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Photostability of Aniline Blue (CI 42755) and Methyl Blue (CI 42780)

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ABSTRACT

Commercially available and purified samples of Aniline Blue and Methyl Blue (both dyes having the synonym "Cotton Blue") in aqueous solution appeared to be reasonably photostable when subjected to simulated sunlight. The compounds were, however, degraded in the presence of hydrogen peroxide (100 vol.), the reaction following second order kinetics with Aniline Blue and first order kinetics with Methyl Blue. Inclusion of mannitol greatly increased the rate constants for both dyes. Irradiation of the dyes separately with 2,2'azobis(2'-amidinopropane) dihydrochloride (ABAP) resulted in rapid degradation of both dyes. Addition of acetaldehyde to both dyes resulted in a reduced rate constant, thus suggesting that the reaction was hydroperoxy radical mediated. When the dyes were treated with singlet oxygen whilst being protected from light or irradiated with simulated sunlight, they underwent decomposition, suggesting that they were singlet oxygen sensitive. These findings may have considerable practical implications when the dyes are used in a diagnostic setting; if they are not protected from light exposure, their staining capability is likely to be considerably reduced. © *1997 Elsevier Science Ltd*

Keywords: Aniline Blue (CI 42755), Methyl Blue (CI 42780), hydroxyl, hydroperoxy, radicals, singlet oxygen.

INTRODUCTION

Aniline Blue WS (Water Soluble; CI 42755; Acid Blue 22; RMM 738) and Methyl Blue (Soluble Blue; Ink Blue; CI 42780; Acid Blue 93; RMM 800), as

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well as a mixture of these two dyes, have previously been known as "Water Blue", "Soluble Blue" and "Aniline Blue, Water Soluble". These are anionic dyes which have application in histological and microbiological staining solutions. The two dyes appear to have identical staining properties.

In histology, aniline dyes are most widely used as constituents of trichrome stains for demonstration of connective tissue elements.[1,2] Both dyes have the synonym "Cotton Blue". The latter is widely used in mycology as a constituent of a formulation referred to as "Lactophenol Cotton Blue".[3] The solution is used as a mounting fluid as well as a stain for fungi. The lactic acid in the formulation acts as a clearing agent and preserves fungal architecture; the phenol is antimycotic; glycerol, another constituent of the preparation, prevents fungal desiccation; and the "Cotton Blue" (usually present as a 1% w/v solution) renders the fungus a blue colour, thus permitting its detection and identification under light microscopy. A modification of this procedure permits permanent mounts of fungal wet preparations or slide mounts to be prepared.[3] The Lactophenol Cotton Blue method has been used with some success for the detection of cysts of *Acanthamoeba*, a free-living protozoan which is the cause of a sight threatening keratitis,[4] in scrapes of corneal epithelium.[5]

The similarities in nomenclature of the two dyes make it difficult to distinguish between them when the CI number is not provided. This is of importance since their structure suggests that they may be susceptible to light-induced degradation. The latter is of consequence since "Cotton Blue" is widely used in countries such as India as a constituent of "Lactophenol Cotton Blue", for rapid identification of fungal causes of suppurative keratitis.[6] There is anecdotal evidence to suggest that, in such circumstances, these dyes when exposed to sunlight become less efficacious as biological stains than those which are stored optimally in light-resistant containers. This also applies to the intensity of colour of stained preparations on glass slides which are exposed to excessive amounts of light.

Thus, the primary purpose of this study was to examine, using an established methodology,[7] the photostability of purified and unpurified samples of Aniline Blue WS and Methyl Blue, to determine if exposure to sunlight would be likely to diminish their staining capacity.

MATERIALS AND METHODS

Purification of Aniline Blue WS and Methyl Blue

A column (55 cm long, 3.5 cm internal diameter) fitted with a glass sinter was three quarters filled with a slurry of Matrex Silica 60 (70–200μ) (Fisons,

U.K.) in a mixture of water (15% v/v) and propan-2-ol (85% v/v). The slurry was allowed to settle. The solvent was run off until the level of the liquid was just at the top of the column of adsorbant, and then eluted with the same solvent mixture. A solution of commercially available dye (50 mg in 15 ml of water) was prepared and this was applied to the column. After separation and evaporation followed by freeze-drying, it was possible to isolate one dye. This was confirmed by thin layer chromatography using silica gel 60_{F254} (Merck), eluting with the same solvent mixture as described above.

