Fast method for estimation of the effect of compounds modulating the radical oxidation of sulfhydryl groups on 4-mercapto-2-nitrobenzoic acid applied as a model substance

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Kinetics of radical oxidation of 4-mercapto-2-nitrobenzoic acid was investigated in the presence of hydrogen peroxide and iron(II) sulfate, *i.e.* a hydroxyl radicals generating system. Initial velocities of radical reaction were established by numerization of the course of kinetic dependences. The value of initial reaction velocity was used as a criterion for evaluation of efficacy of ethylenediaminetetraacetic acid (EDTA) and ascorbic acid as model compounds slowing down the course of radical oxidations. Acting probably *via* chelation of the iron(II) ion, EDTA in lower concentrations stimulated but in higher concentrations inhibited the course of radical oxidation of 4-mercapto-2-nitrobenzoic acid. Ascorbic acid representing a typical scavenger substance was found to inhibit the above radical oxidation reaction in the whole range of concentrations tested.

Исследована кинетика радикального окисления 4-меркапто-2-нитробензойной кислоты в присутствии перекиси водорода и сульфата двухвалентного железа, т. е., в системе, генерирующей гидроокисные радикалы. Начальные скорости радикальной реакции были установлены посредством нумерации хода кинетических зависимостей. Значение начальной скорости реакции использовалось в качестве критерия для оценки эффективности ЭДТА и аскорбиновой кислоты как модельных веществ, замедляющих протекание радикального окисления. Действуя, вероятно, посредством хелатации иона железа(II), ЭДТА в низких концентрациях стимулировала, а в более высоких ингибировала протекание радикального окисления 4-меркапто-2-нитробензойной кислоты. Аскорбиновая кислота, являющаяся типичным акцепторным соединением, была способна ингибировать вышеупомянутую радикальную реакцию во всем интервале испытуемых концентраций.

Reactive forms of oxygen, if present in biological material damage both, the functional and metabolic systems of the cells. In biological material the oxygen free radicals may arise owing to:

a) Action of enzymes (*e.g.* xanthine oxidase) [1, 2];

b) autooxidation of reduced intermediary products according to the following equation [3]

$$AH_2 + O_2 \rightarrow \underset{I}{AH^\bullet + HO_2^\bullet} \rightleftharpoons \underset{II}{AH^\bullet + H^+ + {}^\bullet O_2^-} \qquad (A)$$

where the perhydroxyl radical *I* created is in dissociation equilibrium with the superoxide radical *II* [3]. Generation of the superoxide radical from reduced flavin [4, 5] or from ascorbic acid [6] may be described also by the above mechanism;

c) influence of the environment (*e.g.* ionizing or UV radiation) [7].

In acidic milieu the perhydroxyl radical is succumbed to dismutation according to the equation

$$2{}^\bullet O_2^- + 2H^+ \rightleftharpoons 2HO_2^\bullet \rightarrow H_2O_2 + O_2 \qquad (B)$$

Although this reaction may occur in neutral milieu, too, an expressed generation of hydrogen peroxide was observed only in acidic milieu [8]. In biological systems this reaction may be catalyzed enzymically by superoxide dismutase [9]. By disproportionation of the hydrogen peroxide and superoxide radicals generated and in the presence of iron(II) cation acting as catalyst the extremely reactive hydroxyl radical *III* is produced [10]

$$Fe^{2+} + H_2O_2 \longrightarrow \underset{III}{Fe^{3+} + {}^\bullet OH + OH^-} \qquad (C)$$

$$O_2 \qquad {}^\bullet O_2^-$$

Owing to its high reactivity, hydroxyl radical induces oxidative damage in diversely localized biological material [7, 8] such as in the biological membranes, inhibits the activities of different enzymes and even metabolic pathways, causes mutations of genes, *etc.* [11, 12] and participates in the development of different diseases [13—16]. In pathological states the generation of free radicals may exceed considerably the capacity of their natural enzymic and nonenzymic removal (by diverse reduced intermediary metabolic products). In such cases proved to be useful the therapy utilizing compounds which are capable to reduce the amount of reactive species of oxygen [8]. There are essentially two groups of compounds exhibiting the properties latterly described:

a) Chelating compounds — which decrease the reaction rate of hydroxyl radical generation (eqn (*C*)) by formation of complexes with iron(II) compounds [17, 18];

b) scavengers — which are capable to remove free radicals from solutions [19].

