Effect of phosphate buffer solutions on the reactions of glutathione with hydrogen peroxide and peroxyl radicals

K. M. Zinatullina,[a]★ O. T. Kasaikina,[a] V. A. Kuzmin,[b] N. P. Khrameeva,[b] and L. M. Pisarenko[a]

[a]*N. N. Semenov Institute of Chemical Physics, Russian Academy of Sciences, 4 ul. Kosygina, 119991 Moscow, Russian Federation. Fax : +7 (495) 651 2191. E-mail: karinazinat11@gmail.com*
[b]*N. M. Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, 4 ul. Kosygina, 119991 Moscow, Russian Federation*

Differences in the kinetics and mechanism of the reaction of glutathione (GSH) with hydrogen peroxide (H_2O_2) in deionized water and in phosphate buffer systems with pH ≥ 7 frequently used in biochemical studies were revealed. The formation of GSH dimers and complexes with H_2O_2 in water plays a substantial role in the kinetics of the process, which is manifested as nonlinear dependences of the rate of GSH consumption (W_{GSH}) and the rate of radical formation (W_i) on the reagent concentrations. In phosphate buffer solutions (PBS), the oxidation of GSH by air oxygen is enhanced and the radical formation rate decreases sharply. An effect of NaCl and KCl in PBS on W_{GSH} and W_i was observed, unlike a sodium—potassium phosphate buffer mixture (PB). Under other equivalent conditions, W_{GSH} in PBS is several times lower and W_i is higher than those in PB containing no chlorides. It was found that the rate of the thiol-ene reaction of unsaturated phenol resveratrol (RVT) with GSH initiated by the radicals formed in the presence of H_2O_2 in PBS is nearly three times lower than that in water, whereas in PB resveratrol is not consumed under the same conditions. However, in the reactions with peroxyl radicals formed upon the decomposition of 2,2′-azobis(2-methylpropionamidine) dihydrochloride the GSH consumption rate is the same in both phosphate buffer systems.

Key words: glutathione, hydrogen peroxide, peroxyl radicals, phosphate buffer systems, kinetics, thiol—ene reaction, resveratrol.

Glutathione (GSH) is the most abundant cytosol thiol classified as an endogenic bioantioxidant synthesized directly in living organisms. The concentration of GSH in biological tissues is 0.1—10 mmol L^{-1}, which is significantly higher than the concentrations of other potential bioantioxidants.[1—7] The GSH/GSSG redox pair and H_2O_2 occupy the central place in determining oxidation—reduction homeostasis and redox signalization.[8—13] It is known that the reduction of H_2O_2 by glutathione is accompanied by the formation of disulfide and water[4,14—17]:

$$2\,GSH + H_2O_2 \longrightarrow GSSG + 2\,H_2O. \qquad (1)$$

Detailed studies of the mechanism of this reaction indicate that the complicated process occurred *via* the formation of the intermediate GSH—H_2O_2 complex.[18] It is shown that the rate has the first order with respect to the concentrations of the reactants[4,14,18,19] and depends on the ratio of GSH and H_2O_2 concentrations.[18,20] We have recently found[21,22] that the reaction of GSH and H_2O_2 is accompanied by the formation of radicals with the rate composing fractions of percent of the rate of GSH consumption. However, this rate turned out to be sufficient for initiation of the chain reaction of GSH with unsaturated phenols resveratrol and caffeic acid.[23,24] The rates of radical generation (W_i) were measured by the inhibitor methods using anionic polymethine dye **A** (3,3′-di-γ-sulfopropyl-9-methylthiacarbocyaninebetaine pyridine salt) as an acceptor. This water-soluble dye is inert toward thiols and hydrogen peroxide but reacts actively and stoichiometrically with free radicals.[25] We used bidistilled and deionized water as a reaction medium, whereas the most part of biochemical studies are carried out in phosphate buffer solutions, since biological fluids are characterized by pH 7.2—7.4 and represent buffer systems.

