Lacrimal Gland Uptake and Metabolism of Ascorbic Acid (43529)

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Abstract. **Ascorbic acid is thought to contribute to protection against the potentially damaging effects of radiation, oxygen toxicity, and abrasion in the eye. The anterior surface of the cornea is particularly subject to insult from each threat. We considered the possibility that the lacrimal gland of pigs has transport and/or metabolic capability to sequester the reduced or oxidized form of ascorbic acid and prepare it for secretion in the tears. Slices of fresh lacrimal gland were incubated in a physiologic buffer and exposed to ≤ 12 μM ^{14}C-labeled ascorbic acid or dehydro-L-ascorbic acid over 40-min incubation periods. Dehydro-L-ascorbic acid was taken up to a greater extent than the reduced compound. ^{14}C-Label recovered from the tissue was at least 75% in the form of ascorbic acid after incubation with either substrate. Uptake of both the reduced and oxidized substrates proceeded to a tissue to medium ratio in excess of unity; the former was prevented by the presence of nonlabeled ascorbate in the bathing medium, but was unaffected by the removal of Na^+ from the bath. The uptake of both substrates was less after inhibition of cellular metabolic energy. The lacrimal gland in this diurnal animal species has transport and metabolic capabilities that could serve in secretion of ascorbic acid into tears. This might help to protect the corneal epithelium against various forms of damage.** [P.S.E.B.M. 1993, Vol 202]

Various ocular tissues and fluids have ascorbic acid present at a concentration high in comparison with plasma and most other tissues (1, 2). Ascorbic acid accumulates in human aqueous humor against a 20-fold gradient with respect to plasma (~0.05 mM). Most of the "total" ascorbic acid (reduced plus oxidized) present in the eye appears to be in the reduced form, as is normally true elsewhere in the body. This supports the concept that an important function of ocular ascorbic acid is to protect against reactive free radicals that may be contributing agents in numerous diseases as well as in the general process of aging.

The cornea is uniquely subject to free radical damage due to exposure to radiation, atmospheric oxygen, and other environmental threats. The immediate source of nutrients such as glucose for the cornea is thought to be the aqueous humor (3). It was recently reported (4) that corneal endothelial cells take up both the oxidized and reduced forms of ascorbic acid and have metabolic capability to maintain most of the substrate in the useful reduced form.

Damage to the anterior surface of the cornea from abrasion, sunlight, and chemicals occurs much more abruptly than most threats to internal organs of the body. The unconfirmed report (5) that ascorbic acid is present in the corneal epithelium at >50-fold the plasma level supports the function of this compound here. A stress-induced increase in the supply of ascorbate to the anterior cornea (i.e., through tears) could provide an important defense. We have examined the tissue that produces tears, the lacrimal gland, for which little information on ascorbic acid metabolism exists (6).

We evaluated whether the lacrimal gland recognizes, accumulates, and metabolizes either the reduced or oxidized form of ascorbic acid. The pig is selected as a representative diurnal animal that is likely to use ascorbic acid in protection against radiation and other threats (7). Thin slices of metabolically active lacrimal gland were used based on previous observations that other tissues are effectively evaluated in this way (8). An affirmative finding in this work would indicate potential value in additional studies on the nutritive role of tears, in particular, in terms of ascorbate delivery. The properties of ascorbate transport and metab-

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olism in other ocular tissues (1) and nonocular tissues (9) have recently been reviewed.

Materials and Methods

Tissue Preparation. Pig lacrimal glands were obtained fresh from a local abattoir and transported at 0–4°C to the laboratory. The glands were washed at 4°C in physiologic buffer (in m*M*): 3 KCl, 20 MOPS, 0.2 KH_2PO_4, 1.2 K_2HPO_4, 130 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose, and 1 thiourea, at pH 7.2. The tissue was then sliced into thin sections with a tissue slicer, and each slice was allowed to stabilize 20 min at 37°C in 2 ml of buffer that had been pre-equilibrated with O_2.