The development and testing of such compounds made necessary the elaboration of different methods for quantitative estimation of free radicals in solutions as well as for control of the level of radical oxidation of the biomacromolecules or their functional groups. Methods utilizing the oxidation-induced changes in spectral and fluorescence properties of biomacromolecules were elaborated [7]. However, the greatest importance gained the methods based on direct monitoring of free radicals using the technique of EPR spectroscopy [20]. Nevertheless, still a rapid and sophisticated equipment not pretending screening method is missing for evaluation of the potency of compounds to remove or depress the formation of free radicals. The aim of the present work was the elaboration of a method which would enable to characterize the compounds modulating radical oxidation of SH groups from the description of oxidation kinetics of 4-mercapto-2-nitrobenzoic acid (MNB). Ethylenediaminetetraacetic acid (EDTA) and ascorbic acid were applied as model substances with chelating and reducing properties, respectively.

Experimental

All chemicals applied were of anal. grade and were purchased from Lachema, Brno, or Sigma, St. Louis. Ascorbic acid (Celaskon®) was obtained from Slovakofarma, Hlohovec. MNB was prepared by reduction of 5,5′-dithiobi-2-nitrobenzoic acid with sodium borohydride and by subsequent fast isolation of the reaction product using chromatography on phenoxyhydroxypropylcellulose which was prepared as described earlier [21]. Freshly prepared MNB with spectrophotometrically and chromatographically (TLC) controlled purity was applied in all experiments.

The course of oxidation of MNB was followed spectrophotometrically with the aid of a double-beam spectrophotometer Specord M 40 (Zeiss, Jena). The decrease of absorbance at $\lambda = 412$ nm in phosphate buffer solution ($c = 5$ mmol dm^{-3}) of pH = 7.2 was evaluated. The molar absorption coefficient of $\varepsilon = 13\,600$ cm^{-1} dm^3 mol^{-1} was applied in all calculations. Free oxygen radicals were generated in phosphate buffer solution from H_2O_2 ($c = 0.1$ mmol dm^{-3}) with $FeSO_4$ ($c = 0.016$ mmol dm^{-3}) applied as a catalyst. The influence of EDTA or ascorbic acid on reaction velocity of the radical oxidation of MNB was investigated in the concentration range between 2 and 70 μmol dm^{-3}

Results and discussion

MNB was chosen intentionally as a model substance for investigation of the course of oxidation of thiol groups for the following reasons:

a) Oxidation of the thiol group of MNB induces a marked shift of the absorption maximum from visible (412 nm) to close UV region;

b) 4-mercapto-2-nitrobenzoic acid is characterized by relatively high value of the molar absorption coefficient.

Following concentrations of reactants, *i.e.* H_2O_2, $FeSO_4$, and MNB were applied in the method: 100, 16, and 35 μmol dm^{-3}, respectively. These concentrations resulted from a series of optimalization tests.

