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Light-induced damage to ocular lens cation pump: Prevention by vitamin C

(Rb^+ uptake/ascorbic acid/superoxide/cataract)

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ABSTRACT **The cation pump activity of the ocular lens was damaged by exposure to light in the presence of riboflavin phosphate. The intensity of light was similar to that used for reading purposes. The observed light-induced damage was due to superoxide or its derivatives, the superoxide being produced photochemically. Such damage was attenuated by vitamin C in amounts comparable to that in the aqueous humor. Thus, a new role for the high ascorbate level present in the anterior chamber fluid and the lens has been suggested. Ascorbate in other tissues also might have this novel physiological function of protecting against damage due to superoxide and its derivatives produced during normal cellular oxidation.**

The concentration of ascorbic acid (vitamin C) in the aqueous humor of man, monkey, and many other animals is one of the highest among the various body fluids (1). The prevalence of this high level of ascorbate has been attributed to the ability of the ciliary epithelium to transport this compound actively from the plasma to the posterior chamber through the blood aqueous barrier (2). For reasons undefined at the present time, the concentration of ascorbate is also high in tissues surrounding the aqueous humor—namely, the cornea and the lens. The concentration in the latter tissue in many instances is second only to that in adrenals (1). Although the importance of ascorbic acid in hydroxylation reactions is known in many tissues, the relevance of a high ascorbate level in the aqueous humor and the lens is not precisely known, although it has been suggested that photochemical oxidation of ascorbic acid in the anterior chamber serves to provide hydrogen peroxide to the lens. The peroxide, in turn, may regulate the tissue hexose monophosphate shunt through the following series of interlinked reactions (3).

$$\text{ascorbic acid} + O_2 \rightarrow \text{dehydroascorbate} + H_2O_2$$
$$2\,GSH + H_2O_2 \rightarrow GSSG + 2\,H_2O$$
$$GSSG + 2\,NADPH \rightarrow 2\,GSH + 2\,NADP$$
$$\text{Glucose} + NADP \rightarrow \text{Shunt metabolites} + NADPH + CO_2,$$

in which GSH is reduced glutathione and GSSG is oxidized glutathione.

These reactions have been proposed to constitute a nonenzymatic respiratory link between the aqueous humor and the lens through diffusion of hydrogen peroxide. In the cortex of the lens, ascorbate has been suggested to prevent oxidation of protein-SH (4). However, a crucial role of ascorbic acid in the maintenance of lens physiology has been difficult to demonstrate because scorbutic animals are reported not to develop cataracts (5). In view of the initial high concentration of ascorbic acid in the lens as well as in the aqueous humor, the absence of cataracts in animals who live only for a short time (a month or so), on a diet deficient in vitamin C, does not disprove the importance of this nutrient.

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It would appear that a function of ascorbic acid, if any, in lens and aqueous humor might be linked with photochemical or biological oxidation which may or may not require oxygen. In the former case, quantum considerations require the reactions to proceed through univalent steps of electron transfer (6, 7). The first product of such a step-wise transfer is the generation of superoxide: $O_2 + e \rightarrow \cdot O_2^-$. The superoxide is now known to be produced in aerobic systems. Such radicals are self-dismutated to form H_2O_2 ($\cdot O_2^- + \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). However, the rate of this reaction is not fast enough to provide physiological protection to the tissues against injury by the highly reactive superoxide (8). Such a radical by itself or through certain of its derivatives can impart injury through oxidation of enzymatic and nonenzymatic protein sulfhydryls, oxidation of cell membrane sulfhydryls, polymerization and depolymerization of various small and macromolecular entities, and lipid peroxidation (9). Protection against such deleterious effects of $\cdot O_2^-$ has been suggested to be offered by the universally present enzyme, superoxide dismutase (SOD), EC 1.15.1.1 (8). Whereas this mechanism of protection by SOD against $\cdot O_2^-$ radicals seems applicable to tissues in general, some of the ocular tissues and fluids offer some unusual features. By virtue of their transparency, they provide a unique *in vivo* facility for the photochemical generation of $\cdot O_2^-$ radicals. Such $\cdot O_2^-$ radicals would be additive to that generated by normal oxidation. We hypothesize that the $\cdot O_2^-$ radicals photochemically produced in these transparent tissues and the products consequent to $\cdot O_2^-$ dismutation (H_2O_2 and $\cdot OH$) would be injurious to the integrity of the cell membranes unless the surrounding humoral fluids have appropriate scavengers.

