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Biochemistry

The role of ascorbic acid in senile cataract

(lens aging/vitamin C oxidation/protein–ascorbate interaction)

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ABSTRACT **The reductone ascorbic acid, present in the crystalline lens in concentrations higher than those of glucose, is capable of undergoing nonenzymatic "browning" in the presence of lenticular proteins. We studied the nonenzymatic browning with ascorbate in model systems employing bovine serum albumin and lens crystallins. When bovine serum albumin, α-crystallin, or γ-crystallin was incubated with [^{14}C]ascorbic acid, the formation of yellow and then brown condensation products appeared to correlate with increasing protein-associated radioactivity. The fluorescence spectrum of these products was similar to that of homogenates of human cataractous lenses. We suggest that the nonenzymatic reaction of lens crystallins with ascorbic acid may contribute, at least in part, to the color changes of aging lenses and to the physical lenticular deterioration leading to senile cataract. High dietary intake of ascorbic acid did not affect the fluorescence spectrum of murine lenses; thus, we assume that the speed and extent of the lenticular browning reactions must depend on a deterioration of other factors of the multicomponent antioxidant system of the eye.**

Senescent cataract is a very common spontaneous eye affliction in persons beyond middle age. It becomes progressively more severe and frequent in the elderly, with more than 85% of octagenarians having some degree of lenticular opacification (1). Of particular interest to us is the most common type of senescent cataract, the so-called nuclear sclerosis, in which the central portion of the lens undergoes a gradual increase in density and opacity, usually accompanied by a yellow to dark-brown discoloration (2). What prompted us to undertake these studies was the striking similarity in the appearance of such lenses with the color changes that develop during slow spontaneous autoxidation of ascorbic acid solutions, either in the presence or the absence of proteins. We here report observations on oxidative ascorbic acid–protein interactions *in vitro*. Comparison of our findings with data on cataractous lenses suggests that the biochemical reactions observed by us may play a role in the aging of human lenses.

MATERIALS AND METHODS

All reactions were carried out in 67 mM potassium phosphate buffer (pH 7.4). Sterile solutions of ascorbic acid (Sigma) and proteins (bovine serum albumin, bovine lens α-crystallin, and bovine lens γ-crystallin) were incubated for various times up to 1 month for fluorescence studies, both at room temperature and at 37°C. Bovine serum albumin was obtained (Sigma) as Cohn fraction V, essentially fatty acid-free. Lens crystallins were a gift from T. Chiou (National Taiwan University, Institute of Biochemical Sciences, Taipei, Taiwan). Sterility of the incubation mixtures was monitored periodically by testing aliquots on nutrient agar plates. The fluorescence spectra of these incubation mixtures were obtained with an Aminco Bowman spectrophotofluorometer model 4-8106 after prolonged dialysis of the ascorbic acid-containing protein solutions against large volumes of the buffer. Spectra/Por membrane tubing with a molecular weight cutoff of 12,000–14,000 (Scientific Products) was used.

For binding studies, 25 μCi of [^{14}C]ascorbic acid (New England Nuclear, specific activity 9.9 mCi/mmol; 1 Ci = 37 GBq) was added to sterile protein solutions (100 ml) containing 100 mM unlabeled ascorbic acid. After 1 month of incubation at 37°C in the dark, aliquots of the crystallin/[^{14}C]ascorbate solutions were dialyzed extensively against phosphate buffer at 4°C and radioactivity was measured in a Beckman liquid scintillation counter model LSC 8000. For time-course studies of the binding of ascorbic acid to protein, samples of the bovine serum albumin/[^{14}C]ascorbate solutions were periodically removed, precipitated with 50% (wt/vol) trichloroacetic acid, centrifuged at 11,000 × *g* for 1 min, and washed with 50% trichloroacetic acid until no radioactivity was detectable in the supernatant. The resulting pellet was dissolved in formic acid and radioactivity was measured as described above.

Samples from the various incubations were treated with 1% $NaDodSO_4$/0.5% 2-mercaptoethanol, and electrophoresis was carried out in 10% polyacrylamide slab gels with a 3.5% stacking gel, in the presence of 0.1% $NaDodSO_4$. Fluorograms of ^{14}C-labeled samples were prepared from dried gels that had been impregnated with EN^{3}HANCE (New England Nuclear) by exposing them to preflashed Kodak XAR-2 x-ray film (3). Gels containing unlabeled proteins were silver-stained by the method described by Guevera *et al.* (4).

Thin-layer chromatography of the oxidation products of ascorbic acid was performed on nitrocellulose with a solvent system consisting of acetonitrile/acetone/acetic acid/water (8:5:1:15, vol/vol). The resulting chromatograms were stained with 5% aqueous $AgNO_3$.

