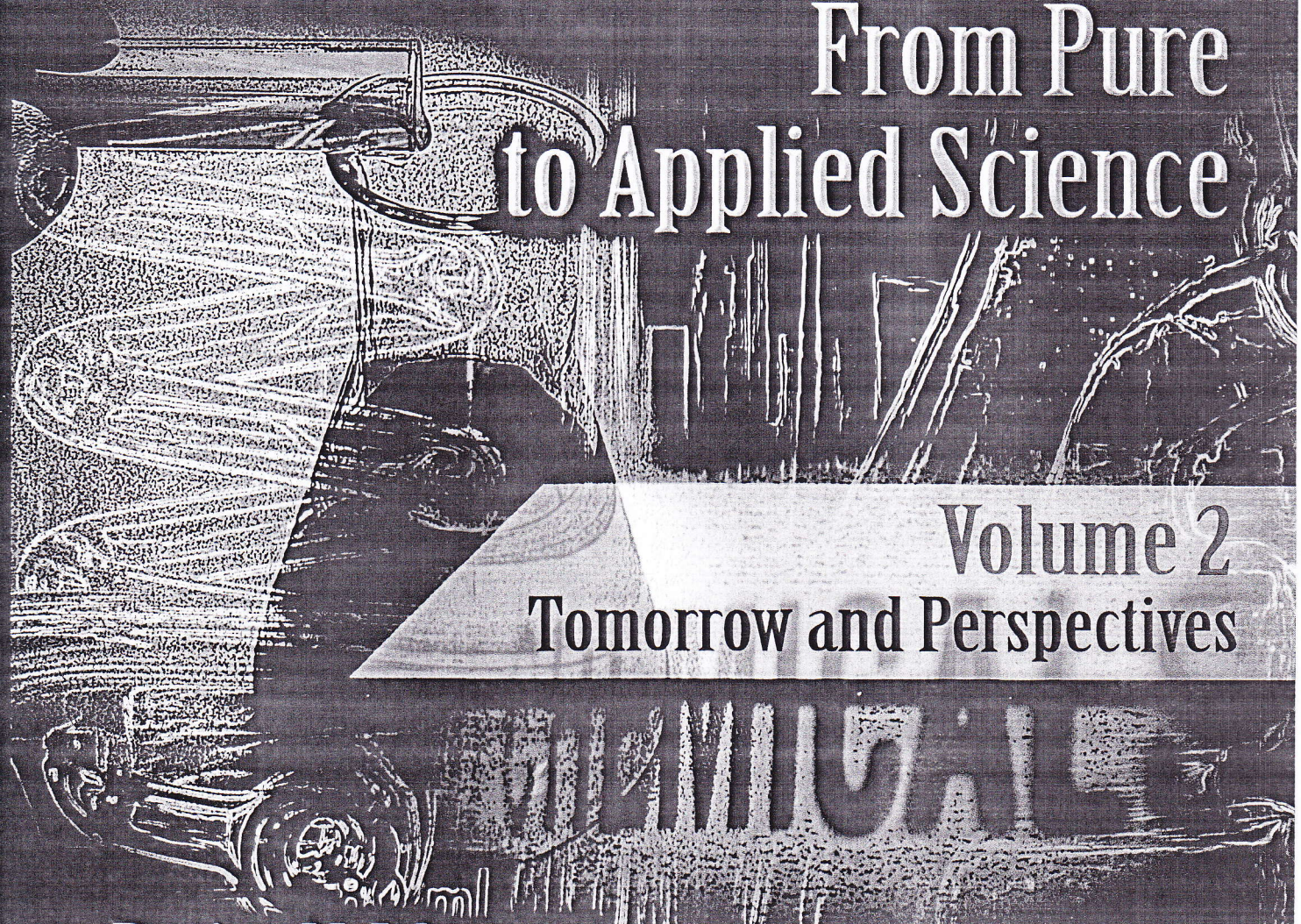
Kinetics, Catalysis and Mechanism of Chemical Reactions

From Pure to Applied Science



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Tomorrow and Perspectives

Regina M. Islamova
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Gennady E. Zaikov
Editors

