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LONG-LIVED FREE RADICALS FORMED IN IRRADIATED DYE SOLUTIONS¹

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Although many free-radical reactions proceed rapidly in aqueous solutions exposed to ionizing radiation, cases are known of radicals produced by other means which are relatively stable even when rapid diffusion is possible. Water-soluble organic radicals such as Wursters Blue and the semiquinone of flavin mononucleotide are well-known examples in which chemical structure and environment can combine to form radical solutions which are relatively stable for days.

Stabilization of organic free radicals could be of particular value in preventing radiation damage in living organisms. In such systems, the free-radical products of water radiolysis initiate radical chain reactions which extend damage beyond the site of initial energy absorption. The interposition of organic compounds capable of forming long-lived radicals in these chains could slow down the propagation of damage until natural repair mechanisms or post-irradiation therapy could take effect. Some naturally occurring compounds may already assume this role during irradiation of an organism. Others might be incorporated with daily food intake prior to irradiation. The present work was directed toward determining chemical structures which did form relatively stable radicals in irradiated aqueous solution and also environmental factors which might improve this stability.

The study of free radical reactions has benefited immensely from the development of electron-spin-resonance (ESR) spectrometry; since by this technique, not only the presence but also, to some extent, the chemical structure of free radicals can be determined. In the present work, a Varian V-4500 spectrometer with 100 kHz modulation was used. A flow system was also utilized which rapidly passed an aqueous solution through an 1800-curie, ^{60}Co source into the resonance cavity of the spectrometer. This is shown schematically in Figure 1 and could be used to explore systems in which the

FIGURE 1

FLOW SYSTEM FOR IRRADIATION OF SOLUTIONS

radical reactions were fairly rapid. Many free radicals are also highly colored, and a Beckman DK-2 recording spectrophotometer was used to follow their reactions, after it had been established that the absorbing species was paramagnetic.

Aromatic dyes were selected for investigation because of their rapid attack by hydrated electrons and hydroxyl radicals formed by water radiolysis and because of the many possibilities for resonance stabilization of free radicals derived from them. This proved to be a very fruitful choice, and the following examples are probably indications that entire classes of organic dye structures form relatively stable free radicals in aqueous solution during irradiation.

FD&C Blue No. 2

This is an indigo dye approved for use as a food and cosmetic coloring, which has the structural formula shown below:

This compound was reported⁽¹⁾ to form a free radical in strongly alkaline, aqueous solutions provided molecular oxygen was excluded from the system. In the present work, it was found that much higher radical yields could be obtained upon reduction of the dye with sodium dithionite so that it could be detected in neutral solutions and also that irradiation of the dye solution produced a very similar if not identical radical.

The ESR spectra of the radical formed from FD&C Blue No. 2 following reduction with dithionite is shown in Figure 2. It is seen to consist of at least 25 groups of lines 0.70 gauss apart with 5 lines, 0.14 gauss apart, within each group. A very similar spectrum was obtained by irradiating a

FIGURE 2
ESR Spectrum of $1.00 \times 10^{-4} M$ FD&C Blue No. 2
After Partial Reduction with Sodium Thionite

deaerated solution of FD&C Blue No. 2 in the continuous flow system. In the latter preparation, elimination of the spectrum by addition of nitrous oxide, an efficient electron scavenger, to the system showed that the radical was formed by reduction of the dye with hydrated electrons. If ethanol rather than nitrous oxide was added to the system, the ESR-signal intensity increased. Since ethanol reacts rapidly with hydroxyl radicals but only slowly, if at all, with hydrated electrons to form the $\text{CH}_3\dot{\text{C}}\text{HOH}$ radicals, this increase in signal intensity indicates that either the $\text{CH}_3\dot{\text{C}}\text{HOH}$ radicals can also reduce the dye or that the back reaction, reoxidation of the dye radical by hydroxyl radicals, is quite significant in the absence of ethanol. Probably both possibilities are true.

