A Comparative Study of Ascorbic Acid Entry into Aqueous and Vitreous Humors of the Rat and Guinea Pig

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The transport rates of radiolabeled ascorbic acid and dehydroascorbic acid, as well as, labeled 3-O-methyl-D-glucose and L-glucose from a central plasma compartment into aqueous and vitreous humors and cerebrospinal fluid were studied in vivo. Normal, male albino Sprague-Dawley rats and English Short Haired guinea pigs were used to explore the mechanism of ascorbic acid entry into ocular humors in a species that can produce ascorbate (the rat) and one that cannot and, like humans, is dependent on dietary sources (the guinea pig). In vivo kinetic studies allowed for the calculation of entry rate constants, \( K \) (min\(^{-1}\)), in double-labeled experiments using L-glucose as an internal passive control. Parallel TLC chromatographic studies were performed to monitor intraocular labeled molecules deriving from the plasma-introduced test molecule. In addition, resting levels of ascorbic acid and D-glucose were determined in order to obtain more reliable data than previously available. Resting levels of D-glucose revealed a consistent pattern of lower levels in aqueous and vitreous humors and CSF than found in plasma for both rat and guinea pig. However, ascorbate levels differed significantly, with the guinea pig demonstrating high ascorbate levels in the aforementioned humors: 58, 77 and 22, respectively, times the circulating plasma value of 0.2 ± 0.2 mg/dl. In contrast, the rat, like the guinea pig, had low plasma ascorbate levels (3.3 ± 0.8 mg/dl) compared to glucose (162 ± 8 mg/dl), with even lower aqueous and vitreous values in a pattern similar to that of D-glucose. In vivo aqueous, vitreous and CSF transport results from the guinea pig indicate active transport mechanisms for ascorbic acid that prefer the ascorbate over the dehydroascorbate moiety and are probably different from the carrier-facilitated diffusion mechanisms for D-glucose, which do not move molecules against a concentration gradient. TLC studies, performed under nitrogen, revealed that only \(^{14}C\)-ascorbic acid was present in aqueous or vitreous humors regardless of whether the radiolabeled pulse was of ascorbic or dehydroascorbic acid. The rat demonstrated little or no carrier involvement, with ascorbic acid crossing into ocular humors at rates very close to those of L-glucose, which is similar in size and is considered to cross the barriers studied via passive diffusion. Saturation studies with unlabeled glucose and glucose inhibitor drugs phloretin (10\(^{-3}\) M) and phloridzin (10\(^{-4}\)) had no apparent effect on ocular entry rates. Dehydroascorbic acid movement was also found to be passive. The rat, which produces ascorbic acid in liver, can supply intraocular humors down a concentration gradient which could arise from intraocular use of ascorbate. Our results suggest that dehydroascorbic acid newly introduced into blood is within 7 min found in intraocular humors to be reduced to ascorbic acid and is in this form available to intraocular tissues of both rats and guinea pigs. Invest Ophthalmol Vis Sci 30:2320-2331, 1989.

Ascorbic acid (vitamin C) appears to be ubiquitous to animal life and, more specifically, to the eye. Species with a genetic inability to synthesize ascorbic acid or ascorbate because they lack the liver enzyme

\[ \text{L-gulonolactone oxidase} \] include humans, lower primates and guinea pigs.\(^{1,2}\) Most others, including the rat, generate ascorbate from D-glucose through gulonic acid. Ascorbate is easily oxidized, both in vivo and in vitro, to dehydroascorbate and further to diketo-L-gulonic acid. Moreover, dehydroascorbic acid also is converted readily back to ascorbic acid.\(^{3}\) This has led to questions concerning which molecular species is transported and which is biologically active.\(^{4-10}\) These questions remain open for many tissue; it is our aim to shed some light on the process of entry into ocular fluids in a species that can synthesize ascorbic acid (the rat) and in the guinea pig, which,
like humans, cannot produce this vitamin. This should lead to a better understanding of the environment and possible requirements of intraocular tissues, including lens, cornea and retina.

There is a general lack of clarity concerning the roles of vitamin C, but some of the most noteworthy hypotheses postulate the vitamin as: a direct chemical co-factor; a link in electron transfer reactions (eg lysine to proline in collagen connective tissue); an antioxidant; a radiation absorber; and a redox coupler involved in stimulating the hexose monophosphate shunt activity. Previous studies have demonstrated some involvement of vitamin C in various intraocular tissues, including retina, iris-ciliary body, retina, and retinal pigment epithelium.

