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The effect of fifteen biocides on formaldehyde-resistant strains of *Pseudomonas aeruginosa*

M. Sondossi[a], H.W. Rossmoore[a,*] and J.W. Wireman[a,b]

[a]*Department of Biological Sciences, Wayne State University, Detroit MI 48202,* and [b]*Biosan Laboratories, Inc., Ferndale, MI 48220, U.S.A.*

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SUMMARY

Evaluation of formaldehyde and fifteen biocides in formaldehyde sensitive (S) and resistant (R) strains of *Pseudomonas aeruginosa* revealed a pattern of response that allowed a comparison of the mode of action of these biocides. The response of these strains to the various biocides, as well as the induction of transient resistance or cross-resistance in the (S) strain, allowed a grouping of biocides based on this pattern of response. Group 1 biocides acted in a manner indistinguishable from formaldehyde for both the (S) and (R) strains. Group 2 biocides were not effective against either the (S) or (R) strains at concentrations calculated to release equimolar concentrations of formaldehyde. However, treatment of the (S) strain with formaldehyde or Group 2 biocides resulted in the development of cross-resistance. Group 3 biocides were equally effective against the (S) and (R) strain, but the (S) strain survivors of treatment with Group 3 biocides were resistant to formaldehyde. Group 4 biocides (controls) had no presumed connection to formaldehyde mode of action. These four groupings, based on pattern of response, also resulted in groupings of biocides based on chemical structure.

INTRODUCTION

The relationship between formaldehyde and a formaldehyde condensate biocide 1,3,5-tris-(hydroxyethyl)hexahydro-*s*-triazine, was confirmed earlier [10], demonstrating that the same metabolic intermediate neutralized the activity of both antimicrobials. In more recent studies, resistance and sensitivity to 1,3,5-tris-(ethyl)hexahydro-*s*-triazine (ET) [17,18] was noted to be a quantitative function of the formaldehyde involved in its synthesis. Resistance was of two types: a transient-induced, and a more permanent-selected variant; both were coupled to an increase in formaldehyde dehydrogenase activity. It appears that the activity of at least the hydroxyethyl- and the ethyltriazine biocides are qualitatively and quantitatively based on their formaldehyde content. However, there is a wide variety of biocides on the market, all formaldehyde adducts, many being referred to as formaldehyde releasers [2] but others not [20].

In this study, the role of formaldehyde in the

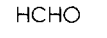
* To whom correspondence should be addressed

Table 1

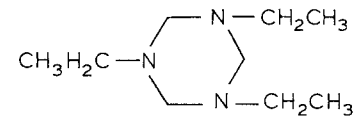
Structural formulae of biocides tested

Group 1 biocides

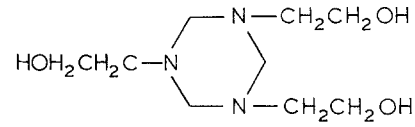
1. Formaldehyde



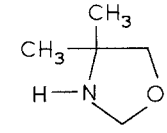
2. 1, 3, 5—tris (ethyl) Hexahydro-S-triazine



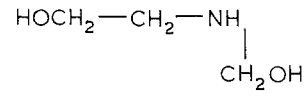
3. 1, 3, 5—tris (2-hydroxyethyl) Hexahydro-S-triazine



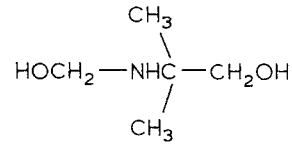
4. 4, 4—Dimethyloxazolidine one of two oxazolidines in commercial mixture



5. 2(Hydroxymethyl) aminoethanol

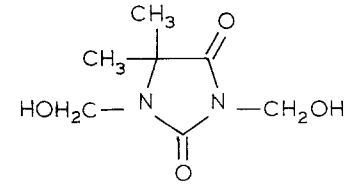


6. 2 (Hydroxymethyl) amino-2-methyl propanol

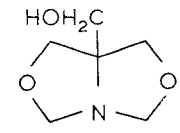


7. A commercial mixture of 3 and 16

8. 1, 3 (Dihydroxymethyl)-5, 5-dimethylhydantoin

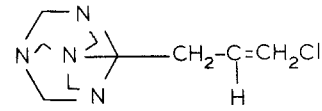


9. 5-Hydroxymethyl-1-AZA-3, 7-dioxabicyclo (3.3.0.) octane one of three cyclo-octanes in commercial mixture

