Free Rad. Res., Vol. 30, pp. 45–57
Reprints available directly from the publisher
Photocopying permitted by license only

Differences in the Reactivity of Phthalic Hydrazide and Luminol with Hydroxyl Radicals

J. SCHILLER[a,*], J. ARNHOLD[a], J. SCHWINN[b], H. SPRINZ[b], O. BREDE[b] and K. ARNOLD[a]

[a]*Institute of Medical Physics and Biophysics, Medical Department, University of Leipzig, Liebigstr. 27, D-04103 Leipzig, Germany;* [b]*Interdisciplinary Group "Time-resolved spectroscopy at the University of Leipzig", Permoserstraße 15, 04318 Leipzig, Germany*

Accepted by Prof. H. Sies

(Received 26 March 1998; In revised form 13 August 1998)

The reactivity of 5-amino-2,3-dihydro-phthalazine-1,4-dione (luminol) and phthalic hydrazide with hydroxyl radicals was studied. $HO^{\bullet}$-radicals were generated by the Fenton reaction as well as by water radiolysis. Both luminol and phthalic hydrazide react with hydroxyl radicals under intense chemiluminescence (CL) emission. However, exclusively the CL arising from phthalic hydrazide oxidation can be quenched by competition (e.g. by the addition of carbohydrates), whereas luminol CL is enhanced.

The reactivities of both compounds with $HO^{\bullet}$-radicals were further studied by time-resolved spectroscopy (pulse radiolysis), competition methods, NMR spectroscopy and mass spectrometry. Whereas only slight differences were detectable by pulse radiolysis, the analysis of competition kinetics in the presence of *p*-nitroso-dimethylaniline (NDMA) gave a two-fold-enhanced reactivity for luminol ($4.8 \times 10^9\,l\,mol^{-1}\,s^{-1}$) in comparison to phthalic hydrazide ($2.0 \times 10^9\,l\,mol^{-1}s^{-1}$).

NMR and mass spectrometric analyses revealed significant differences in the reactivity of $HO^{\bullet}$-radicals: whereas in luminol solutions hydroxylation of the aromatic ring system predominated, hydroxylated products were not detectable upon irradiation of phthalic hydrazide. A hypothetical mechanism is proposed which may explain the observed differences.

Keywords: Hydroxyl radicals, chemiluminescence, NMR spectroscopy, γ-radiolysis, luminol, phthalic hydrazide

INTRODUCTION

5-Amino-2,3-dihydro-phthalazine-1,4-dione (luminol) and analogous phthalic hydrazides are widely used in chemistry,[1,2] biochemistry[3–5] and immunology[6–8] to detect reactive oxygen species by the emission of light. Reactions leading to light emission in the luminol system are extremely complex and consist of a number of oxidation steps and nucleophilic additions to the aromatic ring.[9–12] The involvement of free radical intermediates was also described.[13] Hydrogen peroxide is known to enhance the chemiluminescence (CL) yield of the oxidation

*Corresponding author. Tel.: +49 341 97 15722. Fax: +49 341 97 15709. E-mail: schij@server3.medizin.uni-leipzig.de.

of luminol by hypochlorous acid.[14–17] The amino group in luminol serves as electron-donating group due to its positive mesomeric effect and enhances the CL intensity resulting from reactions on the hydrazide group.[10] The presence of an electron-donating group on the phenyl ring is most important because simple phthalic hydrazide itself does not yield any CL.[18] Thus, primarily an electron-donating group like a hydroxyl residue must be introduced into the aromatic ring by reaction with hydroxyl radicals. This enhances the electron density in the aromatic ring and the resulting hydroxylated phthalic hydrazide can be further converted to emit light.[18]

We focused especially on the reactivity of carbohydrates with hydroxyl radicals generated radiolytically or via Fenton chemistry,[19,20] since hydroxyl radicals are believed to be involved in the pathogenesis of inflammatory diseases, e.g. rheumatoid arthritis.[21,22] As recently shown by ^{1}H NMR spectroscopy, formate and to a lesser extent malondialdehyde are the main products of carbohydrate degradation processes.[20,23] Unfortunately, investigations based on product analysis give only limited information. To perform a detailed analysis, time-resolved techniques such as pulse radiolysis or different competition methods should also be used. Thus, our current aim is to use CL for studying hydroxyl radical-induced carbohydrate degradation processes.[19]

Besides phthalic hydrazide, luminol emits also light upon interaction with hydroxyl radicals. Two different processes, a reaction on the hydrazide group and an addition of hydroxyl radical on the aromatic ring, are assumed to be involved.[12,18] Unfortunately, detailed mechanisms of reactions of hydroxyl radicals with hydrazides remain unknown.

Therefore, here we investigated the reaction of hydroxyl radicals with luminol and phthalic hydrazide using CL, pulse radiolysis, γ-radiolysis, NMR spectroscopy and MALDI-TOF mass spectrometry.

MATERIALS AND METHODS

Materials

Luminol was from Boehringer Mannheim (Germany), whereas phthalic hydrazide (2,3-dihydrophthalazine-1,4-dione) was from Aldrich (Germany). Ferrous chloride, hydrogen peroxide, *p*-nitrosodimethylaniline (NDMA), buffer substances and all chemicals for NMR spectroscopy (sodium 3-(trimethylsilyl)-propane-1-sulphonate (TSP), deuterated water and deuterated dimethylsulphoxide with 99.95% ^{2}H) were purchased from Fluka Feinchemikalien (Neu Ulm, Germany). The concentration of hydrogen peroxide was determined by its UV absorption using $\lambda = 230$ nm and $\varepsilon = 74\,\mathrm{l\,mol^{-1}\,cm^{-1}}$.[24] Carbohydrates (glucose and chondroitin sulphate from bovine trachea with a molecular weight of about 5×10^4 Da) were obtained in highest available purity from Fluka Feinchemikalien (Neu Ulm, Germany). Chondroitin sulphate was dialysed prior to use against 50 mmol/l isotonic phosphate buffer pH 7.4 to remove traces of low-molecular components (mainly acetate and formate).[20,21]

Radiation Experiments

Stationary radiolysis experiments were carried out using the irradiation plant PANORAMA at the Institute for Surface Modification, Leipzig. The applied γ dose rate from a ^{60}Co source was 10.3 kGy per hour. Doses (applied between 4 and 12 kGy) were determined by Fricke dosimetry. All samples were irradiated in the presence of atmospheric oxygen in standard laboratory glass tubes.[20] Subsequently, water was evaporated and the residual material redissolved in perdeuterated DMSO for NMR measurements.

The time resolved (pulse radiolytic) experiments were carried out with the pulse transformer accelerator ELIT generating 20 ns, 100 Gy pulses of 1 MeV electrons. Transients were detected by optical spectroscopy in a continuous flow system. Spectra represent an average of 8 individual single "shots".

