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# Protective effect of ascorbate against oxidative stress in the mouse lens

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**Abstract**

The purpose of this study was to determine if high ascorbate of the human aqueous protects the lens against oxidative stress. Previous studies with the rat lens have been inconclusive because of its fortification with aldose reductase (AR), an important antioxidant. The human lens is deficient in this activity. These studies were hence done with the mouse lens, a species deficient in this enzyme. The reactive oxygen species (ROS)-induced physiological damage to the tissue was assessed in organ culture, by measuring its ability to actively transport $^{86}Rb^{+}$ ions, in the absence and presence of ascorbate. In addition, the status of tissue metabolism and its antioxidant reserve were assessed by quantitating ATP and glutathione (GSH). As expected, ROS decreased the membrane transport activity as well as the levels of ATP and GSH. Ascorbate minimized these toxic effects substantially. The presence of high ascorbate, therefore, appears highly beneficial in protecting the lens against oxidative damage and cataract formation, despite a deficiency of AR. The findings therefore appear to be significant from the point of view of using this nutrient for delaying the onset of cataract development in human beings, therapeutically as well as nutritionally.


*Keywords:* Oxidative stress; Cataract; Ascorbate; $Na^{+}$-$K^{+}$-ATPase; Rubidium transport

## 1. Introduction

Ascorbic acid is an essential nutrient for humans, monkeys and guinea pigs. Its dietary deficiency leads to several pathological manifestations such as perifollicular hyperkeratosis associated with excessive skin exfoliation, subcutaneous hemorrhages, bleeding and inflammation of the gingiva, defects in tooth and bone formation and chronic anemia due to reduced absorption of iron. The protective effect of ascorbate against these and several other related manifestations is primarily because of its role in facilitating physiologically important enzyme catalyzed hydroxylation reactions such as those involved in the synthesis of collagen, norepinephrine, several hydroxylated steroids and the metabolism of certain aromatic essential amino acids. In addition to the enzymatic hydroxylation, ascorbate has been implicated in several non-enzymatic redox reactions such as the reduction of $Fe^{3+}$ to $Fe^{2+}$, facilitating its absorption from the gut, and the conversion of folic to folinic acid. Since ascorbate cannot be synthesized by the species mentioned above, its maintenance at adequate levels in the intracellular as well as extracellular fluids of the tissues is essential for a variety of physiological functions such as those described above. Interestingly, its concentration in all ocular tissues is substantially high [1]. In the case of lens, the maintenance of such high levels is greatly facilitated by its active transport from the plasma across the blood aqueous barrier, to the posterior and then to the anterior chamber. Investigations on its function in relation to the maintenance of lens transparency have therefore been of wide interest. Since its concentration is higher in the diurnal animals as compared to that in the nocturnes, we have previously proposed that its function in the aqueous is to protect the lens against photo-oxidative stress and consequent cataract formation because of a continued light-catalyzed generation of various reactive oxygen species (ROS) such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals in the aqueous and other intraocular compartments [2,3].

It has been demonstrated that it protects the rat lens physiology against oxidative damage under photochemical as well as ambient conditions [3,4]. We have also shown that it protects the lens against oxidative stress leading to cataract formation in vivo [5]. However, the rat lens is endowed with rather high aldose reductase (AR) activity, which also pro-