Methyl Blue was obtained from Aldrich Chemical Co. and Aniline Blue WS from BDH (Merck). It is important to note that both dyes were assigned the name "Aniline Blue, Water Soluble" by their respective suppliers. Infrared spectroscopy was used to confirm the identities of the dyes as Methyl Blue and Aniline Blue WS respectively.

Both dyes are known to change to a red colour in the presence of strong alkali. It was noted that the dyes had a pink fraction on the chromatography column which reverted to blue on acidification, suggesting some alkaline material on the adsorbant.

Photochemical decomposition of Aniline Blue WS and Methyl Blue

A stock solution of each dye, in deionised water, was prepared (0.314 g litre^{-1}). From these solutions a series of dilutions was prepared. Calibration curves for Methyl Blue were constructed using concentrations of 3.5, 6.3, 9.45, 12.6, 15.75, 18.9, 22.05, 25.2 and 28.35 mg litre^{-1}. Linear regression analysis gave a coefficient of 0.9987 [$p = 0.001$]. For Aniline Blue WS the calibration curve was constructed using concentrations 4.6, 9.2, 13.8, 18.4, 23.0, 27.6, 32.2, 36.8, 41.4 and 46.0 mg litre^{-1}. The linear regression coefficient was 0.9956 [$p = 0.001$]. Solutions (30 mg litre^{-1}) of each dye were irradiated using the method previously described,[8] at an ambient temperature of 24 ± 0.2°C. Absorbance readings were taken at 596 nm at time zero and every 5 min thereafter up to a maximum of 5 h. The procedure was repeated with various substances added to the dye solution (Table 1).

Effect of singlet oxygen on photostability

Solutions of both dyes (30 mg litre^{-1}) were prepared using samples from both commercial sources. These solutions were treated with singlet oxygen prepared by a previously reported method.[9] The gas was introduced into the dye solutions at a mean rate of 1 cm^3 min^{-1} through a 5 mm internal diameter tube fitted with a No. 1 sinter frit. Each dye solution was divided into two portions: one was excluded from light by wrapping in aluminium foil, and the other was irradiated with simulated sunlight.[8] In all cases the

absorbance at 596 nm was taken at time zero and at intervals thereafter, up to a maximum of 2 h. Experiments were performed in which the solvent water was degassed by either (1) sonication for 30 min at 20°C or (2) boiling the water for 2 min and then cooling in a closed container before use (Table 2).

TABLE 1
Samples Irradiated in Deionised Water (100 ml)

1. Commercial Aniline Blue (Methyl Blue)[a]
2. Commercial Aniline Blue (Aniline Blue WS)[b]
3. Commercial Aniline Blue (Methyl Blue) + ABAP[a] (100 mg)
4. Commercial Aniline Blue (Aniline Blue) + ABAP[a] (100 mg)
5. Commercial Aniline Blue (Methyl Blue) + ABAP[a] (100 mg) + acetaldehyde (0.1 ml)
6. Commercial Aniline Blue (Aniline Blue) + ABAP[a] (100 mg) + acetaldehyde (0.1 ml)
7. Commercial Aniline Blue (Methyl Blue) + H_2O_2 (1.0 ml)
8. Commercial Aniline Blue (Aniline Blue) + H_2O_2 (1.0 ml)
9. Commercial Aniline Blue (Methyl Blue) + H_2O_2 (1.0 ml) + mannitol (100 mg)
10. Commercial Aniline Blue (Aniline Blue) + H_2O_2 (1.0 ml) + mannitol (100 mg)
11. Purified Aniline Blue WS
12. Purified Aniline Blue WS + H_2O_2 (1.0 ml)
13. Purified Aniline Blue WS + H_2O_2 (1.0 ml) + mannitol (100 mg)
14. Purified Aniline Blue WS + ABAP[a] (100 mg)
15. Purified Aniline Blue WS + ABAP[a] (100 mg) + acetaldehyde (0.1 ml)

[a] ABAP = 2, 2′ – Azobis(2 – amidinopropane) dihydrochloride. H_2O_2 used was 30% v/v. Aniline Blue (Methyl Blue) (Aldrich Chemical Co. Ltd).
[b] Aniline Blue WS (BDH, Merck).

