

Kinetics, Catalysis and Mechanism of Chemical Reactions

From Pure
to Applied Science

Volume 2
Tomorrow and Perspectives

Regina M. Islamova
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Editors

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CHEMISTRY RESEARCH AND APPLICATIONS

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Chapter 2

**RADICAL DEGRADATION OF HIGH-MOLAR-MASS
HYALURONAN INDUCED BY ASCORBATE *PLUS*
CUPRIC IONS: TESTING OF ARBUTIN IN THE
FUNCTION OF ANTIOXIDANT***

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ABSTRACT

Arbutin was tested in the function of a potential anti- or prooxidant in Cu(II) *plus* ascorbate induced degradation of high-molar-mass hyaluronan (HA). The time- and dose-dependences of dynamic viscosity changes of the HA solutions were investigated by the method of rotational viscometry. First, the HA solution was exposed to degradation induced by Cu(II) ions (1 μ M) with various concentrations of ascorbic acid (10, 50, or 100 μ M). Further the action of arbutin (100 μ M) addition into the reaction system, preceding that of ascorbic acid (100 μ M), was inspected. The results obtained clearly indicated prooxidative properties of arbutin in relation to HA free-radical degradation. Both the reduction of the dynamic viscosity of the HA solution and the decrease of the polymer mean molar mass as revealed by the method of size exclusion chromatography

*Dedicated to Professor Radomír Nosál, MD, DSc on the occasion of his 70th birth anniversary.

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proved the tenet that on using the Cu(II) ions *plus* ascorbate, i.e. the Weissberger's oxidative system, the degradation of HA macromolecules is pronounced by added arbutin. Cyclovoltammetric studies of arbutin both in aqueous (0.15 M NaCl) and non-aqueous (0.15 M TBAPF6 in DMSO) solutions confirmed a substantial protonation and hydrolysis reactions of arbutin in aqueous solutions. By applying the method of EPR spectroscopy, the reaction mixture comprising HA, Cu(II) ions, ascorbate, and arbutin indicated the presence exclusively of ascorbyl radicals. A remarkable radical scavenging activity of arbutin was observed on using the ABTS decolorization assay.

Keywords: ABTS assay, cyclic voltammetry, EPR spectroscopy, hydroquinone derivatives, quinone, rotational viscometry, size exclusion chromatography, Weissberger's oxidative system.

INTRODUCTION

Hyaluronan (HA, figure 1) is a linear polysaccharide consisting of repeating disaccharide units of β -1,3-*N*-acetyl-D-glucosamine and β -1,4-D-glucuronic acid. The HA molar sizes in the human organism vary between 2×10^5 and 10×10^6 Da. An adult person weighing 70 kg has ca. 15 g of HA in the body – yet about one-third of this amount turns over daily.

In the human organism, HA is present in two forms: associated with certain glycosaminoglycans and proteins – e.g. in the skin and cartilage, or free/unassociated – in the synovial fluid (SF) and vitreous humor of the eye. The highest contents of (associated) hyaluronan in the human body occurs in the skin in which HA has a rapid turnover rate – with a half-life of 1.5 days. Unassociated high-molar-mass HA in SF confers its unique viscoelastic properties required for maintaining proper functioning of the synovial joints. The half-life of HA within SF is approximately 12 hours [1].

The fast HA turnover in SF of the joints of healthy individuals may be attributed to the oxidative/degradative action of reactive oxygen species (ROS), generated among others by the catalytic effect of transition metal ions on the auto-oxidation of ascorbate [2]. Uninhibited and/or inhibited HA degradation by the action of various ROS has been studied on applying several *in vitro* models. In these studies, the change of the hyaluronan molar mass or a related parameter, such as the HA solution dynamic viscosity, was used as a marker of inflicted damage [3].

Arbutin (termed also hydroquinone- β -D-glucopyranoside, figure 2), a substance used in the function of skin lightening and depigmentation, is a popular drug especially in Japan and other Asian countries, also due to its lower toxicity compared to hydroquinone [4, 5].

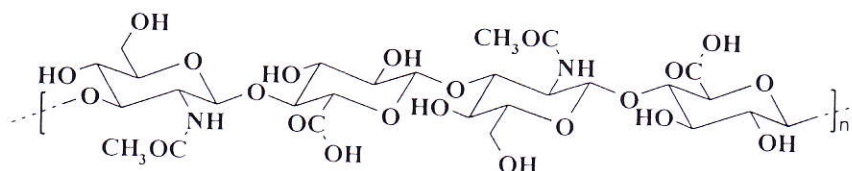


Figure 1. Hyaluronan – acid form of the macromolecule.

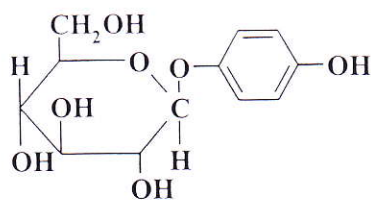


Figure 2. Arbutin structure.

Two anomeric forms, namely α - and β -arbutin, exist from which the latter occurs naturally in plants, e.g. in leaves of bearberry (*Arctostaphylos uva-ursi* Spreng., Ericaceae) and pear trees (*Pyrus communis* L., Rosaceae). The primary mechanism of arbutin action is in inhibiting tyrosinase activity in the skin, resulting in the significantly diminished formation of the brown-colored melanin pigment. Further, arbutin acts as an “anti-aging” agent and a UVB/UVC filter, which protects the skin against deleterious effects caused by photon-generated free radicals. Beyond their cosmetical actions, products containing arbutin are therapeutically used for treating cystitis and kidney stones. Arbutin, a very hygroscopic substance, readily hydrolyzes e.g. by diluted acids yielding D-glucose and hydroquinone in a 1:1 mole ratio [6]. Arbutin-based products are successfully applied also as diuretics: it is anticipated that the drug inhibits/retards the proliferation of pathogens within the urinary tract according to its high electrochemical potential, which within the pH range of 7.5 and 2.0 lies between +466 and +691 mV, respectively [7]. In the body, arbutin is decomposed to D-glucose and hydroquinone. It is a well known fact the latter substance bears strong antimicrobial and disinfectant properties. The greater the pH values of urine, the stronger the bacteria killing efficiency of free (not glucuronided) hydroquinone. However in more acidic urine, hydroquinone undergoes extensive glucuronidation, which is believed to lead to the formation of the antibacterially ineffective hydroquinone-glucuronide [7, 8]. So far no reports have been published devoted to action(s) of arbutin under conditions simulating ROS damaging effects on HA macromolecules. Since the hyaluronan content is so high in various tissues/body fluids – the skin/SF – the presented study is focused on investigating the ability of arbutin to act anti- and/or prooxidatively by monitoring the kinetics of free-radical degradation of high-molar-mass HA. As an effective $\cdot\text{OH}$ radical inducer, the system comprising ascorbate *plus* Cu(II) – the so-called *Weissberger's* oxidative system – was used. Along with monitoring the dynamic viscosity of HA solution by the method of rotational viscometry, further methods such as size exclusion chromatography (SEC), cyclic voltammetry (CV), EPR spectrometry, and standardized decolorization ABTS assay were applied at testing arbutin in the function of preventive and chain-breaking antioxidant.

