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EXPERIMENTAL ANTIOXIDANT ACTIVITY OF A β-CYCLODEXTRIN – HISTOCHROME COMPLEX

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A 2:1 β-cyclodextrin – histochrome complex was synthesized based on a comparison with experimental studies of histochrome and showed antioxidant activity prolonged to 16 d as compared with 6 d for histochrome in tests of the duration of the *in vitro* antioxidant effect. PMR measurements proved that a β-cyclodextrin – histochrome complex formed. Computer modeling of the β-cyclodextrin–histochrome complex showed that the head-to-tail complex corresponded to the energy minimum.

Keywords: histochrome, emoxypine, β-cyclodextrin, antioxidant activity.

The human antioxidant (AO) system plays an important role in neutralizing active species of oxygen, NO, and several other radicals to maintain homeostasis [1]. The last under normal conditions are considered intra- and intercellular signal messengers although they become destructive during pathologies arising in response to internal and external signals [2 – 4].

The human ocular apparatus comprises both enzymatic and non-enzymatic AOs situated in tissue structures and liquid/semi-liquid ocular media, respectively. Thus, superoxide dismutase, glutathione peroxidase, and catalase are found mainly in conjunctiva, cornea, sclera, vascular membrane, lens, retina, and optic nerve. However, ascorbate, urate, L-glutathione, L-cysteine, L-tyrosine, and α-tocopherol prevail in lacrimal fluid and aqueous and vitreous humors [5 – 15]. The aforementioned endogenous AOs maintain homeostasis that is disrupted during pathological conditions because of exhaustion on the cellular level. Homeostasis is normalized using exogenous AOs, the most widely employed of which are histochrome (**I**) and emoxypine (**II**) (Fig. 1). These drugs are used in subconjunctival, parabulbar, retrobulbar, and intramuscular injections. However, their effects are fleeting. Therefore, AOs with modified structures of previously approved drugs should be sought.

The goals of the present work were to synthesize an inclusion complex of β-cyclodextrin and **I** and to study its AO activity as compared with **I**.

EXPERIMENTAL PART

β-Cyclodextrin (β-CD, Sigma-Aldrich, Germany) and medicinal preparations of histochrome (0.02 and 1%, amyl form, Pacific Institute of Bioorganic Chemistry, FEB, RAS, Russia) and emoxypine (1%, amyl form, Moscow Endocrine Plant, Russia) were used in the work.

The complex β-CD–**I** was synthesized by mixing solutions of the starting materials for 2 h at 37°C in dark vials. Solutions of β-CD–**I** were prepared in 1:1 and 2:1 ratios with histochrome molar concentrations 1 mM and 0.5 mM. Solutions were shaken in a 1TZh-0-03 water thermostat (PO Medlabtekhnika, USSR, RF Pat. No. 2,530,886).

AO activity of **I** and **II** was assessed in the range from the initial ampul concentration to a 1/10 dilution of it. The solvent was NaCl solution (0.9%) in distilled H_2O.

AO activity was determined from the effect on free-radical oxidation (FRO) processes using test kits (NX2332 and NX2331, Randox Laboratories, Ltd., Great Britain). The test

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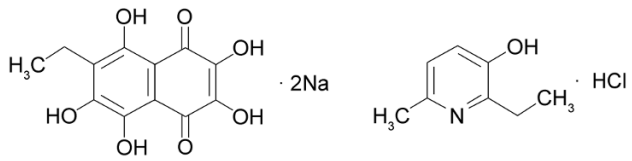

Histochrome (**I**) Emoxypine (**II**)

Fig. 1. Antioxidants for comparative analysis.

was based on spectrophotometric analysis of the AO activity of the studied compounds using the highly active radical cation 2,2'-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonate) as the oxidant and trolox (water-soluble vitamin E derivative) as the control. These were used at the specified concentrations of 0.61 and 2.23 mM, respectively.

Spectrophotometric analysis was performed on an SF-26 spectrophotometer (Lomo, USSR) at 600 nm.