In this study we present evidence suggesting that ascorbic acid enters into aqueous and vitreous humors of the guinea pig against a concentration gradient by an active transport process. Although (14C)-radiolabel following a dehydro-L-ascorbic acid pulse in plasma was found to enter aqueous and vitreous humor faster than expected via simple diffusion (compared to L-glucose) and against a concentration gradient, it did so significantly more slowly than ascorbic acid, and TLC studies indicate that only labeled ascorbic acid (AA) is found in either aqueous or vitreous humors. Our data suggest that ascorbic acid is the preferred form as regards entry into the eye. In contrast, ascorbic acid movement into rat ocular humors was found to be consistent with the idea that movement is via passive diffusion and as a consequence crosses the blood—aqueous and blood—vitreous barriers more slowly than the dehydroascorbic form, which circulates in a more non-ionized, lipid-soluble form at physiologic pH. This statement is based on comparing AA entry rates to those of L-glucose, which is similar in molecular size, as well as saturation studies with cold AA and the lack of response of AA entry rates to transport inhibitor drugs phloretin and phloridzin. As in the guinea pig, TLC radiolabel studies revealed that only the reduced AA form was found to be present in either aqueous or vitreous humors.

Materials and Methods

Transport methods essentially follow those previously published. All experiments were performed in male, albino Sprague-Dawley rats (275—325 g) or English Short Haired guinea pigs (400—500 g) under sodium pentobarbitol (Nembutol) anesthesia (40 mg/kg, ip) using methods conforming to the ARVO Resolution on the Use of Animals in Research.

D-glucose levels in humors and plasma were determined using a D-glucose specific diagnostic kit supplied by Sigma Chemical Co. (St. Louis, MO) that is reliable and uses the coupled reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase and read at 340 nm on a Beckman DU spectrophotometer. Ascorbic acid was determined via modified methods suggested by Ness Pessa based on notes of D. E. Gaasterland, developed from procedures using a 2,6-dichlorophenol-indophenol method. The modifications made by us concerned the instability of ascorbic acid in standard solutions and biologic fluids. Thus, care was taken to insure that samples and standards had not been allowed to degrade appreciably by preparing standard curves using ascorbic acid solutions that were read within 5 min of preparation. Similarly, plasma and humor samples were analyzed and read within 5 min of death and enucleation of the animal. In addition, control blanks were prepared with ascorbate oxidase (Sigma), which allowed for the conversion of ascorbic acid to dehydroascorbic acid (zero ascorbic acid) and corrected for any contribution of test sample proteins, not present in prepared ascorbic acid standards, in decreasing light transmission.

Radiolabeled TLC Chromatographic Studies

Ascorbic acid and its metabolites are unstable in air and in water solutions. Hence, special handling was required to insure that the analysis of radiolabeled molecules in body fluids accurately represented the in vivo situation. TLC silica gel H plates were custom-prepared, incorporating 6% metaphosphoric acid as an ascorbic acid stabilizer (Analtech, Newark, DE). All spotting was performed with freshly obtained samples (within 5 min), under 100% nitrogen (5.0 SLPM), in a specially designed lucite chamber. Glass H plates (20 x 20 cm) were run with 100 ml of solvent in standard glass tanks. Solvent systems used were acetonitrile:butyronitrile:water (66:33:2); and acetic acid:acetone:meethanol:benzene (5:5:20:70). Plates were air-dried (no heat) and later scanned using a Radiomatic Radioscanner RS with RTLC analysis software on a Panasonic AT.

Visualization of spotting standards of ascorbic acid and dehydroascorbic acid was performed with 0.1% dichlorophenol-indophenol in 50% ethanol (Tilman's reagent) and 2,4-dinitrophenyl hydrazine in methanol containing 0.3% HCl. Vapor phase fluorescence was used as a general purpose method for spot development with a VPF chamber using (NH4)HCO3 vapor at 150°C supplied by Analtech, Inc.
Transport Experiment

Each experiment was performed using double-labeling with 5 $\mu$Ci (H) and 10 $\mu$Ci of (14C) label on either L-glucose, 3-O-methyl-D-glucose (mD-glu), ascorbic acid and/or dehydroascorbic acid. Radiolabeled (H)-L-glucose (specific activity (SA): 10.7 Ci/mmol) was supplied by New England Nuclear (Boston, MA). All other forms of radiolabeled glucose, mD-glu (SA: 4.9 Ci/mmol) and ascorbic acid (SA: 17 mCi/mmol) were supplied by Amersham Corp. (Arlington Heights, IL). The labeled dehydroascorbic acid was prepared from ascorbic acid about 20 min before each experiment using modified methods outlined by Heath and Fiddick.\(^{19}\) The ascorbate oxidase used in this conversion was supplied by Sigma. This method is specific and completely converts ascorbate to dehydroascorbate.

In each test animal, the femoral vein was cannulated with PE 50 tubing and connected to a three-way valve on a custom-designed lucite apparatus which allowed for small samples (50–100 $\mu$l) of blood to be withdrawn at will into lightly heparinized collection tubes. The blood samples were centrifuged (1000 $\times$ 10 min) so clear plasma samples could be removed and counted.

At time zero ($t = 0$), a double-labeled bolus of radiolabeled test substances was introduced into the circulation via the cannulated femoral vein using 0.5 ml of saline as the medium. From $t = 0$ to $t = T$, blood samples (50 $\mu$l) were removed from the circulation at 1, 2, 3, 4, 5, 7, 9, 11 and 13 min in the 13 min transport experiments. In longer-term experiments samples were taken at 3–5 min intervals. Tubing samples were sealed with vinyl plastic putty (Cristoseal, Sherwood Med., St. Louis, MO.) and centrifuged (1000 $g$, 10 min) so clear plasma samples could be removed and counted.

