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Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both Gram-negative and Gram-positive bacteria

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Abstract

The photosensitization of microorganisms is potentially useful for sterilization and for the treatment of certain bacterial diseases. Until now, any broad spectrum approach has been inhibited because, although Gram-positive bacteria can be photoinactivated by a range of photosensitizers, Gram-negative bacteria have not usually been susceptible to photosensitized destruction.

In the present work, it has been shown that the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, as well as the Gram-positive bacterium *Enterococcus seriolicida*, can be photoinactivated when illuminated in the presence of a cationic water-soluble zinc pyridinium phthalocyanine (PPC). The degree of photoinactivation is dependent on both the concentration of PPC and the illumination time. In contrast, the three bacteria are not photoinactivated by illumination in the presence of a neutral tetra-diethanolamine phthalocyanine (TDEPC) or negatively charged tetra-sulphonated phthalocyanine (TSPC).

Uptake studies have revealed that the lack of activity of TSPC is due to the fact that it has very little affinity for any of the organisms. However, the issue appears to be more complex than simply the gross levels of cellular uptake, since TDEPC and PPC are both taken up by the organisms but only PPC shows activity. This indicates that the localization and subcellular distribution of the phthalocyanines may be a crucial factor in determining their cell killing potential.

Further analysis of the uptake data has revealed a cell-bound photosensitizer fraction, which remains tightly associated after several washings, and another weakly bound fraction, which is removed by successive washings. Analysis of the cell killing curves, carried out after successive washings of *E. coli* exposed to PPC, has revealed that it is the tightly associated fraction that is involved in the photosensitization.

Taken together with other data, these results suggest that cationic photosensitizers may have a broader application in the photoinactivation of bacterial cells than the anionic or neutral photosensitizers commonly used in photodynamic therapy.

Keywords: Bacteria; Gram-negative; Gram-positive; Cationic; Phthalocyanine

1. Introduction

The use of a photosensitizer and light to effect biological damage has found extensive application in the photodynamic therapy (PDT) of cancer. The application of this approach to the destruction of microorganisms also has considerable medical and technological potential, but is less well advanced. The increase in the antibiotic resistance of bacteria makes the development of alternative antimicrobial techniques an important area of research [1].

Several porphyrins and phthalocyanines have been shown to display a potent photocytotoxic effect against Gram-positive bacteria [2–4]. However, with a few exceptions [5–7], Gram-negative bacteria have been resistant to the photodynamic action of these sensitizers unless substances such as $CaCl_2$, ethylenediaminetetraacetic acid (EDTA) or polymyxin B nonapeptide are employed, which alter the outer membrane permeability, rendering the bacteria photosensitive [8,9].

The reasons for the apparent resistance of Gram-negative bacteria to photosensitization are unclear, but this could be related to the charge on the photosensitizer. Most of the drugs used in PDT, which are ineffective towards Gram-negative bacteria, are negatively charged or neutral, but the potential of cationic photosensitizers in photoinactivating either Gram-negative or Gram-positive bacteria has not been evaluated.

The purpose of this study was to determine the effect of water-soluble zinc phthalocyanines of different charge on the

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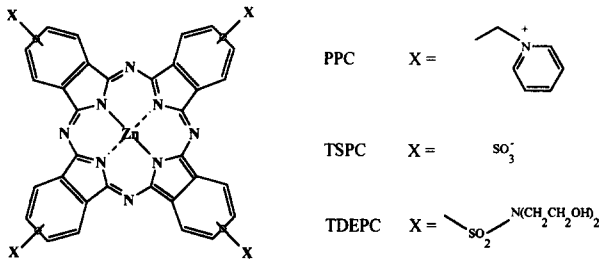

Fig. 1. Structures of the substituted water-soluble zinc phthalocyanines.

photosensitization of the Gram-positive bacterium *Enterococcus seriolicida* and the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and, particularly, to determine whether the resistance to the photosensitization of Gram-negative bacteria could be overcome by the use of a cationic dye.

