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Ascorbate in the Rat Lens: Dependence on Dietary Intake

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Key Words
Ascorbate · Rat lens

Abstract

Purpose: To establish a method for sample preparation to measure ascorbate in whole lenses and to investigate whether lens ascorbate concentration is dependent on dietary ascorbate intake. ***Methods:*** Four groups of 3 young Sprague-Dawley rats each were fed chow containing *L*-ascorbate, either 0.0, 5.7, 57.0 or 114.0 mmol/kg for a duration of 4 weeks. Thereafter, each rat was sacrificed. The lens was extracted, photographed, and lens wet weight was measured. The lens was homogenized in 1.0 ml of 0.25% metaphosphoric acid, the homogenate was centrifuged and the supernatant ultrafiltered. The filtrate was injected into an ion exchange, reversed-phase Polypore H HPLC column equipped with a 254-nm ultraviolet detector. Samples were calibrated against an *L*-ascorbate standard. Polynomial regression analysis was performed on the data. ***Results:*** All lenses were devoid of cataract. A 95% confidence interval for baseline content of ascorbate without any dietary intake was estimated to be 0.16 ± 0.01 μmol/g wet weight of lens. The lens ascorbate concentration increased linearly with dietary ascorbate intake with an increased rate, estimated as a 95% confidence interval of 0.33 ± 0.18 (μmol ascorbate) (g lens)$^{-1}$ (mol ascorbate)$^{-1}$ (kg chow) with $r^2 = 0.62$. ***Conclusion:*** Lens ascorbate concentration linearly increases with dietary ascorbate intake without cataract development in the rat. The currently presented method for sample preparation to measure the whole-lens content of ascorbate is applicable.

Introduction

The purpose of this paper was to develop a method for sample preparation for ascorbate determination in whole lenses and further to investigate whether lens ascorbate concentration in the albino Sprague-Dawley rat lens depends on dietary ascorbate intake.

Ascorbate

Ascorbic acid, or vitamin C (fig. 1a, b), has two ionizable –OH groups with $pK_{a1} = 4.25$ and $pK_{a2} = 11.8$. Ascorbate (fig. 1b) is the favored form at physiological pH [1]. Therefore, we use the name ascorbate throughout.

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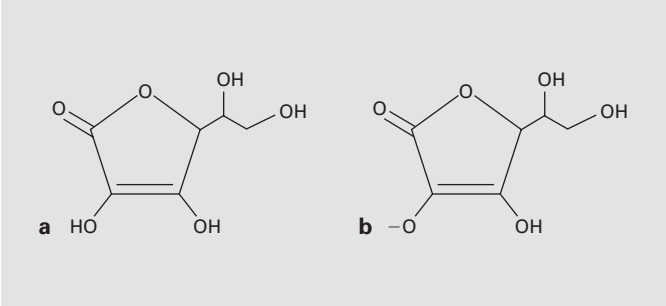


Fig. 1. a Ascorbic acid. **b** Ascorbate.

Ascorbate is an essential nutrient in the human. The rat however is capable of synthesizing ascorbate [2]. In 1961, Long [3] found the ascorbate concentration in the lens to be much higher in diurnal than in nocturnal animals and speculated that this may be due to a protective effect of the ascorbate against eye damage [4]. In the rat, the lens ascorbate concentration was found to be very low, equal to 0.08 mmol/kg lens wet weight [4]. Recently, Reddy et al. [5] have found the ascorbate concentration in the Sprague-Dawley rat lens to be 0.12 m*M* before and 0.16 m*M* 1 h after intraperitoneal ascorbate injection.

Ascorbate is essential for collagen synthesis but is also of particular interest because of its role as an antioxidant in a number of tissues including the lens [4]. By functioning as a water-soluble antioxidant, ascorbate is capable of preventing oxidative damage to protein, lipid and DNA in a number of tissues including the lens [5, 6].

In tissue culture experiments, ascorbate was found to thwart photoperoxidation of lens lipids [7]. Further, in in vitro studies, ascorbate conferred protection against ultraviolet radiation (UVR) B inactivation of rat lens enzymes, including the glycolytic pathway enzyme hexokinase, the pentose phosphate shunt enzyme glucose-6-phosphate dehydrogenase and the cation pump Na/K-ATPase [8, 9].