At $t = T$ the animal was sacrificed with an overdose of pentobarbital. The eyes were quickly enucleated and samples of aqueous, vitreous and CSF were taken within 2–5 min postmortem. Aqueous humor was obtained by making a cut through the cornea and removing 10–15 $\mu$l samples with calibrated glass constriction pipettes. After the aqueous samples were removed, any remaining humor in the anterior chamber was blotted away. A cut was then made into the back of the eye allowing only vitreous to drain on to a clean glass collecting dish, and calibrated 10–20 $\mu$l samples were taken. CSF was removed with a 25-gauge needle via the foramen magnum and 20 $\mu$l samples were taken for counting.

Calibrated aliquots of plasma and samples of aqueous, vitreous and CSF were counted via liquid scintillation spectrometry (Beckman LS8000) using Liquiscint (National Diagnostics, Somerville, NJ) as the scintillation cocktail. Appropriate corrections for channel spillover and quenching were made with computer assistance to obtain DPM/ml counts for each labeled test substance. The plasma isotopic concentration data $C_p(t)$ versus time for each radiolabeled test molecule was fit graphically to a double exponential decay curve of the form:

$$C_p = A + Be^{-bt} + Ce^{-bt}$$

where A, B, C, b\(_1\) and b\(_2\) are determined constants and A has been shown to be the plasma concentration at $t = \infty$, $C_p(\infty)$. The graphically determined constants were used as first-guess approximations for a “best fit” determination of the same constants via curve fitting the above data using Asyntast (Macmillan Software Co., NY). The data analysis software provided “goodness of fit parameters,” one of which was a multiple correlation coefficient. The graphic analysis we previously used generally resulted in a correlation coefficient, $R^2$, of better than 0.98, whereas Asyntast allowed us to improve our estimates to $R^2$ of 0.992 or better. A simplified system equation was used for the plasma–aqueous transport scheme as previously described,$^{30}$

$$\frac{dC_p}{dt} = K_iC_p - K_aC_A$$

where $C_A$ and $C_p$ refer to concentrations of test substances in aqueous (or vitreous, or CSF) and plasma, respectively. $K_i$ and $K_a$ refer to entry and exit rate constants. The concentration versus time appearance function derived from equations (1) and (2) has been shown to be of the form:

$$C_A(t) = A_{A1} + A_{A2}e^{-K_it} + A_{A3}e^{-bt} + A_{A4}e^{-bt}$$

where

$$A_{A1} = A$$

$$A_{A2} = (K_iB)/(b_1 - K_i) + (K_iC)/(b_2 - K_i) - A$$

$$A_{A3} = (K_iB)/(K_i - b_1)$$

$$A_{A4} = (K_iC)/(K_i - b_2)$$

Constants $A$, $B$, $C$, $b_1$ and $b_2$ are derived from the experiment plasma curve and the entry rate constant $K_i$ is obtained via a computer trial and error search process of the above equations with $C_A(t)$ known experimentally for a given time, $T$. The above analysis does not apply to active transport processes. Active transport was considered by comparing ocular concentration ratios found in longer-term experiments (15 to 90 min experiments) with those found with L-glucose. Active transport was considered to be absent for a given test molecule if rising aqueous (or other humor) concentrations did not continue to rise against a concentration gradient, but instead leveled off to values near those of plasma. Rate constant esti-
mates, however, were made only from short-term experiments where the loss of label through metabolism or other route was considered minimal and only back diffusion across the barrier of interest was considered important. Thus, with active transport eliminated, only carrier-mediated or simple passive diffusion is considered by our analysis.

For D-glucose and analogs, including mD-glu and L-glucose, or where transport is via unrestricted passive or facilitated diffusion, the transport steady state is taken as 1.0 for short-term experiments. This has been shown to be valid for rat and guinea pig where glucose diffusion is carrier-facilitated. Ascorbic acid and dehydroascorbic acid transport steady state estimates were made from transport data and determined resting values, and where passive diffusion was indicated, a ratio of 1.0 was used for the rate constant calculation, as reported in Tables 1 to 4. For guinea pig, where no simple or facilitated diffusion mechanisms were indicated for ascorbic acid, the transport constant, \( K \), was estimated by assuming a steady-state ratio value of 10 which represents an experimental approximation derived from in vivo resting levels in aqueous and plasma.

**Saturation and Transport Inhibitor Experiments**

Saturation experiments were performed by introducing unlabeled ascorbic acid (Sigma) along with the bolus of labeled AA and L-glucose. This resulted in elevated plasma cold ascorbate concentrations of varying degrees, depending on the quantity introduced and the plasma volume of the animal. Accordingly, the quantity of unlabeled AA introduced was adjusted to give desired elevations. Resulting concentrations were determined and mean values were estimated for the period during which the 13 min transport experiment was performed, as previously indicated.

