Dyes and Pigments 24 (1994) 305–312

Photochemical Decomposition of Phenol Red (Phenolsulphonphthalein)

J. Hay

Bacteriology Department, Glasgow Royal Infirmary, Castle Street, Glasgow, UK G4 O5F

W. Khan & J. K. Sugden*

Department of Pharmacy, School of Applied Sciences, De Montfort University, The Gateway, Leicester, UK LE1 9BH

(Received 22 November 1993; accepted 6 January 1994)

ABSTRACT

Commercially available Phenol Red, purified using adsorption chromatography, was found to comprise two distinct compounds: pure Phenol Red and a contaminant, which in the solid state was very slightly pink in colour. These two compounds alone and in combination underwent photodegradation via a hydroxyl radical mediated reaction. Photolysis also occurred when the compounds were incorporated into a lauryl sulphate broth medium used in the membrane filtration method for the detection of coliforms and 'faecal' E. coli *in drinking water. These findings support the contention that commercial preparations of Phenol Red should be purified prior to inclusion in growth media for bacteria.*

INTRODUCTION

The triphenylmethane compound Phenol Red (phenolsulphonphthalein) has a wide range of applications in the life sciences. It has been used in pharmacology[1] in a test for renal function, by estimating its rate of excretion in urine after intravenous or intramuscular injection; it has also been used as a drug ingestion indicator, as a marker in drug absorption studies and in a quantitative test of residual urine.

* To whom correspondence should be addressed.

Dyes and Pigments 0143-7208/94/$07.00

Phenol Red possesses weak oestrogenic properties,[2–7] and in cultured porcine thyroid cells it has been found to act as a substrate for thyroperoxidase, which mediates thyroglobulin iodination in the thyroid.[8]

The compound is cholephilic, being effectively taken up and excreted by the liver, and thus has been used for in-vitro cellular assays of the differential effects of drugs on liver transcellular transport.[9] In ophthalmology, Phenol Red has been suggested as an alternative to the Schirmer test for the measurement of tear volume; in this scenario, the compound is impregnated into special threads which are wet by the tears causing a colour change along the thread.[10,11] In diagnostic microbiology, Phenol Red has found wide usage.[12] At a concentration of 50 ppm, it was an effective and selective inhibitor of fungi.[13] It has a pKa of 7·9 and is present in many cell and tissue culture media as a pH indicator. It also has this latter function in a range of bacteriological media, being incorporated into growth systems for the Enterobacteriaceae and other disease causing microbes.[14] It is a constituent of a lauryl sulphate broth (LSB) which is used in the recommended UK membrane filtration (MF) method for enumeration of coliform bacteria, and more specifically 'faecal' coliforms and *Escherichia coli* in drinking water.[15]

However, despite its wide range of applications, little is known regarding the photochemical degradation of the compound, or of its major impurity, and whether such a process could adversely affect the outcome of observations in the various settings described above, especially the MF method, where bacterial colonies must be examined within a few minutes of removal from incubation since they are liable to change colour on cooling and exposure to light,[16] thus providing erroneous results. Therefore, the present study was aimed at elucidating the kinetics of the photochemical decomposition process of Phenol Red and its major contaminant, and at providing a possible mechanism to account for the process.

MATERIALS AND METHODS

Purification of commercial samples of Phenol Red

A column (21·6 cm long by 2·54 cm diameter) fitted with a glass sinter was 3/4 filled with an aqueous slurry of Silica 60 (Merck) and the slurry was allowed to settle. The water was run off until the level was just at the top of the adsorbant column and then 32 ml of a 1% (w/v) solution of commercial Phenol Red (B.D.H.) was added. The dye solution was adsorbed on to the column of silica and the sample constituents eluted with deionised water. The sample constituents were isolated from aqueous solutions by freeze drying (Table 1).

TABLE 1
Purification of Commercial Phenol Red

	Freeze dried	*Yield (g)*	*pH deionised water*	*Rf silica plate (Merck)*
Red solution	Red	0·148	7·44	0·51
Yellow solution	Pink	0·108	7·91	0·40

Photochemical decomposition of purified dye products

Aqueous solutions of purified Phenol Red (2·67 mg in 100 ml) and the major impurity (6·6 mg in 100 ml) were prepared. Calibration curves were constructed for each sample with a concentration range of 0·8, 1·6, 2·4, 3·2, 4·0, 4·8, 5·0, 6·0, 6·4 mg per 100 ml for the Phenol Red and 4·0, 8·0, 16·0, 24·0, 32·0, 40·0, 48·0, 56·0, 60·0, 64·0 mg per 100 ml for the major contaminant. Linear regression analyses yielded regression coefficients of 0·998 and 0·999 respectively.

