Biochemistry (Moscow), Vol. 66, No. 5, 2001, pp. 505-514. Translated from Biokhimiya, Vol. 66, No. 5, 2001, pp. 628-639.

Initiation and Inhibition of Free Radical Processes in H_2O_2–Metmyoglobin (Methemoglobin)–2,2′-Azino-bis-(3-Ethylbenzthiazoline-6-Sulfonic Acid) Systems

D. I. Metelitza[1]*, A. N. Eryomin[1], D. O. Sviridov[2], and V. S. Kamyshnikov[2]

[1]*Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, ul. Kuprevicha 5/2, Minsk, 220141 Belarus; fax: +375 (172) 63-7274; E-mail: enzyme@ns.iboch.ac.by*
[2]*Belorussian State Institute of Physician Improvement, ul. P. Brovki 3, Minsk, 220714 Belarus; fax: +375 (172) 32-2533*

Received July 3, 2000
Revision received December 25, 2000

Abstract—Rates of free radical initiation were determined at 20°C in 10 mM phosphate buffer (pH 7.4) in the systems metmyoglobin (methemoglobin)–H_2O_2 using 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) as the diammonium salt (ABTS). The catalytic activity of MetMb was 2-3-fold higher than that of MetHb. The process can be described by the Michaelis–Menten equation, from which effective values of K_m and V_{max} were calculated. Comparative kinetic studies on the inhibition of ABTS oxidation were carried out using Trolox, propylgallate (PG), polydisulfide of gallic acid (poly(DSG)), polydisulfide of (2-amino-4-nitrophenol) (poly(ADSNP)), and its conjugate with human serum albumin (HSA–poly(ADSNP)). The inhibitors were characterized by inhibition constants K_i and stoichiometric inhibition coefficients f (the number of radicals terminated by a single molecule of inhibitor). The minimum K_i and the maximum f values were obtained for poly(DSG), and in the system of MetHb–H_2O_2–ABTS they were 0.08 μM and 27.5, respectively. According to their antiradical activities, the inhibitors can be arranged as follows: poly(DSG) > poly(ADSNP) > PG > Trolox. PG, poly(DSG), poly(ADSNP), and its conjugate with HSA are suggested as "calibrators", i.e., inhibition standards for evaluation of antioxidant status of biological fluids in possible test systems based on the free radical-generating pair MetMb–H_2O_2 with ABTS as the acceptor of the active radicals.

Key words: metmyoglobin, methemoglobin, hydrogen peroxide, free radical initiation, inhibitors, Trolox, gallic acid polydisulfide, propylgallate, antioxidant status, inhibition constants, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)

Biochemical systems generating oxygen-containing free radicals $O_2^{\cdot-}$, $HO^{\cdot}$ ($RO^{\cdot}$), and $HO_2^{\cdot}$ ($RO_2^{\cdot}$) constantly attract interest because of the important role of these active species in biological systems [1-9]. Direct measurement of free radical concentration *in vitro* (and especially *in vivo*) is usually very difficult [5, 8]. Therefore, the initiation of free radical processes has been mainly studied with inhibitors, i.e., by an approach based on works of Academician N. M. Emanuel and his followers in the 1950-1960s [10-13]. The use of inhibitors even in relatively simple chemical systems is complicated by solvation of radicals and molecules of the inhibitors themselves [11]; the difficulties are increased further in quantitative studies of the effects of inhibitor in biochemical systems [1-9]. The need to determine quantitative characteristics of radical initiation under natural conditions and the requirement for a rapid screening of possible inhibitors (antioxidants) in relatively simple reactions generating active oxygen-containing radicals under mild conditions (20-36°C, aqueous solutions) has stimulated many *in vitro* studies on numerous pairs including H_2O_2 (ROOH) and an iron-containing biocatalyst, where ROOH are organic hydroperoxides or lipid hydroperoxides. The following compounds have been used as biocatalysts: hemin [14-16], its complexes with albumins (methemalbumins) [14-16], MetMb [17, 18], MetHb [17-19], cytochrome P-450-LM2 [17], microperoxidase [20], and iron-containing proteins (ferritin [21-23] and hemosiderin [24]).

Abbreviations: ABTS) 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; MetHb) methemoglobin; MetMb) metmyoglobin; poly(ADSNP)) polydisulfide of (2-amino-4-nitrophenol); PG) gallic acid propyl ester (propylgallate); poly(DSG)) polydisulfide of gallic acid; HSA) human serum albumin; TMB) 3,3′,5,5′-tetramethylbenzidine; PB) phosphate buffer (pH 7.4); InH) inhibitor of free radical reactions.

* To whom correspondence should be addressed.

0006-2979/01/6605-0505$25.00 ©2001 MAIK "Nauka/Interperiodica"

Aliphatic alcohols (methanol, ethanol) [17] and certain typical peroxidase substrates such as ABTS [17], luminol or isoluminol [20, 25], TMB [14-16, 21-23], *o*-phenylenediamine, and other aromatic amines [14-16, 21, 22] can be used as the acceptors of free radical generated in the above-listed systems. ABTS and TMB are especially suitable because the colored products of their oxidation have intense absorption bands in the visible region which do not overlap with the absorption bands of the biocatalysts and other possible components of the reaction mixtures (e.g., inhibitors); this provides for reliable spectrophotometric monitoring of initial oxidation rates of these acceptors [14-19, 21-23]. The use of luminol (isoluminol) provides high accuracy in the evaluation of initial oxidation rates by chemiluminescence [19, 20, 25].

Free radical generation in H_2O_2 (ROOH)–biocatalyst systems can be slowed with various inhibitors including such natural inhibitors as ascorbic acid and α-tocopherol [7, 19] or its soluble analog 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) [18, 19], mannitol, thiourea, α-naphthol [17], polyphenols [2, 26], and new generation antioxidants such as polydisulfides of substituted phenols [16, 23, 27, 28], urea, thiourea, and biuret [23]. Inhibitors with different efficiency compete for free radicals with radical acceptors (ABTS, TMB, luminol, etc.) and decrease the rates of acceptor expenditure, and this is used for testing the antiradical activities of antioxidants [15, 16, 19, 23, 26].

The inhibition of reactions in H_2O_2–biocatalyst–radical acceptor systems is interesting also in connection with the use of such systems in quantitative evaluation of the functional state of antioxidant protection in the human body, in particular, when methods are being developed for determination of the total antioxidative activity of biological fluids, e.g., of blood plasma [18-20, 25]. Available methods for evaluation of antioxidant activity of biological fluids are usually labor-consuming and difficult to apply for mass scale laboratory use: to date a commercial kit (Randox, Great Britain) for determination of total antioxidant status of human blood remains the best solution of this problem [18]. In this case, the H_2O_2–MetMb–ABTS system and Trolox as the antioxidant (the so-called "calibrator") are used, and this allows us to express the total antioxidant activity of serum and other fluids as the equivalent concentration of Trolox (the Trolox equivalent is a compound quantity in moles which is equal in the antiradical activity to 1 mole of Trolox) [18].

