Active Transport of Ascorbate Across the Isolated Rabbit Ciliary Epithelium

Teh-Ching Chu and Oscar A. Candia

The transepithelial transport of ascorbate across the isolated rabbit ciliary epithelium (CE) was investigated. Unidirectional 14 C-ascorbate fluxes were measured in the presence of equal concentrations of ascorbate on both sides of the tissue within the range of 0.025 to 1 mM. The blood to aqueous (Bl → Aq) flux increased from 6 to 95 nmoles/hr and showed nonlinearity and saturation. The aqueous to blood (Aq → Bl) flux increased, for the same range, from 0.5 to 23 nmoles/hr in a linear fashion. The permeability calculated from the Aq → Bl flux was similar to the CE permeability for mannitol suggesting that the Aq → Bl flux is mainly paracellular. The flux ratio Bl → Aq/Aq → Bl was between 4 to 12. Anoxia, ouabain and low Na+ in the media inhibited the Bl → Aq flux indicating that the transport system requires energy and a Na+ gradient. 3-O-methyl-D-glucose, D-isoascorbic acid and phlorizin also inhibited the Bl → Aq flux, suggesting that ascorbate and glucose may share a common carrier mechanism. Although the isolated CE preparation was clearly capable of flux separation and active transport, the rate of ascorbate transport measured in vitro is insufficient to maintain the aqueous ascorbate concentration observed in vivo. Invest Ophthalmol Vis Sci 29:594-599, 1988

Previous studies have shown the existence of a mechanism for active ascorbate transport across the blood-aqueous barrier.1-4 In addition, Becker3 has shown that the in vitro accumulation of ascorbate by the isolated ciliary epithelium (CE) occurs against a concentration gradient, with the tissue-to-medium ratio reaching a value as high as 20 within 1 hr. This process, which required glucose, as well as Na+, K+ and Ca2+ in the medium, exhibited typical saturation kinetics, temperature-dependence and inhibition by ouabain and various metabolic poisons.3 These findings suggest that the ascorbate accumulation by the CE is a carrier-mediated active transport mechanism. Two studies have also shown that the efflux of ascorbate by the CE is by a process of passive diffusion.3,5 These results are comparable to those in other tissues in which ascorbate uptake has been demonstrated to be an energy-dependent Na+-sensitive process.6-15

However, the above studies do not provide information regarding which cell type in the CE contains the ascorbate carrier. Therefore, the translocation steps by which ascorbate moves across the tissue have not been identified. Furthermore, unidirectional and net fluxes have not been measured across the isolated CE in the absence of external driving forces. Since our laboratory can routinely isolate the rabbit CE in an Ussing-type chamber,16,17 the objective of this study was to further characterize the movement of ascorbate across the isolated preparation.

Materials and Methods

Adult female albino rabbits weighing about 3 kg were anesthetized with ketamine (Ketalar, Parke-Davis Co., Detroit, MI) and then sacrificed using air injection into a marginal ear vein. The eyes were promptly enucleated, and the CEs were dissected out and mounted between two Ussing-Zerahn-type chambers with the solution temperature kept at 37°C as previously described.16,17 Animals in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

The basic bathing solution used during the dissection and experiments was as follows (in mM): NaCl, 118; KCl, 4.7; NaH2PO4, 0.8; glucose, 5.5; NaHCO3, 15; CaCl2, 1.8; MgCl2, 1.2; Hepes, 8; Hepes-Na, 8. For experiments requiring a low extracellular Na+ concentration (8.8 mM), an additional solution in which NaCl and NaHCO3 were replaced with choline Cl and choline HCO3, was prepared. The solutions were bubbled with either a 1% CO2-99% air mixture, or a 1% CO2-99% N2 mixture when anoxia was required. The pH of the solutions was 7.5.
Unidirectional aqueous to blood (Aq → Bl) and blood to aqueous (Bl → Aq) ascorbate fluxes were determined under short-circuit conditions at various concentrations of the unlabeled species with thiourea present in the bathing solution. In preliminary experiments in which thiourea was omitted from the solution a net flux of the label could not be measured. It has been demonstrated that thiourea will prevent the oxidation of ascorbate and that the 14C label remains with reduced ascorbate. For these experiments, 2 μCi 14C-ascorbate (New England Nuclear, Boston, MA) were added to the 10 ml of the solution bathing a particular side of the preparation. The concentration of unlabeled ascorbate was either 0.025, 0.05, 0.1, 0.2, 0.5 or 1 mM for a given experiment, and the solution contained thiourea at a concentration level ten times that of ascorbate. The unlabeled ascorbate and thiourea concentrations were the same on both sides of the tissue. After a 30 min equilibration period, samples were taken from both sides of the tissue. Two milliliters were removed from the unlabeled side and replaced with "cold" solution; a 25 μl sample was taken from the labeled side. The unlabeled side was sampled at 15 min intervals for up to 1.5 hr. The flux reached steady state 45 min after addition of the radiolabel.