AO activity was also determined from the effect on lipid peroxidation (LPO) processes. The model system was a suspension of egg-yolk lipoproteins, oxidation of which was activated by adding ferric sulfate. AO activity was assessed from the degree of chemiluminescence (CL) suppression, i.e., emission due to reactions of free radicals [16]. CL was recorded using an CL-003 chemiluminometer (Bashkir State Medical University, MH RF) for 5 min, i.e., the optimal period for assessing LPO activity. The control was distilled H_2O [16].

Acidity of solutions was measured using a pH-150M pH-meter (Gomel Measuring Instrument Plant, Republic of Belarus).

NMR studies were performed at the Agidel CCU at the Institute of Petrochemistry and Catalysis, RAS, using an Avance-400 spectrometer (Bruker, Germany). Spectral data were analyzed using the TopSpin 3.1 program (Bruker, Germany, 2011). Computer modeling used the HyperChem 8.0.8 program (Hypercube, Inc., USA, 2009).

RESULTS AND DISCUSSION

The AO activities of **I** (0.02%) and **II** (1%) were compared in the first stage. Test kits (Randox) were used for the analyses. The results were as follows. Compound **I** at a concentration of 0.02% had the highest AO activity (109.84 ± 2.42)%. The AO activity of **I** decreased by 65.77% ($p < 0.001$) if it was diluted 10 times in normal saline. Compound **II** at 1% had the second highest AO activity (44.54%) that decreased only 27.75% ($p < 0.002$) after a 10-fold dilution (Fig. 2).

An experiment with the LPO model was performed to confirm these results. The AOs were studied at the same doses. It was shown that the control (distilled H_2O) was inactive (100 ± 2.83)% whereas the AO activities of **I** at concentrations of 0.02% and 0.002% were (1.57 ± 0.87)% and

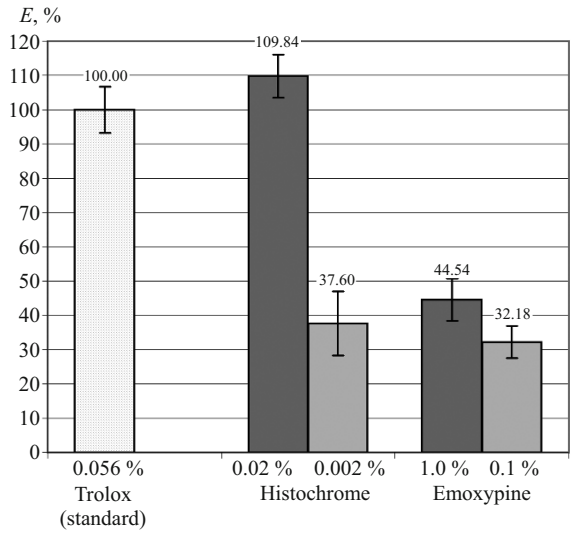

Fig. 2. Comparison of drug AO activities in the FRO model using a Randox kit.

(59.90 ± 1.33)%; **II**, 1.0% and 0.1%, (14.44 ± 0.42)% and (36.54 ± 0.94)%, respectively (Fig. 3).

Like in the preceding model, FRO by **I** (0.02%) was statistically significantly greater than that by **II** (1%) ($p < 0.05$) according to AO activity. The same tendency was observed if the AO concentration was decreased 10-fold. The activity of **I** (0.002%) decreased by 58.33% ($p < 0.002$); **II** (0.1%), only by 22.10% ($p < 0.05$) as compared with the control (Fig. 3). The similar test results for AO activity in the FRO and LPO models enabled further studies to use a chemiluminometer.

Compound **I** (0.02%) had high AO activity in comparison with **II** because of the five OH groups in the molecule. However, the above tests showed that the AO activity of **I** decreased significantly if the concentration was lowered. This necessitated a search for a method to preserve it.