TABLE 2
Samples Treated with Singlet Oxygen and Controls

1. Purified Aniline Blue WS, deionised water, in the dark
2. Purified Aniline Blue WS, deionised water, irradiated
3. Purified Aniline Blue WS, deionised, sonicated water, in the dark
4. Purified Aniline Blue WS, deionised, sonicated water, irradiated
5. Purified Aniline Blue WS, deionised, boiled and cooled water, in the dark
6. Purified Aniline Blue WS, deionised water, with $^1O_2$ in the dark.
7. Purified Aniline Blue WS, deionised water, with $^1O_2$ irradiated
8. Purified Aniline Blue WS, deionised, sonicated water, with $^1O_2$ in the dark
9. Purified Aniline Blue WS, deionised, sonicated water, with $^1O_2$ irradiated
10. Purified Aniline Blue WS, deionised boiled and cooled water, irradiated
11. Purified Aniline Blue WS, deionised boiled and cooled water, with $^1O_2$ in the dark
12. Purified Aniline Blue WS, deionised boiled and cooled water, with $^1O_2$ irradiated
13. Commercial Methyl Blue, deionised water, in the dark
14. Commercial Methyl Blue, deionised water, irradiated
15. Commercial Methyl Blue, deionised sonicated water, in the dark
16. Commercial Methyl Blue, deionised sonicated water, irradiated
17. Commercial Methyl Blue, deionised boiled and cooled water, in the dark
18. Commercial Methyl Blue, deionised water with $^1O_2$ in the dark
19. Commercial Methyl Blue, deionised water with $^1O_2$ irradiated
20. Commercial Methyl Blue, deionised sonicated water, with $^1O_2$ in the dark
21. Commercial Methyl Blue, deionised sonicated water, with $^1O_2$ irradiated

RESULTS AND DISCUSSION

Chromatographic purification of commercial Aniline Blue WS showed that the dye was not contaminated with other compounds. Methyl Blue purification by this method proved unsuccessful.

Examination of Tables 3 and 4 shows that commercial samples of Methyl Blue and Aniline Blue WS are reasonably stable in simulated sunlight, having rate constants of 2.349×10^{-5} and 1.393×10^{-5}, respectively, following second order kinetics.

The reaction of Commercial Methyl Blue irradiated with simulated sunlight, with added hydrogen peroxide as a source of hydroxyl radicals, followed first order kinetics with a rate constant of 155.0×10^{-5}. The reaction with Commercial Aniline Blue WS under identical conditions followed second order kinetics with a rate constant of 154.0×10^{-5}. The incorporation of mannitol and hydrogen peroxide into the solutions caused the photodegradation reactions of both dyes to follow second order kinetics, with Commerical Methyl Blue having a rate constant of 333.0×10^{-5} and Commercial Aniline Blue WS a rate constant of 281.3×10^{-5}.

Experiments with purified Aniline Blue WS and hydrogen peroxide indicated that the dye underwent a photochemical reaction, with second order

TABLE 3
Rate Constants and Reaction Order of Photodecomposition Experiments

Substance irradiated in deionised water	Rate constant ($\times10^{-5}$)	Order of reaction
1. Commercial (Aniline Blue) Methyl Blue	2.349	2nd
2. Commercial Aniline Blue WS	1.393	2nd
3. Commercial (Aniline Blue) Methyl Blue + ABAP[a]	309.0	2nd
4. Commercial Aniline Blue WS + ABAP[a] (100 mg)	197.3	2nd
5. Commercial (Aniline Blue) Methyl Blue + ABAP[a] (100 mg) + acetaldehyde (0.1 ml)	109.8	2nd
6. Commercial Aniline Blue WS + ABAP[a] (100 mg) + acetaldehyde (0.1 ml)	148.2	2nd
7. Commercial (Aniline Blue) Methyl Blue + H_2O_2 (30% v/v, 1.0 ml)	155.0	1st
8. Commercial Aniline Blue WS + H_2O_2 (30% v/v, 1.0 ml)	154.0	2nd
9. Commercial (Aniline Blue) Methyl Blue + H_2O_2 (30% v/v, 1.0 ml) + mannitol (100 mg)	333.0	2nd
10. Commercial Aniline Blue WS + H_2O_2 (30% v/v, 1.0 ml) + mannitol (100 mg)	281.3	2nd
11. "Aniline Blue WS	2.205	2nd
12. "Aniline Blue WS + H_2O_2 (1.0 ml)	5.04	2nd
13. "Aniline Blue WS + H_2O_2 (1.0 ml) + mannitol (100 mg)	6.42	2nd
14. "Aniline Blue WS + ABAP[a] (100 mg)	10270.0	1st
15. "Aniline Blue WS + ABAP[a] (100 mg) + acetaldehyde (0.1 ml)	8866.0	1st