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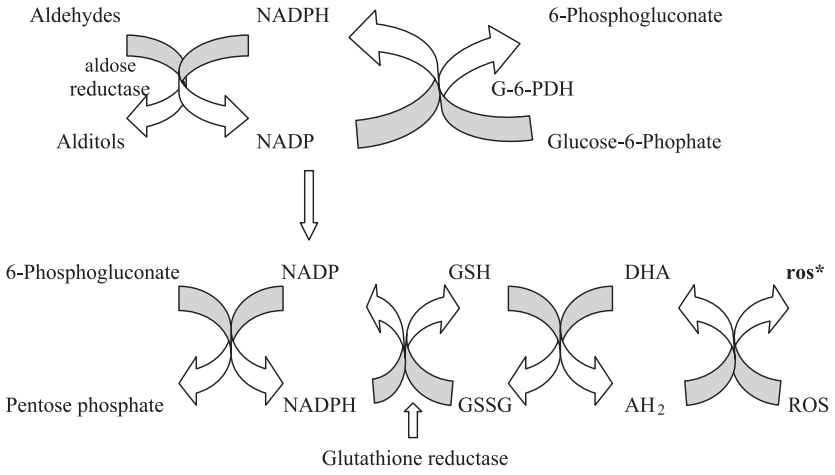


Fig. 1. Integration between AR activity related to detoxication of aldehydes and the maintenance of redox cycle by glutathione and ascorbate. ros*= scavenged reactive oxygen species, DHA = dehydroascorbate, $AH_2$ = ascorbate, G-6-PDH = glucose-6-phosphate dehydrogenase.

tects the tissues against oxidative stress [6]. It does so by converting various toxic carbonyls, produced by many unwanted reactions initiated by oxygen free radicals, to their corresponding alcohols. For example, such derivatives can be produced by lipid peroxidation and oxidative degradation of carbohydrates such as glucose. Additionally, oxidation of ascorbate could also lead to the formation of several toxic carbonyl derivatives, likely to be detoxified by AR (Fig. 1). These NADPH-dependent detoxifying reactions, associated with simultaneous generation of NADP, accelerate the HMP shunt, which is involved in regeneration of GSH from GSSG produced during oxidative stress. The former in turn, while acting as a major antioxidant reserve, is also necessary for regenerating the tissue ascorbate by reducing dehydroascorbate, thereby facilitating the effectiveness of ascorbate in neutralizing various ROS. It was therefore felt necessary to determine the effectiveness of ascorbate in the physiological maintenance of the lens when challenged with oxidative stress using lenses of other species with low AR activity. Such findings could then be useful in further evaluation of a pro/anti-cataractogenic influence of ascorbate in the human lens. The present investigations were therefore conducted using mice lens known to be deficient in this enzyme, similar to that in the human lens. Experiments were also conducted under normal as well as high oxygen tensions. The latter studies were considered especially desirable in view of the known development of cataracts in humans during treatment with hyperbaric oxygen. The results are therefore considered important from the point of view of nutritional as well as therapeutic use of this substance in the prevention of human cataracts, as well as minimizing the other toxicities associated with hyperbaric oxygen therapy.

## 2. Materials and methods

CD-1 mice (weighing 25 g) were obtained from Charles River Laboratories (Wilmington, MA) and housed under standard animal house conditions with 12 h dark/light cycle. Just prior to the experiments, they were sacrificed, eyes enucleated and intact lenses dissected out atraumatically after incising the globe from the posterior side. The effect of ROS on the physiological status of the lens was then determined by culturing it in media containing 0.5 mM xanthine, xanthine oxidase (Sigma X 2252) and uricase (Sigma U 7128) and measuring the uptake of rubidium ions [7]. This was followed by the determination of levels of ATP. That the effects are related to oxidative insult was ascertained further by measurements of glutathione.

Briefly, the experiments were conducted as follows: 1 ml of medium 199 mixed with a trace amount of $^{86}RbCl$ and 0.5 mM sodium xanthine (XA) were pipetted out in a series of test tubes labeled as controls and experimentals. The tubes were then maintained at 37 °C in an incubator gassed with either air/$CO_2$ (95%:5%) or $O_2/CO_2$ (95%:5%). After equilibration, the freshly dissected lenses were transferred contralaterally to the tubes marked controls and experimentals. Lenses from the same animal were used as controls and experimentals. The concentration of ascorbate in the experimental medium was 2 mM. The controls contained 2 mM NaCl instead of ascorbate. Xanthine oxidase (XO, 10 μl) and 2 μl of uricase (U) were then added to all the test tubes and incubation continued for either 5 or 18 h. A set of basal controls were also run simultaneously without the addition of enzymes but with 2 mM NaCl or 2 mM ascorbate. The amount of the enzyme to be added to the medium was predetermined, so that the level of peroxide attained in the medium was ~ 0.3 mM within half an hour. Measurement of $H_2O_2$ was done iodometrically as described previously [4]. Subsequent to incubation, the lenses were collected from the medium by gently transferring the contents of the tube on a porcelain Buchner funnel. The lens retained on the funnel was rinsed with 200 μl of physiological saline to get rid of the adherent medium and its radioactivity determined by gamma counting. The radioactivity of the medium was also simultaneously determined. The uptake of rubidium