In a similar manner, transport inhibitor drugs were introduced into the test animal circulation at the onset of our transport experiment. The required quantity of drug necessary to elevate the plasma to \( 10^{-3} \) M was estimated on the basis that 5% of the body weight was contained in the animal circulation. Neither phloretin nor phloridzin were soluble in water and 40% ethanol was used to dissolve them before combining them with the radiolable bolus. Both phloretin and phloridzin were obtained from Sigma. Some control experiments were performed with 40% ethanol in the bolus; no change in control entry rate constants was observed.

**Results**

Figures 1 and 2 illustrate determined resting levels of D-glucose and ascorbic acid, respectively, in plasma, aqueous humor, vitreous and CSF in Sprague-Dawley rats and English Short Haired guinea pigs. Our results indicate a consistent pattern of lower resting levels of glucose in aqueous, vitreous and CSF than found in plasma for both normal rats and guinea pigs. Since entry of D-glucose into aqueous and vitreous has been shown to be via facilitated diffusion, the fact that aqueous (and vitreous) levels are lower than plasma levels probably reflects metabolic use by intraocular tissue, including lens, cornea and retina.

Resting levels of ascorbate in the plasma of rats and guinea pigs were found to differ, with the guinea pig demonstrating low circulating levels of less than 0.2 ± 0.2 mg/100 ml (n = 13), whereas the rat had circulating levels of 3.4 ± 0.8 mg/100 ml (n = 15). This probably reflects the difference in or lack of metabolic production of this vitamin in the guinea pig. In
contrast, ascorbate levels in both the aqueous and vitreous humors as well as CSF of the guinea pig were found to be significantly higher than the low levels found in plasma. If a guinea pig mean plasma level is taken as 0.2 mg/100 ml, then resting ratios are about 58, 77 and 22 times that of plasma for aqueous, vitreous and CSF, respectively. Figure 2 reveals a different pattern in the rat. Levels of ascorbic acid in the ocular humors of the rat are significantly lower than in its plasma. Moreover, the pattern observed is similar to that seen for glucose—aqueous levels of 0.5–0.7 times that of plasma and vitreous levels of 0.4–0.6 times. In CSF of the rat, levels higher than plasma were observed, suggesting a difference in the handling of the ascorbic acid requirements for CSF formation than that for intraocular fluids.

Table 1 reports our results from in vivo kinetic experiments, using male Sprague-Dawley rats, which followed the movement of radiolabeled test molecules from a circulating blood plasma compartment to an aqueous humor compartment. Experiments were performed using dual labeling with tritium (3H) and carbon 14 (14C) tags in test molecules, with labeled L-glucose used as an estimate of simple passive diffusion. The first column lists actual data of aqueous/plasma concentration ratios determined at 13 min and 60 min. The short experiments (<13 min) blood curve and aqueous/plasma concentration data were used to calculate entry rate constants, K1, whereas the longer-term experiments were used to estimate steady state ratios. Experiments of 120 and 240 min duration also were performed to aid in the estimate of transport steady-state ratios for the various test molecules. The values used as steady-state concentration ratio for transport from plasma to aqueous are also listed. As is consistent with either passive or facilitated diffusion, the transport steady-state ratios for the molecular species listed was taken as 1.0 for rate constant calculations. Thus, if only a nonrestricted diffusion transport process were involved, then steady state values for plasma and aqueous near 1.0 would be expected. This was confirmed by longer-term experiments.

Table 1 reports no significant difference between the entry rates of L-glucose and ascorbic acid. As predicted by similarities in molecular size and the general nature of the side groups on the glucose and ascorbate moiety, our results suggest that the movement of these molecules from blood into aqueous is via a simple passive diffusion mechanism. Moreover, our results with aqueous humor indicate that the passive movements of ascorbic acid and L-glucose occur at effectively the same rate. This could indicate a more general phenomenon in that the passive movement of these two species could or should be similar in crossing other cells and cell membrane barriers.

Our results in Table 1 indicate that dehydroascorbic acid enters aqueous significantly faster than ascorbic acid or L-glucose, but not nearly as fast as 3-O-methyl-D-glucose. This faster diffusion than ascorbic acid’s could reflect the fact that at physiologic pH dehydroascorbic acid circulates in a more non-ionized, lipid-soluble form and thus diffuses across membranes more readily. Our data suggest that although carrier mediation of dehydroascorbic acid cannot be ruled out, the contribution of any carrier involvement would be minimal.