In vivo, ascorbate has been found to protect the lens against oxidatively induced cataract induced by selenite in rats [10]. Recently, it has also been shown to protect against cataract in aldose-reductase-deficient mice in vitro [11] in the xanthine oxidase model. Third, physiological levels of combined antioxidants including ascorbate have been found to increase the viability of UVR-exposed cultured human lens epithelial cells and to maintain transparency of rat lenses exposed to UVR [12].

There is some evidence that oral intake of ascorbate protects against cataract formation in the human lens [13–15]. Further, lenses with increasing degrees of cataract and browning secondary to protein oxidation were associated with a lower ascorbate content [16].

Besides functioning as an antioxidant in the lens, ascorbate in the aqueous humor attenuates UVR, by absorption and conversion of the UVR energy into heat or fluorescence, thereby reducing UVR damage [17].

Pharmacodynamics of Ascorbate

In diurnal animals, the concentration of ascorbate in the aqueous humor is higher than in plasma and further higher in the ocular lens than in the aqueous humor [18]. This concentration gradient is due to active transport [18].

The metabolism of ascorbate in the rat is rapid. In the rat, ascorbate is oxidized to dehydroascorbate. This compound is delactonized to diketogulonate, which can then be decarboxylated to form carbon dioxide, which is exhaled, and a number of other compounds [2].

Measurement of Ascorbate

Biochemists were able to measure ascorbate in eye tissue extracts more than 60 years ago utilizing a color titration method with 2,6-dichlorophenolindophenol [19]. However, the method was not specific for ascorbate since it also assayed for glutathione. In 1979, Omaye et al. [20] determined the ascorbate concentration in animal tissues using a spectrophotometric assay with 2,6-dichlorophenolindophenol. This technique has also been applied to measure ascorbate in the rat lens [2].

Within the last 15 years, a method of measuring intraocular ascorbate utilizing high-performance liquid chromatography (HPLC) with either electrochemical [21–25] or UVR [26–33] detection has been described.

UVR detection is possible because of expressed absorption of UVR by ascorbate. The high molar extinction coefficient for ascorbate at 265 nm (neutral pH) or at 256 nm (acidic pH) allows for quantification of the ascorbic acid peak as it elutes from an HPLC column. The method of HPLC with UVR detection has been used for over a decade to analyze ascorbate from a number of tissues and plasma [34]. HPLC with electrochemical detection has been applied to measure ascorbate in the lens tissue from Emory mice [35], guinea pigs and humans [36]. We decided to apply the technique of HPLC with UVR detection to ascorbate measurement in rat lens samples processed and purified using ultrafiltration. The objective of the paper was to ascertain the relationship between lens ascorbate concentrations with dietary intake.

The current paper intends to evaluate a method for sample preparation of whole lenses for determination of ascorbate content and to determine a possible relationship between oral intake and lens ascorbate content.

Materials and Methods

Animals

Three-week-old female albino Sprague-Dawley rats (B & K Universal AB, Sollentuna, Sweden) were used in the experiment. Ethical approval was obtained from the Northern Stockholm Animal Experiments Ethics Committee. The animals were kept and treated according to the *ARVO Statement for the Use of Animals in Ophthalmic and Vision Research*.

Experimental Procedure

Four groups of 3 rats each were fed ascorbate-free chow (R 36 bas; Analycen, Stockholm, Sweden) supplemented with *L*-ascorbate, either 0.0, 5.7, 57.0 or 114.0 mmol/kg for a duration of 4 weeks. Thereafter, the rat was sacrificed. The animals were not fasted before sacrifice. Each lens was extracted, photographed, wet weight measured, homogenized and centrifuged. The supernatant was ultrafiltered and the ultrafiltrate injected into an HPLC system for ascorbate measurement.

Lens Dissection

After the rats had been sacrificed with carbon dioxide asphyxiation, the lenses were extracted and placed in Ringer's acetate and photographed. Lens wet weights were measured.

Lens Sample Preparation

Each lens was homogenized in 1.0 ml of 0.25% metaphosphoric acid with a tissue grinder [26]. Metaphosphoric acid prevents oxidation of ascorbate to dehydroascorbate because of high acidity and metal ion chelation [34, 37]. The homogenate was centrifuged at 12,000 *g* for 10 min at 4°C. Subsequently, 800 µl of supernatant was ultrafiltered through a 5,000-Da molecular-weight cutoff membrane (Ultrafree CL; Millipore AB, Sundbyberg, Sweden) using centrifugation at 3,830 *g* for 1 h at 4°C.