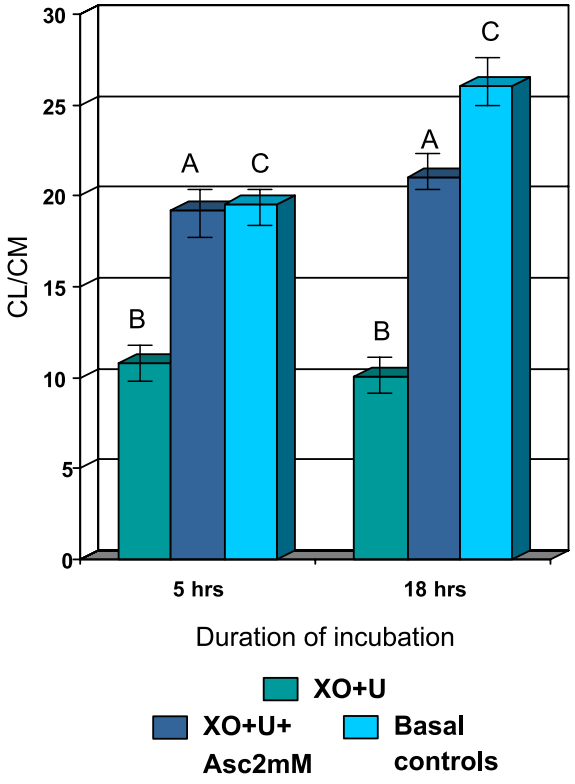


Fig. 2. Effect of ROS on cation ($^{86}Rb^{+}$) transport activity of the lens in culture in the presence or absence of ascorbate. The experiments were conducted in the presence of air/$CO_2$ (95:5). ROS were generated in the culture medium by xanthine–xanthine oxidase reaction. The ordinate represents the distribution ratio of $^{86}Rb^{+}$ between the lens water and the medium (CL/CM) $n \geq 6$. Data are expressed as means ± S.D. The ratios obtained in the presence of ascorbate (A) were significantly higher than in its absence (B) at both time periods. $P$ value $\leq 0.001$. The values in A were also closer to the basal controls (C).

ions was expressed as the distribution ratio of the radioactivity between the lens water (CL) and the medium of incubation (CM).

Following the determination of rubidium uptake, an aqueous extract of the tissue was prepared by homogenizing it in 1 ml of distilled water and centrifugation. The content of ATP was then determined promptly by reacting 50 μl of the supernatant with 200 μl of firefly lantern extract (Sigma FLE 50) [8]. The luminescence produced was then measured in a luminometer (Turner Designs). ATP standards were also determined simultaneously.

For measurement of GSH, the fresh aqueous extract was deproteinized by addition of an aliquot of 100% trichloracetic acid (TCA) to a final concentration of 10% and centrifugation. Three-hundred microliters of 0.6 M disodium phosphate was added to 100 μl of the supernatant. One-hundred microliters of DTNB (5,5′-dithio-bis-2-nitrobenzoic acid) reagent was then added, and the resulting yellow color was read spectrophotometrically at 412 nm. The DTNB reagent was prepared by dissolving 4 mg of DTNB and 100 mg trisodium citrate in 10 ml distilled water. Standard GSH was also run simultaneously [9].

