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Antimicrobial activity of n-alkyltrimethylammonium bromides: influence of specific growth rate and nutrient limitation

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The antimicrobial activity of an homologous series of n-alkyltrimethylammonium bromides has been assessed towards *Escherichia coli* grown at a variety of specific growth rates and under various conditions of nutrient limitation. For each individual set of growth conditions activity was parabolically related to the n-alkyl chain length of the compounds and thus to compound lipophilicity (log P). The compound that showed optimal activity and thereby optimal lipophilicity (log P_o) changed according to growth rate and nutrient limitation. Such changes are related to variations in the gross cell envelope composition of the cultures (phospholipid, lipopolysaccharide, neutral lipids, proteins). The data therefore support the hypothesis that changes in growth rate and nutrient limitation alter the overall lipophilicity of the cell envelope and thereby the optimal value of log P for compounds to traverse it. Additionally, the data suggest that for the compounds examined, the acidic : neutral phospholipid ratios of the cell envelope, also influence the permeation of it.

The specific growth rate of bacteria and the nature of their growth-limiting nutrient greatly influence their susceptibility towards inactivation by chemical antimicrobial agents (Brown 1977; Gilbert & Wright 1987) and antibiotics (De La Rosa et al 1982; Tuomanen et al 1986). Such effects have been reported for a wide range of bacterial species and attributed to alterations in their cellular structure and composition (Tempest & Ellwood 1969; Meers & Tempest 1970; Dean 1972; Dean et al 1976; Broxton et al 1984). With Gram-negative bacteria the changes often involve modification of the outer- and cytoplasmic membranes (Ellwood & Tempest 1972; Nikaido & Nakae 1979; Lugtenberg & Van Alphen 1983). In this respect resistance patterns have been associated with altered phospholipid content (Pechey et al 1974; Imai et al 1975; Teuber & Bader 1976; Ikeda et al 1984), porin-protein composition (Harder et al 1981), lipopolysaccharide content (Tamaki et al 1971) and cation contents (Brown & Melling 1969; Gilleland et al 1974; Melling et al 1974; Nicas & Hancock 1980) of the cell envelopes. These are thought to modify the action of chemical antimicrobial agents through a variety of mechanisms.

1. Where the envelope itself is the primary target for drug action, then reduction in the relative abundance of the target material might reduce overall sensitivity of the cells (Roantree et al 1977; Nikaido & Nakae 1979; Chopra & Ball 1982).

2. Alteration of the bacterial surface, particularly its surface charge, will affect the initial binding of antimicrobial agents (Nadir & Gilbert 1979).

3. Hydrophilic agents must traverse the envelope via its porin-proteins (Nikaido & Nakae 1979), variation in porin-protein content will therefore be reflected in the sensitivity towards such agents (Decad & Nikaido 1976; Harder et al 1981).

4. The cell envelope can be regarded as a series of lipophilic and hydrophilic compartments (Hansch & Clayton 1973; Brown et al 1979). Hydrophobic agents active at the cytoplasmic membrane or cytosol, which are unable to cross the envelope via porins, must partition through these compartments to gain access to their site of action (Hansch & Fujita 1964; Lien et al 1968; Hansch 1970). Partitioning will not only be influenced by the lipophilicity of the agent but also by the lipophilicity of each compartment (Lien et al 1968; Hansch & Clayton 1973). Substances of low water solubility would be unable to penetrate the aqueous layers of the cells and would accumulate in the lipid regions, whereas those of low oil solubility would be unable to cross the lipophilic layers. Compounds beween these two extremes, which possess the optimal balance of properties for traversing the cells barriers must exist. If log P (log of the oil : water partition coefficient) is plotted against the biological activity of a series of compounds, a parabolic relationship ought to occur, log P_o being the value of log P for the compound with optimal activity. Changes in envelope composition,

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by affecting the distribution of an antimicrobial agent throughout the cell, will influence log P_o.