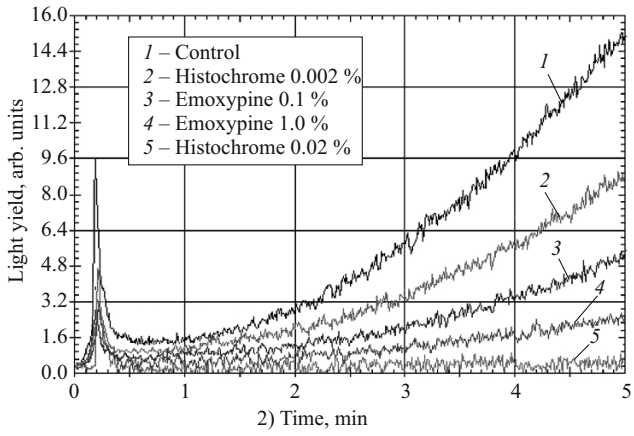

Fig. 3. Comparison of drug AO activities in the LPO model.

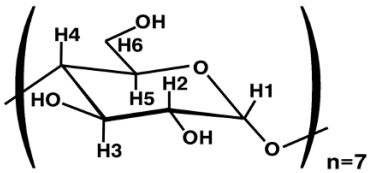

Fig. 4. Glucopyranose unit of β-CD with PMR proton numbering [17].

Compound **I** was modified by creating a complex with β-CD in order to improve its oxidation stability and prolong its AO activity. β-CD is a toroidal molecule consisting of seven glucopyranoses (Fig. 4) in which the outer side is hydrophilic; the inner side, hydrophobic [4]. This allows inclusion complexes (clathrates) to form.

First, experiments were performed to evaluate the effect of the β-CD–**I** (β-CD–H) mole ratio on retention of AO activity. For this, β-CD–H complexes in 1:1 and 2:1 ratios with equimolar concentrations of **I** (1 mM) were synthesized. The AO activity was estimated in the LPO model three days after the β-CD–H complexes were synthesized.

The experimental results showed that the AO activity of the 1:1 β-CD–H complex dropped by (53.34 ± 6.57)% ($p < 0.05$); that of the 2:1 complex, by only (24.12 ± 3.78)% ($p < 0.05$). This allowed a β-CD–H component ratio of 2:1 to be selected for subsequent experiments.

The next step was to compare the AO activities of β-CD–H (2:1) and β-CD itself. The LPO activity was analyzed 24 h after preparing the compounds. It was found that, like distilled H_2O, β-CD was inert to LPO at (100.00 ± 0.11)% and (102.58 ± 1.38)% ($p > 0.1$), respectively. However, the β-CD–H complex was highly active and reduced LPO to (14.33 ± 0.82)% ($p < 0.001$).

The effects of the synthesis temperature regime and β-CD–H component concentrations on the effectiveness of

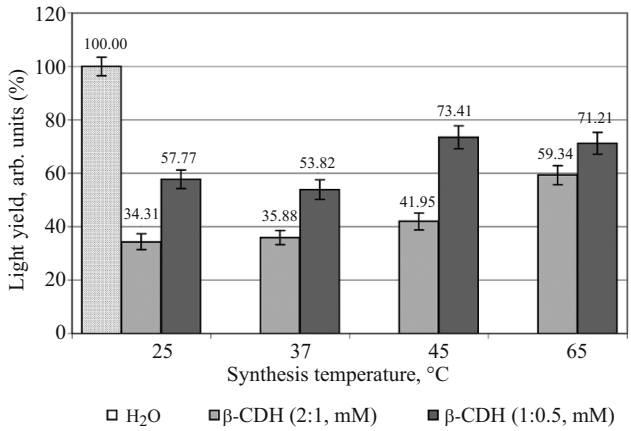

Fig. 5. AO activities in the LPO model of β-CD–H complexes synthesized at various temperatures after storage for 8 d.