2. Materials and methods

2.1. Phthalocyanine solutions

Fig. 1 depicts the structures of the three substituted phthalocyanines of different charge used in this study. These were synthesized in Leeds and have been described previously [10]. They comprised the anionic tetra-sulphonated phthalocyanine (TSPC), the neutral tetra-diethanolamine phthalocyanine (TDEPC) and the cationic pyridinium phthalocyanine (PPC). Analysis of the latter shows this to be a mixture of mono-, di-, tri- and tetra-substituted material. Tryptophan photo-oxidation studies showed that all three phthalocyanines had similar singlet oxygen quantum yields [11]. Samples were dissolved in water to a concentration of 1 mg ml^{-1}, passed through a 0.22 μm filter and stored at −20 °C until required.

2.2. Preparation of microorganism

Escherichia coli (strain DH5) and *Pseudomonas aeruginosa* (strain 12718) were grown in nutrient broth overnight (1% tryptone, 0.5% yeast extract w/v). *Enterococcus seriolicida* was grown overnight in brain heart infusion broth (BDH, UK) supplemented with 2% NaCl. A portion (10 ml) of the culture was aseptically transferred to 200 ml of fresh medium and grown at 37 °C to mid log phase in a shaking incubator. The cells were harvested by centrifugation (5000g × 10 min) and washed once in 0.1 M phosphate buffer (pH 7.0). The cells were then resuspended in the same buffer to $A_{650\,nm} = 0.87$ for *E. coli* and *E. seriolicida* and $A_{650\,nm} = 1.0$ for *P. aeruginosa*, corresponding to $\log_{10}$ colony forming units (CFU) per millilitre of 8.5, 8.6 and 8.8 respectively.

2.3. Cell killing experiment

Samples (25 ml) of the cell suspension were incubated in the dark in foil-covered 250 ml conical flasks with the appropriate concentration of phthalocyanine at 37 °C for 30 min and then placed on an orbital shaker 70 cm below two 400 W metal halide spectral lamps for varying lengths of time. This gives a light intensity of approximately 1 mW cm^{-2} in the range 600–700 nm, which covers the spectral band corresponding to the absorbance of the phthalocyanines. Control experiments were carried out with illumination in the absence of photosensitizer and with photosensitizer in the dark. Irradiated and unirradiated samples were assayed for cell viability by serial dilution in 0.1 M phosphate buffer, and then plated in triplicate on nutrient broth supplemented with 2% agar. Experiments were designed so that between 30 and 300 colonies were formed and these were counted after the plates were incubated at 37 °C for 24 h.

2.4. Growth delay experiment

E. coli and *P. aeruginosa* were grown overnight in Davis minimal medium [12] supplemented with 2 mg ml^{-1} glucose, 1 mg ml^{-1} tryptone and 20 μg ml^{-1} L-tryptophan. A portion (2 ml) of the culture was transferred to 25 ml of fresh minimal media and grown at 37 °C on an orbital shaker. At certain time points, 1 ml samples of the cultures were taken and the turbidity at 550 nm was measured using an SP8-100 UV–visible spectrophotometer. When the cultures reached log phase, the appropriate concentration of phthalocyanines was added and the flasks were placed in the light system described above. Samples were also taken to determine the viability of the cells as described earlier.

2.5. Phthalocyanine binding studies

To determine the amount of photosensitizer tightly associated with the cells, suspension that had been incubated in the dark for 30 min with 10 μg ml^{-1} of the phthalocyanines were centrifuged (5000g × 10 min) and washed successively with 0.1 M phosphate buffer. This was achieved by resuspending the cell pellet in the same volume of buffer as the supernatant discarded, followed by centrifugation (5000g × 10 min). After each wash, samples were taken to determine the amount of bound phthalocyanine, and cell killing experiments were carried out on *E. coli* to investigate the relative importance of cell-bound dye in the photosensitization of the cells.

2.6. Fluorometric estimation of cell-bound phthalocyanine

During the uptake experiments, the amount of cell-bound photosensitizer was determined using the fluorometric approach of Bertoloni et al. [3]. Samples were treated with 2% aqueous sodium dodecyl sulphate (SDS) overnight and the fluorescence emission was measured at 683 nm (excitation at 600 nm) using a Kontron SFM 25 spectrofluorometer. The amount of phthalocyanine present was determined by interpolation from a standard curve of known concentration in 2% SDS.