## 3. Results

The objective of our present studies was to determine the possible effectiveness of ascorbate against cataract induction by ROS generation in the aqueous, using the mouse lens for reasons explained earlier. Several previous studies have reported that a decrease in the $Na^{+}$-$K^{+}$-ATPase-dependent cation transport activity in the lens epithelial cells is procataractogenic [10–12]. The possibility of this happening is more imminent in the case of lens subjected to oxidative stress induced by ROS generation in the aqueous or the culture medium. Hence in the present studies, we have determined the effectiveness of ascorbate against such transport damage to the mouse lens in culture by measuring the post culture distribution ratio of the rubidium ion between the tissue water and the incubation medium.

As shown in Fig. 2, the distribution ratio (CL/CM) of $^{86}Rb^{+}$ in the lenses incubated in the basal medium, under air/$CO_2$ (95%:5%), for a period of 5 h was approximately 20. In the presence of ROS, this was substantially depressed, the ratio in this case being approximately 10. The

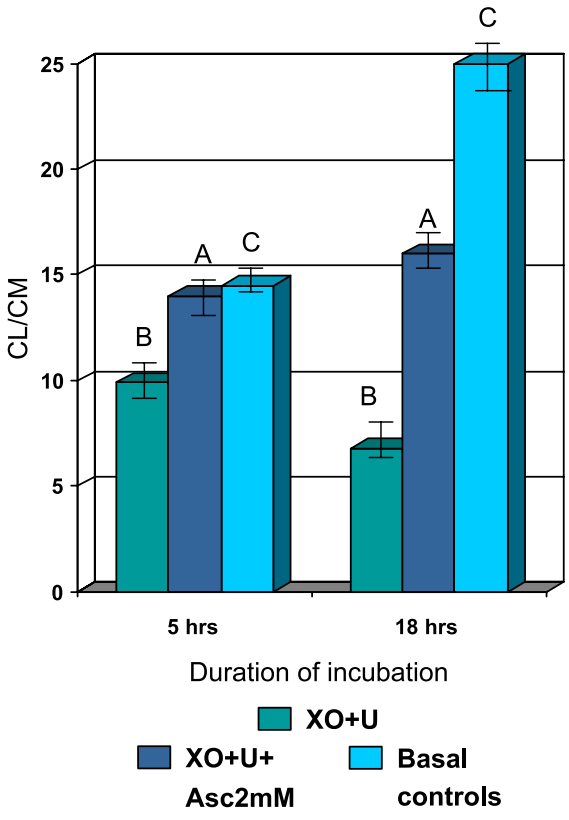


Fig. 3. Effect of ROS on cation transport activity of the lens in culture under $O_2$/$CO_2$ (95:5), in the presence or absence of ascorbate. The experimental details were similar to those described under Fig. 2 and the text. Data are expressed as means ± S.D. $n \geq 6$. All values obtained in the presence of ascorbate and ROS together (A) were significantly higher than those obtained in presence of ROS alone (B). Although the values in A at 18 h were expectedly lower than in the basal controls (C), they were still substantially higher than that observed with the lenses incubated with ROS alone. $P$ values between A and B were $\leq 0.001$.

