ELSEVIER

Dyes and Pigments, Vol. 27, No. 1, pp. 55–61, 1995

0143-7208/95 $9.50 + 0.00

0143-7208(94)00048-4

Photochemical Stability of Sulphorhodamine B

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(Received 1 August 1994; accepted 4 September 1994)

ABSTRACT

Commercially available and purified samples of Sulphorhodamine B in aqueous solution appeared to be photostable over a range of pH when subjected to simulated sunlight. The compound was degraded, however, in the presence of hydrogen peroxide (100 vol.), the reaction following first order kinetics. Inclusion of mannitol into this mixture greatly increased the rate constant. Further addition to the mixture of acetaldehyde rendered the dye non-photodegradable, suggesting that its breakdown with hydrogen peroxide was hydroperoxy radical mediated. The latter reaction was strongly affected by the wavelength of light impinging on the reactants.

INTRODUCTION

Sulphorhodamine B (C.I. Acid Red 52, C.I. 45100), sodium xanthylium-3,6-bis(diethylamino)-9-(2,4-disulphophenyl) hydroxide inner salt, has varied applications. It is a food dye,[1] and it has been used in laser technology[2–4] and as a red fluorochrome for tagging proteins by a sulphamido condensation.[5] The dye can potentiate the antiviral activity of poly r(A-U);[6] it has also found application in *in vitro* anticancer drug screening, providing a sensitive measure of drug-induced cytotoxicity.[7] The latter method has

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been adopted for use in *in vitro* assays aimed at screening for trypanocidal compounds.[8] We have attempted to develop a similar assay system for testing potentially active anti-*Acanthamoeba* compounds to be used for the treatment of keratitis caused by these protozoa.[9]

It is our experience, however, that the commercially available dye tends to fade when exposed to either daylight or to artificial light and a similar effect with this compound has been noted in studies of the photostability of food dyes.[10]

The aim of the present study, therefore, was to investigate the photochemical stability of Sulphorhodamine B in aqueous media of both the commercially available and purified forms in order to establish the usefulness of the compound in antiprotozoal drug testing assay systems.

MATERIALS AND METHODS

Thin layer chromatography

A solution of Sulphorhodamine B (0·1 g ml^{-1}) in double distilled water was spotted on to thin layer chromatography (silica 60 F_{254}) sheets and the dye eluted with various solvents (absolute methanol, ethyl acetate, ethanol, acetone, 2-propanol (GPR grade) and deionized water).

Purification of Sulphorhodamine B

Column chromatography was used. A column (18 mm internal diameter × 30 cm length) was 3/4 filled with an aqueous slurry of silica 60 (Merck) and the slurry allowed to settle. Deionized water was run through the column until the level was at the top of the adsorbant column. A solution of Sulphorhodamine B (0·32 g in 32 ml of double distilled water) was added to the column and the dye adsorbed thereon. When the column was eluted with absolute methanol, three constituents were isolated. The solutions were evaporated under reduced pressure to provide solid materials; the main product was pure Sulphorhodamine B together with very small amounts of two other components, both of which were red dyes but of a gummy consistency.

Photolysis of Sulphorhodamine B

A range of solutions of Sulphorhodamine B in deionized water was prepared (0·1–0·9 mg per 100 ml) and a Beer–Lambert calibration graph constructed. A correlation between concentration and absorbance ($r = 0{\cdot}9983$; $p = 0{\cdot}001$) was obtained. Sulphorhodamine B (1 mg per 100 ml) in deionized water was irradiated using the protocol of Evans *et al.*[11]

TABLE 1

Irradiation of Aqueous Solutions of Sulphorhodamine B (1 mg per 100 ml) with additives[10]