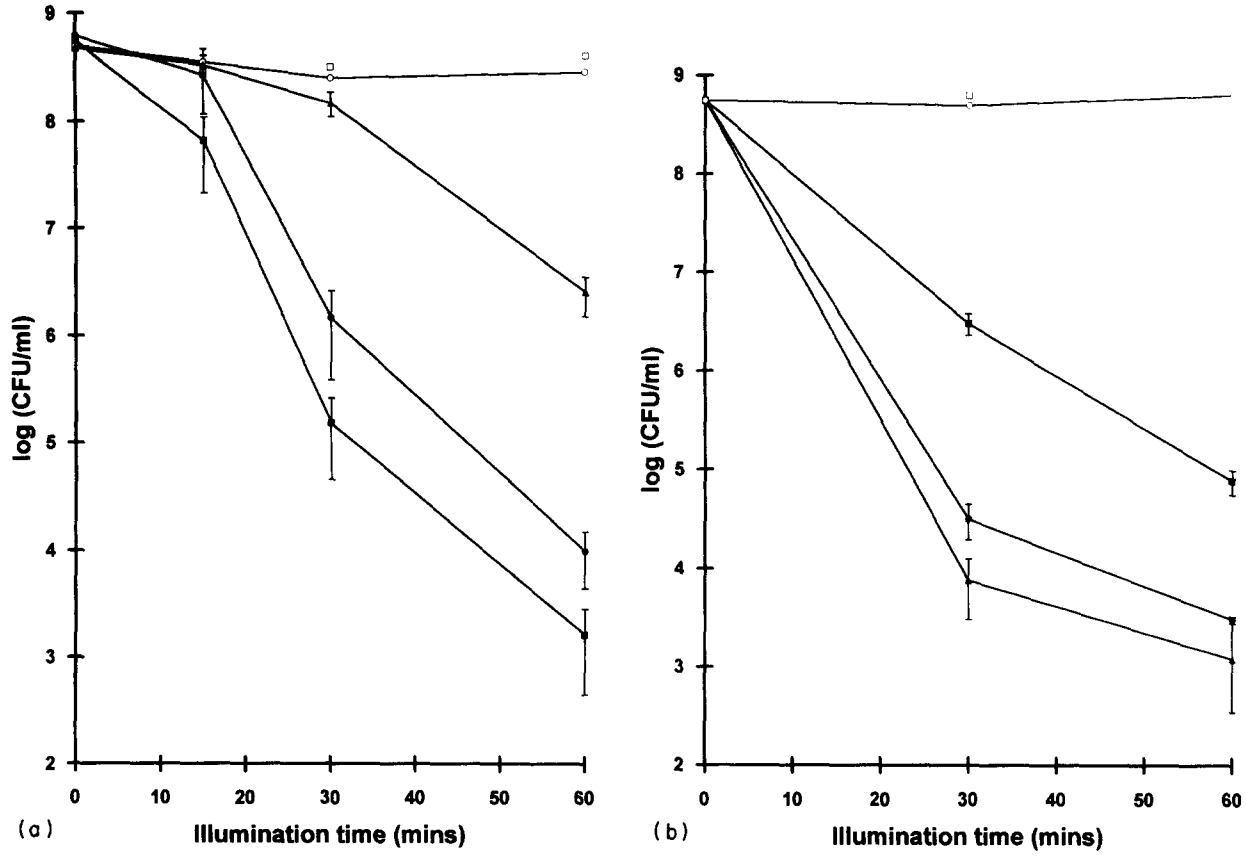

Fig. 2. (a) Survival curves of *E. coli* incubated with 10 μg ml^{-1} TDEPC (□), 10 μg ml^{-1} TSPC (○), 1 μg ml^{-1} PPC (▲), 5 μg ml^{-1} PPC (●) or 10 μg ml^{-1} PPC (■) and illuminated for 0, 15, 30 or 60 min. Each point is the mean of three experiments ± standard deviation. (b) Survival curves of *P. aeruginosa* incubated with 50 μg ml^{-1} TDEPC (□), 50 μg ml^{-1} TSPC (○), 10 μg ml^{-1} PPC (■), 25 μg ml^{-1} PPC (●) or 50 μg ml^{-1} PPC (▲) and illuminated for 0, 30 or 60 min. Each point is the mean of three experiments ± standard deviation.