MATERIAL AND METHODS

Biopolymer

The high-molar-mass hyaluronan sample P9710-2A ($M_w = 808.7$ kDa; $M_w/M_n = 1.63$) used was obtained from Lifecore Biomedical Inc., Chaska, MN, U.S.A. The declared contents

of transition metals in the HA sample given by the Certificate of Analysis are 4 ppm Cu and 13 ppm Fe.

Chemicals

Analytical purity grade NaCl and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ were purchased from Slavus Ltd., Bratislava, Slovakia. Dimethyl sulfoxide (DMSO), L-ascorbic acid, and $\text{K}_2\text{S}_2\text{O}_8$ were the products of Merck KGaA, Darmstadt, Germany. Trolox[®], arbutin, and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Tetrabutylammonium perchlorate (TBAP), LiClO_4 , and ABTS – 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (*purum*, >99%) – were from Fluka, Chemie GmbH, Steinheim, Germany. Redistilled deionized high quality grade water, with conductivity of <0.055 mS/cm, was produced by using the TKA water purification system from Water Purification Systems GmbH, Niederelbert, Germany.

Preparation of Stock and Working Solutions

The HA sample solutions (2.5 mg/mL) were prepared in the dark at room temperature in 0.15 M aqueous NaCl in two steps: First, 4.0 mL of the solvent was added to 20 mg of dry HA powder. Then 3.80, 3.85, or 3.90 mL of the solvent was added after 6 h. The stock solutions of L-ascorbic acid (1.6, 8, 16 mM), arbutin (16 mM), and CuCl_2 (160 μM prepared from 16 mM CuCl_2) were also prepared in 0.15 M aqueous NaCl.

Study of Uninhibited/Inhibited Hyaluronan Degradation

Uninhibited HA degradation was induced by the oxidative system comprising CuCl_2 (1.0 μM) with altering concentrations of L-ascorbic acid (10, 50, 100 μM). The procedure was as follows: the volume of 50 μL of 160 μM CuCl_2 solution was added to the HA solution (7.90 mL) and after 30 s stirring the reaction solution was left to stand for 7.5 min at room temperature. Then 50 μL of L-ascorbic acid (1.6, 8 or 16 mM) were added to the reaction vessel and the solution was gently stirred for 30 s. The reaction mixture was then immediately transferred into the viscometer Teflon[®] cup reservoir.

Inhibited HA degradation: The procedures to test arbutin in the function of (i) preventive and (ii) chain-breaking antioxidant were as follows:

- (i) A volume of 50 μL of 160 μM CuCl_2 solution was added to the HA solution (7.85 mL) and after 30 s stirring the reaction solution was left to stand for 7.5 min at room temperature. Then 50 μL of arbutin (16 mM) were added to the solution and stirred again for 30 s. Finally, 50 μL of L-ascorbic acid (16 mM) were added to the reaction vessel and the solution was gently stirred for 30 s. The reaction mixture was then immediately transferred into the viscometer Teflon[®] cup reservoir;

- (ii) A similar procedure as described above (i) was applied, however, 50 μL of L-ascorbic acid (16 mM) were added to the 7.5 min equilibrated reaction solution (7.90 or 7.85 mL) – comprising hyaluronan *plus* CuCl_2 – and 30 s solution stirring followed. After 1 h, finally 50 or 100 μL of arbutin (16 mM) were added to the solution and stirred again for 30 s. The solution mixture was then immediately transferred into the viscometer Teflon[®] cup reservoir.

Rotational Viscometry

The resulting reaction mixture (8.0 mL) was transferred into the Teflon[®] cup reservoir of a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). The recording of viscometer output parameters started 2 min after the experiment onset. The changes of the dynamic viscosity (η) values of the reaction mixture were measured at 25.0 ± 0.1 °C in 3-min intervals for up to 5 h. The viscometer Teflon[®] spindle rotated at 180 rpm, i.e. at a shear rate of 237.6 s^{-1} .

SEC Analyses

Evaluation of HA molar mass changes was performed with Shimadzu apparatus using a packed HEMA-BIO 1000 column of dimensions $7.8 \text{ mm} \times 250 \text{ mm}$; the packing particle size was 10 μm . The mobile phase 100 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl was pumped by the LC-10AD device at a flow rate of 0.5 mL/min. For calibration of the SEC system used reference hyaluronans ($M_w = 90.2\text{--}1380 \text{ kDa}$) with broader molar-mass distributions ($M_w/M_n = 1.60\text{--}1.88$) were applied. The samples of the volume of 20 μL with the polymer concentrations 1 mg/mL or lower were injected by a 7725i-type Rheodyne valve. The SEC analyses performance was monitored on-line by UV (SPD-10AV, set at 206 nm) and refractive index (RID-10) detectors.

Cyclic Voltammetry

All cyclovoltammetric experiments were performed with HEKA PG 284 (Germany) potentiostat under argon using a standard three-electrode arrangement of a platinum wire as working electrode, a platinum coil as counter electrode, and a Ag/AgCl as reference electrode. The scan rate used was 100 mV/s.