Experiment no.	*Substrate*
1	Purified Sulphorhodamine B
2	Purified Sulphorhodamine B + hydrogen peroxide$_a$
3	Purified Sulphorhodamine B + acetaldehyde$_b$
4	Purified Sulphorhodamine B + mannitol$_c$
5	Purified Sulphorhodamine B + hydrogen peroxide$_a$ + mannitol$_c$
6	Purified Sulphorhodamine B + chlorhexidine digluconate$_d$
7	Purified Sulphorhodamine B + chlorhexidine digluconate$_d$ + mannitol$_c$
8	Purified Sulphorhodamine B + dequalinium chloride$_e$
9	Purified Sulphorhodamine B + trichloroacetic acid$_f$
10	Purified Sulphorhodamine B + Tris buffer (pH 10·7)
11	Purified Sulphorhodamine B + hydrogen peroxide$_a$ + red film†
12	Purified Sulphorhodamine B + hydrogen peroxide$_a$ + green glass
13	Purified Sulphorhodamine B + hydrogen peroxide$_a$ + blue manganese glass‡
14	Commercial Sulphorhodamine B
15	Commercial Sulphorhodamine B + hydrogen peroxide$_a$
16	Commercial Sulphorhodamine B + acetaldehyde$_b$
17	Commercial Sulphorhodamine B + mannitol$_c$
18	Commercial Sulphorhodamine B + dequalinium chloride$_e$*
19	Commercial Sulphorhodamine B + hydrogen peroxide$_a$ + mannitol$_c$ + acetaldehyde$_b$
20	Dequalinium chloride$_e$*

Subscripts: a = 1 ml in 100 volume (30% v/v); b = 1 ml per 200 ml (0·067%); c = 1 mg per 100 ml (0·01%); d = 1 ml 20% w/v solution per 100 ml (0·2%); e = 20 mg per 100 ml (0·02% w/v); f = 1 mg per 100 ml (0·1% w/v).

* Assayed at 326 nm for dequalinium chloride.

† Rosco Supergel red plastic film, λ_{max} = 410 nm, 13% transmission.

‡ Blue manganese glass λ_{max} = 834 nm and 890 nm, with 17·8% and 17·9% transmission, respectively.

The absorbance was measured at 553 nm (measured λ_{max} of purified Sulphorhodamine B) at different time intervals. There was, however, no change in absorbance; this indicated that the dye was stable under these conditions. Subsequent experiments performed under the same conditions are shown in Table 1. Results were treated by the method of Patel and Sugden[12] and the order of reaction and rate constants calculated.

RESULTS AND DISCUSSION

The order and rate constants of the photochemical reactions are shown in Table 2. Commercially available or purified Sulphorhodamine B in

TABLE 2
Reaction Order and Rate Constants of Photolysis Reaction of Sulphorhodamine B with Additives

Experiment no.	*Order of reaction*	*Rate constant*
1	No reaction	
2	First	$27{\cdot}6 \times 10^{-3}$
3	No reaction	
4	No reaction	
5	First	$2{\cdot}17 \times 10^{-1}$
6	No reaction	
7	No reaction	
8	No reaction	
9	No reaction	
10	No reaction	
11	Zero	$20{\cdot}93 \times 10^{1}$
12	First	$5{\cdot}59 \times 10^{-3}$
13	First	$180{\cdot}0 \times 10^{-3}$
14	No reaction	
15	First	$26{\cdot}9 \times 10^{-3}$
16	No reaction	
17	No reaction	
18	No reaction	
19	No reaction	
20	No reaction	

aqueous solution was found to be stable to simulated sunlight as provided by the method of Evans *et al.*[11] Irradiation with stimulated sunlight in the presence of hydrogen peroxide induced photochemical decomposition of the dye ($k = 27{\cdot}6 \times 10^{-3}$ min^{-1}) for purified dye. The reaction followed first order kinetics. Incorporation of mannitol into the reaction mixture resulted in a greatly increased rate constant ($2{\cdot}17 \times 10^{-1}$ min^{-1}), but this reaction also follows first order kinetics. The irradiation of an aqueous solution of commercially available Sulphorhodamine B with hydrogen peroxide, mannitol and acetaldehyde did not photo-degrade the dye; this indicates that the reaction may be mediated by the action of hydroperoxy radicals (HO_2). Ho *et al.*[13] have shown that acetaldehyde is a potent scavenger of the hydroperoxy radical. Hydrogen peroxide on irradiation with simulated sunlight forms hydroxyl radicals.[12,14] The interrelationship between the hydroxyl and hydroperoxy radicals is illustrated in Fig. 1.

