An Alternative Standard for *Trolox*-Equivalent Antioxidant-Capacity Estimation Based on Thiol Antioxidants. Comparative 2,2′-Azinobis[3-ethylbenzothiazoline-6-sulfonic Acid] Decolorization and Rotational Viscometry Study Regarding Hyaluronan Degradation

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Comparison of the effectiveness of antioxidant activity of three thiol compounds, D-penicillamine, reduced L-glutathione, and 1,4-dithioerythritol, expressed as a radical-scavenging capacity based on the two independent methods, namely a decolorization 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] assay and a rotational viscometry, is reported. Particular concern was focused on the testing of potential free-radical scavenging effects of thiols against hyaluronan degradation, induced by hydroxyl radicals. A promising, solvent-independent, antioxidative function of 1,4-dithioerythritol, comparable to that of a standard compound, *Trolox*®, was confirmed by the 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] assay. The new potential antioxidant 1,4-dithioerythritol exhibited very good solubility in a variety of solvents (*e.g.*, H_2O, EtOH, and DMSO) and could be widely accepted and used as an effective antioxidant standard instead of a routinely used *Trolox*® on 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] assay.

Introduction. – Reactive free-radical species (neutral radicals, radical anions, and radical cations), due to their various redox reactions [1], play, in general, a key role in intra- and intercellular signaling preventing biomolecular systems (cells, tissues, organs) from great many of stress factors. Nevertheless, owing to the disruption of homeostasis by increased oxidative stress induced by catalytic actions of transition metal ions, their function can promote initiation of the pathways leading to undesirable oxidation of important biomacromolecules [2].

Hyaluronic acid/hyaluronan (HA; *Fig. 1*) is a unique biocompatible polysaccharide among glycosaminoglycans (1–10% of the cartilage) with a repeating disaccharide unit, *i.e.*, D-glucuronate and *N*-acetyl-D-glucosamine, containing no sulfate in structure. Being a high-molar-mass polymer (up to 10 Mda), it is designated to function, especially, in synovial fluid, at the concentration range of 2–4 mg/ml, as a highly viscoelastic lubricating agent. Due to joint inflammation-related diseases, initiated by a deleterious action of free oxygen- and nitrogen-derived radical species, the rheological properties of the biopolymer are unfavorably affected.

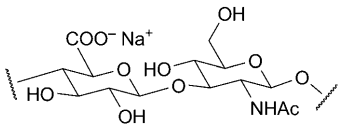

Fig. 1. *Disaccharide unit of the sodium salt of hyaluronic acid*

Several thiol compounds have attracted much attention from pharmacologists because of their reactivity toward endobiotics such as hydroxyl radical-derived species: alkyl, alkoxyl, and hydroperoxyl radicals [3][4], protecting thus important biomacromolecules (DNA, enzymes, proteins) against undesirable oxidation of their SH groups [5]. One representative of the thiols used in this study is a well-known drug, D-penicillamine (D-pen; *Fig. 2*,*a*). It exhibits a dual behavior producing and scavenging H_2O_2. This fact might partially explain its pro- and antioxidative properties observed *in vitro* [6][7]. Reduced L-glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH; *Fig. 2*,*b*) is the most ubiquitous intracellular low-molar-mass non-protein endobiotic thiol compound, found in either millimolar concentrations, *i.e.*, intracellularly (2–8 mM), or in micromolar concentrations, *i.e.*, extracellularly (5–15 μM), respectively [8]. A redox potential of the GSH/GSSG (oxidized glutathione) couple is −0.24 V [9]. GSH, at the concentrations over 10 μM, demonstrated a total inhibition of the degradation of HA macromolecules *in vitro*, whereas D-pen indicated different chemistry when tested in the function to scavenge hydroxyl radical-derived species [4]. An aqueous solution (12 μM) of GSH was reported as not effective in the suppression of the 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical cation ($ABTS^{\bullet+}$) absorbance at 734 nm within 4 min [10]. 2,2′-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) decolorization assay, though limited, is one of the many fast and convenient tests that give reliable results provided that compounds tested are well H_2O-soluble [10][11].