Each solution was irradiated by simulated sunlight in a standard apparatus[17] at 23 ± 1°C and absorbance readings were taken at 432 nm at time zero and at regular time intervals thereafter up to a maximum of 5 h. The procedure was then repeated with various additives (Table 2) included with the two compounds, and with the commercially available

TABLE 2
Samples Irradiated

Purified Phenol Red alone
Purified Phenol Red + H_2O_2 (equimolar)
Purified Phenol Red + acetaldehyde (equimolar)
Purified Phenol Red + H_2O_2 (equimolar) + mannitol 5%
'Pink Dye' (impurity)
'Pink dye' + H_2O_2 (30% v/v; 0·1 ml)
'Pink dye' + acetaldehyde (0·1 ml)
Purified Phenol Red + 'pink dye' 1 : 1
Purified Phenol Red + 'pink dye' 1 : 1 + H_2O_2 (0·1 ml)
Commercial Phenol Red in lauryl sulphate broth[a,b,c]
Purified Phenol Red in lauryl sulphate broth[a,b]
'Pink dye' in lauryl sulphate broth[a,b,c] [13,14]

Peptone	39·0 g litre^{-1}	Lactose	30·0 g litre^{-1}
Sodium lauryl sulphate	1·0 g litre^{-1}	Yeast extract	6·0 g litre^{-1}
[a] Dye	0·2 g litre^{-1}	Lactose	30·0 g litre^{-1}

[b] Irradiation time up to 239 h.
[c] Measured against a blank of broth irradiated under the same conditions as the broth with added dyes.

Phenol Red; the dyes were tested alone and in combination with LSB, as used in the MF method.

The order of reaction and the rate constants were calculated by application of linear regression analyses.[18]

Investigation of the nature of the pink impurity

Refluxing the 'pink dye' with ethanol (50 ml) for 30 min gave a white insoluble solid and a pink solution. The solid was shown to be a mixture of inorganic compounds, which could be eluted from the column with deionised water with and without the presence of dyes, by comparison of Fourier Transform IR spectra. Evaporation of the ethanolic solution under reduced pressure yielded a minute trace of an intractable red tar which had a yellow colour in dilute aqueous solution. This material was regarded as being the source of the pink colour.

RESULTS AND DISCUSSION

Table 3 shows that the photodecomposition of purified Phenol Red followed first-order kinetics and had a rate constant $1{\cdot}3 \times 10^{-3}$. The addition of an equimolar amount of hydrogen peroxide produced a rise in the rate constant to $11{\cdot}9 \times 10^{-3}$, but the reaction kinetics remained first order. Incorporation of mannitol (5%) into the reaction mixture resulted in a diminution of the rate constant to $3{\cdot}7 \times 10^{-3}$. These findings suggest that this photodecomposition of purified Phenol Red is substantially hydroxyl radical mediated. This notion is supported by the observation that the incorporation of acetaldehyde did not result in a

TABLE 3
Rate Constant and Reaction Order of Photodecomposition Experiments

Sample irradiated	*Rate constant* $\times 10^{-3}$	*Reaction order*
Purified Phenol Red	1·3	First
Purified Phenol Red + H_2O_2	11·9	First
Purified Phenol Red + acetaldehyde	3·9	First
Purified Phenol Red + H_2O_2 + mannitol 5%	3·7	First
'Pink impurity' control	15·5	First
'Pink impurity' + H_2O_2	73·9	First
'Pink impurity' + acetaldehyde	16·1	First
'Pink impurity' + H_2O_2 + mannitol 5%	14·9	First
Purified Phenol Red + 'pink impurity' 1 : 1	3·2	First
Purified Phenol Red + 'pink impurity' 1 : 1 + H_2O_2	32·2	First

reduction in the rate constant, which might be expected if the reaction proceeded by a triplet sensitisation reaction brought about by the action of the dye on water. Photochemical degradation of the pink dye, isolated from the commercial sample of Phenol Red, also followed first-order kinetics, but here the rate constant was much higher at $15{\cdot}5 \times 10^{-3}$. Addition of hydrogen peroxide also resulted in a substantial increase in the rate constant, which was recorded at $73{\cdot}9 \times 10^{-3}$. The addition of acetaldehyde did not result in a reduction in the rate constant, indicating that this dye, as for Phenol Red, did not act by a triplet sensitisation mechanism.