We investigated this possibility in the lens. Because this tissue, being avascular, derives most of its basic constituents from transport activity, injury of its anterior cell membranes may interfere with the maintenance of cellular dynamics. Experiments reported herein revealed that indeed the lens membrane activity as measured by the efficacy of its cation pump is adversely affected by $\cdot O_2^-$ generated photochemically, and this adverse effect could be attenuated substantially by ascorbic acid in physiological amounts.

MATERIALS AND METHODS

The cation pump efficacy was assessed by determining *in vitro* uptake of $^{86}Rb^+$ by the lenses of Sprague–Dawley rats when incubated in physiological medium labeled with $^{86}RbCl$ (10). Rat lenses were used in these experiments to minimize the effect of endogenous ascorbate, the native level of ascorbate in the lenses of this species being negligible (11). Animals weighing 100 g were killed by decapitation and the lenses were dissected out after enucleation. The dissection technique started with letting the anterior side (cornea) of the enucleated eye rest on a filter paper and cutting open the eyeball along the four

Abbreviations: $\cdot O_2^-$, superoxide radical; SOD, superoxide dismutase; CL, counts in less water; CM, counts in incubation medium.

equidistant meridians (postero-anterior) until the limbus was reached. The four resulting flaps were then stretched out on the filter paper, and the exposed lens was loosened from the ciliary muscles by gently cutting the zonules. The intact lens was then scooped out with a smooth plastic blade avoiding contact with the anterior surface, quickly weighed on a Mettler scale balanced with parafilm to avoid trauma, and transferred to a 30 × 10 mm plastic petri dish containing 4 ml of prewarmed (37°C) incubation medium. The medium used was a modification of TC199 (12), which contained 141 mM Na^+, 4.94 mM K^+, 2.48 mM Ca^{2+}, 0.5 mM Mg^{2+}, 26.8 mM HCO_3^-, 123.4 mM Cl^-, 0.5 mM SO_4^{2-}, 0.28 mM $H_2PO_4^-$, 0.21 mM HPO_4^{2-}, 5.14 mM amino acids, and 5.55 mM glucose. Other organic constituents accounted for 0.45 mM. Less than 500 μl of $^{86}RbCl$ (specific activity of 11 mCi/mg; 1 Ci = 3.7×10^{10} becquerels) was added to the medium (1500 counts per 0.1 ml) so that the ionic concentration was very minimally influenced. The stock solution of $^{86}RbCl$ was 1 μCi/ml. In medium containing ascorbic acid (2.5 mM), the amount of NaCl was decreased by 1.25 mM to minimize alteration in tonicity, which was maintained at 295 ± 5 mosM as measured by an osmometer (Model 3W from Advanced Instruments, Needham Heights, MA). Sigma bovine blood SOD S-8254 and Sigma bovine liver catalase C-10, EC 1.11.1.6 were used in concentration of 15 units/4 ml of incubation medium. Bovine serum albumin in amounts equivalent to SOD (5 μg/4 ml) or catalase (10 mg/4 ml) when used as control did not mimic the protective effect of the enzyme. Incubation of lenses in the media described above was conducted in a water-jacketed incubator (Bellco Glass, Vineland, NJ) gassed with 95% air/5% CO_2 and maintained at 37°C. The period of incubation was 21 hr. The incubator was fitted with a light box containing two 15-W fluorescent day-light tubes (legend of Fig. 1), such that the measured light intensity at the surface of the petri dishes was 130 footcandles (1 footcandle = 10.76 lux).

After incubation, the medium along with the lens was poured gently over a Buchner funnel (1-inch diameter), and the lens retained on the filter disc was rinsed with 0.25 ml of isotonic saline (0.9% NaCl) to eliminate radioactivity adherent to the surface of the tissue. The lens was then extracted in 1 ml of 10% aqueous trichloroacetic acid. The extract was centrifuged at 1500 × *g* to obtain a clear supernatant, and 100 μl of the supernatant was used for liquid scintillation counting and determination of radioactivity in the lens water (60% of wet weight) (12). Medium was also counted simultaneously. The results were expressed as the distribution ratio of $^{86}Rb^+$ (cpm/1.0 ml) between lens water (CL) and an equal volume of incubation medium (CM) attained at the end of incubation period. The ratio is abbreviated as CL/CM. Because the specific activity of $^{86}Rb^+$ is the same in the medium of incubation and the tissue water, CL/CM also represents the distribution ratio of nonradioactive rubidium.