Incubation Conditions. [^{14}C]Ascorbic acid (6–20 mCi/mmol) was checked routinely for purity by high-performance liquid chromatography (10). To study ascorbic acid uptake, [^{14}C]ascorbic acid was added to the bathing media to provide a final ascorbic acid concentration of ≤ 12 μM. [^{14}C]Dehydro-L-ascorbic acid was made from [^{14}C]ascorbic acid at 0°C by addition of an aliquot of Br_2; the Br_2 was immediately removed with a stream of N_2 gas. Because of the lability of ascorbic acid and its derivatives, the bath was replaced with a fresh duplicate halfway through the 40-min incubation.

To evaluate the Na^+ dependence of ascorbic acid and dehydro-L-ascorbic acid uptake mechanisms, NaCl was replaced with LiCl to provide final Na^+ concentrations of 130 m*M* (control), 86 m*M*, 43 m*M*, and 0 m*M*. MOPS was replaced with HEPES (20 m*M*) in these studies to facilitate pH maintenance. Tissue slices were preincubated in the appropriate media for 20 min before incubation with ^{14}C-tracers.

After 40 min of incubation each tissue slice was removed, blotted on tissue paper, weighed, and extracted overnight in 1 ml of 10% phosphoric acid. This technique recovers most of the radiolabeled compound absorbed by the gland. Samples of the bathing media were taken immediately after addition of radiolabel and at the end of the incubation period, and stabilized in 10% phosphoric acid. For evaluation of the chemical form of ^{14}C-label recovered from either the tissue or the bath, samples were analyzed by high-performance liquid chromatography (10). All quantitation of ^{14}C-label was performed in a Beckman LS 5000TD scintillation counter.

All results mentioned in tables and text are mean ± SE. Statistical comparisons in tables were made using the pooled *t* test.

Results

Comparison of Reduced and Oxidized Ascorbate Uptake. Values of ascorbic acid and dehydro-L-ascorbic acid accumulation in surviving slices of lacrimal gland *in vitro* are presented in Table I. In both cases, a tissue to bathing medium (T:M) ratio of radiolabeled compound exceeding unity was demonstrated (Table I). Dehydro-L-ascorbic acid appears to be the preferred form for uptake because it accumulates against the greatest gradient.

Table I. Uptake and Metabolism of Ascorbic Acid and Dehydro-L-Ascorbic Acid by Surviving Lacrimal Gland *In Vitro*[a]

Compound presented to tissue	Final tissue concentration (μM)	Final bath concentration (μM)	T:M
^{14}C-AA (9)	6.4 ± 0.6	4.1 ± 0.5	1.6 ± 0.2
^{14}C-DHAA (4)	7.3 ± 0.3	2.5 ± 0.4	3.2 ± 0.6[b]

[a] Lacrimal gland slices were incubated with either [^{14}C]ascorbic acid (^{14}C-AA) or [^{14}C]dehydro-L-ascorbic acid (^{14}C-DHAA) for 40 min. Values are means ± SE. Number of studies is shown in parentheses. The final bath concentration of DHAA is lower than AA because this compound is more labile than the reduced molecule.

[b] Tissue to medium concentration ratio (T:M) was significantly greater when DHAA was presented ($P < 0.05$).