HPLC with UVR Detection

Ascorbate was separated on an ion-exchange, reversed-phase column, 220 mm × 4.6 mm, 10 µm (Polypore H, Applied Biosystems, Foster City, Calif., USA) using 2 m*M* sulfuric acid, pH 2.4, as the mobile phase. A 20-µl ultrafiltrate sample was injected by a refrigerated autoinjector set at 6°C (CMA/200 Microsampler, CMA/Microdialysis AB, Stockholm, Sweden) using a 15.4-µl sample loop. The mobile phase was delivered at 0.3 ml/min using an isocratic HPLC pump (CMA 250; CMA/Microdialysis AB). The exploring beam of the detection module consisted of radiation from a deuterium lamp that was spectrally limited with a monochromator to 254 nm. The absorbance of the column exit was measured using a UVR detector (Lachrom Merck Hitachi L7400, Darmstadt, Germany).

For HPLC measurements, a calibration procedure was run. The calibration procedure consisted of running a 10-equidistance level calibration curve for the measurement range intended, to assure linearity. Then, the absorbance was obtained for 2 samples of 10 µ*M* *L*-ascorbate (Merck, Darmstadt, Germany) diluted in 2.5% metaphosphoric acid and prepared from a stock solution. Finally, the absorbance of a 2.5% metaphosphoric acid-only blank was determined. Before each series, the average absorbance for the 2 samples of 10 µ*M* *L*-ascorbate was used to set sensitivity and with this, ascorbate was calculated for each measured unknown sample considering a first-order relationship between concentration of ascorbate and absorbance, omitting the zero order term.

Recovery of Ascorbate in Measurements

Two rats were sacrificed, and lenses (n = 3) were extracted. Lenses were homogenized in 1.0 ml 0.25% metaphosphoric acid and centrifuged at 12,000 *g* for 10 min at 4°C. Ascorbate was measured in the supernatant (800 µl). Then, the pellet was washed with 1.0 ml 0.25% metaphosphoric acid and centrifuged. Ascorbate was measured in the supernatant (800 µl). Finally, the pellet was washed a second time with 1 ml 0.25% metaphosphoric acid and centrifuged. Ascorbate was then measured in the supernatant. Ascorbate was measured using HPLC with UVR detection.

Spectrophotometry

UVR spectra for 1 m*M* ascorbate and 1 m*M* dehydroascorbate solutions in 2.5% metaphosphoric acid were obtained using a spectrophotometer (Unicam UV/Vis, Cambridge, UK). The UVR wavelength range used for measurement was 200–400 nm.

Experimental Design

Four groups of 3 rats each were fed chow supplemented with varying amounts of ascorbate. One ascorbate measurement was obtained for each lens sample. Ascorbate was measured in both lenses. The mean ascorbate concentration was averaged for each animal.

Statistical Parameters

The significance levels and confidence coefficients were set to 0.05 and 0.95, respectively.

