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Assaying for Hydroxyl Radicals: Hydroxylated Terephthalate is a Superior Fluorescence Marker than Hydroxylated Benzoate

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Generation of hydroxyl radicals in terephthalate (benzene-1,4-dicarboxylic acid) solution yields fluorescent 2-hydroxy-terephthalate. The reaction product is stable for hours and can readily be assessed using standard fluorimeters. The efficiency, i.e. the relative increase of fluorescence per •OH radical, is about three times higher than that of the formation of salicylate (2-hydroxy-benzoate) from benzoic acid and approximately hundred-fold higher than that of the hydroxylation of phenylalanine. As the terephthalate molecule is symmetric with respect to ring-hydroxylation, only one isomer is formed; hence, mechanistic interpretation of the hydroxylation reaction is facilitated.

The scavenging rate constant of terephthalate for •OH yielding the hydroxycyclohexadienyl adduct as first intermediate is close to the diffusion controlled limit ($k = 3.3 \times 10^9\,M^{-1}\,s^{-1}$). Therefore, competition of the detector molecule with biomolecules being present under physiological conditions is expected to be efficient. The assay can be used to detect 'free' •OH radicals produced by the radiolysis of water as well as 'hydroxyl analogous species' that have been suggested to arise from the interaction of complex-bound reduced metal with either oxygen or hydrogen peroxide, e.g. from Fenton reactions.

Based on calibration with radiolytically generated hydroxyl radicals the detection limit of the method is estimated to be around 50 nmol/dm^3. Terephthalate is classified non-toxic and hence may also prove useful for microdialysis and continuous flow experiments as observation of fluorescence is 'non-destructive' and the reporter substance does not necessarily have to be subjected to HPLC.

Keywords: Fluorescence assay, hydroxyl radical, Fenton reaction, autoxidation, metal catalysis, benzoate–salicylate dosimetry

INTRODUCTION

Detection of hydroxyl radicals in physiological environments and their quantification is one of the most demanding tasks in biological research. Due to its extreme reactivity the •OH radical is able to attack almost any molecule at almost any site. Hence, with most organic compounds a large manifold of isomers may be formed. Terephthalic acid (TA) is a rare example of a molecule that,

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because of its symmetric configuration, will not result in differently hydroxylated stereoisomers. Attack at any of the four unsubstituted ring carbons yields the same initial •OH-adduct, attack at benzoic acid (BA), in contrast, may occur at *ortho-*, *meta-* or *para*-positions.

Terephthalate as well as benzoate are non-fluorescent, whereas their ortho-hydroxylated compounds (2-OH-TA) and salicylic acid (2-OH-BA) are highly fluorescent. Such hydroxylation reactions have therefore been used to detect hydroxyl radicals produced by radiolysis,[1] sonolysis,[2–4] UV-photolysis,[5] or in metal-catalyzed Fenton-type processes.[6] The hydroxylation of salicylate is often used as a marker for •OH radicals in microdialysis experiments[7–9] but in this case fluorescence measurements are only applicable to the decrease of salicylate fluorescence whereas the hydroxylated products 2,3- and 2,5-OH-BA, which are non-fluorescent, are generally assessed by HPLC analysis using electrochemical detection. Many other reactions are known to yield fluorescent derivatives from non-fluorescent parent compounds,[10] e.g. hydroxylation of coumarin-3-carboxylic acid[11,12] or phenylalanine.[13,14] Especially the generation of o,o′-dityrosine from tyrosine[15,16] has found wide interest due to the rather high concentration at which this amino acid occurs in biological systems.

Some of these methods, however, have considerable drawbacks for experimental use: they are either rather insensitive, as e.g. the hydroxylation of phenylalanine, or as is the case for tyrosine, are hampered by the fact that the parent compound itself is fluorescent and therefore the determination of the fluorescence of newly formed o,o′-dityrosine above a high background level of initial fluorescence is rather difficult. A general disadvantage inherent in HPLC methods is that the OH-probe, after having been forced through the HPLC column, has to be discarded because of dilution by the HPLC solvent. For many purposes it would be preferable to recycle the detector solution in a closed loop in order to gain time-dependent information on hydroxyl radical generation in the investigated source.

In our search for compounds which allow to avoid complications of the above kind we decided to compare in detail the terephthalate and benzoate reactions for the quantification of hydroxyl radicals generated by radiation and/or Fenton processes.