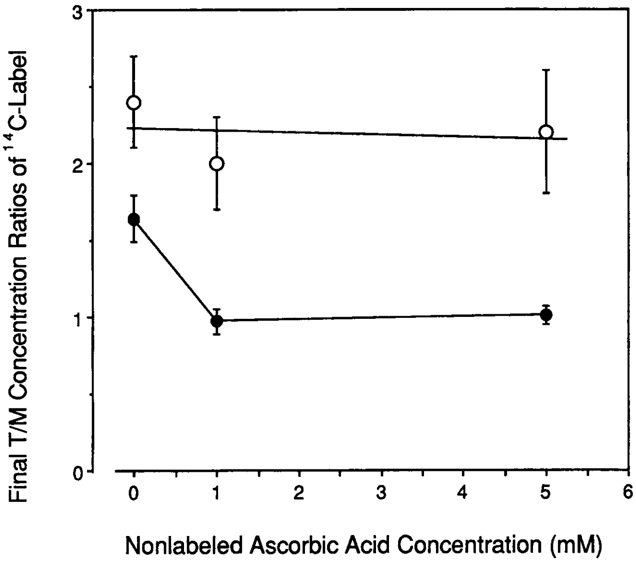

Figure 1. The effect of nonlabeled ascorbic acid on tissue to medium concentrations of [^{14}C]ascorbic acid (●) and [^{14}C]dehydro-L-ascorbic acid (○) evaluated at the end of lacrimal gland incubations. Nonlabeled ascorbic acid was present at 0, 1.0, and 5.0 m*M*. A significant difference in [^{14}C]ascorbic acid accumulation from control (0 m*M* nonlabeled ascorbic acid) was seen at 1 and 5 m*M* ascorbic acid (by one-way analysis of variance with *F* test; $P < 0.01$). Values are mean ± SE of determinations on at least three glands.

It was of interest to evaluate whether a specific transport mechanism participates in the uptake of ascorbic acid and dehydro-L-ascorbic acid. Evidence of competition between chemically similar molecules for a fixed number of transporters is taken as one line of evidence of carrier-mediated uptake. As seen in Figure 1, the presence of nonlabeled ascorbic acid at 1 or 5 m*M* in the incubation medium resulted in the T:M ratio of [^{14}C]ascorbic acid being reduced to a value not significantly different from unity. There was no significant effect on uptake of [^{14}C]dehydro-L-ascorbic acid.

An evaluation was made of whether the accumulation of ^{14}C-label discussed above is dependent upon intact supplies of cellular metabolic energy. Tissue slices were preincubated 20 min in buffer that was equilibrated with 100% N_2 in place of O_2; the buffer also contained NaCN at 1 m*M*. After 20 min, [^{14}C]ascorbic acid or dehydro-L-ascorbic acid was added and the incubation proceeded as above, except for the continued absence of oxygen and presence of CN. The results (Table II) show that the tissue no longer accumulated ascorbic acid against a gradient and had less capacity to take up dehydro-L-ascorbic acid. T:M ratios that exceed 1.0 under control conditions but approach unity with inhibition of cellular metabolism are interpreted to indicate good tissue viability in the control state.

The properties of ascorbic acid uptake were further evaluated by replacing Li^+ for Na^+ in the bathing medium. The results indicate that there was no statistically significant change in the mean value of ascorbic acid or dehydro-L-ascorbic acid accumulation (Table III) after reduction of the Na^+ concentration. In additional studies, it was found that isosmotic replacement of mannitol for NaCl also had no statistically significant effect on ascorbic acid accumulation (control T:M, 1.65; test T:M, 1.39; $n = 3$, $P > 0.1$).

Metabolism of Dehydro-L-Ascorbic Acid. The data available allow an evaluation of [^{14}C]ascorbic acid formation by the lacrimal gland. This is possible because the oxidation of ascorbic acid by bromine is virtually complete and there is no detectable [^{14}C]ascorbic acid present initially. Also, there is no detectable spontaneous generation of ascorbic acid in the bathing medium. Thus, when the tissue is exposed to [^{14}C] dehydro-L-ascorbic acid, any [^{14}C]ascorbic acid recovered in the tissue is assumed to have been formed there. As seen in Table IV, approximately 78% of the ^{14}C-label recovered in the tissue is in the form of ascorbic acid, whether the isotope was supplied in the reduced or oxidized form.

A comparison of the bathing solution profiles of ^{14}C-label taken at Time 0 and 40 min under the incubation conditions of Table IV allows a determination of any increase in bathing media [^{14}C]ascorbic acid (data not shown). Because no increase is seen in the absence of tissue, the bath content of [^{14}C]ascorbic acid is attributed to intracellular reduction of [^{14}C]dehydro-L-ascorbic acid, followed by leakage or secretion to the extracellular space. Of the total dehydro-L-ascorbic acid that was taken up and reduced by the tissue, approximately 23% was released to the bath.