Earlier incubation studies have demonstrated that $^{86}Rb^+$ and $^{42}K^+$ are handled by the lens cation pump in analogous fashion (10, 13). Either of the two isotopes can thus be used in these cation-uptake studies. However, the use of $^{86}Rb^+$ offers practical advantages due to its longer half-life and lower gamma emission.

RESULTS AND DISCUSSION

CL to CM ratios attained in various experiments are indicated in Fig. 1. The experimental variations are given in the legend of Fig. 1. The CL to CM ratio after incubation of the lens in control medium in darkness (bar A) was 24 ± 1. Exposure of these controls to light had no effect. Addition of riboflavin phosphate (50 μM) was also without any effect if incubation was conducted in darkness. In light, however, riboflavin phosphate had a very pronounced effect on Rb^+ uptake, the CL to CM ratio in this case being approximately 4.5 (bar B), only 20% of the controls. In addition to a decreased CL/CM, light and riboflavin phosphate together resulted in a loss of clarity and transparency as compared to controls. Phosphate alone in equivalent amounts had no effect, either in dark or light, indicating that the observed effect of light in the presence of riboflavin phosphate is essentially dependent on the riboflavin moiety. Previous studies have shown that the riboflavin moiety undergoes photoreduction upon exposure to room light in the presence of potential electron donors and the photoreduced riboflavin produces superoxide radicals by interaction with the ambient oxygen (14). These radicals can dismutate to yield H_2O_2. The H_2O_2 so produced could give rise to highly reactive hydroxyl radicals through the iron-catalyzed Haber–Weiss reaction (15):

$$\cdot O_2^- + H_2O_2 \xrightarrow{Fe} OH^- + \cdot OH + O_2.$$

The observed damage to the lens pump could thus be related to $\cdot O_2^-$, OH^-, and H_2O_2 (16). They could be acting individually or in combinations.

Inclusion of SOD in the medium led to a CL/CM of 18 ± 1 (bar C), 75% of the control. The pump inactivation in presence of light and riboflavin was thus substantially abolished by SOD. Catalase in similar amounts abolished the inactivation (bar E). Because SOD, which scavenges $\cdot O_2^-$ and generates H_2O_2, protected the cation pump, we can conclude that $\cdot O_2^-$ was an important agent of lens injury and that H_2O_2 alone was insufficient to cause the injury. Similarly, because catalase, which scavenges H_2O_2 but not $\cdot O_2^-$, also protected the cation pump, we conclude