The purpose of this work is to reveal the effect of aqueous buffer solutions, isotonic sodium phosphate buffer PBS (pH 7.4), and sodium—potassium phosphate buffer PB (pH 7.2, used in electrochemical methods of evaluation of antioxidants[26,27]) on the kinetics of GSH consumption in the reaction with H_2O_2 and on the yield of radicals in this reaction. We also have studied the possibility of occurrence of the thiol—ene reaction of glutathione with resveratrol. The latter is an exogenic phenol antioxidant

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 7, pp. 1441—1444, July, 2019.
1066-5285/19/6807-1441

that comes to the organism with feeding products and biologically active additives,[28—31] and the reaction with resveratrol proceeds *via* the chain mechanism in the presence of H_2O_2 in an aqueous medium.[23,24]

Experimental

Glutathione, Ellman reagent (5,5′-dithio-bis(2-nitrobenzoic acid), DTNB (both from Sigma-Aldrich), hydrogen peroxide H_2O_2 (Usol′khimprom, Russia), azoinitiator 2,2′-azobis(2-methylpropionamide) dihydrochloride (AAPH, Fluka), and *trans*-resveratrol (RVT, ABCR GmbH) were used as received.

Bidistilled deionized water and two phosphate buffer solutions, PBS and PB, were used as reaction media. Buffer PBS (pH 7.4) is an isotonic phosphate—salt buffer solution containing sodium and potassium chlorides, sodium hydrophosphate, and potassium dihydrophosphate in which the concentration of ions in the solution corresponds to the concentration in the human blood; PB is a buffer mixture of KH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$ with pH 7.2. The reactions of GSH with H_2O_2 were carried out at a physiological temperature of 37 °C in a temperature-maintained vessel with magnetic stirring, from which samples (9 μL) were taken during the reaction. The taken samples were diluted in 3 mL of phosphate buffer (PBS, pH 7.4) containing the Ellman reagent (0.1 mmol). The concentration of formed 2-nitro-5-thiobenzoic acid equal to GSH concentration was determined spectrophotometrically: $\varepsilon = 0.14 \cdot 10^5$ L mol^{-1} cm^{-1} at $\lambda_{max} = 412$ nm.[32—34]

The rate of radical formation in the reaction GSH + H_2O_2 (W_i) was determined by the inhibitor method. Polymethine dye **A** was used as an acceptor of free radicals, and its concentration was detected spectrophotometrically: $\varepsilon = 0.77 \cdot 10^5$ L mol^{-1} cm^{-1} at λ_{max} = 543 nm. The reaction was carried out directly in the temperature-maintained cell of a Ultraspec 1100 Pro spectrophotometer (l = 1 cm). The thiol—ene reaction of GSH with unsaturated phenol RVT was conducted in the spectrophotometer cell in which the RVT consumption was detected ($\varepsilon = 0.3 \cdot 10^5$ L mol^{-1} cm^{-1} at λ_{max} = 304—308 nm).

Bidistilled water was used to prepare basis solutions of acceptor **A**, and basis solutions of RVT were prepared in ethanol (Medkhimprom, Russia). The basis solutions were added to the reaction mixture in amounts of 1—10 μL. The concentration of H_2O_2 (in the absence of glutathione) was monitored by the iodometric method. The error in rate measurements did not exceed 10%.

Results and Discussion

Reaction of glutathione with peroxyl radicals. The kinetic curves of consumption of anionic polymethine dye **A** in the reaction with peroxyl radicals derived from AAPH decomposition in water and in phosphate buffers PBS and PB are presented in Fig. 1. The kinetic characteristics of the reaction of **A** with the formed peroxyl radicals have been determined previously[25]: stoichiometric coefficient f = 1 and rate constant of the reaction with peroxyl radicals $k_{ROO} = 5.4 \cdot 10^5$ L mol^{-1} s^{-1}.