MATERIALS AND METHODS

Solutions were prepared with water from a Millipore water-purification system using compounds as supplied from Fluka (sodium benzoate, Fe(II) sulfate, and tyrosine), from Serva (p-aminobenzoic acid), from Merck (sodium salicylate), from Ventron (phenylalanine), from Sigma (acetylsalicylic acid), and from Aldrich (terephthalic acid (disodium salt)). Gases for saturating the solutions were obtained by Linde (Unterschleissheim, Germany) and were of at least 99.99% quality.

Gamma radiolysis was carried out with ^{60}Co-radiation in a GammaCell 220 (Atomic Energy of Canada, Ontario, Canada) at a dose rate of 12 Gy/min. Erlenmeyer flasks containing the experimental solution were stoppered with silicone stoppers and continuously bubbled with the respective gas 30 min before and during the whole irradiation procedure.

The set-up for pulse radiolysis experiments has been described earlier.[17] In brief, for obtaining the oxygen dependence of terephthalate hydroxylation in the inset of Figure 3 oxygen-saturated and nitrogen-saturated solutions were mixed in the respective ratio and immediately subjected to pulse irradiation with 1.8 MeV electrons (generating approx. 7 μmol of •OH radicals per dm^3).

For fluorescence measurements two different procedures were adopted: the samples were either sucked into the fluorescence cuvette of a Perkin-Elmer luminescence spectrometer LS 30, or, for kinetic determinations, a cyclic flow-through system was installed, which allowed

continuous transport of fluorescent liquid through the detector cuvette of the LS 30 by means of a peristaltic pump. For each of the investigated compounds a suitable absorption/emission wavelength-pair was separately determined, e.g. hydroxylated terephthalate was measured at 323/435 nm, salicylic acid at 309/420 nm and benzoic acid at 308/433 nm.

Transfer time from the reaction vessel into the fluorescence cuvette was about 3 s, hence kinetics in the second time-scale could be resolved. Occasionally the circulating test solution was also guided through the flow-through quartz cell of a diode array spectrophotometer (X-dap, Polytec, Germany) in order to determine fluorescence and absorption changes within the same sample in a continuous-flow experiment.

Unless otherwise stated curves shown in the figures represent single, representative experiments out of at least four different runs.

RESULTS AND DISCUSSION

Figure 1 compares the fluorescence behavior of a variety of phenolic compounds that are commonly used to detect •OH radicals. Terephthalate showing the steepest positive slope, and salicylate with the steepest negative slope, are clearly the best suited compounds with respect to sensitivity, i.e. change in fluorescence units per •OH radical. Considering the fact that a rise of newly appearing fluorescence is more accurate to observe than small decrements of an initially high level of fluorescence, terephthalate seems to be superior to salicylate. The other compounds are less well suited and phenylalanine, due to very small absolute fluorescence yield, is unsuitable for detection of low levels of •OH.

As evident from Figure 1 the fluorescence increase of TA exceeds that of BA by a factor of about three. The hydroxylation reaction is almost independent of pH in the range of pH 5–8 (data