NMR-measurements

High resolution proton NMR measurements were conducted at ambient temperature on a Bruker AMX-300 spectrometer operating at 300.13 MHz for ^{1}H. Typically 0.40 ml of the aqueous solutions from the radiation experiments was placed in a 5 mm diameter NMR tube and 50 µl of D_2O was added to provide a field frequency lock. Water signal suppression was performed by presaturation, i.e. a 2 s pulse was applied on the water resonance frequency before data acquisition. One hundred and twenty eight free induction decays were averaged with a total delay between two pulses of 8 s to allow full spin-lattice relaxation (T_1) of the protons in the sample.[20] In some experiments to detect exchangeable protons, samples of both hydrazides were evaporated to dryness in a vacuum centrifuge (Jouan, Germany) and the sample was subsequently redissolved in perdeuterated dimethylsulphoxide (DMSO-d_6), which was carefully dried over molecular sieves.

All spectra were recorded with a spectral width of 4000 Hz, according to approximately 13 ppm. No line-broadening or Gauss-broadening was applied to the free induction decay (FID). Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propane-1-sulphonate in a final concentration of 500 µmol/l,[25] which was added after irradiation. In the case of DMSO, the remaining proton resonance of incompletely deuterated DMSO ($\delta = 2.49$ ppm) was used as internal reference.[26]

CL Measurements

CL measurements were performed on a Auto-Lumat LB 953 (Berthold, Wildbad, Germany) in polypropylene vessels (1 ml measuring volume) using 0.05 mol/l phosphate buffer, pH 7.4. The following concentrations were used: 1×10^{-5} mol/l luminol or phthalic hydrazide, which was added immediately prior to measurement, hydrogen peroxide and freshly prepared ferrous chloride in a concentration of 2.5×10^{-6} mol/l. The latter two substances were injected by an injector device.[19]

Ferrous ion solutions were prepared in double-distilled water under an atmosphere of argon. This procedure was necessary to avoid the formation of oxidation products like $Fe(OH)_3$ and $FePO_4$ under alkaline conditions and in the presence of oxygen.[27]

Photons were counted over a period of 10 s. Experiments were repeated at least three times.

MALDI-TOF Mass Spectrometry

Since luminol and phthalic hydrazide are strongly UV-absorbing chemicals, they can be easily analysed by MALDI-TOF mass spectrometry. An aqueous solution of luminol or phthalic hydrazide was treated with different ferrous ion concentrations, whereas the hydrogen peroxide concentration was kept constant. Subsequently all solutions were evaporated to dryness and the residual material redissolved in a small amount of DMSO. Since both hydrazides absorb at the laser wavelength, no additional matrix was used in these experiments and the samples were directly applied to the sample plate. Samples were allowed to crystallize under a warm stream of air under standard conditions.

MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry workstation (PerSeptive Biosystems, Framingham, UK). The extraction voltage used was 20 kV. Pressure in the ion chamber was maintained between 1×10^{-7} and 4×10^{-7} torr. To enhance the signal-to-noise ratio 128 single shots from the nitrogen laser (337 nm) were averaged for each mass spectrum. The laser strength was kept about 10% over threshold setting to obtain the best signal-to-noise ratio; spectra were run in the reflector mode.

Colorimetric Measurements

A stock solution (2×10^{-4} mol/l) of NDMA was prepared in 50 mmol/l phosphate buffer.[28]

Because of its light sensitivity this solution was kept in the dark. For all determinations it was further diluted to 4×10^{-5} mol/l. Hydrogen peroxide, followed by ferrous chloride were added to yield both final concentrations of 2×10^{-3} mol/l. Absorption of remaining NDMA was measured at 440 nm[29] in the absence and the presence of luminol or phthalic hydrazide to determine the rate constants of the reaction between these compounds and hydroxyl radicals. All colorimetric measurements were performed on a Hitachi U-2000 spectrophotometer in plexiglas cuvettes.

RESULTS

CL Measurements

Since luminol is one of the most commonly used chemiluminescent hydrazides,[8] CL experiments were first performed with luminol and the complete Fenton reagent (hydrogen peroxide and ferrous chloride). The generation of hydroxyl radicals is assumed in this oxidation reaction, although Fenton chemistry is still under discussion and ferryl species are also thought to be involved.[30–32]

Upon treatment of a phosphate buffered solution of luminol with hydrogen peroxide and ferrous ions, a flash of light is emitted. As shown in Figure 1(a), using a fixed concentration of luminol (1×10^{-5} mol/l) and ferrous chloride (2.5×10^{-6} mol/l), the light emission upon luminol oxidation depends on hydrogen peroxide concentration. With increasing hydrogen peroxide concentrations, CL yield increases up to ferrous ions and hydrogen peroxide are present in the same molar ratio, but CL decreases again with larger hydrogen peroxide concentrations.[30] Therefore, a 1:1 molar ratio between Fe^{2+} and H_2O_2 was used to obtain a maximum yield of CL.

In Figure 1(b), the time course of the reaction between luminol and the Fenton reagent is shown; counts were integrated over a 50 ms interval. The generation of light is completed after about 0.5 s after mixing luminol with the Fenton reagent. In accordance with Figure 1(a), light emission is most expressed, when an equimolar ratio between hydrogen peroxide

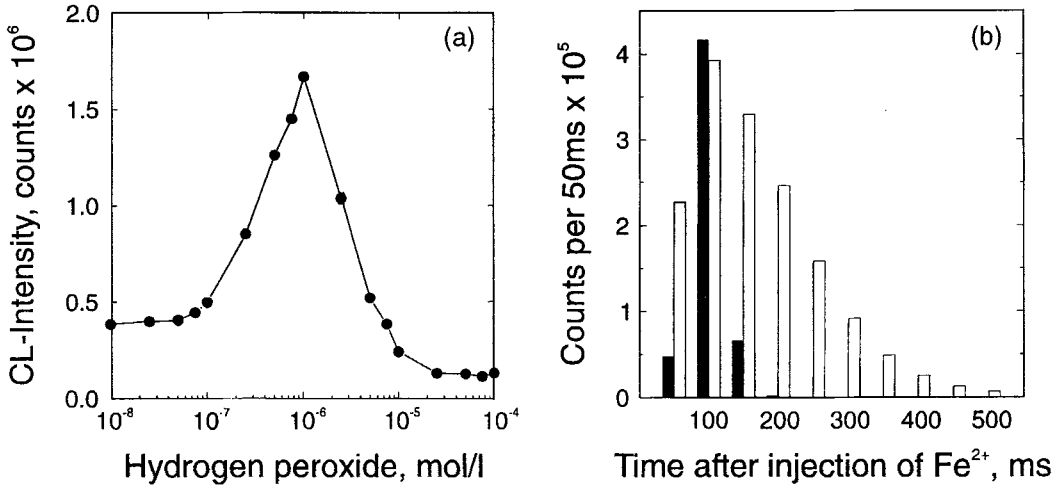


FIGURE 1 Intensity of luminol CL (a) and the corresponding time course (b). In (a) luminol (10^{-5} mol/l) was mixed with ferrous chloride (2.5×10^{-6} mol/l). Photons were counted for 10 s immediately after addition of varying amounts of hydrogen peroxide. (b) Shows the time course of CL-generation (1×10^{-5} mol/l luminol and 2.5×10^{-6} mol/l Fe^{2+}). Hydrogen peroxide was used at 1×10^{-6} mol/l (■) and 2.5×10^{-6} mol/l (□). CL yield was determined over 50 ms.

and ferrous ions is used, and luminescence yield decreases when the stoichiometry is altered. At lower concentrations of hydrogen peroxide, the reaction is much faster and is already completed after 0.2 s.

