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Photostability of Crystal Violet (CI 42555)

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ABSTRACT

Commercially available Crystal Violet, when subjected to adsorption chromatography, was shown to contain Crystal Violet, Methyl Violet 2B and Methyl Violet 6B. All three compounds were stable on irradiation with simulated sunlight. In the presence of equimolar amounts of hydrogen peroxide these dyes were rapidly decomposed by light, the reaction following second-order kinetics. Addition of acetaldehyde inhibited the photochemical degradation of the dyes, suggesting some hydroperoxy radical involvement in this reaction. Incorporation of mannitol reduced the rate constants of the reactions when hydrogen peroxide was present: this suggests some hydroxyl radical mediation. These findings may have considerable implications when Crystal Violet (pure or impure) is used in bacteriological assays which involve differentiation of microbial colonies by colour detection alone.

INTRODUCTION

Crystal Violet (CI 42555; Basic Violet 3, hexamethylpararosaniline chloride; Methyl Violet 10B) has wide application in microbiology. It is used therapeutically as an antiseptic, both in its own right and combined with pentamethylpararosaniline chloride (and other ill-defined rosanilines) as Gentian Violet. The latter has been used as an antiseptic for infested wounds, serous and mucous membranes, for pleural cavity and joint affections and also as an anthelminthic agent against the nematode *Strongyloides*.[1] Crystal

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Violet binds to DNA by ionic attraction and hydrogen bonding.[2] The dye causes collapse of the inner mitochondrial membrane potential of the parasitic protozoan *Trypanosoma cruzi*.[3]

Crystal Violet (and other pararosaniline dyes such as Methyl Violet 6B) is used in the Gram's method, the most important staining procedure in diagnostic bacteriology.[4] Tissue elements such as fibrin, keratin and calcium may also be demonstrated by inclusion of Crystal Violet in this method.[5] Crystal Violet is also used as a constituent of Ryu's stains for bacterial flagellae[6] and in Neisser's stain for metachromatic (volutin) granules.[7] The outer membrane of *Brucella sp.* does not present a barrier to the uptake of Crystal Violet,[8] but the dye inhibits α-glutathione transferase, undergoing decolorisation due to adduct formation.[9] The loss of colour of dye induced by hydrophobic oleophilic bacteria appears primarily due to reduction to the leuco form.[10] The dye undergoes degradation to 4',4'-bis(dimethylamino)-benzophenone under the action of *Bacillus subtilis*.[11]

A Crystal Violet test has been used in an attempt to differentiate diverse strains of coagulase negative staphylococci.[12] A Crystal-Violet-containing agar is used to cultivate bacteria in this test and resultant colonies may be purple, yellow or white; the coloration may be of value in defining the origin and pathologenicity of the bacteria. It is however, uncertain whether the coloration is consequent upon impurities in the commercially available Crystal Violet used in these assays undergoing photochemical degradation when they are exposed to conditions of light whilst being examined. This phenomenon is known for other dyes such as Phenol Red, which are also used to distinguish bacteria.[13] Thus, as a prelude to a bacteriological investigation of the significance of this notion, for colour differentiation of CNS-sensitivity to glycopeptide antibiotics, a study was performed aimed at examining the reaction of impure and purified Crystal Violet to exposure by photogenerated hydroxyl radicals.

MATERIALS AND METHODS

Purification of Crystal Violet

A column (21·6 cm long by 2·45 cm internal diameter) fitted with a glass sinter was three-quarters filled with a slurry of Silica 60 (Merck) in acetone and concentrated hydrochloric acid (1:1) and the slurry allowed to settle. The solvent was run off until the level was just at the top of the adsorbant column and then eluted with deionised water. A solution of commercial Crystal Violet (509 mg in 100 ml deionised water was applied to the column, and after separation and evaporation of the solvent under

TABLE 1
Purification of Crystal Violet

Dye	*Colour*	*Yield (g)*
Crystal Violet	Blue	0·960
Substance A	Blue	0·0122
Substance B	Blue	0·0065

A number of separations were carried out to accumulate sufficient material for subsequent analysis.

reduced pressure, followed by freeze drying of a concentrated aqueous solution, it was possible to isolate three distinct compounds (Table 1).

Crystal Violet (CI 42555), Methyl Violet 6B (CI 42536) and Methyl Violet 2B (CI 42535) were supplied by BDH Merck.

Photochemical decomposition of purified Crystal Violet and associated dyes

A stock solution of each dye in deionised water (0·0198 g in 25 ml) was prepared. From this a series of dilutions in deionised water was prepared. Calibration curves were then constructed for the dye using concentrations of