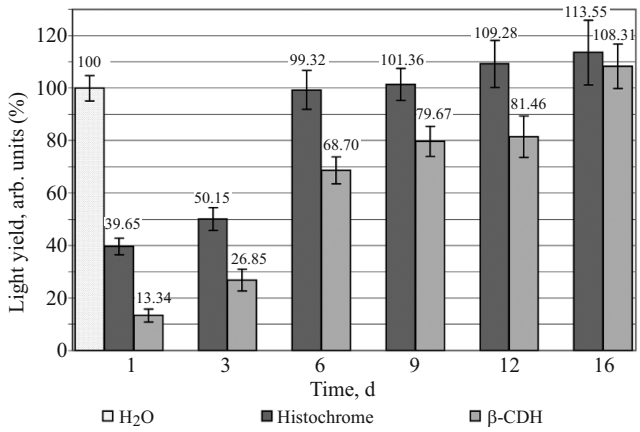

Fig. 6. Dynamics of AO activity of histochrome and β-CD–H complex (2:1) in the LPO model after storage for 16 d.

retaining AO activity were also evaluated. Experiments used solutions of β-CD–H synthesized at 25, 37, 45, and 65°C. The AO activity of solutions stored in dark vials at 25°C was estimated on the eighth day after the synthesis.

It was found that 2:1 β-CD–H complexes that were synthesized at 25 and 37°C retained their AO activity. The AO activity tended to decrease at 45°C ($p < 0.1$) and decreased at 65°C ($p < 0.05$) (Fig. 5). The AO activity was retained for 1:0.5 (mM) β-CD–H complexes synthesized at 25 and 37°C whereas it dropped already at 45°C ($p < 0.05$) (Fig. 5). The results indicated that the 2:1 (mM) β-CD–H complex was efficiently synthesized at 25 – 45°C.

In the next stage, the durations of the AO activities of **I** and the 2:1 β-CD–H complex in normal lighting were estimated and compared.

It was found that the AO activity of **I** disappeared by the 6th day of daily illumination for 60 sec by a 60-W incandescent lamp at a distance of 30 – 35 cm; that of β-CD–H, on the 16th day (Fig. 6). Thus, the prolonged AO activity confirmed that forming the β-CD–H complex was effective.

The acidity of an aqueous solution of the 2:1 (mM) β-CD–H complex was studied for a preliminary evaluation of the irritating effect of instilling it into the conjunctival cavity. The irritating effect is known to increase from 1 to 99% as the pH decreases from 7.3 to 5.9 or increases from 9.7 to 11.4 [18]. It was found that the acidity of an aqueous solution of β-CD–H was tilted toward the acid side at (6.73 ± 0.06). This could be the cause of a slight irritating effect.

PMR spectroscopy of D_2O solutions was used to refine and compare the molecular structures of the β-CD–H complex and β-CD.

The PMR spectrum (D_2O) of β-CD showed classical resonances for glucopyranose (δ, ppm): 5.01 (d, J 3.6 Hz, 1H, H-1); 3.90 (t, J 9.5 Hz, 1H, H-3); 3.81 (m, 2H, H-6); 3.79 (m, 1H, H-5); 3.59 (d, J 9.5 Hz, d, J 3.6 Hz, 1H, H-2); 3.52 (t, J