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Chapter 3

FREE-RADICAL DEGRADATION OF HIGH-MOLAR-MASS HYALURONAN INDUCED BY ASCORBATE *PLUS* CUPRIC IONS: ANTI-OXIDATIVE PROPERTIES OF THE PIEŠŤANY-SPA CURATIVE WATERS FROM HEALING PELOID AND MATURATION POOL

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ABSTRACT

High-molar-mass hyaluronan was exposed to oxidative free-radical chain degradation induced by the action of ascorbate *plus* Cu(II) ions, the conditions that simulate an early phase of acute synovial joint inflammation. Changes of dynamic viscosity of hyaluronan solutions were monitored *via* rotational viscometry. Potential anti-oxidative effects of the Piešťany-spa curative waters from healing peloid and maturation pool were tested against the hyaluronan degradation. Despite a significant content of transition metal ions, remarkable anti-oxidative effects of the spa-water samples were found. These findings could be, in part, ascribed to the anti-oxidative properties of sulphur-based natural compounds present in the Piešťany-spa curative waters.

Keywords: natural curative resources, sulphuric compounds, hyaluronan, rheumatoid arthritis, rotational viscometry.

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INTRODUCTION

Health Spa Piešťany, in the South-West Slovakia, owes its worldwide reputation mainly to curative natural springs of thermal mineral water rising from the depth of over 2000 m through tectonic breaks. This water is the main factor in the formation of curative sulphur peloid, which is the result of perpetual long-lasting reactions of thermal water with local soft rocks and specific bacterial microflora. The peloid base is a homogenized sediment being continuously precipitated in the Váh river by-pass branch on the site of the current hot springs of the sulphuric thermal mineral waters [1].

Peloids consist of humus and minerals formed over a very long period by physical, chemical, biologic, and geologic processes. They are used in treatment either in their original form or after fermentation. Peloid extracts have great reducing properties. The redox potential of humic substances corresponds approx. to that of ascorbic acid. In peloid extracts, with dichlorphenol-indophenol, ascorbic acid is destroyed four-fold more rapidly than in a control water solution of ascorbic acid. The trace elements found in peloids include boron, cobalt, copper, iodine, and manganese [2].

The Piešťany-spa curative waters are natural, weakly mineralized calcium-sodium, hypotonic sulphuric thermal waters. The water contains about 1,500 mg of mineral elements per liter of water, and is trapped at the depth of 60 m what gives the water its constant chemical composition. The water temperature of the springs at the outlet varies from 67 to 69 °C, and it is used for baths and drinking cures [1].

For the treatment of diseases of locomotive system, the most important component is sulphur, present in the high content (from 6 to 10 mg per liter), which occurs in the water in various forms and chemical compounds (sulphates, sulphites, sulphides, thiols/mercaptanes). High content of hydrogen sulphide is also important – on average 4.7 mg per liter of water. Positive curative effects were for centuries documented at the treatment of diseases/disorders such as chronic rheumatoid disorders, rheumatoid arthritis (RA), Bechterew's disease, as well as conditions following reactive arthritic disorders [1].

One of the important physical effects of thermal mineral waters is the hyperthermic reaction of the body. The nervous, cardiovascular, respiratory, endocrine, and immunity systems are involved in this reaction. Apart from physical effects, chemical effects of the sulphuric waters are important as well. Curative effect occurs likely *via* dermatic penetration of sulphids from the water into the skin structures. Sulphur inhibits degradation of elastine, collagen and hyaluronan. It reduces blood pressure, also through dilatation of the blood vessels.

The overall effect of the bath is a total relaxation and tranquilizing action. Temperature of the mineral sulphuric bath is 36–38 °C. It is usually applied for 20 minutes with a final dry wrap up to 15 min. As sulphur is capable to create deposits in the human skin, sulphuric substances may penetrate quite deep into the tissues. Sulphur exerts also keratolytic, keratoplastic, vasodilating, and antiseptic effects, which are used for treatment of certain skin disorders [1].

