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On the mechanism of OH· scavenger action

O_2 in aqueous solution is fairly unreactive and many of its damaging effects have been attributed to formation of hydroxyl radical, OH· (for reviews see Halliwell & Gutteridge, 1984; Slater, 1984). The damage observed, however, can be brought about by a number of oxidizing species not identical with OH·, including perferryl radical (Svingen *et al.*, 1978) or ferryl radical (Rush & Koppenol, 1986). The involvement of OH· in a given system is frequently inferred by the prevention of the studied reaction by known scavengers of OH· such as mannitol, formate, thiourea and ethanol (Tien *et al.*, 1982; Gutteridge, 1984; Halliwell & Gutteridge, 1985).

If we search the literature on the use of OH· scavengers in biological systems, we find that the protective effect provided by these scavengers is not necessarily what is expected from chemically determined rate constants. Several hypotheses were suggested to explain the reason for these deviations (Gutteridge, 1984, 1987; Henglein & Kormann, 1985; Moorhouse *et al.*, 1985). Recently we have conducted a study on the mechanism of action of some of the classical OH· scavengers.

We studied the effect of OH· scavengers both on the formation of thiobarbituric acid-reactive material from deoxyribose caused by the Fenton reaction ($Fe^{2+} + H_2O_2$) and on Fe^{2+} oxidation by H_2O_2. The buffering species Mes and Mops, which have low affinities for metals (Good *et al.*, 1966), were used to facilitate the interpretation of these metal ion dependent reactions. The concentration of the buffer was kept rather low (5 mM) to limit the possible interference due to its reaction with OH·. Damage to 2.8 mM-deoxyribose by Fenton reaction (200 μM-H_2O_2 and 150 μM-$FeCl_2$) was detected by thiobarbituric acid-reactive material according to Tien *et al.* (1982). Fe^{2+} determination was made by the *o*-phenanthroline method of Mahler & Elowe (1954).

In the absence of OH· scavengers, OH· damage to deoxyribose is higher at pH 6 compared with pH 7 and 7.4. At acid pH, deoxyribose is apparently able to inhibit Fe^{2+} oxidation by H_2O_2. This effect of the sugar is greatly decreased at pH 7 and 7.4. Deoxyribose is known to bind iron salts (Gutteridge, 1987) and Fe^{2+} bound on this detector molecule would catalyse a site-specific production of OH·. This system thus generates OH· on the substrate and there immediately converts the resulting radical to stable product by oxidation by the metal just oxidized in the OH·-forming process. In the presence of deoxyribose an apparent decreased Fe^{2+} oxidation by Fenton reaction would occur. Oxidations of this type were postulated in the original schemes of Haber & Willstätter (1931), confirmed by Walling (1975). The effect of pH can be ascribed to a different absorption of

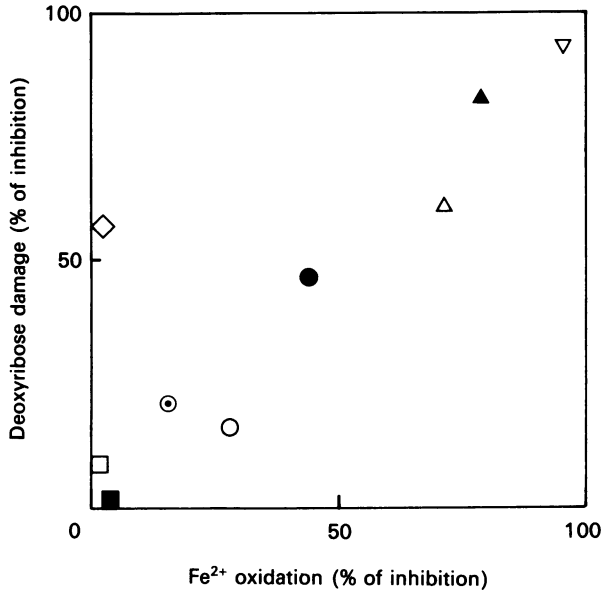

Fig. 1. Inhibition of deoxyribose damage and Fe^{2+} oxidation by OH· scavengers

17 mM-Ethanol (○), 15 mM-butan-1-ol (⊙), 20 mM-methanol (●), 20 mM-formate (◇), 0.1 mM- (△) and 1 mM- (▽) thiourea, 10 mM-mannitol (▲), 40 mM-acetate (□) and 10 mM-benzoate (■) were used.

Fe^{2+} to the substrate detector molecule due to a replacement of bound ligand by hydroxyl anions.