EPR Spectroscopy

The generation of free radicals during hyaluronan degradation was examined by spin trapping technique using an EPR X-band EMX spectrometer (Bruker, Rheinstetten, Germany) at ambient temperature [9, 10]. The sample preparation conditions were identical to those described under the paragraph “Study of uninhibited/inhibited hyaluronan degradation” – see

the section "Uninhibited" as well as that of (i) within the section "Inhibited HA degradation". Yet the prepared total sample volume was only 800 μL and the addition of L-ascorbic acid solution (the last reactant) when appropriate was replaced by addition of the aqueous NaCl-diluent (5 μL).

The volume of 250 μL of each sample solution was thoroughly mixed with 5.0 μL of DMPO spin trap prior to each experimental set carried out in a thin flat EPR quartz cell. The operational parameters of the equipment were adjusted as follows: Centre field 3354 G, sweep width 100 G, time constant 81.92 ms, conversion time 20.48 ms, receiver gain $5\text{e}+5$, microwave power 10 mW, and modulation amplitude 2 G.

ABTS Assay – Determination of the TEAC Value

The $\text{ABTS}^{+\cdot}$ radical cations were pre-formed by the reaction of an aqueous solution of $\text{K}_2\text{S}_2\text{O}_8$ (3.3 mg) in water (5 mL) with ABTS (17.2 mg). The resulting bluish-green radical cation solution was stored overnight in the dark below 0 °C. Before experiment, the solution (1 mL) was diluted into a final volume (60 mL) with water [11].

Arbutin solution was prepared as specified under the paragraph "Preparation of stock and working solutions". The investigated samples comprised 2 mL of the diluted $\text{ABTS}^{+\cdot}$ solution with addition of 50 μL of working arbutin solution (1 mM).

A modified ABTS assay was run applying a UV/VIS S2000 spectrophotometer (Sentronic, Germany). UV/VIS spectra were taken up to 120 min. The relative differences (ΔA) in the light absorbance at 731 nm in the 10th and 120th min compared to the reference experiment using Trolox[®] were used to calculate the values of TEAC – Trolox Equivalent of Antioxidant Capacity.

ABTS assay – Determination of the IC₅₀ Value

The $\text{ABTS}^{+\cdot}$ radical cation solution (250 μL) – prepared from 7 mM ABTS and 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$, 1:1 v/v ratio of water solutions – was added to 2.5 μL of the arbutin solution and the absorbance of the sample mixture was measured at 734 nm after six minutes. The radical scavenging capacity of arbutin was investigated at the drug concentration range 2–1000 μM .

The light absorbance was measured quadruplicately in 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) with Tecan Infinite M 200 reader (Tecan AG, Austria). The IC₅₀ value was calculated with CompuSyn 1.0.1 software (ComboSyn, Inc., Paramus, USA).

RESULTS AND DISCUSSION

Establishing a Proper HA Oxidative Degradation System

The HA macromolecules were exposed to free-radical degradation induced by Cu(II) ions (1 μM) and ascorbate at various concentrations (10, 50, or 100 μM) where the primary

variable – the concentration of cupric ions – was set to such a value which may occur at the early stages of acute joint inflammation. The second variable, the concentration of ascorbate, covers that range which is characteristic in SF of a human being. As shown in Figure 3, the curve coded 1, the reaction system Cu(II):ascorbate equaling 1:10 was during the whole time period of 5 h ineffective to induce a relevant HA degradation. The observed minor growing of the solution η value represents the well known effect – called rheopexy – which is characterized by orientations and attractive self-interactions of dissolved HA macromolecules in the space between the viscometer reservoir wall and the rotating spindle [2].

By increasing the ascorbate concentration to 50 μM (cf. figure 3, curve coded 2), a gradual decrease of the dynamic viscosity of HA solution was evidenced during the first 90 min. Then a slower HA degrading process was observed, which continued up to the end of monitoring the solution η values – 5 h. The attained decrease of the HA dynamic viscosity from its initial value of 9.93 to 8.01 mPa·s was, however, classified as not sufficiently effective for our further investigations.

Increasing the ascorbate concentration to 100 μM resulted in an approximately 60-min time interval during which “no degradation” of HA macromolecules was registered. Yet as evident from the curve coded 3 in Figure 3, this stationary/lag phase was continued by a stepwise decrease of the solution dynamic viscosity, achieving at 5 h the value of 6.53 mPa·s. As proven by using the method of EPR spectroscopy [12] under the experimental conditions represented in Figure 3, curve coded 3, the signal detected during the earlier time intervals (≤ 60 min) belonged exclusively to ascorbyl radical. However as confirmed by applying the DMPO spin-trapping method, at later time intervals, the generated $\cdot\text{OH}$ radicals “exceeded” the scavenging effectivity of the actual ascorbate (AscH^\cdot) level and thus they represented the radical species which reacted with the HA chains leading to polysaccharide fragmentation.

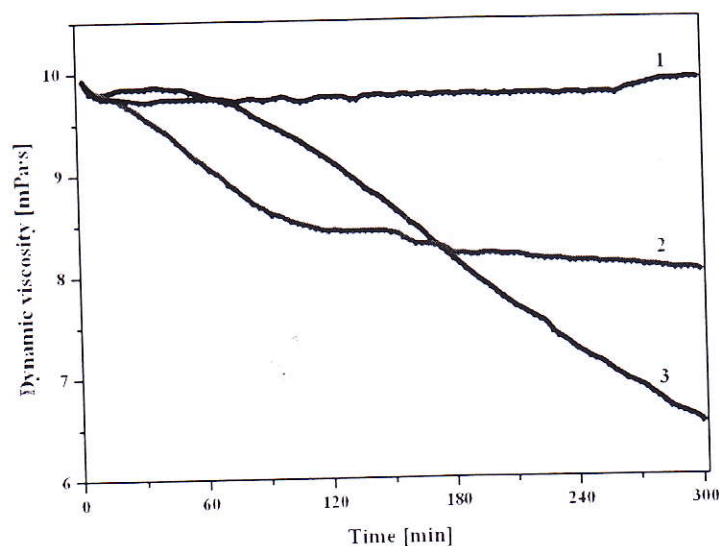
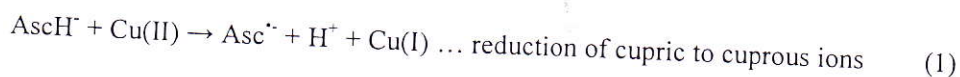


Figure 3. Time dependences of dynamic viscosity values of the HA solution in the presence of 1.0 μM CuCl_2 plus ascorbate at concentrations 10 (1), 50 (2), or 100 (3) μM .