Irradiation of a solution containing equal amounts of purified Phenol Red and the pink dye demonstrated a significant rise in the rate constant as compared to purified Phenol Red alone. However, the rate constant was less than that recorded for the pink dye control. The mean value of the rate constants for purified Phenol Red and the 'pink dye' was $(1{\cdot}3 + 15{\cdot}5)/2 = 8{\cdot}4 \times 10^{-3}$, which was less than that of the 'pink dye' alone. These results suggest that the photochemical decomposition of Phenol Red was unlikely to be synergistic as a consequence of the presence of the impurity. A similar conclusion can be reached when considering the rate constant recorded for the combined solution irradiated with equimolar amounts of hydrogen peroxide; the mean value was $42{\cdot}9 \times 10^{-3}$.

Tables 4 and 5 show that the photochemical decomposition of Phenol Red in LSB followed second-order kinetics and that there was a very small difference between the rate constants of the commercial Phenol Red and the purified Phenol Red.

However, findings from the 'pink impurity' alone revealed that the photochemical decomposition of this compound also followed second-order kinetics (Table 6), but the rate constant was significantly lower than those of either the commercial sample or the purified Phenol Red.

TABLE 4
Photochemical Decomposition of Commercial Phenol Red in Lauryl Sulphate Broth

Time (h)	*Absorbance*	*% dye left*	*Log% dye left*	*1% dye left*
0	4·6809	100·00	2·0000	0·0100
24	4·6809	100·00	2·0000	0·0100
93	3·4126	72·90	1·8627	0·0137
168	2·4839	53·06	1·7248	0·0188
239	2·1174	45·23	1·6554	0·0221
		$r = -0{\cdot}9809$	$r = -0{\cdot}9913$	$r = 0{\cdot}9938$
				Slope = 5·387
				Rate constant = 5·39

TABLE 5
Photodecomposition of Purified Phenol Red in Lauryl Sulphate Broth

Time (h)	*Absorbance*	*% dye left*	*Log% dye left*	*1% dye left*
0	4·680 9	100·00	2·000 0	0·010 0
24	4·680 9	100·00	2·000 0	0·010 0
93	3·425 2	73·17	1·864 3	0·013 6
168	2·472 8	52·82	1·722 8	0·018 9
239	2·196 4	46·92	1·671 3	0·021 3
		$r = -0.976\,6$	$r = -0.986\,8$	$r = 0.990\,6$
				Slope = 5·135
				Rate constant = 5·13

The pink impurity also appeared to be more stable as far as the first degradation product was concerned. When the LSB system, incorporating a commercially available Phenol Red, is used to grow coliform bacteria, the stability of the resulting yellow colour of the colonies is capricious.[15,16] This could suggest that the organisms produce a compound which facilitates the decomposition of the dye.

Oxygen radicals are known to be primarily responsible for oxygen-dependent toxicity to *E. coli B* by photosensitive dyes, represented in one study by thiazines, xanthines, acridines and phenazines; one adaptive response of this bacterium to the threat of dye mediated oxidative response is to induce superoxide dismutase (SOD).[19] Hydrogen peroxide formed by SOD and by the uncatalysed reactions of hydroperoxy radicals[20] is scavenged by catalase which converts hydrogen peroxide into water and molecular oxygen. Thus, the photoability of the Phenol Red in the MSB medium[15,16] may be a consequence of the biochemical response to the intracellular aggregation of the dye molecules; the hydrogen peroxide, when exposed to light, provides an increased rate of oxygen radical generation