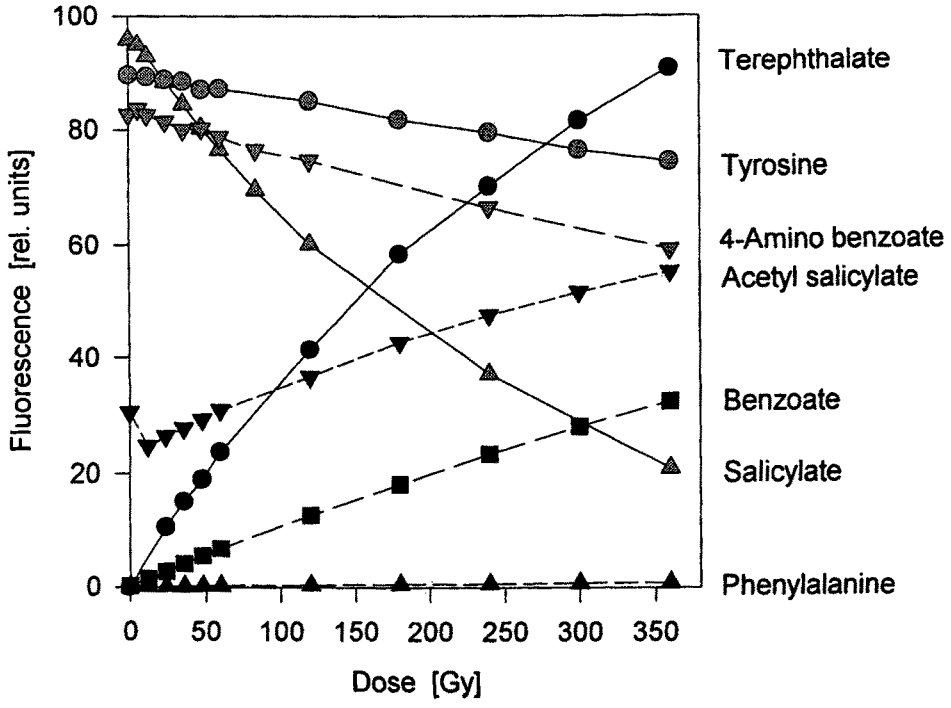


FIGURE 1 Comparison of the fluorescence of various phenolic compounds for the detection of hydroxyl radicals. All substances were 323 μM in 50 mM phosphate buffer at neutral pH, constantly purged with air; a constant flux of hydroxyl radicals (280 nM/Gy) was generated by gamma irradiation.

not shown) which makes TA suitable for •OH detection in the pH range of interest for biological experiments. The only restriction is that TA has a pK at 4.82[18] and precipitates at lower values; it can, therefore, not be used at acidic conditions.

Hydroxylated terephthalate is chemically stable, i.e. fluorescence variations are less than +3% during 36 h (data not shown) and the probes can thus be reliably assessed up to several days after the experiment.

A serious problem with •OH detection is that detector molecules may be destroyed *during* the experiment. Figure 1 shows that this is also the case for BA-OH and TA-OH: the negative slope of the *disappearance* of salicylate is about twice the positive slope of its *buildup* by benzoate hydroxylation. Figure 2 exemplifies this further: the