The development of methods for determination of the total antioxidative activity of biological fluids based on H_2O_2 (ROOH)–biocatalyst–radical acceptor systems is associated with some problems and complications: first, the rates of radical initiation in these systems should be rather high and have a level required; second, the presence in the system of additional organic solvents (dimethyl sulfoxide, dimethylformamide, ethanol, etc.) is undesirable because of their high antiradical activities [15]; third, the expenditure kinetics of radical acceptors (ABTS, TMB, *o*-phenylenediamine, etc.) in the presence of inhibitors are often characterized by an induction period ($\Delta\tau$) [14-16, 18, 26]; therefore, it is necessary to choose an approach for quantitative characterization of the inhibitor effect by the $\Delta\tau$ value or by a decrease in the initial rate of the acceptor expenditure Δv_o; fourth, depending on the specific features of the inhibitor, it can differently interact with biocatalysts and other protein components of the system resulting in dissimilarity of the antiradical activity of the inhibitor [15, 16, 29]; fifth, all components of the system should be well soluble in the reaction medium and compatible to each another; sixth, only such system is technologically promising that consists of time-stable components with characteristics reproducible during the oxidation (initial rate of acceptor oxidation, direct relation of $\Delta\tau$ values with inhibitor concentrations, etc.).

The purpose of this work was to compare the kinetic characteristics of the catalytic the H_2O_2–metmyoglobin (methemoglobin) systems using the radical acceptor ABTS and polyphenol antioxidants such as propylgallate, gallic acid polydisulfide, polydisulfide of (2-amino-4-nitrophenol), and its conjugate with human serum albumin, and also to compare them with the widely used inhibitor Trolox.

MATERIALS AND METHODS

Human serum albumin from Reanal (Hungary) was used without additional purification; horse heart metmyoglobin was from Sigma (USA). Methemoglobin was isolated from cattle blood, lyophilized, and kindly presented to us by A. P. Vlasov (Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Minsk). Diluted perhydrol (DIAplus, Russia) was used as an oxidant and the diammonium salt of ABTS (Sigma) was used as a radical acceptor.

Inhibitors. Trolox was from Aldrich (USA) and propylgallate was from Reakhim (Russia). Poly(DSG) with mean molecular weight ~1760 daltons and containing seven monomer units [HOOC-$C_6(OH)_3$-S-S-] was synthesized as described earlier [27]. The UV spectrum of poly(DSG) solution in distilled water was characterized by two maxima with absorption coefficients ε_{215} and ε_{266} equal to 421 and 126 $mM^{-1}\cdot cm^{-1}$, respectively. An aqueous solution of poly(DSG) (2.25 mM) was used; it was stored at ~6°C. Poly(ADSNP) with mean molecular weight of ~1400 daltons and containing ~7 monomer units [HO-C_6H_3-(NO_2)-NH-S-S-] was synthesized as described earlier [28]. The UV spectrum of poly(ADSNP) solution in distilled water was characterized by maxima with absorption coefficients ε_{220} and ε_{255} equal to 79 and 63 $mM^{-1}\cdot cm^{-1}$, respectively. Poly(ADSNP) solution (1.62 mM) in ethanol was used.

Reagent concentrations were determined spectrophotometrically with a Specord M-40 spectrophotometer (Germany) using the absorption coefficients presented below: $\varepsilon_{230} = 72.4\ M^{-1}\cdot cm^{-1}$ for H_2O_2 [30]; $\varepsilon_{280} = 35\ mM^{-1}\cdot cm^{-1}$ for HSA [31]; $\varepsilon_{417} = 119\ mM^{-1}\cdot cm^{-1}$ for MetMb [32]; $\varepsilon_{405} = 151\ mM^{-1}\cdot cm^{-1}$ for MetHb [33]; $\varepsilon_{340} = 36\ mM^{-1}\cdot cm^{-1}$ for ABTS [34]; $\varepsilon_{292} = 3.26\ mM^{-1}\cdot cm^{-1}$ for Trolox [35].

Synthesis of HSA–poly(ADSNP) conjugate. Poly(ADSNP) (355 μM) and HSA (7.1 μM) were dissolved in 5.0 mM bicarbonate buffer (pH 9.0) containing 31% ethanol, and the mixture was kept for two days at ~16°C. The conjugate resulting through thiol–disulfide exchange was washed from the unbound polydisulfide first with water–ethanol solution and then with distilled water using a PAN-20 ultrafiltration membrane (MIFIL, Minsk) with retention limit of 20-kD protein. The conjugate was stored in aqueous solution at ~6°C. The concentration of poly(ADSNP) covalently bound to HSA was calculated by the difference in light absorption of the experimental and control specimens at 364 nm at the absorption coefficient of poly(ADSNP) $\varepsilon_{364} = 31.0\ mM^{-1}\cdot cm^{-1}$. The calculation showed that in the conjugates of HSA there is, on average, ~1.5 polydisulfide molecules per molecule of HSA.

Oxidation of ABTS with hydrogen peroxide with the involvement of MetMb and MetHb in the absence and in the presence of inhibitors was carried out at 20°C in 10 mM PB (pH 7.4). Concentrations of catalysts, ABTS, H_2O_2, and of inhibitors are shown in the figure captions and in the text. The oxidation of ABTS was initiated by addition of biocatalyst to the medium and was followed by increase in the absorption of the ABTS oxidation product at 734 nm (Specord M-40). Note that the absorption band of the ABTS oxidation product does not overlap with absorption bands of transformation products of the inhibitors used, i.e., in all cases the monitoring of ABTS oxidation was correct. In the figures the initial accumulation rates of the ABTS oxidation product are expressed in relative units which are equal to the change per second in the absorption of this product. The initial rates of ABTS oxidation were calculated using the absorption coefficient of the oxidation product $\varepsilon_{734} = 13.8\ mM^{-1}\cdot cm^{-1}$, which was 2.6-fold lower than the absorption coefficient of the cation-radical $(ABTS)^{\cdot+}$, $\varepsilon_{340} = 36.0\ mM^{-1}\cdot cm^{-1}$ [34].

Quantitative characteristics of inhibitor efficiency. The inhibitor efficiencies in the oxidation of ABTS were compared by the inhibition constants K_i: to provide this, dependences of the inverse initial rate of the inhibited reaction were plotted against the inhibitor concentration (Dixon's coordinates) only at one concentration of the substrate (250 μM), and the value of the intercept [InH]* on the abscissa axis was determined. The [InH]* value is known to be related with K_i by the Eq. (1) [36]:

$$[InH]^* = K_i \cdot ([S]/K_m + 1). \quad (1)$$

With knowledge of the ABTS concentration (250 μM) and the K_m value in the absence of the inhibitor (Table 1), the K_i value was calculated from Eq. (1).

The inhibited oxidation of ABTS in each case was characterized by the induction period Δτ in seconds. Stoichiometric coefficients *f* of inhibitors which describe the number of radicals destroyed per inhibitor molecule were calculated based on the theory of Emanuel et al. [10-13]. According to this theory, at the constant rate of radical initiation v_i and with linear chain breaking by the inhibitor, the induction period is determined by Eq. (2) provided that [InH] = 0:

$$\Delta\tau = f \cdot [InH]_o/v_i. \quad (2)$$

From Eq. (2) the rate of radical initiation can be evaluated with accuracy to the 1/*f* coefficient. At the stationary oxidation of substrate, the initial rate of the reaction is close to the rate of radical initiation:

$$v_o \approx v_i = f \cdot (v_i/f). \quad (3)$$

On calculating the value of v_i/f from Eq. (2), the value of *f* can be obtained from Eq. (3) by measurement of v_o, i.e., of the initial rate of ABTS oxidation in the absence of the inhibitor. Higher *f* values correspond to stronger antioxidant effect under specific conditions of a radical process.