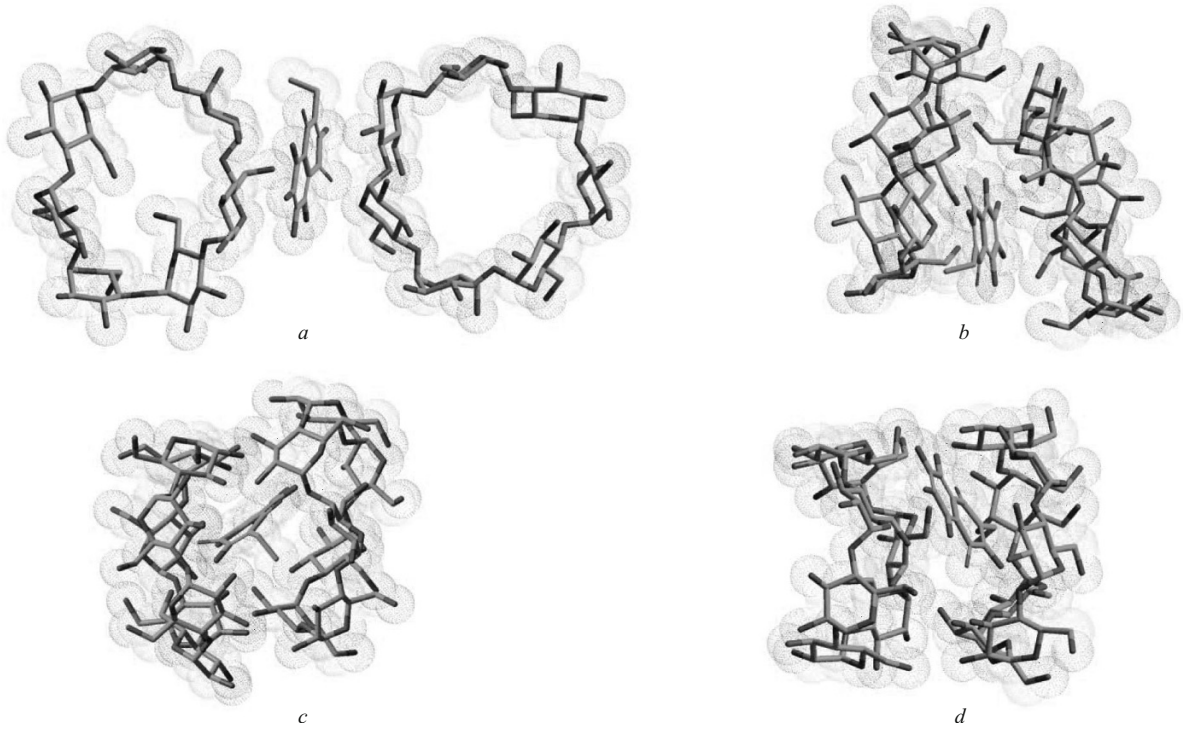

Fig. 7. Structural models of the β-CD–H complex: side-to-side (a), tail-to-tail (b), head-to-head (c), and head-to-tail (d).

9.5 Hz, 1H, H-4). (Fig. 4). The PMR spectrum (D_2O) of synthesized β-CD–H gave resonances (δ, ppm): 4.99 (d, J 3.7 Hz, 1H, H-1); 3.89 (t, J 9.6 Hz, 1H, H-3); 3.79 (m, 3H, H-6); 3.56 (d, J 9.9 Hz, d, J 3.7 Hz, 1H, H-2); 3.51 (t, J 9.6 Hz, 1H, H-4); 2.58 (q, J 7.2 Hz, 2H, $-CH_2$); 2.43 (q, J 7.2 Hz, 2H, $-CH_2$); 1.05 (t, J 7.2 Hz, 3H, $-CH_3$); 0.99 (t, J 7.2 Hz, 3H, $-CH_3$).

Resonances of H-6 shifted to strong field after formation of a H-bond between **I** and β-CD, ΔδH6 = 0.02 ppm. Apparently, this was due to the formation of a H-bond by H-6 with **I** encapsulated inside the β-CD cavity. Resonances in the spectral range of **I** showed the main peaks and doubled resonances at 0.97 – 1.00 and 2.40 – 2.46 ppm that were due to conformational isomers in aqueous solutions of the monosodium salts of **I** [2]. Weak peaks resulting from destruction of 12 – 18% of **I** in the solution were observed in parallel at 1.09 – 1.12 (t, 3H, CH_3). Thus, the obtained PMR spectra were consistent with complexation without inclusion of **I** in the β-CD cavity.

Finally, computer modeling of β-CD–H used HyperChem 8.0.8 and molecular mechanics calculated in MM+ mode to a gradient of <0.001. Four models with **I** positioned differently between two β-CD cavities in a side-to-side fashion (Fig. 7*a*), with a narrow entrance to each other (tail-to-tail) (Fig. 7*b*), with a wide entrance to each other (head-to-head) (Fig. 7*c*), and with a wide entrance to a narrow one (head-to-tail) (Fig. 7*d*) were formulated.