Discussion

Ocular tissues are susceptible to damage from a variety of sources and might receive protection from ascorbic acid. The role of the lacrimal gland in supplying ascorbic acid to the corneal epithelium through the tears has not previously been investigated. One advantage in having a source of ascorbate in tears would be that in the event of abrupt damage to the corneal epithelium, the supply of ascorbate could be increased within a few seconds. The slow rate of aqueous humor turnover and the considerable diffusion barrier that the corneal stroma represents between the epithelium and endothelium are major impediments to the epithelium deriving an adequate supply of ascorbate via the aqueous humor.

An initial step in the processing of plasma ascorbic acid (or dehydro-L-ascorbic acid) to tears would be uptake into the secretory epithelial acinar cells. A second step might be metabolism of the substrate so that it is in the preferred form to be transported in the tears. The present studies indicate that both the oxidized and reduced molecules are taken up by the gland, although dehydro-L-ascorbic acid is preferred. Because both substrates accumulated in the tissue water at a concentration higher than that at which they were included in the bathing media, it is concluded that they are sequestered in the acinar cells, the prevalent cell type in the gland. The process by which ascorbic acid is taken up into cells is likely to be a transport event at the cell membrane, based on the observation that uptake is inhibited by the presence of nonlabeled ascorbic acid. Additional details about this transport step would be

Table II. Effect of Cellular Metabolism Inhibition on Uptake of Ascorbic Acid and Dehydro-L-Ascorbic Acid in Lacrimal Gland[a]

Compound presented	Incubation condition	Tissue concentration (μM)	Bath concentration (μM)	T:M
^{14}C-AA	Control (4)	15.7 ± 4.5	5.9 ± 1.2	2.5 ± 0.3
^{14}C-AA	CN + N_2 (4)	6.1 ± 1.7	5.9 ± 1.4	1.0 ± 0.1[b]
^{14}C-DHAA	Control (3)	20.6 ± 6.3	4.4 ± 1.4	4.8 ± 0.4
^{14}C-DHAA	CN + N_2 (3)	7.1 ± 2.6	5.4 ± 1.6	1.2 ± 0.1[b]

[a] Lacrimal gland slices were incubated with [^{14}C]ascorbic acid (^{14}C-AA) or [^{14}C]dehydro-L-ascorbic acid (^{14}C-DHAA) under control conditions or in buffer that had been gassed with N_2 and contained CN at a final concentration of 1 m*M*. Values are mean ± SE. The higher bath concentration of ascorbic acid compared with Table I reflects our use of lower specific activity (and higher molarity) [^{14}C]ascorbic acid in latter studies. Note that all studies within this and each table made use of the same batch of [^{14}C]ascorbic acid.

[b] There was a significant difference in final T:M ratio from control ($P < 0.01$).

Table III. Effect of Incubation Medium Na^+ Concentration on Tissue Accumulation of Ascorbic Acid and Dehydro-L-Ascorbic Acid[a]

Incubation medium Na concentration (m*M*)	Tissue:media ^{14}C from AA	Tissue:media ^{14}C from DHAA
130 (control)	1.6 ± 0.3	2.9 ± 0.4
86	1.5 ± 0.2	3.1 ± 0.6
43	1.4 ± 0.4	3.0 ± 0.7
0	1.4 ± 0.3	4.2 ± 1.2

[a] Tissue slices were preincubated 20 min in buffer with the Na^+ concentration indicated. Fresh buffer of the same composition was then added along with ^{14}C-labeled ascorbic acid (AA) or dehydro-L-ascorbic acid (DHAA). Values are mean ± SE on three determinations.

Table IV. Identification of ^{14}C-Label in Lacrimal Gland after Incubation with [^{14}C]Ascorbic Acid or [^{14}C]Dehydro-L-Ascorbic Acid[a]

Compound presented	Ascorbic acid (%)	Dehydro-L-ascorbic acid (%)	Diketogulonic acid (%)
[^{14}C]Ascorbic acid	76.8 ± 3.9	11.4 ± 1.7	10.3 ± 2.2
[^{14}C]Dehydro-L-ascorbic acid	79.0 ± 1.2	12.0 ± 0.7	7.3 ± 0.7

[a] Incubation conditions were as in Table I. Values are mean ± SE of determinations on six glands.

more easily derived from an isolated membrane vesicle preparation, as used previously in the study of kidney, intestine, etc. Most of the ascorbate molecule remains in the reduced state upon entering the tissue; the value of 78% reported above is likely an underestimate in that some degradation occurs during extraction, storage, and assay.