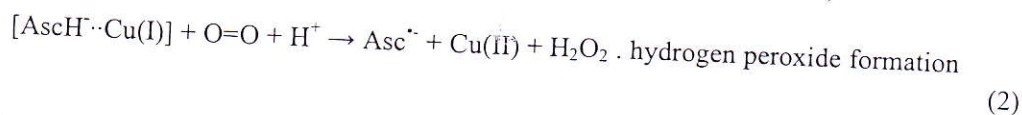
As seen in Figure 3, curve coded 3, during the time interval between 60 and 300 min the continual stepwise decrease of the solution η values covers a range equaling to 3.25 mPa.s.

The earliest time interval of the reaction: Let us first analyze the potential chemical processes which resulted in the observations represented by the curve coded 3 in Figure 3 – namely the time interval up to approximately 60 min: Under aerobic conditions, the bicomponent system comprising ascorbate and a trace amount of cupric ions is a well known Weissberger's oxidative system generating the strong oxidant – hydrogen peroxide. The following flow chart can be primarily used to characterize the individual reaction steps, which however take place simultaneously in a concerted action

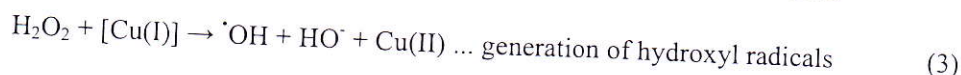


Note 1: It is a well known fact that AscH^- donates a hydrogen atom (H^{\bullet} or $\text{H}^+ + \text{e}^-$) yielding the resonance-stabilized tricarbonyl ascorbate free radical. Since the latter has a $\text{p}K_a$ value of -0.86, it is not protonated and thus in aqueous solution it will be present as $\text{Asc}^{\bullet-}$ [13].

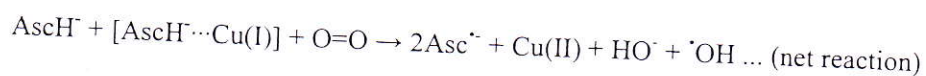
As the cuprous ions are unstable, namely they can quickly undergo a disproportional reaction $\text{Cu(I)} + \text{Cu(I)} \rightarrow \text{Cu(0)} + \text{Cu(II)}$, they are charge-stabilized by an excess of the present ascorbate as follows $\text{AscH}^- + \text{Cu(I)} \rightarrow [\text{AscH}^{\bullet-} \cdots \text{Cu(I)}]$. This reaction intermediate participates in the next bi-electron reduction of the dioxygen molecule ($\text{O}=\text{O}$)



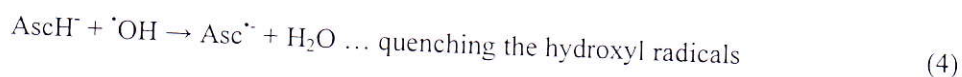
and one may suppose that the nascent H_2O_2 molecule is decomposed immediately by the "uncomplexed"/complexed cuprous ion(s) according to a Fenton-like reaction



In summary, we could express the following final/net reaction



Yet the conditions of the above flow chart should be taken into account: Since within the reaction system there is a real ascorbate excess, the nascent hydroxyl radicals are continually quenched and due to these facts the EPR spectrometer monitors exclusively the ascorbyl radicals



According to our previous findings obtained by using the method of EPR spectroscopy [12] under the experimental conditions represented also in Figure 3, curve coded 3, during the earlier time intervals (≤ 60 min) the monitored ascorbyls – $\text{Asc}^{\bullet-}$ – indicate that any potentially generated hydroxyl radical is quenched immediately at its phase of nascence.

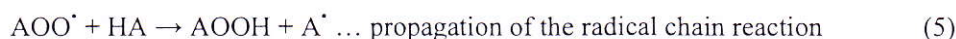
The informed reader knows that (two) ascorbyls undergo a redox disproportional reaction yielding back a molecule of ascorbate and a not charged dehydroascorbate (DHA). The DHA

molecule however hydrolyzes yielding an intermediate, namely 2,3-diketo-L-gluconic acid, and/or several final products such as L-xylose, and L-xylonic-, L-lyxonic-, L-threonic-, as well as oxalic acids [13]. Study of the participation of the above mentioned intermediate as well as of the final products within the reaction flow charts (cf. reactions 1–4) was however beyond the scope of the present investigation.

The later time interval of the reaction: Let us continue the analysis of the potential chemical processes which could result in the observations represented by the curve coded 3 in Figure 3 – namely the result within the time interval from approximately 60 to 300 min:

As anticipated according to the sequential depletion of the ascorbate the nonquenched hydroxyl radicals can react with the HA macromolecule by abstracting a hydrogen radical (H^\bullet), resulting in the formation of a C-macroradical represented in Figure 4 and further denoted as A^\bullet .

Under aerobic conditions, the alkyl-type macroradical – A^\bullet – reacts rapidly with the molecule of dioxygen (O_2) yielding a peroxy-type macroradical, hereafter denoted as AOO^\bullet . The intermediate peroxy-type macroradical formed may react with an adjacent hyaluronan macromolecule (HA), and thus the radical chain reaction propagates quickly



After its “collision” with an HA macromolecule (cf. reaction 5), the generated peroxy-type macroradical yields a high-molar-mass hydroperoxide, which subsequently, mostly induced by the present Cu(I) ions $\{AOOH + Cu(I) \rightarrow AO^\bullet + HO^- + Cu(II)\}$, yields an alkoxy-type macroradical (AO^\bullet). This is a presumed intermediate of the main chain splitting, resulting in biopolymer fragments whose solution is characterized by a reduced dynamic viscosity (cf. Scheme 1 [14]).

The attack of hydroxyl radicals on D-glucuronate or N-acetyl-D-glucosamine moieties of HA can also lead to the “opening” of rings without breaking the polymer chain [15, 16].