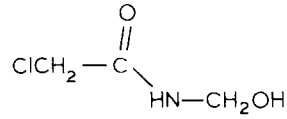


Group 2 biocides

10. 1-(3-Chloroallyl)-3,5-triaza-1-azoniaadamantane

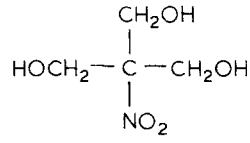


11. N-Methylolchloroacetamide

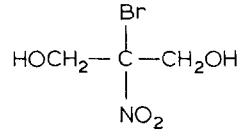


Group 3 biocides

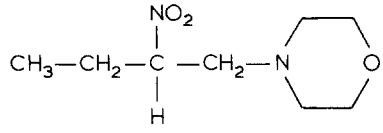
12. Tris(hydroxymethyl) nitromethane



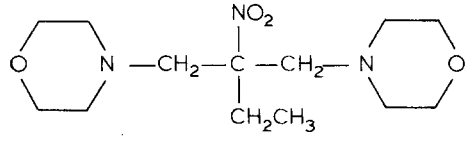
13. 2-Bromo-2-nitro-1,3-propanediol



14. 4-(2-Nitrobutyl) morpholine

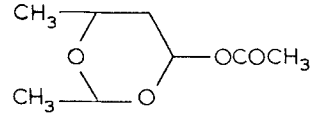


4,4'(2-Ethyl-2-nitrotrimethylene)dimorpholine

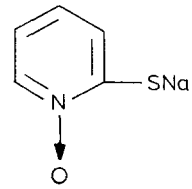


Group 4 biocides

15. 6-Acetoxyl-2,4-dimethyl-m-dioxane



16. Sodium 2-pyridinethiol-1-oxide



activity spectrum of biocides synthesized with formaldehyde was examined. It should be emphasized that minimal inhibitory concentrations (MIC) or efficacy are frequently relative and practical terms based on the percentage of active compounds in a commercial mixture. In assessing the role of formaldehyde in the antimicrobial activity of a compound, it is more relevant to base comparisons of formaldehyde with the biocides on the percentage of those biocide molecules attributable to their formaldehyde-derived moieties. This is the approach that is followed in this study.

MATERIALS AND METHODS

Media and culture conditions

All cultures were maintained on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI), grown in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) 12 h, and transferred to fresh medium for 3–5 h prior to use to ensure that inocula were in exponential phase. Population levels of inocula were between 1 and $5 \cdot 10^7$ cfu/ml.

All experiments were carried out in 250-ml flasks with a total suspension volume of 100 ml (with the exception of inductions of the sensitive (S) strain with formaldehyde which were done in final volume of 1–2 l). Flasks were incubated at 30°C with rotary shaking at 200 rpm. Agar plates were incubated at 30°C for 48 h.

Organisms and selection of resistant strains

An isolate of *Pseudomonas aeruginosa* obtained from contaminated metalworking fluid [16] was the formaldehyde (S) strain in this study. MIC of formaldehyde by tube dilution was 5 mM in TSB for the (S) strain. This strain was used to compare the initial sensitivity of biocides with formaldehyde. Resistance of the (S) strain survivors increases after exposure to formaldehyde [17]. Cells pretreated with formaldehyde were used to evaluate cross-resistance development to other biocides. The (S) strain was also induced upon exposure to sublethal concentrations of other biocides and subsequently was tested for resistance development to formaldehyde. Sequential treatment of the (S) strain with increasing concentrations of ET resulted in selection of a stable formaldehyde-resistant (R) strain [18]. The MIC of formaldehyde for the (R) strain was 3 times greater than for the original strain.

Biocide selection and treatment

In the selection of biocides for this study, consideration was given to their method of synthesis, chemical structure, and binding site of reacted formaldehyde in the adduct. The names and structures of all biocides used are shown in Table 1. For more information and their trade names, refer to previous publications [11,12,15,20].

