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Uptake and distribution of some isothiazolone biocides into *Escherichia coli* ATCC 8739 and *Schizosaccharomyces pombe* NCYC 1354

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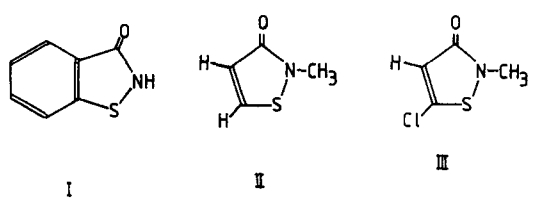
Summary

Uptake onto and distribution within *Escherichia coli* and *Shizosaccharomyces pombe* of three ^{14}C-radiolabelled isothiazolone biocides, benzisothiazolone (BIT), *N*-methylisothiazolone (MIT) and 5-Cl-*N*-methylisothiazolone (CMIT) has been investigated. 'C-type' adsorption was observed in all cases at low concentrations of biocide. For *E. coli* suspensions, saturation of binding sites was indicated by S/L-type adsorption patterns at elevated biocide levels. Levels of uptake by the yeast suspension were twice that by *E. coli* and 4–5 times greater for CMIT than for BIT or MIT. Of the absorbed biocide, greater fractions of CMIT (4%), than MIT or BIT (2%) were associated with the yeast envelope. Similar fractions of all three biocides were associated with Gram-negative envelopes (6–8%). Autoradiographs of envelope and cytosolic fractions taken from biocide-treated suspensions failed to demonstrate specific target proteins, rather uniform, low level binding was associated with all of the detectable proteins.

Introduction

Isothiazolone biocides such as benzisothiazolone (BIT, **I**), *N*-methylisothiazolone (MIT, **II**) and 5-chloro-*N*-methylisothiazolone (CMIT, **III**) are widely used as industrial biocides (Singer, 1976; Andrykovitch and Neihof, 1987). Mixtures of CMIT and MIT (Kathon, Rohm and Haas Inc.) are also used in the preservation of cosmetic products (Zeelie and McCarthy, 1983; Law et al., 1984, 1987). BIT is a skin sensitising agent, a property which prevents its use in pharmaceutical, cosmetic and toiletry preparations. CMIT, a chemically related compound, lacks such sensitising properties and is now widely used in this range of products. This is in spite of its action as a primary skin irritant (>25 μg/ml; Weaver et al., 1985) and its Ames positivity (Monte et al., 1983). Concentrations employed within topical products are below those



I II III

Scheme 1.

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likely to act as primary skin irritants, observation of mutagenicity (Ames test) however is indicative of potential carcinogenic properties which would prevail at all concentrations.

The antimicrobial activity of all three agents is strongly antagonised by exogenous thiol-containing agents. The biocides are thought to interact oxidatively with accessible thiols such as glutathione, within the cell (Fuller et al., 1985; Collier et al., 1990a). Whilst thiol interactive agents are not noted for their biocidal properties, CMIT, but not BIT or MIT has been observed to be potently fungicidal towards *Shizosaccharomyces pombe*. None of these agents possess significant bactericidal property (Collier et al., 1990a). Recent studies (Collier et al., 1990a) show the activity of CMIT, but not that of BIT or MIT to be neutralised by the presence of histidine and valine. Patterns of growth inhibition for CMIT and morphological changes associated with its action are reminiscent of inhibition of initiation of DNA replication.