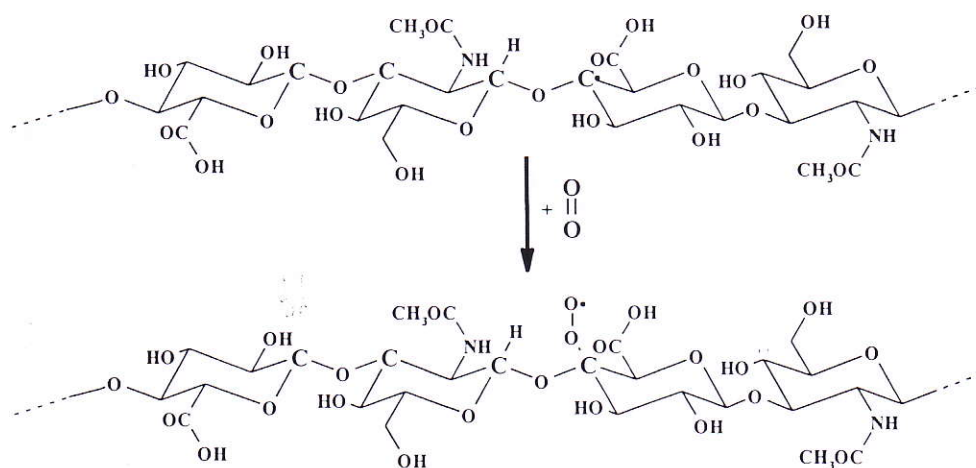
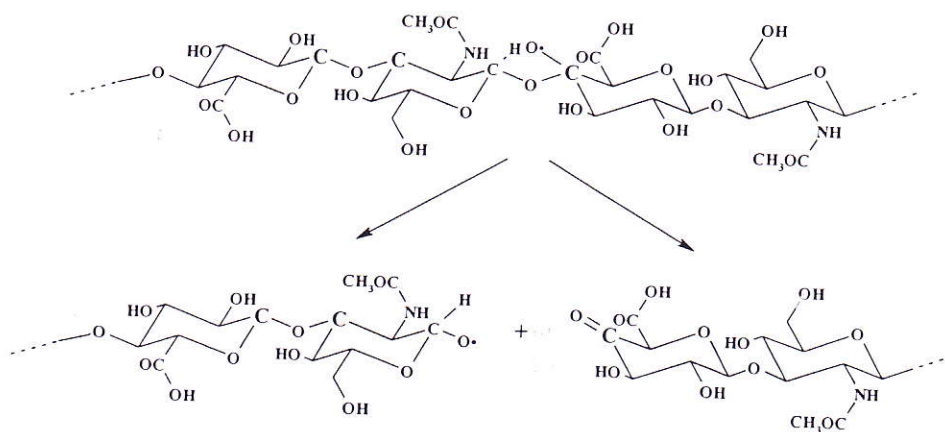


Figure 4. The hyaluronan C-type macroradical, due to its high reactivity, immediately traps a molecule of dioxygen yielding a peroxy-type macroradical.



Scheme 1. Fragmentation of the alkoxy-type macroradical (AO').

Investigating Pro- and Anti-Oxidative Action of Arbutin

Based on its chemical structure (cf. figure. 2), the natural compound arbutin can be grouped among O-alkyl derivatives of hydroquinone, which are generally classified as efficient antioxidants [17]. To assay arbutin action in its function as a preventive antioxidant, the drug was loaded into cupric ions containing HA solution, directly before ascorbic acid admixing – as described in procedure (i) under the paragraph “Study of uninhibited/inhibited hyaluronan degradation”.

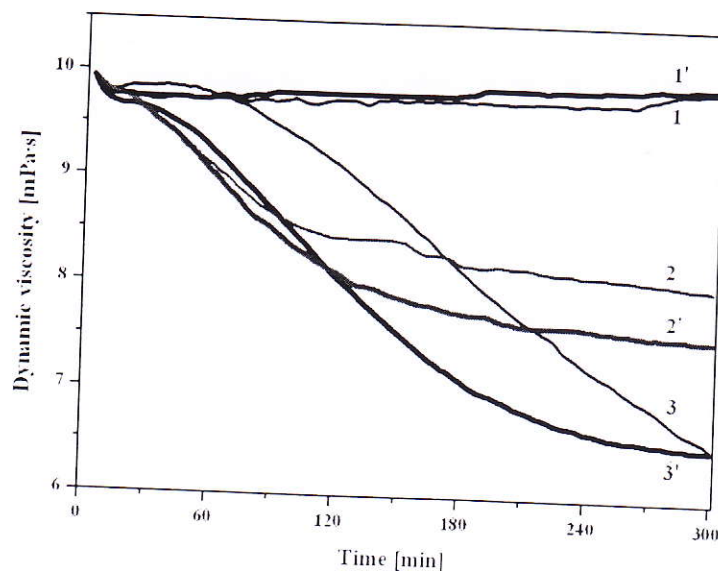
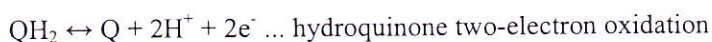


Figure 5. Time dependences of dynamic viscosity values of the HA solution in the presence of 1 μM CuCl_2 , 100 μM arbutin, *plus* ascorbate at the concentrations 10 (1'), 50 (2'), or 100 (3') μM , as well the dependences observed under similar experimental condition without any arbutin – curves 1, 2, and 3 – relate to ascorbate concentrations of 10, 50, and 100 μM , respectively.

As indicated in Figure 5, contrary to the expected inhibition/retardation of the degradation of HA macromolecules, on introducing the drug – resulting in its concentration of 50 or 100 μM (cf. curves coded 2' or 3' in figure 5) – a remarkable reduction of the η value of the solution monitored was recorded at the earliest time interval. While the shape of curve 3 (cf. figure 5), relating to the reaction mixture without arbutin, indicates a stationary/lag phase of the HA degradation process during the first 60 min, addition of the arbutin drug tested (100 μM) shows both elimination of the reaction lag phase and significant acceleration of the decay of HA macromolecules.