[a] Purified Aniline Blue WS.

kinetics, with a rate constant of 5.04×10^{-5}. The reaction with hydrogen peroxide and mannitol also followed second order kinetics and had a rate constant of 6.42×10^{-5}, exhibiting the same trend as the commercial dye. This finding was considered unusual, since mannitol is normally a hydroxyl radical scavenger. Thus a reduction in the rate constant might have been predicted. The results suggest that there was more than one chemical reaction taking place.

Aniline Blue WS has some structural features in common with xanthen dyes which are known to be singlet oxygen sensitisers.[10] Both of the dyes tested undergo decomposition in light which does not occur in the dark. In light, the rate constants for Commercial Aniline Blue WS and Commercial Methyl Blue were 1.393×10^{-5} and 2.349×10^{-5}, respectively.

In the presence of singlet oxygen, and the absence of light, Purified Aniline Blue WS and Commercial Methyl Blue gave rate constants of 1.61×10^{-5} and 1.86×10^{-5} respectively and followed second order kinetics. Treatment of the dyes with singlet oxygen in the presence of simulated sunlight resulted in respective rate constants of 3.76×10^{-5} and 1.24×10^{-5} and also followed second order kinetics. Irradiation of Purified Aniline Blue WS and

TABLE 4
Rate Constants and Reaction Orders of Photodecomposition with Singlet Oxygen

Substance irradiated in deionised water	Rate constant ($\times10^{-5}$)	Order of reaction
1. [a]Aniline Blue WS, dark		No reaction
2. [a]Aniline Blue WS, sonicated water, dark		No reaction
3. [a] Aniline Blue WS, sonicated water, dark		No reaction
4. [a]Aniline Blue WS, sonicated water, irradiated		No reaction
5. [a]Aniline Blue WS, boiled and cooled water, dark		No reaction
6. [a]Aniline Blue WS, with $^1O_2$ dark	1.61	2nd
7. [a]Aniline Blue WS, with $^1O_2$ irradiated	3.76	2nd
8. [a]Aniline Blue WS, sonicated water, with $^1O_2$ dark	2.15	2nd
9. [a]Aniline Blue WS, sonicated water, with $^1O_2$ irradiated	3.94	2nd
10. [a]Aniline Blue WS, boiled and cooled water, irradiated		No reaction
11. [a]Aniline Blue WS, boiled and cooled water, with $^1O_2$ in the dark		No reaction
12. [a]Aniline Blue WS, boiled and cooled water, with $^1O_2$ irradiated		No reaction
13. Commercial Methyl Blue, dark		No reaction
14. Commercial Methyl Blue, irradiated		No reaction
15. Commercial Methyl Blue, sonicated water, dark		No reaction
16. Commercial Methyl Blue, sonicated water, irradiated		No Reaction
17. Commercial Methyl Blue, boiled and cooled water, dark		No reaction
18. Commercial Methyl Blue, with $^1O_2$ dark	1.86	2nd
19. Commercial Methyl Blue, with $^1O_2$ irradiated	1.24	2nd
20. Commercial Methyl Blue, sonicated water with $^1O_2$ dark	6.47	2nd
21. Commercial Methyl Blue, sonicated water, with $^1O_2$ irradiated	5.10	2nd

[a] Purified Aniline Blue WS.

Commercial Methyl Blue in sonicated water with singlet oxygen gave rate constants of 3.94×10^{-5} and 5.10×10^{-5}, respectively, both reactions following second order kinetics, suggesting that all the air had not been removed or was re-introduced by the sampling procedure. Treatment of the dyes in sonicated water with singlet oxygen in the dark showed decomposition following second order kinetics with rate constants of 2.15×10^{-5} and 6.47×10^{-5} for Purified Aniline Blue WS and Commercial Methyl Blue, respectively. The use of boiled and cooled water as a solvent resulted in no reaction with either dye in the presence of singlet oxygen. These results provide evidence of the photodegradation of the dyes, being facilitated by light and singlet oxygen, thus suggesting that they may be acting as singlet oxygen sensitisers. However, the increased rate constants recorded for reactions with mannitol and hydrogen peroxide suggests that the reactions are being influenced by the presence of singlet oxygen which has not been scavenged by mannitol. In this case the oxygen could be provided from air dissolved in the water used as a solvent.