It is apparent that the hydroperoxy radical can be formed from the hydroxyl radical. Consequently, it is probable that in the photochemical reaction of Sulphorhodamine B with hydrogen peroxide, some of the

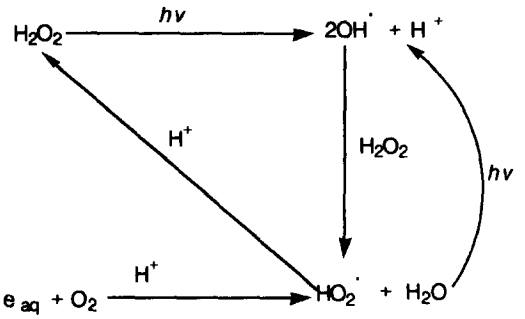

Fig. 1. Inter-relationship between hydroxyl and hydroperoxy radicals.

hydroxyl radicals are transformed into hydroperoxy radicals. These are not scavenged by mannitol but by acetaldehyde. The irradiation of an aqueous solution of Sulphorhodamine B with dequalinium chloride did not induce photodegradation of either the dye or the quaternary ammonium salt. This indicates that Sulphorhodamine B did not promote photochemical degradation of the dequalinium chloride, nor did it undergo self-degradation. It is worthy of note that many dyes act as triplet sensitizers and can promote photodegradation by a triplet mechanism.[14]

We have observed the formation of a blue precipitate on admixture of aqueous solutions of commercially available Sulphorhodamine B with chlorhexidine digluconate at concentrations of 50 μg ml^{-1} and above; this phenomenon was not observed with chlorhexidine digluconate and purified Sulphorhodamine B. This is important, since cationic antiseptics are now being utilized clinically for treatment of amoeba-induced keratitis[9] and, as stated above, the drug-screening assay for these organisms requires the compound in suspension and not precipitated by the indicator dye. Irradiation of these mixtures with simulated sunlight did not result in any photochemical degradation; neither was any visible precipitate formed.

Irradiation of aqueous solutions of Sulphorhodamine B either in the presence of Tris buffer (pH 10·7) or trichloroacetic acid (pH 1·4) did not result in any observed photochemical reaction, thus indicating that the dye is stable over a wide range of pH. Studies of the photostability of aqueous solutions of Sulphorhodamine B in the presence of hydrogen peroxide with filtered parts of the simulated solar spectrum were performed. In containers shielded with Rosco Supergel red plastic film (T = 13%; λ_{max} = 410 nm) the photochemical reaction followed zero order kinetics with a rate constant of 20·93 min^{-1}, giving a change in reaction order compared to that of the control (Experiment 2, Table 1). The use of a blue 'manganese' glass bottle gave a rate constant of 180 $\times$ 10^{-3} min^{-1}; the reaction followed first order kinetics. This rate

constant was approximately six times as large as that of the material irradiated in borosilicate glass (Experiment 2, Table 1). This is strongly suggestive that the light reaching the solution is more effective at promoting the formation of hydroxyl and hydroperoxy radicals. It has been suggested that Methylene Blue in acid solution can photo-oxidize water by a two photon process and that the quantum yield of leuco-methylene blue exceeded 10^{-3} in red light.[16] In the present study the light reflected from blue glass is blue, so light transmitted will be at the red end of the spectrum, and thus may facilitate the formation of hydroxyl and hydroperoxy radicals.

Experiment 12 (Tables 1 and 2) showed that a similar solution irradiated in green glass gave a rate constant of $5{\cdot}59 \times 10^{-3}$ min^{-1} and also followed first order kinetics. The rate constant of this reaction was twice that of the control. Green glass excludes both extremes of the simulated solar spectrum and thus the intermediate part of the spectrum may be less suitable for promoting the formation of oxidative radicals. In order to protect solutions of Sulphorhodamine B in oxidative conditions it would be necessary to use a filter which restricted the incident light to a relatively narrow band of the simulated solar spectrum or to use green glass of more than 2 mm thickness as found in medicinal bottles. For our drug assays we have found dark yellow-green plastic sheeting (Rosco Supergel 90, $T = 13\%$; $\lambda_{max} = 510$ nm) to be eminently suitable.

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