The number of formaldehyde molecules theoretically available in each biocide was estimated from stoichiometric synthetic data and chemical structures. Stock solution of biocides in distilled water (10% active ingredient, w/v) were used. The concentrations of biocides in the test system were calculated to give 3 mM of potential formaldehyde. Formalin (37.7% reagent grade sol.) was used to obtain 3 mM final concentration of formaldehyde.

Resistance induction

Induction of resistance of (S) strain to formaldehyde was carried out in flasks containing 1–2 l of suspension ($1–5 \cdot 10^7$ cfu/ml) with a final 3 mM formaldehyde concentration. Regrown populations from survivors of sublethal treatment (induction) were harvested by centrifugation [17] and resuspended in fresh TSB at $1–5 \cdot 10^7$ cfu/ml. The induced population was then challenged with various biocides, all equivalent to 3 mM formaldehyde. For example, the synthesis of ET involves 3 mol of formaldehyde, and it is expected to release 3 mol upon chemical hydrolysis; therefore, 1 mM of ET equals 3 mM of formaldehyde.

Alternatively, (S) strain resistance induction was done with 100 ml final volume in 250 ml flasks with biocides. Regrown populations were harvested, as described above, and challenged with 3 mM of formaldehyde.

Measurement of resistance

Resistance and sensitivity of populations were

determined by the change in number of cfu/ml with time. The number of cfu was determined on TSA plates after 48 h and rechecked for late colony formation after 5 days. All of the data presented have been replicated one or more times.

RESULTS

The data obtained in this study were used to group biocides according to the following parameters: (a) pattern of formaldehyde equivalence effect of biocide; (b) differential effect on the (S) and (R) strain; and (c) induction of resistance and cross-resistance of the (S) strain.

Group 1 biocides

These biocides (see Biocides 1–9 in Table 1) show antibacterial activity indistinguishable from formaldehyde when concentrations yielding equivalent formaldehyde levels were used (based on N-bound formaldehyde). The (S) strain was equally sensitive to the biocide and formaldehyde. The (R) strain was totally resistant to the same concentration of the biocides (Fig. 1A). When the (S) strain was induced with 3 mM of formaldehyde and transferred to the above concentrations of biocide (3 mM available formaldehyde), a decrease in sensitivity was clearly shown (Fig. 1B). Alternatively, induction with the biocides (3 mM available) also shows a decrease in sensitivity to formaldehyde