The data in Fig. 1 show that in buffer solutions **A** is consumed with the same rate, which is halved compared

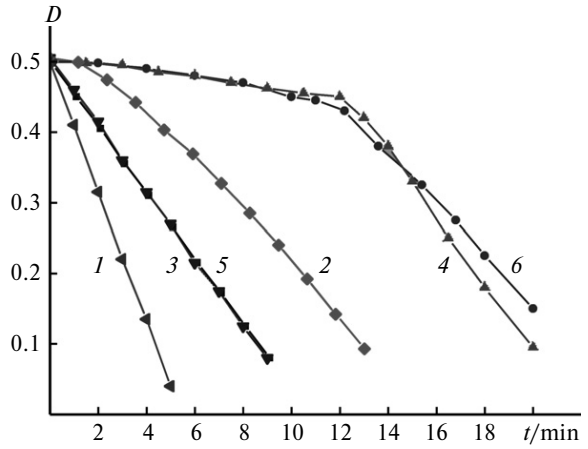


Fig. 1. Kinetic curves of consumption of acceptor **A** (0.0065 mmol L^{-1}) in the reaction with the radicals formed upon the decomposition of AAPH (18 mmol L^{-1}) in water (*1*, *2*), PBS (pH 7.4) (*3*, *4*), and phosphate buffer solution PB (pH 7.2) (*5*, *6*) in the absence of glutathione (*1*, *3*, *5*) and with the addition of glutathione: 0.015 (*2*), 0.012 (*4*), and 0.011mmol L^{-1} (*6*).

to the rate of consumption of **A** in water and to the rate of radical initiation (W_i). The latter remained almost unchanged in the range of pH 5—8.[35,36] This means that in PBS and PB for **A** $f = W_i/(-d[\mathbf{A}]/dt) = 2$. The addition of GSH (see Fig. 1, curve *2*) decreases the consumption rate of **A** in water, indicating the competition between **A** and GSH. The rate constant of the reaction of GSH with peroxyl radicals formed upon the decomposition of AAPH in an aqueous medium at 37 °C (k_{ROO} = $= 0.84 \cdot 10^5$ L mol^{-1} s^{-1}) was determined[22] by the method of competing reactions. The additives of GSH to PBS and PB lead to induction periods (τ = 12 min), after the end of which **A** is consumed nearly with the rate of the non-inhibited reaction (see Fig. 1, *cf.* curves *4* and *3*, *6* and *5*). The pronounced induction periods in the presence of small concentrations of GSH ([GSH] < [**A**]) indicate that in PBS and PB glutathione reacts with radicals more actively than with **A**, and both buffer solutions affect the antiradical activities of **A** and GSH in the same way. Since the equation $[GSH]_0/\tau \cong W_i$ is fulfilled, the stoichiometric coefficient for GSH in PBS and PB is equal to 1.

Reaction of glutathione with H_2O_2. The effect of buffer solutions PBS and PB on the consumption of GSH in the reaction with H_2O_2 is presented in Fig. 2. The influence of buffer solutions PBS and PB on the yield of radicals in this reaction, which was measured by the inhibitor method from the consumption of acceptor **A** is shown in Fig. 3.

It is seen that in phosphate buffer solutions the rate of GSH consumption (W_{GSH}) increases and the rate of radical formation decreases sharply. The oxidation of GSH by air oxygen is a reason for increasing W_{GSH} in PBS and especially in PB. In buffer solutions GSH was consumed in a larger amount (5 mmol L^{-1}) than it is required ac-

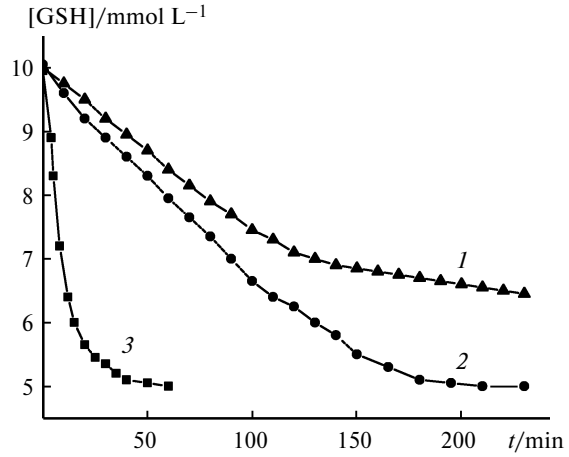


Fig. 2. Kinetic curves of consumption of glutathione (10 mmol L^{-1}) in the reaction with H_2O_2 (2 mmol L^{-1}) in water (*1*), PBS (*2*), and phosphate buffer PB (*3*).

