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# Ascorbic acid and protein glycation *in vitro*

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ABSTRACT

The aim of the study was to compare the effects of ascorbic acid (AA) *in vitro* in the absence and in the presence of cell-dependent recycling. In a cell-free system, AA enhanced glycoxidation of bovine serum albumin (BSA) by glucose and induced BSA glycation in the absence of sugars. On the other hand, AA did not affect erythrocyte hemolysis, glycation of hemoglobin and erythrocyte membranes, and inactivation of catalase, protected against inactivation of acetylcholinesterase of erythrocytes incubated with high glucose concentrations and enhanced the loss of glutathione. These results can be explained by assumption that AA acts as a proglycating agent in the absence of recycling while is an antiglycating agent when metabolic recycling occurs.



## 1. Introduction

One of unavoidable consequences of metabolism are non-enzymatic reactions of proteins with reducing sugars (glycation) leading finally to formation of Advanced Glycation End Products (AGEs) [1]. AGEs formation takes place under normal physiologic conditions but is accelerated in hyperglycemia [2]. Glycation alters the structure and functional properties of proteins, which affects adversely cellular metabolism. Accumulation of glycation products is observed in human and animal tissues during aging and is associated with various diseases including, first of all, diabetes and diabetic nephropathy, microangiopathy and atherosclerosis [3].

The possibility of reducing glycation and tissue AGEs is an approachable target for delaying or preventing the onset of diabetic complications. Various natural compounds have been tested for inhibition of glycation, first of all antioxidants and metal chelators. From among antioxidants, AA is of special importance as one of main antioxidants in the human body and an easily bioavailable compound, nontoxic even at high doses [4].

The chemical and physical properties of AA are related to its structure. AA functions as an electron donor and scavenges free radicals such as superoxide radicals and hydroxyl radicals *in vitro* [5]. At physiological pH, AA is entirely dissociated to the ascorbate radical and reacts as a one-electron donor in free radical scavenging. After donating an electron, ascorbate is oxidized to a semidehydroascorbate radical (SDA), a less reactive compound. The SDA radical can either be recycled back to AA or can be transformed to dehydroascorbate (DHA), the fully oxidized form of AA [6]. DHA is taken up into cells by glucose transporters [7,8]. The reduced form of AA is imported by an active mechanism, requiring one of two sodium-dependent vitamin C transporters (SVCT1 and SVCT2). The transport of DHA is mediated by glucose transporters GLUT1, GLUT3 and possibly GLUT4. Initial rate of uptake of both AA and DHA is saturable with increasing external substrate concentration [9]. A recent study suggests that GLUT1, the primary glucose transport protein in human erythrocytes, transports DHA as its primary substrate and that only a subpopulation of GLUT1 transports sugars [10].

DHA is reduced spontaneously by glutathione, as well as enzymatically in reactions using glutathione or NADPH [11]. Under chronic oxidative stress (e.g. diabetes, UV-light, aging, etc.) increased formation of DHA has been reported and the inability of glutathione to completely reduce DHA back to AA brings about the rapid formation of highly reactive carbonyls from DHA [12,13].

*In vitro* AA can exhibit both antioxidant or pro-oxidant activities. The pro-oxidant activity is related to the maintenance of iron as $Fe^{2+}$ and, consequently, in a redox state that can generate reactive species [14]. Moreover, in this reaction ascorbyl radicals are produced [15]. Mixtures of AA and copper or iron have been used for decades to induce oxidative modifications of lipids, proteins and

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DNA. AA may contribute to oxidative damage formation by reducing ferric $Fe^{3+}$ to ferrous $Fe^{2+}$ ions (and $Cu^{2+}$ to $Cu^{+}$), which in turn can reduce hydrogen peroxide ($H_2O_2$) to hydroxyl radicals. However, in general these vitamin C-mediated Fenton reactions should be controlled in the human body due to efficient iron sequestration by metal binding proteins such as ferritin and transferrin [16]. Chen et al. found that AA generated detectable levels of $H_2O_2$ in extracellular medium in the presence of trace serum protein but not in whole blood. The findings indicate that ascorbate at pharmacologic concentrations in blood may be a pro-drug for $H_2O_2$ delivery to tissues, with major therapeutic implications [17].

*In vivo* AA has been shown to have a diversity of antioxidant properties, protecting the organism from oxidative damage [18–21]. However, data on the effects of ascorbic acid on protein glycation are contradictory, most of them pointing to the enhancement or even induction of glycation by AA *in vitro* [13,22–26] and its inhibition *in vivo* [20,21,27–29].

Reports on the impact of AA on *in vitro* glycation have been inconclusive. Both pro-glycating and antiglycating properties have been attributed to AA with a prevalence of proglycating evidence [22–24,26,30,31]. The mechanism by which AA can cause protein glycation probably originates from DHA, which is known to cross-link protein [24,26,30,32]. The study conducted by Reihl et al. revealed that the modification of lysine and arginine side chains by DHA is a complex process and could involve a number of reactive carbonyl species [32].