Results

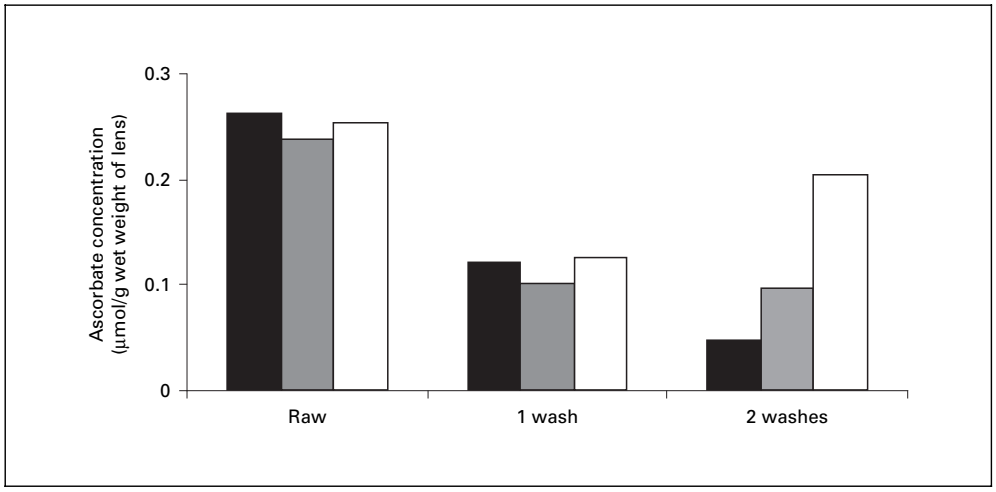
Recovery of Ascorbate in Measurements

The ascorbate concentration in the supernatant from the ultracentrifugation of grinded lens in metaphosphoric acid was twice that of the ascorbate concentration in the second and third supernatants obtained after re-extracting and centrifuging the pellets. The data are plotted in figure 2.

Ascorbate and Dehydroascorbate Spectra

The UVR spectra for 1 m*M* ascorbate and 1 m*M* dehydroascorbate solutions in 2.5% metaphosphoric acid are shown (fig. 3). The pH for each solution was: 1 m*M* ascorbate, 0.75 pH; 1 m*M* dehydroascorbate, 0.60 pH; 2.5% metaphosphoric acid, 1.10 pH.

Using the absorbance at 254 nm, the UVR absorbance for 1 m*M* dehydroascorbate was calculated as 4.1% of the absorbance of 1 m*M* ascorbate.



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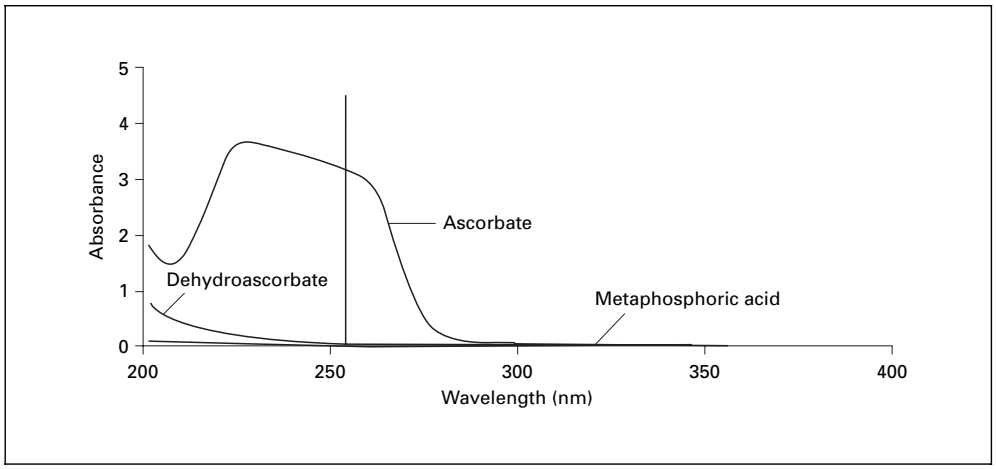

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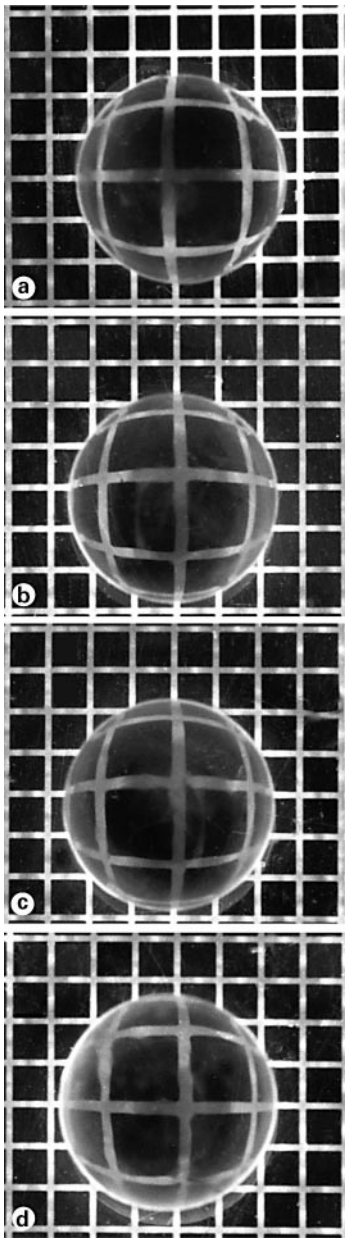

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Fig. 2. Recovery of ascorbate in measurements. Raw = Supernatant after centrifugation of lens homogenate in 0.25% metaphosphoric acid; 1 wash = supernatant after centrifugation of 1 wash of the raw pellet with 0.25% metaphosphoric acid; 2 washes = supernatant after centrifugation of pellet after second wash. The 3 columns indicate each individual lens.