All samples were mixed with 10 ml of modified Bray's solution (National Diagnostics, Highland Park, NJ), and the activity of 14C was counted in a Beckman (Berkeley, CA) beta scintillation counter. Fluxes are expressed as nmol/hr per preparation.

Unidirectional Bl → Aq and Aq → Bl 14C-ascorbate fluxes were also measured in the presence of the ascorbate concentrations reported for the rabbit blood and aqueous humor. For this experiment, 0.05 mM ascorbate was placed in the blood-side and 1 mM ascorbate was placed in the aqueous-side solution with each side containing appropriate thiourea levels. The sampling procedure was conducted as described above.

The pharmacological agents used in flux experiments were: ouabain, 3-O-methyl-D-glucose (3-O-MG), D-isoascorbic acid (DIA) and phlorizin (all from Sigma Chemical Co., St. Louis, MO); and amphotericin B (E. R. Squibb, Princeton, NJ). Concentrated stock solutions of these drugs were prepared with the bathing solution, prewarmed to 37°C, and were added in a 10 μl volume. Five 15 min interval samples were taken prior to the addition of a given agent to the chamber, and the calculated flux was compared to four flux determinations made after the drug addition.

The effects of low extracellular Na⁺ levels on the unidirectional fluxes were determined after sampling five control periods with the Na⁺-rich solution. For the experimental periods, the low Na⁺ solution replaced the control solution in the chamber, the labeled ascorbate was added to the appropriate side of the preparation, and the sampling procedure was restarted after a 30 min re-equilibration period. Due to a small volume of the Na⁺-rich solution remaining in the chamber after the washout, the final Na⁺ concentration in the experimental solution as measured by flame photometry was about 10 mM instead of 8.8 mM.

### Results

Unidirectional ascorbate fluxes were measured across the isolated rabbit CE under short-circuit conditions within a range of ascorbate concentrations in the bathing solutions from 0.025 to 1 mM. For each determination the ascorbate concentration was the same in both bathing solutions. The results are shown in Table 1. There was a significant difference between the ascorbate fluxes in the two opposite directions with a net flux existing in the Bl → Aq direction. As the ascorbate concentration was increased from 0.025 to 1 mM, the Bl → Aq flux rose and saturated at about 90 nmol/hr when the bathing solution concentration was 0.5 mM. For the Aq → Bl ascorbate fluxes, a relatively linear relationship with respect to the ascorbate concentration was observed. At all ascorbate concentrations, the ratio of unidirectional flux from Bl → Aq versus Aq → Bl was greater than 4. The functional relations of the two fluxes are shown in Figure 1. It could be inferred from this Figure that both unidirectional fluxes would be equal when the aqueous concentration is 1 mM and blood concentration is about 0.1 mM.