The degradation of the native HA sample after 5-h treatment by the system comprising Cu(II):arbutin:ascorbate in the molar concentration ratio of 1:100:100 was unequivocally proved by SEC analysis (cf. figure 6, UV and RI traces). As evident from Figure 6, the polymer sample at 5 h had a lower molar mass ($M_{\text{peak}} \approx 695 \text{ kDa}$) as compared to that of the original hyaluronan.

To explain the above observations, the reader's attention is to be switched to the reversible redox processes known for the pair of hydroquinone and quinone (denoted hereafter shortly QH_2 and Q), which can be described as follows [18]



As claimed above, arbutin is readily hydrolyzed by diluted acids yielding QH_2 and D-glucose in a 1:1 mole ratio [6]. The latter tenet was supported also by our cyclic voltammetric experiments performed both in DMSO/TBAPF6 and aqueous 0.15 M NaCl solution, as shown in Figure 7.

Clear differences are seen on comparing DMSO and water solutions. In water solution, the products of hydrolysis as well as protonation of arbutin are observable already during the first CV scan, in contrast to DMSO where only a small increase of arbutin degradation products is seen. In water solution, the strong increase of the CV peak, characteristic of Q/QH_2 redox couple during repetitive redox cycling, indicates that compared to non-oxidized arbutin, the oxidized form of arbutin strongly hydrolyzes (decomposes). Thus due to the addition of ascorbic acid to arbutin, rapid drug hydrolysis occurred.

Reaction chemistry investigated by spin-trapping EPR spectroscopic method: Figure 8 represents typical records registered on applying DMPO in the function of radical trapping within the reaction system comprising (a) HA and Cu(II); (b) HA, Cu(II), and arbutin; as well

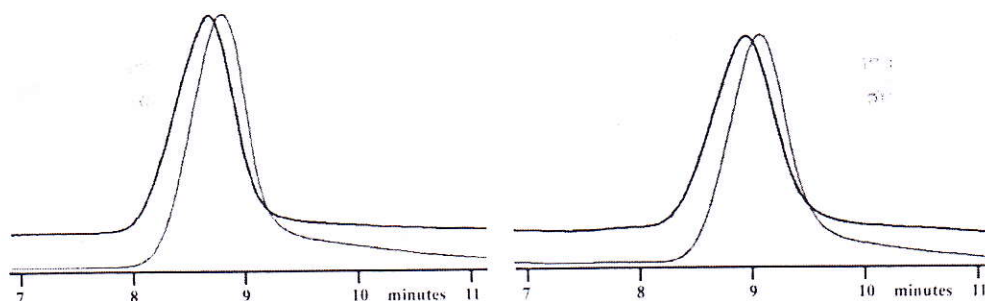


Figure 6. SEC analyses of the native HA sample and of that degrading over 5 h. (The native HA peaked earlier.) Left panel – UV traces at 206 nm; right panel – RI records.

as (c) HA, Cu(II), arbutin, and ascorbate, where the reactants were applied at such concentration ratios which were identical to those described under the paragraph "Study of uninhibited/inhibited hyaluronan degradation".

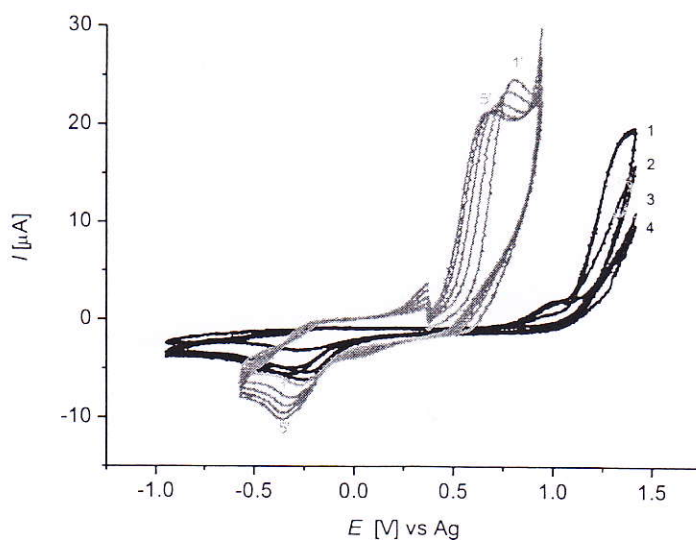


Figure 7. Cyclic voltammetry (repetitive cycling, CV scans are indicated with numbers) of arbutin in DMSO/TBAPF6 (black lines 1–4) or in H₂O/NaCl (gray lines 1'–5').

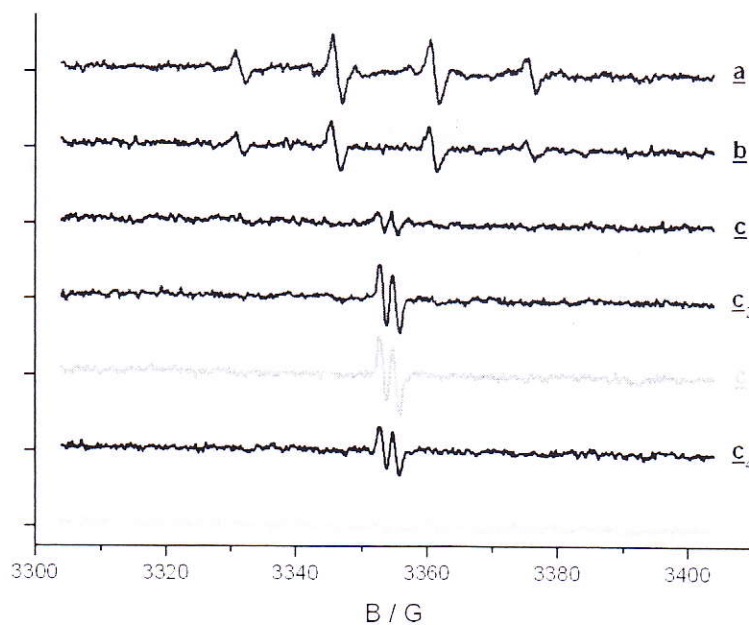


Figure 8. The DMPO-OH signals registered 1 min after mixing the DMPO spin trap with the sample solution containing HA and Cu(II) – trace a; and with the solution comprising HA, Cu(II), and arbutin – trace b. The signals for ascorbyl detected at time intervals of 10, 30, 60, 90, and 120 min are represented by the respective traces from c1 to c5.