In this study, the antimicrobial activity of a homologous series of n-alkyltrimethylammonium bromides has been evaluated. Their biocidal activity has been previously demonstrated to be parabolically related to the length of the n-alkyl chain substituent (Hansch & Fujita 1964; Al-Taae & Gilbert 1986) and thus to a compound's lipophilicity. Maximal activity was demonstrated by compounds with n-alkyl chain lengths of between 16 and 18 (Hansch & Clayton 1973; Al-Taae & Gilbert 1986). Variation in the sensitivity of bacterial cells towards cetrimide (C_{16} TAB) has been previously reported (Klemperer et al 1980) and related not only to changes in the lipophilicity of the various envelope compartments but also to alterations in the acidic phospholipid content. If the biocidal activity of the compounds is reflected by alteration in the relative abundance of a common target, such as acidic phospholipid, then the biocidal activity of individual compounds of an homologous series ought to vary to a similar extent for the different homologues. On the other hand, if the biocidal activity change reflects exclusion of the biocide by the envelope, then the biocidal activity of the different homologues ought to have different dependencies upon envelope composition according to their own lipophilicities. This study assesses the changes in lipophilicity required for optimal envelope permeation, associated with alteration of the composition of the cell envelope by growth rate and nutrient limitation.

MATERIALS AND METHODS

Organisms and chemicals

Escherichia coli ATCC 8739 was used as it represents a non-capsulated strain and is one of the organisms used in the USP 'Antimicrobial agents effectiveness test'. Stock cultures were maintained on nutrient agar (Oxoid CM3) slopes at room temperature (20 °C) in the dark, after overnight incubation at 35 °C. n-Alkyltrimethylammonium bromides (C_nTAB) were obtained from the Sigma Chemical Co. for n-alkyl chain lengths of C = 12, 14 and 16 and synthesized from trimethylamine and octadecylbromide for C = 18. Purity, as checked by NMR, chemical ionization mass spectrometry and elemental analysis, was in all cases better than 99%. Phospholipid standards were obtained from Lipid Products Ltd, South Nutfield, Surrey, UK. All other reagents were of the purest available grade and obtained from BDH Ltd, London, UK.

Preparation of cell suspensions

Chemically defined liquid media, based on those described by Al-Hiti & Gilbert (1980), were used. In these, the growth of the cultures ultimately ceased at an optical density (E_{470}) of 1·0, as a result of depletion of glycerol (C-lim), magnesium (M-lim), $(NH_4)_2SO_4$ (N-lim) or phosphate (P-lim). All other nutrients ($(NH_4)_2Fe(SO_4)_2$, NaCl, and KCl) were present in excess and the media were buffered to pH 7·5 with 3-(*N*-morpholino)propane sulphonic acid ($2{\cdot}5 \times 10^{-2}$ M).

Cultures were grown in small volume (50 mL) glass fermenters (Gilbert & Stuart 1977), at a variety of growth rates, corresponding to dilution rates of 0·05–0·6 h^{-1}, using the methods described by Gilbert & Brown (1978).

Cells were harvested by centrifugation at 35 °C (15 000*g*, 15 min), washed three times and finally resuspended to an optical density (E_{470}) of 1·0 in sterile distilled water. Alternatively, cell pellets were collected and freeze-dried before chemical analysis.

Drug sensitivity

The bactericidal activity of C_{12}, C_{14}, C_{16} and C_{18}TAB was assessed individually against washed suspensions of cells prepared at six separate growth rates under C-lim, N-lim, M-lim and P-lim. Aliquots of freshly prepared cell suspension were held in 5 mL Pyrex tubes, maintained at 35 °C, to which were added various concentrations of C_nTAB (1 mL). At 15 min intervals, 0·1 mL aliquots were withdrawn and added to a lecithin/Tween 80 neutralizer mixture (3% w/v lecithin, 2% w/v Tween 80, 0·9 mL). Further dilutions were prepared in distilled water and appropriate 0·1 mL amounts plated onto the surfaces of triplicate predried nutrient agar plates which were incubated at 35 °C for 16 h; viable counts were then made. All experiments were in duplicate on at least two separate occasions. Results were expressed as mean percentages relative to untreated control suspensions. Preliminary experiments indicated that the procedure enabled the effective recovery of small numbers of microorganisms and efficiently neutralized the bactericidal activity of the biocides.