Similar results were seen (Table 2) across the blood–vitreous barriers of the rat. Again, L-glucose and ascorbic acid movements are slow and similar in magnitude, while dehydroascorbic acid movement is somewhat faster. These results suggest a passive entry, in the rat, of all the mentioned species except for the D-glucose analog, mD-glu. Our longer-term experiments give some indication that, as regards ascorbic acid and dehydroascorbic acid movement into vitreous humors, processes other than simple diffusional entry may become more important with time. That is, although our entry rate calculations based on short-term experiments clearly indicate simple passive entry, the fact that more label is seen at

<table>
<thead>
<tr>
<th>Table 1. Blood–aqueous transport: Sprague-Dawley rats*</th>
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<tbody>
<tr>
<td><strong>Data ([Ca/Cp])</strong></td>
</tr>
<tr>
<td>13 min</td>
</tr>
<tr>
<td>60 min</td>
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<tr>
<td><strong>Transport steady state</strong></td>
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<tr>
<td><strong>Entry rate K1 (min⁻¹)</strong></td>
</tr>
<tr>
<td>L-glucose</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>3-O-methyl-D-glucose</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
</tr>
<tr>
<td>11</td>
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<tr>
<td>Dehydroascorbic acid</td>
</tr>
<tr>
<td>7</td>
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</tbody>
</table>

* Values listed as mean ± SD with the number of experiments in parenthesis.
Table 2. Blood–vitreous transport: Sprague–Dawley rats

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<th>Blood–vitreous transport: Sprague–Dawley rats</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>L-glucose</td>
<td>3-O-methyl-D-glucose</td>
<td>L-ascorbic acid</td>
</tr>
<tr>
<td></td>
<td>Data (Cv/Cp)</td>
<td>Data (Cv/Cp)</td>
<td>Data (Cv/Cp)</td>
</tr>
</tbody>
</table>
|        | 13 min                                        | 60 min           | Transport steady state | Entry rate K
|        | 0.19 ± 0.02                                   | 0.44 ± 0.06      | ~1.0             | 0.0001 ± 0.0013 |
|        | (12)                                          | (7)              |                  | (12)               |
|        | 0.44 ± 0.04                                   | 0.88 ± 0.04      | ~1.0             | 0.0046 ± 0.003 |
|        | (11)                                          | (8)              |                  | (11)               |
|        | 0.30 ± 0.04                                   | 0.84 ± 0.09      | ~1.0             | 0.0083 ± 0.0017 |
|        | (13)                                          | (5)              |                  | (13)               |
|        | 0.39 ± 0.06                                   | 0.85 ± 0.06      | ~1.0             | 0.0013 ± 0.004 |
|        | (7)                                           | (5)              |                  | (7)                |

* Values listed as mean ± SD with the number of experiments in parenthesis.

longer times (60 min) may reflect the importance of some type of use or metabolism of ascorbic acid or dehydroascorbic acid after it enters the vitreous compartment.

Figure 3 illustrates our results where plasma ascorbic acid concentrations were elevated during the period when entry rate constants were determined. As previously, L-glucose was used as a passive transport marker. Entry rates for L-glucose and AA were determined for various mean ascorbic acid concentrations from 3–250 mg/100 ml plasma. The 3 mg/100 ml value was our control value where 0 ascorbic acid had been introduced and, thus, represented resting levels. As expected, we found no significant change in L-glucose plasma–aqueous or plasma–vitreous entry rate constant. If ascorbic acid entry was in any way carrier-mediated, then a decrease in ascorbic acid entry rate with high plasma ascorbate should be expected. We found that our calculated rate constants did not change significantly even with 200 mg/dl ascorbic acid circulating in plasma. At higher plasma AA levels, an apparent (not significant, P > 0.02) drift in entry constant to higher levels could reflect an osmotic increase in passive permeability, but this remains unclear.

Table 3 reports our results with the glucose transport inhibitors phloretin and phloridzin. We saw no significant (P < 0.001) decrease in either passive L-glucose or ascorbic acid entry rates with relatively high plasma levels of these drugs, which are considered to affect both facilitated glucose transport and sodium-sensitive active glucose transport. This would suggest that AA movement into aqueous humor is independent of these transport inhibitors.

Figure 4 illustrates rate constants calculated on the basis of experiments of different time duration, T. Each rate constant was calculated using a specific plasma decay curve, specific ratio at end time T, and transport steady-state estimate. Our results suggest that the rate constants calculated by us are not strictly independent of the length of the transport experiment. This probably reflects the fact that the assum-
tions made in our transport modeling, such as complete mixing and no metabolism of the test molecule, are not exactly true and become less valid as time proceeds. Nevertheless, results on a comparative basis for the different molecular species involved hold up quite consistently for both rats and guinea pigs. Thus, the transport of L-glucose and ascorbic acid for all experiments was nearly identical while that of dehydroascorbic acid was, as previously indicated, faster than ascorbic acid, but still likely to have occurred via a passive diffusion mechanism.