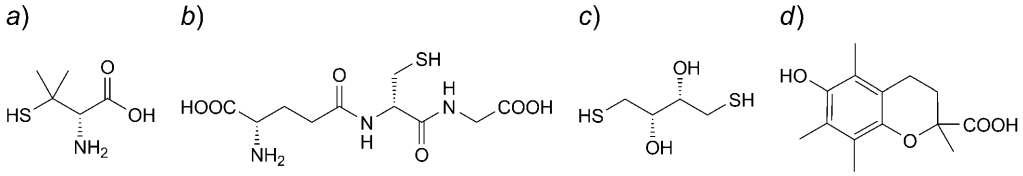

Fig. 2. *Antioxidants used on 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] decolorization study.* pK_a Value of the SH group for *a*) D-penicillamine: −7.90 [7]; *b*) reduced L-glutathione: −8.75 [7]/8.83 [12]/9.20 [13]; *c*) 1,4-dithioerythritol: −9.0 (pK_{a1}) and 9.90 (pK_{a2}) [14]; *d*) *Trolox*®.

Comparison of the effectiveness of antioxidant activity of three thiol compounds such as D-pen, GSH, and 1,4-dithioerythritol (DTE; *Fig. 2*,*c*) expressed as the radical-scavenging capacity based on the two independent methods, namely the decolorization ABTS assay and a rotational viscometry, is reported. Particular emphasis was focused on the testing of potential free-radical-scavenging effects of thiols against hyaluronan degradation, induced by hydroxyl radicals. DTE, widely accepted and used as an effective antioxidant in the field of enzyme and protein oxidation, is a new potential antioxidant standard exhibiting very good solubility in a variety of solvents.

Results and Discussion. – A spectrophotometric ABTS decolorization assay, based on the reduction of $ABTS^{\bullet+}$ to the parent ABTS (*Fig. 3*) is applicable to both hydrophilic and lipophilic putative antioxidant compounds. This assay is generally used for the screening of scavenging efficiency of various antioxidants such as flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants [10]. $ABTS^{\bullet+}$ (see inset in *Fig. 3*) exhibits a bluish-green color with maximum absorbance values at 645, 734, and 815 nm [10], which rapidly decreased after addition of investigated thiol compound as shown in *Figs. 3*, *6*, and *7*. GSH, D-pen, and DTE were found to display similar $ABTS^{\bullet+}$ scavenging effectiveness. DTE (aq. soln.) exhibited efficient suppression of the $ABTS^{\bullet+}$ absorbance within 1 min (*Fig. 3*); the absorption spectra were identical within 1, 5, or 10 min, respectively. As evidenced from the spectral data (*Fig. 4*), DTE has proven to be a promising antioxidative, highly effective standard (total oxidant scavenging capacity (TOSC) 65% within 1 min; *Trolox*-equivalent antioxidant capacity (TEAC) 0.9 mmol *Trolox*/l; *Table*), comparable with that of *Trolox*® (TOSC 70% within 1 min; TEAC 1.0 mmol *Trolox*/l; *Table*). TEAC Parameter was calculated from the standard calibration plot (*Fig. 5*). Independently on solvent used (*Fig. 4* and *Table*), there was almost no difference between the scavenging effectiveness of $ABTS^{\bullet+}$ by this thiol group-based species within 10-min interval.