TABLE 2
Samples Irradiated

1. Purified Crystal Violet
2. Purified Crystal Violet + H_2O_2 (equimolar amounts)
3. Purified Crystal Violet + acetaldehyde (equimolar amounts)
4. Purified Crystal Violet + H_2O_2 + mannitol (equimolar amounts)
5. Methyl Violet 2B
6. Methyl Violet 2B + acetaldehyde 0·1 ml
7. Methyl Violet 2B + H_2O_2
8. Methyl Violet 6B
9. Methyl Violet 6B + acetaldehyde (0·1 ml)
10. Methyl Violet 6B + H_2O_2
11. Purified Crystal Violet + Methyl Violet 2B
12. Purified Crystal Violet + Methyl Violet 6B
13. Purified Crystal Violet + Methyl Violet 2B + H_2O_2
14. Purified Crystal Violet + Methyl Violet 6B + H_2O_2
15. Commercial Crystal Violet
16. Commercial Crystal Violet + H_2O_2 (equimolar amounts)
17. Commercial Crystal Violet + H_2O_2 + mannitol (equimolar amounts)
18. Commercial Crystal Violet + acetaldehyde (equimolar amounts)
19. Commercial Crystal Violet + H_2O_2 + acetaldehyde

0·792, 1·58, 2·38, 3·16, 3·96, 4·75 and 7·92 mg ml^{-1}. Linear regression analysis for Crystal Violet was 0·999. The values for Methyl Violet 2B and Methyl Violet 6B, the likely candidates for substances A and B (Table 1) were 0·994 and 0·999 respectively. The infrared spectra of substances A and B were compared with those of Methyl Violet 2B and 6B respectively, and found to be superimposible.

Each solution was irradiated with simulated sunlight using the apparatus and methods described previously[11] at 38 ± 1°C. Absorbance readings were taken at 590 nm at time zero and hourly thereafter up to a maximum of 5 h. The procedure was repeated with various substances added to the sample (Table 2).

RESULTS AND DISCUSSION

The results of the photochemical experiments (Table 3) show that these dyes undergo photodegradation under the conditions of test.

Table 3 shows that purified Crystal Violet alone does not undergo photochemical degradation on irradiation with simulated sunlight.[14] It has been previously shown that Crystal Violet undergoes photodecomposition on

TABLE 3
Rate Constants and Reaction Order of Photodecomposition Experiments

Substance irradiated	*Rate constant* $\times 10^{-3}$	*Order of reaction*
1. Purified Crystal Violet	No reaction	—
2. Purified Crystal Violet + H_2O_2	40·00	2nd
3. Purified Crystal Violet + H_2O_2 + mannitol	2·89	2nd
4. Purified Crystal Violet + acetaldehyde	No reaction	
5. Methyl Violet 2B alone	1·10	2nd
6. Methyl Violet 2B + acetaldehyde	No reaction	
7. Methyl Violet 2B + H_2O_2	8·31	2nd
8. Methyl Violet 6B alone	1·075	2nd
9. Methyl Violet 6B + acetaldehyde	1·59	2nd
10. Methyl Violet 6B + H_2O_2	66·00	2nd
11. Crystal Violet + Methyl Violet 2B	No reaction	—
12. Crystal Violet + Methyl Violet 2B + H_2O_2	1·10	2nd
13. Crystal Violet + Methyl Violet 6B	No reaction	—
14. Crystal Violet + Methyl Violet 6B + H_2O_2	1·16	2nd
15. Commercial Crystal Violet alone	No reaction	—
16. Commercial Crystal Violet + H_2O_2	0·2776	1st
17. Commercial Crystal Violet + H_2O_2 + mannitol	4·56	2nd
18. Commercial Crystal Violet + H_2O_2 + acetaldehyde	0·619	2nd
19. Commercial Crystal Violet + acetaldehyde	No reaction	—

irradiation with short wavelength light at very low temperatures; this reaction gives rise to propan-2-ol as well as 4-dimethylaminophenylmethyl radicals.[15] Natural sunlight exposure induces oxidation and partial reduction of Crystal Violet; this leads to the formation of 4-dimethylaminobenzophenone,[16] a product also produced under the action of *B. subtilis*.[11] The reactions leading to the formation of this ketone are inhibited by low concentrations of copper (II) sulphate, but enhanced by high concentration of this salt.[17]

In the presence of hydrogen peroxide the dye decomposes with a rate constant of $40{\cdot}00 \times 10^{-3}$. This rate constant is reduced to $2{\cdot}89 \times 10^{-3}$ on the addition of an equimolar amount of mannitol, indicating that the reaction is hydroxyl radical mediated. This observation provides support for the previous findings that ultrasound could induce colour fading of Crystal Violet solutions;[18] sonic energy is a means of producing hydroxyl radicals.[19,20] Addition of acetaldehyde, a triplet-quenching agent, to a Crystal Violet solution, did not induce photochemical decomposition of the dye. This suggests that, at least under the conditions of testing, Crystal Violet does not decompose via a triplet state in the absence of hydrogen peroxide. With added hydrogen peroxide and acetaldehyde, however, there was a very slow reaction following second-order kinetics (rate constant $0{\cdot}619 \times 10^{-3}$), indicating that the acetaldehyde is scavenging the radicals produced. This would suggest that hydroperoxy radicals are being generated in this process. It has been reported[21] that Crystal Violet undergoes colour fading in phosphate buffer (pH 6·0) in the presence of ascorbic acid (a free-radical scavenger) with added tripaflavin, and that this reaction involves an energy transfer via ascorbic acid.