At physiological pH, MIT, BIT and CMIT interact oxidatively with most thiol-containing compounds (e.g. cysteine, glutathione) to form disulphide adjuncts. Further interaction with thiols causes the release of oxidised thiol dimers (e.g. cysteine, glutathione disulphide) and reduced, ring-opened forms of the biocides (mercaptoacrylamide). In an analogous fashion to the initial reaction, the mercaptoacrylamides are able to react with further molecules of biocide to give biocide dimers (Collier et al., 1990b). NMR spectral studies indicate that for CMIT, the mercaptoacrylamide will tautomerise to give a highly reactive thioacyl chloride, capable of rapid reaction, not only with thiols, but also with amines and water. Such reactivity probably accounts for much of the enhanced activity of CMIT and CMIT-containing products such as Kathon (Collier et al., 1990b). What is not explained by such reactions are the especial differences in bactericidal and fungicidal property of the two groups of compound. This might relate to physiological differences between prokaryotic and eukaryotic cells or to the structure/function of their cell envelopes which would contribute towards accessibility of the agents to cytosolic targets. In this respect, initial interactions between drugs and target cells are adsorptive. Since drug action at the immediate, interactive surface of cells is rare, adsorption is inevitably followed by absorption and diffusion to other sites. For thiol-interactive agents the major interactive sites are within the cytosol (e.g. glutathione, cysteine etc.), with relatively little thiol being situated at the cell surface/membrane. In prokaryotic cells many important cellular functions are located at their cell envelope which is enriched, relative to eukaryotic cells, in thiol groups. Formation of a thio-acyl chloride from CMIT might increase reactivity at the cell envelope for bacterial cells and enhance the biological effect. For BIT the reversibility of thiol-adjunct formation would favour partitioning of the agent to the cytosol. The primary objective of the present study was to examine the binding to and distribution within *Escherichia coli* and *S. pombe* cells of the three isothiazolone biocides. Results from such a study might further elucidate the differences in biological properties between these otherwise closely related compounds, particularly in the light of the suggested toxicity of the chlorinated compounds.

Materials and Methods

Organisms and chemicals

E. coli ATCC 8739 and *S. pombe* NCYC 1354 were obtained from the respective culture collections and maintained on Nutrient agar (Oxoid CM3) and Sabouraud Dextrose agar (Oxoid CM41) slopes respectively, at room temperature in a darkened cupboard. *E. coli* and *S. pombe* were incubated throughout the study at 37 and 30°C, respectively.

5-Chloroisothiazol-3-one (CIT), isothiazol-3-one (IT), *N*-methylisothiazolone (MIT) and 5-Cl-*N*-methylisothiazolone (CMIT) were synthesised according to the methods described by Collier et al. (1990a). Uniformly ^{14}C-radiolabelled 1,2-benzisothiazol-3-one (BIT; 500 μCi/g) and unlabelled BIT were the kind gifts of ICI plc (Organics Division, Manchester). ^{14}C-radiolabelled methyl iodide was obtained from Amersham International (Bucks, U.K.). All other reagents were obtai-

ned from either Sigma (Poole, U.K.) or BDH (Poole, U.K.).

Preparation of [^{14}C]methylisothiazolones

Equimolar amounts of CIT (0.0224 g), potassium carbonate (0.0224 g) and [^{14}C]methyl iodide (0.03 g, 200 μCi/g) were dissolved in 5 ml acetone contained in a round bottomed flask and stirred constantly in a water bath at 40°C for 16 h. The presence of a single product, 5-chloro-*N*-methylisothiazolone (CMIT) was confirmed in the reaction mixture by TLC. CMIT was extracted twice with chloroform and the extracts subjected to flash chromatography utilising a petroleum ether (40–60°C):chloroform dilution range. Flash chromatographic fractions were tested for the presence of CMIT by TLC and HPLC. Positive fractions were bulked and dried, by rotary evaporation, to produce an oily deposit. Addition of HCl (2 N) facilitated crystallisation. Crystals were washed, dried and submitted to spectroscopic analysis.

Identical procedures were employed for the synthesis of radiolabelled MIT except that the reaction was scaled up by a factor of 10 and IT was used in place of CIT.