Since carbohydrates do not react with hydrogen peroxide or ferrous ions alone[20] and, thus, the stoichiometry of the reaction leading to CL is not altered in the presence of carbohydrates, CL intensity should be reduced depending on the carbohydrate concentration due to a competition of carbohydrates with hydrazides for hydroxyl radicals.[19] Four typical CL curves obtained with the physiologically relevant polysaccharide chondroitin sulphate and glucose are shown in Figure 2 using luminol (open symbols) as well as phthalic hydrazide (solid symbols) as light-generating compounds.

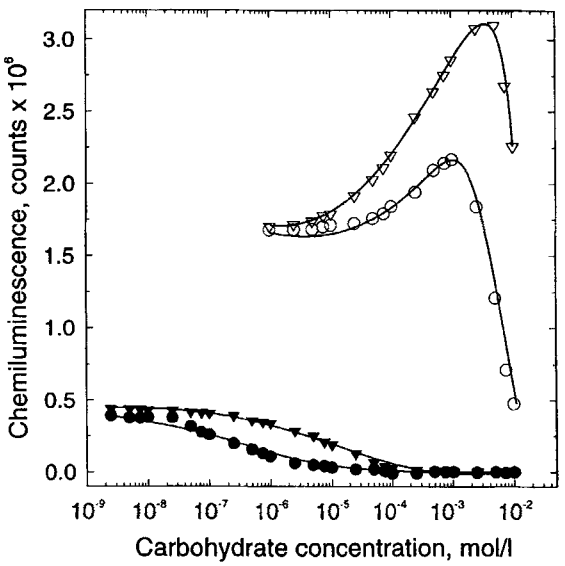


FIGURE 2 Comparison of CL curves recorded with luminol (open symbols) and phthalic hydrazide (solid symbols) upon the addition of the Fenton reagent. CL was recorded in the presence of chondrotin sulphate (▽) and glucose (○). Experiments were carried out in phosphate buffer, pH 7.4 using 1×10^{-5} mol/l of the corresponding hydrazides and hydrogen peroxide as well as ferrous chloride (2.5×10^{-6} mol/l). Photons were counted over 10 s. The means of four independent experiments are shown: standard deviations are in the range of symbol size.

Differences in the shape of competition curves and the corresponding CL intensities are detectable. First, CL intensities obtained by the use of luminol are enhanced about five times in comparison to the light intensity using phthalic hydrazide. Additionally, the shape of the curves is different for both compounds. A diminution of CL intensities with increasing concentrations of carbohydrates (due to competition of phthalic hydrazide with carbohydrates for hydroxyl radicals) is only found in the presence of phthalic hydrazide.

However, strongly enhanced CL intensities result over a wide carbohydrate concentration range (up 1 mmol/l to) when luminol is used. Whereas the differences in light intensity can be explained by the electron-donating amino group in luminol,[10] the enhanced CL intensities in the presence of luminol are not yet understood (see discussion).

Time Resolved Radiolysis Studies

Phthalic hydrazides exhibit two possible targets for HO•-radicals, on the aromatic and on the N-substituted heterocyclic ring system. Although Baxendale[33] did pioneering pulse radiolysis studies on luminol, this work did not show any evidence for the generation of hydroxylated aromatic species. However, hydroxylation is expected upon radiolysis of aqueous solutions of aromatic species.[34]

In Figure 3 the time-resolved UV-spectra of N_2O-saturated solutions of both compounds are shown. In pulse radiolysis methodology, a short high-energy electron pulse[35,36] from an accelerator is passed through a cuvette containing the corresponding sample and the optical spectrum recorded a few μs after this pulse. In the case of dilute aqueous solutions, energy is practically exclusively spent in the production of reactive oxygen species from the water and the following particular reactions can be assumed:

$$H_2O \rightarrow H_2O^{\bullet+} + e^-, H_2O^* \qquad (1)$$

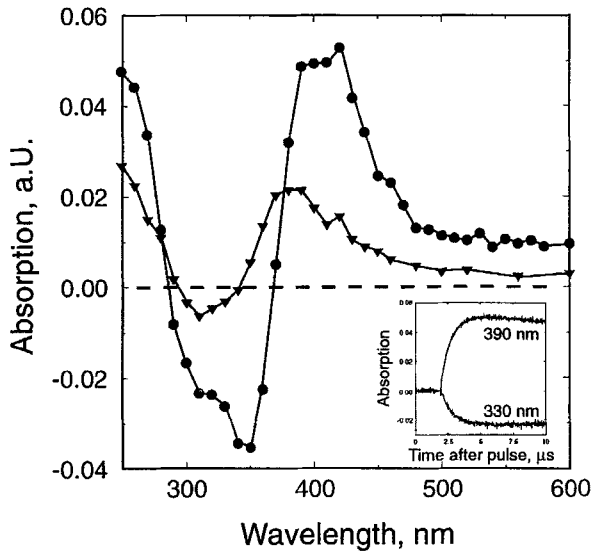


FIGURE 3 Time-resolved spectrophotometric study of the reaction between luminol (●) and phthalic hydrazide (▼) in a final concentration of 1×10^{-4} mol/l in 0.05 mol/l phosphate buffered solution, flushed with N_2O. Spectra were recorded 5 µs after a 5.6 krad electron pulse. The inset shows the decay at 330 nm and the formation of a new absorption at 390 nm.

$$H_2O^{\bullet+}(+nH_2O) \rightarrow HO^{\bullet} + H^{+} \quad (2)$$

$$e^{-}(+nH_2O) \rightarrow e^{-}_{aq} \quad (3)$$

$$H_2O^{*} \rightarrow H^{\bullet} + HO^{\bullet} \quad (4)$$

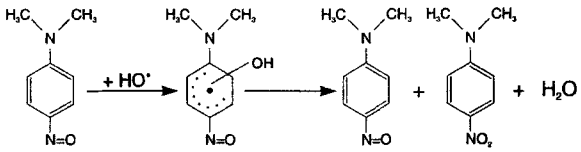
N_2O-saturated solutions are often used, where the N_2O serves for the conversion of electrons into hydroxyl radicals. Thus, the yield of hydroxyl radicals is enhanced:

$$e^{-}_{aq} + N_2O + H_2O \rightarrow HO^{\bullet} + HO^{-} + N_2 \quad (5)$$

In our experiments, spectra were obtained 5 µs after a 5.6 krad electron pulse. In both cases, the absorption at about 330 nm is diminished markedly upon the electron pulse, and this diminution is accompanied by the formation of an additional absorption at about 390 nm according to the formation of the hydroxy-cyclohexadienyl radical (see insert). From the formation of the strong absorption at 390 nm, second-order rate constants can be derived for the reaction between HO•-radicals and both hydrazides. The analysis according to the formation of the cyclohexadienyl radical yields 7.4×10^9 l mol^{-1} s^{-1} for luminol and 5.3×10^9 l mol^{-1} s^{-1} for phthalic hydrazide, indicating only small differences in the reactivity.