Quantitative fluorescence measurements were carried out on homogenates of 10 lenses from five mice that had been fed a diet containing 8.3% (wt/wt) ascorbic acid for a period of at least 1 year (from the age of 7 weeks). The results were compared with those of lenses from age-matched control animals fed standard laboratory chow *ad libitum*. Human lenses afflicted with senescent cataract were obtained immediately after their surgical removal. Lenses (human or murine) were homogenized with a Brinkman Instruments Polytron tissue homogenizer model PT 10/55 at a setting of 4, for 1 min in 10 volumes of 67 mM potassium phosphate buffer (pH 7.4), and fluorescence measurements were made on these homogenates. The instrument employed for these determinations was the same used for studies on the ascorbic acid/protein mixtures, described above.

RESULTS

Autoxidation of Ascorbic Acid Solutions. In the presence of atmospheric oxygen, a progressive yellow discoloration of an initially colorless, sterile 1 M ascorbic acid solution in

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phosphate buffer (pH 7.4) occurs over a period of several days at 20°C. There is a simultaneous drop in pH to about 4.5 if the buffer strength is weak; this acidification results in a slowing of the oxidation process. However, further oxidation, yielding a light brown solution, will take place within a month. Acceleration of this discoloration is brought about by either intermittent exposure of the reaction mixture to 100% oxygen or addition of Cu^{2+} in micromolar concentrations; either of these manipulations result in a further darkening of the solution (unpublished observations). Isolation and characterization of the oxidation products was not carried out. Oxidation of ascorbic acid for short periods of time has been extensively studied (5). Most of the numerous attempts to identify the many breakdown products of dehydroascorbic acid have been frustrating and inconclusive because of the formation of a large number of labile, short-chained molecules, as well as larger polymers (5–7). Thin-layer chromatography of 1-month-old solutions of ascorbic acid on nitrocellulose revealed at least six compounds detectable by silver staining (data not shown).

Autoxidation of Ascorbic Acid/Protein Mixtures. Prolonged incubation of protein with ascorbic acid [bovine serum albumin (0.01 or 10 mg/ml) with 1.0 M ascorbic acid or bovine lens α- or γ-crystallin (1 μg/ml) with 0.1 M ascorbic acid] in sterile 67 mM phosphate buffer (pH 7.4) confirmed our previous observation that ascorbic acid oxidation is retarded by serum albumin but that within days a yellow discoloration of the solution occurs if air has access to the reactants (7). Within one month, this process yields a light brown solution that has an odor reminiscent of caramel.

Binding of Ascorbic Acid Oxidation Products to Proteins. Fig. 1 shows the increase in radioactivity derived from [^{14}C]ascorbate bound to bovine serum albumin with increasing incubation time. After 1 month at 37°C, ≈75% of the protein had precipitated as brown particulates. These precipitates were analyzed by NaDodSO$_4$/PAGE (data not shown) and appear to be protein polymers that have been crosslinked in a reaction reminiscent of that which increases the non-disulfide-crosslinked protein in cataractous lenses (8). Neither albumin nor lens crystallins produced such precipitates when incubated in the absence of ascorbic acid. Extensive dialysis of the ascorbate/albumin mixtures yielded light-brown retentates. This color was not lost on treatment with 2-mercaptoethanol and urea or on precipitation of the protein with 50% trichloroacetic acid. When albumin was incubated in the presence of ascorbic acid and Cu^{2+}, considerable breakage of protein molecules rapidly occurred, as revealed by NaDodSO$_4$/PAGE after <24 hr of incubation. This, after several days, is followed by formation of brown particulates similar to those present in 1-month-old Cu^{2+}-free ascorbic acid/protein solutions.

When lens crystallins (1 μg/ml) were incubated with [^{14}C]ascorbic acid (100 mM, 25 μCi/mmol) for 1 month and then dialyzed extensively against phosphate buffer, the α- and γ-crystallin retentates contained, respectively, 750 ± 39 and 60 ± 8 mmol of ascorbic acid (based on specific radioactivity of ascorbate) per mole of protein. Retention of the yellow color correlated with retention of radioactivity.

Fluorescence Spectra. Fig. 2 shows the fluorescence spectra of a human cataractous lens homogenate and of an albumin/ascorbic acid incubate. These spectra are similar to those observed by Monnier and Cerami with lens proteins incubated with glucose for 10 months at 37°C (9). The excitation maximum for lens chromophores, which was obtained on samples prepared as described by Monnier and Cerami (9), was found to be 380 nm and that for dialyzed albumin/ascorbic acid solutions, 385 nm. The emission maxima are similar for both mixtures (450 nm). Fluorescence of murine lens homogenates was not affected by a prolonged diet high in ascorbic acid (Table 1). The relative fluorescence (excitation, 380; emission, 450 nm) of homogenates of lenses from mice that had been fed a diet containing 8.3% ascorbic acid is not significantly different from that of lens homogenates of age-matched controls. The blood ascorbate levels of the mice fed the high ascorbate diet were 2.5–3.0 mg/100 ml, as compared to normal levels of 1 mg/100 ml.

