N-Acetylcysteine is an effective analog of glutathione in reactions with reactive oxygen species

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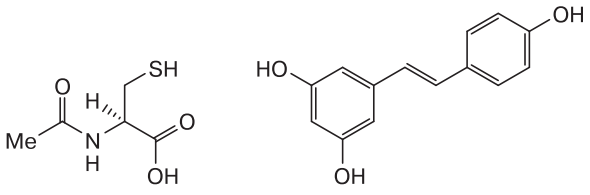
The kinetic characteristics of the interaction of *N*-acetylcysteine (ASH) with reactive oxygen species (ROS), peroxyl radicals and hydrogen peroxide were determined. It was found that in terms of activity ASH in these reactions is similar to glutathione GSH, the main endogenous bioantioxidant. The kinetics of heat release in the interaction of GSH and ASH with H_2O_2 was studied for the first time by isothermal calorimetry. It is shown that the kinetic curves of heat release and changes in specific heat release rates practically coincide for both thiols taken in the stoichiometric ratio in the known reaction 2 TSH + H_2O_2 → TSST + 2 H_2O.This indicates the relative autonomy of the S—H and S—S bonds in thiols and disulfides, which are not affected by other groups in the molecule. At pH<7, ASH, like GSH, interacts with H_2O_2 to form thiyl radicals, which initiate thiol-ene reactions with unsaturated phenol resveratrol. Under the same conditions, ASH ensures nearly the same radical initiation rates as GSH, and thiyl radicals from ASH are close in activity to GS$^{\bullet}$ in chain propagation reactions.

Key words: *N*-acetylcysteine, glutathione, hydrogen peroxide, peroxyl radicals, thiyl radicals, thiol-ene reaction, resveratrol.

Reactions with reactive oxygen species and thiol-disulfide metabolism involving thiol SH-groups of cysteine in proteins and low-molecular-weight peptides play an important role in the vital functions of living beings and the formation of the immune system. The pandemic of 2020 and 2021 has led to an increasing research interest in the biochemical reactions of endogenous thiol glutathione (GSH) and, in particular, synthetic *N*-acetylcysteine (ASH), that was used in treatment of the early stages of COVID-19.[1—3] *N*-Acetylcysteine has been used since the late 1980s as a mucolytic and anti-inflammatory drug, and also in conditions of oxidative stress with a decrease in the level of endogenous glutathione[4,5], which is considered as the main bioantioxidant. The presence of thiol group in the structure of the ASH molecule provides its antioxidant properties and enables the reduction of disulfide bonds. This may lead to the cleavage of mucopolysaccharide chains and depolymerization of sputum mucoproteins. In these processes ASH, apparently, is more active than GSH. Fragmentary data suggest[6—9] that it has noticeable therapeutic effects in conditions characterized by decreased GSH levels or oxidative stress, such as HIV, cancer, heart disease, smoking and (recently) COVID-19.



Earlier[10—16], we studied in detail the reaction mechanism of GSH with H_2O_2 in deionized water. The interaction of GSH with H_2O_2 is accompanied by the formation of thiyl radicals.[16] The yield of radicals is low (~1%), but even in this amount they can initiate chain thiol-ene processes. The thio-ene reaction between GSH and unsatured phenols in the presence of hydrogen peroxide was studied[17] with resveratol (RVT), the properties and chemical behavior of which are of interest for medicine and pharmacy.[18—20]

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The aim of this work is to study the reaction of ASH with reactive oxygen species (ROS): peroxyl radicals and hydrogen peroxide. It was also of interest to study the heat release kinetics during the reaction of GSH and ASH with H_2O_2 and carry out comparative analysis of the thiol-ene reactions of ASH and GSH with RVT in the presence of H_2O_2 in aqueous media.

Experiment

Reagents *N*-acetylcysteine (ASH, Acros Organics), glutathione, Ellman reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma—Aldrich), hydrogen peroxide (H_2O_2, PanReacAppliChem) and *trans*-resveratrol (abcrGmbH) were used without additional purification. Peroxyl radicals were generated with azo initiator, 2,2′-azobis(2-methyl propionamidine) dihydrochloride (AAPH, Fluka) similar to the method previously described.[11,13]

Deionized water (Direct-Q UV Millipore, 18 mΩ cm, pH 7) was used as the reaction medium. The initial solutions of ASH and GSH were prepared in deionized water, RVT in DMSO, then they were added into the reaction mixture with an electronic pipette. The concentration of H_2O_2 (in the absence of thiols) was controlled by iodometry. The concentration of GSH and ASH was measured by the Ellman method.[21]

The kinetics of heat release in the exothermic reaction of thiols with H_2O_2 was studied by isothermal calorimetry (DAK-1-1) at 37.7 °C in sealed ampoules.