(a, b): By admixing DMPO into the reaction vessel containing already the two components – HA and Cu(II) – a signal characteristic for the 'DMPO-OH adduct was recorded, however the following arbutin application diminished somewhat the signal abundance (cf. figure 8, traces coded **a** and **b**). The simplest explanation of these observations could be that DMPO added in a great excess reduced Cu(II) ions to cuprous ones, which subsequently reacted with dioxygen, yielding superoxide anion radicals ($O_2^{\cdot-}$). These ROS spontaneously dismutate and the dismutation product H_2O_2 is decomposed by the reduced copper ions in a Fenton-like reaction.

Note 2: It should be pointed out, however, that systems comprising HA and Cu(II) or HA, Cu(II), and arbutin did not induce any significant degradation of hyaluronan macromolecules (not shown). (c): It should be pointed out here that the mixing of all the reaction components, namely DMPO with the sample solution containing HA, Cu(II), arbutin, and ascorbate, took but approximately 1 min during which time the sample was not scanned by the EPR spectrometer. However, after a short time, a signal related to $Asc^{\cdot-}$ emerged, whose intensity varied: During the first time interval – up to approximately 60 min – the intensity of the recorded signal rose, then it declined and after 120 min the spectrometer monitor was “silent” (cf. figure 8, traces **c₁–c₅**). The EPR spectroscopic observations at a later time interval, namely the less and less abundant signal related to $Asc^{\cdot-}$ between 60 and 120 min, as well as later on the signal disappearance, coincide well with the results represented in Figure 5, curve coded 3' at similar time intervals.

By admixing DMPO into the reaction vessel containing already the two components – HA and Cu(II) – a signal characteristic for the 'DMPO-OH adduct was recorded, however the following arbutin application reduced the EPR signal of 'DMPO-OH adducts by about 20–30 percent (cf. figure 9).

The simplest explanation of these observations could be that hydroperoxides already present in the initial HA solution [9, 19, 20] rapidly decompose due to the presence of

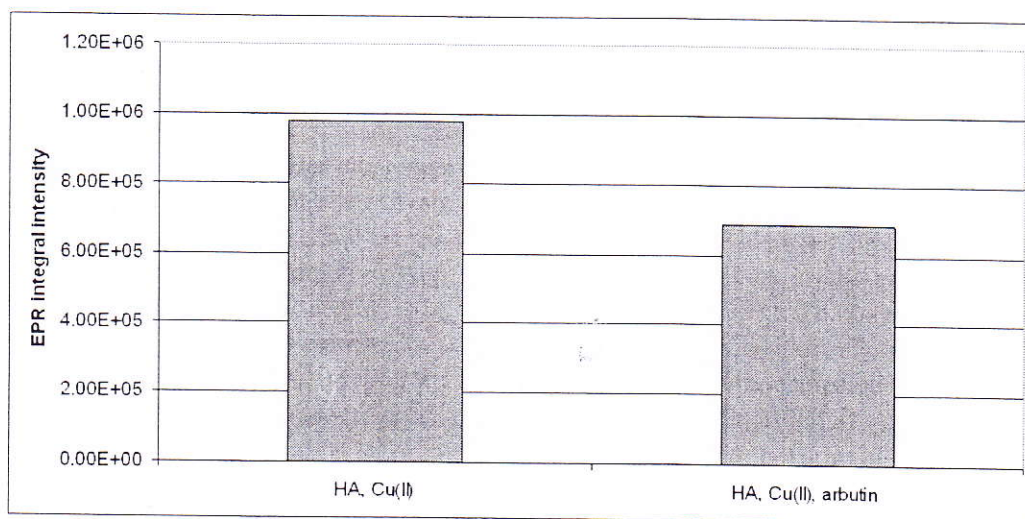
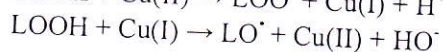
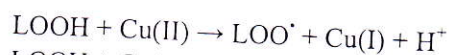


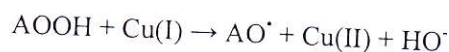
Figure 9. Integral EPR intensity of 'DMPO-OH adducts registered 1 min after mixing the DMPO spin trap with sample solution containing HA and Cu(II) and with the solution comprising HA, Cu(II), and arbutin.

Cu(II)/Cu(I) ions (cf. also Scheme 1 above), as already well established in the field of peroxidation of lipids (denoted here as L)



Note 3: It is well known that the presence of peroxy and alkoxy radicals as well as their DMPO spin adducts in water solutions leads mostly to the formation of $\cdot\text{OH}$ radicals generating the signal of $\cdot\text{DMPO-OH}$ adduct. Arbutin is probably able to eliminate reactive alkoxy and peroxy radicals before their spin trapping.

In the case of the system comprising a trace amount of oxidized hyaluronan, or more precisely the HA sample containing already a pre-formed macrohydroperoxides (AOOH), the two electrons gained according to reaction (6) could reduce Cu(II) ions to Cu(I). The generated cuprous ions are then involved in the following reaction



Thus a short-living (ca. 1 μs) alkoxy-type macroradical was only present in our experimental system. This macroradical was extremely unstable and degraded quickly to fragments with lower molar mass (cf. Scheme 1).

Note 4: It should be pointed out, however, that systems comprising HA and Cu(II) or HA, Cu(II), and arbutin did not induce any significant degradation of hyaluronan macromolecules (not shown).

ABTS assay of arbutin: The determined TEAC values, i.e. those for the 10th min 1.22 mmol Trolox/L and for the 120th min 1.59 mmol Trolox/L (cf. Figure 10) prove a really high reductant power of arbutin in aqueous solutions.