Table 1
Cell killing results from the growth delay experiments of (a) *E. coli* and (b) *P. aeruginosa*

(a)

Time after addition of drug (min)	1 μg ml^{-1} PPC (CFU ml^{-1})	Cell survival (%)	5 μg ml^{-1} PPC (CFU ml^{-1})	Cell survival (%)	10 μg ml^{-1} PPC (CFU ml^{-1})	Cell survival (%)
0	2.5×10^{8}	100	1.36×10^{8}	100	1.42×10^{8}	100
30	1.59×10^{7}	6.4	1.36×10^{5}	0.1	5.92×10^{4}	0.044
60	8×10^{5}	0.32	6.4×10^{4}	0.047	3.6×10^{3}	2.53×10^{-3}
120	2.3×10^{5}	0.09	2.4×10^{3}	1.76×10^{-3}	5.8×10^{3}	4.26×10^{-3}

(b)

Time after addition of drug (min)	10 μg ml^{-1} PPC (CFU ml^{-1})	Cell survival (%)	25 μg ml^{-1} PPC (CFU ml^{-1})	Cell survival (%)	50 μg ml^{-1} PPC (CFU ml^{-1})	Cell survival (%)
0	1×10^{9}	100	$9{,}8\times10^{8}$	100	8.53×10^{8}	100
30	8.36×10^{6}	0.84	3.26×10^{5}	0.033	1.8×10^{5}	0.021
60	5.1×10^{5}	0.051	4.45×10^{5}	0.045	1.3×10^{5}	6×10^{-3}

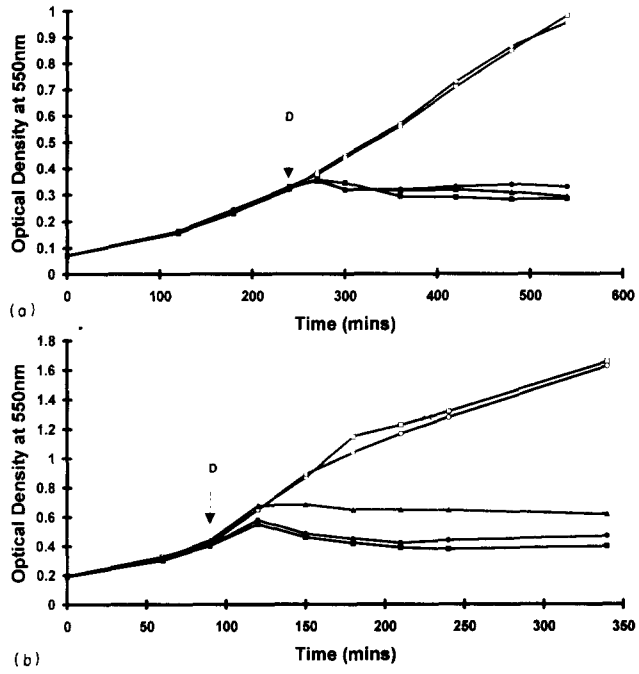

Fig. 3. (a) Growth delay curves of *E. coli* incubated with 10 μg ml^{-1} TDEPC (□), 10 μg ml^{-1} TSPC (○), 1 μg ml^{-1} PPC (▲), 5 μg ml^{-1} PPC (●) or 10 μg ml^{-1} PPC (■). D marks the time point where the appropriate concentrations of the phthalocyanines were added. (b) Growth delay curves of *P. aeruginosa* incubated with 50 μg ml^{-1} TDEPC (□), 50 μg ml^{-1} TSPC (○), 10 μg ml^{-1} PPC (■), 25 μg ml^{-1} PPC (●) or 50 μg ml^{-1} PPC (▲). D marks the time point where the appropriate concentrations of the phthalocyanines were added.

3. Results and discussion

Control experiments showed that the viability of *E. seriolicida* and *E. coli* was unaffected by 2 h of illumination alone or by 2 h dark incubation with 10 μg ml^{-1} of the photosensitizers or 50 μg ml^{-1} of the photosensitizers for *P. aeruginosa* cells.

