

## Interaction of natural thiols and catecholamines with reactive oxygen species

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Natural thiols (TSH) cysteine, glutathione, and homocysteine, as well as catecholamines (CA) dopamine, noradrenaline and adrenaline are known as multifunctional biologically active compounds with antioxidant potential, i.e. bio-antioxidants, which play an important role in the regulation of the redox status and free radical formation – utilization in living organisms. The kinetic characteristics of interaction of TSH and CA with peroxy radicals, RO<sub>2</sub>•, formed from the azo-initiator AAPH in aqueous solutions at 37°C by the method of competing reactions were determined. The kinetics of radical formation in the reactions of TSH with H<sub>2</sub>O<sub>2</sub> was studied by the inhibitors method. The polymethine dye (A, pyridine salt of 3,3'-di-γ-sulphopropyl-9-methylthia-carbocyanine betaine) was used as a radical scavenger. CA demonstrated the highest antiradical activity ( $k_i > 10^6(\text{M}\cdot\text{s})^{-1}$ ), whereas TSH possess moderate activity ( $k_i \sim 10^5(\text{M}\cdot\text{s})^{-1}$ ).

**Keywords:** Free radical generation, Thiols, Catecholamines, Caffeic acid, Kinetics

### INTRODUCTION

Oxidative stress is characterized by an increased content of reactive oxygen species (ROS) and reflects an imbalance between the rates of formation of ROS and their utilization [1-4]. The generation of free radicals by biochemical redox reactions is part of the normal cellular metabolism and cells have evolved a variety of mechanisms for scavenging them. Natural thiols (TSH) cysteine, CSH, [5-7], glutathione, GSH, [8-10], and homocysteine, HSH, [11-15], as well as catecholamines (CA) dopamine, DA, norepinephrine, NE, and adrenaline, epinephrine EN, [16-20] are known as multifunctional biologically active compounds with antioxidant potential, i.e. bio-antioxidants, which play an important role in the regulation of the redox status and free radical formation – utilization in living organisms. Catecholamines (CA) compose the group of biogenic amines containing 3,4-dihydroxyphenyl (catechol) as a common structural fragment, which exhibits antioxidant properties in free radical oxidation reactions. Both TSH and CA are water-soluble compounds. Catecholamines are neurotransmitters and neurohormones in animals and humans, and they also function as endogenous antioxidants in the nervous system. In a number of studies, TSH and CA are considered together as compounds, which affect the redox situation in the nerve cells and have potential relevance to age-related diseases [21-23]. In [21] the antioxidant and pro-oxidant capacity of catecholamines (CA) and related compounds were analyzed using the oxygen radical absorbance capacity (ORAC) assay, in which

2,2-azobis (2-amidino-propane) dihydrochloride (AAPH) was a peroxy radical generator. The antioxidant effects of CA and glutathione (GSH) were in the order: dopamine (DA) > norepinephrine (NE) >> GSH. The comparative assay of antioxidant potential of TSH made in [24] showed that their antiradical activity decreased in the order: CSH > HSH > GSH, and the recovery of hydrogen peroxide by thiols was found to be accompanied by a low yield of radicals [24, 26].

The first goal of this study was to evaluate and to compare the antioxidant and pro-oxidant nature of CA and TSH in the presence of AAPH using the method of competing reactions with the polymethine dye A (pyridine salt of 3,3'-di-γ-sulphopropyl-9-methylthia-carbocyanine betaine) in aqueous solution. The second goal was to estimate the effect of H<sub>2</sub>O<sub>2</sub> on the GSH behavior towards the phenol antioxidants resveratrol and caffeic acid known as having immune-modulatory, anti-inflammatory activity and inhibitory effect on cancer cell proliferation [27-30] and having in the molecule a double bond conjugated with the phenolic ring.