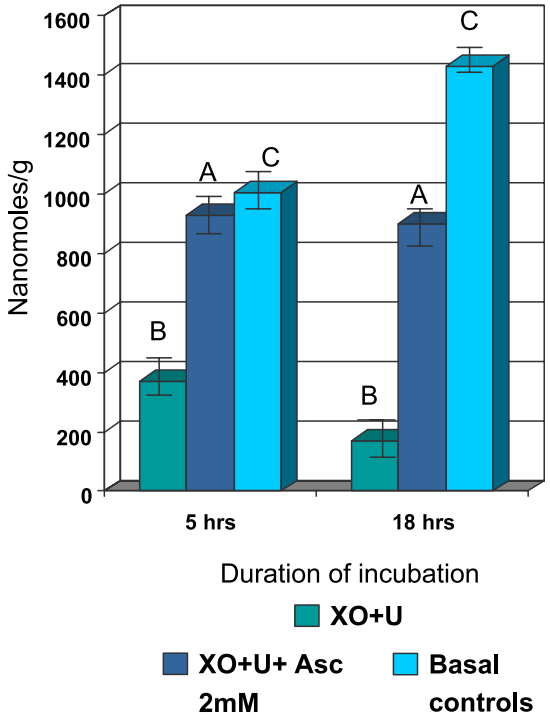


Fig. 4. ATP levels in the lenses. Effect of ROS and ascorbate. Incubations were done in the atmosphere of air/$CO_2$ (95:5). ATP was determined in the fresh aqueous extract of the tissue as described in the text. Data are expressed as means ± S.D. $n \geq 6$. The $P$ values: between B and A $\leq 0.001$; between C and A, not significant and between C and B $\leq 0.001$.

lenses in this group were also less clear as compared to the basal controls. The experimental set-up was hence found reliable for assessing the anti-cataractogenic potential of ascorbate. As further shown in Fig. 2, addition of this substance to the ROS-generating medium had a substantial beneficial effect, the distribution ratio (CL/CM ~ 19) in this case being close to the basal controls. The clarity of the lens in this group was also better maintained. It was interesting to find that further incubation of the lenses in the ROS medium for up to a period of 18 h did not increase the extent of pump damage any further, perhaps because of the ability of the lens to sustain itself at least to a certain extent. The effectiveness of ascorbate still remained substantial, as indicated by maintenance of lens transparency as well as the distribution ratio which in this case was still 80% of the basal controls. This was twice of that obtained in the lenses incubated with ROS for identical period.

In order to assess the effectiveness of ascorbate under more severe oxidative conditions, subsequent experiments were done in the presence of 95% $O_2$:5% $CO_2$ instead of air/$CO_2$. It was notable that with this level of $O_2$, the basal ratio (CL/CM ~ 14) after 5 h was significantly less than that obtained in the presence of normal $O_2$ environment (CL/CM ~ 20). The presence of excess oxygen by itself therefore has a damaging effect on the lens. Simultaneous generation of ROS depressed the ratio even further (CL/CM ~ 10) (Fig. 3), with loss of transparency of the lens. However, as postulated, in the presence of ascorbate, the ratios were maintained closer to the basal controls (CL/CM ~ 14). The lenses also remained relatively clear. The substance was therefore found to be effective even in conditions of hyperoxia. Also, in experiments extended to 18 h, the ratio in the presence of ascorbate (CL/CM ~ 16) was about three times higher than that in the lenses incubated without ascorbate (CL/CM ~ 6) but in the presence of ROS. However, in these extended experiments, the protective effect of ascorbate although highly significant was not full, the CL/CM being 67% of the basal controls (CL/CM ~ 26). The decreased effect of ascorbate with extended time is obviously due to its depletion as well as the cumulative increase in oxidative stress, suggesting the need of greater supplementation with ascorbate in situations with higher $O_2$ tensions, such as in hyperbaric treatments.

That the effectiveness of ascorbate against oxidative stress to the tissue transcends down to the intracellular compartment was also observed. As summarized in Fig. 4, the levels of ATP in the controls incubated for 5 and 18 h in the normal oxygen conditions were 371 ± 158 and 170 ± 50 nm/g tissue weight, respectively. The basal values varied between 1000 and 1500 nm/g in various experiments. ROS therefore had a significant depressive effect on tissue metabolism also. Incubation with ascorbate had a reversal