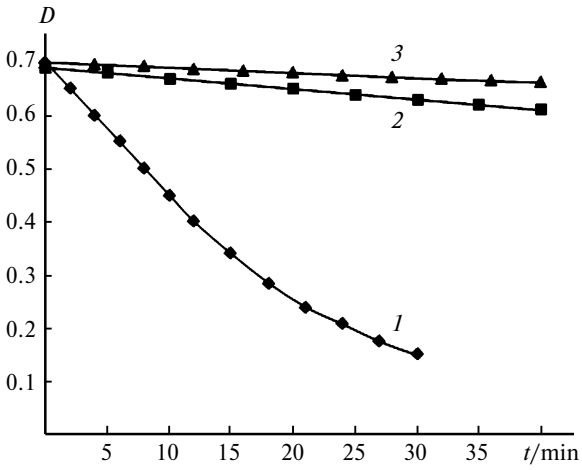
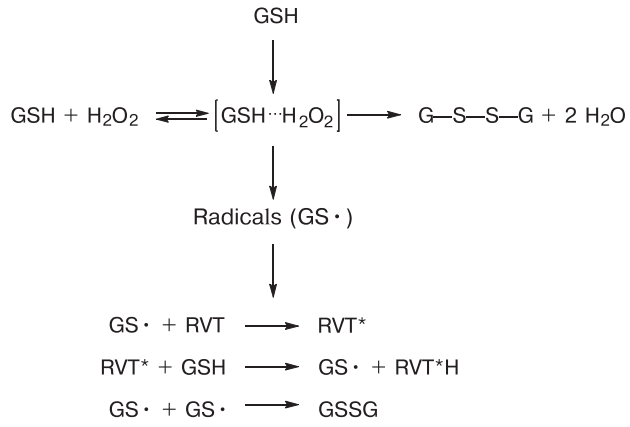


Fig. 3. Kinetic curves of consumption of acceptor **A** (0.009 mmol L^{-1}) in the reaction of GSH (10 mmol L^{-1}) with H_2O_2 (2 mmol L^{-1}) in water (*1*), PBS (*2*), and phosphate buffer PB (*3*).

cording to the stoichiometry of Eq. (1), whereas less than 4 mmol L^{-1} were consumed in water. A substantial effect of NaCl and KCl in PBS (unlike sodium—potassium phosphate buffer mixture PB) on the rate of GSH consumption and W_i is remarkable. Under other equivalent conditions, W_{GSH} in PBS is seven times lower than that in PB containing no chlorides, while W_i is three times higher.

Thiol—ene reaction of glutathione with resveratrol in the presence of H_2O_2. Thiyl radicals, which are formed with relatively low rates in the reactions of GSH with H_2O_2, can initiate chain processes of *cis—trans*-isomerization and thiol—ene reaction of GSH with unsaturated compounds. We have previously shown[23,24] that the reaction of GSH with unsaturated phenol RVT in water is satisfactorily described by Scheme 1, where RVT* is the alkyl radical, the product of thiyl radical addition to the double bond of phenol.

Scheme 1



In buffer solutions PBS and PB the radical generation rates are substantially lower than those in water and, hence, a decrease in the rate of the thiol—ene reaction of GSH and RVT can be expected. The consumption rates of GSH, acceptor **A**, and RVT at the same initial concentrations of the reactants are compared in Table 1. The consumption rate of acceptor **A** in water and its doubled rate in buffer solutions PBS and PB determine the rate of radical initiation (W_i), and the W_{RVT}/W_i ratio characterizes the chain length (ν) of the thiol—ene reaction. The data in Table 1 show that the rate of RVT consumption decreased less than by three times in spite of the strong decrease in W_i in PBS. However, RVT is not consumed at all in phosphate buffer PB.

