptake of L-ascorbate into brush border vesicles from rat kidney cortex begins to deviate from linearity already at 5 s (similar results were obtained with vesicle preparations from guinea pig and rabbit kidney cortex). At shorter incubation times, the radioactivity taken up was too small to yield reliable measurements. Thus, the initial, linear range of unidirectional flux could not be used for the determination of the overall kinetic parameters, as we have suggested elsewhere for D-glucose uptake [13]. As the next best solution, we resorted to measuring the apparent K_m and V values at incubation times as short as possible, i.e., at 5 s (Table I) (See Discussion).

Thiocyanate inhibits L-ascorbate uptake in this system (see below), whereas it affects little, if at all, the transport system in guinea pig small intestinal vesicles. (In Refs. 7 and 8 most experimental work was carried out in the presence of SCN^-). Thus, a

* The equipment is now available from Innovativ-Labor AG., CH-8134 Adliswil, Switzerland.

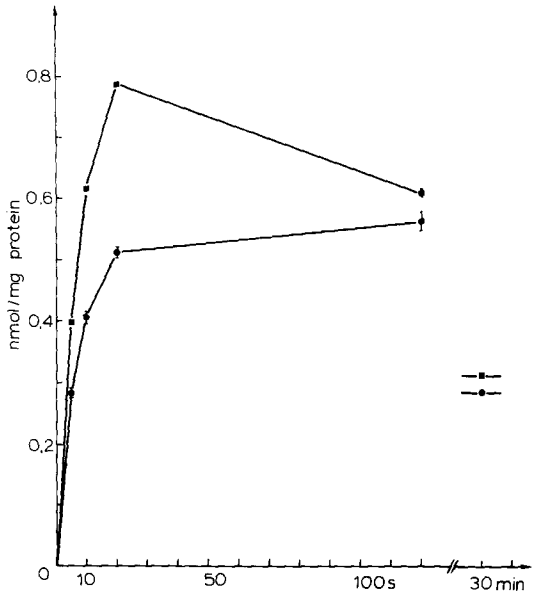

Fig. 1. L-Ascorbate uptake under the influence of a gradient of Na^+. The incubation media contained 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5), 0.02% KN_3 (2.5 mM), 0.3 mM L-[^{14}C]ascorbate, 0.15 mM DTT and the following sodium salts: 100 mM NaN_3 (▪) or 100 mM sodium cyclamate (•). The vesicles had been prepared in a solution containing 25 mM choline sulphate in addition to the usual constituents (300 mM mannitol, 10 mM Tris-Hepes (pH 7.5), 0.02% KN_3). Incubation temperature was 23°C. In this and the following figures the bars indicate S.D. When not drawn, the S.D. values were smaller than the symbols used.

precise comparison between the data in Table I and the corresponding data in Ref. 7 cannot be made. It is clear, however, that the V of L-ascorbate uptake in kidney membrane vesicles from the rat and guinea pig is much larger than in small intestinal brush border membrane vesicles from the guinea pig.

In most of the work to be reported below, rat kidneys were used, although kidneys from guinea pigs or rabbits were also used in some experiments for comparison, yielding essentially identical results.

2. Binding vs. transport across the membrane

The ratio between the substrate concentration in the medium and the substrate associated with the vesicles at equilibrium (30 min) was essentially the same for L-ascorbate and for D-glucose and was independent of the kind of salt present and of ionic strength: in the various experiments this ratio for either substrate was essentially identical, ranging between 700 and 1000 ml per mg protein. Thus,

TABLE I

K_m AND V OF L-ASCROBATE UPTAKE AND K_i OF D-ISOASCORBATE IN MEMBRANE VESICLES FROM RAT AND GUINEA PIG KIDNEY

The kinetic overall constants were determined from 5-s uptake values measured at 23°C in the presence of an initial NaSCN or a Na_2SO_4 gradient (out → in). The membrane vesicles were exposed to media of the following compositions: 300 mM mannitol; 10 mM Hepes/Tris, pH 7.5; 0.02 or 0.03% NaN_3 (all these components were present both in the intra- and extra-vesicular media). At the start of the incubation the outer medium contained, in addition, variable concentrations of L-[^{14}C]ascorbate (from 0.3 to 1 mM), DTT (at one half the concentration of L-ascorbate), and, finally, either 100 mM NaSCN or 50 mM Na_2SO_4.

Species	Salt gradient initially (out → in)	K_m (mM)	V (pmol per mg protein per 5 s)
Rat	NaSCN	0.288 ± 0.024	371 ± 19
	Na_2SO_4	0.114 ± 0.012	760 ± 47
Guinea pig	NaSCN	0.747 ± 0.142	177 ± 19
	Na_2SO_4	0.351 ± 0.073	221 ± 18

the amount of L-ascorbate bound to the membrane was negligible small as compared to that present in the osmotic space. The volume of the vesicles in Fig. 1 as calculated from these ratios was approx. 1 μl per mg protein, i.e., it was not much different from the volume of similar vesicles prepared from small intestinal brush border membranes (e.g., Ref. 7).