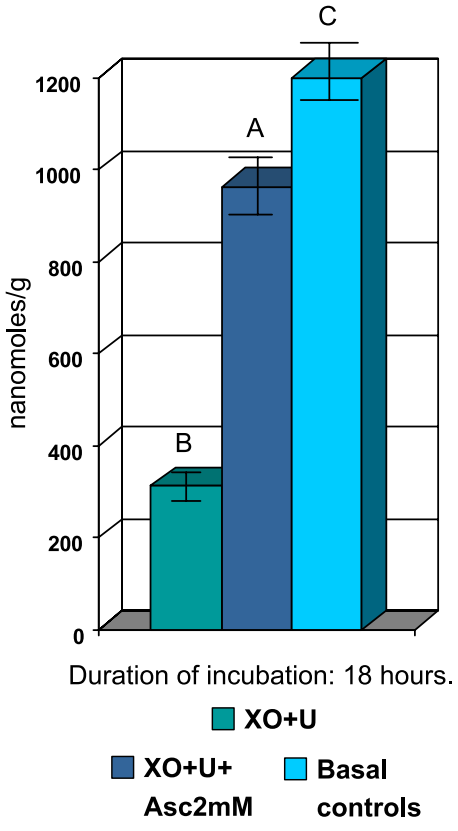


Fig. 5. ATP levels in lenses incubated in presence of oxygen/$CO_2$ (95:5). $n \geq 6$. Data are expressed as means ± S.D. The values in A and C are significantly higher than B, $P \leq 0.001$.

effect, the ATP levels in this case being 929 ± 350 and 930 ± 200 nm/g at the end of 5 and 18 h, respectively. Overall, the metabolic status of the tissue was reduced to 30% and 10% of the basal controls at the end of 5 and 18 h, respectively, in the presence of ROS, with substantial restoration in the presence of ascorbate.

In the presence of higher oxygen (95%) (Fig. 5), the ATP levels were depressed to 315 ± 230 as compared to ~ 1200 nm/g in the basal controls. The value in the presence of ascorbate with ROS was 960 ± 200 nm/g. The preventive effect of ascorbate against metabolic deterioration was therefore remarkable even under conditions of excessive oxidative stress.

The level of GSH in the lenses incubated with ROS decreased to at least about 1/10 of the basal controls. This again was substantially preventable by the addition of ascorbate. The effect was held up in experiments lasting 5 as well as 18 h, in the presence of normal as well as hyperoxic conditions (Figs. 6 and 7). The higher level of GSH in basal controls in hyperoxic conditions as compared to that in normoxic conditions is consistent with previous observations [13,14].

Overall, the results strongly suggest that even in the relative deficiency of AR as well as excessive oxygen, the effectiveness of ascorbate against oxidative stress to the tissue is fairly significant and unequivocal.

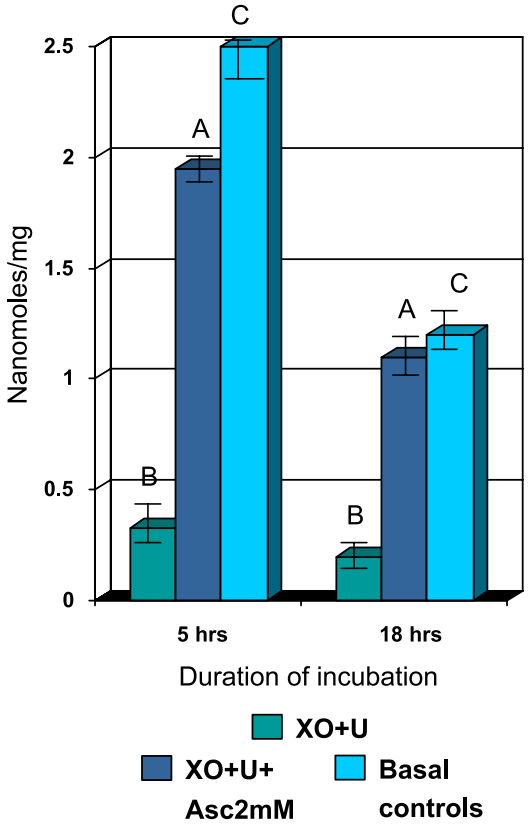


Fig. 6. GSH levels in the lenses incubated in the presence of air/$CO_2$ (95:5). The values are expressed as means ± S.D. $n \geq 6$. The values in A are significantly higher than that in B, although they are significantly lower than C related to the depletion of ascorbate in the extended period.