In vivo, the concentration of ascorbate in the aqueous is considerably higher than in the plasma. An aqueous ascorbate concentration 20 times higher than the plasma level has been reported for the rab-

### Table 1. Unidirectional 14C-ascorbate fluxes as a function of ascorbate concentration in the bathing solutions

<table>
<thead>
<tr>
<th>Ascorbate concentration (mM)</th>
<th>Fluxes</th>
<th>Flux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(both sides)</td>
<td>Bl → Aq</td>
<td>Aq → Bl</td>
</tr>
<tr>
<td>1.0</td>
<td>95 ± 7 (21)</td>
<td>23 ± 2* (21)</td>
</tr>
<tr>
<td>0.5</td>
<td>91 ± 12 (5)</td>
<td>16 ± 2* (8)</td>
</tr>
<tr>
<td>0.2</td>
<td>51 ± 7 (4)</td>
<td>4.0 ± 0.5* (4)</td>
</tr>
<tr>
<td>0.1</td>
<td>14 ± 1 (16)</td>
<td>1.4 ± 0.3* (14)</td>
</tr>
<tr>
<td>0.05</td>
<td>10 ± 1 (4)</td>
<td>1.0 ± 0.4* (4)</td>
</tr>
<tr>
<td>0.025</td>
<td>6 ± 1 (3)</td>
<td>0.5 ± 0.2* (3)</td>
</tr>
</tbody>
</table>

Flux values are expressed as nmol/h per preparation and are given as the means ± SE with the number of experiments in parenthesis. Ratio: Bl → Aq/Aq → Bl. * Significantly smaller than the corresponding Bl → Aq flux at each ascorbate concentration, P < 0.01.
The Aq/Aq flux was 6 ± 2 nmoles/hr giving a net flux in the Aq → Bl direction; a result predictable from Figure 1. Thus, the in vitro preparation could not maintain the ascorbate concentration gradient observed in vivo.

Table 2. Effects of low Na⁺, phlorizin and amphotericin B on the unidirectional ¹⁴C-ascorbate fluxes with 0.1 mM ascorbate in the bathing solutions

<table>
<thead>
<tr>
<th>Fluxes</th>
</tr>
</thead>
<tbody>
<tr>
<td>( B_1 \rightarrow \text{Aq} )</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Low Na⁺ (10 mM)</td>
</tr>
<tr>
<td>Phlorizin (1 mM) Blood-side†</td>
</tr>
<tr>
<td>Phlorizin (1 mM) Aqueous-side‡</td>
</tr>
<tr>
<td>Amphotericin B (0.01 mM) Blood-side§</td>
</tr>
<tr>
<td>Amphotericin B (0.01 mM) Aqueous-side‡</td>
</tr>
</tbody>
</table>

Flux values are expressed as nmoles/hr per preparation and are given as the means ± SE with the number of experiments in parenthesis. Ratio: \( B_1 \rightarrow \text{Aq} / \text{Aq} \rightarrow B_1 \).

Table 3. Addition of ouabain to both bathing solutions produced a reduction of the unidirectional \( B_1 \rightarrow \text{Aq} \) ascorbate flux to 42% of the control, while the \( \text{Aq} \rightarrow B_1 \) flux increased by 39%. The ratio of the \( B_1 \rightarrow \text{Aq}/\text{Aq} \rightarrow B_1 \) flux became 1.3. Under anoxia, the \( B_1 \rightarrow \text{Aq} \) flux decreased to a level similar to that obtained after ouabain treatment while the \( \text{Aq} \rightarrow B_1 \) flux did not change. The combination of ouabain plus N₂ produced the largest inhibitory effect; the \( B_1 \rightarrow \text{Aq} \) flux fell to 31% of the control and there was no significant net ascorbate flux. The nonmetabolizable glucose, 3-O-MG, elicited a decrease of the \( B_1 \rightarrow \text{Aq} \) flux of 35% while the \( \text{Aq} \rightarrow B_1 \) flux did not change. DIA, a competitive inhibitor of ascorbic acid uptake, inhibited the \( B_1 \rightarrow \text{Aq} \) flux by half while the \( \text{Aq} \rightarrow B_1 \) flux did not change. The ratio of the fluxes was reduced to 53% of the control.