3. Fully-competitive inhibition and counterflow by erythorbate

The epimer of L-ascorbate at C_5, i.e., D-erythorbate (D-isoascorbate) inhibited L-ascorbate uptake into vesicles of rat cortex (K_i, approx. 16 mM). The inhibition was fully-competitive (data not shown). Fig. 2 shows a counterflow experiment in which D-erythorbate was used as the elicitor. The experiment was carried out in the presence of both Na^+ and monactin, 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 accumulation of L-

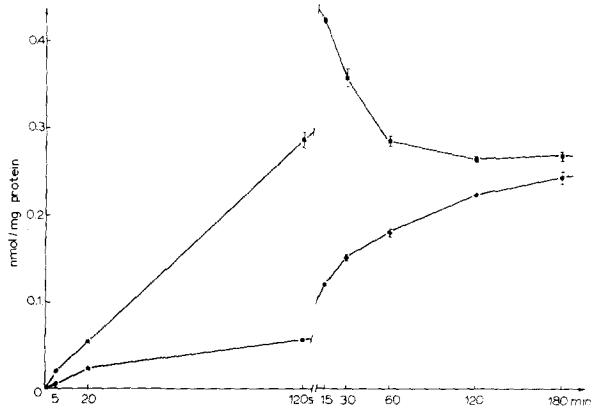

Fig. 2. Counterflow experiment in rat kidney vesicles with D-isoascorbate as elicitor in the internal compartment. The vesicles were pre-incubated in 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5), 100 mM $NaNO_3$, 10 μg monactin per mg protein and either 150 mM Tris-erythorbate (▪) or 150 mM mannitol (•) for 60 min at 23°C. 5 μl vesicles were then mixed with 45 μl of a solution with the following composition: 300 mM mannitol, 10 Tris-Hepes (pH 7.5), 100 mM $NaNO_3$, 0.33 mM L-[^{14}C]ascorbate and 0.165 mM DTT.

ascorbate could not be ascribed to indirect coupling (e.g., via movements of elicitors and/or Na^+ via transporters other than that of L-ascorbate) and must be due to a counterflow mechanism. We concluded that D-erythorbate was, in all likelyhood, another substrate of L-ascorbate transport system (we had no labeled erythorbate at our disposal to test this conclusion directly).

Clearly, the L-ascorbate transport system in kidney cortex membrane vesicles fulfils the classical kinetic criteria of a 'mobile' carrier (i) saturation by the substrate (previous section); (ii) competitive inhibition and (iii) counterflow. In agreement with current ideas we do not imply a 'rotating' or 'diffusing' carrier.

4. Cation dependence

The experiments of Table II and Fig. 3 show that Na^+ present in the outer compartment strongly accelerates L-ascorbate uptake into kidney membrane vesicles from rats or rabbits. Similar results were obtained with vesicles from the small intestine of either guinea pig [7] or man [8], but not of either rabbits [7] or rats (Murer, H., personal communication). The observed acceleration of uptake might be explained in either of two ways: (i) by an indirect, electrical coupling: the entry of the L-ascorbate anion

TABLE II

Na^+-DEPENDENCE OF L-ASCORBATE UPTAKE IN THE PRESENCE OF SALT GRADIENTS

The incubation media were the same as those in the experiments in Table I, except for the additions at time zero, which were as follows: 0.3 mM L-[^{14}C]ascorbate, 0.15 mM dithiothreitol and the salts indicated.

Species	Salts in the medium	L-ascorbate uptake (pmol per mg protein per 5 s)
Rabbit	100 mM $NaNO_3$	155 ± 10
	100 mM KNO_3	9 ± 3
Rat	25 mM Na_2SO_4	111 ± 3
	25 mM K_2SO_4	8 ± 1

would be braked by the negative membrane potential which it builds up. The entry of Na^+ would make collapse this $\Delta\psi$. Alternatively, Na^+ for wich the membrane has a larger permeability than for L-ascorbate, might 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 ascorbate would be transported by the same transport agency.

Mechanism (i) can be ruled out from the experiment shown in Table II: in fact, in indirect coupling, cations other than Na^+ should be able to drag ascorbate into the vesicles provided that the permeability for them is large enough; this is, however, not the case. Even in the presence of valinomycin potassium (100 mM outside, 0 mM inside) does not increase the small residual uptake of ascorbate observed in the absence of Na^+. The movement of L-ascorbate is therefore directly coupled with that of Na^+.