RESULTS AND DISCUSSION

Kinetics of ABTS oxidation in H_2O_2–MetMb (MetHb) systems. Dependences of the initial rate of ABTS oxidation on initial concentrations of biocatalysts are presented in Fig. 1 (curves *1* and *2*). Within a certain concentration range of both catalysts, a first-order reaction is observed; however, at the MetMb (curve *1*) concentration >3 μM the reaction accelerates, and its order in [MetMb] becomes higher than the first. This is suggested to be associated with increasing autooxidation of the reduced myoglobin with molecular oxygen that will be discussed later. In the presence of Trolox as the inhibitor, the reaction remains first-order in the concentrations of both biocatalysts (curves *3* and *4*).

In the presence of Trolox, kinetic curves of accumulation of ABTS oxidation products are characterized by induction periods which strongly decrease with increase in concentrations of both biocatalysts (curves *5* and *6*). Note, that after complete expenditure of Trolox, the initial rate of ABTS oxidation is lower that in its absence: in the presence of 5 μM MetMb or MetHb v_o values decrease 1.1- and 1.5-fold, respectively, i.e., during the catalysis the heme proteins are partially inactivated by radicals generated during the decomposition of H_2O_2.

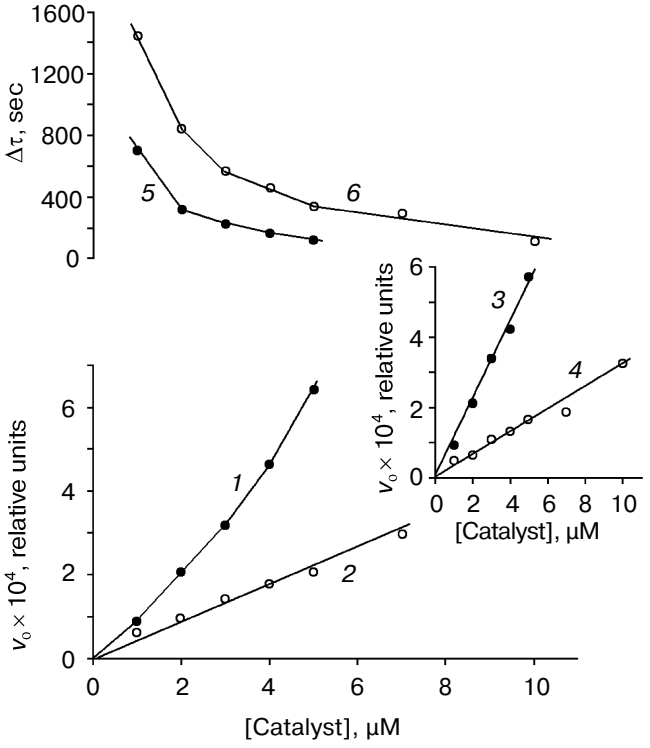

Fig. 1. Dependences of the initial rate of ABTS (150 μM) oxidation with hydrogen peroxide (75 μM) and of the induction periods Δτ on initial concentrations of MetMb (*1*, *3*, *5*) and MetHb (*2*, *4*, *6*) without the inhibitor (*1*, *2*) and in the presence of 4.38 μM Trolox (*3*, *4*, *5*, *6*) in 10 mM PB (pH 7.4) at 20°C.

Under comparable conditions, the catalytic activities of the heme proteins are significantly different: MetMb is 2.7-fold more active than MetHb in the absence of Trolox and 3.5-fold more active in its presence. It is likely that radicals cause a stronger destruction of the protein in the case of degradation of four peroxide complexes of oligomeric MetHb than in the case of monomeric MetMb, and this results in a nonproductive expenditure of H_2O_2 for destruction of the biocatalyst itself specific for many heme proteins [4, 37].

Figure 2 shows dependences of the initial rate of ABTS oxidation on its concentration during the reactions with MetMb (curves *1* and *3*) and MetHb (curves *2* and *4*) in the presence of Trolox (curves *3* and *4*) and in its absence (curves *1* and *2*). Values of Δτ strongly decrease with increase in the concentration of ABTS (curves *5* and *6*). The dependences in double reciprocal coordinates have breaks, and the features of these dependences suggest that two sites should exist for ABTS binding to both MetMb (curves *1* and *3*) and MetHb (curves *2* and *4*). From the data presented in Fig. 2, the pairs of V_{max} and K_m values were determined for both biocatalysts: at $[ABTS]_o > 90$ μM, the two kinetic parameters are significantly increased (Table 1). The presence of Trolox (4.38 μM) does not change the character of $1/v_o$ dependences on $1/[ABTS]_o$ but increases K_m and decreases V_{max}; thus, during ABTS oxidation with the involvement of both heme proteins Trolox displayed a mixed-type effect that was similar to effects of two other inhibitors, poly(DSG) and poly(ADSNP) (Table 1). This type of inhibition is caused by competition between the inhibitors and substrate for active radicals and by interaction of the inhibitors with the biocatalysts themselves that affects their activities: e.g., a direct interaction of poly(DSG) with the proteins was shown for catalase by CD spectroscopy [38] and for HSA and BSA by fluorescence spectroscopy [29].

Figure 3 presents dependences of $1/v_o$ on $[H_2O_2]_o$ during the oxidation of ABTS (250 μM) with the involvement of MetMb (curves *1* and *3*) and MetHb (curves *2*

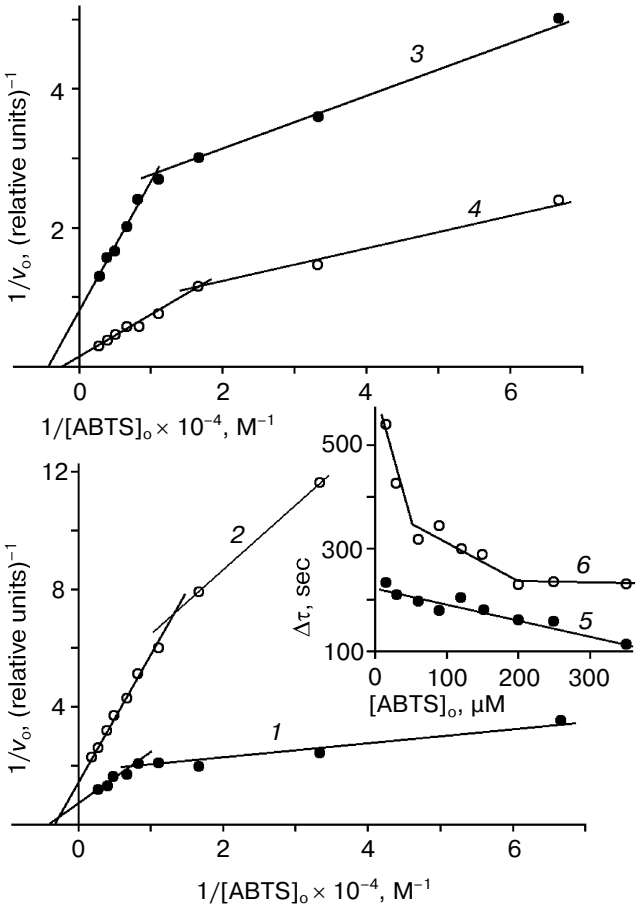

Fig. 2. Dependences of the initial rate of ABTS oxidation with hydrogen peroxide (75 μM) on the initial substrate concentration in double reciprocal coordinates and of the induction periods Δτ on $[ABTS]_o$ with the involvement of 5 μM MetMb (*1*, *3*, *5*) and 5 μM MetHb (*2*, *4*, *6*) without the inhibitor (*1*, *2*) and in the presence of 4.38 μM Trolox (*3-6*) in 10 mM PB (pH 7.4) at 20°C.