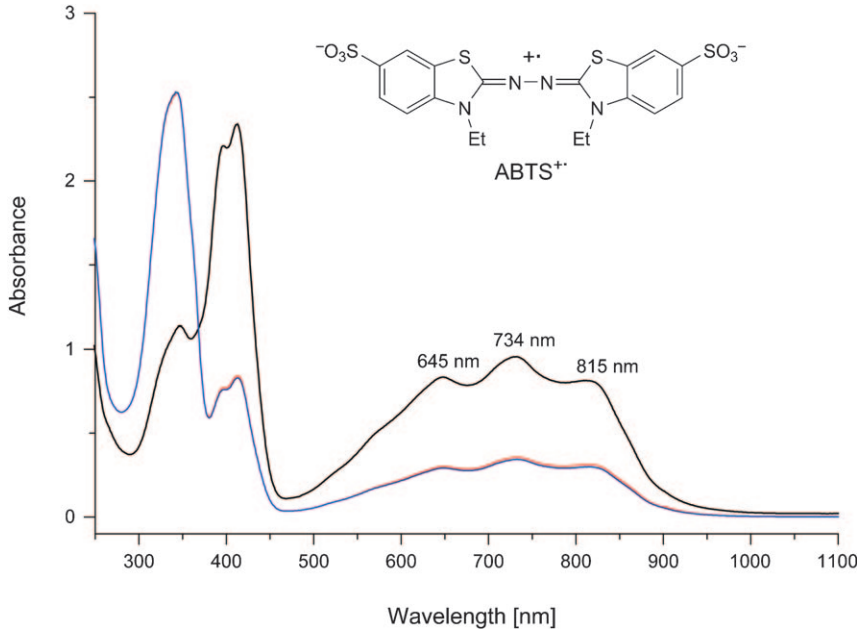

Fig. 3. *Representative 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] decolorization test of antioxidant-efficiency of 1,4-dithioerythritol.* Reaction conditions: 50 µl of 1 mM aq. soln. of the 1,4-dithioerythritol sample added to 2 ml of an aqueous solution of 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical cation; 1-cm quartz cuvette, ambient temperature. Time interval tested within 1 min (red line), 5 min (green line), or 10 min (blue line), respectively; reference experiment (black line): aqueous solution of 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical cation.

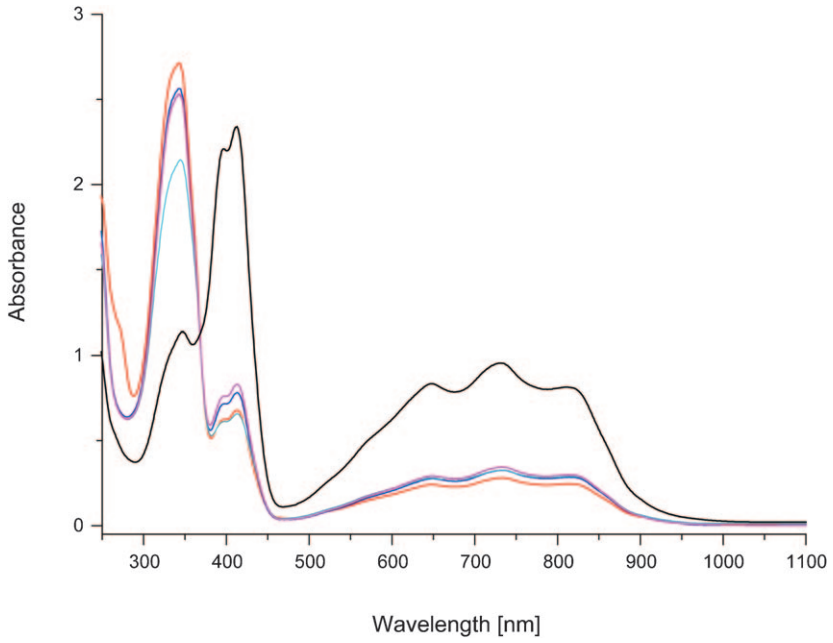

Fig. 4. *Comparative 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] test for* Trolox-*equivalent antioxidant-capacity* ([mmol *Trolox*/l]) *determination of a tested thiol compound related to the standard compound* Trolox® *within 10-min interval.* Reaction conditions as given for *Fig. 3. Trolox*® (wine line: in EtOH/H_2O 1:1); 1,4-dithioerythritol (violet line: in H_2O; turquoise line: in EtOH; blue line: in DMSO); reference experiment: aqueous solution of 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical cation (black line).