Hyaluronan (HA), a linear high-molar-mass glycosaminoglycan (GAG), is part of the extracellular matrix (ECM). It is the only GAG not attached to a core protein, and the only unsulphated one. This biopolymer consists of repeating disaccharide units (D-glucuronic acid

and *N*-acetyl-D-glucosamine) linked *via* alternating β-1,4 and β-1,3 glycosidic bonds. In the synovial fluid (SF) of healthy individuals, the HA molar mass can reach 6 to 8 MDa. The HA found in ECM can also reach large size of polymeric chains – between 10^5 and 10^7 Da [3]. These high-molar-mass HAs function as indicators of intact normal tissue, while fragmented forms of HA are usually mediators indicating oxidative stress.

Solutions of high-molar-mass HAs form highly-viscous networks within the tissue important for molecular exclusion, flow resistance, tissue osmosis, lubrication, and hydration. Importantly, at sites of inflammation, there is an accumulation of low-molar-sized HA fragments that are either synthesized *de novo* by HA synthases or generated by enzymatic cleavage of native high-molar-mass HAs by ECM-hyaluronidases and/or oxidative degradation mediated by ROS [4].

Indeed, various sized-HA fractions have been found to possess a number of various functions, and may represent an information system. HA influences proliferation, migration, and adhesion of cells *via* its interaction with the cell surface where it binds to the receptors such as trans-membrane glycoprotein – CD44. Increased evidence has been gathered that low-molar-mass HA fragments have different activities when compared to that of the native high-molar-mass biopolymer. Large matrix HA polymeric network exhibit anti-inflammatory, spacefilling, anti-angiogenic, and immunosuppressive properties. On the other hand, the intermediate-sized HA polymer fragments, comprising more than 100 saccharide units, are inflammatory, immunostimulatory, and highly angiogenic. They can induce apoptosis in cultured cells, and inflammation *in vivo*. The low-molar-mass oligosaccharidic fragments of about 25–50 disaccharide units are anti-inflammatory, and appear to function as endogenous stress signals. HA is also being widely used as a promising component of artificial matrices and in bioengineering for tissue scaffolding applied in regenerative medicine [5,6].

The HA turnover is rather fast. In the 70-kg human body comprising approx. 15 g of HA, it is about 5 g per day. The $t_{1/2}$ of HA in the circulation is between 2 and 5 min. In the epidermis of the skin, where ~ 50% of the extracellular HA is found, it is one to two days, whereas in an apparently inert tissue – the cartilage – it is approx. one to three weeks. On the other hand, the $t_{1/2}$ of HA is approx. 12 h in the SF [5,6].

Rheumatoid arthritis involves the synovial fluid HA degradation, which is accompanied with the loss of the viscoelastic properties of SF. Elevated HA concentrations in the blood of RA patients are usually observed. It has been shown that the serum levels of HA are also increased in a number of diseases, including scleroderma and some liver disorders. The quantitation of HA in tissues and body fluids is thus emerging as a useful adjunct to an effective diagnostics [7].

It was demonstrated that ROS are involved in cartilage degradation associated with inflammatory joint disease. Inflammation of the articular joints is accompanied by a decrease in the viscosity of SF, in which HA is the major macromolecule and imparts viscosity [8].

The aim of our study was to investigate the potential anti-oxidative efficacy of some sulphur compounds-containing water samples, namely the curative waters from healing peloid and maturation pool collected in Health Spa Piešťany. As a reference experiment – degradation of a high-molar-mass HA sample induced by ascorbate *plus* Cu(II) ions – was implemented throughout the studies.

Experimental Parts

Biopolymer: The high-molar-mass hyaluronan P0207-1 (M_w = 1.07 MDa; M_w/M_n = 1.60) was from Lifecore Biomedical Inc., Chaska, MN, U.S.A.

Chemicals: Analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were purchased from Slavus Ltd., Bratislava, Slovakia. L-ascorbic acid was the product of Merck KGaA, Darmstadt, 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.

Study of uninhibited HA degradation: The HA sample (2.5 mg/mL) was prepared in the dark at room temperature in two steps: first 4.0 mL of the solvent (0.15 M aqueous NaCl) was added and after 6 h the second portion of the solvent was added. After adding of the tested compound, a final volume reached 8 mL. The stock solutions of L-ascorbic acid (16 mM) and cupric chloride (16 mM diluted to a 160 μM solution) were prepared in 0.15 M aqueous NaCl. The final Cu(II) ion and ascorbate concentrations were 1 μM and 100 μM, respectively. Their mode of addition into the HA solution was already published [9–14].