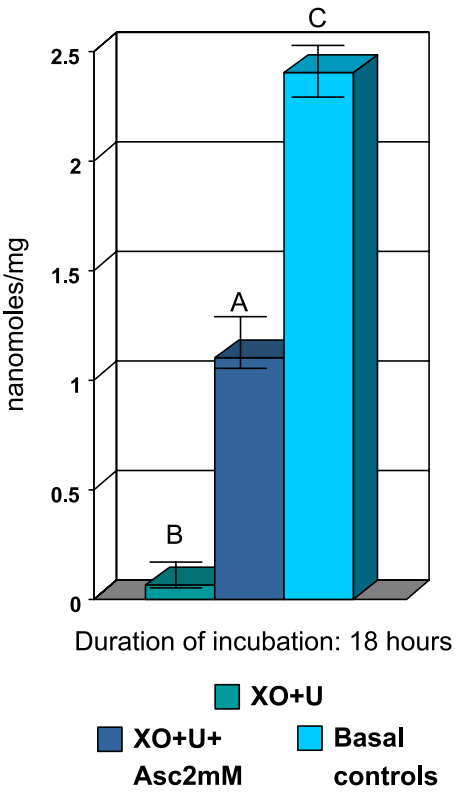


Fig. 7. GSH levels in the lenses incubated in the presence of $O_2/CO_2$ (95:5). The values are expressed as means ± S.D. $n \geq 6$. The values in A are significantly higher than that in B, although they are significantly lower than C. $P$ values between B and A, A and C, and B and C ≤ 0.001.

## 4. Discussion

The concentration of ascorbate in the ocular tissues is relatively high in comparison to most other body tissues with the possible exception of adrenals and leucocytes. Most interestingly, its concentration is higher in the aqueous humor and the lens of the diurnals as compared to the nocturnals. In view of the fact that the incidence of cataract is higher in the population exposed to a greater level of solar radiation, it has been proposed that this may be related to the photocatalyzed conversion of molecular oxygen in its ground state to its excited states of very high reactivity such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radical, etc. [2,15]. However, despite the possibility of a continuous production of these species in the aqueous and lens because of an incessant penetration of light during the photopic vision, the latent period of frank cataract development is very long, clinically significant opacity in the lens becoming apparent in the late 50s and 60s on a global scale. We have previously attributed this latency to the presence of high levels of ascorbate in the aqueous and consequently in the lens maintained on account

of its active transport from the plasma to the aqueous. Chemically, ascorbate is an effective scavenger of hydroxyl radicals as well as superoxide ions. The rate constants of these reactions [4] are sufficiently high to merit investigations into the physiological systems. Such investigations have indeed yielded highly significant results. Its effectiveness is strikingly apparent by the protection it offers against ROS-induced damage to the lens in vitro, in photochemical as well as ambient environment. The high ascorbate in the aqueous could also serve as a filter, preventing penetration of UV light into the lens and thereby protecting the tissue against its direct adverse effects on the protein and nucleic acid structures [16,17]. Limited evidence of this has also come from in vivo studies with animal models and certain human epidemiological studies. For example, it has been found to prevent cataract formation induced by sodium selenite administration to young rat pups [5]. Although the cataract formation in this model has been shown to be related to its oxidative effect reflected by the tissue GSH depletion and increased lipid peroxidation, the validity of these findings in adult animals including humans is still in question. This is because cataract induction by sodium selenite in the pups is very age-specific and takes place only in very early stages of postnatal development. Further experimental evidence of the possible effectiveness of ascorbate against oxidative stress and cataract formation is obviously desirable. Additionally, experiments with isolated proteins have shown that the oxidation products of ascorbate can cross-link lens crystallins, producing high molecular weight species capable of causing light scattering, characteristic of cataracts [18,19].