The rate constants for the reaction of both Methyl Violet 2B and Methyl Violet 6B with hydrogen peroxide were markedly different, ($8{\cdot}31 \times 10^{-3}$ and $66{\cdot}00 \times 10^{-3}$ respectively), Methyl Violet 6B being the more readily reacted of the two. In the presence of purified Crystal Violet and hydrogen peroxide, both of these impurities give notably reduced rate constants, ($1{\cdot}10 \times 10^{-3}$ and $1{\cdot}16 \times 10^{-3}$) respectively. This suggests that there is no synergistic action with either of the impurities isolated when used individually, and that purified Crystal Violet has a marked protective effect on the photochemical reaction of both impurities by hydroxyl radicals. In the unpurified (commercial) sample of Crystal Violet, which contained both impurities, however, there was no observable reaction when the dye alone was irradiated. When the unpurified dye was irradiated with hydrogen peroxide the rate constant was $0{\cdot}2776 \times 10^{-3}$, the reaction following first-order kinetics, and most of the dye was degraded within 5 h of initiation of irradiation. The reaction in the presence of both hydrogen peroxide and mannitol gave a rate constant of $4{\cdot}56 \times 10^{-3}$. The photochemical degradation of Crystal Violet in alcohols

and acetonitrile indicates that ultraviolet light induces greater decomposition than does visible light. The nature of the solvent is also of importance, the dye being oxidised in aerated solutions of acetonitrile but reduced in aerated solutions in alcohols.[22]

Crystal Violet is decomposed by the extracellular fluid from lignolytic cultures of *Phanerochaete chrysosporium* provided that a hydrogen-peroxide-generating system is present.[23] The photochemical studies presented here show that Crystal Violet is degraded by hydroxyl and hydroperoxy radicals. This suggests that, as a means of the detection of bacteria, capable of generating hydroxyl and hydroperoxy radicals, the use of Crystal Violet might be inappropriate, since such organisms could decompose the dye and give erroneous colour reactions in Crystal-Violet-containing media.

REFERENCES

1. Martindale, *The Extra Pharmacopoeia* (30th edn) ed. J. E. F. Reynolds. The Pharmaceutical Press, London, 1993, p. 793.
2. Pal, M. K. & Ghosh, J. K., *Spectrochimica Acta Part A*, **50A**(1) (1994) 119.
3. Vercesi, A. E., Barnardes, C. F., Hoffmann, M. E., Gadhela, F. R. & Docampo, R., *J. Biol. Chem.*, **266** (1991) 14431.
4. Cruikshank, R., Duguid, J. P., Marmion, B. P. & Swain, R. H. A., *Medical Microbiology*, 12th edn, Vol. 2: *The Practice of Medical Microbiology*. Churchill Livingstone, Edinburgh, 1975.
5. Conn, H. J., Darrow, M. A. & Emmel, V. M., *Staining Procedures used by the Biological Stain Commission*, 2nd edn. Williams and Wilkins, Baltimore, 1960.
6. Kodaka, H., Armfield, A. Y., Lombard, G. L. & Dowell, V. R., *J. Clin. Microbiol.*, **16** (1982) 948.
7. Neisser, M., *Hyg. Rundschau*, **13** (1903) 705.
8. Martinez de Tejada, G. & Moriyon, I., *J. Bacteriol.*, **175** (1993) 5273.
9. Debnam, P., Glanville, S. & Clark, A. G., *Biochem. Pharmacol.*, **45** (1993) 1227.
10. Roth, P., Sattler, K., Berger, R. & Vinz, M., *Zentrabl. Mikrobiol.*, **147** (1992) 409.
11. Yatome, C., Ogawa, T. & Matsui, M., *J. Environ. Sci. Health Part. A.*, **A26** (1991) 75.
12. Freeman, R., Burdess, D. & Smith, S., *J. Clin. Pathol.*, **47** (1994) 283.
13. Hay, J., Khan, W. & Sugden, J. K., *Dyes and Pigments*, **25** (1994) 305.
14. Evans, P. G. E., van Abbe, N. J. & Sugden, J. K., *Pharm. Acta Helv.*, **50** (1974) 94.
15. Kozlov, Yu. I. & Shigorin, D. N., *Dokl. Akad. Nauk. SSSR*, **161** (1965) 871.
16. Desai, C. M. & Vaidya, B. K., *J. Indian Chem. Soc.*, **31** (1954) 261.
17. Desai, C. M. & Vaidya, B. K., *J. Indian Chem.* Soc., **31** (1955) 717.
18. Azami, T. & Hinshara, T., *J. Chem. Soc. Japan. Pure Chem. Sect.*, **72** (1951) 515.
19. Anbar, M. & Pecht, I., *J. Phys. Chem.*, **71** (1967) 1246.
20. Parkinson, H., Stanford, J. B. & Sugden, J. K., *Int. J. Pharm.*, **51** (1989) 189.
21. Tomita, G. & Takeyama, N., *Kagaku (Tokyo)*, **29** (1959) 662.
22. Nakamura, R. & Hida, M., *Sen'i Gakkaishi*, **28** (1982) T183.
23. Bumpus, J. A. & Brock, B. J., *App. Environ. Microbiol.*, **54** (1988) 1143.