DISCUSSION

The lens is relatively inactive metabolically, with most of its metabolic activity being of the anaerobic type. This may in part be due to the slow turnover of lens proteins, with most of lenticular energy metabolism being expended on the maintenance of transport processes (10). Appropriately, the lenticular concentration of glucose is only about 15% that of the surrounding aqueous humor because of the lack of need

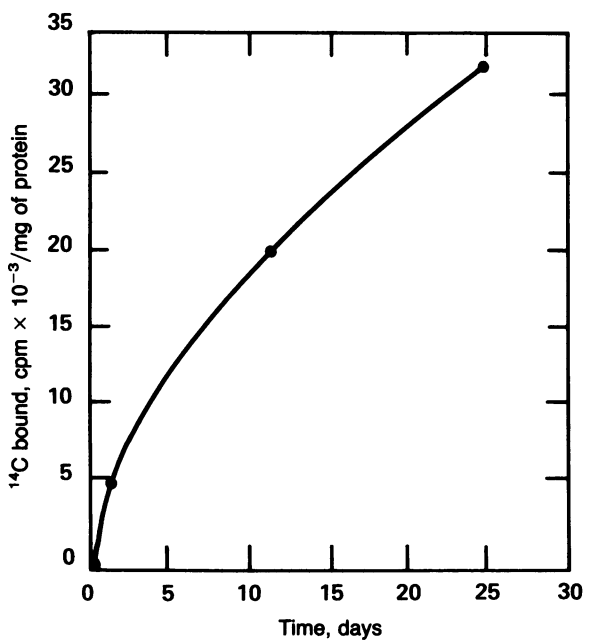

FIG. 1. Time-course study showing the binding of [^{14}C]ascorbate (100 mM) to bovine serum albumin (0.01 mg/ml).

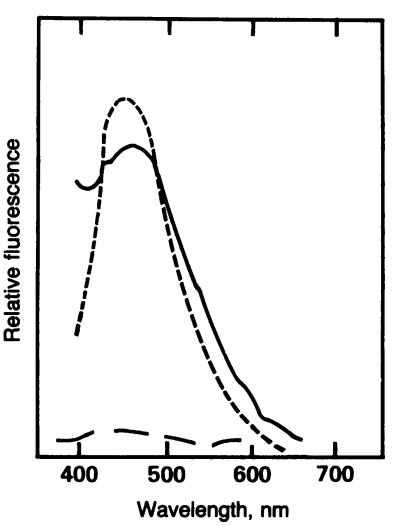

FIG. 2. Comparison of fluorescence spectrum of bovine serum albumin after incubation with ascorbic acid for one month (----) with that of a homogenate of human cataractous lenses (——); the fluorescence of bovine serum albumin was negligible (---). Fluorescence scans were performed with an Aminco Bowman spectrofluorometer model 4-8106 at an excitation wavelength of 360 nm.

Table 1. Relative fluorescence of lens homogenates from mice fed high levels of ascorbic acid

Animals	Relative fluorescence
Control	20.6 ± 2.8
Ascorbic acid-fed	20.7 ± 4.9

Fluorescence was measured with an Aminco Bowman spectrofluorometer model 4-8106. Excitation and emission wavelengths were 380 nm and 450 nm, respectively. Relative fluorescence was based on comparison with a standard of quinine sulfate (1 μg/ml; relative fluorescence, 1000 units). Values represent mean ± SD for 10 lenses (control) or 10 lenses (ascorbic acid-fed).

for extensive generation of high-energy phosphates by aerobic glycolysis (11). A major advantage of anaerobic glycolysis, although inefficient, is that the necessity for complex organellar arrangements such as for the electron transport chain is greatly reduced.

The lens contains several mechanisms capable of protecting itself from oxidative damage brought about by oxygen- and photooxidation-derived radical species. The most significant of these appears to be the glutathione system in conjunction with glutathione peroxidase, superoxide dismutase, and ascorbic acid (12). In mammals, ascorbate is present in the lens in unusually high concentration, as much as 30–35 times the blood level; in man, the lens ascorbate concentration is even greater than the already high level present in the aqueous humor (13, 14). However, little is known about the role of ascorbic acid in lens function and in cataractogenesis. There is an early drop to low levels of ascorbic acid in microwave-induced cataract (15), and ascorbic acid inhibits lens darkening *in vitro* in experimental ultraviolet light-induced cataract (16). The ascorbic acid level in lenses of patients with senile cataract, even in moderately advanced cases, is significantly below normal (14). Conversely, an unexplained increase in the lenticular Cu^{2+} ion concentration appears to accompany the reduced ascorbic acid levels (17).