Study of influence of curative water samples on HA degradation: Effects of the Piešťany-spa curative water samples from healing peloid and maturation pool on the oxidative HA degradation system, containing 1 μM cupric chloride *plus* 100 μM ascorbate as the probe reference, were investigated by adding 50 or 500 μL of the tested spa-water sample into the HA-Cu(II)-ascorbate system either before the onset of the reaction or 1 h after the initiation of the HA degradation.

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}.

Results and Discussion

Figure 1 illustrates changes of the dynamic viscosity of the HA solution due to pro-oxidative action of two reactants, namely cupric chloride and ascorbic acid. After a few minute-induction period, the viscous solution, having the initial value of η = 11.5 mPa·s, started to gradually decrease its viscosity. At the 5th hour, a final η equaled 4.65 mPa·s. As already proved, the decrease of the HA solution viscosity is a result of the degradation of high-molar-mass HA, whose initial M_w value equals 1.07 MDa. The M_w decreased up to about one tenth of the original molar mass given for the intact HA sample [14]. The decrease of the η value in basic experimental set is sufficiently high to be used as the probe reference. The reference curve (Figure 1) is depicted in all the other figures (Figures 2–4).

It can be expected that by lowering a concentration ratio of cupric chloride/ascorbate, e.g. applying higher concentrations of $CuCl_2$ solutions, the curve representing the loss of the HA

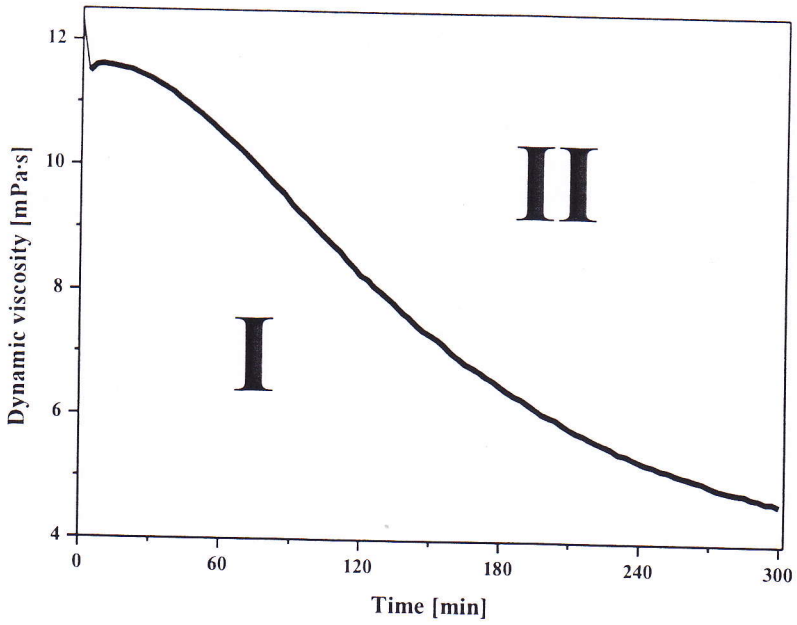

Figure 1. Uninhibited HA degradation by the action of the pro-oxidative system containing 1 μM cupric chloride *plus* 100 μM ascorbate.

solution viscosity (Figure 1), would turn to lower η values of the HA solutions than that of 4.65 mPa·s as given for our probe reference.

By the way, any pro-oxidative action of a substance added into the reference oxidative system would result in a more radical slope of the curves situated within the area I (Figure 1). Contrary to this, any anti-oxidative effect of a compound added into the same system would cause a retardation of the η drop. In the limiting situation providing that the high-molar-mass HA would be completely protected against any degradation, we can speak about total inhibitory action of the admixed anti-oxidative compound with resulting curves situated in the area II (Figure 1). The extent of the HA degradation or inhibition of the HA degradation is dose-dependent. Hence, the area I represents preferential action of the compounds acting in pro-oxidative mode, while the area II is represented by preferential action of the compounds acting in anti-oxidative mode. The task to be solved regarding classification of pro- and anti-oxidative properties – in our case – curative spa waters from healing peloid and maturation pool seems to be indeed marvelous.