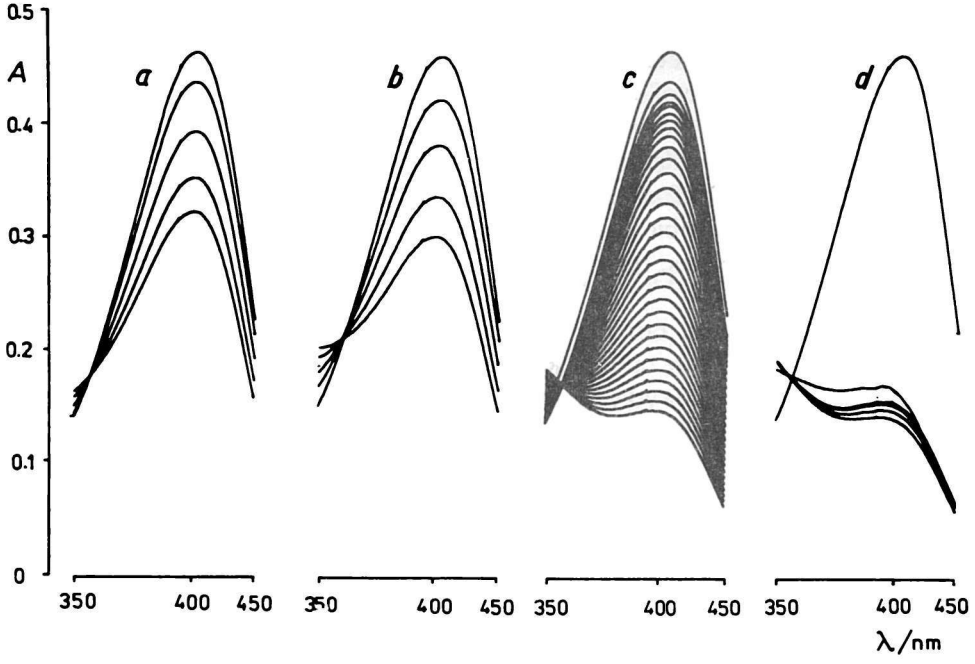

Fig. 1. Spectrophotometric recording of oxidation of 4-mercapto-2-nitrobenzoic acid. Applied substances and concentrations: MNB 35 μmol dm^{-3}, $FeSO_4$ 16 μmol dm^{-3}, H_2O_2 100 μmol dm^{-3}. *a*) Spontaneous oxidation — spectral peaks were recorded in 0, 15, 30, 45, and 60 min of reaction (see from top to the bottom); *b*) oxidation of MNB in the presence of $FeSO_4$ (time intervals similar to those in *a*); *c*) oxidation of MNB in the presence of H_2O_2. Spectra were registered in 3 min intervals (see from top to the bottom); *d*) oxidation of MNB in the presence of $FeSO_4$ and H_2O_2. Peaks were registered in 1, 3, 5, 7, 9, and 11 min of reaction (see from top to the bottom).

The course of MNB oxidation registered as a decrease in the absorption maximum at $\lambda = 412$ nm is shown in Fig. 1. A considerable increase in the rate of oxidation of MNB occurred only if both $FeSO_4$ and H_2O_2 were present (Fig. 1*d*). In the presence of sole H_2O_2 a slight acceleration of oxidation reaction could be observed only (Fig. 1*c*). The above described trends appear more

expressively in Fig. 2, where the kinetic dependences of MNB oxidation are demonstrated. Fig. 2 reveals that up to 1 h of reaction time the spontaneous oxidation of MNB as well as oxidation of the substance in the presence of $FeSO_4$ or H_2O_2 follows the zeroth reaction order. Reaction rates of spontaneous MNB oxidation and oxidation of the substance in the presence of $FeSO_4$ amounted to

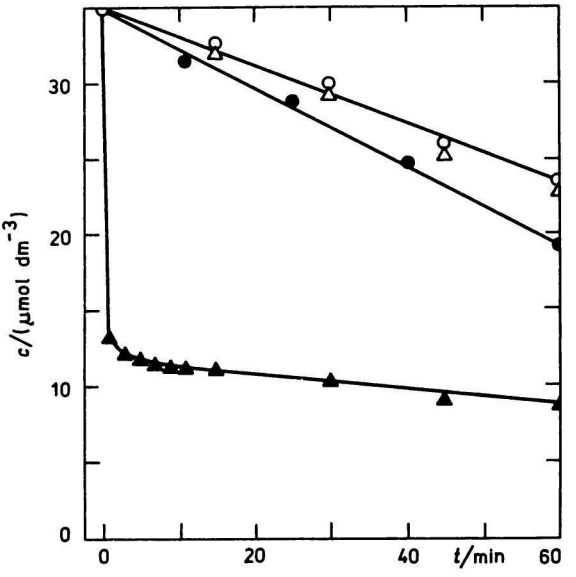

Fig. 2. Kinetics of oxidation of 4-mercapto-2-nitrobenzoic acid.
○ Spontaneous oxidation of MNB; △ oxidation of MNB in the presence of $FeSO_4$; ● oxidation of MNB in the presence of H_2O_2; ▲ oxidation of MNB in the presence of H_2O_2 and $FeSO_4$. Concentrations of reactants are similar to those in Fig. 1. Single points represent means from five independent measurements with standard deviation of arithmetic mean δ never exceeding 3 % of the means.