As stated previously, studies on the preventive aspect of ascorbate against in vitro as well as in vivo damage to rat lens could be of limited implications because of the presence of excessive AR activity as compared to the humans and other primates. Hence, it remains unclear if ascorbate could also be effective in situations such as that prevalent in the human lens. To this effect, the present studies with mouse lens were considered more informative. Fortuitously, our results described in this communication convincingly demonstrate that even in those situations, the efficacy of ascorbate remains undiminished in low as well as high oxygen situations. At the levels prevailing in the human aqueous humor, this compound shows significant protection against the physiological damage to the tissue even when it was exposed to oxygen at concentrations much beyond the normal. As summarized in Results, incubation of lenses in low as well as high oxygen situations in the presence of ROS depressed the uptake of $^{86}Rb^{+}$ substantially when compared to the uptake in the basal controls. The magnitude of the decrease in the uptake process was such that the protective effect of ascorbate could be studied unequivocally. The distribution ratio in the normal oxygen situation decreased to approximately 10 CL/CM units over the period of 5 and 18 h. A decrease beyond 10 units did not occur, understandably due to the contribution of endogenous protective mechanisms. In the presence of high oxygen tension however, as expected, the pump damage was greater than in the normal oxygen tension by the end of 18 h. As apparent from Figs. 1 and 2, addition of ascorbate was protective both in the normal as well as high oxygen situations. The protection offered was nearly complete in the low oxygen situation. Under high oxygen tension, the protection remained complete till the end of 5 h, declining in later periods because of the continued depletion of ascorbate. Still, the protection offered was substantial. The immediate site of reaction of the ROS produced in the medium would obviously be the cell membrane as reflected by the decrease or damage to its ability to transport the $^{86}Rb^{+}$ against its concentration gradient. However, hydrogen peroxide can also penetrate the cells and cause intracellular damage. This was ascertained by the measurements of GSH and ATP. The levels of both the compounds decreased in the presence of ROS. The decreases were greatly minimized by ascorbate.

The possibility that some oxidation products of ascorbate could be cataractogenic remains uncertain because of their very short half-lives as well as the prompt reduction of dehydroascorbate by glutathione, by enzymatic as well as non-enzymatic reactions. It has been proposed that ascorbate could exert a cataractogenic effect by glycating certain lens proteins [20]. However, evidence of this possibility, either in vivo or in organ culture experiments, is very limited, if any. Indeed, some of the degradation products of ascorbate such as methylglyoxal have recently been shown to enhance the chaperone activity of certain heat shock proteins including the alpha crystallins involved in maintaining the companion tissue proteins in their native undenatured states [21,22]. Additionally, dehydroascorbate by itself is a peroxide scavenger by virtue of its action as an alpha-ketoacid after its hydrolysis to the carboxylate form. The observations suggesting its possible antioxidant-cum-anti-cataractogenic functions essentially because of its ROS scavenging properties are also in line with several epidemiological studies most of which show a lower incidence of cataracts in population consuming greater amounts of vitamin C [23,24]. We have previously demonstrated that dehydroascorbate can also prevent oxidative damage [25]. This could be attributed to the acceleration of the HMP shunt caused by this compound as well as through its direct antioxidant action after its reduction by glutathione [26]. Acceleration of the shunt has a direct effect of transferring the reducing equivalents from the basal glucose to ROS, thereby rendering them inactive.

The results on the measurement of $^{86}Rb^{+}$ uptake as well as of ATP and GSH are therefore strongly suggestive that ascorbate in the aqueous humor exerts an overall protective effect against oxidative damage to the cell membrane as well as the indirect intracellular effects ROS might exert. The presence of high ascorbate concentrations in the aqueous is therefore considered physiologically beneficial in preventing or delaying the formation of senile cataracts.

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