Illumination of *E. coli* cells which had been exposed to PPC caused a considerable decrease in cell survival. Fig. 2(a) illustrates that cells exposed to 10 μg ml^{-1} PPC and illuminated for 60 min have greater than a 5 $\log_{10}$ decrease in cell survival. Fig. 2(b) illustrates that another Gram-negative bacterium, *P. aeruginosa*, is also photoinactivated using PPC. *P. aeruginosa* is less sensitive to photosensitization than *E. coli*, requiring exposure to 25 μg ml^{-1} PPC and illumination for 60 min to show a greater than 5 $\log_{10}$ decrease in cell survival. In contrast, both bacteria illuminated in the presence of 50 μg ml^{-1} of the neutral TDEPC or negatively charged TSPC showed no appreciable decrease in cell survival. Figs. 2(a) and 2(b) also show that the degree of cell killing increases with both the concentration of PPC and the illumination time.

Although previous experiments by Bertoloni et al. [9] involved a similar system to that described above, it is possible that Gram-negative bacterial cells suspended in phosphate buffer for a prolonged period have an altered outer membrane structure or an altered function of some of the pores [13]. Therefore growth delay experiments were carried out in medium to ensure that photosensitization was still possible when the cells were not under starvation conditions or the potential damaging effects of phosphate buffer washing. Fig. 3(a) shows that, under these conditions, growth was arrested when *E. coli* was exposed to PPC. After 30 min illumination in the presence of 1, 5 or 10 μg ml^{-1} PPC, the cells no longer appeared to be growing as measured by turbidity. *E. coli* cells exposed to 10 μg ml^{-1} PPC in the dark or 10 μg ml^{-1} TSPC or TDEPC in the light showed no growth delay compared with controls. Furthermore, Fig. 3(b) illustrates a similar result for *P. aeruginosa* exposed to 10, 25 or 50 μg ml^{-1} PPC. The small apparent increase in the optical density at 50 μg ml^{-1} PPC is due to the absorbance of the added phthalocyanine at 550 nm. Once again, no effect was seen of PPC in the dark or TSPC and TDEPC in the light.

Table 1 illustrates the results found when the cell viability was assayed during the growth delay experiments. The extent of cell killing of *E. coli* and *P. aeruginosa* increased with increasing concentration of PPC or illumination time. For example, *E. coli* exposed to 10 μg ml^{-1} PPC and illuminated for 60 min shows greater than 99.997% cell kill. Thus the data illustrate that the observed growth delay is due to a bactericidal effect on the cells rather than a bacteriostatic effect.

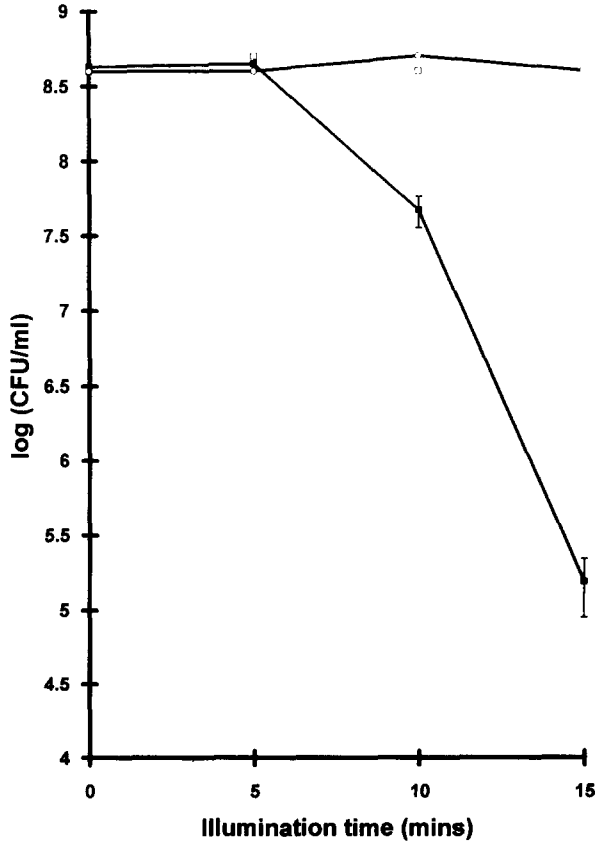

Fig. 4. Survival curves of *E. seriolicida* incubated with 10 μg ml^{-1} TDEPC (□), 10 μg ml^{-1} TSPC (○) or 10 μg ml^{-1} PPC (■) and exposed to visible light for 0, 5, 10 or 15 min. Each point is the mean of three experiments ± standard deviation.