Since the samples of curative spa waters contain really wide repertoire of native trace elements and substances, the experiments performed should be classified as a "black box" study. If the anti-oxidative effects of the tested spa-waters prevailed over those of the pro-oxidative ones, a considerable retardation or even inhibition of the HA degradation should be observed. The pro-oxidative effect of the system, containing 1 μM cupric chloride *plus* 100 μM ascorbate as the probe reference, on the HA degradation is illustrated in Figure 2, curve coded A. The results (Figure 2) indicated that the tested curative spa water sample from the healing peloid added into the system comprising of hyaluronan, Cu(II) ions and ascorbate, even in a small volume of 50 μL, acted protectively (Figure 2, curve coded B).

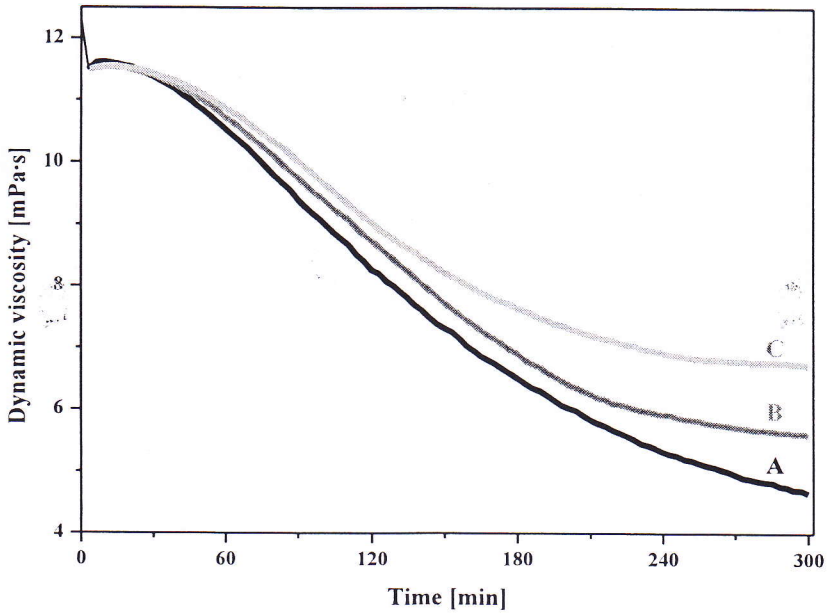

Figure 2. Effect of the curative spa water sample from the healing peloid on the high-molar-mass hyaluronan degradation induced by 1 μM cupric chloride *plus* 100 μM ascorbate. Volumes of the spa-water, added into the system before the onset of the HA degradation, in μL: B – 50; C – 500. Reference experiment: A – nil addition of the spa-water.