Table. *Comparison of Scavenging Efficiency of Thiol Compounds with the Standard Compound* Trolox® *within 10-min Interval*

Sample	ΔA [a])	TOSC [b]) [%]	TEAC [b]) [mmol *Trolox*/l]
Reference sample (H_2O)	0.02	2	0.01
DTE [b]) (H_2O)	0.61	64	0.90
DTE (EtOH)	0.62	65	0.90
DTE (DMSO)	0.62	65	0.90
Trolox® (EtOH/H_2O 1:1)	0.67	70	1.00
D-Pen [b]) (H_2O)	0.64	67	0.94 (1.03) [c])
GSH [b]) (H_2O)	0.79	83	1.18 (1.45) [c])

[a]) ΔA*:* Difference between the absorbance of the control 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical cation, and *Trolox*® or the tested thiol sample, respectively. [b]) Abbreviations: TOSC: total oxidant scavenging capacity; TEAC: *Trolox*-equivalent antioxidant capacity; DTE: 1,4-dithioerythritol; D-Pen: D-penicillamine; GSH: reduced L-glutathione. [c]) Within 35-min Interval.

As known from the literature, both thiol isomers, 1,4-dithioerythritol (*cis*-configuration) and 1,4-dithiothreitol (DTT; *trans*-configuration; known as *Cleland*'s redox reagent) are unusually powerful reducing agents used to protect biological probes

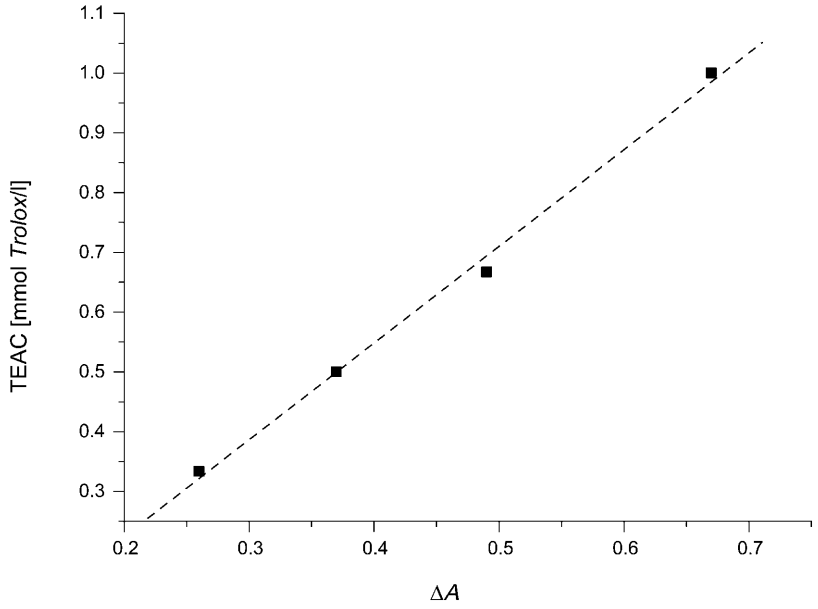

Fig. 5. *Standard calibration plot, based on* Trolox®, *applied for the experimental* Trolox-*equivalent antioxidant-capacity determination of various thiol compounds*

(mostly proteins/enzymes) from undesirable oxidation. DTE exhibits, however, slightly less effective reductive behavior than the other one, as a consequence of presumably, steric repulsion of its OH groups rendering the cyclic disulfide formation less favorable. However, DTE exhibits better storage stability than that of DTT [15].

