

Degradation of High-Molar-Mass Hyaluronan by Ascorbate *plus* Cupric Ions: Effects of D-Penicillamine Addition

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Pro- and anti-oxidative effects of an anti-rheumatoid drug, D-penicillamine (D-PN), on the kinetics of high-molar-mass hyaluronan (HA) degradation were monitored using the method of rotational viscometry. The degradation of the dissolved HA macromolecules was attained by applying the Weissberger's system comprising ascorbic acid *plus* cupric ions. Electron paramagnetic resonance (EPR) spectroscopy was used to identify the generated free radicals. The results obtained indicate that the initial anti-oxidative action of D-PN is followed by induction of pro-oxidative conditions due to the generation of reactive free radicals. It is speculated, however, that the latter situation may be considered as an advantageous property of D-PN. Hydroxyl radicals formed in this way may participate in decomposition of proteinases, which are believed to be responsible for the destruction of joint cartilage under rheumatoid arthritic conditions.

Introduction. – Hyaluronan (HA; also called hyaluronic acid, hyaluronate; *Fig. 1*) is a polyelectrolyte component of the synovial fluid (SF). The concentration of HA in human SF is 2.5 mg/ml on average.

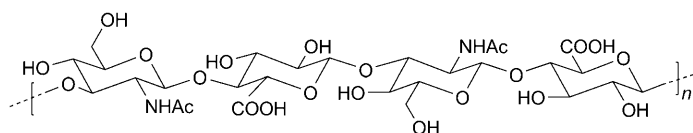


Fig. 1. Hyaluronan – the acid form

In SF of healthy subjects, the molar mass of HA is of the order of several mega-Daltons. In patients suffering from rheumatoid arthritis (RA), the mean molar mass of HA is, however, significantly reduced [1]. Among other factors, reactive oxygen species were established as being co-responsible for the reduction of the biopolymer molar mass [2–4].

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D-Penicillamine (D-PN; *Fig. 2*) has been used to treat patients with severe active RA. This compound is classified as an immunomodulating/third-line disease-modifying anti-rheumatoid drug [5].

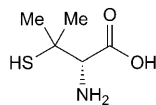


Fig. 2. Chemical structure of D-penicillamine (D-PN)

One of the reasons for D-PN administration was the finding that SF of RA patients contains a three-fold higher concentration of Cu ions than that of the healthy persons [6]. Since this drug chelates Cu ions, its positive action may be partly ascribed to Cu complexation. Yet, as has been claimed, under aerobic conditions, the D-PN–copper complex can generate H_2O_2 [7]. On the other hand, D-PN has been assigned to the scavengers of H_2O_2 . The opposing properties of D-penicillamine, namely its ability to produce as well as to scavenge H_2O_2 may be relevant to the drug actions in rheumatoid diseases [8].

Thus, to expand our knowledge of these counteractive phenomena, the method of rotational viscometry applied to high-molar-mass HA and the electron paramagnetic resonance (EPR) spin-trapping spectroscopic technique were exploited in the present study.

Results and Discussion. – *Fig. 3* (curve marked 0) demonstrates that the addition of Cu^{II} , followed by the admixture of reductant – ascorbic acid – results in a gradual decline of the dynamic viscosity value of the B22157 sample solution. It should be, however, pointed out that addition of either CuCl_2 or D-PN alone to the HA solution (within 5 h) did not induce any degradation of HA [9][10]. On the other hand, addition of one single reactant, namely ascorbic acid, to the B22157 solution resulted in a significant reduction of the sample viscosity value [11], which is a marker of degradation of HA macromolecules [12][13].

As can be seen in *Fig. 3* (curves marked 50, 100, and 200), addition of D-PN dose-dependently prolonged the period of complete inhibition of the degradation of HA macromolecules. However, after a certain time, namely 30 min at 50 μM D-PN, 40 min at 100 μM , or *ca.* 90 min at 200 μM D-PN, a rapid uni- (curves marked 50 and 100) or biphasic (curve marked 200) reduction of the sample dynamic viscosity was evidenced. The method of spin-trap EPR spectroscopy was employed to provide a deeper insight into the chemistry of the processes presented in *Fig. 3*.

