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Communication

Modification of the deoxyribose test to detect strong iron binding

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Deoxyribose test has been widely used for determination of reactivities of various compounds for the hydroxyl radical. The test is based on the formation of hydroxyl radical by Fe^{2+} complex in the Fenton reaction. We propose a modification of the deoxyribose test to detect strong iron binding, inhibiting participation of Fe^{2+} in the Fenton reaction, on the basis of examination of concentration dependence of deoxyribose degradation on Fe^{2+} concentration, at a constant concentration of a chelating agent.

Key words: chelation, deoxyribose test, desferrioxamine, DETAPA, EDTA, Fenton reaction, hydrogen peroxide, hydroxyl radical, iron, superoxide

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; DETAPA, diethylenetriaminepentaacetic acid; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl

INTRODUCTION

The deoxyribose test is based on the use of a simple system, in which an iron complex reacts with hydrogen peroxide in the presence of ascorbic acid, presumably forming hydroxyl radicals. Hydroxyl radicals attack deoxyribose forming products that, upon heating with thiobarbituric acid (TBA) at low pH, yield a pink chromogen. The test has been proposed to determine the rate constants of reactions of various compounds with the hydroxyl radicals since hydroxyl radical scavengers compete with deoxyribose for the hydroxyl radicals and diminish chromogen formation. A rate constant for reaction of the scavenger with hydroxyl radical can be deduced from the extent of inhibition of color formation. It is suggested that the deoxyribose assay is a simple and cheap alternative to pulse radiolysis for determination of rate constants for reaction of most biological molecules with hydroxyl radicals (Halliwell & Gutteridge, 1981; Gutteride & Halliwell, 1982; Halliwell *et a*l., 1987). Reactivities of various substances for the hydroxyl radical have been estimated using this test (Bhat *et al*., 2001; Lapenna *et al*., 2002; Manoj & Aravindakumar, 2003). The deoxyribose test has also been used to evaluate antioxidant activities of compounds and extracts (De *et al*., 2008; Guedes *et al*., 2013; Mokdad-Bzeouich *et al*., 2015). The test has been widely used and modified, i. a. to detect both antioxidant and prooxidant properties of compounds (Chobot, 2010). The mechanism of the test has been the subject of further studies. They demonstrated, i. a., that Fe^{3+}, product of the reaction, contributes to deoxyribose degradation (Genaro-Mattos *et al*., 2009), that in reactions of hydroxyl radicals with deoxyribose five different deoxyribose radicals are formed, only one of which is transformed into malondialdehyde-like products reactive with TBA and that relative activity of antioxidants depends on the rate constants of many secondary reactions of antioxidants (Rachmilovich-Calis *et al*., 2009).

Moreover, the very basic assumption, i. e. that hydroxyl radical is the oxidant formed in the system and responsible for the deoxyribose degradation, has been questioned by various researchers. Winterbourn (1991) suggested that deoxyribose may be oxidized by iron(IV) species formed from H_2O_2 and Fe^{2+}, but concluded that "the system is too complex for definitive identification of the Fenton oxidant". Similarly, hydroxylation of terephtalic acid by Fe^{2+} was ascribed to "cryptohydroxyl radical" (Fe^{2+}/buffer complex) rather than to hydroxyl radical by Saran and coworkers (2000). It has also been suggested that at concentration ratios of $[O_2]/[H_2O_2]>100$ (prevailing in almost all cell compartments), hydrogen peroxide contributes negligibly to biological free radical oxidations and a non-identified "Fe-O" complex outcompetes H_2O_2-dependent oxidation pathways (Qian & Buettner, 1999).

The Fenton system used for the induction of deoxyribose degradation in the deoxyribose test consists of hydrogen peroxide, Fe^{2+} ions, ethylenediaminetetraacetic acid (EDTA) chelating ferrous and ferric ions and ascorbate needed to recycle ferric ions produced in the Fenton reaction. Thus, the protection against deoxyribose degradation may be due not only to scavenging of the oxidant formed in the system, but also to prevention of the Fenton reaction by strong chelation of iron preventing its participation in the Fenton reaction. In this study, we attempted to find conditions for identification of the second possibility, which should be useful to detect compounds capable of strong iron binding, preventing the participation of ferrous ion in the Fenton reaction.