Preparation of washed cell suspensions

Overnight, liquid cultures were prepared in chemically defined liquid media as described by Gilbert and Brown (1978) for *E. coli* and in Sabouraud Liquid Medium (Oxoid CM 147) for *S. pombe*. These were incubated in a shaking incubator (150 oscillations/min) at 35 and 30°C, respectively. Cells were harvested, in mid-log phase of growth, by centrifugation (4000 × *g*, 10 min) at room temperature, washed twice and resuspended in sterile phosphate buffer (0.05 mmol/l, pH 7.0) to an appropriate absorbance.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cultures of *S. pombe* and *E. coli* were centrifuged (10000 × *g*, room temperature, 15 min) and supernatants discarded. Cell pellets were resuspended in phosphate buffer (Sørensens, 0.1 M, pH 7.0) and disrupted by sonication (Soniprep 150, MSE, Crawley, Sussex) for 90 s total time (*E. coli*) or 10 min total time (*S. pombe*). Disrupted cell suspensions were centrifuged (4000 × *g*, 4°C, 10 min) and unbroken cells discarded (pellet). Cell-envelopes and cytosol (supernatant) were further centrifuged (220000 × *g*, 4°C, 60 min; Sorvall OTD 65 B ultracentrifuge). Cytosol (supernatant) and cell envelopes (pellet) were separated and stored (−25°C). Pellets were resuspended in sarkosyl (2.5%, 2 ml) and incubated at 35°C for 30 min. This procedure solubilises inner membranes which may then be removed as supernatants after ultracentrifugation (220000 × *g*, 4°C, 60 min). The remaining pellets (outer membrane/wall) were resuspended in buffer (1 ml). All samples were stored at −25°C. Wall and envelope fractions were separated by SDS-PAGE according to the methods of Smith (1984).

Uptake and distribution of radiolabelled isothiazolones within E. coli and S. pombe

^{14}C-radiolabelled isothiazolones (0.1 ml; 1.0 μCi) were added to volumes (10 ml) of washed cell suspensions (1.5×10^{10} cells/ml, *E. coli*; 1.5×10^{7}, *S. pombe* in 50 mM phosphate buffer, pH 7.0) held in test tubes at 30 or 37°C in a shaking water bath. After 10 min cells were removed by centrifugation (10000 × *g*, 15 min, room temperature) and duplicate samples (0.2 ml) of the supernatants transferred to plastic scintillation vials. Preliminary experiments had shown that 10 min incubation was sufficient to allow for equilibration of drug solution and uptake. Cell pellets were resuspended (10 ml, 50 mM phosphate buffer, pH 7.0) and aliquots (0.2 ml) removed to scintillation vials. The remaining cell suspensions were sonicated on ice (90 s for *E. coli*; 10 min for *S. pombe*) in order to disrupt the cells and the sonicates centrifuged (4000 × *g*, 10 min, 4°C) to remove unbroken cells. Duplicate samples (0.2 ml) were removed from these supernatants and transferred to scintillation vials. Pellets, containing cell debris, were resuspended in 1 ml of water and duplicate samples (0.2 ml) removed to scintillation vials. The supernatants were further centrifuged (220000 × *g*, 1 h, 4°C), to separate the envelope and wall material (pellet) from the cytosol (supernatant), and the cytosolic supernatant fractions were sampled (0.2 ml) into scintillation vials as was the envelope pellet, after resuspension (1 ml, 50 mM phosphate

buffer, pH 7.0). Each of the scintillation vials was filled with Optiphase 'HiSafe' liquid scintillation fluid (4 ml; Fisons), the contents mixed and ^{14}C counts determined by liquid scintillation counting using a 1218 Rackbeta liquid scintillation counter (LKB, Wallac). The counts for each duplicate set of samples were averaged as disintegrations/minute (dpm) and related to drug concentration. Applied and equilibrium drug concentrations could be determined, as could the proportionate uptake by envelope and cytosol.