Aldoses and similarly reactive carbohydrates are capable of modifying proteins by undergoing nonenzymatic adduct formation with amino groups (6). This process, the Maillard reaction and the subsequent stages of the so-called "browning" reaction, eventually results, via formation of aldimine bonds and their Amadori rearrangement, in stable yellow-brown products (6). *In vivo*, nonenzymatic glycosylation occurs particularly in long-lived organelles and with compounds that turn over relatively slowly, such as erythrocyte membranes, hemoglobin, collagen, and several other proteins including lens crystallin (18–20). Pathologic glucose levels, such as present in diabetics, greatly accelerate this type of glycosylation (21). The role of this condensation reaction in the aging of the lens has been repeatedly explored under experimental conditions (20, 22). Prolonged incubation of extracts of normal bovine lenses with glucose results in browning of the incubation mixture, as borne out by the appearance of ultraviolet absorption and fluorescence peaks (18). Crosslinked aggegrates of the lenticular crystallins also appear under such *in vitro* conditions.

The color changes and the appearance of fluorescence during lens aging and the development of senile cataract have prompted numerous attempts to isolate and characterize the responsible compound(s) and their possible role as crosslinks between crystallins (23–30). Most of the findings have been inconclusive. This is not surprising because the Maillard and subsequent reactions produce a variety of colored compounds, not all bound to protein, during the browning process of foods (6, 28, 31, 32). These compounds fall into three groups: simple sugar dehydration/fragmentation products, simple amino acid degradation products, and volatile products. We, as others have before, found it to be a major task to characterize analogous compounds that form on autoxidation of ascorbic acid alone by a process referred to as nonamino browning of reductones (6). The principal aim of our studies, however, was to examine the behavior of ascorbic acid in a setting conducive to adduct formation and to compare the findings with relevant data from cataractous lenses.

Our findings indicate that ascorbic acid indeed forms condensation products with bovine serum albumin as well as with lens crystallins and that this process occurs at a rate faster than that with common aldoses. This is not surprising, because ascorbic acid oxidation proceeds via formation of very labile and highly reactive enediols with conjugated carbonyl groups. Studies with aldoses (33) suggest that during the Maillard reaction, glucose fragmentation with formation of two-carbon intermediates occurs and that also cationic radicals are formed prior to the Amadori rearrangement (i.e., prior to the formation of a stable, covalent bond between two molecules). It is reasonable to postulate that during browning, fragmentation of the carbon chain of dehydroascorbic acid generates several highly reactive breakdown products. The latter may interact not only with each other and unaltered ascorbic acid but also with the amino and hydroxyl groups of proteins, eventually leading to fragmentation of protein molecules as well as to bridging between adjacent molecules. This would explain the formation of precipitates that takes place in the later stages of nonenzymatic browning. Although the mechanisms resulting in this protein aggregation are not clear, it appears not to be caused only by the formation of disulfide bridges between molecules of serum albumin or crystallin. The not infrequent, irregular but sharply circumscribed black spots in cataractous lenses most likely represent foci of advanced protein condensation and precipitation; focal enhancement of the process could occur on the basis of a localized chain reaction. Stable α-hydroxycarbonyl groups will greatly accelerate browning as will copper, such as present in copper–protein complexes, leading to the formation of an amorphous fluorescent product in the presence of ascorbic acid (34).

Evidence that similar, if not identical, adducts form during lens aging as form during the interaction of ascorbic acid with bovine serum albumin was provided by spectrophotometric absorption and fluorescence measurements. An identical fluorescence-excitation pattern was obtained for the ascorbic acid adducts formed *in vitro* with bovine serum albumin and with crystallin; a similar fluorescence pattern was seen with homogenates of cataractous lenses. These findings are analogous with those of other investigators who had analyzed lens proteins after prolonged incubation with glucose (9). Thus, our results suggest that ascorbic acid, present in higher concentration than glucose in the healthy lens and more prone to oxidative decay, may play a major role in lens aging and cataract formation. *In vivo*, accelerated ascorbic acid oxidation and subsequent reactions may be secondary to a progressive malfunctioning of the protective, complex lenticular reduction–oxidation system; for instance, the known decrease in glutathione reductase in aging lenses may thus eventually result in an oxidative chain reaction (12, 35). Free radical formation occurs during the Maillard reaction and also can be expected to take place in the low-oxygen environment of the crystalline lens. Our lack of knowledge of the different roles of the lenticular antioxidative compounds and enzymes, their mutual balance, and their regeneration and redundancy does not permit us yet to assign a specific function to the lenticular ascorbic acid, but it may well be that of an abundantly available, rapidly acting, free radical scavenger.

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