### EXPERIMENTAL

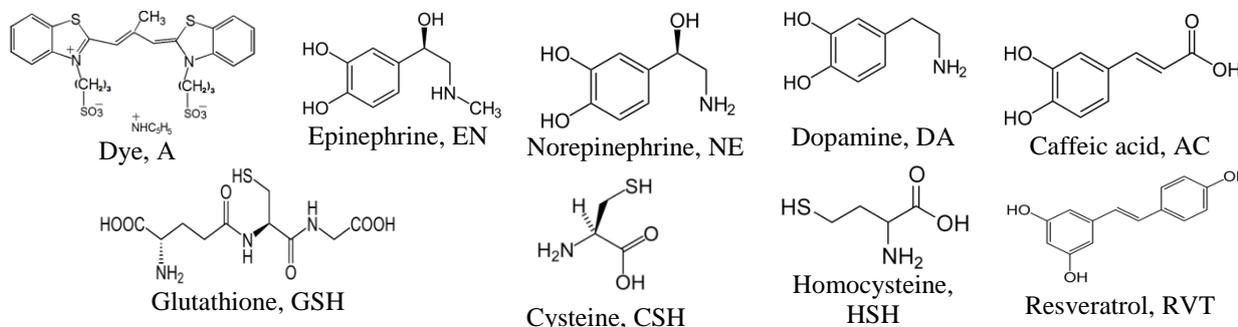
Commercially available natural thiols glutathione (GSH), homocysteine (HSH) and cysteine (CSH), catecholamines epinephrine (EN), norepinephrine (NE), and caffeic acid (AC) (Sigma-Aldrich), trans-resveratrol, RVT (abcr GmbH), hydrogen peroxide (Usolehimprom), dopamine (DA) (Fluka) (Fig. 1), azo-initiator AAPH (2,2'-azobis (2-methylpropionamide dihydrochloride, Fluka) were used as purchased. The polymethine dye (A, pyridine salt of 3,3'-di-γ-sulphopropyl-9-methylthia-

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carbocyanine betaine) (Fig. 1) was used in the method of competing reactions as a reference free radical scavenger with known spectral-kinetic characteristics:  $\epsilon = 0.77 \cdot 10^5 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$  at  $\lambda_{\text{max}} = 546 \text{ nm}$ ; the rate constant of the reaction of A with peroxy radicals derived from AAPH is equal to  $5,4 \cdot 10^4 \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$  and the stoichiometric coefficient

$f = 1$  at  $37^\circ\text{C}$  [25]. The concentration of A, RVT and AC was determined spectrophotometrically. All reactions were carried out in redistilled water at the physiological temperature of  $37^\circ\text{C}$  directly in a temperature-controlled cell ( $l = 1 \text{ cm}$ ) of Ultraspec 1100. The determination error of the kinetic characteristics was about/less than 10%.



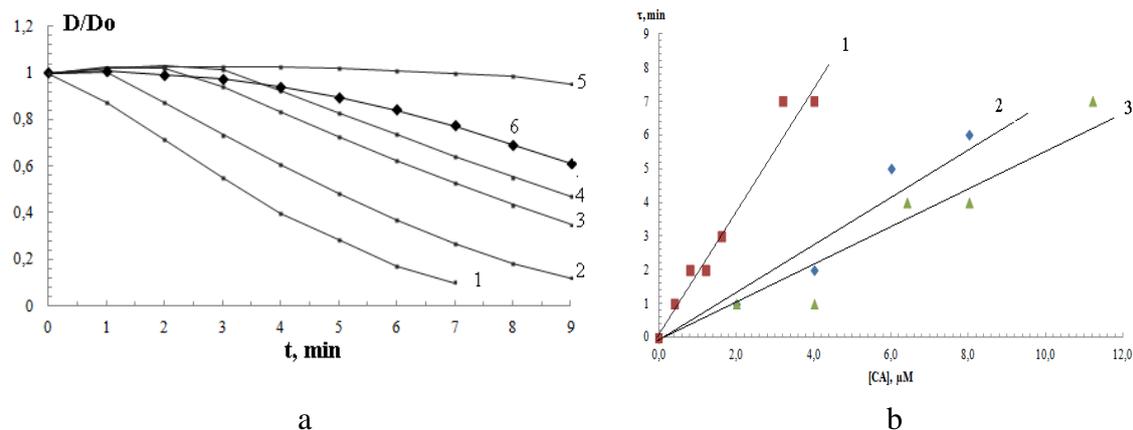
**Figure 1.** Structural formulas of the dye (A), catecholamines, natural thiols, resveratrol and caffeic acid

## RESULTS AND DISCUSSION

### *Antiradical activity of catecholamines and natural thiols*

Figure 2a shows that small additives of DA, more than an order of magnitude less than the

concentration of dye (A) leads to dose-dependent induction period ( $\tau$ ) in A consumption in the reaction with peroxy radicals ( $\text{RO}_2^\bullet$ ) generated by AAPH. After the end of the induction period, the dye is consumed with a rate of noninhibited reaction.