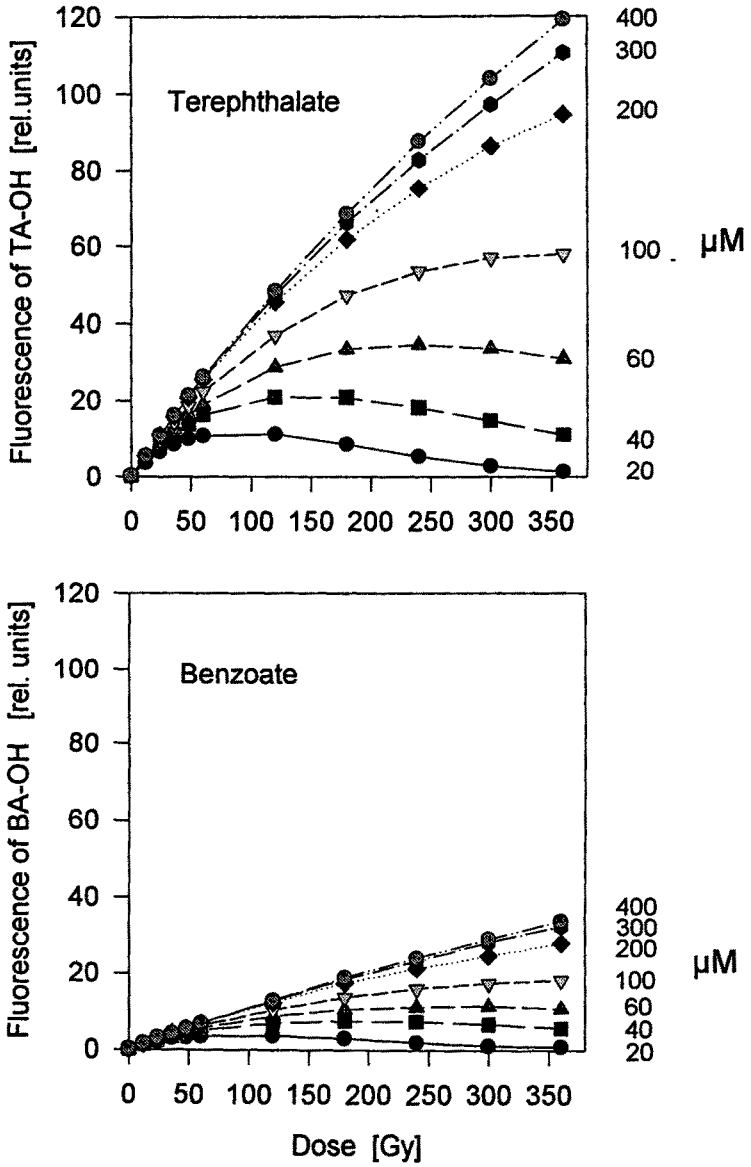


FIGURE 2 Concentration dependence of the generation of fluorescence by hydroxylation of terephthalate and benzoate by radiolytically generated hydroxyl radicals (conditions as in Figure 1).

lower the concentration, the earlier the curves start to deviate from linearity; this means that after a certain amount of •OH radicals has reacted with terephthalate the probability rises that newly formed •OH degrades already existent TA-OH rather than reacting with unreacted TA generating new fluorescent TA-OH. The general conclusion from Figure 2 is that above 200 μM of detector substance the dose response curve becomes essentially linear and the use of about 300 μM of terephthalate will allow to accurately detect up to 100 μM of hydroxyl radicals.

Figure 3 addresses the possible disturbance of the detection method by variations in oxygen concentration by comparing oxygen- to nitrogen-saturated solutions; the inset quantifies this for different O_2/N_2 ratios. According to the inset, the observed fluorescence yield rises by about a factor of 2 when zero oxygen is compared to 100% oxygen. For *in vivo* conditions, however, one only has to consider the region left of the arrow in the inset; i.e. uncertainties in fluorescence determinations due to possible variations in oxygen concentration may be maximally around 20%. A long-standing matter of dispute in radical research is the postulate that radiolytically generated •OH radicals might behave differently than those being produced in Fenton-type processes; the possibility has been discussed that crypto-OH'[19] or high valency iron-oxo states[20] may result in different products than 'free' •OH radicals. We therefore compared the results obtained with gamma radiolysis (Figure 2) and pulse radiolysis (Figure 3) to those with an •OH source that depends on a reaction of complexed metal with oxygen (Figure 4) i.e. the generation of •OH by phosphate complexes as reported by Reinke *et al.*[21] or Biaglow and coworkers.[22,23]

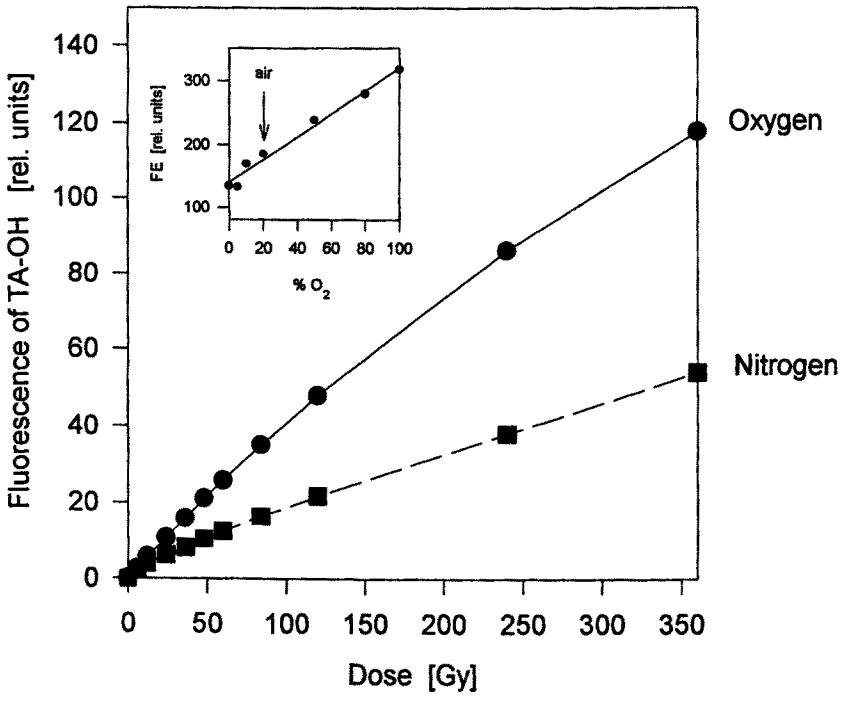


FIGURE 3 Oxygen dependence of the formation of hydroxylated terephthalate (conditions as in Figure 1). The inset shows TA-OH fluorescence when oxygen- and nitrogen-saturated solutions were mixed in respective ratio and 7.2 μM of hydroxyl radicals were generated by a short pulse of 1.8 MeV electrons.