Thiol-ene reactions of ASH and GSH with RVT in the presence of H_2O_2 were carried out at 37 °C in thermostatically controlled cell of the SF-2000 spectrophotometer (OKB Spectr LLC, Russia), where the RVT consumption was recorded (at λ_{max} = 304—308 nm, $\varepsilon = 0.3 \cdot 10^5$ L mol^{-1} cm^{-1}). The antiradical activity of ASH and RVT was determined by the chemiluminescent method in a model oxidation reaction of ethylbenzene initiated by azobisisobutyronitrile (AIBN) at 50 °C similar to the previously published technique.[22] AIBN was purified by double recrystallization from ethanol. The reaction rate constant of ASH and RVT with the ethylbenzene peroxyl radical was determined from the slope of the extrapolated linear branch (tgφ) of the kinetic chemiluminescence curve (CL) at the luminescence rise after the inhibitor was consumed at the inflection point[22]:

$$k_{inh} = \sqrt{2k_t}\,\mathrm{tg}\varphi \big/ (\sqrt{W_i} \cdot 0.273), \qquad \textbf{(I)}$$

where W_i is the rate of initiation due to decay of AIBN; $2k_t = 1.6 \cdot 10^7$ L mol^{-1} s^{-1} is the rate constant of disproportionation of ethylbenzene peroxyl radicals.

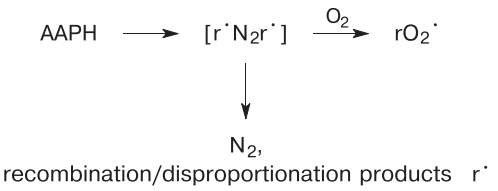
The measurement error of the consumption rates of GSH, ASH and RVT did not exceed 15%.

Results and Discussion

The reactivity of acetylcysteine in reaction with peroxyl radicals. In the air, tertiary peroxyl radicals are generated *via* the decay of AAPH in an aqueous medium (Scheme 1).

In the reaction with peroxyl radicals ($rO_2^{\bullet}$), ASH occupies an intermediate position between GSH and homocysteine (HSH), rate constants of the reaction of thiols with $rO_2^{\bullet}$ ($k_{inh} \cdot 10^{-5}$) are 0.84, 2.16 and 1.5 L mol^{-1} s^{-1} for GSH, HSH and ASH, respectively. The stoichiometric inhibition coefficient for ASH equals $f = W_i/\mathrm{d[ASH]}/\mathrm{d}t = 1$.

Scheme 1



Determination of the antiradical activity of ASH by the CL method in an ethylbenzene medium at 50 °C has led to the following values: $f < 1$, $k_{inh} = 2.8\ 10^4$ L mol^{-1} s^{-1}. Probably, at an elevated temperature, ASH reactions with the formed hydroperoxides and oxygen, reduce the value of *f*. In addition, hydrophilic ASH almost does not dissolve in nonpolar hydrocarbons, and in the "CL mixtures" it can form clusters that simulate dissolution, but complicate reactions with peroxyl radicals. Under similar conditions hydrophilic glutathione does not migrate into an organic phase (chlorobenzene containing 20% of ethylbenzene) and does not affect the kinetics of CL, for RVT, which is soluble in aromatic hydrocarbons, under the same conditions, $f = 2$, $k_{inh} = 2.3 \cdot 10^5$ L mol^{-1} s^{-1}. In an aqueous solution at 37 °C with $rO_2^{\bullet}$ from AAPH $k_{inh} = 1.1 \cdot 10^5$ L mol^{-1} s^{-1}.[13]

Reaction of acetylcysteine and glutathione with H_2O_2. Reactions of thiols with hydroperoxides in living beings proceed in the presence of glutathione peroxidase enzymes, but with H_2O_2 thiols can react directly. Reduction of H_2O_2 with thiols (TSH) is described by the overall reaction (1).[10,23—29] However, practically, thiols reacts with H_2O_2, as in the case of GSH, with a complex mechanism, including the formation of intermediate complexes GSH—GSH,[10,17] GSH—H_2O_2,[23,27—29] and radical generation.[13,16]

$$2\,\mathrm{TSH} + \mathrm{H_2O_2} \longrightarrow \mathrm{TSST} + 2\,\mathrm{H_2O} \qquad (1)$$