**Figure 2.** a) Kinetic curves of the consumption of the  $10 \mu\text{M}$  dye (A) in reaction with peroxy radicals with additives of DA and GSH; [DA]  $\mu\text{M}$ : 1 – 0; 2 – 0.8; 3 – 1.6; 4 – 2; 5 – 4; 6 – 4  $\mu\text{M}$  GSH; [AAPH] 18 mM; b) Dependences of the induction periods observed in consumption of dye (A) on the concentration of catecholamines: 1-DA; 2-EN, and 3-NE. [AAPH] 18 mM; [A]  $10 \mu\text{M}$ .

Induction periods are observed in the presence of EN and NE as well, but they are shorter than  $\tau$  in the case of DA (Fig. 2b). The main kinetic characteristics of antiradical activity of a radical scavenger (X) are the rate constant for its reaction with radicals ( $k_X$ ) and the stoichiometric coefficient  $f$ , which largely determines the duration of the induction period with the known rate of radical initiation ( $W_i$ ) [31-34]:

$$\tau = f [X]_0 / W_i \quad (1)$$

In the case of dopamine, the dependence of the induction period observed in dye (A) consumption at  $W_i = 1.8 \cdot 10^{-8} \text{ M/s}$  (Fig. 2b, curve 1) is linear and the stoichiometric coefficient  $f = 2$ , calculated according to eqn. (1).

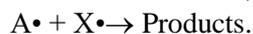
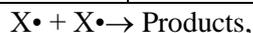
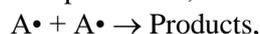
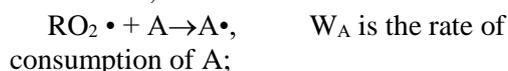
**Table 1.** Kinetic characteristics of antiradical activity of catecholamines, thiols and resveratrol in aqueous solution

Kinetic Characteristic	DA	EN	NE	GSH [24]	HSH [24]	CSH [24]	RVT [35]
f	2	0.8	0.6	1	1	1	2
$k_{RO_2 \cdot} \cdot 10^{-5} M^{-1} \cdot s^{-1}$	>10	>10	>10	0.84	2.2	4.4	1.1
$\varpi \cdot 10^3, M^{-1} \cdot s^{-1}$	-	-	-	0.07	0.07	2.1	-

The induction periods for adrenaline (curve 2) and noradrenaline (curve 3), whose molecules in contrast to DA contain a hydroxyl group in  $\alpha$ -position to the aromatic ring, are significantly shorter. Evaluation of stoichiometric coefficients, calculated for the catecholamines according to Fig. 2b and eqn. (1), gives values of 0.8 and 0.6 for EN and NE, accordingly (Table 1).

It is known from the literature that catecholamines are highly active in reactions with different radicals. In [36], by means of the stop-flow method, the reaction rate constants were determined for the reaction of DA, EN and NE with low-reactive tocopheroxyl ( $k_{Toc} \sim 10^2 M^{-1} \cdot s^{-1}$ ) radicals in 2-propanol/water medium. In [37], by pulsed laser photolysis method, the rate constant for the reaction of CA with cumyloxyl radicals ( $k_{RO_2 \cdot} \sim 10^7 M^{-1} \cdot s^{-1}$ ) was determined in alcohol media. It must be noted that the stoichiometric coefficients for DA, EN and NE in [36, 37] are equal to those determined in this work (table 1). The rate constants in reactions with various radicals including phenoxyl and alkoxy radicals decrease in the order: DA > EN > NE. Antioxidant properties of CA were tested in [19] in the PC liposomes oxidation initiated by AAPH. It was found that for DA, EN, and NE  $f = 2$  in phosphate buffer of pH 7.2. In aqueous solution  $f = 2$  for DA and NE, but  $f = 4$  for EN and in aqueous solution EN oxidizes to pink adrenochrome. May be, these results can be explained by interactions of CA with negatively charged model bio-membranes, found in [38, 39].