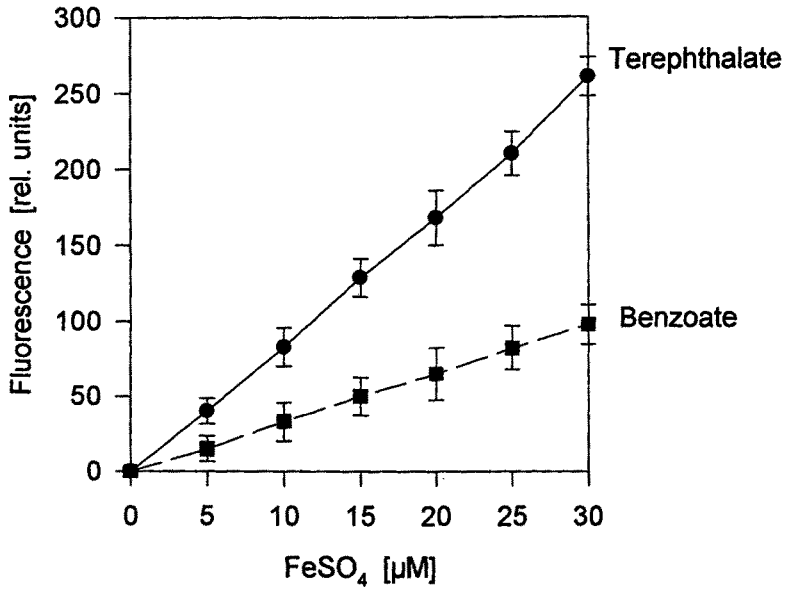


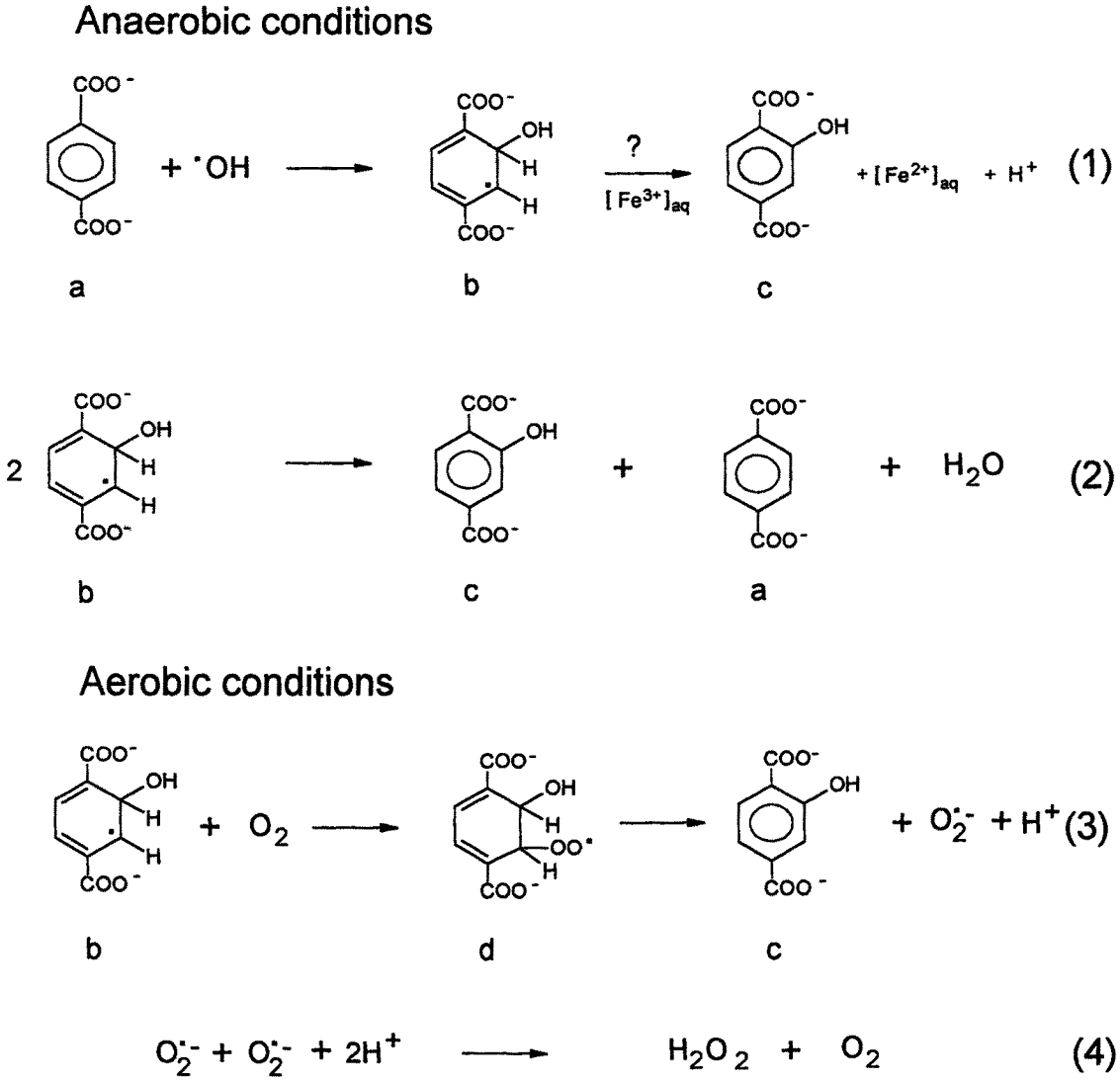
FIGURE 4 Concentration dependence of the generation of fluorescence in terephthalate and benzoate solution by hydroxyl radicals generated in a 'Fenton-type' process. Boli of 5 μM $FeSO_4$ were injected into 50 mM phosphate buffer containing 400 μM TA or BA, respectively; solution was purged with air. (Data are means ± standard deviation for $n = 5$.)