Cell envelope composition

Total readily extractable lipid (REL), phospholipid (PL), fatty and neutral lipid (FAN) and lipopolysaccharide (LPS) content of the various cell suspensions was assessed quantitatively in freeze-dried samples of cells using the methods described by Gilbert & Brown (1978). Protein composition of envelope and

outer-membrane fractions was examined by SDS-PAGE using the methods described by Filip et al (1973) and Williams et al (1984).

RESULTS AND DISCUSSION

Time-survival data of the bactericidal activity of the various C_nTABs, against washed suspensions prepared from nutrient broth (Oxoid CM1)-grown cultures, showed initial pseudo-first order kinetic reductions in viable count which reached a plateau to give a steady-state viability after 30 min contact. Inactivation rate constants for these initial periods gave concentration exponents for the compounds of about 1·0. The volume of experimental work entailed in this study prevented isotoxic concentrations of each agent being determined for each set of conditions. Concentrations which gave steady-state 90% reductions in viability after 30 min contact with biocide, for nutrient broth-grown suspensions, were therefore calculated (C_{12}TAB, 26 mM; C_{14}TAB, 12 mM; C_{16}TAB, 4·1 mM, C_{18}TAB, 3·8 mM) and used in subsequent experiments to assess the sensitivity of the variously grown chemostat cultures. Activities are presented as steady-state viabilities (30 min) following contact with the biocides.

Figs 1 and 2 show the effects of n-alkyl chain length and specific growth rate upon the activity of the compounds towards cell suspensions prepared under four nutrient limitations. Fig. 3 summarizes the effects of nutrient limitation and growth rate upon the activity of cetrimide USP, (C_{16}TAB). In all instances, steady state viability increased as growth rate was increased from 0·05 to about 0·2 h^{-1}, reached a maximum over a narrow growth rate band, then decreased as growth rate increased further.

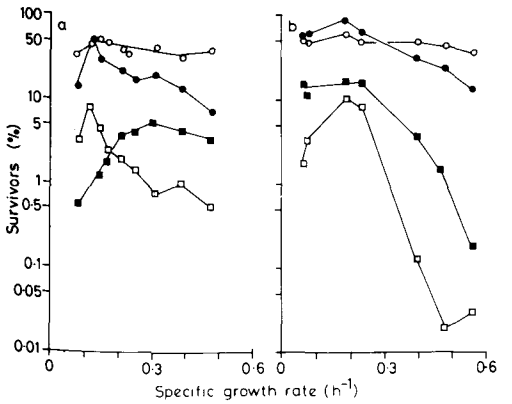

FIG. 1. Steady-state viability (30 min), following exposure to C_{12}TAB (26 mM, ○), C_{14}TAB (12 mM, ●), C_{16}TAB (4·1 mM, □) and C_{18}TAB (3·8 mM, ■), of *E. coli*, previously grown in a chemostat under (a) magnesium- and (b) nitrogen-limitation at various specific growth rates.

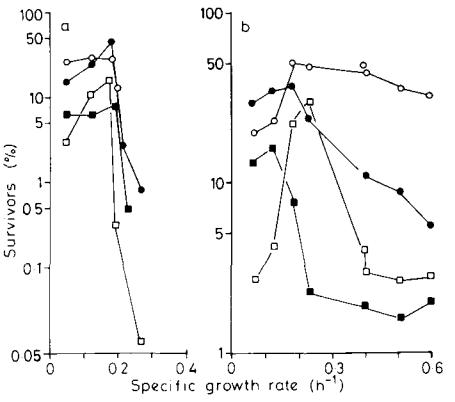

FIG. 2. Steady-state viability (30 min), following exposure to C_{12}TAB (26 mM, ○), C_{14}TAB (12 mM, ●), C_{16}TAB (4·1 mM, □) and C_{18}TAB (3·8 mM, ■), of *E. coli*, previously grown in a chemostat under (a) phosphate- and (b) carbon-limitation at various specific growth rates.