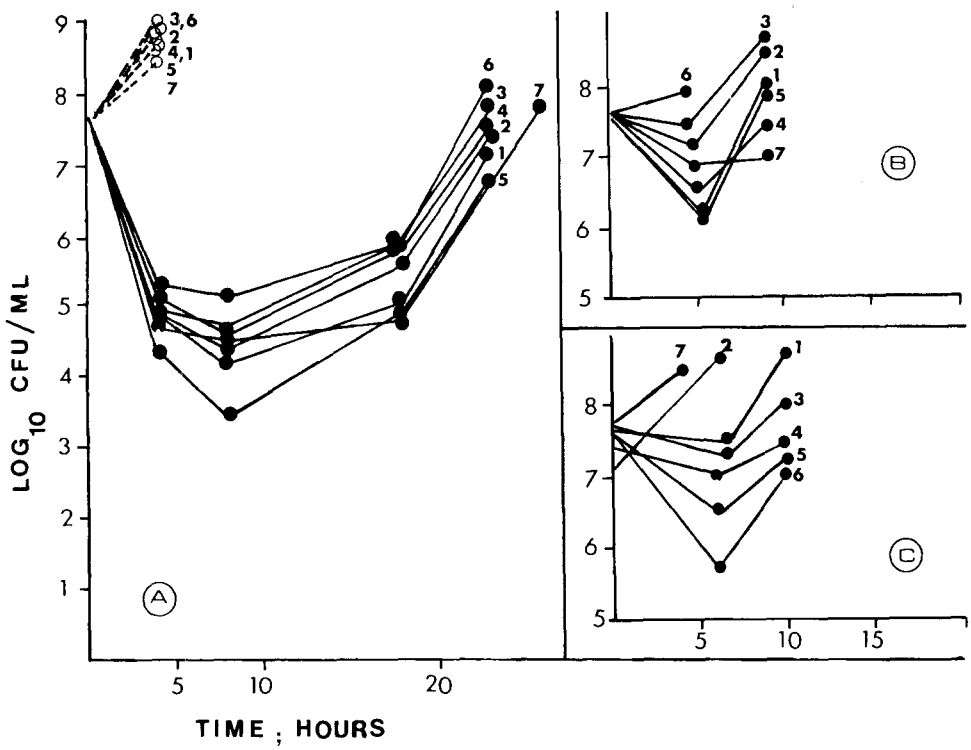


Fig. 1 (A) Exponentially growing sensitive (S) and resistant (R) strains of *Pseudomonas aeruginosa* were exposed to 3 mM of formaldehyde and equimolar concentration of biocides (3 mM available formaldehyde). 1, formaldehyde; 2, 1,3,5-tris-(ethyl)hexahydro-*s*-triazine; 3, 1,3,5-tris-(2-hydroxyethyl)hexahydro-*s*-triazine; 4, 4,4-dimethyloxazolidine and 3,4,4-trimethyloxazolidine, a commercial mixture; 5, 2-(hydroxymethyl)aminoethanol; 6, 2-(hydroxymethyl)amino-2-methyl propanol; 7, a commercial mixture of 3 and 16 (in Table 1); S, ●——●; R, O-----O. (B) The sensitive (S) strain was induced with 3 mM of formaldehyde. Then cells were exposed to equimolar concentrations (3 mM available formaldehyde) of biocides. (C) The sensitive (S) strain was induced with biocides (3 mM available formaldehyde), then exposed to 3 mM of formaldehyde.