TABLE 6
Photodecomposition of 'Pink' Impurity in Lauryl Sulphate Broth

Time (h)	*Absorbance*	*% dye left*	*Log% dye left*	*1% dye left*
0	1·249 6	100·00	2·000 0	0·010 0
24	1·233 2	98·68	1·994 2	0·010 1
93	0·473 6	37·90	1·578 6	0·026 3
168	0·382 4	30·60	1·485 7	0·032 6
		$r = -0.936\,0$	$r = -0.953\,9$	$r = 0.973\,2$
				Slope = 1·479 5
				Rate constant = 1·48

which overwhelms the abilty of the bacterial catalase to process them. This notion may also explain the biodegradation of Phenol Red by sewage sludge.[21]

Alternatively, there may be selective uptake, or enhanced uptake, into the multiplying bacteria of the pink impurity, which is actually yellow in aqueous solution (Table 1). On exposure to light, the phenolsulphonphthalein would be more rapidly photodecomposed, leaving only the colour of the impurity to be observed.

The findings obtained from the present study indicate that the presence of the pink impurity in the unpurified dye mixture in the LSB medium does not affect the kinetics of photodecomposition when compared with the purified Phenol Red. However, the presence of multiplying bacteria in the system would clearly affect the findings, as has previously been reported.[15,16] Thus, purified Phenol Red only should be incorporated into bacteriological growth media.

REFERENCES

1. Martindale, *The Extra Pharmacopoeia*, (29th edn), ed. A. Wade. The Pharmaceutical Press, London, 1989, p. 944.
2. Berthois, Y., Katzenellenbogen, J. A. & Katzenellenbogen, B. S., *Proc. Natl. Acad. Sci., (Cell Biol.) USA*, **83** (1986) 2496–500.
3. Hubert, J. F., Vincent, A. & Labrie, F., *Biochem. Biophys. Res. Comm.*, **141** (1986) 885–91.
4. Hofland, L. J., Van Koetsveld, P., Koper, J. W., Den Holder, A. & Lamberts, S. W. J., *Mol. Cell. Endrocrinol.*, **54** (1987) 43–50.
5. Ravdin, P. M., Van Beurden, M. & Jordan, V. C., *Breast Cancer Res. Treat.*, **9** (1987) 151–4.
6. Richards, J., Imogawa, W., Balakrishnan, A., Edery, M. & Nandi, S., *Endocrinology*, **123** (1988) 1335–40.
7. Welshons, W. V., Wolf, M. F., Murphy, C. S. & Jordan, V. C., *Mol. Cell. Endocrinol.*, **57** (1988) 169–78.
8. Gruffat, D., Gonzalvez, S., Mauchamp, J. & Chabaud, O., *Mol. Cell. Endocrinol.*, **81** (1991) 195–203.
9. Aoyama, N., Ohya, T., Chandler, K., Gresky, S. & Holzbach, R. T., *Hepatology*, **14** (1991) 1–9.
10. Hamano, H. *et al.*, *CLAO J.*, **9** (1983) 281–9.
11. Hamano, T. *et al.*, *CLAO J.*, **16** (1990) 57–61.
12. Lennette, E. H., Spaulding, E. H. & Truant, J. P., *Manual of Clinical Microbiology* (2nd edn). American Society for Microbiology, Washington, DC, 1974.
13. Bragulat, M. R., Abarca, M. L., Bruguera, M. T. & Cabanes, F. J., *Appl. Environ. Microbiol.*, **57** (1991) 2777–80.
14. Bridson, E. Y., *The Oxoid Manual* (6th edn). Unipath Ltd, Basingstoke, UK.

15. Department of the Environment, *The Bacteriological Examination of Drinking Water Supplies 1982, Methods for Examination of Water and Associated Materials.* Reports on Public Health and Medical Subjects No. 71. HMSO, London, 1983.
16. Hay, J., Khan, W., Mead, A. J. C., Seal, D. V. & Sugden, J. K., *Lett. Appl. Microbiol.*, **18** (1994) 117–19.
17. Evans, P. G. E., Sugden, J. K. & Van Abbe, N. J., *Pharm. Acta. Helv.*, **50** (1975) 94–9.
18. Patel, R. & Sugden, J. K., *Pharmazie*, **47** (1993) 113–15.
19. Martin, J. P. & Logsdon, N., *J. Biol. Chem.*, **262** (1987) 7213–19.
20. Czapski, G., *Isr. J. Chem.*, **10** (1972) 987–97.
21. Shukla, N. & Pandey, G. S., *Ind. J. Environ. Prot.*, **11** (1991) 110–11.