Incorporation of ABAP resulted in a very rapid loss of colour and decomposition of the commercial dyes with rate constants of 309.0×10^{-5} for Commercial Methyl Blue and 197.3×10^{-5} for Commercial Aniline Blue WS. ABAP has been shown to provide a source of hydroperoxy radicals in the presence of light.[11,12] Confirmatory evidence of this effect was provided by incorporating acetaldehyde, a scavenger of hydroperoxy radicals, into the reaction mixture. The latter caused a reduction in the rate constant from 309.0×10^{-5} to 109.8×10^{-5} for Commercial Methyl Blue and from 197.3×10^{-5} to 148.2×10^{-5} for Commercial Aniline Blue WS.

The reactions using singlet oxygen suggest the possibility that the Commercial Aniline Blue WS could act as a singlet oxygen sensitizer and facilitate the formation of singlet oxygen from air in the deionised or sonicated water solvents. The rapid reactions observed with ABAP may be due to a combination of the generation of hydroperoxy radicals and the formation of singlet oxygen from atmospheric oxygen and that dissolved in the water solvent. The small movements involved in taking absorbance readings may well be sufficient to incorporate some air which would, of course, provide a source of oxygen.

In the experiment with Purified Aniline Blue WS the reaction with ABAP followed first order kinetics as did that with ABAP and acetaldehyde, with rate constants of $10\,270\times10^{-5}$ and 8866×10^{-5}, respectively, indicating that the impurities removed by chromatography had some 'antioxidant' effect under the conditions of testing. Table 4 shows that Purified Aniline Blue WS did not undergo degradation in either treated solvent in simulated sunlight or in the dark. These findings suggest that the presence of dissolved air was essential for the reaction which led to loss of colour. This provides further

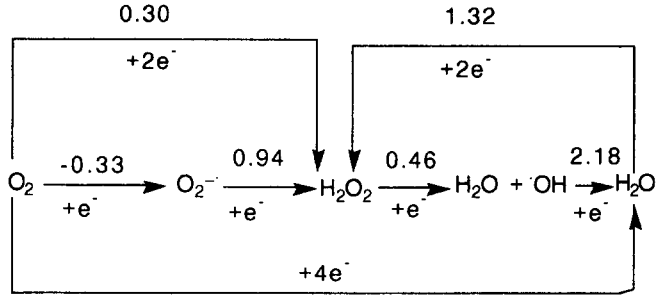

Fig. 1. Reduction of oxygen by microorganisms. Numerical values expressed in volts.

evidence to suggest that both dyes had some singlet oxygen sensitising activity. It was surprising that the experiments performed in sonicated water did not exhibit degradation of the dyes, since sonication is known to be a source of hydroxyl radicals.[13]

The findings from the present study may have implications when Aniline Blue WS is used as a constituent of bacteriological media. Aniline Blue WS has been added to cultures of *Legionella* bacteria, colour changes and colonial appearance used as presumptive markers between species.[14] Certain microorganisms can reduce oxygen.[15] This occurs according to the scheme shown in Fig. 1.

Thus microorganisms which generate oxygen radicals which are then present in the medium can be expected to cause loss of colour of either Aniline Blue WS or Methyl Blue. Such colour loss may reduce the visualisation of the organisms. Clearly the photolability of these dyes raises doubt as to their suitability for inclusion in microbiological tests involving organisms which generate oxygen radicals, or in aerated media where there will be loss of colour due to oxidative degradation. Both of these effects render these dyes less suitable for inclusion in diagnostic assays since the degradative reactions observed are facilitated by light and this would make visual observations more difficult if they were dependent on colour detection within a microbe. Furthermore, it is apparent that inclusion of these dyes into a formulation, such as Lactophenol Cotton Blue for demonstration of fungi or *Acanthamoeba* in ocular tissue, could be associated with certain drawbacks. *Acanthamoeba* trophozoites are known to generate superoxide during phagocytosis,[16] leading to production of hydrogen peroxide which causes inhibition of bacterial growth.[17] Storage of these dyes in light-resistant containers is considered essential, as is stained preparation on glass slides. We would not recommend the inclusion of the dyes into media to be used for identification or speciation of bacteria such as *Legionella* since superoxide and hydrogen peroxide can be generated in the medium used to cultivate the bacteria.[18]

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