5. $\Delta\psi$-dependence of renal Na^+-dependent L-ascorbate transport

In previous papers we have studied the Na^+-dependent L-ascorbate transport in brush border membrane vesicles from guinea pig [7] or human small intestine [8]. In both membranes it was found that Na^+-dependent L-ascorbate transport was electroneutral, i.e., in the presence of Na^+ it was unaffected by manipulations of the compositions of the media known to produce $\Delta\psi$ across the membranes, either negative or positive inside the vesicles. This lack of response to

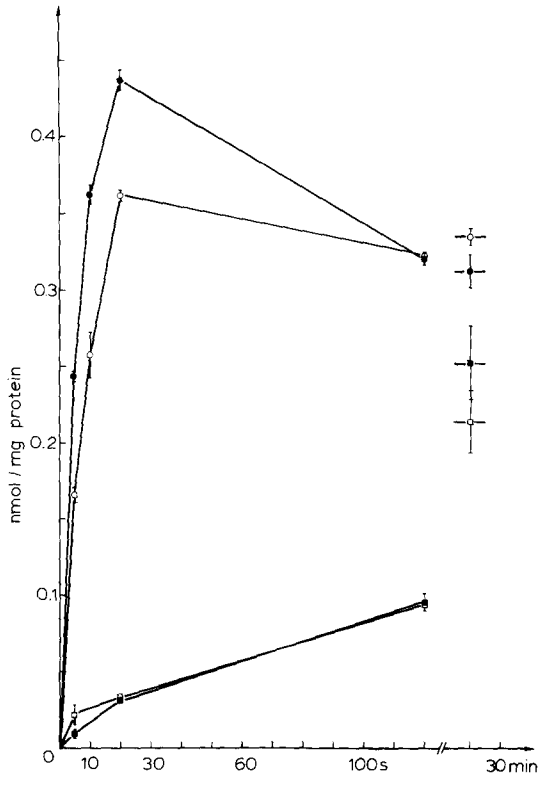

Fig. 3. L-Ascorbate uptake in rat kidney vesicles in the presence and absence of Na^+ and at different membrane potentials. □ and ○, the vesicles were preincubated with 10 μg valinomycin per mg protein for 60 min at 23°C (valinomycin was dissolved in ethanol; the final ethanol concentration in the preincubation was 2%). Control vesicles (■ and ●) were treated the same way with ethanol being added instead of the valinomycin solution. Final composition of the incubation media: 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5), 0.02% KN_3 0.15% DTT, 0.3 mM L-[^{14}C]ascorbate and the following salts (initial concentrations in the outer media only): 100 mM NaN_3 + 50 mM K_2SO_4 (●, ○) or 50 mM K_2SO_4 (■, ○).

$\Delta\psi$ clearly indicated an Na^+ ascorbate$^-$ flux ratio of one in the small intestinal brush border membrane. In contrast, the Na^+-dependent ascorbate transport across membrane vesicles from kidney tubuli of guinea pigs, rats or rabbits is affected by $\Delta\psi$, albeit in a rather complex manner, which depends on the type of the anions present (see section 6 below). Membrane potential differences ($\Delta\psi$) were modulated in three different ways:

(a) *Negative $\Delta\psi$ produced by the diffusion of K^+ (in → out) in the presence of valinomycin.* Table IIIA shows that the presence of valinomycin, which can be presumed to increase the diffusion potential of K^+ and thus lead to a $\Delta\psi$ (negative inside) accelerated the uptake of L-ascorbate. Note, however, that in the absence of N_3^- (Table IIIA) no acceleration is observed (see more below).

(b) *Negative $\Delta\psi$ produced by anion diffusion potentials (out → in) (Table IIIB).* At constant Na^+ concentration gradients (out → in) L-ascorbate uptake depended on the nature of the anion present, the more lipophilic the anion, the more effective being is gradient. (Additional phenomena related with N_3^- and SCN^- will be mentioned below.)

(c) *Reduction of the $\Delta\psi$-acceleration of L-ascorbate uptake by reduction of the $\Delta\psi$.* If the acceleration of L-ascorbate uptake by gradients of highly permeant anions (as described under (b)) is indeed due to a $\Delta\psi$, negative inside, a reduction, or even inversion, of this membrane potential difference via a gradient (out → in) of a highly permeant cation should entail a reduction of this acceleration. Indeed, this was found to be the case: if the N_3^--diffusion (out → in) potential (negative inside the vesicles) was reduced by a parallel valinomycin-induced diffusion of K^+ (out → in) the uptake of L-ascorbate was less accelerated (compare in Fig. 3, ● and ○).

It seems beyond doubt, therefore, that L-ascorbate uptake into kidney brush border vesicles is accelerated by a $\Delta\psi$ (negative inside). For the system to respond to $\Delta\psi$, Na^+ must be present.