Results and Discussion

Rates of uptake and times of equilibration for the absorption of ^{14}C radiolabelled isothiazolones by *E. coli* and *S. pombe*, were determined. Steady-state absorption was obtained for all the biocides within three minutes. Rates of uptake into porin-deficient strains of *E. coli* were found to be not significantly different from those by the porin-sufficient *E. coli* strains (Fig. 1). This indicated that the absence of the porin transport route across the outer membrane did not influence either the rate of absorption or the overall level of isothiazolone uptake. Subsequent experiments utilised a fixed contact time of 10 min and related uptake at equilibrium, to the applied biocide concentration (Giles et al., 1974a,b). *S. pombe* suspensions exhibited absorption patterns for BIT, MIT and CMIT (Figs 2a,b and 3b) which approximated to C-type isotherms (Giles et al., 1974a,b). C-type adsorption/absorption suggests that uptake

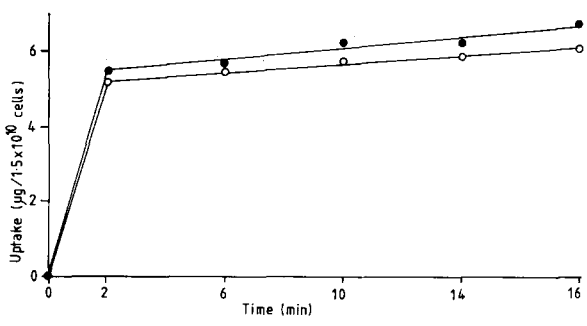

Fig. 1. Uptake of ^{14}C-radiolabelled BIT over time by *E. coli* ATCC 8739 (•) and a porin-deficient strain of *E. coli* (○).

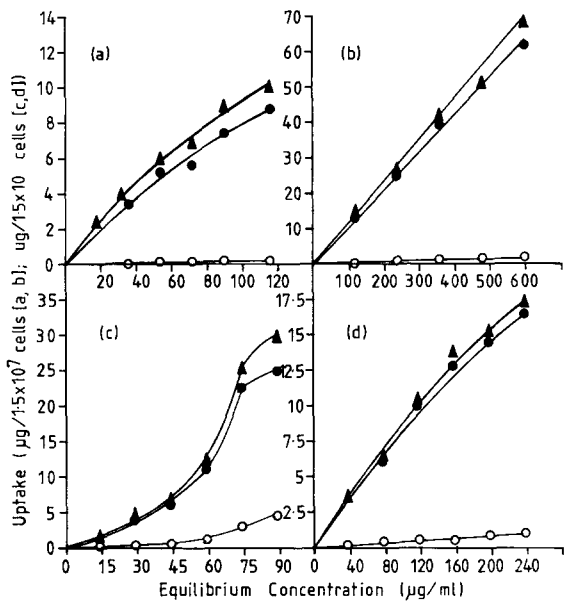

Fig. 2. Uptake isotherms of MIT (b, d) and BIT (a, c) by washed whole cell suspensions of *E. coli* (c, d) (1.5×10^{10} cells/ml) and *S. pombe* (a, b) (1.5×10^7 cells/ml), both in phosphate buffer (pH 7.5, 50 mM). Total uptake (▲), cytosol (•) and cell envelope (○).

of the isothiazolones by the yeast is directly proportional to the drug concentration applied and related to general partitioning into various compartments of the cell. Relative uptake of the biocides corresponded to 11% (MIT and BIT) and 35% (CMIT) of the applied concentration. Of such absorbed material, 97% was located within the cytosol and 3% within the cell envelope. Isotherms for absorption into the envelope and cytosol fractions (Figs 2a,b and 3b) demonstrated C-type uptake once again.

Patterns of absorption by *E. coli* cell suspensions, on the other hand, exhibited S-type uptake isotherms for BIT (Fig. 2c), possible S-type/L-type for MIT (Fig. 2d) and C-type for CMIT (Fig. 3a). S/L-isotherms are indicative of facilitated uptake (Giles et al., 1974a,b) and suggest binding of the unchlorinated isothiazolones to *E. coli* to be monopolar and to facilitate the binding of subsequent drug molecules. Such an adsorptive mechanism is compatible with our knowledge of the chemical reactivity of these drugs (Collier et al., 1990b), in that they react initially with sulphydryl