Contrary to CA, the additive of GSH (Fig. 2a curve 6) does not show an induction period, but it results in the measured decrease of A consumption rate. In [24], the kinetics of A consumption in the presence of competitive radical scavenger (X) in respect to thiols was described by the following reactions:



The latter three reactions of recombination/disproportionation of radicals  $A \cdot$  formed from the dye and recombination of thiyl radicals  $X \cdot$  formed from thiols proceed with high rates [24, 40] and provide stoichiometric coefficients  $f = 1$  for dye (A) and thiols in the reactions with peroxy radicals. At sufficient concentrations of the scavengers under steady-state conditions, i.e.  $W_i = W_A + W_X$  the rate of dye consumption upon addition of X is equal to  $W_A = k_A[A][RO_2 \cdot] = (k_A[A]W_i) / \{k_A[A] + k_X[X]\}$ . To analyze the experimental data, eqn. (1) was transformed into the form (2):

$$1/W_A = [1 + (k_X/k_A) \cdot ([X]/[A])] / W_i \quad (2)$$

From the slope of the dependence of  $1/W_A$  on the concentration ratio  $[X]/[A]$ , the rate constants for all the natural thiols were determined (table 1). This approach [24] is suitable for determining rate constants for reactions with peroxy radicals in aqueous media for inhibitors of medium strength ( $10^3 < k_{RO_2 \cdot} < 10^6 M^{-1} \cdot s^{-1}$ ). It was applied in [35] to determine  $k_{RO_2 \cdot}$  and  $f$  for resveratrol (table 1) which characterized RVT as a moderate scavenger of free radicals.

#### Free radical formation in the reaction of thiols with $H_2O_2$

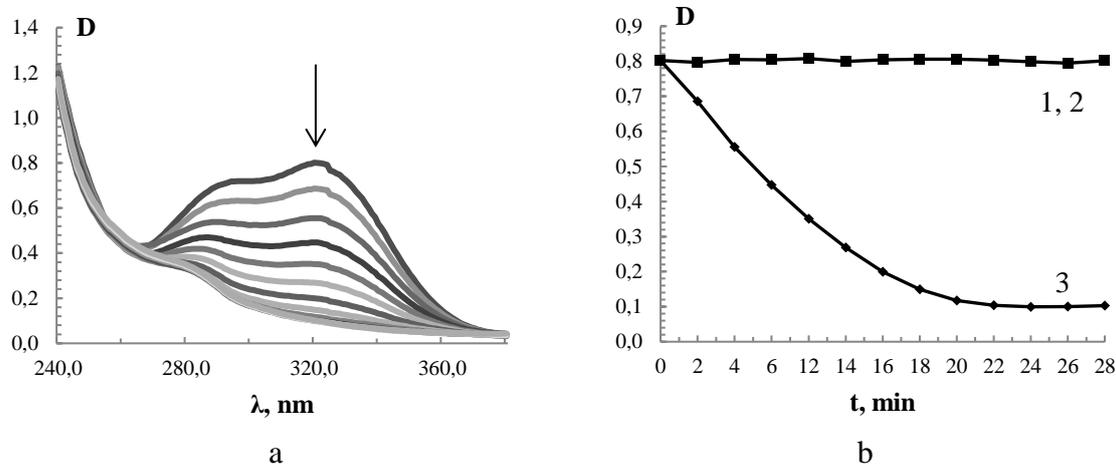
The polymethine dye A was used as the radical scavenger to study the free radical generation upon the reaction of thiols with  $H_2O_2$  [24]. The specific rates of radical generation equal to  $\varpi_i = W_i / ([TSH][H_2O_2])$  are presented in table 1. The yield of radicals in the reaction of TSH with  $H_2O_2$  is very small <1%, however, this value of free radical formation may be sufficient to initiate chain processes, especially in multiphase systems. We have established [35] that the well-known antioxidant resveratrol (RVT), which has in the molecule a double bond activated by conjugation with two phenolic rings, interacts with glutathione (GSH) and cysteine (CSH) in aqueous solutions at 37°C. Reaction of RVT with thiols (thiol-ene reaction) proceeds by a chain mechanism and is accelerated in the presence of  $H_2O_2$ . In this study, we investigated the interaction of caffeic acid (AC) with

glutathione. The reactions were carried out in aqueous solution at 37°C.

The behavior of AC was controlled via the UV absorption spectra. When GSH and H<sub>2</sub>O<sub>2</sub> were added separately, the AC spectrum did not change (Fig. 3b, curves 1 and 2). However, when GSH and H<sub>2</sub>O<sub>2</sub> were