6. Effect(s) of N_3^-

This anion had a complex and unexpected effect on L-ascorbate transport in renal brush border membrane vesicles. First of all, its presence (at least 2 mM) was necessary for the L-ascorbate carrier to be accelerated by $\Delta\psi$ in the presence of Na^+. Indeed, in the absence of azide (sulfate or isethionate being the principal anion) ascorbate uptake was inhibited rather than accelerated by $\Delta\psi$, inside negative (Table IIIA). (The Na^+-independent component of L-ascorbate uptake was small and $\Delta\psi$-insensitive and thus cannot account for this inhibition). Azide was apparently necessary for the carrier to respond to $\Delta\psi$, when it was produced by way of a cation-diffusion potential (in → out). (Fig. 3 and Table IIIA). Chloride (100 mM) could substitute for N_3^- in this kind of experiment (data not shown).

N^-_3 stimulated Na^+-dependent L-ascorbate trans-

TABLE IIIA

INFLUENCE OF A K^+/VALINOMYCIN DIFFUSION POTENTIAL ON L-ASCORBATE UPTAKE, IN THE PRESENCE AND ABSENCE OF 10 mM KN_3

The vesicles were preequilibrated with 50 mM K_2SO_4 during 90 min at 20°C. Valinomycin was added to the vesicles to give a concentration of 3.5 μg/mg vesicle protein (0.9% ethanol). Control vesicles were exposed to the equivalent amount of ethanol. At the beginning of the incubation the vesicles were diluted 1 : 10. The incubation media contained: 300 mM mannitol, 10 mM Hepes/Tris (pH 7.5) (all these components were present both in the intra- and extra-cellular media). At the start of the incubations the following were added: 0.2 mM L-[^{14}C]ascorbate, 100 mM sodium isethionate, 5 mM K_2SO_4 and 0 or 10 mM KN_3. Incubation time 5 s.

Initial concentrations in the outer medium (mM)		Valinomycin	Expected $\Delta\psi$ negative inside the vesicles	L-Ascorbate taken up (pmol per mg protein per 5 s)
Sodium isethionate	KN_3			
100	10	–	small	340 ± 4
100	10	+	large	417 ± 5
100	0	–	small	72 ± 6
100	0	+	large	54 ± 2

port also in the absence of $\Delta\psi$, i.e., the effect of N_3^- is not confined to mediating the response to $\Delta\psi$ of the Na^+-dependent L-ascorbate carrier. The stimulation was usually 2- to 5-fold and was half maximal at azide concentrations in the 2–10 mM range. In the presence of an initial 100 mM to 0 Na^+ gradient it was independent of the nature of the other anion present and was found also in vesicles which had been preequilibrated with 10 mM K_2SO_4 in the presence of valinomycin and FCCP (Table IV, under these conditions any $\Delta\psi$ established by an N_3^- gradient would have been short circuited by the ionophores). In addition, the stimulation by N_3^- was independent on the pH; the addition of FCCP neither affected L-ascorbate uptake nor abolished its N_3^- activation. Therefore, activation by N_3^- is not related to its properties of 'uncoupled' or proton-carrier.

The major parameter affecting the stimulation of

TABLE IIIB

EFFECT OF ANION GRADIENTS ON L-ASCORBATE UPTAKE

Uptake was measured at 4 s in solutions of the following compositions: 300 mM mannitol, 10 mM Hepes/Tris (pH 7.5), 0.2 mM L-[^{14}C]ascorbate, 0.1 mM dithiothreitol and the Na^+ salt gradients listed in the table.

Na^+ salt added to vesicles at t = 0 along with L-ascorbate		L-Ascorbate taken up (pmol per mg protein per 4 s)
NaN_3	100 mM	252 ± 7
$NaNO_3$	100 mM	105 ± 3
NaCl	100 mM	91 ± 3
Na_2SO_4	50 mM	64 ± 6
Sodium isethionate	100 mM	64 ± 2
Sodium cyclamate	100 mM	51 ± 2

TABLE IV

STIMULATION OF L-ASCORBATE UPTAKE BY 2.5 mM KN_3 IN VESICLES EQUILIBRATED WITH 20 mM K^+, VALINOMYCIN AND THE UNCOUPLER FCCP

The vesicles were preequilibrated with 100 mM choline sulfate and 10 mM K_2SO_4 for 1 h at room temperature, then valinomycin (20 μg/mg protein) and FCCP (10 nmol/mg protein) were added dissolved in ethanol. Controls received the equivalent amount of ethanol. Composition of the incubation medium: 300 mM mannitol, 10 mM Hepes/Tris (pH 7.5), 10 mM K_2SO_4, 50 mM Na_2SO_4, 0.2 mM L-[^{14}C]-ascorbate and 0 or 2.5 mM KN_3.