Table 1. Kinetic characteristics of ABTS peroxidation with the involvement of 5 μM MetMb or MetHb in 10 mM phosphate buffer (pH 7.4) at 20°C

Substrate/antioxidant	MetMb		MetHb	
	K_m, μM	$V_{max} \times 10^8$, M · sec^{-1}	K_m, μM	$V_{max} \times 10^8$, M · sec^{-1}
From dependences of v_o on $[ABTS]_o$				
ABTS				
< 90 μM	14.6	4.23	69.9	2.04
> 90 μM	243.9	10.60	322.6	5.25
ABTS + 4.38 μM Trolox				
< 90 μM	17.2	3.12	31.3	0.96
> 90 μM	266.7	9.78	357.1	4.58
From dependences of v_o on $[H_2O_2]_o$				
H_2O_2	78.1	10.60	167.0	7.10
+ 10 μM Trolox	158.7	10.70	—	—
+ 0.5 μM poly(DSG)	99.0	3.00	—	—
+ 0.5 μM poly(ADSNP)	149.3	10.10	—	—

and *4*) in the presence of Trolox (curves *3* and *4*) and in its absence (curves *1* and *2*). In all cases, the dependences are described by the Michaelis–Menten equation. The values of V_{max} and K_m (Table 1) were calculated from the data presented in Fig. 3. With increase in the H_2O_2 concentration, the Δτ values decreased monotonically (Fig. 3, curves *5* and *6*). Table 1 shows that Trolox, poly(DSG), and poly(ADSNP) increase K_m and change V_{max}. Values of V_{max} for MetMb obtained from the $1/v_o$ dependence on $1/[ABTS]_o$ ($1.06 \cdot 10^{-7}$ M·sec^{-1}) and from the $1/v_o$ dependence on $1/[H_2O_2]_o$ ($1.01 \cdot 10^{-7}$ M·sec^{-1}) were virtually the same, whereas values of v_o obtained for MetHb in two experimental series were different ($5.2 \cdot 10^{-6}$ and $7.1 \cdot 10^{-6}$ M·sec^{-1}) that was associated with a pronounced nonproductive expenditure of H_2O_2 during the oxidation with the involvement of MetHb.

Thus, under comparable conditions MetMb and MetHb clearly displayed pseudoperoxidase activity during ABTS oxidation with hydrogen peroxide. The process is formally described by the Michaelis–Menten equation: the comparison of V_{max} values in two experimental series (Table 1) shows that the catalytic activity of MetMb is 1.5-2.0-fold higher than that of MetHb. Values of the catalytic constants for MetMb and MetHb (0.021 and 0.0105 sec^{-1}) rank significantly below these constants for peroxidases [4] but are quite comparable to the catalytic constants for ferritin during oxidation of aromatic amines (0.18-3.33 sec^{-1}) [22] and are of the same order as the catalytic constants of *o*-phenylenediamine oxidation with the involvement of hemin (0.013 sec^{-1}) or of TMB oxidation with the involvement of hemin or methemalbumin (0.017 and 0.0116 sec^{-1}) [16]. According to the catalytic activities during the peroxidation of amines, biocatalysts

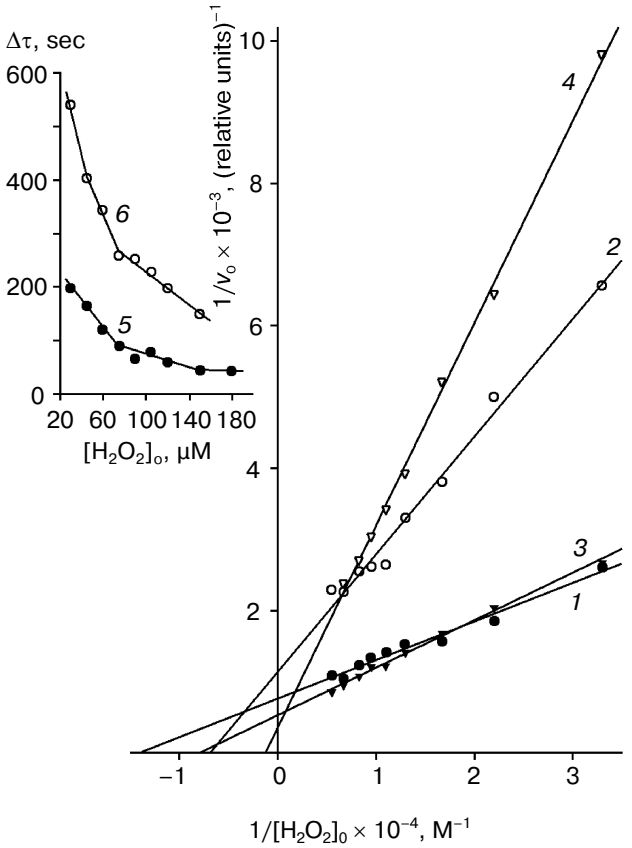

Fig. 3. Dependences of the initial rate of ABTS (250 μM) oxidation on the initial concentration of hydrogen peroxide in double reciprocal coordinates and of the induction periods Δτ on $[H_2O_2]_o$ with the involvement of MetMb (*1*, *3*, *5*) and MetHb (*2*, *4*, *6*) without the inhibitor (*1*, *2*) and in the presence of 4.38 μM Trolox (*3*-*6*) in 10 mM PB (pH 7.4) at 20°C.

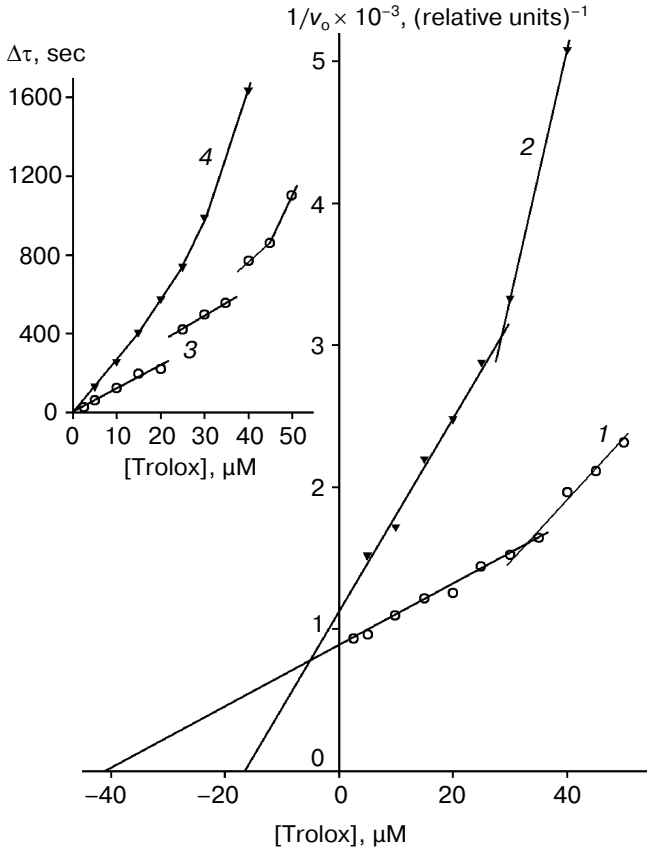

Fig. 4. Dependences of the inverse initial rate of ABTS (250 μM) oxidation with hydrogen peroxide (150 μM) and of the induction periods Δτ on the concentration of Trolox with the involvement of MetMb (*1*, *3*) and MetHb (*2*, *4*): 5 μM catalyst in 10 mM PB (pH 7.4) at 20°C.

can be arranged as follows: peroxidases >> ferritin > MetMb > MetHb ≈ methemalbumin > hemin.