MATERIALS AND METHODS

All the reagents were from Sigma-Aldrich (Poznań, Poland). In a simplified version of the test, the samples contained 50 mM phosphate buffer, 5 mM deoxyribose, pH 7.4, 80 μM FeCl2 and variable amounts of the compounds tested, or 80 μM of a compound tested and variable amounts of Fe^{2+}. In a full version of the test, 100 μM ascorbic acid and 1 mM hydrogen peroxide were also present. The mixtures were incubated at 37°C for 1 h, then mixed with 250 μl of 2.8% trichloroacetic

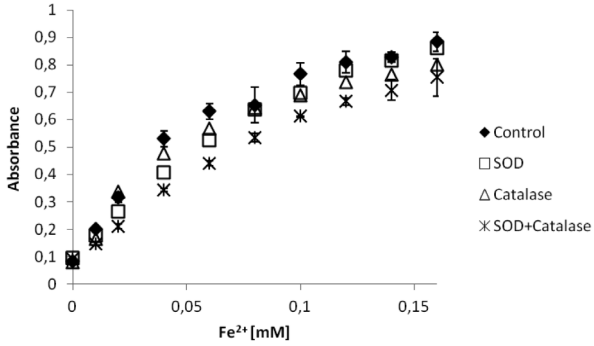


Figure 1. Effect of superoxide dismutase (SOD) and catalase and SOD+catalase on the deoxyribose degradation by 80 μM Fe^{2+} in 50 mM phosphate buffer, pH 7.4.
Enzyme concentrations: 10 μg/ml. For better transparency, S.D. is shown only for the extreme plots.

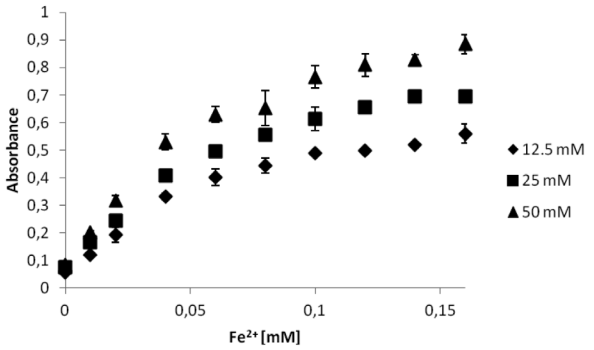


Figure 2. Effect of phosphate concentration (12.5, 25 and 50 mM) on the deoxyribose degradation by 80 μM Fe^{2+} in 50 mM phosphate buffer, pH 7.4.

acid (TCA) and 250 μl of 1% TBA (prepared in 0.1 M NaOH) and heated for 10 min at 100°C. After cooling to room temperature, absorbance of the samples was measured at 532 nm.

RESULTS AND DISCUSSION

Fe^{2+} induced deoxyribose degradation in the absence of chelators, both in the simplified and in the full version of the test. Deoxyribose degradation by Fe^{2+} in the simplified version of the test can be explained by a simple assumption of autoxidation of Fe^{2+} leading to formation of superoxide and, by its decomposition, hydrogen peroxide. Reaction of hydrogen peroxide with non-reacted Fe^{2+} would produce hydroxyl radical (Eqn. 1–3).

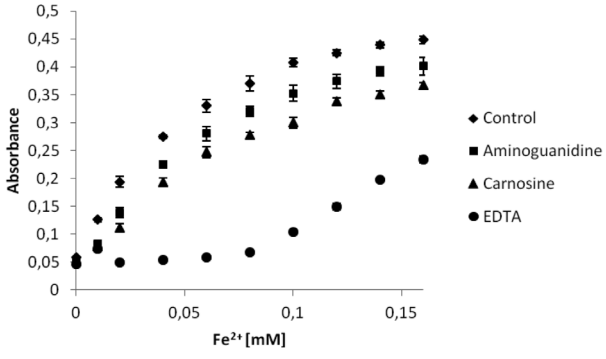
$$Fe^{2+}+O_2 \leftrightarrow Fe^{3+}+O_2^{\bullet} \quad (1)$$