Table 2
Uptake of PPC, TDEPC and TSPC into (a) *E. coli*, (b) *P. aeruginosa* and (c) *E. seriolicida* after dark incubation with 10 $\mu g\ ml^{-1}$ of the phthalocyanines for 30 min followed by repeated washing (ND, not determined)

(a) *E. coli*

Number of washes	PPC cell uptake (%)	TDEPC cell uptake (%)	TSPC cell uptake (%)
	100 ± 4.6	100 ± 4.7	100 ± 2.0
	37.9 ± 4.0	77.8 ± 3.6	1.3 ± 0.2
	29.4 ± 1.0	58.1 ± 3.2	0.33 ± 0.01
	27.3 ± 2.6	47.8 ± 6.8	0.22 ± 0.01
	21.8 ± 2.7	30.6 ± 1.4	0.2 ± 0.01
	17.3 ± 0.5	20.1 ± 1.1	0.12 ± 0.01

(b) *P. aeruginosa*

Number of washes	PPC cell uptake (%)	TDEPC cell uptake (%)	TSPC cell uptake (%)
	100 ± 1.1	100 ± 1.4	100 ± 5
	74.8 ± 4.9	74.4 ± 4.4	4.5 ± 2.4
	55.3 ± 7.3	54.2 ± 3.5	0.47 ± 0.1
	48.5 ± 12.5	41.1 ± 1.6	0.23 ± 0.01
	46.0 ± 11.9	26.1 ± 1	0.06 ± 0.03
	35.2 ± 12.9	20.4 ± 1.6	ND

(c) *E. seriolicida*

Number of washes	PPC cell uptake (%)	TDEPC cell uptake (%)	TSPC cell uptake (%)
	100 ± 2.7	100 ± 1.4	100 ± 0.33
	33.3 ± 1.4	28.1 ± 4.4	1.01 ± 0.08
	22.5 ± 1.2	23.5 ± 3.5	0.17 ± 0.01
	19.7 ± 1.4	13.8 ± 1.6	ND
	17.1 ± 1.6	9.8 ± 1	ND
	15.0 ± 0.6	9.2 ± 1.6	ND

E. seriolicida exposed to PPC shows a dramatic decline in cell viability. Fig. 4 illustrates that incubating *E. seriolicida* with 10 $\mu g\ ml^{-1}$ PPC and irradiating for 15 min leads to a 4 log_{10} decrease in cell survival. The sensitivity to photoinactivation for this Gram-positive bacterium is more than that for either of the Gram-negative bacteria described earlier. Exposure to 10 $\mu g\ ml^{-1}$ TDEPC or TSPC and 15 min irradiation resulted in no cell killing effect. This observation is a little surprising since there have been several reports of the use of neutral or anionic porphyrins or phthalocyanines to photoinactivate Gram-positive bacteria [2–4].

The results of successive washings with 0.1 M phosphate buffer of *E. coli*, *P. aeruginosa* and *E. seriolicida*, which had been incubated with 10 $\mu g\ ml^{-1}$ of the phthalocyanines, are shown in Table 2. The results indicate that the failure of TSPC to show any photosensitizing activity on the bacteria is most probably due to its failure to bind to them. In the case of PPC, it seems to have a binding affinity for all three of the organisms and shows a large photosensitization effect on each. However, this is not the case for TDEPC, which shows binding affinity to all three bacteria, but exhibits no PDT effect. Thus the binding data indicate that it is necessary for the photosensitizer to bind to the cells to exert its photocytotoxic effect. However, the fact that TDEPC exhibits no effect on the three organisms illustrates that it is not sufficient to expect that a gross cellular uptake of the photosensitizer will render the bacteria susceptible to PDT. It implies a level of subcellular distribution and localization that is critical if a photosensitizer is to inactivate these organisms.