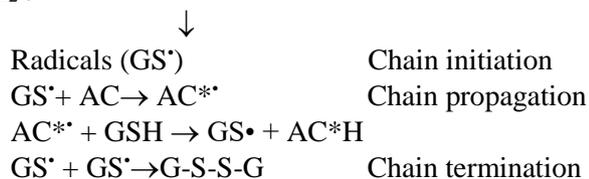
added together, AC consumption was observed (Fig. 3a and curve 3 Fig. 3b) with a rate equal to  $W_{AC} = 4.9 \cdot 10^{-8} \text{ M} \cdot \text{s}^{-1}$ . The rate of radical initiation in the reaction GSH + H<sub>2</sub>O<sub>2</sub> can be calculated as follows:

$$W_i = \varpi[\text{GSH}][\text{H}_2\text{O}_2] = 0.07 \cdot 10^{-3} \times 5 \cdot 10^{-3} \times 8.6 \cdot 10^{-3} = 3 \cdot 10^{-9} \text{ M} \cdot \text{s}^{-1}.$$



**Figure 3** a) Change of the UV absorption spectra of caffeic acid in the reaction with glutathione initiated by H<sub>2</sub>O<sub>2</sub>: [GSH] 5 mM; [H<sub>2</sub>O<sub>2</sub>] 8.6 mM; [AC] 0.053 mM; b) Changes of optical density of caffeic acid at 321 nm in reaction with GSH and H<sub>2</sub>O<sub>2</sub>. 1: [AC] 0.053 mM, [GSH] 5 mM; 2: [AC] 0.053 mM, [H<sub>2</sub>O<sub>2</sub>] 8.6 mM; 3: [AC] 0.053 mM, [GSH] 5 mM, [H<sub>2</sub>O<sub>2</sub>] 8.6 mM.

The comparison of the rates  $W_{AC}$  and  $W_i$  reveals that similar to the reaction of resveratrol with GSH [35], the reaction of caffeic acid with GSH in the presence of H<sub>2</sub>O<sub>2</sub> proceeds by a chain mechanism with rather long chain length equal to  $\nu = W_{AC} / W_i = 16$ .



Here, AC<sup>\*•</sup> is alkyl radical resulted from GS<sup>\*</sup> addition to the double bond of AC.

### CONCLUSION

Using the polymethine dye (A, pyridine salt of 3,3'-di-γ-sulphopropyl-9-methylthia-carbocyanine betaine) as the reference radical scavenger, the kinetic characteristics of interaction of natural thiols (TSH) and catecholamines (CA) with peroxy radicals, RO<sub>2</sub><sup>•</sup>, formed from azo-initiator AAPH in aqueous solutions at 37°C by the method of competing reactions were determined. CA have demonstrated the highest antiradical activity ( $k_{ROO} > 10^6 (\text{M} \cdot \text{s})^{-1}$ ), whereas TSH possess moderate activity ( $k_{ROO} \sim 10^5 (\text{M} \cdot \text{s})^{-1}$ ). The kinetics of radical formation in the reactions of TSH with H<sub>2</sub>O<sub>2</sub> was studied by the inhibitors method and specific rates of these

reactions were determined. Due to radical formation in the reaction of GSH with H<sub>2</sub>O<sub>2</sub>, we have established for the first time that the reaction of caffeic acid with glutathione in the presence of H<sub>2</sub>O<sub>2</sub> proceeds by a chain mechanism with rather long chain length.

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## ВЗАИМОДЕЙСТВИЕ НА ПРИРОДНИ ТИОЛИ И КАТЕХОЛАМИНИ С РЕАКТИВНИ ФОРМИ НА КИСЛОРОДА

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(Резюме)

Природните тиоли (TSH) цистеин, глутатион и хомоцистеин, както и катехоламините (СА) допамин, норадреналин и адреналин са известни като мултифункционални биологично активни съединения с антиоксидантен потенциал, т.е. био-антиоксиданти, които играят важна роля за регулиране на редокс статуса и образуването и използването на свободни радикали в живите организми. Определени са кинетичните характеристики на взаимодействието на TSH и СА с пероксилните радикали RO<sub>2</sub>•, образувани от азоинициатора ААРН във водни разтвори при 37°C по метода на конкурентните реакции. Кинетиката на радикалообразуването при реакциите на TSH с H<sub>2</sub>O<sub>2</sub> е изследвана по инхибиторния метод. Полиметиновото багрило (А, пиридинова сол на 3,3'-ди-γ -сулфопропил-9-метилтиа-карбоцианин бетаин) е използвано като радикалоуловител. СА проявяват висока антирадикалова активност (k<sub>i</sub> > 10<sup>6</sup>(M·s)<sup>-1</sup>, докато активността на TSH е умерена (k<sub>i</sub> ~ 10<sup>5</sup>(M·s)<sup>-1</sup>).