$$O_2^{\bullet}+O_2^{\bullet}+2H+ \leftrightarrow O_2+H_2O_2 \quad (2)$$

$$H_2O_2+Fe^{2+} \leftrightarrow {}^{\bullet}OH+HO^-+Fe^{3+} \quad (3)$$

If this simple scheme was true, superoxide dismutase (SOD) producing hydrogen peroxide (reaction 2) and catalase dismutating it to O_2+H_2O should increase and decrease, respectively, the deoxyribose degradation. Alternatively, SOD could decrease the rate of reaction if superoxide is able to reduce Fe^{3+} formed. Experimental results do not conform with these predictions as SOD did not affect the degradation, catalase slightly decreased it and SOD+catalase produced a definite but small decrease of the extent of degradation (Fig. 1). It can be concluded that reactions in the system are more complicated than those presented by Equations (1)–(3); perhaps there is a significant contribution of direct degradation of deoxyribose by Fe^{3+} (Genaro-Mattos *et al.*, 2009).

We studied the effect of phosphate concentration on the extent of deoxyribose degradation and found it to increase with the phosphate concentration (Fig. 2). Apparently, weak chelation of Fe^{2+} by phosphate increases the activity of ferrous ions. We checked various modifications of the test to distinguish between compounds binding weakly or not binding ferrous ions and those strongly binding these ions. Testing concentration dependence of deoxyribose degradation using constant Fe^{2+}concentration and variable concentrations of potential chelators was not sufficiently discriminative (not shown). However, examination of the dependence of the extent of degradation on the Fe^{2+} concentration at a constant (80 μM) concentration of a potential chelator yielded two types of dependencies. In the absence of any chelator, a hyperbolic dependence was observed (Fig. 3, control). The same type of dependence was found for compounds known not to bind iron tightly (aminoguanidine, citrate, carnosine and rutin). In contrast, diethylenetriaminepentaacetic acid (DETAPA) and EDTA produced a concave plot demonstrating that until reaching a 1:1 stoichiometry, i. e. saturation of the binding capacity of the compound tested, the presence of the chelator decreased the participation of Fe^{2+} in the Fenton reaction (Fig. 3). The hyperbolic-type dependence was also found for 4-amino-TEMPO, captopril, carnosine, 4-cyano-1-hydroxycinnamic acid, cysteamine, ellagic acid, ferulic acid, gallic acid, genistein, 1-hydroxycinnamic acid, 4-hydroxy-TEMPO, kempferol, metformin, naringin, propyl gal-



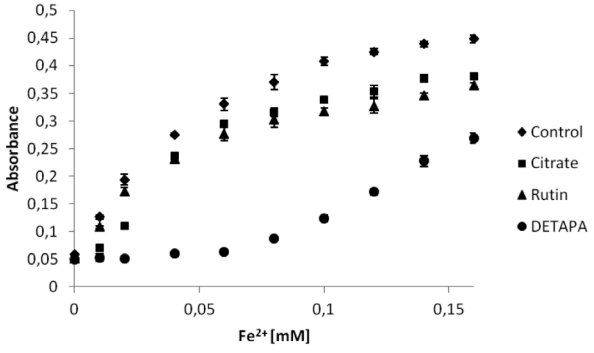


Figure 3. Dependence of deoxyribose degradation on the concentration of Fe^{2+} for various potential chelators in a simplified detection system. Concentration of potential chelators: 80 μM.

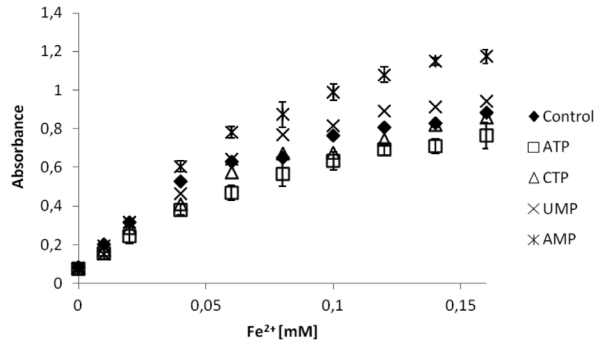


Figure 4. Dependence of deoxyribose degradation on the concentration of Fe^{2+} for nucleoside phosphates potential chelators in a simplified detection system.
For better transparency, S.D. is shown only for the extreme plots.

late, pyridoxine quercitrin, quinic acid, rutin, spermidine, TEMPO and tiron, while desferrioxamine and *o*-phenanthroline yielded a concave-type plot.