The data in Table 2 also indicate that two cell-associated fractions of photosensitizer exist. For example, the first washing of *E. coli* exposed to PPC caused the removal of greater than 60% of the original cell-bound dye but, after four washings, little further photosensitizer was removed, even though in excess of 17% remained bound. It would therefore appear that there are two fractions of photosensitizer associated with the cells, one a tightly bound fraction, which is not removed on washing, and the other a weakly bound fraction, which is removed by washing.

Analysis of the survival curves of the cell killing experiments carried out after each wash for *E. coli* (shown in Fig. 5) shows that there is no substantial decrease in the degree of cell killing. It appears, therefore, that it is the tightly bound photosensitizer rather than the weakly bound photosensitizer that is involved in the photosensitization of *E. coli*.

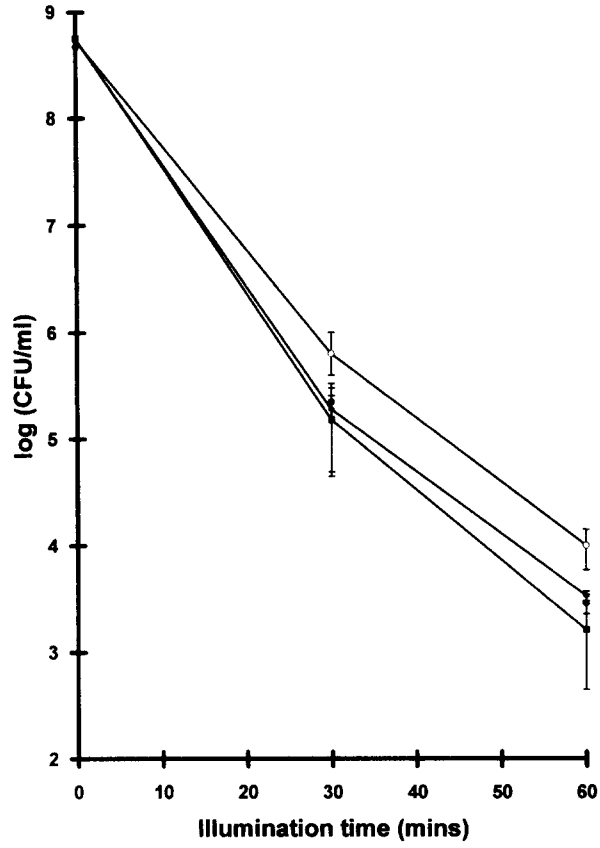

Fig. 5. Survival curves of *E. coli* unwashed (■) or washed with phosphate buffer for one (○), three (♦) or five (●) times before exposure to visible light for 0, 30 or 60 min. Each point is the mean of three experiments ± standard deviation.

4. Conclusions

This paper reports the first use of any phthalocyanine to photoinactivate Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. Our results illustrate that *E. coli*, *P. aeruginosa* and the Gram-positive bacterium *E. seriolicida* can be photoinactivated using the cationic PPC, but not with the neutral TDEPC or anionic TSPC. The cell photosensitization with PPC is dependent on both the illumination time and dye concentration. Furthermore, growth delay experiments carried out in medium indicate that the bacteria are rapidly photoinactivated in an environment free from the possible damaging effects of phosphate buffer washings.

Parallel studies by Merchat et al. [14] have obtained similar data with cationic porphyrins. However, the reasons why cationic materials should be effective are not yet clear. The issue appears to be more complex than simply the levels of gross cellular uptake, since the neutral TDEPC exerts no photocytotoxic effect, and in the studies referred to above [14], the anionic porphyrins appeared to be taken up by Gram-negative bacteria, but not to induce photosensitization. An important factor could be the subcellular distribution and localization of the photosensitizer within the Gram-negative organisms. Preliminary subcellular localization studies with PPC indicate that substantial quantities of the cell-bound dye are associated with the inner membrane and cytosolic fractions [15]. Theoretically, there may be a possible subcellular target within one of these structures which renders the bacteria more sensitive to cationic dyes rather than the neutral or anionic dyes.

If cationic phthalocyanines or porphyrins, such as PPC, can be shown to photoinactive Gram-negative and Gram-positive bacteria in general, then photosensitization could be considered as a possible alternative to current broad spectrum antibacterial methods, where local treatment is required.

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