$[N_3^-]$ (mM)	Ionophores present	L-Ascorbate taken up (pmol per mg protein per 5 s)
0	None	47 ± 5
2.5	None	85 ± 7
0	valinomycin, FCCP	50 ± 2
2.5	valinomycin, FCCP	111 ± 8

L-ascorbate uptake by N_3^- was found to be the concentration of Na^+. Indeed, at $Na^+ \leqslant 20$ mM, azide failed to activate and even inhibited L-ascorbate uptake (Fig. 4). As a consequence, the Na^+ activation curve was markedly sigmoid in the presence of N_3^-. Azide increased the maximum transport velocity V approx. three to four times (not shown). As a whole, therefore, azide seems to increase critically the overall mobility of the renal L-ascorbate carrier and, probably as a consequence, to make it more responsive to the Na^+ gradient or to the combined Na^+ and membrane potential gradient. It is as if N_3^- would mediate or modulate the interaction of the carrier with Na^+. Be as it may, this activation by N_3^- prevails over the short-circuiting effect of this lipophilic anion on the $\Delta\psi$ elicited by cation diffusion (Table IIIA).

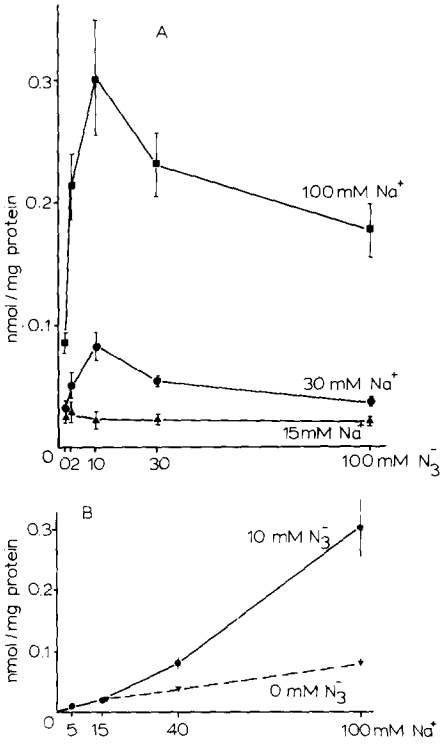

Fig. 4. (A) Stimulation of L-ascorbate uptake by different concentrations of N_3^-, at various initial outer concentrations of Na^+ (15, 40 and 100 mM). Incubation time was 5 s. The vesicles were suspended in 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5), 35 mM choline sulphate. Composition of the incubation media: 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5), 0.2 mM L-[^{14}C]ascorbate, 0.2 mM DTT, KN_3 at the concentrations indicated on the abscissa and (■) 50 mM Na_2SO_4, (●) 15 mM Na_2SO_4 + 35 mM choline sulphate, (▲) 7.5 mM Na_2SO_4 + 42.5 mM choline. Incubation temperature: 20°C. (B) Na^+-concentration dependence of L-ascorbate uptake in the presence of 0 mM (▾) or 10 mM (●) N_3^-. The values are taken from the same experiment as in Fig. 4A.

The mechanism of this action of N_3^- is ill-understood. It does not involve a slow change in the carrier molecule, since N_3^- activation of L-ascorbate is present also in 0.8-s incubations and the effect of N_3^- is immediately reversed upon dilution. It does not involve ferric ions or other transition metals (for which N_3^- is a good ligand), because a similarly good ligand, CN^-, at 10 mM concentration fails to affect L-ascorbate uptake. A chemical modification of L-ascorbate by N_3^- in the presence of membrane vesicles could also be ruled out.

7. Is renal ascorbate uptake driven by proton gradient or proton-coupled?

Some of the observations presented in the previous section made an involvement of H^+ in renal ascorbate uptake unlikely. However, it was of some interest to investigate this problem directly, particularly in view of the Na^+-H^+ exchange system present in this membrane. The experiments in Fig. 5 rule out H^+ fluxes or H^+ gradients as major factors in the transmembrane movement of ascorbate in vesicles from the proximal tubuli. In fact ascorbate uptake in the presence or in the absence of an Na^+ gradient is unaffected by the H^+-carrier FCCP, and an (out → in) H^+ gradient does not accelerate ascorbate uptake. (In the pH range 5–8 ascorbate uptake is not affected by the pH). In addition, the possible effect of an (in → out) gradient was also tested, but none was found (data not shown).

8. Miscellaneous observations

In all inhibition studies it was always made sure that the amount of L-ascorbate found to be associated with the vesicles at long incubation times was not affected by the inhibitors. This ruled out the possibility that non specific damage of the vesicles may have simulated inhibition of transport.

Thiocyanate. Another anion often used in probing the effect of $\Delta\psi$ on transport systems, SCN^-, inhibits L-ascorbate uptake (data not shown). This inhibition which, much as the activation by N_3^-, is not due to a chemical modification of the substrate, was not subjected to detailed investigation.