Determination of Rate Constants by the Bleaching of *p*-nitrosodimethylaniline

Due to the superposition of absorbances, competition kinetics by pulse radiolysis with e.g. NDMA[28,29] are difficult to determine. Thus, we have performed additional, stationary experiments using the competition between the hydrazides and NDMA. This dye is known to react with hydroxyl radicals under the formation of colourless products.[29] The reaction between HO• and the dye can be monitored by the "bleaching" of the absorption at 440 nm, where the following reactions are assumed:[29]



(6)

Figure 4(a) shows the differences in bleaching of the dye in the presence of luminol or phthalic hydrazide. In the absence of any additional compound, NDMA is the sole available target for HO• which induces intense bleaching, i.e. a low residual absorption at 440 nm. The observed absorption difference upon the addition of the Fenton reagent is about 0.9 under our experimental conditions. When additional components (luminol and phthalic hydrazide) compete with NDMA for hydroxyl radicals, a reduced bleaching is observed with increasing concentrations of the hydrazides. It is evident that both compounds show slightly different competition curves (Figure 4(a)), corresponding to different second-order rate constants.

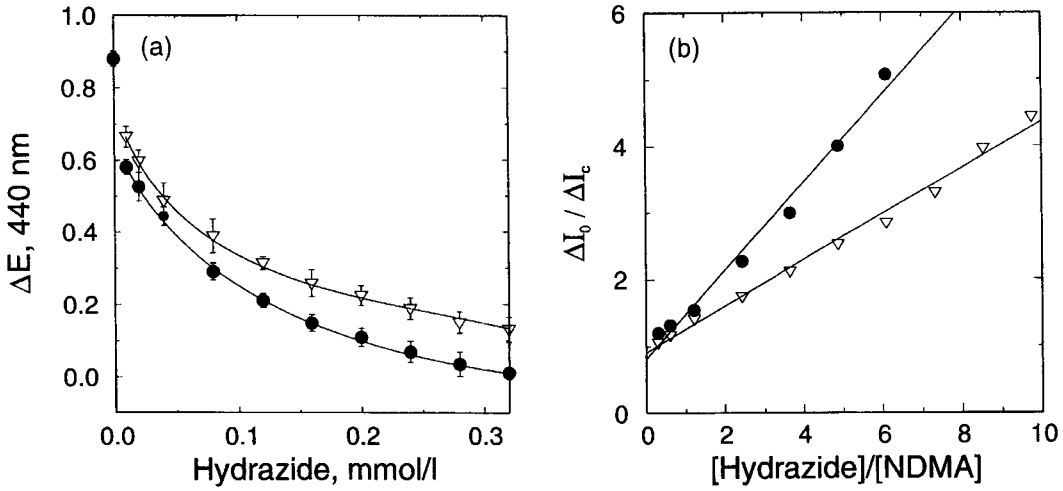


FIGURE 4 UV-absorption at 440 nm of a solution of 4×10^{-5} mol/l NDMA. Hydrogen peroxide, followed by ferrous chloride were added to yield final concentrations of 2×10^{-3} mol/l in the presence or absence of luminol (●) or phthalic hydrazide (▽, a). Stern–Volmer plots are given.

Quantitative analyses were also performed using a Stern–Volmer plot:[29]

$$\frac{\Delta I_0}{\Delta I_c} = 1 + \frac{k_b[\text{hydrazide}]}{k_a[\text{NDMA}]}. \qquad (7)$$

With the second-order rate constant for the reaction between HO• and NDMA, the corresponding values for the hydrazides can be calculated. ΔI_0 and ΔI_c denote the absorption differences at 440 nm in the absence or the presence of the corresponding hydrazides, respectively, where k_a and k_b are the rate constants of the reaction of NDMA and the corresponding hydrazide with hydroxyl radicals.[36]

Straight lines with an intercept at 1 are obtained (Figure 4(b)), when the ratio $\Delta I_0/\Delta I_c$ is plotted versus the concentration ratio [hydrazide]/[NDMA]. The second-order rate constant for the reaction between HO• and NDMA was determined to be 1.25×10^{10} l mol^{-1} s^{-1};[29] this value is about two-fold higher than one would expect for a diffusion-controlled reaction. On the other hand, the determination by pulse radiolysis gave 6.9×10^9 l mol^{-1} s^{-1}. This value seems more suitable and was used for the calculation of the second-order rate constants, 4.8×10^9 l mol^{-1} s^{-1} for luminol and 2.0×10^9 l mol^{-1} s^{-1} for phthalic hydrazide. This is roughly in agreement with the value for luminol, determined by pulse radiolysis (8.7×10^9 l mol^{-1} s^{-1}).[33] Thus, the presence of the amino group in luminol markedly changes the reactivity against HO•-radicals in comparison to phthalic hydrazide.[11,12]

NMR Spectroscopic Studies

Figure 5 shows the NMR spectra of luminol and phthalic hydrazide in DMSO-d_6. This solvent was chosen since it allows (in contrast to water) the detection of acidic protons (the –NH-groups of the hydrazide group and the amino group in luminol).[37] Both hydrazides are scarcely soluble in water or buffers, but easily soluble in DMSO.[38] The spectrum of luminol (Figure 5(a)) exhibits three well-resolved resonances for the C–H-protons of the aromatic ring and two broad signals for the exchangeable protons of the hydrazide (11.25 ppm) and the amino group (7.32 ppm). In contrast, the spectrum of phthalic hydrazide (Figure 5(b)) consists only of three well-resolved resonances, in good agreement with Ref. [38]. It should be mentioned that the