Fig. 3. UVR spectra for 1 m*M* ascorbate and 1 m*M* dehydroascorbate in 2.5% metaphosphoric acid. Absorbance for all three spectra at 254 nm is denoted by the vertical line.

Fig. 4. Lens photographs for rats fed chow supplemented with varying amounts of ascorbate: 0.0 (**a**), 5.7 (**b**), 57.0 (**c**) and 114.0 mmol/kg (**d**). The grid square diameter is 0.79 mm.

Lens Analysis

All lenses were devoid of cataract. Figure 4 shows lenses for each of the four groups of dietary ascorbate.

Figure 5 shows a typical chromatogram for ultrafiltered whole lens homogenate.

Retention time for ascorbate was 5.7 min under the HPLC conditions used in the experiment.

Lens ascorbate concentration, expressed as micromoles ascorbate per gram wet weight of lens, increased linearly with dietary ascorbate intake (moles per kilogram chow; fig. 6).

The data were fitted to a first-order polynomial assuming a baseline content of ascorbate without any dietary intake, A_0 (micromoles per gram wet weight of lens), and that the content of ascorbate in the lens per wet weight of lens, A_{lens} (micromoles per gram wet weight of lens) is directly proportional to the dietary intake, I (moles per kilogram chow), with the proportionality constant k [(micromoles ascorbate) (gram lens)$^{-1}$ (mol ascorbate)$^{-1}$ (kilogram chow)] (equation 1):

$$[A_{lens}] = A_0 + k\,I$$

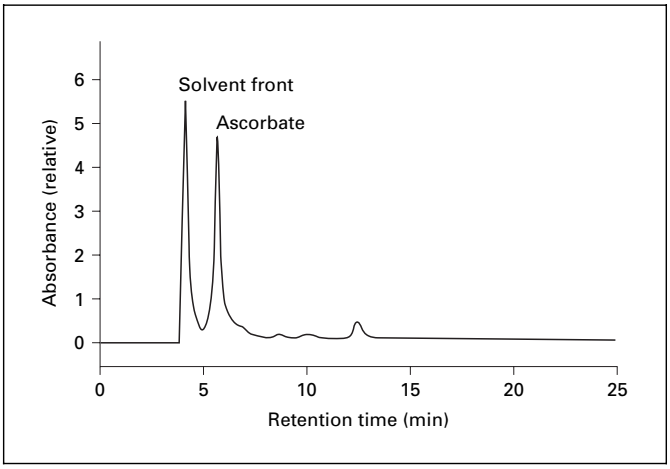

Fig. 5. Chromatogram for ultrafiltered whole-lens homogenate. UVR detection at 254 nm.