Tables 4 and 5 report analogous but quite different transport results with the guinea pig. Our results indicate a carrier-facilitated mechanism for D-glucose entry into aqueous and vitreous humor of the guinea pig. The entry rate constant for mD-glu is significantly greater \( (P < 0.001) \) than for L-glucose, even though the mD-glu molecule is encumbered with an extra methyl group. The concentrations of both L-glucose and D-glucose in the ocular humors never exceeded those in plasma, suggesting the absence of active transport processes. Thus, glucose transport mechanisms for entry into the aqueous and vitreous humors demonstrate a degree of similarity in both the rat and the guinea pig, which would be consistent with carrier-facilitated diffusion. In contrast, no similarity was noted with ascorbic acid movement into either ocular humor in these species. By 20 min, the concentration of radiolabeled L-ascorbic acid in the aqueous humor of the guinea pig is 56% greater than in the plasma from which it originated. Labeled ascorbic acid entry into the aqueous is much faster than via simple diffusion, as indicated by the dramatic difference in concentration ratio when compared to L-glucose, which is similar in size and atomic composition. More importantly, the concentration of labeled ascorbic acid continues to increase with time and even against a concentration gradient. Thus, the presence of a specific active transport mechanism for the transport of ascorbic acid from blood to aqueous or vitreous humors in the guinea pig can be inferred from these data.

Since transport is active, no clear transport steady state could be achieved that would allow us to estimate a simple entry rate constant. To overcome this apparent difficulty, we noted that the dominant process in accumulating the label in the aqueous humor at these experimental times (20 min) is entry rate-limited. Thus, the actual steady state need not be an important factor in our entry rate calculation. In fact, Figure 5 shows that as long as we are not near steady state and the collecting compartment is relatively large, the actual value taken for the transport steady state is not essential for estimating an entry rate constant. As noted previously, resting ratios of ascorbic acid to plasma concentration for ascorbic acid are high enough to warrant the approximation that accumulation is entry rate-limited. Thus, a steady state ratio of 10 was used in our calculation of entry constant, \( K_i \), reported in Tables 4 and 5. As indicated by the high resting levels of ascorbic acid in both aqueous and vitreous humors and low plasma levels

### Table 4. Blood–aqueous transport: English Short Haired guinea pigs

<table>
<thead>
<tr>
<th></th>
<th>Data ((Ca/Cp))</th>
<th>Transport steady state</th>
<th>Entry rate (K_i) ((min^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 min</td>
<td>60 min</td>
<td></td>
</tr>
<tr>
<td>L-glucose</td>
<td>0.24 ± 0.02</td>
<td>0.42 ± 0.04</td>
<td>~1.0</td>
</tr>
<tr>
<td></td>
<td>(9)</td>
<td>(7)</td>
<td></td>
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<tr>
<td>3-O-methyl-D-glucose</td>
<td>0.57 ± 0.04</td>
<td>0.89 ± 0.04</td>
<td>~1.0</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(6)</td>
<td></td>
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<tr>
<td>L-ascorbic acid</td>
<td>1.56 ± 0.11</td>
<td>2.95 ± 0.26</td>
<td>~10.0</td>
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<tr>
<td></td>
<td>(7)</td>
<td>(5)</td>
<td></td>
</tr>
<tr>
<td>Dehydroascorbic acid</td>
<td>0.59 ± 0.06</td>
<td>1.47 ± 0.15</td>
<td>~10.0</td>
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<td></td>
<td>(7)</td>
<td>(5)</td>
<td></td>
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</tbody>
</table>

* Values listed as mean ± SD with the number of experiments in parenthesis.
Table 5. Blood–vitreous transport: English Short Haired guinea pigs

<table>
<thead>
<tr>
<th></th>
<th>20 min</th>
<th>60 min</th>
<th>Transport steady state ratio</th>
<th>Entry rate K* (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Data (Cv/Cp)*</td>
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<td></td>
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<tr>
<td>L-glucose</td>
<td>0.081 ± 0.009</td>
<td>0.118 ± 0.012</td>
<td>~1.0</td>
<td>0.0041 ± 0.0005</td>
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<td></td>
<td>(9)</td>
<td>(7)</td>
<td></td>
<td>(10)</td>
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<tr>
<td>3-O-methyl-D-glucose</td>
<td>0.41 ± 0.03</td>
<td>0.85 ± 0.04</td>
<td>~1.0</td>
<td>0.031 ± 0.003</td>
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<td></td>
<td>(8)</td>
<td>(6)</td>
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<td>(11)</td>
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<tr>
<td>L-ascorbic acid</td>
<td>0.73 ± 0.08</td>
<td>1.62 ± 0.11</td>
<td>~10.0</td>
<td>0.023 ± 0.002</td>
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<tr>
<td></td>
<td>(7)</td>
<td>(5)</td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>Dehydroascorbic acid</td>
<td>0.39 ± 0.05</td>
<td>1.11 ± 0.10</td>
<td>~10.0</td>
<td>0.014 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(5)</td>
<td></td>
<td>(7)</td>
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</table>

* Values listed as mean ± SD with the number of experiments in parenthesis.

of the guinea pig, the ratio we used for calculation purposes was a minimal level beyond which the resulting entry rate constant remained essentially constant.