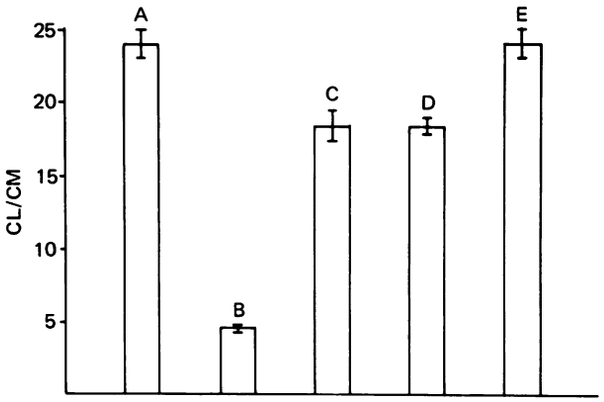

FIG. 1. Lenses isolated from rats weighing 100 ± 5 g were individually incubated for 21 hr in 4 ml of culture medium contained in a 35 mm × 10 mm plastic petri dish. The medium used was modified TC199 labeled with $^{86}Rb^+$ as described in the text. After incubation, $^{86}Rb^+$ in the lens was determined by liquid scintillation counting of an aliquot of protein-free supernatant prepared by homogenization of the tissues in 10% trichloroacetic acid and centrifugation. The distribution of $^{86}Rb^+$ in the lens was calculated by assuming that 60% of the wet weight represented water (12). The ratio of CL to CM was obtained. Bars: A, control with or without riboflavin in the dark and without riboflavin in the light; B, with riboflavin in the light; C, with riboflavin and SOD in the light; D, with riboflavin and ascorbic acid in the light; E, with riboflavin and catalase in the light. Riboflavin concentration in various experiments was 50 μM. The concentrations of SOD and catalase were 15 units/4 ml. Ascorbate concentration was 2.5 mM. The light source consisted of two 15-W (18 × 1 inch) fluorescent daylight tubes enclosed in a box open at one end. The opening was covered by a 0.5-inch-thick plastic opaque sheet to decrease exposure to UV light as well as to make illumination more uniform. The illumination provided by this arrangement was 130 footcandles per foot^2 (1 ft = 0.3048 m) on the covers of petri dishes. At least 10 lenses were incubated in each group. The I on the bars represents SD.

that H_2O_2 appears to contribute to the lens damage and that $\cdot O_2^-$ alone was not sufficient. It follows that both $\cdot O_2^-$ and H_2O_2 are simultaneously needed to cause the observed damage. The iron-catalyzed Haber–Weiss reactions (15, 17)—Fe (III) + $\cdot O_2^-$ → Fe (II) + O_2 and Fe (II) + H_2O_2 → Fe (III) + OH^- + $\cdot OH$ could explain these results. Precise localization of radical or molecular species involved in the pump damage needs further investigation using specific $\cdot OH$ scavengers. These studies, nevertheless, indicate that $\cdot O_2^-$ and its derivatives produced in the aqueous humor must be scavenged rapidly for proper maintenance of the cation pump. Because SOD or catalase would not ordinarily be present in extracellular fluids, the function of these enzymes in the aqueous humor must be taken up by other possible substances. The substance examined here was ascorbic acid.

Incubation of lenses in light in riboflavin-containing medium fortified with ascorbic acid near physiological concentration (2.5 mM) led to a CL to CM ratio of approximately 18 (bar D). This is substantially higher than that attained when the lenses were incubated in light in medium containing the riboflavin and not fortified with ascorbic acid (bar B). Obviously, ascorbate protects the lens pump substantially against damage by $\cdot O_2^-$ or its derivatives. The extent of protection provided by ascorbate (bar D) is similar to that provided by SOD (bar C).

The riboflavin concentration (50 μM) used in these *in vitro* experiments is possibly close to that found in the lens epithelium (12 μM) (18). Its concentration in the aqueous humor is probably much lower (30 nM) (19). It will, however, vary greatly with the dietary status as well as the time lapse after meals when aqueous humor is collected for analysis. Consequent to the presence of riboflavin and other photosensitive entities in lens epithelium as well as in the aqueous humor (20), there is a possibility of the incessant presence of $\cdot O_2^-$ in the anterior vicinity of the lens, at least during the photopic vision. A cumulative, long-term adverse effect of $\cdot O_2^-$ and its derivatives on the lens and also in the adjoining tissues, in the absence of a scavenger, is feasible, particularly in persons receiving high amounts of photoreactive substances through diet, supplementation with vitamins having strong light-absorbing property, and long term use of psychoactive drugs (phenothiazines). High aqueous ascorbate thus appears useful for protecting the surface of epithelial membranes, especially in an avascular lens, against damage concomitant to photochemical generation of $\cdot O_2^-$ radicals under various conditions. These findings further emphasize the concept of the importance of essential nutrients in prevention of certain forms of cataracts (21, 22).

The concentration of ascorbic acid in the aqueous humor of man, monkey, and rabbit is low prenatally, the level of the nutrient in early fetal aqueous humor being close to that in their plasma ($\approx$0.05 mM) (23). Postnatally, the concentration in the aqueous humor is about 20- to 40-fold higher than that in the corresponding plasma; the actual aqueous humor concentrations range between 1 and 1.8 mM in all three species (24). In some species like rat, cat, and galago the level remains low in adult aqueous humor also, the respective levels being trace, 0.07 mM, and 0.03 mM (24). In some instances lenticular ascorbate concentration may also be low. In rat lens, for example, it is barely detectable (11). In view of the proposal that aqueous humor ascorbate functions as a scavenger of photochemically produced $\cdot O_2^-$ and its derivatives, the mechanism of such scavenging in low ascorbate situations is intriguing. The low ascorbate, however, appears to be associated primarily with a predominantly nocturnal habitat (galago, rat, and cat) (25) or with *in utero* situations in which the animals are exposed to much less light than are diurnal and arrhythmic animals. Seemingly, a lower aqueous humor ascorbate level in nocturnal or *in utero* animals meets the physiological needs. On the other hand, a high ascorbate level is of obvious advantage in habitat adjustments. This variation with habitat in ascorbate level further exemplifies the importance of this substance in relation to photochemistry of the anterior chamber of the eye.