**Discussion**

Some of the previous studies have clearly shown that the ascorbate concentration of the aqueous is
much higher than the plasma, and others report that the isolated iris-ciliary body preparation accumulates ascorbate. The latter studies do not prove by themselves a transepithelial net transport (unless the system is asymmetrical) but in conjunction with the former do provide the necessary evidence for net active transport. Thus, the main results from this study were predictable, but nevertheless provide the first direct measurements of transepithelial ascorbate fluxes in the isolated rabbit preparation in the absence of external driving forces and under a variety of ascorbate concentrations. Our previous measurements of unidirectional Na+ and Cl− fluxes as well as determinations from other laboratories gave large unidirectional fluxes but small or nonsignificant net fluxes. These findings led us to suspect that the isolated preparation may be leaky or that it does not perform as efficiently as it does in situ. In this study the unidirectional ascorbate flux from blood to aqueous was, under equivalent conditions, up to 12 times larger than the opposite flux, indicating the nonsymmetrical behaviour of the isolated preparation. We have previously measured mannitol fluxes across this preparation as an indicator of paracellular permeability and a value of 9 × 10−1 cm/sec was obtained which was comparable to the paracellular mannitol permeability reported for other epithelia. The permeability of the ciliary epithelium to ascorbate can also be calculated from the unidirectional flux and the medium concentration. When this is done for the Aq → Bl value of 10 × 10−7 cm/sec which is similar to the mannitol permeability, is obtained. This suggests that most of the Aq → Bl ascorbate flux is paracellular and that the isolated CE is reasonably “tight” to ascorbate. The Aq → Bl ascorbate movement was also a linear function of the bathing concentration, further indicating the passive nature of this flux. The Bl → Aq ascorbate flux was larger than the Aq → Bl flux within a broad range of concentrations and thus the amount in excess above the passive component must represent transepithelial movement. It should be noted that the flux ratios shown in Tables 1–3 are minimum estimates. If the paracellular component of the fluxes was precisely known and was subtracted from the total measured values, the flux ratios would be considerably larger. Other characteristics of the fluxes were also indicative of carrier-mediated active transport. The Bl → Aq flux showed a typical saturation curve with a Vmax of 104 nmoles/hr and a Km of 0.37 mM. This flux was inhibited by ouabain and low Na+ in the medium, suggesting that the ascorbate flux is coupled to the Na+ movement and driven by the Na+ gradient. The Aq → Bl flux slightly increased in low Na+. This probably reflects a change in paracellular permeability produced by a variation in cell volume as a result of the low Na+ concentration. When net Bl → Aq fluxes, rather than unidirectional fluxes, are used to calculate the transport parameters, a Vmax of 83 nmoles/hr and a Km of 0.37 mM are obtained. These values probably are a better representation of the transepithelial movement of ascorbate.

Previous work showed that DIA, the optical isomer of ascorbic acid, could be used to inhibit the ascorbate uptake by the guinea pig CE. It has also been shown that 3-O-methyl-D-glucose inhibited the transport of ascorbate into the retinal capillary pericytes and retinal pigment epithelial cells. Our studies showed that both the nonmetabolizable glucose and the ascorbate analogue inhibited the Bl → Aq flux, results which are consistent with carrier stereospecificity. Phlorizin, an inhibitor of Na+-glucose cotransporter, also reduced the Bl → Aq flux. Therefore, ascorbate and glucose may share a common carrier mechanism for transport into the isolated rabbit CE. Of all inhibitors tested, ouabain, N2 and their combination were the most potent, indicating the energy-dependency of ascorbate transport.

The CE is composed of two cell layers and the ascorbate carrier could be located in one of four cell membranes. The sidedness of effects of phlorizin and amphotericin B provides some clues as to its location. These drugs were ineffective from the aqueous-side but inhibited transport from the blood-side solution. These effects are consistent with the ascorbate carrier located at the basolateral membrane of the pigmented cells and reinforce previous histochemical studies which have shown that ascorbate was concentrated in the pigmented epithelial cell layer. After its accumulation in the pigmented cells, ascorbate could probably reflect a change in paracellular permeability produced by a variation in cell volume as a result of the low Na+ concentration. When net Bl → Aq fluxes, rather than unidirectional fluxes, are used to calculate the transport parameters, a Vmax of 83 nmoles/hr and a Km of 0.37 mM are obtained. These values probably are a better representation of the transepithelial movement of ascorbate.

### Table 3. Effects of ouabain, 3-O-MG, DIA and N2 on the unidirectional 14C-ascorbate fluxes with 1 mM ascorbate in the bathing solutions.