Conclusions about the mechanism of dehydro-L-ascorbic acid uptake are more speculative. There was no clear demonstration that the uptake process is inhibited by the presence of ascorbic acid (Fig. 1). It is concluded that dehydro-L-ascorbic acid and ascorbic acid are not taken up by a common transporter. We cannot make additional firm statements about whether an ascorbic-acid-independent transport mechanism exists for uptake of dehydro-L-ascorbic acid in the lacrimal gland. This inconclusiveness is common in studies on uptake of dehydro-L-ascorbic acid into surviving tissue (9); this is in large part because a series process of transport followed by enzymatic metabolism is very difficult to evaluate with standard kinetic studies. The possibility of demonstrating competitive inhibition (i.e., saturation kinetics) of transport is complicated by the cell membrane toxicity property of this substrate (11–14) when present at concentrations above approximately 200 μM. No specific inhibitor of dehydro-L-ascorbic acid transport has been found.

The lack of any significant effect of Na^+ depletion from the incubation medium on ascorbic acid or dehydro-L-ascorbic acid uptake suggests that the transport mechanism is distinct from the familiar sodium-coupled mechanism suggested by Crane (15) that appears to mediate ascorbate uptake in the small intestine (16, 17), kidney (18), retinal pigment epithelial cells (19), iris-ciliary body (20), and ciliary epithelium (21, 22). The lacrimal gland is more similar to other tissues, e.g., placenta (23), corneal endothelium (4), and neutrophils (24), that preferentially take up dehydro-L-ascorbic acid by a sodium-independent process. In that dehydro-L-ascorbic acid does not accumulate in the tissue against an electrochemical gradient, there is no evidence that an active transport process participates in uptake.

The observation that ^{14}C-label originally in the form of dehydro-L-ascorbic acid is taken into the tissue against a concentration gradient has the following possible explanation: the molecule diffuses across the cell membrane and is then metabolized to a form that can be directed to a secretory mechanism at the apical surface of the acinar cell. This is supported by the data in Table III, and would be comparable to net transport of this molecule across the small intestinal epithelium or the renal tubular cells (9). Details might be derived with the use of additional experimental approaches.

The nearly complete reduction of dehydro-L-ascorbic acid that has been recently taken up in the lacrimal gland is similar to results on comparable tissues evaluated *in vitro*, such as the corneal endothelium (4), iris-ciliary body (A. M. Bode, Y. L. Gomez, and R. C. Rose. Ascorbate metabolism by bovine iris ciliary body. Submitted for publication.), colonic mucosa (25), placenta (23), and kidney (26). In most instances, a high molecular weight compound with properties of an enzyme was involved. Others have proposed that reducing agents such as glutathione are present at a sufficiently high concentration that the process of dehydro-L-ascorbic acid reduction proceeds as a nonenzymatic step. This possibility was specifically applied to the lens (27), where the endogenous glutathione concentration is quite high. We are unaware of any estimates of the glutathione concentration in the lacrimal gland, and cannot speculate on whether nonenzymatic reduction here is feasible. Additional studies that assess endogenous levels of reducing agents in lacrimal gland and the possibility of protein-catalyzed dehydro-L-ascorbic acid reduction would be helpful.

The physiologic significance of having preferential uptake of the oxidized molecule followed by its immediate reduction is not clear. It may be that each tissue that functions in this way contributes to maintenance of the ascorbate redox state of its nutritive fluid by selectively removing dehydro-L-ascorbic acid. It must also be recognized that although the oxidized molecule was preferentially taken up under our experimental conditions, the reduced molecule is prevalent in most biological fluids; thus, most uptake *in vivo* might be attributed to this form of the molecule. Through some combination of the two uptake processes, the lacrimal gland accumulates ascorbate from plasma against a concentration gradient (R. Dreyer and R. C. Rose, unpublished observations). This process, followed by subsequent release into the lacrimal duct, might account for the significant content of ascorbic acid found in human tears (2).

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