Nonenzymatic scavenging of $\cdot O_2^-$ or its derivatives by ascorbate will obviously be slower than enzymatic scavenging. A high concentration of ascorbate will, nevertheless, compensate for the sluggishness of the nonenzymatic process. The presence of high levels of ascorbate in lens and aqueous humor is thus again in conformity with the hypothesis that vitamin C functions to protect the lens against damage by interacting with $\cdot O_2^-$ (26, 27) or its derivatives, $\cdot O_2^-$ (the primary damaging agent) being produced either photochemically or by nonphotochemical oxidation.

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1. Pirie, A. & Van Heyningen, R. (1956) in *Biochemistry of the Eye* (Thomas, Springfield, IL), pp. 24–28.
2. Becker, B. (1967) *Invest. Ophthalmol.* **6**, 410–415.
3. Pirie, A. (1965) *Nature (London)* **205**, 500–501.
4. Pirie, A. (1972) in *Contemporary Ophthalmology*, ed. Bellows, J. G. (William & Wilkins, Baltimore, MD), pp. 494–501.
5. Monjukowa, N. & Fradkin, M. (1935) *Albrecht von Graefes Arch. Ophthalmol.* **133**, 328–338.
6. Russel, G. (1959) *J. Chem. Ed.* **36**, 111–118.
7. Taube, H. (1965) *J. Gen. Physiol.* **49**, 29–52.
8. Fridovich, I. (1978) *Science* **201**, 875–880.
9. Zimmermann, R., Floche, L., Weser, U. & Hartman, H. J. (1973) *FEBS Lett.* **29**, 117–120.
10. Becker, B. (1962) *Invest. Ophthalmol.* **1**, 502–506.
11. Bellows, J. G. (1975) in *Cataract and Abnormalities of the Lens* (Gruen & Stratton, New York), p. 75.
12. Merola, L. O., Kern, H. L. & Kinoshita, J. H. (1960) *Arch. Ophthalmol.* **63**, 830–835.
13. Kinoshita, J. H. (1974) *Invest. Ophthalmol.* **13**, 713–724.
14. Beauchamp, C. & Fridovich, I. (1971) *Anal. Biochem.* **44**, 276–287.
15. Haber, F. & Weiss, J. (1934) *Proc. R. Soc. London Ser. A* **147**, 333–351.
16. Fukui, H. N., Epstein, D. L. & Kinoshita, J. H. (1973) *Exp. Eye Res.* **15**, 249–253.
17. Day, E. D. & McCord, J. M. (1978) *FEBS Lett.* **86**, 139–142.
18. Kinsey, V. E. & Frohman, C. E. (1951) *AMA Arch. Ophthalmol.* **46**, 536–541.
19. Philpot, F. J. & Pirie, A. (1943) *Biochem. J.* **37**, 250–254.
20. Harris, J. E., Gruber, L., Talman, E. & Hoskinson, G. (1959) *Am. J. Ophthalmol.* **48**, 528–535.
21. Heffley, J. D. & Williams, R. J. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4164–4168.
22. Varma, S. D., Mikuni, I. & Kinoshita, J. H. (1975) *Science* **188**, 1215–1216.
23. Kinsey, V. E. & Jackson, B. (1949) *Am. J. Ophthalmol.* **32**, 374–378.
24. Davson, H. (1962) *The Eye* (Academic, New York), p. 90.
25. Duke-Elder, S. (1958) in *System of Ophthalmology* (Mosby, New York), Vol. 1, p. 605.
26. Halliwell, B. & Foyer, C. H. (1976) *Biochem. J.* **155**, 697–700.
27. Varma, S. D., Ets, T. K. & Richards, R. D. (1977) *Ophthalmic Res.* **9**, 421–431.