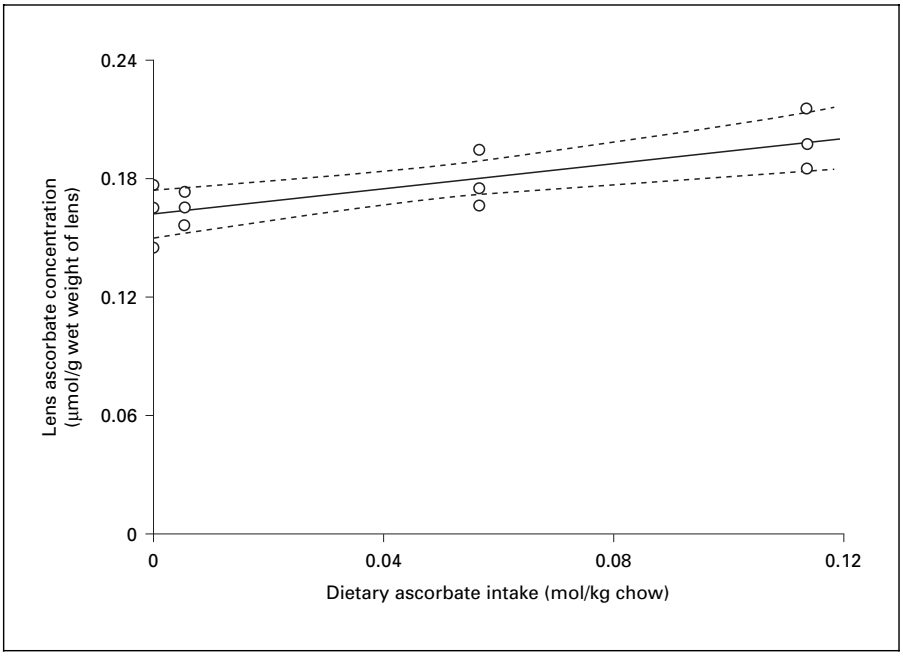

Fig. 6. Lens ascorbate concentration as a function of dietary intake. Each data point is the average of 2 lenses from the same animal. The solid line is the best-fit first-order polynomial. Dotted lines demarcate the 95% confidence interval for the regression line.

The baseline lenticular content of ascorbate (A_0 was, estimated as a 95% confidence interval, 0.16 ± 0.01 µmol/g wet weight of lens. The fact that the confidence interval excludes zero indicates a statistically significant amount of ascorbate in the lens, even without oral intake.

The relative increase in lenticular ascorbate per ascorbate intake *(k)* was, estimated as a 95% confidence interval, 0.33 ± 0.18 (µmol ascorbate) (g lens)$^{-1}$ (mol ascorbate)$^{-1}$ (kg chow) with $r^2 = 0.62$. The exclusion of zero in the confidence interval indicates a statistically significant linear increase in lenticular ascorbate due to oral intake of ascorbate.

Discussion

The current study aimed at evaluating a technique for sample preparation to determine the whole-lens content of ascorbate and to investigate whether lens ascorbate concentration depends on dietary ascorbate intake.

Since our goal was to elevate lens ascorbate concentration, we chose to supplement the chow with high concentrations of ascorbate. The reason for trying to elevate lens ascorbate concentration is to study whether lens ascorbate modulates the UVR-induced cataract in vivo. Our group has studied the rat animal model extensively.

Because ascorbate has a short biological half-life (peak exhalation at 2–3 h), we chose to feed the rats with chow supplemented with ascorbate for a period of 4 weeks rather than to acutely increase ascorbate concentration through intraperitoneal injection [38]. The goal of feeding for 4 weeks was to achieve a steady-state lens ascorbate concentration.

Our selection of the method for ascorbate measurement among other methods published [20] was based on the finding that HPLC allows accurate separation and UVR provides specific detection with high sensitivity for ascorbate in biological tissues [26]. The absorbance ratio for the oxidized form of ascorbate, dehydroascorbate, and ascorbate was found to be 4.1% at 254 nm. This implies that 96% of the signal at 254 nm is ascorbate.

The spectrophotometically derived absorbance spectrum for ascorbate is broad and within the linear range of the instrument. Because of the broad shape of the ascorbate peak, it may appear to lack distinguishable features. However, UVR detection using HPLC is ideal for ascorbate since ascorbate is distinguishable from dehydroascorbate at 254 nm (fig. 3) and in addition since the ion exchange, reversed-phase HPLC column used in the experiment sufficiently separates ascorbate from other compounds based on charge (fig. 5).

The adapted method for sample preparation of ascorbate was found to be reliable, indicating that this method is adoptable in the future. The current HPLC method has the advantage over previously described non-HPLC methods of detecting small amounts of ascorbate in biological tissues, less than 1 pmol per injected sample [26].

When processing the lens sample for ascorbate measurement, it is important to add an agent to the sample to prevent metal-catalyzed oxidation of ascorbate. The lens samples in the study were prepared in metaphosphoric acid, which prevents oxidation of ascorbate to dehydroascorbate because of high acidity and metal ion chelation [34, 37].