Antioxidant properties of studied thiols were compared with that of the *Trolox*® standard compound (*Fig. 2*, *d*), *i.e.*, the vitamin E-derived chroman compound with a COOH group at C(2) *in lieu* of a phytyl chain ($C_{16}H_{33}$) of its precursor exhibiting more hydrophilic behavior. The antioxidant properties of *Trolox*®, commonly used on ABTS tests, are ascribed to the ability of OH group to interact with free-radical species [16]. A total oxidant-scavenging capacity or *Trolox*®-equivalent antioxidant capacity assay is generally used for a quantitative comparison of the scavenging efficiency of antioxidants toward various oxidants (hydroxyl or peroxyl radicals, peroxynitrite, *etc.*) [3]. Owing to the solubility in polar solvents, *Trolox*® was efficient in electrochemical determination of amino acids, using a voltammetric method [16]. Routinely used as a traditional standard compound on ABTS assays, *Trolox*® function is highly influenced by its very limited solubility in H_2O (0.5 mg/ml), the fact which is frequently overlooked [17]. On the other hand, *Trolox*® is highly soluble in EtOH (160 mg/ml) [17], and in DMSO or DMF (20 mg/ml) [18], respectively. *Trolox*® is usually, prior to experiment, dissolved in DMSO improving thus its solubility in H_2O or aqueous buffer solutions [19]. As a result, ambiguous results varying from laboratory to laboratory might be obtained, because the solvation effect and traces of residual organic solvent may interfere with the accuracy of the ABTS method. Summary of the advantages and disadvantages of the ABTS assay application was reported in [20].

Our results (*Table*) show that GSH is a much slower, however, more effective ABTS$^{\bullet+}$ scavenger (see inset in *Fig. 6*) than that of D-pen (see inset in *Fig. 7*). TEAC determined for GSH within 35 min (1.45 mmol *Trolox*/l) is substantially higher in comparison to D-pen within the same time (1.03 mmol *Trolox*/l). GSH is a bulky tripeptidic molecule whose mobility in aqueous solution, due to the phenomenon of hydration, is more suppressed. In addition, no remarkable effect was observed on D-pen testing within a prolonged time of 210 min (*Fig. 7*).

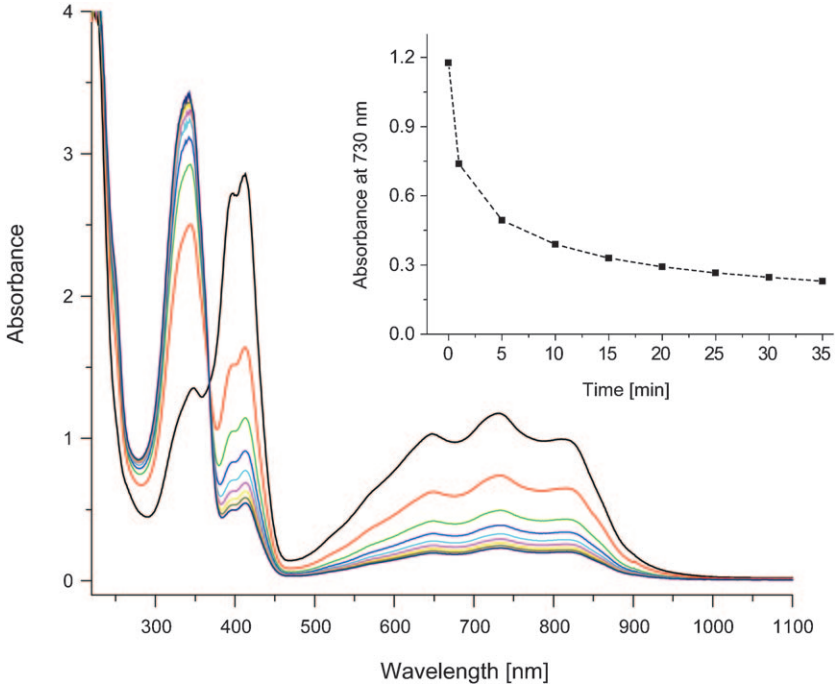

Fig. 6. *2,2′-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical-cation scavenging by reduced* L-*glutathione* (aq. soln). Reaction conditions as given for *Fig. 3.* Time interval tested in min and the spectrum color given in the parentheses: 1 (red), 5 (green), 10 (blue), 15 (turquoise), 20 (violet), 25 (yellow), 30 (olive), 35 (dark blue); reference experiment: aqueous solution of 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical cation (black line).