<table>
<thead>
<tr>
<th>Fluxes</th>
<th>Bl → Aq</th>
<th>Aq → Bl</th>
<th>Flux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95 ± 7 (21)</td>
<td>23 ± 2 (21)</td>
<td>4.1</td>
</tr>
<tr>
<td>Ouabain (1 mM)</td>
<td>40 ± 5* (7)</td>
<td>32 ± 5* (6)</td>
<td>1.3</td>
</tr>
<tr>
<td>N2</td>
<td>41 ± 2* (3)</td>
<td>24 ± 1 (2)</td>
<td>1.7</td>
</tr>
<tr>
<td>Ouabain + N2</td>
<td>30 ± 4* (5)</td>
<td>33 ± 2* (2)</td>
<td>0.9</td>
</tr>
<tr>
<td>3-O-MG (1 mM)</td>
<td>62 ± 5* (6)</td>
<td>24 ± 3 (6)</td>
<td>2.6</td>
</tr>
<tr>
<td>DIA (1 mM)</td>
<td>48 ± 4* (5)</td>
<td>22 ± 1 (5)</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Flux values are expressed as nmoles/hr per preparation and are given as the means ± SE with number of experiments in parenthesis. Ratio: Bl → Aq/Aq → Bl.

* Difference significantly from the control group, P < 0.01.
+ After the addition of each of the pharmacological agents presented in this table, a 30 min re-equilibration period elapsed prior to the reinitiation of the sampling procedure.
diffuse across the other cell membranes into the aqueous, driven by a concentration gradient.

It is interesting to compare the $V_{\text{max}}$ and $K_m$ for the ascorbate transport system obtained in this study with those reported by Becker. In this study $V_{\text{max}}$ was 83 nmoles/hr and $K_m$ was 0.37 mM compared to 12 nmoles/hr and 0.28 mM respectively in the Becker study. They are within the same order of magnitude despite the fact that Becker used guinea pig and the measurements were of accumulation rather than of unidirectional transepithelial flux. It seems clear that transepithelial ascorbate transport occurs by active Na+-coupled uptake at the basolateral membrane of the pigmented cells followed by diffusion across the other cell membranes. Diffusion must be the rate-limiting factor; otherwise the tissue would be unable to accumulate ascorbate.

It should be noted that the rate of ascorbate transport which we measured in the isolated preparation is insufficient to maintain the in vivo ascorbate concentration in the aqueous chamber. In vivo, the rate of flow of aqueous humor in the rabbit eye determined by fluorophotometry is about 1.9 μl/min which represents a loss of ascorbate through the outflow pathways of 114 nmoles/hr. Thus, a $\text{Bl} \rightarrow \text{Aq}$ transport of 114 nmoles/hr is necessary to maintain the steady state ascorbate concentration in the aqueous humor. At 0.05 mM—the plasma concentration—the ascorbate flux was only a fraction of the in vivo value. Even at 1 mM the observed flux was less than the in vivo flow. It is possible that when ascorbate is presented to the processes by perfusion through the capillaries it could be more effectively transported than when it is simply part of the bathing solution, since diffusion gradients could be a limiting factor. Of course, the isolated preparation may not be operating as effectively as it does in vivo. That the in vitro transport is less than that observed in vivo was also observed, but not discussed by Becker. The $V_{\text{max}}$ required in vivo to maintain aqueous ascorbate concentration in guinea pig aqueous humor was 180 nmoles/hr whereas the $V_{\text{max}}$ for the in vitro accumulation was only 12 nmoles/hr, a larger discrepancy than with our flux studies in the rabbit. Although the in vitro preparation may not function exactly as the in vivo situation, it is also possible that an additional source of aqueous ascorbate secretion exists in vivo. The ascorbate concentration of the vitreous is also considerably higher than its concentration in plasma. Ascorbate uptake by the retina has been studied, but a unidirectional net ascorbate transport across the blood-vitreous barrier has never been measured. The possibility for ascorbate transport across the isolated retinal pigment epithelium needs to be investigated.

**Key words:** rabbit, ciliary epithelium, ascorbate flux, active transport

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**References**