The current HPLC method is highly precise in the measurement range for rat lenses, based on experiments with both external and internal controls. The external controls, in which ascorbate solutions ranging from 0 to 20 μ*M* were prepared and measured on HPLC, followed a linear increase with regression of $r^2 > 0.99$ (data not shown). We also used internal controls to verify the precision and accuracy of the method. Known amounts of ascorbate ranging from 0 to 20 μ*M* were added to a portion of pooled, processed, whole-rat lens ultrafiltrate solution and measured on HPLC. As with the external controls, the increase was linear with regression of $r^2 > 0.98$ (data not shown). The ascorbate concentration in the rat lens ultrafiltrate was with an internal standard addition technique determined to be 1.87 μ*M* and with the external calibration technique to be 1.95 μ*M*. Considering the insignificant difference between the two methods, it was decided to only use external calibration for future experiments.

The HPLC-measured lens sample concentrations of ascorbate obtained in the experiment ranged from 4.4 to 6.7 μ*M*. The sample concentrations were close to 3 of the 11 concentrations of external standards, 4, 6 and 8 μ*M*, used for calibration and calculation in the experiment.

The ascorbate concentration in the supernatant after re-extracting the pellet was 46% of that in the supernatant of the lens homogenate. This finding indicates that ascorbate in the pellet is released into solution upon re-extraction.

The baseline lenticular content of ascorbate (fig. 6) is in agreement with the fact that the rat is capable of synthesizing ascorbate [2]. The presently found baseline content of ascorbate is in agreement with a previous study of the content of ascorbate in the lens, which found the baseline lenticular content to be approximately 0.12 m*M* or 0.08 μmol/g wet weight of lens [5].

The linear relationship between lenticular content of ascorbate and dietary intake (fig. 6) supports previous findings indicating that, in the rat, ascorbate enters the aqueous humor from the plasma and the lens from the aqueous humor by passive diffusion [18, 39]. This finding is important since it shows that it is possible to alter the lenticular ascorbate concentration with dietary intake. The linear dependence of lens ascorbate concentration on dietary intake in the rat reported in this study is different from the dependence reported in the guinea pig [36]. In the guinea pig, the increase is nonlinear and saturable. The difference between the rat and the guinea pig may be explained by a difference in mechanism of ascorbate transport into the lens [18], that being passive diffusion in the rat and saturable active transport in the guinea pig.

The dietary ascorbate intake listed in the graph (fig. 6) is the concentration in the chow. Since the drinking water contained no ascorbate, and since the chow ingredients contained no ascorbate, the amount of ascorbate supplemented in the chow comprised the dietary intake. Assuming a rat weight of approximately 200 g and a corresponding approximate dietary chow intake of 16 g/day, 8% of body weight/day, the actual total amount of ascorbate intake per animal in each group was: 0 mmol/day (0.0 mmol/kg chow group); 0.09 mmol/day (5.7 mmol/

kg); 0.91 mmol/day (57.0 mmol/kg), and 1.82 mmol/day (114.0 mmol/kg).

Our finding also corresponds with the previous finding that, in the rat, an intraperitoneal injection of ascorbate increases concentrations in aqueous humor and lens [5]. The lens ascorbate concentration of 0.10 μmol/g lens, after intraperitoneal injection of a dose of 1.1 mmol [5], corresponds to the lens ascorbate concentration of 0.18 μmol/g lens obtained in our experiment from the group of animals receiving a comparable daily amount of ascorbate, 0.91 mmol/day.

The increase in lenticular ascorbate concentration with dietary supplementation in the rat was prominent, approximately 25% from group 1 to group 4. The increase is biologically significant since ascorbate serves as an important antioxidant in the lens. The dependence of lens ascorbate concentration on dietary intake in the rat has not been studied previously. However, our data do correspond with an experiment analyzing the effect of a single intraperitoneal injection of an ascorbate load into the rat [5]. The finding that the lens ascorbate concentration depends on dietary intake is important since one may use it to study the preventive effect of lenticular ascorbate against oxidatively and photochemically induced cataract in vivo using the model.

In conclusion, grinding of the lens in metaphosphoric acid, ultrafiltration of the lens homogenate and subsequent HPLC with UVR detection can be used to measure ascorbate in the rat lens. The rat lens has a baseline ascorbate content even without any intake. The lens ascorbate concentration follows a linear dependence on dietary ascorbate intake without cataract development in the rat.

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