Despite the increased radical yield in irradiated solutions of FD&C Blue No. 2 caused by addition of ethanol, the signal intensity of the ESR spectrum could not be increased to a point where more than the center 17 groups of lines could be observed. Since line intensity falls off rapidly on either side of this center core, it is not surprising that the outer lines are not apparent in the spectrum of the irradiated dye, or were not seen in strongly alkaline solution by the other investigators.⁽¹⁾ In order to further identify the radiation-produced radical with the dithionite-produced radical, optical absorption spectra of the solutions were measured; and the radicals obtained by either method were found to have the same properties. At pH 12, aqueous solutions of the radicals are red with absorption peaks at 565, 760 and 900 nm. As the pH is lowered to 7, the color shifts to green and absorption peaks occur at 608 nm, a peak for the original dye, and at 410 nm, a peak for the completely reduced dye. It was concluded that disproportionation of the radical is a reversible process in any of its protolytic forms, but occurs more rapidly at neutral pH due to less electrostatic repulsion between the radicals. This conclusion was also supported

by the decrease in ESR spectrum intensity with decreasing pH. While correct interpretation of the ESR spectrum of the radical will help define the structure of the radical, this interpretation is still not clear. As a result, the reaction scheme shown below is proposed to account for the formation and reactions of the radical in irradiated aqueous solutions of FD&C Blue

No. 2:

In the absence of oxygen, the radicals formed from FD&C Blue No. 2 were stable for days even at pH7 and room temperature. They disappeared rapidly, however, by reaction with molecular oxygen; but this process was considerably slower in concentrated neutral salt solutions such as 1.2M Na_2SO_4 . In the later case, the solubility of oxygen is only one-third that possible in pure water while the viscosity of the solution is three times that of pure water. These factors not only slow down the radical-oxygen reaction in Na_2SO_4 but would also stabilize the radicals in living tissue where large salt and other solute concentrations are maintained.

Thionine and Methylene Blue

Both thionine and methylene blue are phenothiazine dyes characterized by the structure:

Thionine: $R=H$

Methylene Blue: $R=CH_3$

Several reports in the literature have suggested that methylene blue forms relatively stable radicals during irradiation in strongly acid solution but that these radicals disappear extremely rapidly in neutral solutions. In one report⁽²⁾, formation was accomplished in $23N$ H_2SO_4 ; and the radical was quite stable in the absence of oxygen. Its 4-line ESR spectrum and other properties suggested that its structure was:

Pulse radiolysis studies on methylene blue solutions revealed⁽³⁾ stable optical absorption spectra in strongly acid solutions; but in neutral solutions, transient spectra attributed to methylene blue radicals disappeared with a rate constant of $3.0 \times 10^9 M^{-1} sec^{-1}$.

In the present work, it was found that relatively stable radicals could be formed by irradiating methylene blue or thionine even in neutral, oxygen-free solutions. Figure 3 shows the ESR spectra of the radicals formed. In both cases, the solutions were made $0.5M$ in ethanol to convert any hydroxyl radicals to $CH_3\dot{C}HOH$ radicals. The second-order disappearance rate of methylene blue was found to be $0.3M^{-1} sec^{-1}$ in sharp contrast with the large value reported previously. The stability of the radicals was also increased by irradiating methylene blue in either $2M$ Na_2SO_4 or 60%

FIGURE 3

ESR Spectra of Irradiated Dye Solutions
Initial Dye Concentration = 1.00×10^{-4} M
Absorbed Dose = 1.0×10^5 rads

sucrose solutions. In these media, the radical concentrations were found to decrease by less than 10% even after 60 hours. This increased stability also allowed higher concentrations of methylene blue radical to be attained in irradiated solutions as shown in Figure 4. While concentrations eventually decreased as a result of prolonged irradiation, the initial radical yields in all the media were identical, $G=1.0$ radicals per 100 eV absorbed.