Comparative characteristics of inhibiting effects of Trolox and polyphenol antioxidants in MetMb (MetHb)–H_2O_2–ABTS systems. Figure 4 presents dependences of the inverse initial rate of ABTS oxidation with hydrogen peroxide on increasing Trolox concentration (Dixon's coordinates [36]) with the involvement of MetMb (*1*) and MetHb (*2*). The linear dependences are characterized by fractures which reflect the binding of Trolox to proteins by at least two sites, and this results in a dissimilarity of the bound inhibitor with respect to free radicals and affects values of the Trolox inhibition constants calculated by Eq. (1) (see "Materials and Methods"): as follows from Table 2, the K_i value for Trolox is about twofold lower in the case of MetHb than in the case of MetMb. Afterwards, we determined and analyzed for all inhibitors only K_i values which characterize the protein binding sites with increased affinity for the inhibitor (up to the breaks in the Dixon's dependences).

Figure 4 (curves *3* and *4*) presents dependences of the induction periods Δτ on the Trolox initial concentration for MetMb (*3*) and MetHb (*4*): in accordance with the theory of the inhibitor method [10-13], up to certain concentrations of Trolox a strict linear dependence of Δτ on $[InH]_o$ is observed. Therefore, the dependences (*3*, *4*) up to the Trolox concentration of 20 μM and Eqs. (2) and (3) were used at first for calculation of v_i/f values and then the stoichiometric coefficients for the inhibitor were obtained: ~0.83 (MetMb) and 1.7 (MetHb); i.e., in the radical-initiating systems of MetMb (MetHb)–H_2O_2, Trolox reacts with one or two radicals, which is specific for many known inhibitors [39]. The difference in f in the case of the two biocatalysts seems to be caused by the different location of the protein-bound Trolox relative to the active center of free radical generation (the peroxide complex of heme iron in the case of the two hemoproteins). Note a "jump-like" dependence of Δτ on the Trolox concentration in the case of MetMb (Fig. 4, curve *3*) that is suggested to be associated with changes in the state of the inhibitor itself with the increase in its concentration because of micelle formation, though in this case it is unclear why this dependence is smoothed for MetHb (Fig. 4, curve *4*).

Table 2. Quantitative parameters of ABTS oxidation with hydrogen peroxide with the involvement of MetMb and MetHb (250 μM ABTS, K_m values for MetMb and MetHb are 244 and 322 μM, respectively)

InH	Biocatalyst	$v_0 \times 10^8$, M·sec^{-1}	$(v_i/f) \times 10^8$, M·sec^{-1}	f	[InH]*, μM	K_i, μM
Trolox	MetMb	8.3	10.0	0.83	40.7	20.1
	MetHb	5.6	3.3	1.70	17.3	9.8
PG	MetMb	4.4	2.2	2.00	6.2	3.07
	MetHb	3.1	1.9	1.63	10.8	6.08
Poly(ADSNP)	MetMb	5.6	0.4	14.0	3.0	1.5
Poly(DSG)	MetMb	4.4	0.17	25.8	0.32	0.16
	MetHb	3.3	0.12	27.5	0.15	0.08

Note: Values of K_i are calculated by Eq. (1); values of f are calculated using Eqs. (2) and (3).

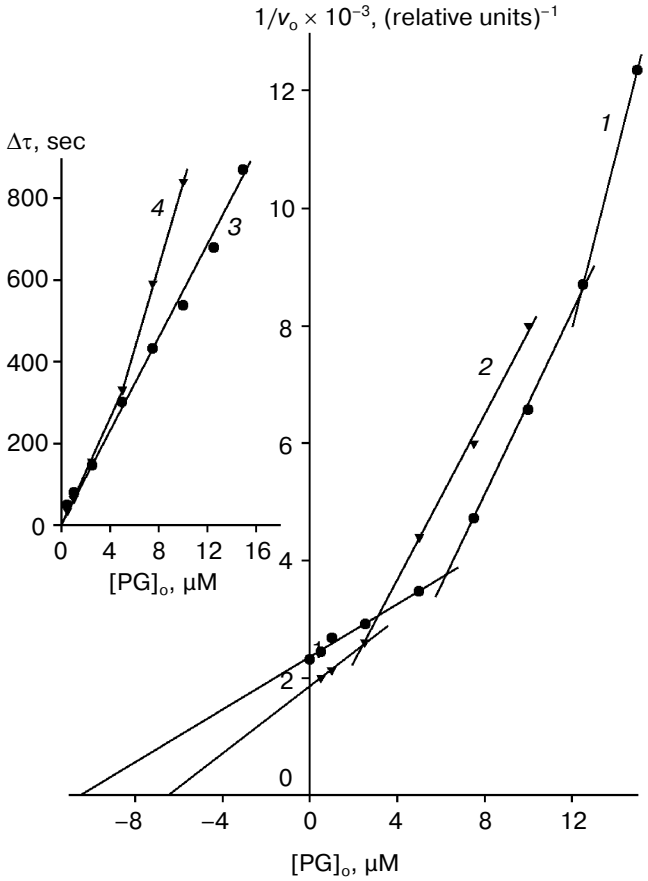

Fig. 5. Dependence of the inverse initial rate of ABTS (250 μM) oxidation with hydrogen peroxide (75 μM) and of the induction periods Δτ on the initial concentration of propylgallate with the involvement of MetMb (*1*, *3*) and MetHb (*2*, *4*): 5 μM catalyst in 10 mM PB (pH 7.4) at 20°C.

Dependences for propylgallate and polymer inhibitors poly(DSG), poly(ADSNP), and its conjugate HSA–poly(ADSNP) were obtained and processed similarly.

Figure 5 (curve *1*) presents in Dixon's coordinates the dependence for PG in the MetMb–H_2O_2 system; and from this dependence the [InH]* value of 10.8 μM was determined graphically, and then by the Eq. (1) the value of K_i = 6.08 μM (Table 2) was obtained. The lower value of K_i = 3.07 μM was obtained for PG in the MetMb–H_2O_2 system. Figure 5 (curves *3* and *4*) shows that in certain concentration ranges there is a linear correlation between Δτ and the increasing concentration of PG: from the dependences *3* and *4* by Eqs. (2) and (3) values of v_i/f and f were calculated; these were 2.0 and 1.63 in the case of MetMb and MetHb, respectively, which is specific for many polyphenol inhibitors [39]. The relatively low value of K_i for PG in the MetMb–H_2O_2 system seems to be caused by the coordination of propylgallate with the heme iron that prevents the MetMb complexing with peroxide and markedly decreases the rate of radical initiation, i.e., in addition to the inhibitor competition with substrate for active radicals there is another way for inhibition of the ABTS oxidation that has mixed character (Table 1).

Figure 6 presents in Dixon's coordinates dependences for the polymeric inhibitor poly(DSG) in the systems with MetMb (*1*) and MetHb (*2*): obviously, the polymeric antioxidant, similarly to its monomeric analog PG, bound to the protein by two sites that corresponded to different concentrations of InH. From the data presented in Fig. 6 (curves *1* and *2*) and Eq. (1), the K_i values were found to be 0.16 and 0.08 μM for MetMb and MetHb, respectively (Table 2), i.e., poly(DSG) is the most active inhibitor with antiradical activity 122-125 times higher than the activity of Trolox (Table 2).