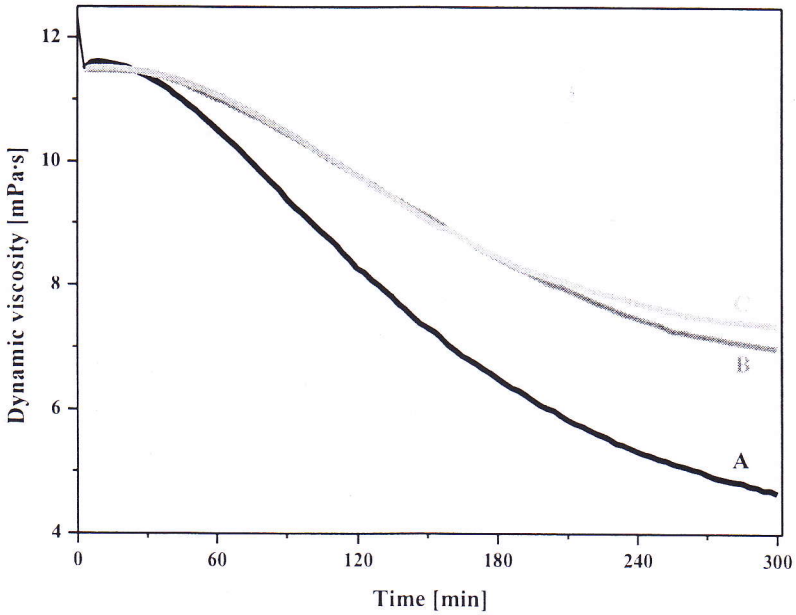

Figure 3. Effect of the curative spa water sample from the maturation pool on the high-molar-mass hyaluronan degradation induced by 1 μM cupric chloride *plus* 100 μM ascorbate. Volumes of the spa-water, added into the system before the onset of the HA degradation, in μL: B – 50; C – 500. Reference experiment: A – nil addition of the spa-water.

The greater the volume of the spa-water the greater was the anti-oxidative effect (Figure 2, curve coded C). In this special case, we interpreted the effect of the curative spa water from the healing peloid as that of preventive antioxidant. Practically identical results were obtained (Figure 3) documenting a remarkable anti-oxidative effect of the curative spa water from the maturation pool, whose effect was less profoundly dose-dependent. Both volumes (50 and 500 μL) of the spa-water samples (Figure 3; curves coded B and C), applied in the system, resulted in similar protection against the HA degradation as stated in the case of the curative spa water from the healing peloid.

The experimental sets represented in Figures 2 and 3 are suitable for testing preventive anti-oxidative effects of the curative spa waters from the healing peloid and the maturation pool in the initiation phase of the HA degradation. Figure 4 shows how the curative spa waters were tested in the already-running HA degradation. During the 1-hour propagation phase, HA macromolecules are degraded *via* free-radical chain-breaking processes. This phase can thus be classified as one of the most destructive. The observed retardation of the η value drop as a result of the anti-oxidative effect of the curative spa water samples, is indeed a remarkable finding. Although the total protective effect of both tested samples (Figure 4) may be less evident, one should take into account that in clinical practice, the patients are treated by multiple curative procedures. That means the sum of all the anti-oxidative effects are undoubtedly significant for their recovery from locomotive system disorders.

In conclusion, our findings, though indirectly, proved that the Health Spa Piešťany curative waters from the healing peloid and the maturation pool acted not only as preventive antioxidants but also as chain-breaking antioxidants with a potential of healing action in the ongoing pathology.

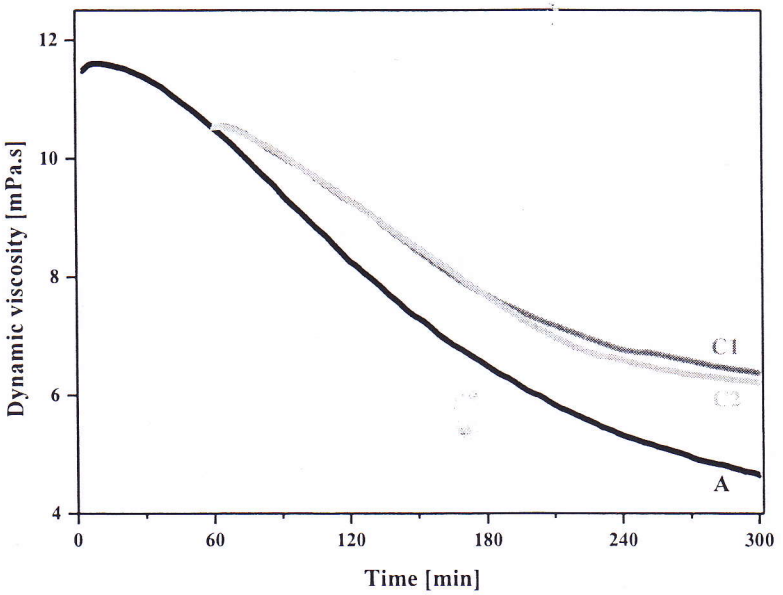

Figure 4. Effects of the curative spa-water sample from the healing peloid (C1) and the maturation pool (C2) on the high-molar-mass hyaluronan degradation induced by 1μM cupric chloride *plus* 100 μM ascorbate. Volumes of the spa-waters added into the system 1 h after the initiation of the HA degradation, in μL: C1 and C2 – 500. Reference experiment: A – nil addition of the spa-water.

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