Results shown in *Fig. 4*, curve marked Cu, support the assumption that addition of CuCl_2 into the reaction mixture containing HA and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) leads to the generation of $\cdot\text{OH}$ radicals. This observation, which does not fully correspond to the situation when no sample viscosity reduction was observed (*cf.* [9]), might indicate that DMPO alone or with participation of the polysaccharide can serve as ‘a reducing medium’ for Cu^{II} . Subsequently, the formed Cu^{I} ions, by a sequence of *Fenton*-like reactions, generate $\cdot\text{OH}$ radicals, whose presence was unequivocally proved by DMSO addition into the reaction mixture (*cf.* *Fig. 5*). EPR Spectrum of $\cdot\text{DMPO-R}$ adducts confirms the formation of C-centered radicals when DMSO was

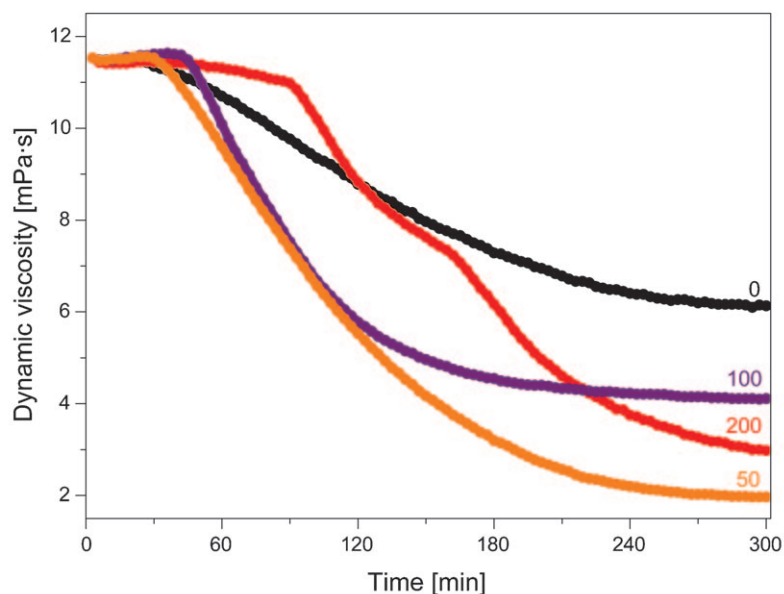


Fig. 3. Effects of D-PN (0, 50, 100, or 200 μM) on HA degradation induced by a system containing 1.0 μM CuCl_2 plus 100 μM ascorbic acid

included in the reaction mixture. These C-centered radicals are likely formed due to proton abstraction from DMSO. DMSO is well-known to be $\cdot\text{OH}$ scavenger and is recognized to form C-centered radicals, which can be spin-trapped and are often used as detecting molecules in $\cdot\text{OH}$ assays [14].

Due to consecutive reactions with molecular oxygen present in the sample, $\cdot\text{OR}$ radicals are also simultaneously formed and trapped as O-centered $\cdot\text{DMPO-OR}$ adducts. On mixing the reagents – HA, Cu^{II} , and D-PN – with DMPO (*cf.* Fig. 4, curve marked Cu + D-PN), a significant increase of the signal corresponding to the $\cdot\text{OH}$ radicals was observed. On the contrary, the reaction mixture containing also ascorbic acid (100 μM) did not reveal the presence of $\cdot\text{OH}$ radicals (*cf.* curves marked 1 and 20) within the time interval < 60 min. At longer time intervals, namely 90 and 120 min, a gradual increase of the intensity of EPR signal corresponding to the $\cdot\text{OH}$ radicals was unequivocally recorded (*cf.* Fig. 4, curves marked 90 and 120). This observation is in an excellent accordance with the result documented in Fig. 3, *i.e.*, that after a complete inhibition period lasting *ca.* 90 min, an extensive degradation of HA biopolymer is evidenced. To explain the zone of complete inhibition shown in Fig. 3, the result represented in Fig. 6 is of a great importance. As evident, within the early time interval < 20 min (*cf.* Fig. 4, curves marked 1 and 20 – position of the arrows), the added ascorbic acid quenched efficiently reactive radicals ($\cdot\text{OH}$, $\text{RS}\cdot$) yielding ascorbyl radicals [15]. However, it should be noted that $\cdot\text{DMPO-OH}$ spin adduct can be simultaneously reduced by ascorbate to give EPR-silent species leading to some overestimation of ascorbate's capacity to scavenge ROS [16].