Figure 6 (curves *3* and *4*) shows that at certain concentrations of the polymeric inhibitor, Δτ has a linear correlation with $[InH]_o$. In accordance with the inhibitor method theory, the v_i/f values and coefficients f were calculated for linear regions of the dependences *3*, *4* (Table 2): the f values for poly(DSG) are many times higher than the standard values (1-2), similarly to the case of another polymeric inhibitor, poly(ADSNP); the effect of the lat-

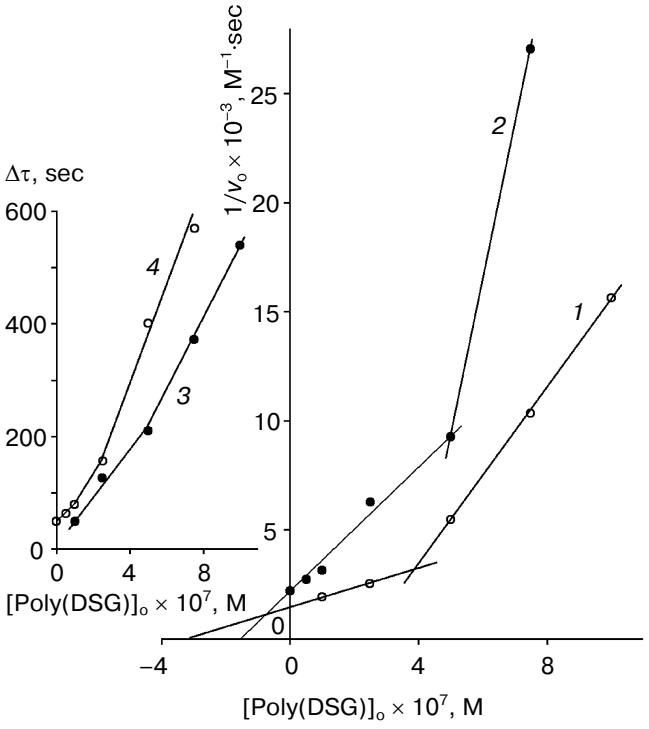

Fig. 6. Dependences of the inverse initial rate of ABTS (250 μM) oxidation with hydrogen peroxide (75 μM) and of the induction periods Δτ on the initial concentration of poly(DSG) with the involvement of MetMb (*1*, *3*) and MetHb (*2*, *4*): 5 μM catalyst in 10 mM PB (pH 7.4) at 20°C.

ter is presented in the diagram in Fig. 7. On referring the *f* values to the number of monomer units in polymeric inhibitors, we obtain for the monomer unit of poly(DSG) ($n = 7$) *f* equal to 3.7-3.9, and for the monomer unit of poly(ADSNP) ($n \approx 7$) $f \approx 2$. Thus, the high antiradical activity of poly(ADSNP) is explained by a simple additivity of the effects of its monomer units, whereas the antiradical effectiveness of poly(DSG) is significantly higher than the summarized effect of propylgallate which is an analog of its monomer units: on the calculation, $f = 2 \times 7 = 14$, whereas the experimental value of *f* for poly(DSG) is 25.8-27.5 (Table 2), i.e., poly(DSG) demonstrates a so-called "intramolecular" synergic effect that has been shown by us for the radical-initiating system of ferritin-H_2O_2 [23]. Based on the findings in the MetMb (MetHb)–H_2O_2 systems (Table 2), the coefficient of intramolecular synergism of poly(DSG) can be determined by comparison of the experimental and calculated *f* values (25.8-27.5 and 14.0, respectively): the resulting coefficient is 1.84-1.96, i.e., about 2.

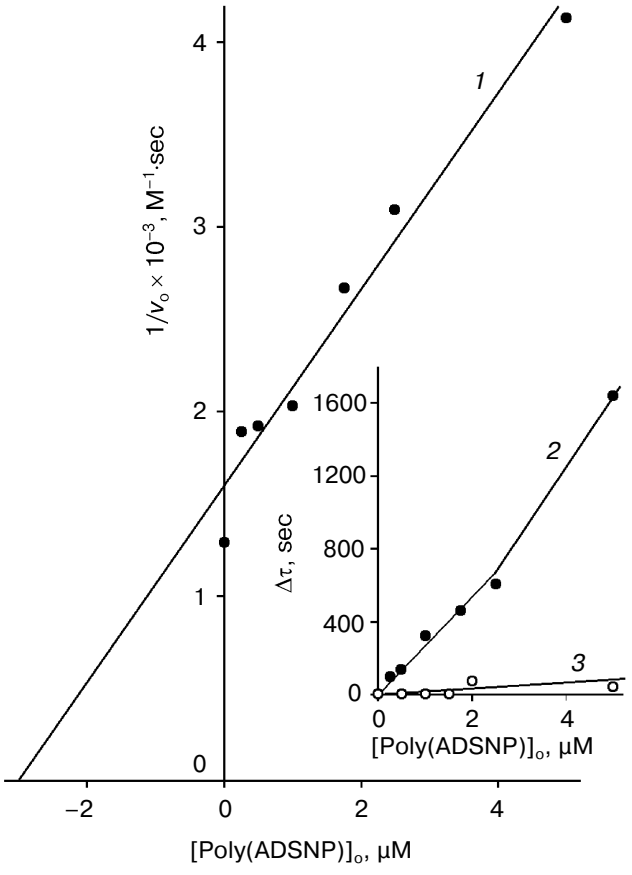

Fig. 7. Dependences of the inverse initial rate of ABTS (250 μM) oxidation with hydrogen peroxide (75 μM) and of the induction periods Δτ on the initial concentrations of poly(ADSNP) (*1*, *2*) and of HSA–poly(ADSNP) (*3*) with the involvement of 5 μM MetMb; 10 mM PB (pH 7.4) at 20°C.

The high *f* value for polydisulfides of substituted phenols can be caused, in particular, through the exchange reaction between aminyl radicals and phenols that was discovered by Emanuel and coworkers [40, 41]:

$$AmNH^{\cdot} + PhOH \leftrightarrow AmNH_2 + PhO^{\cdot}. \qquad (4)$$

If the reaction goes from the right to the left, a phenol inhibitor is repeatedly regenerated during the oxidation; if the reaction goes from the left to the right, an amine component is regenerated. In the MetMb (MetHb)–H_2O_2 systems, the regeneration of poly(DSG) and PG is very likely to occur during the interaction of the corresponding phenoxyls with ABTS through the exchange reaction presented above.

It has been shown by special experiments that the covalent binding of poly(ADSNP) with HSA about 2.6-fold decreases the inhibiting effect of the polydisulfide: the initial rate of ABTS oxidation in the MetMb–H_2O_2 system becomes half as high at the concentrations of poly(ADSNP) 0.77 μM and of HSA–poly(ADSNP) 2.0 μM; thus, HSA in the conjugate prevents the interaction of MetMb with the inhibitor, and this decreases its antiradical activity. It is important that when HSA–poly(ADSNP) is used as an inhibitor, the initial rate of ABTS oxidation decreases, but the induction period Δτ is observed only at the conjugate concentration 5 μM and more (Fig. 7, curve *3*).