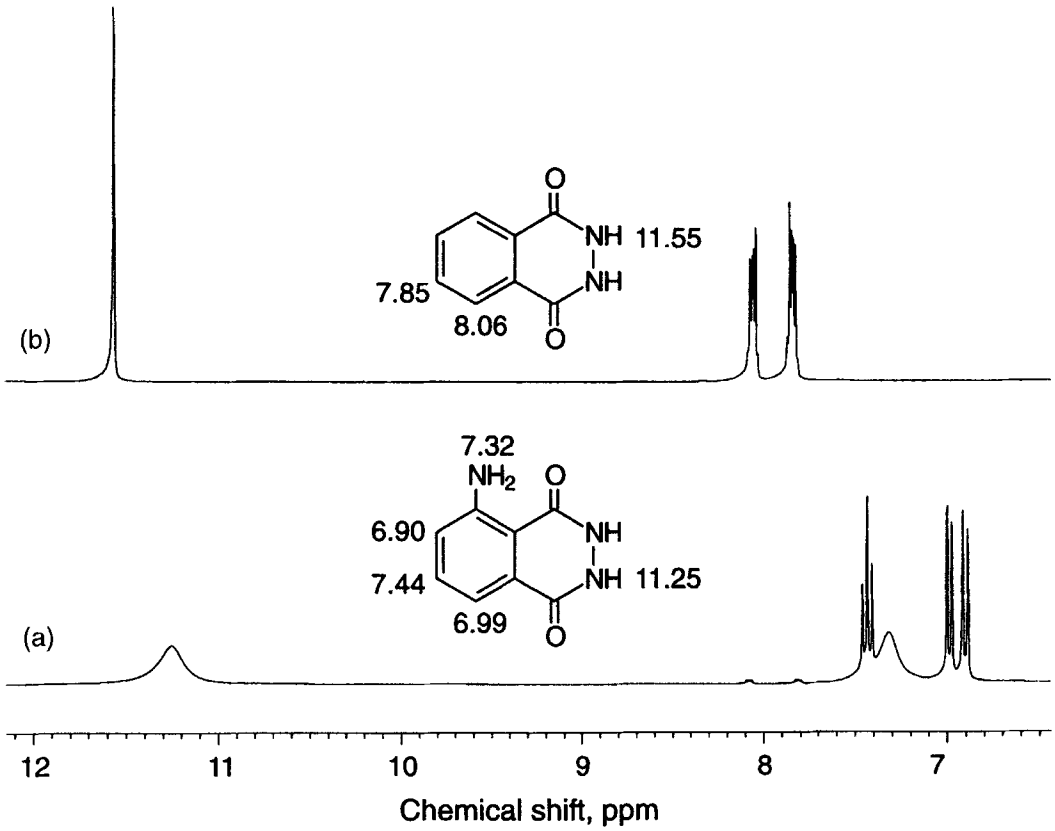


FIGURE 5 Proton NMR spectra of a 0.01 mol/l solution of luminol (a) and phthalic hydrazide (b) dissolved in DMSO-d_6. The assignment of the different resonances to the corresponding protons is given.

resonances are influenced by the residual water content in the DMSO (data not shown), particularly the resonances at 6.90 and 6.99 ppm.

Regarding the shape of the resonance of the hydrazide group (11.25/11.55 ppm), it is sharp in phthalic hydrazide, and it is rather broad in the luminol spectrum. Additional data from ^{13}C NMR investigations indicate an expressed tautomerization between the keto- and the enol-form (data not shown) which results in a considerable line-width of the hydrazide protons. An exchange of the NH protons with the residual small quantity of water cannot be excluded, whereby luminol is more influenced by the presence of water than phthalic hydrazide. When the residual water resonance in a luminol solution (in DMSO containing a small quantity of water) is suppressed by presaturation, the resonance of the amino group at 7.29 ppm also vanishes (data not shown). This indicates that a small quantity of water is in direct contact with the amino group of luminol and a fast exchange with the amino group takes place.[39]

Figure 6 shows the low-field end of the NMR spectra of a solution of luminol in DMSO-d_6 in dependence on the applied radiation dose. The following doses were applied: (a) blank sample, (b) 4 kGy, (c) 8 kGy, and (d) 12 kGy. In (a) the influence of the water concentration on the luminol resonances is clearly visible. Since irradiation has to be carried out in water, the aqueous solutions were evaporated to dryness after irradiation and the residual material was redissolved in DMSO. Obviously, this procedure

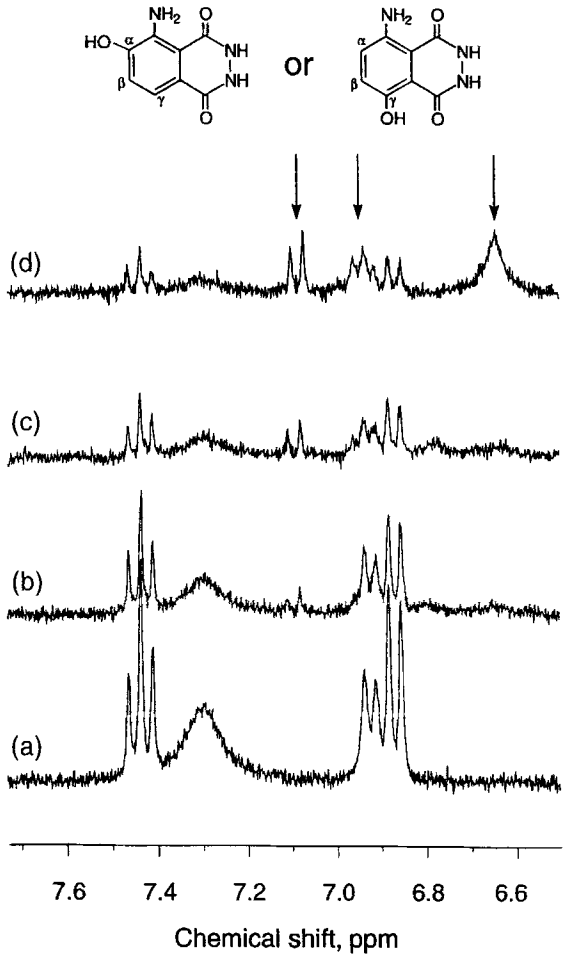


FIGURE 6 Proton NMR spectra of irradiated samples of luminol dissolved in DMSO-d_6. The following radiation doses were applied: (a) No irradiation, (b) 4 kGy, (c) 8 kGy, and (d) 12 kGy. Samples were irradiated in water. Subsequently, water was evaporated and replaced by DMSO-d_6.

confers an enhanced water content in the sample and, thus, the intensities of resonances are changed in comparison to a luminol sample dissolved in absolute DMSO (Figure 5(a)). The limited solubility of luminol in water also leads to a poor signal-to-noise ratio in the spectra of irradiated samples.

Spectra change markedly with increasing radiation doses, whereby all resonances of luminol are markedly diminished and some new resonances arise. A doublet at 7.09 ppm gets already detectable at the lowest radiation dose ((b), 4 kGy) and increases over the applied radiation dose. A doublet is obviously caused by the coupling with a further C–H-proton. The characteristic coupling constant $^3J(^1H^1H) = 8.3$ Hz indicates that both protons are directly ortho-coupled.[37] This can only result from a hydroxylation in α- or γ-position to the amino group (see Figure 6). These positions exhibit the highest negative charge density[40] and are, thus, the preferred sites of hydroxylation. Unfortunately, the expected second doublet of the hydroxylated product was not detected, most likely because of a superposition of this new resonance with the residual luminol resonance at 6.99 ppm.

The hydroxylation of the aromatic ring is also monitored by the appearance of a broad resonance at 6.65 ppm most likely corresponding to the OH-proton of the introduced hydroxyl group. This resonance is only detectable upon irradiation with 12 kGy where luminol is nearly completely converted into the hydroxylated product. Surprisingly, changes on the hydrazide group at 11.25 ppm were not detectable. Here, only a drastic diminution of signals occurs without the formation of further resonances.

In contrast, the NMR spectra of phthalic hydrazide remain nearly unchanged upon irradiation (data not shown). A continuous decrease of all resonances indicates a complete breakdown of phthalic hydrazide. Only at the highest applied radiation dose (12 kGy) very small additional resonances between 7.3 and 7.6 ppm become detectable.