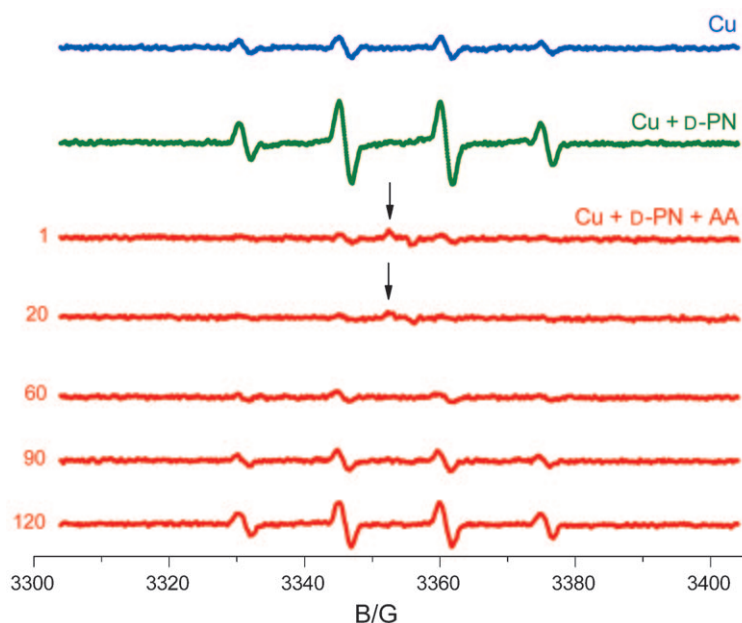
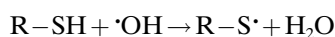


Fig. 4. EPR Spectra of the following samples: Cu, Cu + D-PN, and Cu + D-PN + AA in the 1st, 20th, 60th, 90th, and 120th min after addition of ascorbic acid. 250 μ l of the corresponding sample (HA content 2.5 mg/ml) was mixed with 5 μ l of DMPO spin trap, and the EPR spectra were immediately recorded in the thin flat EPR quartz cell (EPR signal of the ascorbyl anion radical is marked with an arrow).

At physiological concentrations of HA and ascorbate (*ca.* 2.5 mg/ml and 100 μ M), addition of Cu^{II} ions induces generation of H_2O_2 [17]. As can be seen, the $\cdot\text{OH}$ radicals responsible for degradation of HA macromolecules are incompletely scavenged by ascorbate (curve marked 0 in Fig. 3). When using analogous tricomponent mixture of HA, D-PN, and Cu^{II} ions, no reduction in the dynamic viscosity was registered within 5 h [10], although the EPR signal corresponding to a $\cdot\text{DMPO-OH}$ adduct has been recorded (*cf.* Fig. 5 and curve marked Cu + D-PN in Fig. 4).

In the experiments partly simulating the conditions occurring in RA, both ascorbate and D-PN participate in quenching $\cdot\text{OH}$ radicals, which are generated in the presence of redox couple $\text{Cu}^{\text{II}}/\text{Cu}^{\text{I}}$. Yet, after the period classifiable as a complete inhibition of degradative conditions related to the production of $\cdot\text{OH}$ radicals (*cf.* Fig. 3, curves marked 50, 100, and 200 – the time period *ca.* 30, 40, and 90 min), a substantial decrease of the HA solution dynamic viscosity was registered. The employment of the DMPO spin-trap (as documented in Fig. 4, curves marked 90 and 120) corroborates the assumption of the abundant presence of $\cdot\text{OH}$ radicals in the studied solution.