Our results in the guinea pig suggest that the entry rate of ascorbic acid into aqueous humor is faster than either 3-O-methyl-D-glucose or dehydroascorbic acid. We further note that although entry of labeled dehydroascorbic acid also is fast and results in accumulation in both the aqueous and vitreous humor, with 60 min concentration ratios significantly greater than 1.0, it is significantly slower \( (P < 0.001) \) than that of ascorbic acid. A similar situation was encountered for entry into vitreous humor (Table 5), with the clarification that ascorbic acid movement was not as fast there as that of mD-glucose.

Figure 6 (top) illustrates a normalized (10,000 DPM/spot:10–100 mm running length) radioscan of fresh \( (^{14}C) \)-ascorbic acid track which resulted in a major peak at 16 ± 1 mm and a minor peak of about 2–3% at 3 mm. Figure 6 (middle) illustrates the change in radioscan resulting when \( (^{14}C) \)-ascorbic acid is converted to dehydroascorbic acid with ascorbate oxidase. Thus, only one major peak is present, running at 28 ± 1 mm. The minor peak at 3 mm remains unaffected by the ascorbic oxidase. Figure 6 (bottom) illustrates the instability of ascorbic acid in water solutions. After sitting at room temperature (24°C) for 1 hr, the ascorbic acid solution of Figure 6 shows three major peaks and two minor peaks. Thus,
2,3 diketogulonic acid, ascorbic acid and dehydroascorbic acid were seen with the radioscan and further with specific color visualization methods outlined in Methods. The identity of the minor peaks at 50 and 70 mm resulting from ascorbic acid oxidation remains unresolved.

Figure 7 illustrates a normalized radioscan resulting from an 8 µl spot (10,000 DPM/spot; 100 mm running length) of rat aqueous (vitreous in insert) obtained at the end of our 13 min kinetic pulse experiment with (C\textsuperscript{14})-ascorbic acid. Note the major peak is identified as (C\textsuperscript{14})-ascorbic acid; no oxidation products are seen. The small peak at 2 mm is likely 2,3 diketogulonic acid and may have already been present in the ascorbic acid bolus. Also in Figure 7 is a radioscan of rat aqueous and vitreous humors after a pulse of (C\textsuperscript{14})-dehydroascorbic acid. Prior to introduction into the rat circulation a radioscan similar to that in Figure 6 (middle) was seen. Aqueous and vitreous humors reveal that the dehydroascorbic acid had been converted to ascorbic acid during our kinetic pulse experiment. Only ascorbic acid was seen in the eye, regardless of whether the starting label material in our bolus was ascorbic acid or dehydroascorbic acid.

Figure 8 illustrates that a similar situation was found with guinea pigs. Only ascorbic acid was found in either aqueous or vitreous humors. There is a minor peak at about 2 mm corresponding reasonably with 2,3 diketogulonic acid. Since a small peak was already noted at this position with (C\textsuperscript{14})-ascorbic acid, it seems unlikely that it represents a further oxidation product.

**Discussion**

Our results concerning resting levels of ascorbic acid in aqueous humor of guinea pigs are supported by previous reports noting that aqueous levels are high\(^5\); however, these cited data are dated (1934–37) and range from 5 to 25 mg/100 ml. No clear vitreous data were found. In addition, our data support the observation that ascorbic acid levels in the rat relative to diurnal species are low in plasma, aqueous and vitreous, but not as low as the aqueous value of 0.0 mg/100 ml previously reported.\(^5\) The lack of clear agreement in determining ascorbic acid levels may be explained by the instability of the ascorbic acid molecule in water and biological solutions. In fact, we noted that levels of ascorbic acid measured in freshly obtained aqueous humor was decreased 10% by the first 10 min, about 25% by the first hour, and by 90%+ at the first 24 hr. Factors such as temperature and light also play an important role. Thus, we took care to perform the ascorbic acid determinations quickly and efficiently; determinations were completed within 10 min of death and enucleation. Moreover, all analyses of humors and plasma samples were performed together using standardized control animals and reported results represent from 12 to 22...
animals for each value reported, thereby minimizing biologic variation.

Therefore, we note that in the rat ascorbic acid clearly is present in plasma and the ocular humors. Our data suggest that ascorbic acid entry into aqueous and vitreous is probably via simple passive diffusion. This is based on the observations that AA entry rates are similar to those of the passive marker, L-glucose. Moreover, as with L-glucose, entry rates of AA are not saturable by unlabeled AA and nor are they decreased by the known transport inhibitors, phloretin and phloridzin. Thus, if a carrier-mediated component is present, moving AA into aqueous and vitreous, it cannot be very large compared to the passive component.

Ascorbic acid is available, therefore, to intraocular tissues of both rat and guinea pig. Our observation that aqueous levels are lower than plasma levels in the rat could well be explained by and taken to indicate some form of metabolic use by intraocular tissue, namely lens and cornea. The rate of movement of ascorbic acid into aqueous is not transport-limited and thus probably is dependent upon a general production rate by the liver and the utilization rate at specific ocular tissue loci.