To conclude, these results explain in part why in numerous publications on glutathione almost nobody mentions the formation of radicals in the reaction of GSH with H_2O_2 and the thiol—ene reaction with unsaturated phenols. The unexpected substantial differences in effects of PBS and PB, which are close in ionic strength, on the

Table 1. Consumption rates of GSH, acceptor **A**, and RVT upon the interaction of glutathione (10 mmol L^{-1}) and H_2O_2 (2 mmol L^{-1}) in various aqueous media

Medium	Consumption rate /mol L^{-1} s^{-1}			ν*
	GSH	**A**	RVT	
Bidistilled water	$8\cdot10^{-7}$	$5.3\cdot10^{-9}$	$7.8\cdot10^{-8}$	14.7
Phosphate buffer—NaCl, pH 7.4 (PBS)	$11\cdot10^{-7}$	$4.5\cdot10^{-10}$	$2.9\cdot10^{-8}$	32
Phosphate buffer pH 7.2 (PB)	$70\cdot10^{-7}$	$1.6\cdot10^{-10}$	~0	~0

* Chain length of the thiol—ene reaction.

kinetics of the reactions of GSH with H_2O_2 and of GSH with RVT indicate a high sensitivity of GSH and GSH—H_2O_2 pair to a change in the composition of the medium, due to which they occupy the central place in determining oxidation—reduction homeostasis and redox signalization.[8—13] Perhaps, two COOH groups in the GSH molecule play a certain role in the sensitivity to the composition of the cations.

This work was financially supported by the Russian Foundation for Basic Research (Project No. 18-33-00742) and in the framework of the state task of the Federal Agency for Scientific Organizations of Russia (Project No. 0082-2018-0006, registration No. AAAA-A18-118020890097-1).