The study applying a rotational viscometry was focused on the *in vitro* generation of degradative conditions in a hyaluronan solution by the *Weissberger*'s oxidative system (ascorbate *plus* Cu^{II} ions) [21]. Antioxidant efficiency of the thiol compounds, namely, D-pen, GSH, and DTE, was tested by the above mentioned system. This standardized method is, in general, used to mimic the pathophysiological situation, which may occur at the early stage of acute joint inflammation. The chief advantage of rotational viscometry is the possibility to continuously carry out measurements at a given shear rate over extended periods of time.

On application of *Weissberger*'s system, the hyaluronan degradation was evidenced by the gradual decline of its dynamic viscosity. Addition of the thiol to the system before the onset of the reaction resulted in a time-dependent inhibition of hyaluronan

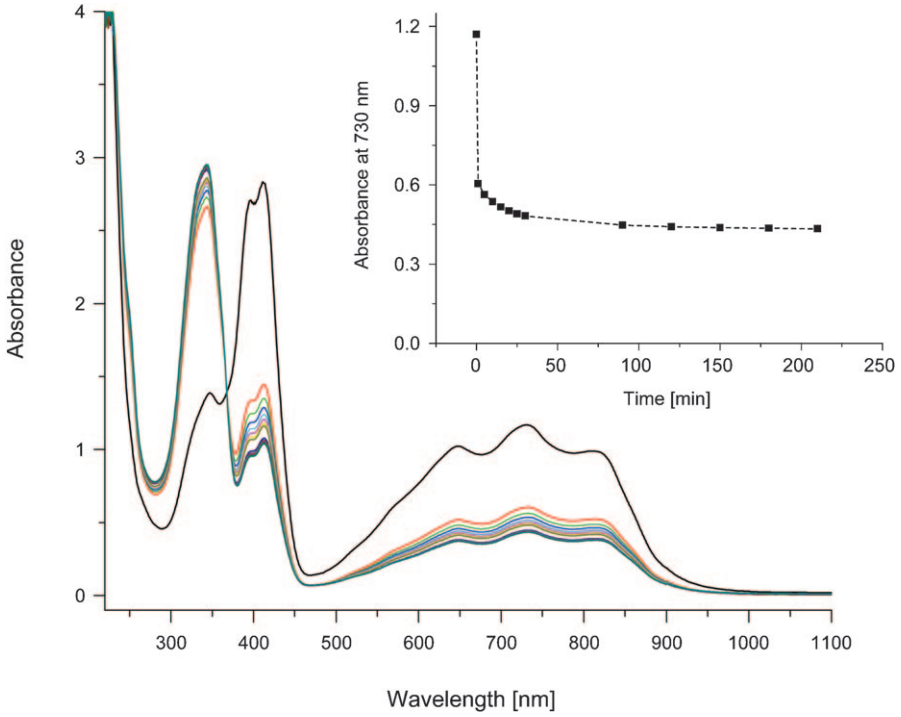

Fig. 7. *2,2′-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical-cation scavenging by* D-*penicillamine* (aq. soln). Reaction conditions as given for *Fig. 3.* Time interval tested in min and the spectrum color given in the parentheses: 1 (red), 5 (green), 10 (blue), 15 (turquoise), 20 (violet), 25 (yellow), 30 (olive), 90 (dark blue), 120 (dark violet), 150 (brown), 180 (dark green), 210 (dark turquoise); reference experiment: aqueous solution of 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] radical cation (black line).

degradation (*Fig. 8*). Interestingly, on application of thiol compounds, it was shown that rotational viscometry output (*Fig. 8*) confirms the results obtained by the ABTS decolorization assay (*Figs. 3*, *6*, and *7*) within an initial phase of hyaluronan degradation that is 1 h. An expected antioxidative effect of the thiol compound tested *via* rotational viscometry is, however, more evident than in the case of the ABTS method. The entire time of radical scavenging, using the ABTS assay, is usually prolonged due to a strong effect of hydration caused by H-bonds.