Although they result from different means, levels in the guinea pig also insured that an adequate supply of ascorbic acid is available to intraocular tissues. In the guinea pig, however, the task appears to be performed by an active transport process that continues to pump ascorbic acid from a very low plasma concentration with an apparent disregard for the already high aqueous concentration. This is clearly an energy-requiring process and likely performed by the ciliary process.\(^2\)\(^1\) It may be of some importance that a significant difference exists between intraocular ascorbate levels of the rat, a nocturnal animal, and guinea pig, a diurnal animal. G. R. Reiss\(^3\)\(^8\) suggests that ascorbic acid in the aqueous may play a protective role in those animals most exposed to light. Although our results could be taken as support for this notion, we caution any conclusions since a good deal of interspecies variation was encountered by Reiss. Importantly, resting levels encountered give no indication as to the various processes that may result only in an apparent stable level. It could be that delivery rates at equilibrium with utilization processes mask differences between species and that resting levels may have little to do with any role for ascorbic acid.

Our work helps clarify two major issues that appear unresolved in the literature.\(^5\)\(^8\): first, is ascorbate a general requirement of interior ocular tissues that needs to be supplied via ocular humors in all species? Pirie (1962) and most recently Varma (1987) suggest that ascorbate may be required in lens metabolism, but Cole has also argued that “this cannot be universally true, since it is practically absent from aqueous of the rat”.\(^5\)\(^17\)\(^33\) Our results suggest that ascorbate enters both aqueous and vitreous humors of the rat passively, down a concentration gradient, and is available to intraocular tissues. The rate of entry depends on the resting plasma level and the utilization rate in eye. Furthermore, since intraocular resting levels, like those of D-glucose, are lower than circulating plasma levels, metabolic utilization by intraocular tissues is a clear possibility. Thus, both species we studied have means of adequately supplying intraocular tissue with ascorbic acid. These representative vertebrates could reflect a more general requirement for ascorbate by ocular tissue of other mammalian species, including humans.

A second unresolved question concerns which molecular species, ascorbate or dehydroascorbate, was the transported and which was the required species? Our data indicate that in species such as the guinea pig, (and possibly humans), the carrier-specific active transport mechanism prefers the reduced form of the vitamin, ascorbic acid. Our transport results indicate that dehydroascorbic acid, although entering the eye faster than via simple diffusion and with some carrier involvement, is not favored by the ascorbic acid carrier. We report that in the rat, there is no significant difference in the entry rate constants of L-glucose and ascorbic acid. This suggests that movement into aqueous and vitreous is passive. Moreover, in this species, where there was no ascorbic acid carrier involvement, dehydroascorbic acid was found to cross into both ocular humors significantly faster than ascorbic acid. This could well be due to differences in molecular charge and lipid or membrane solubility at physiologic pH. Thus, our results indicate that where there is carrier involvement, ascorbate movement across biological barriers is faster than that of the dehydro form, and where there is no carrier, dehydroascorbate moves faster. Our TLC studies clearly confirm the importance of ascorbic acid in that only the reduced form was found in either aqueous or vitreous humor, regardless of whether the transport bolus pulse was either labeled ascorbic or dehydroascorbic acids.

In the guinea pig, the source of the relatively fast movement of the dehydroascorbate moiety could be a less-than-optimal use or borrowing of the ascorbate carrier or, more likely, the prior conversion of the dehydroascorbate moiety to the reduced form and then its carrier transport into aqueous. This carrier is probably not the D-glucose carrier since this carrier is not able to move molecules against a concentration
gradient, but only facilitates movement down an already existing concentration gradient. Thus, it is likely that the dehydroascorbate moiety enters into the ocular humors as the reduced form of ascorbic acid. Where the conversion of dehydroascorbate to ascorbic acid takes place remains unclear, especially as to whether ocular tissues are involved in this conversion.

In addition, we note that for both rat and guinea pig our results indicate the presence of an active transport mechanism whereby ascorbic acid enters CSF. It seems reasonable that whatever role ascorbic acid plays in normal CSF physiology, light should not be involved. In the eye, because of the presence of ascorbate in aqueous humor of many species, it has been suggested that a connection may exist between light and/or light oxidation and ascorbic acid. We suggest that perhaps in the eye, as in CSF (where there is an absence of light), light may not be directly involved in any role for ascorbic acid as an antioxidant. This does not, however, rule out any role for ascorbic acid in anaerobic glycolysis or other related metabolic pathways since both CSF and intraocular fluids are both relatively distant from oxygen sources. This relative remoteness from the oxygen source could be the basis for the hypothesized commonality of purpose and the reason for the presence of ascorbic acid in CSF and ocular humors.

**Key words:** ascorbic acid, dehydroascorbic acid, aqueous humor, vitreous, vitamin C, transport, guinea pig, thin layer chromatography

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**References**


**Correction**

In the article by Woodward et al., "Prostaglandin F$_2$\alpha Effects on Intraocular Pressure Negatively Correlate with FP-Receptor Stimulation," which appeared in the August 1989 issue of Investigative Ophthalmology and Visual Science, Figure 4 (page 1841) should be corrected as shown below.