As documented by other authors [18–20], the reaction of D-penicillamine – a thiol-type (R-SH) compound – with $\cdot\text{OH}$ radicals yields thiyl radicals



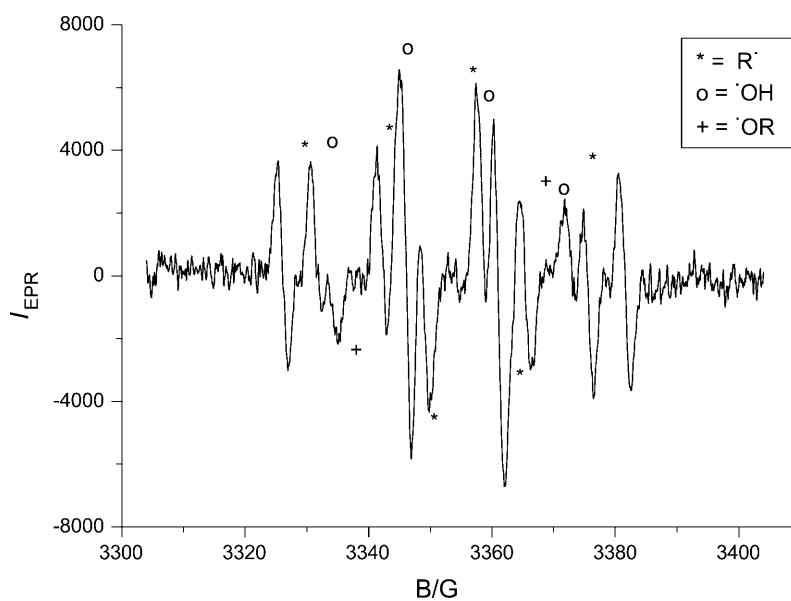


Fig. 5. EPR Spectrum recorded in the system containing HA sample B22157, $CuCl_2$ ($1.0\ \mu M$), D-PN ($200\ \mu M$), and ascorbic acid ($100\ \mu M$) after addition of $100\ \mu l$ of DMSO, followed by the addition of $5\ \mu l$ of DMPO. The spectrum confirms the production of $R\cdot$, $\cdot OH$, and $\cdot OR$ radicals trapped onto DMPO.

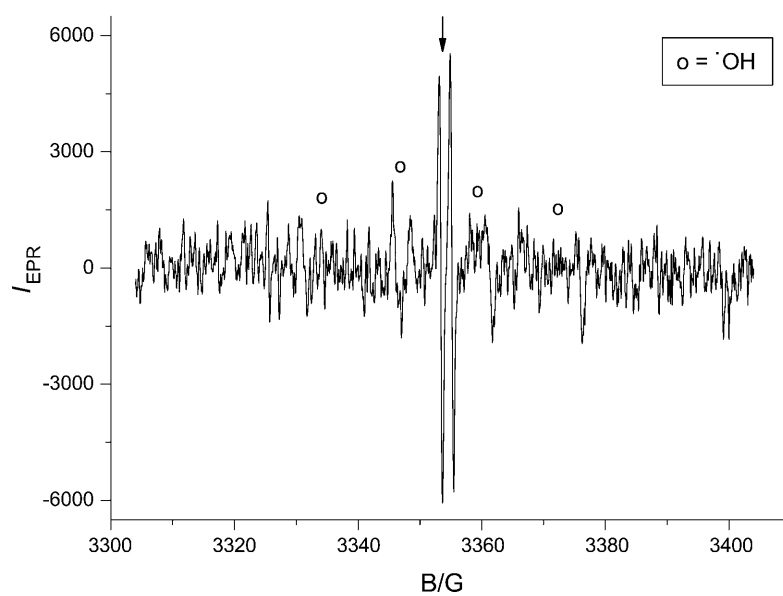
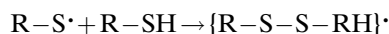
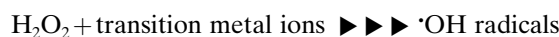
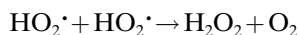
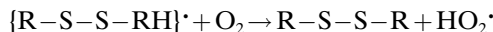


Fig. 6. Detail of the ascorbyl anion radical EPR spectrum (marked by arrow) recorded for the sample $Cu + D-PN + AA$ in the 5th min of reaction (empty circles correspond to the $\cdot DMPO-OH$ adduct)

These, in turn, react with the excess of the parent D-penicillamine



giving rise under aerobic conditions to the disulfide radicals that are responsible for a 'secondary' massive $\cdot\text{OH}$ radical flow



Thus, using the combination of the two methods – spin-trap EPR spectroscopy and rotational viscometry – significant evidence has been obtained that the initial anti-oxidative action of D-PN is followed by extensive pro-oxidative conditions. The latter situation may be, however, considered as an advantage in the treatment of RA. Hydroxyl radicals generated in this way may participate in *in situ* decomposition of proteinases, which are believed to be responsible for the destruction of joint cartilage in severe RA cases.