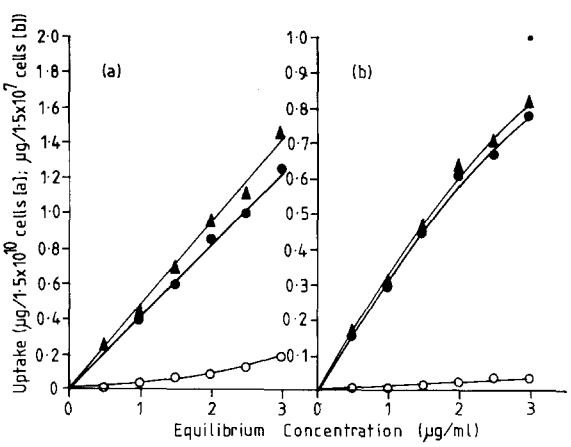

Fig. 3. Uptake isotherms of CMIT by washed whole cell suspensions of (a) *E. coli* (1.5×10^{10} cells/ml) and (b) *S. pombe* (1.5×10^7 cells/ml), both in phosphate buffer (pH 7.5, 50 mM). Total uptake (▲), cytosol (●) and cell envelope (○).

groups to form mixed disulphides which may further react, either with incoming biocide or with further sulphydryl groups. The initial drug/cell surface interactions of BIT and MIT with *E. coli* might therefore, be mixed disulphide formation with thiol containing envelope targets, such as the outer membrane proteins. Mid-phase levels of uptake from solvent corresponded to 7 and 5% of the applied BIT and MIT, respectively. Of this, 11% (BIT) and 5% (MIT) were associated with cell envelope. Greater association with the cell envelope of *E. coli* than *S. pombe* might relate to the absence of an outer membrane in *S. pombe* and hence reduced sulphydryl content. The presence of thiol-containing outer membrane proteins in *E. coli* may result in enhanced binding of the isothiazolones to these cells and a resultant S-type adsorption pattern.

Uptake isotherms for CMIT by *E. coli* suspensions (Fig. 3a) on the other hand showed C-type adsorption patterns. CMIT has been suggested to react initially with sulphydryl groups to form mixed disulphides (mercaptoacrylamide) which on further interaction with either biocide or additional thiol liberate thioacyl chloride tautomers (TAC). These are potent acylating agents and especially reactive. Formation of the TAC within the envelope will cause it to be irreversibly bound within the envelope through non-specific secondary reactions (Collier et al., 1990b). Such reaction reduces the probability that biocide will react with bound biocide. Differences in uptake pattern between the yeast and *E. coli* might therefore reflect differences in envelope content. Such a hypothesis was given support through analysis of binding proteins in the *E. coli* suspensions. Proteins from the cytosol and inner and outer membranes of [^{14}C]CMIT treated *E. coli* were separated by SDS-PAGE and examined by a variety of techniques, including linear-plate analysis and liquid scintillation counting. No indications were found that any particular protein or group of proteins bound CMIT more tightly than others. A generalised pattern of non-specific binding in the envelope was apparent for all three biocides.

In conclusion, no specific binding proteins were observed for any of the compounds. Since they react with thiols and since the binding distributions between envelopes and cytosol differed for chlorinated and non-chlorinated compounds then secondary, non-thiol interactions must be occurring for CMIT and its adjuncts within the cell envelope. For the bacteria such interactions will have greater consequences to the physiology of the cell since many essential functions are membrane and envelope associated. Thus, CMIT, whilst not being bactericidal, is several orders of magnitude greater in growth inhibitory activity towards bacteria than BIT or MIT (Collier et al., 1990a). These differences in activity are much reduced with respect to antifungal activities. Such data are compatible with the proposal of thio-acyl chloride formation through CMIT/thiol interactions (Collier et al., 1990a,b) being associated with the particular toxicological properties of CMIT.

Possible toxicological problems associated with the use of chlorinated isothiazolones as preservatives of topical products have been long recognised (Zeelie and McCarthy, 1983; Weaver et al., 1985) but largely overlooked through a general lack of understanding of the mechanisms involved. The present study together with those of Collier et al. (1990a,b) present a consistent and plausible explanation of both the mode of action and toxicity profiles demonstrated within this group.

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