Like oxidation reactions by oxygen, the reaction of thiol oxidation with hydrogen peroxide is exothermic. Figure 1 compares the kinetic curves of heat release during the reaction of GSH and ASH with H_2O_2, taken in stoichiometric quantities of the reaction (1). It can be seen that the kinetic heat release curves and changes in specific rates for these thiols coincide, thus indicating the relative autonomy of the S—H and S—S bonds in thiols and disulfides, which are not affected by other groups in the molecule.

Generation of thiyl radicals in the reactions of GSH and ASH with H_2O_2 was found by ESR method us-

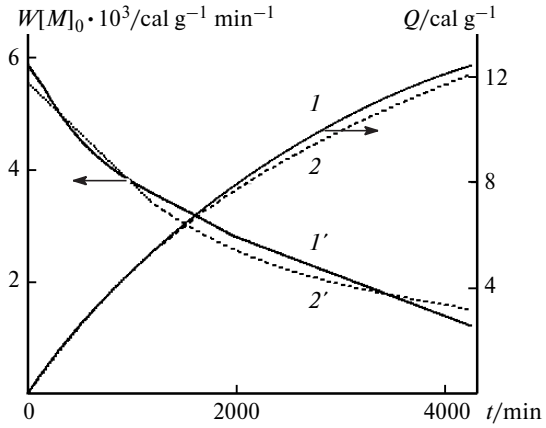


Fig. 1. Kinetics of heat release (*1, 2*) and specific heat release rate (*1′*, *2′*) in the reactions of *N*-acetylcysteine (*1*, *1′*) and glutathione (*2*, *2′*) with H_2O_2 in deionized water at 37.7 °C; thiol concentrations are 0.1 mol L^{-1}, H_2O_2 concentration is 0.05 mol L^{-1}.

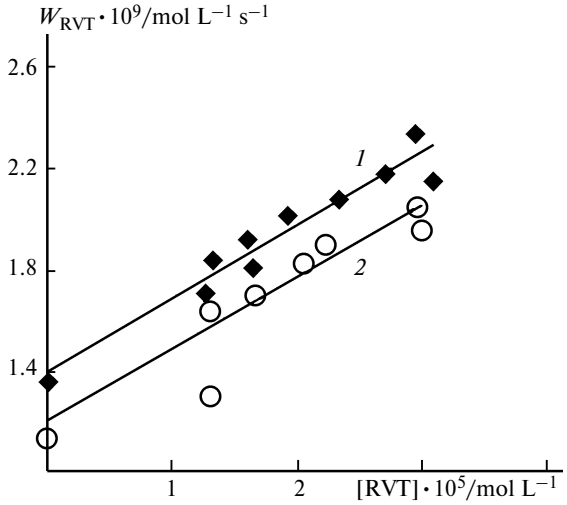


Fig. 2. The RVT consumption rate (W_{RVT}) as a fuction of the RVT concentration in the reaction mixture of H_2O_2 (1.5 mmol L^{-1}) with thiol (2.5 mmol L^{-1}): glutathione (*1*), *N*-acetylcysteine (*2*).

ing DMPO (5,5-dimethyl-1-pyrrolidine *N*-oxide) as a spin trap.[16]

The rate of radical generation (W_i) in the ASH reaction with H_2O_2 in deionized water were measured in a wide range of reagent concentrations by inhibitor method using a water-soluble acceptor (polymethine dye) with known spectral and kinetic properties.[15] Empirical functions of W_i from thiol concentrations and H_2O_2 for GSH (see lit.[10]) and ASH can be represented as:

$$W_{iGSH} \approx k[GSH]^{0.75}[H_2O_2]^{0.75} \quad (II)$$

$$(k = (1.3\pm0.2) \cdot 10^{-5}\ L^{0.5}\ mol^{-0.5}\ s^{-1}),$$

$$W_{iASH} \approx k[ASH][H_2O_2]^{1.5} \quad (III)$$

$$(k = (7.9\pm0.7) \cdot 10^{-3}\ L^{1.5}\ mol^{-1.5}\ s^{-1}).$$

The yield of radicals is low ($W_i/W_{TSH} > 1\%$), however these radicals can initiate chain processes. In the case of glutathione, the thiol-ene chain reaction was studied in detail taking the reaction with unsaturated phenol RVT in the presence of H_2O_2 as an example.[17]

Thiol-ene reaction of thiols with RVT in the presence of H_2O_2. RVT is not consumed in the reaction with an individuel thiol or H_2O_2, and its consumption is observed only in the simultaneous presence of both thiol and H_2O_2.