From the practical standpoint, it is important to compare the ABTS oxidation in the MetMb (MetHb)–H_2O_2 systems over a wide range of hydrogen peroxide concentrations in the presence of Trolox and polyphenol inhibitors. Such a comparison is presented in Figs. 8 and 9. Over the whole range of H_2O_2 concentrations the initial reaction rates in the presence of 10 μM Trolox and 0.5 μM poly(ADSNP) are comparable, whereas the initial rate of the process inhibited with 0.5 μM poly(DSG) is much lower (Fig. 8, curve *3*). At comparable initial rates of ABTS oxidation inhibited in the presence of Trolox and poly(ADSNP), the Δτ value is much lower in the presence of polydisulfide (compare the dependences b, *1* and b, *2*); thus, under comparable conditions, the use of poly(ADSNP) in the possible test system for evaluation of the antioxidant status of biological fluids is significantly more advantageous than the use of Trolox: the polydisulfide concentration is 20-fold lower than that of Trolox, and the Δτ value is much lower, which can accelerate the analytical procedures with Δτ value used as a quantitative parameter. Poly(DSG) is also better than Trolox: its concentration is 20-fold lower than that of Trolox and the Δτ value is significantly lower over the whole range of H_2O_2 concentrations.

Similar dependences for the MetHb–H_2O_2 system are presented in Fig. 9: the general appearance of the dependences resembles that of the dependences obtained for the MetMb–H_2O_2 system, and the advantages of

poly(ADSNP) and poly(DSG) compared to Trolox are quite evident.

Based on our experimental findings (Figs. 4-9, Table 2) and on previous results [14-16, 21-23], practical recommendations for designing possible test systems for evaluation of total antioxidant status of biological fluids are proposed.

1. The rates required for initiation of active free radicals can be provided with the systems MetMb (MetHb)–H_2O_2, methemalbumins–H_2O_2 (ROOH) [14-16], hemin–H_2O_2 (ROOH) [14-16], and ferritin–H_2O_2 (ROOH) [21-23].

2. Water-soluble forms of ABTS as diammonium salt or TMB as dihydrochloride are the best for use as radical acceptors [14-16, 21-23] because the use of poorly soluble ABTS and TMB requires the aqueous medium to contain cosolvents (dimethyl sulfoxide, dimethylformamide, etc.), and this is undesirable because of their ability to accept free radicals.

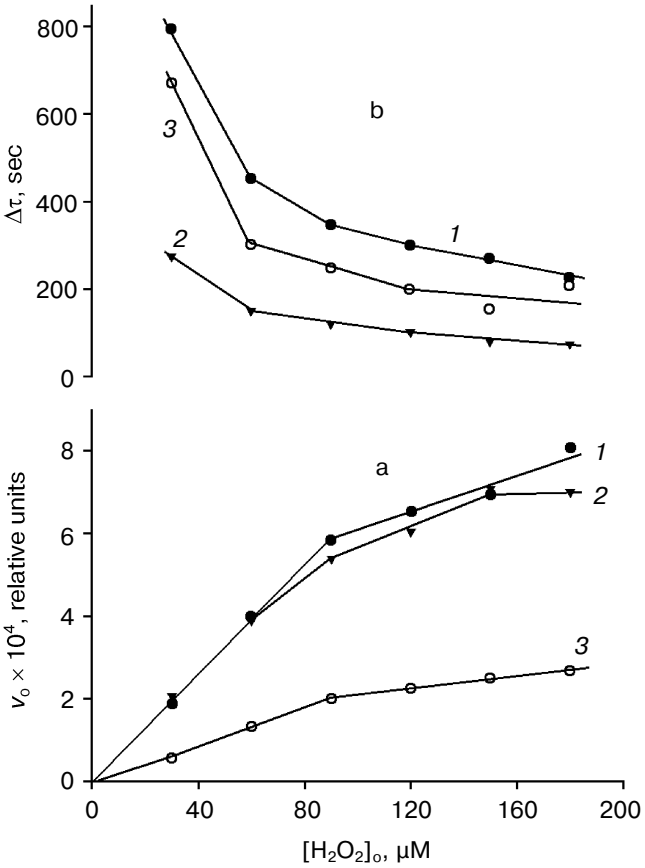

Fig. 8. Dependences of the initial rate of ABTS (250 μM) oxidation on the initial concentration of hydrogen peroxide (a) and of the induction periods Δτ on $[H_2O_2]$ (b) with the involvement of 5 μM MetMb in the presence of 10 μM Trolox (*1*), 0.5 μM poly(ADSNP) (*2*), and 0.5 μM poly(DSG) (*3*); 10 mM PB (pH 7.4) at 20°C.

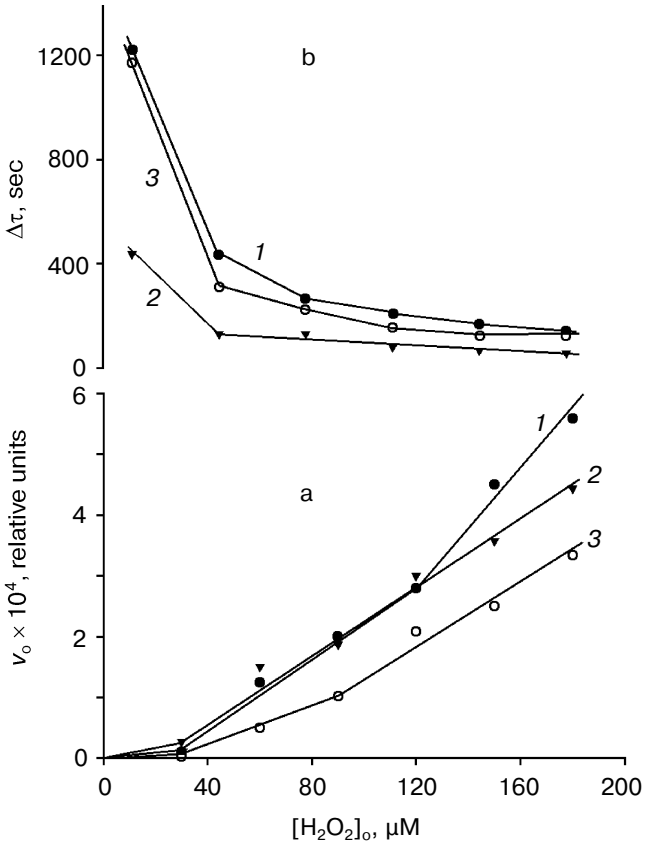

Fig. 9. Dependences of the initial rate of ABTS (250 μM) oxidation on the initial concentration of hydrogen peroxide (a) and of the induction periods Δτ on $[H_2O_2]_o$ (b) with the involvement of 5 μM MetHb in the presence of 10 μM Trolox (*1*), 0.5 μM poly(ADSNP) (*2*), and 0.5 μM poly(DSG) (*3*); 10 mM PB (pH 7.4) at 20°C.

3. To be used for inhibitors and possible "calibrators" for the test systems, PG, poly(DSG), or poly(ADSNP) are the best. They can be used by plotting linear calibration dependences in the coordinates of Δτ versus the inhibitor concentration, as shown in Figs. 4 (*3*, *4*), 5 (*2*, *4*), 6 (*3*, *4*), and 7 (*2*), and expressing the antioxidant potential of the plasma (in μl) analyzed in molar equivalents of the calibrator used.

4. Calibration dependences can be used in the coordinates of $1/v_o$ versus the inhibitor concentration (Figs. 4 (*1*, *2*), 5 (*1*), 6 (*1*, *2*), and 7 (*1*)), using HSA–poly(ADSNP) conjugate as the antioxidant when induction periods are virtually absent (Fig. 7, *3*). In this case, the initial rates of the substrate (ABTS) expenditure are compared, and this is promising for rapid testing of many samples of blood serum and for automation of the whole analytical procedure.