References

1. R. Kheirabadi, M. Izadyar, *J. Phys. Chem. A.*, 2016, **120**, 10108; DOI: 10.1021/acs.jpca.6b11437.
2. E. C. Kritzinger, F. F. Bauer, W. J. du Toit, *J. Agric. Food Chem.*, 2013, **61**, 269; DOI.org/10.1021/jf303665z.
3. S. Saito, J. Kawabata, *J. Agric. Food Chem.*, 2004, **52**, 8163.
4. C. C. Winterbourn, D. Metodieva, *Methods Enzymol.*, 1995, **252**, 81.
5. A. Gambuti, G. Han, A. L. Peterson, A. L. Waterhouse, *Am. J. Enol. Vitic.*, 2015, **66**, 411.
6. Y. Wang, M. Qiao, J. J. Mieyal, L. M. Asmis, R. Asmis, *Free Radical Biol. Med.*, 2006, **41**, 775.
7. F. Q. Schafer, G. R. Buettner, *Free Radical Biol. Med.*, 2001, **30**, 1191.
8. S. C. Albrecht, A. Barata, J. Großhans, A. A. Teleman, T. P. Dick, *Cell Metabolism.*, 2011, **14**, 819; DOI 10.1016/j.cmet.2011.10.010.
9. S. Weschawalit, S. Thongthip, P. Phutrakool, P. Asawanonda, *Clin. Cosmet. Investig. Dermatol.*, 2017, **10**, 147.
10. A. Altıntaşa, K. Davidsena, C. Gardea, U. H. Mortensena, J. C. Brasen, T. Sams, C. T. Workman, *Free Radical Biol. Med.*, 2016, **101**, 143.
11. H. S. Marinho, C. Real, L. Cyrne, H. Soares, F. Antunes, *Redox Biol.*, 2014, **92**, 535.
12. H. Sies, *Redox Biol.*, 2017, **11**, 613; DOI.org/10.1016/j.redox.2016.12.035.
13. C. C. Winterbourn, M. B. Hampton, *Free Radical Biol. Med.*, 2008, **45**, 549.
14. J. C. Deutsch, C. R. Santhosh-Kumar, J. F. Kolhouse, *J. Chromatogr. A.*, 1999, **862**, 161.
15. H. Petzolda, P. J. Sadler, *Chem. Commun.*, 2008, 4413; DOI: 10.1039/b805358h.
16. B. Singh, R. S. Das, R. Banerjee, S. Mukhopadhyay, *Inorg. Chim. Acta*, 2014, **418**, 51.
17. C. Chatgilialoglu, V. W. Bowry, *J. Org. Chem.*, 2018, **83**, 9178; DOI: 10.1021/acs.joc.8b01216.
18. Z. Abedinzadeh, M. Gardes-Albert, C. Ferradini, *Can. J. Chem.*, 1989, **67**, 1247.
19. M. Takashima, M. Shichiri, Y. Hagihara, Y. Yoshida, E. Niki, *Biofactor; 2012 International Union of Biochemistry and Molecular Biology*, *Inc.*, 2012, **38**, 240.
20. K. M. Zinatullina, O. T. Kasaikina, V. A. Kuzmin, N. P. Khrameeva, *Kinet. Catal.*, 2019, **60**, 266.
21. K. M. Zinatullina, O. T. Kasaikina, V. A. Kuzmin, N. P. Khrameeva, B. I. Shapiro, *Russ. Chem. Bull.*, 2017, **66**, 1300.
22. K. M. Zinatullina, N. P. Khrameeva, O. T. Kasaikina, V. A. Kuzmin, *Russ. Chem. Bull.*, 2018, **67**, 726; DOI 10.1007/s11172-018-2129-0.
23. K. M. Zinatullina, N. P. Khrameeva, O. T. Kasaikina, V. A. Kuzmin, B. I. Shapiro, *Russ. Chem. Bull.*, 2017, **66**, 2145; DOI 10.1007/s11172-017-1995-1.
24. K. M. Zinatullina, N. P. Khrameeva, O. T. Kasaikina, *Bulg. Chem. Comm.*, 2018, **50**, 25.
25. K. M. Zinatullina, O. T. Kasaikina, V. A. Kuzmin, N. P. Khrameeva, B. I. Shapiro, *Russ. Chem. Bull.*, 2016, **65**, 2825.
26. A. V. Ivanova, E. L. Gerasimova, Kh. Z. Brainina, *Crit. Rev. Anal. Chem.*, 2015, **45**, 311; DOI: 10.1080/10408347.2014.910443.
27. Kh. Z. Brainina, A. V. Ivanova, E. N. Sharafutdinova, E. L. Lozovskaya, E. I. Shkarina, *Talanta*, 2007, **71**, 13.
28. P. Langcake, R. J. Pryce, *Physiol. Plant Pathol.*,1976, **9**, 77.
29. L. Frémont, Life Sci., 2000, **66**, 663.
30. S. Renaud, M. de Lorgeril, *Lancet*, 1992, **339**, 1523.
31. M. Jang, L. Cai, G. O. Udeani, K. V. Slowing, C. E. Thomas, C. W. Beecher, H. H. Fong, N. R. Farnsworth, A. D. Kinghorn, R. G. Mehta, R. C. Moon, J. M. Pezzuto, *Science*, 1997, **275**, 218.
32. G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70.
33. C. D. Pereira, N. Minamino, T. Takao, *Anal. Chem.*, 2015, **87**, 10785.
34. C. K. Riener, G. Kada, H. J. Gruber, *AnalBioanalChem*, 2002, **373**, No. 4—5, 36; DOI: 10.1007/s00216-002-1347-2.
35. B. Frei, R. Stocker, B. N. Ames, *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 9748.
36. V. A. Roginsky, *Arch. Biochem. Biophys.*, 2003, **414**, 261.

Received February 9, 2019;
in revised form March 21, 2019;
accepted May 23, 2019