Bovine lens proteins are glycated 6.5-fold higher by AA than by glucose [33]. AA, but not glucose, fructose, ribose or erythrulose, caused the aggregation of calf lens proteins to proteins ranging from $2.2 \times 10^6$ up to $3.0 \times 10^8$ Da. Linetsky et al. showed that this aggregation was likely due to the glycation of lens crystallins because [U–$^{14}$C] ascorbate was incorporated into the aggregate fraction and because $NaCNBH_3$, which reduces the initial Schiff base, prevented any protein aggregation. Reactions of AA with purified crystallin fractions showed little or no aggregation of α-crystallin, significant aggregation of β(H)-crystallin, and rapid precipitation of purified β (L)- and γ-crystallin [13].

Some authors identified ascorbate as a precursor of pentosidine [34,35]. The yield of pentosidine was about 30 x increased during incubation of ascorbate with basic amino acids [34]. AA was reported to inhibit the glycation of bovine serum albumin (BSA) by a mixture of 25 mM glucose/fructose [36]. In another study it slightly inhibited glycation but decreased the inhibitory effect of $Zn^{2+}$ on glycation of BSA. It should be noted, however, that in that study BSA was glycated at 37 °C for 150 days in sealed tubes [26] while in most studies much shorter incubation times were employed in the presence of air.

In the study of Monacelli et al., AA was found to be an effective inducer of human serum albumin (HSA) glycation, somewhat weaker than ribose but the combination AA + ribose significantly counteracted the effects of ribose [37]. The effects of AA depended on the parameter studied: AA reduced ribose-induced pentosidine generation, but was an effective inducer of AOPP production and induced more Maillard-related fluorescence (370/440 nm) than ribose. The metal chelator diethylenetriaminepentaacetic acid (DTPA) efficiently inhibited AA-induced glucation. UVA treatment of human lens homogenates in the presence of AA lead to oxidation of ascorbate mediated by products of tryptophan photo-oxidation [38].

In the presence of atmospheric oxygen, a progressive yellow discoloration of an initially colorless, sterile AA acid solution in phosphate buffer (pH 7.4) occurs over a period of several days at 20 °C. Further oxidation, yielding a light brown solution, takes place within a month. Intermittent exposure of the reaction mixture to 100% oxygen or addition of micromolar $Cu^{2+}$ accelerates this process. Attempts to identify the many breakdown products of the primary oxidation product, 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. The fluorescence spectra of products of AA reactions with BSA, α-crystallin, or γ-crystallin were similar to that of homogenates of human cataractous lenses; on this basis, it was proposed that reactions of products of AA autoxidation are responsible for cataract formation *in vivo* [39].

*In vivo*, it has been reported in a mouse model that high levels of vitamin C may mediate cataract formation through protein glycation. If so, high cellular concentrations of vitamin C can inflict damage in other tissues with high activity of the SVCT2 transporter, such as the hippocampus and the adrenal glands, in which AA concentrations reach up to 10 mM. Both tissues can be severely affected in their function during aging, and thus the possibility emerges that excess AA in hippocampal neurons may contribute to age-related neurodegenerative diseases [40]. The main degradation products of dehydroascorbic acid in human lens were: 2,3-diketogulonic acid, 3-deoxythreosone, xylosone, and threosone. 3-Deoxythreosone in water-soluble fraction of the lens showed positive linear correlation with age [41].

However, data from other animal models and from human studies do not support this observation and numerous antiglycating effects of AA were reported. Single intake of vitamin C significantly decreased AGEs level in saliva [42]. Low serum levels of the vitamin have been associated with increased risk of cataractogenesis in the human [43], and various studies suggest that supplementation of the vitamin is beneficial for decreasing the of risk of cataractogenesis [44], in particular under conditions that favor oxidant stress [45,46]. The level of glycated hemoglobin was found to decrease in response to AA supplementation [47]. Short-time administration of AA (1000 mg of AA in the form of Re-Natured Vitamin C® for a period of 4 weeks) decreased the level of glycation of serum proteins by 46.8% [36].

In our opinion, one reason for this discrepancy between the *in vitro* and *in vivo* effects of AA on protein glycation may be linked to the possibility of metabolic recycling of oxidation products of AA. Therefore, the aim of the present study was to compare the effect of AA on glycation in a pure cell-free *in vitro* system of BSA and in a cellular system of human erythrocytes, in which AA recycling is possible.

## 2. Materials and methods

### 2.1. Chemicals and equipment

All basic reagents were from Sigma–Aldrich Company (Poznan, Poland). All reagents used were of analytical reagent grade. All the spectrophotometric analyses were done using a Varian Cary 50 UV–Vis spectrophotometer (Varian Inc., Cary, NC, USA) except for acetylcholinesterase assay which, like fluorescence measurements, was read using an Infinite 200 PRO multimode reader (Tecan Group Ltd., Männedorf, Switzerland).

### 2.2. Glycation of bovine serum albumin

BSA (BioShop® Canada Inc.; 90 μM) was incubated with 0.5 M glucose (POCh, Gliwice, Poland), fructose (Park Scientific Limited, Northampton, UK) or ribose (BioShop® Canada Inc) in 0.1 M phosphate buffer, pH 7.4, in the absence or in the presence of 0.05, 0.1, 0.2, 0.5 and 1 mM AA (Sigma–Aldrich), at 37 °C for 6 days. BSA was also incubated with various concentrations of AA alone, without sugars, under the same conditions. The samples were