The authors are grateful to Yu. P. Losev (School of Chemistry, Belorussian State University) for providing gallic acid and 2-amino-4-nitrophenol polydisulfides and to A. P. Vlasov for providing methemoglobin.

This work was supported in part by INTAS (Brussels, Belgium), project No. 99-1768.

REFERENCES

1. Emanuel, N. M. (1977) *Kinetics of Experimental Tumors* [in Russian], Nauka, Moscow.
2. Burlakova, E. B., Alessenko, A. V., Molochkina, E. M., Pal'mina, N. P., and Khrapova, N. G. (1975) *Bioantioxidants in Radiation Damage and Malignant Growth* [in Russian], Nauka, Moscow.
3. Zhuravlev, A. I. (1982) *Bioantioxidants in Regulation of Metabolism in Health and Disease* [in Russian], Nauka, Moscow.
4. Metelitza, D. I. (1984) *Modeling of Oxidative-Reducing Enzymes* [in Russian], Nauka i Tekhnika, Minsk.
5. Vladimirov, Yu. A., Azizova, O. A., Deev, A. I., Kozlov, A. V., Osipov, A. N., and Roshchupkin, D. I. (1991) *Advances in Science and Technology. Biophysics*, *Vol. 29, Free Radicals in Living Systems* [in Russian], VINITI, Moscow.
6. Dubinina, E. E., and Shugalei, I. V. (1993) *Usp. Sovr. Biol.*, **113**, 71-81.
7. Poli, G., Albano, E., and Dianzani, M. U. (eds.) (1993) *Free Radicals*: *from Basic Science to Medicine*, Birkhauser Verlag, Basel-Boston-Berlin, pp. 365-523.
8. Pryor, W. (ed.) (1979) *Free Radicals in Biology* [Russian translation], Vols. 1, 2, Mir, Moscow.
9. Thomas, C. E., and Kalyanaraman, B. (eds.) (1998) *Oxygen Radicals and the Disease Process*, Harwood Academic Publishers, Wisconsin.
10. Emanuel, M. N., Denisov, E. T., and Maizus, Z. K. (1965) *Chain Reactions of Organic Substance Oxidation in Liquid Phase* [in Russian], Nauka, Moscow.
11. Emanuel, N. M., Zaikov, G. E., and Maizus, Z. K. (1973) *The Role of Environment in Radical Chain Oxidation Reactions of Organic Compounds* [in Russian], Nauka, Moscow.
12. Emanuel, N. M., and Buchachenko, A. L. (1982) *Chemical Physics of Polymer Aging and Stabilization* [in Russian], Nauka, Moscow.
13. Knorre, D. G., Maizus, Z. K., Obukhova, L. K., and Emanuel, N. M. (1957) *Usp. Khim.*, **26**, 416-430.
14. Rus', O. B., Puchkaev, A. V. and Metelitza, D. I. (1996) *Biochemistry (Moscow)*, **61**, 1813-1824.
15. Metelitza, D. I., Rus', O. B., and Puchkaev, A. V. (1997) *Zh. Prikl. Khim.*, **70**, 1713-1720.
16. Rus', O. B., Puchkaev, A. V., Losev, Yu. P., and Metelitza, D. I. (1998) *Zh. Prikl. Khim.*, **71**, 842-848.
17. Metelitza, D. I., and Plyugachyova, E. I. (1985) in *Cytochrome P-450*, *Biochemistry*, *Biophysics*, *and Induction*, Akademiai Kiado, Budapest, pp. 57-61.
18. *Great Britain Patent*, No. 2250819A, published 17.06.1992.
19. Klebanov, G. I., Teselkin, Yu. O., Babenkova, I. V., Lyubitskii, O. B., and Vladimirov, Yu. A. (1999) *Vestn. Ros. Akad. Med. Nauk*, No. 2, 15-22.
20. Hirayama, O., Takagi, M., Hukumoto, K., and Katoh, S. (1997) *Analyt. Biochem.*, **247**, 237-241.
21. Metelitza, D. I., and Arapova, G. S. (1996) *Biochemistry (Moscow)*, **61**, 308-321.
22. Metelitza, D. I., Arapova, G. S., and Eryomin, A. N. (1997) *Biochemistry (Moscow)*, **62**, 460-470.
23. Metelitza, D. I., Arapova, G. S., Eryomin, A. N., and Losev, Yu. P. (1999) *Biochemistry (Moscow)*, **64**, 1200-1209.
24. O'Connell, M., Halliwell, B., Morehouse, L. A., Aruoma, O. I., Baum, H., and Peters, T. J. (1986) *Biochem. J.*, **234**, 727-731.
25. Teselkin, Yu. A., Babenkova, I. V., Lyubitskii, O. B., Klebanov, G. I., and Vladimirov, Yu. A. (1997) *Vopr. Med. Khim.*, **43**, 87-93.
26. Metelitza, D. I., Rus', O. B., Puchkaev, A. V., and Shadyro, O. I. (1997) *Biochemistry (Moscow)*, **62**, 275-284.
27. Losev, Yu. P., Losev, V. I., Fedulov, A. S., Oleshkevich, F. B., Klimkovich, V. A., and Biryukova, N. M. (1989) *USSR Author's Certificate* No. 1452087, MKI 4C 08 G 75/14, A 61 K 31/795, published 17.04.1989, *Byull. Izobret.*, No. 4.
28. Losev, Yu. P., Losev, V. I., Shonorov, V. I., Ivanina, N. I., and Lebedev, V. T. (1989) *USSR Author's Certificate* No. 1621484, MKI C 08, L 23/06, C 08 K 5/37.
29. Rus', O. B., Puchkaev, A. V., Ivanov, A. I., and Metelitza, D. I. (2000) *Prikl. Biokhim. Mikrobiol.*, **36**, 36-47.
30. Nikolskii, B. P. (ed.) (1967) *A Handbook on Chemistry* [in Russian], Vol. 4, Khimiya, Leningrad, p. 919.
31. Chmelik, T., Kadlecek, T., and Kalons, N. (1979) *J. Electroanalyt. Chem.*, **99**, 245-250.
32. Volkenstein, M. V. (1975) *Molecular Biophysics* [in Russian], Nauka, Moscow, p. 424.
33. Andreyuk, G. M., and Kisel, M. A. (1999) *Biochemistry (Moscow)*, **64**, 867-874.
34. Childs, R. E., and Bardsley, W. (1975) *Biochem. J.*, **145**, 93-103.
35. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1991) *Data for Biochemical Research* [Russian translation], Mir, Moscow, p. 117.
36. Keleti, T. (1990) *Basic Enzyme Kinetics* [Russian translation], Mir, Moscow, pp. 183-188.
37. Eryomin, A. N., Litvinchuk, A. V., and Metelitza, D. I. (1996) *Biochemistry (Moscow)*, **61**, 483-494.
38. Eryomin, A. N., Losev, Yu. P., and Metelitza, D. I. (2000) *Biochemistry (Moscow)*, **65**, 298-308.
39. Denisov, E. T. (1995) *Handbook of Antioxidants: Bond Dissociation Energies*, *Rate Constants*, *Activation Energies*, *and Enthalpies of Reactions*, CRC Press, Times Mirror International Publishers Ltd., Boca Raton.
40. Karpukhina, G. V., Maizus, Z. K., and Emanuel, N. M. (1963) *Dokl. Akad. Nauk SSSR*, **152**, 110-114.
41. Karpukhina, G. V., Maizus, Z. K., and Emanuel, N. M. (1963) *Dokl. Akad. Nauk SSSR*, **160**, 158-162.