Figure 4 corroborates that with Fenton-type hydroxylation terephthalate is again superior to benzoate. Results with the 'true' Fenton-system, i.e. Fe(II) + hydrogen peroxide, were congruent to those in Figure 4.

The Scheme summarizes the main reaction pathways leading to hydroxylated products.[3] Under anoxic conditions the initially formed hydroxycyclohexadienyl radical (b) may either form TA-OH by reacting with spurious reductants (second step of reaction 1) or by disproportionation (reaction 2). In the presence of oxygen intermediate peroxyl radicals (d) are eventually formed, which, after elimination of the perhydroxyl radical $HO_2^{\bullet}$, rearrange to stable fluorescent TA-OH. $HO_2^{\bullet}$, which rapidly dissociates above p*K* 4.8, may dismutate in a pH-dependent reaction to H_2O_2 (reaction 4). Other pathways, not being discussed in the scheme, lead to decarboxylation and ring fragmentation. Matthews[1] reported a ratio of the yields of hydroxylated product/CO_2/peroxide of 1/1.3/2.8 which suggests that decarboxylation and H_2O_2 generation are rather important pathways. As the produced CO_2, however, does not influence further reactions[1] and ring fragmentation does not lead to any fluorescent product[3] severe disturbances of the overall fluorescence assay by these side reactions are not likely to be relevant.

H_2O_2 from reaction 4, in contrast, in the presence of metal contaminants, may be assumed to participate in further hydroxylations through Fenton-type reactions and hence may contribute to the detected fluorescence. This secondary effect, however, certainly occurs with all phenolic compounds in a similar manner.

The *absolute* sensitivity of the assay depends on the available equipment and parameters such as fluorescence self-quenching. Based on the known *G*-value of 280 nmol/dm^3 of $^{\bullet}$OH radicals produced by 1 Gy of radiation our set-up should be able to detect as low as 50 nmol/dm^3 of hydroxyl radicals.



SCHEME 1 Reaction pathways of terephthalate hydroxylation. Under anaerobic conditions *ortho*-hydroxylated terephthalate (c) may be formed from terephthalate (a) via intermediary hydroxycyclohexadienyl radicals (b) by direct reduction through metal contaminants (reaction 1) or via dismutation of two hydroxycyclohexadienyl radicals (reaction 2). In the presence of oxygen intermediary peroxyl radicals (d) may yield hydroxylated terephthalate by elimination of a perhydroxyl radical $HO_2^{\bullet}$ (reaction 3) which dissociates and dismutates to hydrogen peroxide and oxygen (reaction 4).

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