MALDI-TOF Mass Spectrometric Analysis

MALDI-TOF (matrix-assisted laser desorption and ionization time of flight) mass spectrometry was originally developed for the investigation of proteins and other biopolymers,[41] where an appropriate UV-absorbing matrix component transfers the laser energy to the sample. This method can also be applied for the analysis of low-molecular compounds which show an

UV-absorbance, without any additional matrix compound. Changes in the negative ion mass spectra of luminol (left) and phthalic hydrazide (right) upon the action of Fenton reagent (hydrogen peroxide and ferrous chloride) are given in Figure 7. Pure luminol and phthalic hydrazide yield a single peak at 176 or 161, respectively, corresponding to the molecular mass of their anions. The treatment with hydrogen peroxide alone (b) did not cause any changes in the mass spectra of both hydrazides.

However, some minor peaks arise at higher molecular weights after the addition of $FeCl_2$ (traces c and d). An additional peak at 192 is formed under these experimental conditions in luminol solution. In accordance with NMR experiments this peak corresponds to hydroxylated luminol. Other products were not detectable in this sample. On the other hand, a more complicated product spectrum is observed in phthalic hydrazide treated with the Fenton reagent. In addition to the phthalic hydrazide (161) there is a number of less intense peaks. The peak at 177 corresponds to the hydroxylated product as in the case of luminol. The hydroxylation of the aromatic ring in hydrazides shifts the molecular weight by 16 units. There is a further peak at 193 with a shift of 32 units towards the phthalic hydrazide. It seems very unlikely that a second hydroxylation site exists because NMR experiments did not reveal signs of hydroxylation. Thus, we assume that subsequent to the abstraction of a hydrogen atom from the hydrazide group either by Fe^{3+} or by the hydroxyl radical, molecular oxygen can be added to the molecule. Finally, the peak at 209 may result from a combination of both effects, i.e. addition of molecular oxygen to the hydrazide ring and hydroxylation of the aromatic site.

Thus, a hydroxylated product is exclusively formed in the luminol solution upon the action of Fenton reagent, but several different products are formed in phthalic hydrazide. Another conclusion from these experiments is that mainly hydroxyl radicals must be formed by the Fenton reaction. The involvement of ferryl species would result in the formation of higher oxidation products, which should show a marked molecular weight distribution of iron isotopes. However, such products were not detectable.

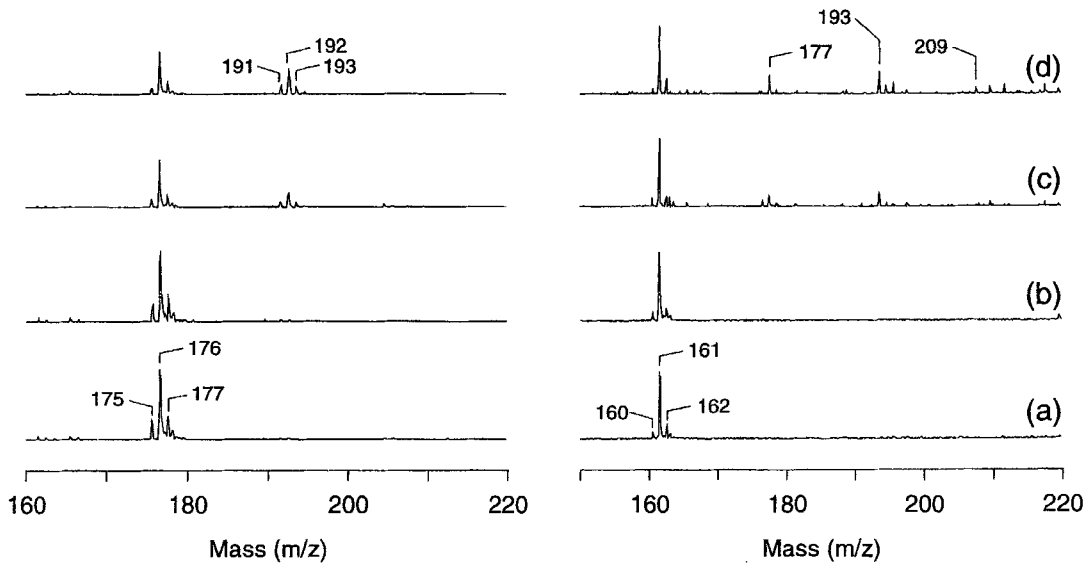


FIGURE 7 MALDI-TOF mass spectra of luminol (left) and phthalic hydrazide (right). For each sample a 10 mmol/l solution of the corresponding hydrazide was used. (a) shows the reference sample, whereas (b) was recorded upon the addition of a 1 : 1 molar ratio of hydrogen peroxide. Ferrous chloride was added to the samples: 5 mmol/l (c), and 10 mmol/l (d).

DISCUSSION

The amino substituted derivative of phthalic hydrazide, known as luminol,[42] and its unsubstituted analogue, phthalic hydrazide[43,44] were compared towards their reactivity with hydroxyl radicals. The second-order rate constants for the initial reaction with hydroxyl radicals determined by pulse radiolysis and, more expressed, by competition kinetics with NDMA are about two-fold higher for luminol than for phthalic hydrazide.

Using ^{1}H NMR spectroscopy we were able to detect marked differences in the radiation behaviour. Whereas luminol is clearly hydroxylated by HO$^\bullet$, hydroxylation was not observed using phthalic hydrazide in good agreement with MALDI-TOF experiments. With this technique, hydroxylation of luminol was detectable, but hydroxylated phthalic hydrazide was only detectable to a low extent. In the latter case, the addition of molecular oxygen to the hydrazide site of the molecule predominates.[45]

Whereas differences in p*Ka* values of luminol (1.46/6.35/15.21)[46] and phthalic hydrazide (5.87/14.75)[47] are not likely to cause such differences, electronic effects of the amino group obviously markedly influence the reactivity of luminol and phthalic hydrazide. Due to its positive mesomeric effect, electron density is enhanced in the aromatic rings of luminol.

The presence of the amino group also determines the structure of luminol, conferring differences in the NMR spectrum in comparison to phthalic hydrazide. This especially concerns the line-width of the hydrazide protons and the interaction of the amino group with water. We assume that a water molecule is very close to the amino group, since upon saturation of the water resonance, the amino group also vanishes. Additionally, a fast equilibrium between the keto- and enol-forms of luminol is assumed, since the line-width of the hydrazide protons in luminol is considerably larger than in unsubstituted phthalic hydrazide. On the other hand, an aggregation of phthalic hydrazide in aqueous solution cannot be excluded.