The reaction between the methylene blue radical and oxygen was found to have a bimolecular rate constant of $6.0M^{-1}sec^{-1}$ in water. In $2M Na_2SO_4$ solution, the corresponding reaction rate constant was found to be $2.8M^{-1}sec^{-1}$. While these rate constants are far smaller than those expected for encounter-controlled reactions, they are still inversely proportional to the viscosities of the media. The concentration of radicals in over-irradiated solutions of either methylene blue or thionin could be increased by careful admission of oxygen to the solution, while continued exposure of the dye to oxygen restored the original, diamagnetic dye molecules. On the basis of this evidence, it was concluded that the formation and reactions of the radicals in solutions of these dyes could be represented as follows:

FIGURE 4

Concentration of Methylene Blue Radicals
as a Function of Radiation Dose

The higher concentration of radicals obtainable in irradiated Na_2SO_4 solutions allowed further resolution of their ESR spectrum as shown in Figure 5. Like the spectrum obtained for FD&C Blue No. 2 radicals, however, resolution was not sufficient to unequivocally determine the radical structure.

FD&C Blue No. 1 and FD&C Green No. 1, No. 2 and No. 3

These are all sulfonated triphenylmethane dyes with the following structures:

Free radicals were found to be formed in aqueous solutions of each of these dyes, except FD&C Green No.2, during irradiation; but the lifetimes of the radicals were considerably shorter than those described previously in this paper. In each case, oxygen-free solutions containing 0.5M $\text{C}_2\text{H}_5\text{OH}$ were irradiated; and broad-singlet ESR spectra were obtained as illustrated in Figure 6. While radicals were detected in irradiated neutral solutions of FD&C Blue No. 1, their ESR signal disappeared rapidly after irradiation. Saturation of the solution with Na_2SO_4 did not slow down this disappearance

*ESR SPECTRA OF IRRADIATED $10^{-3}M$
SOLUTIONS OF METHYLENE BLUE.*

rate; however, an increase in acidity was effective as shown in the table below.

Table 1. Rate Constants for the Disappearance of Triphenylmethane

Dye Radicals in Aqueous Solutions at 22°C

<u>Dye</u>	<u>Solvent</u>	<u>k</u>
FD&C Blue No. 1	aqueous 1N H_2SO_4 , 0.5M $\text{C}_2\text{H}_5\text{OH}$	$3.8\text{M}^{-1}\text{sec}^{-1}$
FD&C Blue No. 1	aqueous 10N H_2SO_4 , 0.5M $\text{C}_2\text{H}_5\text{OH}$	$0.32\text{M}^{-1}\text{sec}^{-1}$
FD&C Green No. 3	aqueous 1N H_2SO_4 , 0.5M $\text{C}_2\text{H}_5\text{OH}$	$2.1\text{M}^{-1}\text{sec}^{-1}$
FD&C Green No. 1	aqueous 1N H_2SO_4 , 0.5M $\text{C}_2\text{H}_5\text{OH}$	$10\text{M}^{-1}\text{sec}^{-1}$

Because of the transient nature of these radicals, their structures and properties are still obscure. Since ethanol was needed for their production and a trace of oxygen caused immediate disappearance of the ESR signals, it was concluded that the radicals are probably the reduction product of the dye resulting from addition of a hydrated electron to the triphenylmethane structure. The large differences in disappearance rates resulting from small alterations in the distribution of ring substituents in the dye structures indicates the sensitivity of radical stability to chemical structure.

The above examples show that organic chemical structures can be found which can exist as free radicals for days. In general, protolytic forms of the radicals which possess a high net charge are more stable, probably due to the effect of coulombic repulsion between radicals slowing down their rates of dimerization and disproportionation. Environments such as aqueous 2M Na_2SO_4 and aqueous 60% sucrose were found to slow down disproportionation and reactions with molecular oxygen in some cases, and further investigation of the effects of these and other environments will be investigated. Finally, it seems quite possible that some of the dyes discussed could form relatively stable free radicals in living tissue if incorporated prior to irradiation. Such a possibility provides a new avenue of research into ways of protecting living systems from radiation damage.

FIGURE 6

ESR Spectrum of Irradiated $1.00 \times 10^{-3} \text{M}$ FD&C Blue No. 1
in Aqueous 0.5M CH_3OH , 1.0N H_2SO_4

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