11.4 ± 0.2 and 12.2 ± 0.3 μmol dm^{-3} h^{-1}, respectively; in the presence of H_2O_2 the rate of oxidation was increased to 15.8 ± 0.3 μmol dm^{-3} h^{-1}. The latter increase may be ascribed to weak oxidation properties of the hydrogen peroxide which inhibits the thiol enzymes predominantly by oxidizing the SH groups [8]. In the presence of both, H_2O_2 and $FeSO_4$ the reaction of MNB oxidation is considerably accelerated and it is shifted to a higher, probably not full number reaction order.

In initial phase (up to 10 min approximately) the course of the reaction kinetics could be linearized when plotted $(c_0 - c)^{-1}$ *vs.* t^{-1} (Fig. 3). In this plot the reciprocal value of slope of the regression line ascertains the value of initial reaction velocity [22] as $12\,500 \pm 325$ μmol dm^{-3} h^{-1}. The rate of oxidation of MNB established by this method was further applied as a parameter for inves-

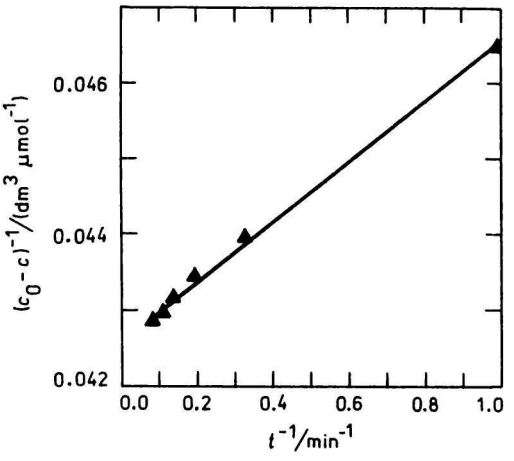

Fig. 3. Linearization of the time course of 4-mercapto-2-nitrobenzoic acid oxidation in the presence of H_2O_2 and $FeSO_4$.
c_0 Initial concentration of MNB; c — concentration of MNB in time t. Single points represent means from five independent measurements with standard deviation δ values never exceeding 3 % of the means.

tigation of slowing down of the radical oxidation process in the presence of ascorbic acid (a scavenger chosen intentionally) and EDTA (typical chelator). Results obtained are summarized in Table 1. At low concentration ratios of EDTA to $FeSO_4$ a stimulation of radical oxidation of MNB was observed. At an increase in concentration ratio of EDTA to $FeSO_4$ to 0.5 and over it the oxidation reaction is already inhibited by EDTA. Similar effects were observed also with nonsaturated fatty acids of phospholipids [23]. Ascorbic acid as a typical scavenger substance inhibited the oxidation of MNB in the full range of concentrations applied.

Table 1

Influence of EDTA and ascorbic acid on the course of radical oxidation of 4-mercapto-2-nitrobenzoic acid

[EDTA or ascorbic acid] : [$FeSO_4$][a]	Reaction rate of MNB oxidation/%[b]	
	EDTA	Ascorbic acid
0.15	134.9 ± 3.1	63.1 ± 4.8
0.25	117.5 ± 4.2	37.2 ± 3.5
0.50	79.4 ± 2.9	22.9 ± 3.4
1.00	50.1 ± 4.5	14.8 ± 2.8
2.00	28.2 ± 2.6	7.9 ± 1.6
4.00	14.8 ± 2.4	5.1 ± 1.9

a) The concentration of $FeSO_4$ applied amounted to 16 μmol dm^{-3}; *b*) % from the initial velocity (12.5 ± 0.3 mmol dm^{-3} h^{-1}) established in the absence of EDTA and ascorbic acid.

From the results it may be concluded that the method proposed appears to be suitable for fast screening estimation of action of compounds which are modulating radical oxidation of the thiol groups. The results obtained indicate that after further elaboration the method may be suitable for distinguishing whether the tested substance behaves as a chelator or as a scavenger.

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