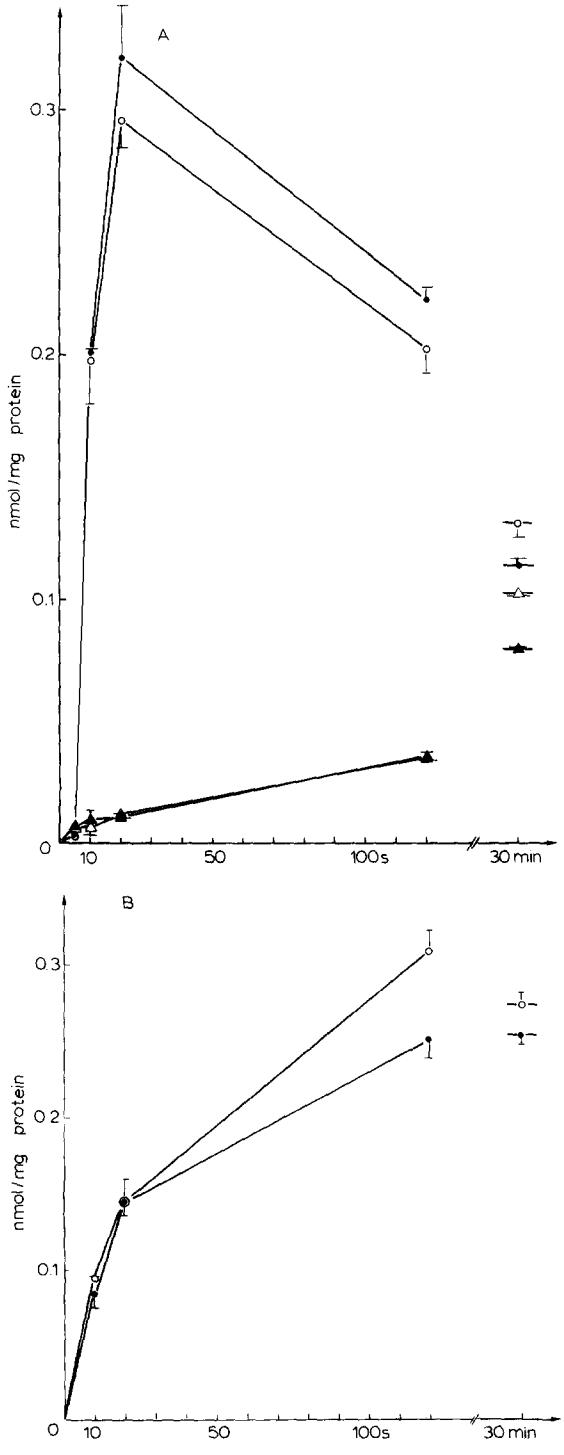

Fig. 5. Lack of effect of FCCP on L-ascorbate uptake. (A) In the presence of a Na_2SO_4 (○, ●) or a K_2SO_4 (△, ▲) gradient (50 mM out, zero in). Conditions: 10 mM Tris-Hepes (pH 7.5), 300 mM D-mannitol, 0.02% KN_3, 0.3 mM DTT, 0.6 mM L[^{14}C]ascorbate and 10 μM FCCP (filled symbols) or without FCCP (open symbols). (B) In the presence of a H^+ gradient and an Na_2SO_4 gradient. Conditions, as in (A) except that the vesicles were preincubated in 50 mM Tris-Hepes (pH 7.5) and incubated in 50 mM Tris-Mes (pH 5.25). ●, with 80 μM FCCP; ○, without FCCP.

Intravesicular K^+. The accelerating effect of K^+ gradients (in → out) has been discussed in the previous section as being mediated by the membrane potential. In principle, however, a direct coupling should also be considered, particularly in view of the recent reports [16,17] that the driving force of L-glutamate uptake into kidney membrane vesicles is provided by the combined gradients of Na^+ (out → in) and of K^+ (in → out). In the case of L-ascorbate transport, however, a similar role of intravesicular K^+ is very unlikely (Fig. 6): in membranes electrically short-circuited with NO_3^-, the outwardly directed K^+ concentration gradient does not stimulate L-ascorbate uptake.