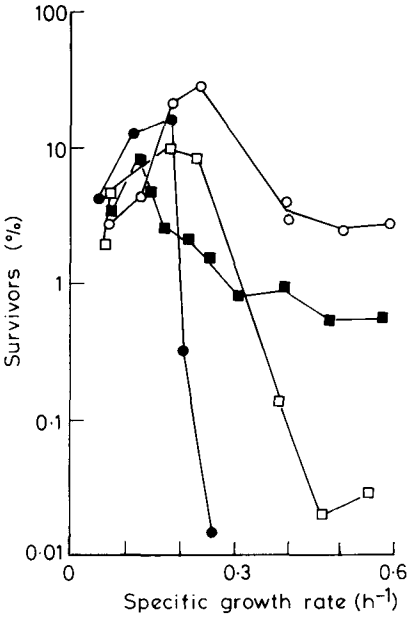

FIG. 3. Steady-state viability (30 min), following exposure to C_{16}TAB (4·1 mM), of *E. coli*, previously grown in a chemostat under (■), magnesium-, (○) carbon-, (●) phosphate- and (□) nitrogen-limitation at various specific growth rates.

Such increases in steady-state viability indicate an increase in the resistance of the test strain. The compounds studied represent one side of a parabolic relationship between log P and biological activity where, for nutrient broth grown-cells, activity increased with increasing n-alkyl chain length to a maximum for C_{16}TAB. The decreased activity of all the compounds over the slow growth rates (0·05–0·2

h^{-1}) suggests an overall increase in envelope lipophilicity followed by a steady decrease with growth rate increase. The effects of changes in envelope lipophilicity upon biocidal activity would be expected to be greatest for those compounds with log P closest to log P_o (C_{16}TAB). This is compatible with the observation that dependence of activity upon growth rate was greatest for the higher n-alkyl chain length compounds. C-lim cultures were generally the most resistant to the agents and showed a 10-fold variation in sensitivity over the range of growth rates tested. Cells grown under P-lim were the most sensitive and demonstrated an approximate 1000-fold change in sensitivity over the range of growth rate studied.

Analysis of gross cellular composition revealed that whilst REL remained relatively constant with changes in growth rate (11·4 ± 0·76% w/w), the amounts of FAN generally increased with increasing growth rate, while PL decreased (Fig. 4). This was

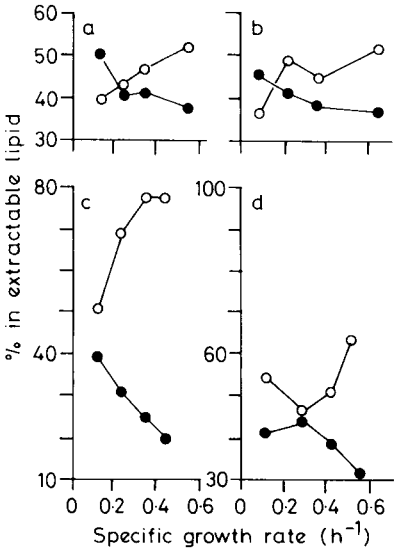

FIG. 4. Interrelation of phospholipid (solid symbols) and fatty and neutral lipid (open symbols) content of *E. coli* cells grown in a chemostat under (a) carbon-, (b) nitrogen-, (c) phosphate- and (d) magnesium-limitation at a variety of specific growth rates.

particularly marked for those cells grown under P-lim and might account for the unusually high dependence of their sensitivity upon growth rate. Changes in abundance of the major phospholipids (phosphatidylethanolamine, PE; phosphatidylglycerol, PG and diphosphatidylglycerol, DPG) is illustrated in Fig. 5. The amounts of all these components relative to one another remained constant, except