Figure 2 shows the initial rates of RVT consumption (W_{RVT}) as function of its concentration in reactions with GSH and ASH, which were determined at the equal concentrations of thiols and H_2O_2. Thus, for both thiols, W_{RVT} increases linearly with increasing RVT concentration. It is known[30] that thiyl radicals easily add to unsaturated bonds with rate constants $k \approx 10^5$ L mol^{-1} s^{-1} and rapidly recombine ($k \approx 10^9$ L mol^{-1} s^{-1}).[31] The rate of radical-chain consumption of RVT in such a process is linearly releated to its concentration:

$$W_{RVT} = W_i + a[RVT]W_i^{0.5}, \quad (IV)$$

where a is a parameter that characterizes the relative activity of the leading chain radicals in the chain proragation and termination reactions ($a = k_p/(2k_t)^{0.5}$). According to equation (IV), segments equal to W_i (the rates of radical initiation) are marked on the ordinate axis.

Table 1 compares the kinetic properties of RVT consumption when reacting with both thiols in the presence of H_2O_2. Under the same conditions at the ratio of concentrations of thiol and H_2O_2, close to stoichiometric ($1 < [TSH]/[H_2O_2] < 2.5$), due to the presence of ASH nearly the same rates of radical initiation as with GSH can be achieved. In addition, the thiyl radicals from ASH have a similar to GS$^{\bullet}$ activity in chain propagation. Table 1 shows the pH values measured in the reaction mixture after the reagents were added into deionized water, which indicate the effect of thiols on the pH of the medium. Both thiols contain carboxyl groups and form acidic solutions

Table 1. Kinetic characteristics of resveratrol consumption during reaction with thiols (2.5 mmol L^{-1}) in the presence of H_2O_2 (1.5 mmol L^{-1}); aqueous solution, 37 °C

Thiol	pH	W_i/mol L^{-1} s^{-1}		a /L$^{0.5}$ mol$^{-0.5}$ s$^{-0.5}$
		*A**	*B***	
GSH	3.28	$1.50 \cdot 10^{-9}$	$1.4 \cdot 10^{-9}$	0.7
ASH	3.31	$1.15 \cdot 10^{-9}$	$1.2 \cdot 10^{-9}$	0.8

* According to a Eqs (I) and (II).
** Determined using the data in Figure 2.

in water, thus, depending on the concentration of thiols, they can shift the pH of buffer solutions to the acidic values.[16] Previously,[32] it was found that with an increase in pH, the rate of GSH consumption in the reaction with H_2O_2 increases, the rate of radical formation decreases, and at pH ≥7, no radicals are formed.

The vast majority of studies on biochemistry of GSH and other natural thiols are carried out under conditions close to physiological, i.e. in buffer solutions with pH 7.2—7.4. Under such conditions, the reaction rates of thiols with ROS is largely determined by the contribution of thiolate anion in these processes and depends on the value of pK_a of S—H bond. A review[33] has been published that summarized and analyzed in detail the data on the antioxidant and recovery activity of ASH available in the literature. At the same time, it was concluded[33] that the reaction rate constants of ASH and endogenous thiol-containing compounds with respect to various oxidants are arranged in the following sequence: cysteine > GSH > > ASH. An explanation of the sequence is that the antioxidant activity of thiol is caused by thiolate anion, the relative concentration of which is regulated by the acidity of the thiol.

This work presents the results of a study on the reactivity of GSH and ASH towards ROS (peroxyl radicals and hydrogen peroxide) in deionized water (pH 7). The obtained data show that under these conditions, thiols demonstrate similar activity. It is possible that the generation of radicals during the interaction of TSH with H_2O_2 which we were able to detect, as well as the reactions of TSH with unsaturated phenols, play an important role in the physiology of plants where intracellular and intercellular fluids are characterized by relatively low pH values (≤7). These reactions may also be important on using thiols in winemaking, cosmetics and pharmaceuticals.

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This paper does not contain descriptions of studies on animals or humans.

The authors declare no competing interests.

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