Phthalic hydrazide competes efficiently with other compounds for hydroxyl radicals, detectable by the inhibition of CL (see Figure 2). This approach can be used to determine second-order rate constants for hydroxyl radical reactions with carbohydrates.[19] Due to its p*Ka*-value of 6.35 there is also a fast exchange between the protonated and the unprotonated form of luminol under mild alkaline conditions, and, thus, both forms cannot be discriminated.[46,47] Two different reaction sites for HO$^\bullet$ on luminol have been proposed (Figure 8). First, HO$^\bullet$ is added as a powerful electrophilic oxidant to the aromatic ring (compare NMR and MALDI results). The resulting radical interacts with molecular oxygen under the formation of a peroxyl radical. This product is subsequently converted into a hydroxylated compound under removal of

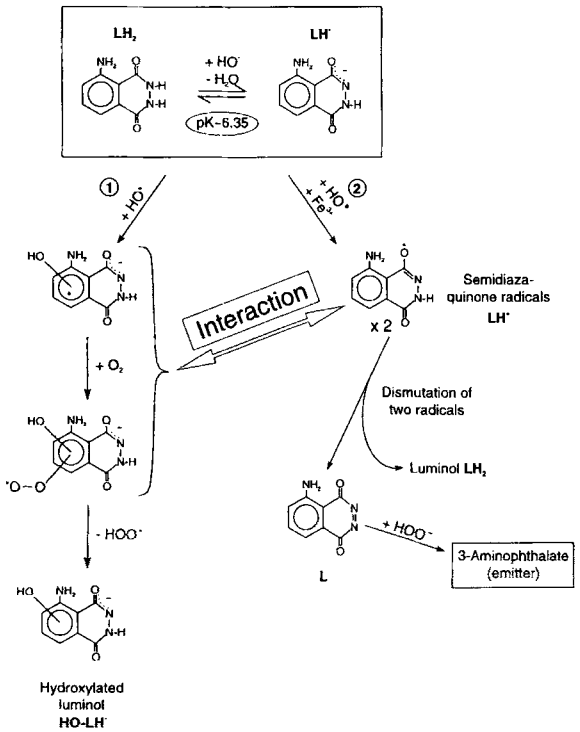


FIGURE 8 Schematic representation of the molecular events involved in luminol CL.

superoxide.[18,48] Secondly, HO• is also known to abstract a hydrogen atom from the hydrazide group yielding a semidiazaquinone radical.[12,45] This second pathway leads to the formation of 3-aminophthalate, the emitter of luminol CL.[12] It is assumed that only about 30% of hydroxyl radicals contribute to the second pathway, whereas most hydroxyl radicals cause a hydroxylation of the aromatic ring.[12] This is in agreement with our MALDI experiments. Since CL is formed solely from the diazaquinone, and hydroxylated luminol acts as an efficient scavenger, higher amounts of hydroxylated luminol may quench CL.[18]

On the other hand, ferric ions have also been shown to induce the formation of semidiazaquinone radicals, resulting in CL.[49] Since the Fenton reaction yields two different species (hydroxyl radicals, ferric ions) which both react with luminol, CL-generation is extremely difficult to describe and depends on a lot of different conditions.

From our experiments we propose a mechanistic scheme to explain the differences in the quenching ability of luminol and phthalic hydrazide; both, hydrogen peroxide as well as carbohydrate concentration are known to influence CL patterns. At low concentrations of oxidizing species (HO•, Fe^{3+}), pathway 2 of luminol oxidation is preferred. At higher concentrations, the contribution of pathway 1 increases and an inhibition of luminescence-generation is observed. Thus, scavenging of hydroxyl radicals by carbohydrates leads to the diminution of oxidizing species and conditions favouring pathway 2, whereby an enhancement of CL occurs.

Thus, we must conclude that the CL properties of luminol are different from its unsubstituted derivative. These differences should be carefully taken into account when using CL as a competition method to estimate effects of hydroxyl radicals on selected substances. Phthalic hydrazide seems to be the compound of choice for this purpose.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (INK 23/A1-1, Graduiertenkolleg "Mechanisms and applications of non-conventional oxidation reactions"). J. Schiller was supported by a DFG-Forschungsstipendium (Schi 476/1-1). Helpful advice from Dr. Haferburg (Faculty for Biosciences, Pharmacy and Psychology, University of Leipzig) is gratefully acknowledged.

References

[1] H. Obata, H. Karatani and E. Nakayama (1993) Automated determination of iron in seawater by chelating resin concentration and chemiluminescence detection. *Analytical Chemistry*, **65**, 1524–1528.
[2] L.L. Klopf and T.A. Nieman (1983) Effect of iron (II), cobalt (II), copper (II), and manganese (II) on the chemiluminescence of luminol in the absence of hydrogen peroxide. *Analytical Chemistry*, **55**, 1080–1083.
[3] R.C. Allen (1987) Phagocyte oxygenation activities: quantitative analysis based on luminescence of hypochlorous acid: Implication for microbicidal mechanisms of leukocyte myeloperoxidase. *Proceedings of the National Academy of Sciences of the USA*, **78**, 210–214.
[4] C. Rathakrishnan, K. Tiku, A. Raghavan and M.L. Tiku (1992) Release of oxygen radicals by articular chondrocytes: A study of luminol-dependent chemiluminescence and hydrogen peroxide secretion. *Journal of Bone and Mineral Research*, **7**, 1139–1148.
[5] J. Arnhold, K. Sonntag, H. Sauer, H. Häntzschel and K. Arnold (1994) Increased native chemiluminescence in granulocyes isolated from synovial fluid and peripheral blood of patients with rheumatoid arthritis. *Journal of Bioluminescence and Chemiluminescence*, **9**, 79–86.
[6] C. Dahlgren, P. Follin, H. Lundqvist and C. Sjolin (1993) Quantitative slot-blot chemiluminescence assay for determination of myeloperoxidase from human granulocytes. *Analytical Biochemistry*, **214**, 284–288.
[7] C.E. Olesen, J.J. Fortin, J.C. Voyta and I. Bronstein (1997) Detection of beta-galactosidase and beta-glucuronidase using chemiluminescent reporter gene assays. *Methods in Molecular Biology*, **63**, 61–70.
[8] L.J. Kricka (1991) Chemiluminescent and bioluminescent techniques. *Clinical Chemistry*, **379**, 1472–1481.
[9] G. Merényi and J.S. Lind (1980) Role of a peroxide intermediate in the chemiluminescence of luminol. A mechanistic study. *Journal of the American Chemical Society*, **102**, 5830–5835.
[10] S. Ljunggren, G. Merényi and J.S. Lind (1983) A plausible mechanism for the chemiexcitation of cyclic hydrazides. The "double-crossing" model. *Journal of the American Chemical Society*, **105**, 7662–7666.
[11] E. Würzberg and Y. Haas (1979) Radiolysis study of the chemiluminescence of some luminol-like molecules. *Journal of Physical Chemistry*, **83**, 2687–2692.