We then studied OH· scavenger effects. When the experiments were conducted in Mes buffer, pH 6, a close relationship (Fig. 1) was found between the effects of OH· scavengers on the two parameters measured. Damage to deoxyribose was inhibited to various extents by all OH· scavengers tested (ethanol, butan-1-ol, methanol, formate, thiourea and mannitol) but not by benzoate and acetate. The inhibition of Fe^{2+} oxidation by OH· scavengers was qualitatively and quantitatively similar to that exerted on deoxyribose damage. Formate was the only exception. The decreased oxidation of Fe^{2+} observed in these experimental conditions was neither due to inhibition by OH· scavengers of the Fenton reaction nor to a direct reaction of OH· scavengers with H_2O_2. This was demonstrated by determining H_2O_2 concentration by the horseradish peroxidase reaction of H_2O_2 with the fluorescent dye scopoletin (Loschen *et al.*, 1971). These results contrast with the reported reaction between thiourea and H_2O_2 (Cederbaum *et al.*, 1979) which was suggested to explain the abnormal behaviour of thiourea as OH· scavenger (Moorhouse *et al.*, 1985). To explain the correlation between OH· scavenger effects on deoxyribose damage and Fe^{2+} oxidation, a mechanism

similar to that suggested for deoxyribose can be invoked. The OH· scavengers would bind the metal and compete for it with the detector molecule. This would result in the decreased site-specific generation of OH· on deoxyribose, in the formation of the OH· scavenger radical and in its successive oxidation by the Fe^{3+} generated by the Fenton reaction. This mechanism explains the apparent reaction of thiourea with H_2O_2 reported by Cederbaum *et al.* (1979), as in that paper H_2O_2 concentration was measured by the thiocyanate method, i.e. measuring Fe^{3+} produced by Fenton reaction. The lack of correlation observed in the presence of formate may be due to the insufficient reactivity to induce Fe^{3+} of the secondary radical produced by OH· attack on this scavenger. This agrees with the known inertness of carbonyl radicals to oxidation by Fe^{3+} (Walling, 1975). The effects of OH· scavengers on deoxyribose damage and Fe^{2+} oxidation are both pH-dependent. When the reactions are conducted in Mops buffer, pH 7 or 7.4, a parallel decreased inhibition, by all OH· scavengers, of the two phenomena is observed. However the dependence of the effects of the various OH· scavengers on the pH greatly differs. A rough order of sensitivity is ethanol, butan-1-ol, methanol, formate > thiourea > mannitol.

In summary, our results indicate that the ability of classical OH· scavengers to inhibit deoxyribose degradation correlates with their ability to decrease Fe^{2+} oxidation by H_2O_2. If the mechanism that can explain the present findings is that proposed by Haber & Willstätter (1931) and by Walling (1975), some conclusions can be drawn. Not only mannitol and thiourea but other classical OH· scavengers can affect OH·-dependent damage to detector molecules by binding Fe^{2+}. All these OH· scavengers act predominantly by this mechanism, as they are practically unable to protect against the damage produced by OH· radical generated at specific sites (Gutteridge, 1984) in experimental conditions where they do not interact with Fe^{2+}. As it appears more and more evident that OH· generation by the Fenton reaction in biological systems is site-specific, the suggested use of OH· scavengers to indicate the involvement of OH· in such systems (Halliwell & Gutteridge, 1985) becomes questionable.

Bruna TADOLINI and Luciana CABRINI

Dipartimento di Biochimica, Universita' di Bologna, Via Irnerio 48, 40126 Bologna, Italy

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The deoxyribose assay: an assay both for 'free' hydroxyl radical and for site-specific hydroxyl radical production

The interaction of iron ions with hydrogen peroxide in biological systems can lead to formation of a highly-reactive tissue-damaging species that is thought to be the hydroxyl radical, ·OH [1,2]. Various reactive iron–oxygen complexes may also exist, such as ferryl, perferryl and $Fe^{2+}/Fe^{3+}/O_2$ species (reviewed in [3–5]). There has thus been considerable interest in the development of methods for assaying ·OH and related species in biological systems (reviewed in [6]).

The pentose sugar 2-deoxyribose is attacked by ·OH radicals to yield a mixture of products (reviewed in [7]). On heating with thiobarbituric acid at low pH, some or all of these products react to form a pink chromogen that can be measured by its absorbance at 532 nm; this chromogen is indistinguishable from a thiobarbituric acid–malondialdehyde (TBA–MDA) adduct [8]. Generation of a TBA–MDA adduct from deoxyribose was thus introduced in 1981 [8,9] as a simple assay for ·OH generation in biological systems, provided that suitable control experiments are performed. The assay has been widely used [8–10].

If deoxyribose is incubated with H_2O_2 and an Fe^{2+}–EDTA complex (or an Fe^{3+}–EDTA complex in the presence of a reducing agent such as ascorbate or superoxide, O_2^-), the resulting deoxyribose degradation is inhibited by any added scavenger of ·OH to an extent that depends only on the concentration of scavenger relative to deoxyribose, and on the scavenger's second-order rate constant for reaction with ·OH [11–13]. It seems that, when ·OH is generated by reaction of Fe^{2+}–EDTA with H_2O_2, any ·OH that escapes scavenging by the EDTA itself [14] enters 'free solution' and is equally accessible to deoxyribose and to any added scavenger. Indeed, the deoxyribose assay in the presence of Fe^{3+}–EDTA, H_2O_2 and a reducing agent has been proposed as a simple 'test-tube' method for determining rate constants for the reaction of substrates with ·OH [11,12].

If deoxyribose is incubated with H_2O_2 and Fe^{2+} (or Fe^{3+} plus a reductant) in the absence of EDTA, it is still degraded into products that can react to form a TBA–MDA chromogen [6,8,9]. However, some ·OH scavengers (such as ethanol, formate, dimethyl sulphoxide and Hepes) no longer inhibit the deoxyribose degradation whereas others, such as mannitol, thiourea and hydroxychloroquine, still do [11,13,15]. Two possible explanations of this observation have been advanced.