On the basis of our results, due to an excellent solubility of DTE in H_2O and its antioxidant ability to protect thiol group-based biomacromolecules against undesirable oxidative effects, we may recommend as a promising scavenger DTE/DTT couple that should be favorably chosen as a novel standard compound on the ABTS decolorization assays for testing other thiol compounds.

It should be noted that effectiveness of various endogenous thiols, which contribute to the intracellular antioxidant defense system, such as GSH, cysteine, homocysteine, cysteinyl-glycine, and γ-glutamyl-cysteine, tested to scavenge $ABTS^{\bullet+}$ radical cation, is strongly dependent on the pK_a value of their SH group. The higher pK_a value at pH 7.4,

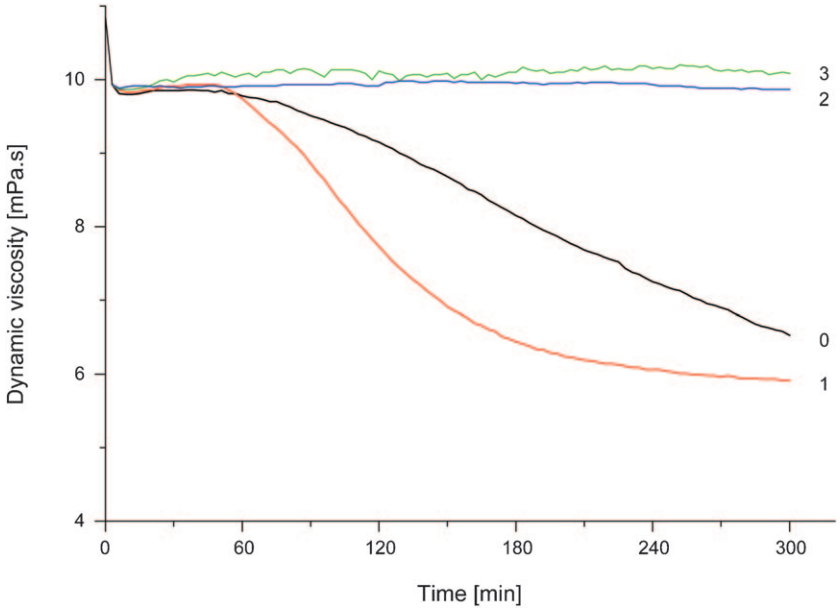

Fig. 8. *Comparison of the antioxidant efficiency of* D-*penicillamine* (*1*), *1,4-dithioerythritol* (*2*), *and reduced* L-*glutathione* (*3*) *on the degradation of hyaluronan sample* (P9710-2A; 2.5 mg/ml) *induced by the system containing 1.0* μM $CuCl_2$ *plus 100* μM *ascorbate using a rotational viscometry.* The concentration of the thiol compounds, added before the reaction onset, in μM: 100. Reference experiment (*0*): – nil thiol concentration.

the higher tendency for the SH group to exist in a protonized form, that means the H-atom transfer, in this context, could be decisive. As assumed, the length of the aliphatic chain might also play a role [22]. Redox potentials of thiol/disulfide pairs, strictly ruled by temperature, pH, and ionic strength of solution, also influence the $ABTS^{\bullet+}$-scavenging effectiveness.

Conclusions. – Antioxidant properties of a series of thiols were compared with that of the *Trolox*® standard. We found that 1,4-dithioerythritol is a unique, nontoxic, and easy-to-handle radical scavenger which can be favorably chosen as a novel standard in the ABTS decolorization assay. Scavenging efficiency of DTE is comparable with that of *Trolox*® (0.9 mmol *Trolox*/l), and DTE can be applied in a variety of solvents. Particular emphasis was focused on the testing of potential free-radical-scavenging effects of thiols against hyaluronan degradation induced by hydroxyl radicals formed using *Weissberger*'s system. The hyaluronan degradation was evidenced by the gradual decline of its dynamic viscosity. Addition of the thiol to the system before the onset of the reaction resulted in a time-dependent inhibition of hyaluronan degradation. It was shown that rotational viscometry output confirms the results obtained by the ABTS decolorization assay within the initial phase of degradation of hyaluronan macromolecules.