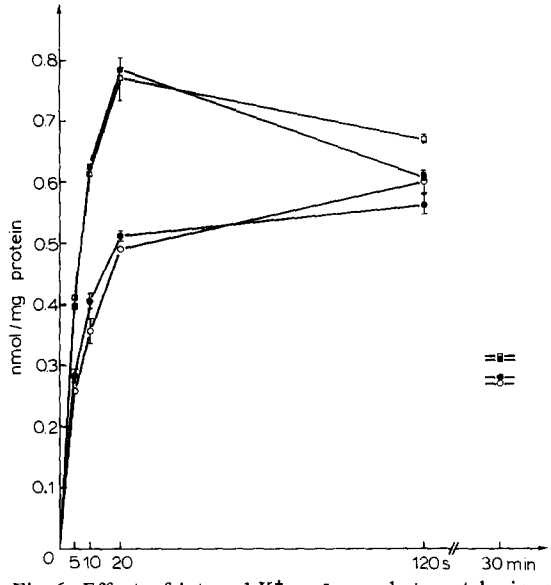

Fig. 6. Effect of internal K^+ on L-ascorbate uptake in membrane vesicles of rat kidney. The vesicles were preincubated in 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5), 0.02% KN_3 and 25 mM choline sulphate (□, ○) or 25 mM K_2SO_4 (■, ●) for 60 min at 23°C. To start the incubation, the preincubated vesicles were diluted 10-fold with a solution of the following composition: 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5), 0.02% KN_3, 0.33 mM L[^{14}C]ascorbate, 0.165 mM DTT, and 110 mM NaN_3 (■, □) or 110 mM sodium cyclamate (●, ○).

D-Glucose and phlorizin. The transporter of L-ascorbate is not identical with that (those) of D-glucose. In fact, phlorizin, a well-known inhibitor of D-glucose uptake in kidney tubuli [18,19], failed to inhibit (when tested at 0.2 mM concentration) the uptake of L-ascorbate. However, D-glucose did inhibit L-ascorbate uptake, the inhibition being detectable at 0.2 mM and maximal (approx. 30%) at approx. 100 mM glucose. This effect of glucose was not present or was strongly reduced if phlorizin was present (0.25 mM). This indicated that glucose inhibition of L-ascorbate uptake probably occurred via the glucose transport system(s). Although not studied further, this phenomenon was likely to be one more example of the heterologous inhibition, which is often found among Na^+-dependent, electrogenic transport systems [20–22] and may be mediated by the glucose-coupled Na^+ entry [22,23]. Alternatively, D-glucose may interact with the L-ascorbate carrier at the intravesicular side, i.e., after having been taken up if the substrate inhibitor specificity of this carrier is different at the two sides of the membrane. (The monosaccharide carrier of the erythrocyte membrane e.g., does show this difference in substrate-inhibitor specificities).

Dehydroascorbate. This substance (0.3 mM) was taken up very little, if at all, by kidney membrane vesicles (less than 5% of L-ascorbate). It did not significantly inhibit L-ascorbate uptake.

Discussion

1. General features

The determination of overall kinetic parameters of substrate uptake into vesicles requires some important conditions to be fulfilled. Perhaps the most important is that the trans concentration(s) of substrate(s) be negligible small. In fact, trans effect cannot always be satisfactorily accounted for a priori, particularly since some substrates can act as modulators at one side of the membrane, but not at the other (e.g., Ref. 27) *. Ideally, initial rates of uptake should be determined. Furthermore the driving force(s) should be kept constant. Naturally, a driving force such as $\Delta\psi$ tends to dissipate quickly; ** it follows that incubations must be as short as possible and be limited to that very initial period of time during which the effect of the dissipation of the driving force(s) is negligible small. The experimental precautions and the theoretical background have been discussed at some length elsewhere in connection with Na^+, D-glucose cotransport [11,13], and will not be repeated here.

The L-ascorbate uptake system studied in the present work is even less sensitive to $\Delta\psi$ than D-glucose transport; however, the low capacity of the system and the rather low specific radioactivity of the substrate made reliable measurements impossible at incubation times shorter than 2–5 s. As a result, the overall kinetic parameters in Table I should be regarded as fairly good approximations but not as exact measures of initial K_m and V. At any rate, there is no doubt that the renal L-ascorbate transport system shows saturation and that it is inhibited fully-competitively by D-erythorbate.

2. Renal vs. intestinal ascorbate transport

The renal ascorbate carrier has some features in common with the intestinal carrier of this subsgance (in guinea pig [7] and in man [8]). It has the kinetic characteristics of a 'mobile carrier' (Table I; Fig. 2); it is activated by external Na^+ (Table II); it is inhibited competitively by D-erythorbate (D-isoascorbate) ($K_i \simeq 16$ mM) which is in all likelyhood another substrate

* Others in a congress report [28] have propounded the use of tracer exchange in short-circuited membrane vesicles for measuring 'true' K_m values. In addition to the complications arising from the high trans-concentrations of the substrate(s) (see above), it should be noted that the tracer exchange rates reported follow neither a zero nor a first-order kinetics, probably due to the heterogeneity of the vesicles both in size and form. This, plus the very high 'leak' rate required sizeable corrections, which were introduced either on the basis of the exchange rates observed in the presence of an inhibitor, or by manipulating the data so as to obtain 'the best fits'. In the latter procedure the gratuituous assumption was made that the tracer exchange rates depend on the substrate concentrations according to a single, strict Michaelian function. As a whole, therefore, equilibrium tracer exchange rates do not show any serious advantage over initial rates, even in the case of the high capacity Na^+,D-glucose transport system.