Experimental Part

General. The high-molar-mass HA sample – B22157 (1.34 MDa) [21] – was kindly donated by *Genzyme Corporation*, Cambridge, MA, USA. The declared content of total heavy metal contaminants in the biopolymer was 2 ppm. The anal. purity grade NaCl and $\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$ were from *Slavus Ltd.*, Bratislava, Slovakia; dimethyl sulfoxide (DMSO) and L-ascorbic acid were purchased from *Merck KGaA*, D-Darmstadt; D-PN and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were both the products of *Sigma-Aldrich Chemie GmbH*, D-Steinheim. High-purity-grade H_2O , with conductivity of $\leq 0.055 \mu\text{S/cm}$, was prepared by using the TKA water purification system (*Water Purification Systems GmbH*, D-Niederelbert).

Preparation of Stock and Working Solns. The stock solns. of ascorbic acid (16.0 mM), CuCl_2 (16.0 mM), and D-PN (32.0 mM) were prepared in 0.15M aq. NaCl. The working soln. of HA sample (2.5 mg/ml) was prepared in the dark at r.t. in 0.15M aq. NaCl in two steps: first, HA (20.0 mg) was swollen in 4.0 ml of 0.15M aq. NaCl, and after 6 h, another 3.85 ml of the solvent was added.

Study of Hyaluronan Degradation. The appropriately diluted stock CuCl_2 soln. (50 μl) was admixed into the 7.85 ml HA working soln. Then, after 7.5 min equilibration, the working solns. of D-PN (50 μl) and after 30 s ascorbic acid (50 μl) were added. On assessing behavior of the HA soln., after adding only one or both reagents, the volume of the reaction mixture was adjusted to 8.0 ml by the solvent (0.15M NaCl). Addition of each reactant was followed by a 30-s mild stirring.

Rotational Viscometry. The resulting reaction mixture (8.0 ml) was transferred into the *Teflon*[®] cup reservoir of the *Brookfield LVDV-II + PRO* digital rotational viscometer (*Brookfield Engineering Labs., Inc.*, Middleboro, MA, USA). Recording of the viscometer output parameters started 2 min after the onset of the experiment. The changes of dynamic viscosity of the system was measured at $25.0 \pm 0.1^\circ$ in 3-min intervals for up to 5 h. The viscometer *Teflon*[®] spindle rotated at 180 rpm, *i.e.*, at the shear rate equaling 237.6 s^{-1} .

EPR Spectroscopy. EPR-Spectroscopic experiments employed DMPO as the spin trap. Spectra were recorded with a EPR X-band *EMX* spectrometer (*Bruker*, D-Rheinstetten) at r.t. for the following samples: Cu (1 μM CuCl_2 and 2.5 mg/ml HA in 0.15M aq. NaCl), Cu + D-PN (1 μM CuCl_2 , 200 μM D-PN,

and 2.5 mg/ml HA in 0.15M aq. NaCl), and Cu + D-PN + AA (1 μ M CuCl₂, 200 μ M D-PN, 100 μ M ascorbic acid, and 2.5 mg/ml HA in 0.15M aq. NaCl). In the case of the latter sample, spectra were recorded at the 1st, 20th, 60th, 90th, and 120th min after the addition of ascorbic acid. Sample soln. (250 μ l) was mixed with 5 μ l of DMPO spin trap, and the measurements were immediately carried out in a thin flat EPR quartz cell. The following operational parameters were set: center field 3354 G, sweep width 100 G, time constant 81.92 ms, conversion time 20.48 ms, receiver gain 5e+5, microwave power 10 mW, modulation amplitude 2 G.

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