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Experimental Part

Biopolymer and Chemicals. The high-molar-mass hyaluronan sample *Lifecore P9710-2A*, kindly donated by *Lifecore Biomedical Inc.*, Chaska, MN, USA (M_r 808.7 kDa; $M_r/M_n = 1.63$), was used on experiments. The anal. purity grade NaCl and $CuCl_2 \cdot 2\,H_2O$ (*Slavus Ltd.*, SK-Bratislava); D-pen and GSH (*Sigma-Aldrich Chemie GmbH*, D-Steinheim); DTE (99%; *Aldrich-Europe*, B-Beerse); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (*Trolox*®; 97%; *Aldrich-Chemie*, D-Steinheim); L-ascorbic acid and $K_2S_2O_8$ (*p.a.* purity, max 0.001% nitrogen; *Merck*, Germany); 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS; *purum*, >99%; *Fluka*, Germany) were used. Deionized high-purity grade H_2O, with conductivity of ≤ 0.055 μS/cm, was produced by using the TKA H_2O purification system (*Water Purification Systems GmbH*, D-Niederelbert). EtOH for UV/VIS spectroscopy was purchased from *Microchem*, SK-Pezinok. DMSO (0.05% of H_2O content) was purchased from *Merck*, Germany.

Procedures. For the $ABTS^{\bullet+}$ decolorization assay, the radical cations were pre-formed by the reaction of an aq. soln. of $K_2S_2O_8$ (3.30 mg) in H_2O (5 ml) with ABTS (17.2 mg). The resulting bluish-green radical cation soln. was stored overnight in the dark below 0°. Before experiment, the soln. (1 ml) was diluted into a final volume (60 ml) with H_2O. The thiol compound of interest (1.0 mM) was prepared as an aq. stock soln. except for DTE (1.0 mM), which was prepared in H_2O, EtOH, or DMSO, resp. The stock soln. of *Trolox*® (1.0 mM) was prepared in H_2O/EtOH soln. (50%). A modified ABTS assay [11] was used to test the radical-scavenging efficiency applying a UV/VIS *S2000* spectrophotometer (*Sentronic*, Germany). The UV/VIS spectra were recorded in 1-min intervals from 1 to 210 min, depending on compound used (*Figs. 3*, *4*, *6*, and *7*), in 1-cm quartz UV cuvette after mixing of aq./non-aq. soln. of antioxidant sample (50 μl) with an $ABTS^{\bullet+}$ soln. (2 ml).

For the rotational viscometry measurements, a hyaluronan soln. (2.5 mg/ml) was prepared in the dark, standing at r.t. in an aq. NaCl soln. (0.15 M) in two steps: first, the solvent (4.0 ml) was added to a hyaluronan powder (20 mg), and, within 6 h, the same solvent (3.85 ml) was added. The stock solns. (16 mM) of ascorbic acid, D-pen, GSH, and DTE, as well as the soln. of $CuCl_2$ (160 μM) were dissolved in the same solvent. Before starting to monitor the kinetics of the hyaluronan degradation *via* rotational viscometry, the mixture (8.0 ml), consisting of the biopolymer soln. of the concentration as stated above, ascorbic acid (100 μM), thiol compound (100 μM), and Cu^{II} ions (1.0 μM), was transferred into the *Teflon*® cup reservoir of the *Brookfield LVDV-II+PRO* digital rotational viscometer (*Brookfield Engineering Labs., Inc.*, Middleboro, MA, USA). Data acquisition of the viscometer output parameters was performed by recording within 2 min after the onset of the experiment. Time-dependent changes of the dynamic viscosity values of the system were measured at 25.0 ± 0.1° within 3-min intervals for up to 5 h. The viscometer *Teflon*® spindle rotation rate was 180 rpm, *i.e.*, at the shear rate equaling 237.6 s^{-1} [23][12].

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