** It should be noted, though, that if $\Delta\psi$ is described by Goldman's equation, the initial $\Delta\psi$ does remain essentially constant (i.e., equal to approximately $(RT/F \ln (P_{Na^+}/P_{anion^-}))$, as long as the intravesicular concentrations of both Na^+ and the lipophilic anion are negligibly small as compared to the respective outer concentrations.

in some respects: whereas the Na^+-dependent L-ascorbate carrier of guinea pig and human small intestine is $\Delta\psi$-insensitive and electroneutral (and thus presumably has an ascorbate: Na^+ flux ratio equal to 1), that of renal cortex is accelerated by manipulations known to produce a $\Delta\psi$, negative inside the vesicles in the presence of Cl^- or of low concentrations of N_3^-.

3. The acceleration by $\Delta\psi$

In principle, the response of a membrane transport system to $\Delta\psi$ can result from one of at least two mechanisms (or a combination thereof): (i) thermodynamic mechanism: the system may involve the net transfer of one or more positive charges across the membrane, i.e., it is 'electrogenic' (or 'rheogenic'); (ii) kinetic mechanism(s): the system may involve no overall net transfer of charges across the membrane (i.e., be electroneutral), but its rate-limiting step may respond to $\Delta\psi$; e.g., the rate-limiting step may involve the movement of a charged portion of the carrier (or of one of the carrier-substrate(s) forms), or be accelerated in some other way by the $\Delta\psi$. We did not meet with success in our attempts to distinguishing between thermodynamic and kinetic mechanisms: in fact, experiments aiming at producing ascorbate accumulation solely with $\Delta\psi$ as driving force (in vesicles preequilibrated with Na^+ and labelled ascorbate) did not yield a clear-cut answer. The 'accumulation' achieved was small and not statistically different from controls with small $\Delta\psi$. On the other hand, this difficulty to detect an unequivocal accumulation did not rule out an electrogenic process, either: in Na^+-equilibrated vesicles ascorbate fluxes generally are small due to a large trans-inhibition by internal Na^+ (compare e.g., Figs. 1 and 2); the potential might therefore have dissipated before a significant accumulation beyond the equilibrium concentration was achieved.

The responsiveness of renal L-ascorbate transport to $\Delta\psi$ was established (see Results) by manipulating the $\Delta\psi$ by way of diffusion potentials, e.g., with valinomycin plus K^+ (in → out) or with gradient of anions (out → in) of different permeabilities. The effect of anions, however, deserves a comment. The uptake of D-glucose into these vesicles via the well defined electrogenic Na^+, D-glucose cotransport system is most effectively promoted by gradients of SCN^-, NO_3^-, N_3^-, and less effectively by Cl^-, SO_4^{2-}, isethionate and cyclamate [15], a sequence which we could confirm (data not shown). Na^+-dependent L-ascorbate uptake also was found to be accelerated by an anion-diffusion $\Delta\psi$, negative inside (Table IIIB), the efficiency of the anion gradients being: $N_3^- \gg NO_3^- > Cl^- > SO_4^{2-} \simeq$ isethionate $\simeq$ cyclamate $\simeq SCN^-$. On the whole, therefore, this sequence is similar to that observed for the Na^+-dependent, electrogenic D-glucose transport and supports the notion that renal Na^+-dependent L-ascorbate transport responds to $\Delta\psi$ (at least in the presence of N_3^- or Cl^-; see Results). As compared with the sequence for D-glucose uptake, the different positions of N_3^- and of SCN^- are remarkable: they are due to the accelerating effect of the former and the inhibitory effect of the latter, respectively, which produce a distortion over the sequence related to their permeabilities.

4. Points of possible physiological significance

One difference between kidney and intestine (not in respect to the single transport system but rather the organ as a whole) is that also those species which are devoid of an intestinal Na^+-dependent L-ascorbate carrier (i.e., rat, rabbit) can reabsorb L-ascorbate from the glomerular filtrate. In addition, the transport capacity in the renal vesicles is much larger than in the intestinal vesicles isolated from guinea pig or human intestines. This fast absorption in the kidney and possibly also the acceleration by the membrane potential (in the presence of Cl^-) may be of physiological significance. It is important that the ascorbate present in the filtrate be totally reabsorbed during the 10 s or so of its transit time in the proximal tubuli [24]. Insofar as the conditions in vitro can be compared with those in vivo, the K_m values in vitro (Table I) are also reasonably close to the L-ascorbate concentration in the blood (1.4 mg%: Ref. 25) and thus in the glomerular filtrate.

Finally, a potential pathological significance may probably be attributed to the competitive inhibition by D-erythorbate: it is possible that this substance, which is easily absorbed in man [26] and thus appears in blood and glomerular filtrate, may preclude a complete reabsorption during the short time of contact of primary urine with the luminal membrane of proximal tubuli of ascorbate.

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