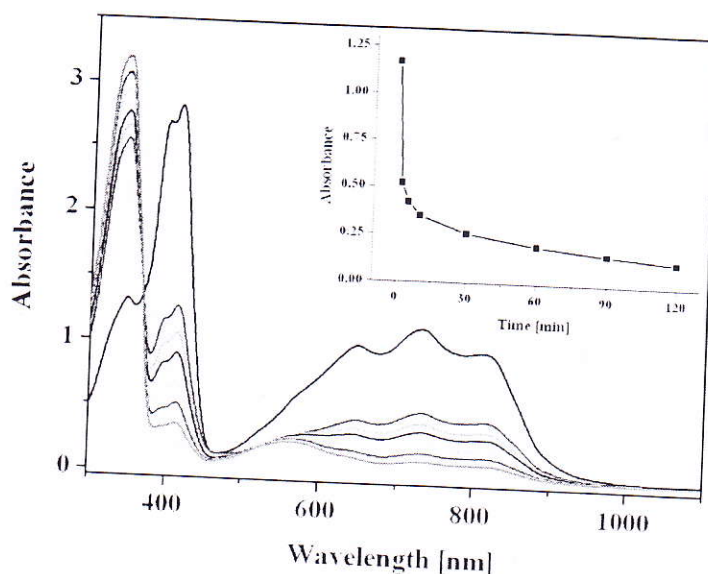


Figure 10. The UV/Vis records documenting arbutin scavenging of the $\text{ABTS}^{\bullet+}$ radical cation. Inset: Time dependence of the absorbance at 731 nm.

The arbutin scavenging activity represented by its established IC_{50} value $5.43 \mu M$ should be classified as relatively high, comparable to that value of quercetin ($IC_{50} = 2.86 \mu M$) – the substance used as a standard effective natural antioxidant [21, 22].

The reaction of decolorization of the $ABTS^{\bullet+}$ radical cation solution is based on a simple reaction during which a proper reductant provides an electron to the acceptor – the $ABTS^{\bullet+}$ radical cation. Although the ABTS assay is still one of the primary choices, the reader should take into account that the results described by either the TEAC or IC_{50} values represent just values determined at the experimental conditions used; by applying e.g. another electron acceptor (such as that of $DPPH^{\bullet}$ – the 1,1-diphenyl-2-picrylhydrazyl radical) different IC_{50} values are established.

Note 5: On applying the DPPH assay, the determined IC_{50} value for arbutin ($\approx 768 \mu M$) represents a 176-times lower antioxidant capacity than the one determined for quercetin ($4.36 \mu M$). Although the above arbutin IC_{50} value correlates well with those published by other investigators applying similar DPPH-assay conditions [23], it should be pointed out that there is an experimental “conflict”: The DPPH assay, which uses alcohols (methanol) as the 1,1-diphenyl-2-picrylhydrazyl-radical dissolvent, is preferably used to investigate the antioxidant capacity of lipophilic (alcohol soluble) substances. Yet arbutin is classifiable as a hydrophilic substance, perfectly soluble in water. As calculated, the arbutin vs. quercetin solubility in water, 39.1 vs. 0.26 g/L, closely correlates to the logP – lipophilicity values of these substances, namely -1.36 vs. $+1.07$ [24], respectively. Thus, the IC_{50} value for arbutin ($\approx 768 \mu M$) determined by using the DPPH assay does not primarily indicate the very low reduction power of the drug, but most plausibly, this value reflects also a much poorer arbutin solubility compared to that of quercetin when methanol was the solvent used.

Investigating Arbutin in the Function of a Chain-Breaking Antioxidant

There is a discrepancy of arbutin classification as an efficient antioxidant/reductant – based on the ABTS assays – and on the contrary a pro-oxidant investigated by the drug action when introduced into the running free-radical HA degradation during the phase of the reaction propagation. Under the experimental conditions specified under the paragraph “Study of uninhibited/inhibited hyaluronan degradation” – section (ii) – the results represented in Figure 11 should be assessed as bi-phasic. As evident (cf. figure 11, curves coded 100 and 200), addition of the arbutin solution after 1 h of the reaction onset, immediately increased the degradation rate of HA macromolecules. This process however continued by a step-wise slowing-down of the solution dynamic viscosity with the final values 8.43 and 7.33 mPa·s, proving the inhibitory action of arbutin compared to uninhibited free-radical HA degradation represented by the curve coded 0 in Figure 11.

Based on the above results (cf. figure 11), one may conclude that arbutin functions as a chain-breaking antioxidant. Yet it should be pointed out again that such a statement is valid only under the experimental conditions used. On applying the drug for skin lightening, one should take care since the pro-oxidative action of arbutin may result in damaging the tissues involved. The skin contents of HA, ascorbate, and the ubiquitous redox-active (transition) metals [18] – including Cu ions – predispose the processes documented by the above observations.

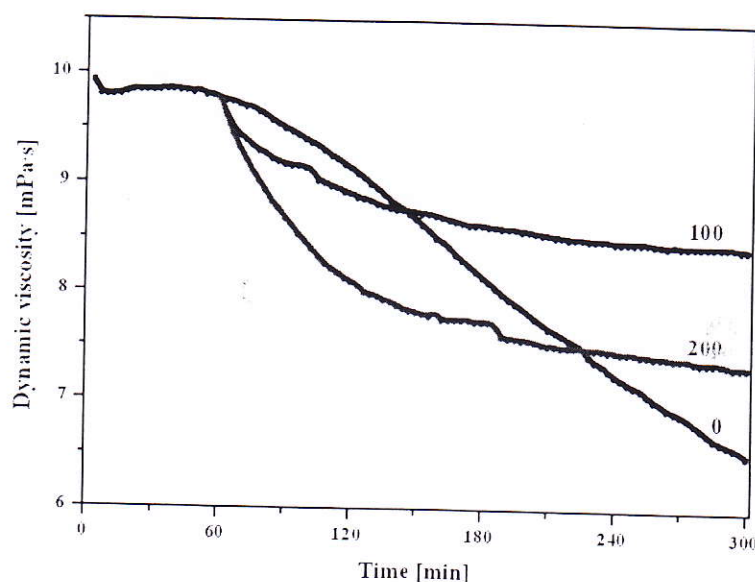


Figure 11. Time dependences of dynamic viscosity values of HA solution in the presence of 1 μM CuCl_2 plus 100 μM ascorbate (0) and after addition of arbutin in the concentration of 100 (100) and 200 (200) μM .

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