The lowest energy according to the molecular mechanics calculations belonged to the head-to-tail complex; second lowest, head-to-head. The energy difference from the head-to-tail complex was 4.05%. The tail-to-tail complex fell in third place with an energy difference of 11.34%. The side-to-side complex took last place with an energy difference of 20.62%. Thus, formation of the head-to-tail complex was found to be energetically most favorable and; therefore, most stable with respect to the other forms of β-CD–H complexation.

Thus, preliminary studies showed that the optimum synthetic conditions for β-CD–H were 25 – 45°C and a 2:1 (mM) β-CD:**I** mole ratio. The functional advantages of the β-CD–H complex included of prolonged AO activity of **I**. The PMR spectrum of β-CD–H in D_2O showed complexation without inclusion of **I** in the β-CD cavity.

REFERENCES

1. Z. A. Alieva, M. Yu. Sultanov, R. V. Gadzhiev, et al., *Vestn. Oftal'mol.*, **101**(3), 35 – 38 (1985).
2. V. P. Glazunov, D. V. Berdyshev, and V. L. Novikov, *Izv. Akad. Nauk, Ser. Khim.*, No. 1, 44 – 55 (2010).
3. A. E. Gubareva, *Lipid Metabolism / Biochemistry* [in Russian], E. S. Severin (ed.), (2003).
4. N. A. Nikitin, *Vopr. Biol., Med. Farm. Khim.*, **6**, 3 – 11 (2015).
5. M. M. Bikbov, N. A. Nikitin, et al., RU Pat. 2,530,886, Oct. 20, 2014; *Byull. Izobret.*, 29 (2014).

6. N. P. Chesnokova, E. V. Ponukalina, and M. N. Bizenkova, *Usp. Sovrem. Estestvozn.*, No. 7, 37 – 41 (2006).
7. G. Bartosz, *Biochem. Pharmacol.*, **77**, 1303 – 1315 (2009).
8. R. F. Brubaker, W. M. Bourne, L. A. Bachman, et al., *Invest. Ophthalmol. Visual Sci.*, **41**(7), 1681 – 1683 (2000).
9. J. Cejkova, T. Ardan, M. Filipec, et al., *Histol. Histopathol.*, **17**(3), 755 – 760 (2002).
10. C. K. Choy, P. Cho, W. Y. Chung, et al., *Invest. Ophthalmol. Visual Sci.*, **42**(13), 3130 – 3134 (2001).
11. C. Cotagliola, G. Iuliano, M. Menzione, et al., *Exp. Eye Res.*, **43**(6), 905 – 914 (1986).
12. R. K. Crouch, P. Goletz, R. A. Snyder, et al., *J. Ocul. Pharmacol. Ther.*, **7**(3), 253 – 258 (1991).
13. J. V. Fecondo and R. C. Augusteyn, *Exp. Eye Res.*, **36**(1), 15 – 23 (1983).
14. C. J. Kennedy, P. E. Rakoczy, and I. J. Constable, *Eye*, **9**, 763 – 771 (1995).
15. D. V. Ratnam, D. D. Ankola, V. Bhardwaj, et al., *J. Controlled Release*, **113**(3), 189 – 207 (2006).
16. R. R. Farkhutdinov and V. A. Likhovskikh, *Chemiluminescent Methods for Studying Free-Radical Oxidation in Biology and Medicine* [in Russian], Ufa (1995).
17. F. B. Pessine, A. Calderini, and G. L. Alexandrino, in: *Magnetic Resonance Spectroscopy*, D.-H. Kim (ed.), InTech (2012), pp. 237 – 264.
18. K. J. Falahee, C. S. Rose, S. S. Olin, and H. E. Seifried, *Eye Irritation Testing*, Washington (1981).