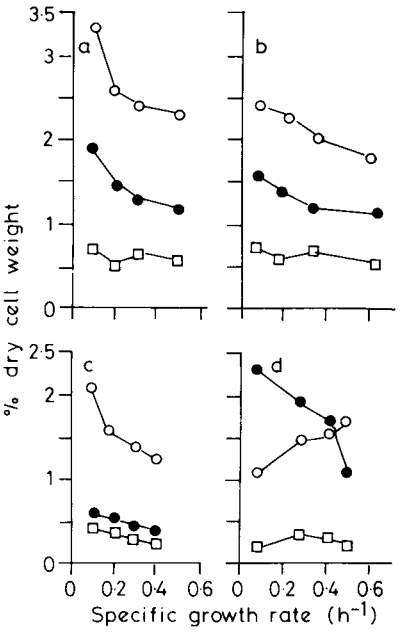

FIG. 5. Interrelation of phosphatidylglycerol (●), phosphatidylethanolamine (○) and diphosphatidylglycerol (□) content of *E. coli* cells grown in a chemostat under conditions of (a) carbon-, (b) nitrogen-, (c) phosphate- and (d) magnesium-limitation.

under conditions of M-lim when PE:PG ratios increased markedly with increasing growth rate. Such changes in the composition of cell-envelope phospholipid accompanying changes in growth rate and nutrient limitation are well documented (Melling et al 1974; Gilbert & Brown 1978; Klemperer et al 1980), and relate to adaptation of the cells to particular nutrient deficiencies and growth rates. In the present study there was no apparent relationship between individual phospholipids or groups of phospholipids and sensitivity towards the test agents.

Nutrient limitation had little effect upon the LPS content of the cells at slow rates of growth, as indicated by the presence of 2-keto-3-deoxyoctonic acid (KDO), but became significant as growth rate was increased (Fig. 6). For C-lim, M-lim and N-lim cultures the KDO content reached a maximum at specific growth rates of 0·2 h^{-1} (ca 14–17 μg mg^{-1} dry wt) with decreases to ca 12 μg mg^{-1} dry wt as growth rate was further increased. P-lim cultures, on the other hand, showed a more or less continual trend of decreasing KDO with increased growth rate. LPS gave the best single correlation with sensitivity to C_nTAB, the increased resistance towards the biocides being demonstrated by those

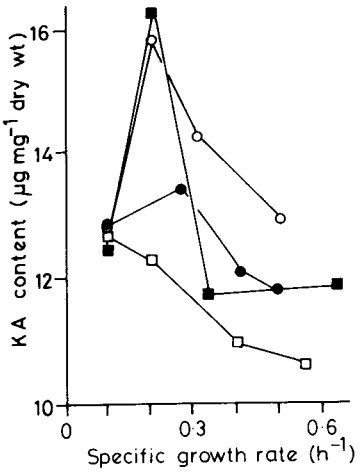

FIG. 6. Variation in the amounts of LPS, as indicated by ketodeoxyoctonic acid level, with growth rate and nutrient limitation, (■) nitrogen-, (○) carbon-, (□) phosphate- and (●) magnesium-limitation.

cells having increased levels of KDO. This is a similar observation to that for *Pseudomonas aeruginosa* (Gilbert & Brown 1978); for this organism it was demonstrated that the LPS acted as an exclusion barrier preventing both binding and penetration of phenolics. Similar barrier properties for LPS towards hydrophobic antibiotics have been reported (Lien et al 1968; Pechey et al 1974; Roantree et al 1977). Greater dependence of biocidal activity upon growth rate for P-lim than for other nutrient limitations cannot be explained by the LPS content alone, it is more likely that a combination of change in LPS and acidic : neutral phospholipid ratio is involved (Tuomanen et al 1986).

SDS-PAGE profiles of the major outer membrane proteins showed little variation with growth rate. Under C-lim, however, the 17·5 kDal protein was repressed at slowed growth-rates. A 50 kDal protein was repressed at all growth rates under N-lim and M-lim but not those under C-lim or P-lim, whilst a 21 kDal protein H1, associated with polymyxin resistance (Nicas & Hancock 1980), was greatly increased under P-lim and M-lim. No relationship between outer-membrane protein composition and drug sensitivity was apparent in the present study.

The results of this study therefore support the hypothesis that growth rate and nutrient limitation alter the overall lipophilicity of the cell-envelope and thereby the optimal value of Log P for compounds to traverse it. Additionally, some involvement of the acidic : neutral phospholipid ratio is implicated for these agents.

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