Our results indicate that the flavonoids tested, considered to be relatively strong iron chelators, do not bind Fe^{2+} strong enough to make the chelates unable to participate in the Fenton reaction. Similarly, nucleoside phosphates, considered to be relatively strong biological iron chelators, behaved like weak chelator in our test. Interestingly, white ATP and CTP decreased the extent of deoxyribose degradation, AMP and UMP increased it (Fig. 4). This property of nucleoside phosphates may be of biological relevance for iron-induced degradation *in vivo*. It could be suspected that chelators affect autoxidation of Fe^{2+} (reaction 1) rather than prevent participation of Fe^{2+} in the Fenton reaction (3). However, the same concentration dependence was observed in a full Fenton system, which is independent of Fe^{2+} autoxidation (Fig. 5).

Various tests have been proposed to evaluate binding of Fe^{2+} by pure substances, extracts and complex biological material including blood plasma, based mostly on spectral changes induced by the binding or on the inhibition of formation of Fe^{2+}-ferrozine complex (Dinis *et al.*, 1994; Khokhar & Apenten, 2003; White & Flashka, 1973). However, they do not allow for differentiation between compounds that are able or not to prevent participation of ferrous ions in the Fenton reaction, which is of considerable biological importance. The induction

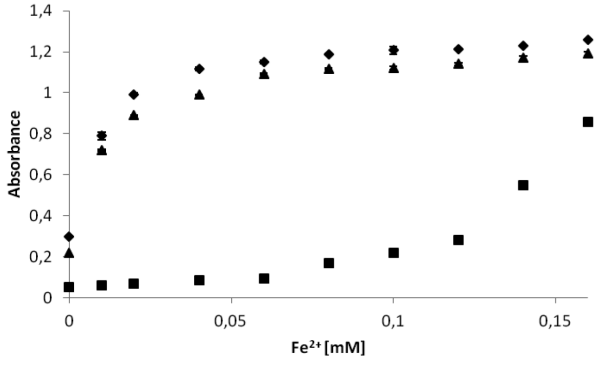


Figure 5. Dependence of deoxyribose degradation on the concentration of Fe^{2+} for various potential chelators in the full version of the test.
(◆) Control; (▲) Citrate, (■) DETAPA. Concentration of potential chelators: 80 μM.

of deoxyribose degradation by iron salts and protective effects of various substances has been studied by Halliwell and Gutteridge (1981). This study pointed to a significant inhibition of the degradation by iron chelators (EDTA, DETAPA and desferrioxamine) and •OH scavengers but did not point to the way of discrimination between both groups of substances. Such discrimination is difficult since virtually all substances, including iron chelators, scavenge •OH. In our opinion the modification of the deoxyribose assay proposed here, though qualitative only, may be useful in this respect and allow for identification of strong iron chelators among newly synthesized compounds.

SIMPLE PROTOCOL TO DETECT STRONG IRON BINDING

Reagents: (i) 50 mM sodium phosphate buffer, pH 7,4; (ii) 20 mM deoxyribose in (i); (iii) 1 mM $FeCl_2$ in 1 mM HCl (prepare fresh before use); (iv) 1 mM substance tested in (i) or another solvent, e. g. DMSO; (v) the solvent if different from (i); (vi) 2.8% of trichloroacetic acid (TCA); (vii) 1% thiobarbituric acid (TBA) in 50 mM NaOH.

Procedure: Pipette 125 μl of deoxyribose, (335-x) μl of buffer (i), 40 μl of 1 mM solution of the compound tested (iv) and increasing volumes (x) of 1 mM Fe^{2+} solution (x=0, 5, 10, 20, 30, 40, 50, 60, 70 and 80 μl) to successive Eppendorf tubes. Blank: 125 μl of deoxyribose and 375 μl of buffer (i). Incubate at 37°C for 1 h. Then add 250 μl of TCA solution (vi) and 250 μl of TBA solution (vii). Heat at 100°C for 10 min. Cool to room temperature, measure absorbance at 532 nm against a blank in a spectrophotometer or microplate reader. Plot absorbance *vs* Fe^{2+} concentration.

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