[12] G. Merényi, J. Lind and T.E. Eriksen (1990) Luminol chemiluminescence: chemistry, excitation, emitter. *Journal of Bioluminescence and Chemiluminescence*, **5**, 53–56.
[13] M. Candida, B.L. Shohoji, H.M. Novais and B.J. Herold (1988) Electron spin resonance study of radicals derived from cyclic hydrazides. *Journal of the Chemical Society, Faraday Transactions II*, 1259–1261.
[14] S. Mueller and J. Arnhold (1995) Fast and sensitive determination of the H_2O_2 concentration in stimulated human neutrophils. *Journal of Bioluminescence and Chemiluminescence*, **10**, 229–237.
[15] J. Arnhold, S. Mueller, K. Arnold and K. Sonntag (1993) Mechanisms of inhibition of chemiluminescence in the oxidation of luminol by sodium hypochlorite. *Journal of Bioluminescence and Chemiluminescence*, **8**, 307–313.
[16] E.P. Brestel (1985) Co-oxidation of luminol by hypochlorite and hydrogen peroxide: implications for neutrophil chemiluminescence. *Biochim. Biophys. Res. Commun.* **126**, 482–488.
[17] J. Arnhold, S. Mueller, K. Arnold and E. Grimm (1991) Chemiluminescence intensities and spectra of luminol oxidation by sodium hypochlorite in the presence of hydrogen peroxide. *Journal of Bioluminescence and Chemiluminescence*, **6**, 189–192.
[18] T. Reitberger and J. Gierer (1988) Chemiluminescence as a means to study the role of hydroxyl radicals in oxidative processes. *Holzforschung*, **42**, 351–356.
[19] J. Arnhold, J. Schiller and K. Arnold (1997) Interaction of carbohydrates with hypochlorous acid and hydroxyl radicals. In: *Bioluminescence and Chemiluminescence. Molecular Reporting with Photons* (eds. J.W. Hastings, L.J. Kricka and P.E. Stanley) Wiley, Chichester, 323–326.
[20] J. Schiller, J. Arnhold, J. Schwinn, H. Sprinz, O. Brede and K. Arnold (1998) Reactivity of cartilage and selected carbohydrates with hydroxyl radicals – An NMR study to detect degradation products. *Free Radical Research*, **28**, 215–228.
[21] M. Grootveld, E.B. Henderson, A. Farrell, D.R. Blake, H. Parkes and P. Haycock (1991) Oxidative damage to hyaluronate and glucose in synovial fluid during exercise of the inflamed rheumatoid joint. *Biochemical Journal*, **273**, 459–467.
[22] H.G. Parkes, M.C. Grootveld, E.B. Henderson, A. Farrell and D.R. Blake (1991) Oxidative damage to synovial fluid from the inflamed rheumatoid joint detected by ^{1}H NMR spectroscopy. *Journal of Pharmaceutical & Biomedical Analysis*, **9**, 75–82.
[23] C. von Sonntag (1985) *The Chemical Basis of Radiation Biology*. Taylor & Francis, London.
[24] R.F. Beers and I.W. Sizer (1952) A spectrometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* **195**, 133–140.
[25] M. Kriat, S. Confort-Gouny, J. Vion-Dury, M. Sciacky, P. Viout and P.J. Cozzone (1992) Quantitation of metabolites in human blood serum by proton magnetic resonance spectroscopy. A comparative study of the use of formate and TSP as concentration standards. *NMR in Biomedicine*, **5**, 179–184.
[26] H.-O. Kalinowski, S. Berger and S. Braun (1984) ^{13}C-*NMR Spektroskopie*. Georg Thieme Verlag, Stuttgart.
[27] G. Bottu (1989) The effect of quenchers on the chemiluminescence of luminol and lucigenin. *Journal of Bioluminescence and Chemiluminescence*, **3**, 59–65.
[28] I. Kraljić and C.N. Trumbore (1965) p-Nitrosodimethylaniline as an OH radical scavenger in radiation chemistry. *Journal of the American Chemical Society*, **87**, 2527–2550.
[29] W. Bors, C. Michel and M. Saran (1979) On the nature of biochemically generated hydroxyl radicals. *European Journal of Biochemistry*, **95**, 621–627.
[30] P. Wardman and L.P. Candeias (1996) Fenton chemistry: An introduction. *Radiation Research*, **145**, 523–531.
[31] S. Goldstein, D. Meyerstein and G. Czapski (1993) The Fenton reagents. *Free Radical in Biology and Medicine*, **15**, 435–445.
[32] X. Shen, J. Tian, X. Li and Y. Chen (1992) Formation of the excited ferryl species following Fenton reaction. *Free Radical in Biology and Medicine*, **13**, 585–592.
[33] J.H. Baxendale (1973) Pulse radiolysis study of the chemiluminescence from luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) *Journal of the Chemical Society, Faraday Transactions* **69**, 1665–1677.
[34] X.-M. Pian and C. von Sonntag (1990) OH-radical-induced oxidation of benzene in the presence of oxygen: $R^\bullet + O_2 \rightleftharpoons RO_2^\bullet$ equilibria in aqueous solution. A pulse radiolysis study. *Zeitschrift für Naturforschung*, **45b**, 1337–1340.
[35] C. von Sonntag and H.-P. Schuchmann (1994) Pulse radiolysis. *Methods in Enzymology*, **233**, 3–20.
[36] A. Henglein, W. Schnabel and J. Wandenberg (1969) *Einführung in die Strahlenchemie*. Verlag Chemie, Weinheim.
[37] H. Friebolin (1988) *Ein- und zweidimensionale NMR-Spektroskopie*. VCH Verlagsgesellschaft, Weinheim.
[38] L.R. Caswell and G. Casavos (1995) Cyclic Imides. 16. Hydroxy and methoxy derivatives of aminophthalimide and phthalhydrazide. *Journal of Heterocyclic Chemistry*, **32**, 907–914.
[39] J. Lee and H.H. Seeliger (1970) Spectral characteristics of the excited states of the product of the chemiluminescence of luminol. *Photochemistry and Photobiology*, **11**, 247–258.
[40] H. Beyer and W. Walter, *Lehrbuch der Organischen Chemie*, Hirzel Verlag Stuttgart, 1984.
[41] F. Hillenkamp and M. Karas (1991) Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Analytical Chemistry*, **63**, 1193A–1203A.
[42] K. Faulkner and I. Fridovich (1993) Luminol and lucigenin as detectors for $O_2^{\bullet-}$. *Free Radical in Biology and Medicine*, **15**, 447–451.
[43] E.H. White and D.F. Roswell (1970) The chemiluminescence of organic hydrazides. *Accounts of Chemical Research*, **3**, 54–62.
[44] J. Stauff und G. Hartmann (1965) Über den Mechanismus der Chemilumineszenz von Phthalhydrazid. *Berichte der Bunsengesellschaft für Physikalische Chemie*, **69**, 145–155.
[45] G.N.R. Tripathi (1998) Electron-transfer component in hydroxyl radical reactions observed by time resolved resonance raman spectroscopy. *Journal of the American Chemical Society*, **120**, 4161–4166.
[46] Beilstein Handbuch der Organischen Chemie, E V 25/16, p. 7.
[47] Beilstein Handbuch der Organischen Chemie, E V 24/7, p. 560.
[48] C. von Sonntag and H.-P. Schuchmann (1991) Aufklärung von Peroxyl-Radikalreaktionen in wässriger Lösung mit strahlenchemischen Techniken. *Angewandte Chemie*, **103**, 1255–1279.
[49] P.B. Shevlin and H.A. Neufeld (1970) Mechanism of the ferricyanide-catalyzed chemiluminescence of luminol. *Journal of Organic Chemistry*, **35**, 2178–2182.