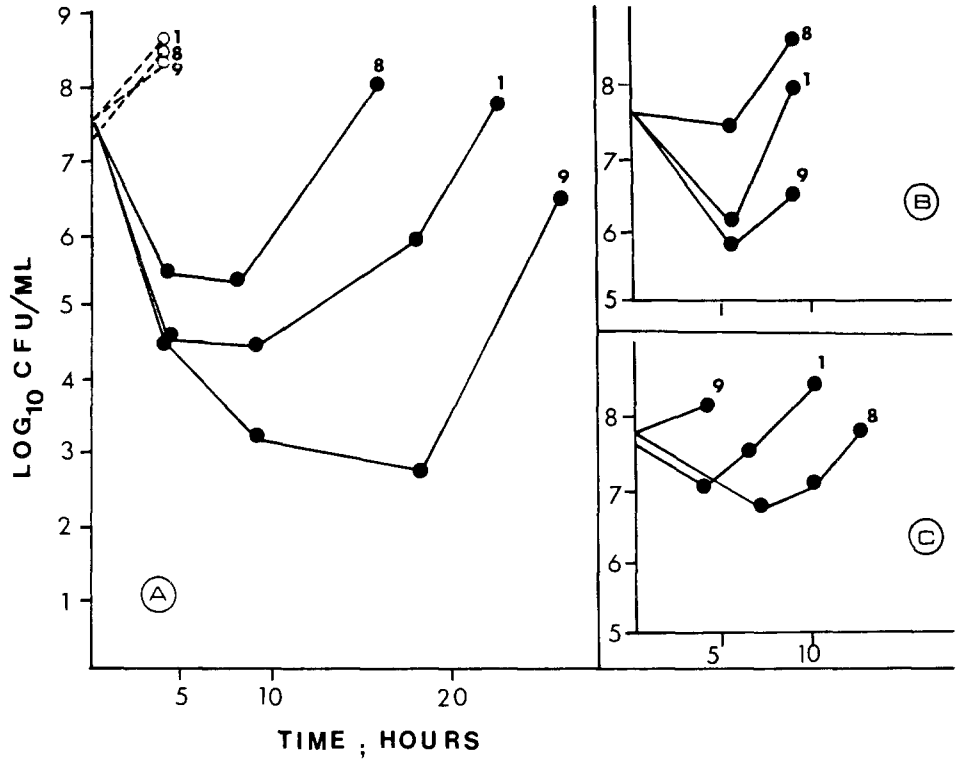


Fig. 2. (A) Exponentially growing sensitive (S) and resistant (R) strains of *Pseudomonas aeruginosa* were exposed to 3 mM of formaldehyde and calculated equimolar concentration of biocides (calculated 3 mM available formaldehyde); 1, formaldehyde; 8, 1,3-(dihydroxymethyl)-5,5-dimethyl hydantoin; 9, a commercial mixture of 5-hydroxymethoxymethyl-1-aza-3,7-dioxabicyclo(3.3.0.)octane, 5-hydroxymethyl-1-aza-3,7-dioxabicyclo(3.3.0.)octane and 5-hydroxypoly[methyleneoxy (C_2,C_3,C_4,C_5)] methyl-1-aza-3,7-dioxabicyclo(3.3.0.)octane; S, ●——●; R, O-----O. (B) The sensitive (S) strain was induced with 3 mM of formaldehyde. Then cells were exposed to equimolar concentrations (3 mM available formaldehyde) of biocides. (C) The sensitive (S) strain was induced with biocides (3 mM available formaldehyde), then exposed to 3 mM of formaldehyde.

(Fig. 1C). The behavior of Biocide 7 in Fig. 1 may be attributable to the presence of pyridinethion (Biocide 16) as part of a commercial mixture.

Two other biocides in this group (Biocides 8 and 9 in Table 1) showed significant differences in effectiveness versus the (S) strain when used at equimolar concentrations (calculated 3 mM available N-bound formaldehyde) (Fig. 2A). The effect of these two biocides on the (R) strain and the development of cross-resistance follow the same pattern as the other Group 1 biocides (Figs. 2B and 2C).

Group 2 biocides

These biocides (Biocides 10 and 11 in Table 1) were not effective against the (S) strain and do not mimic formaldehyde at 3 mM potentially available formaldehyde. This may be due to their degree of hydrolysis (amount of formaldehyde released) and/or interference from either non-formaldehyde additives present in high levels in both commercial products. In order to produce a lethal effect on the (S) strain, the concentration used was increased (Fig. 3A). Interestingly, the (R) strain was markedly more resistant at the same concentration used against the (S) strain (Fig. 3A). Furthermore, induction with formaldehyde and/or biocides resulted in the development of cross-resistance in the (S) strain (Figs. 3B and 3C). Thus, despite the lack of a response quantitatively similar to formaldehyde, there seems little doubt that formaldehyde is involved in the activity of Group 2 biocides.

Groups 3 and 4 biocides

Group 3 biocides (Biocides 12, 13 and 14 as shown in Table 1). Tris-(hydroxymethyl)nitromethane (Biocide 12) is described as a slow formaldehyde releaser and effective in metalworking fluids, and an analog, 2-bromo-2-nitro-1,3-propanediol (Biocide 13), is believed to have some activity due to formaldehyde release. Biocide 14 is synthesized with a mixture of nitropropane, morpholine, and formaldehyde, but it is not thought to be a formaldehyde-releasing compound. Therefore, the concentration used of this biocide was based on the amount formaldehyde used in its synthesis (3 mM formaldehyde equivalents).

Surprisingly, tris-(hydroxymethyl)nitromethane was totally ineffective at concentrations based on putative release of one, two or three molecules of formaldehyde. Furthermore, the failure of this biocide in the test system continued even at higher doses, 13.24 mM equal to 2000 ppm active ingredient (Fig. 4A); nevertheless, it did induce resistance in the (S) strain to 3 mM of formaldehyde (Fig. 4B).

The bromonitro analog was extremely effective on (S) and (R) strains (Fig. 4A). On the other hand, the morpholine-based biocide mixture was effective initially on both the (S) and (R) strains (Fig. 4A). When the (S) strain was treated with this biocide however, there was development of resistance to formaldehyde (Fig. 4B).

A fourth group of biocides (Biocides 15 and 16 in Table 1) with no presumed formaldehyde connection appeared to act totally different from formaldehyde and the formaldehyde condensate biocides, since the (S) and (R) strains are equally sensitive (Fig. 5).

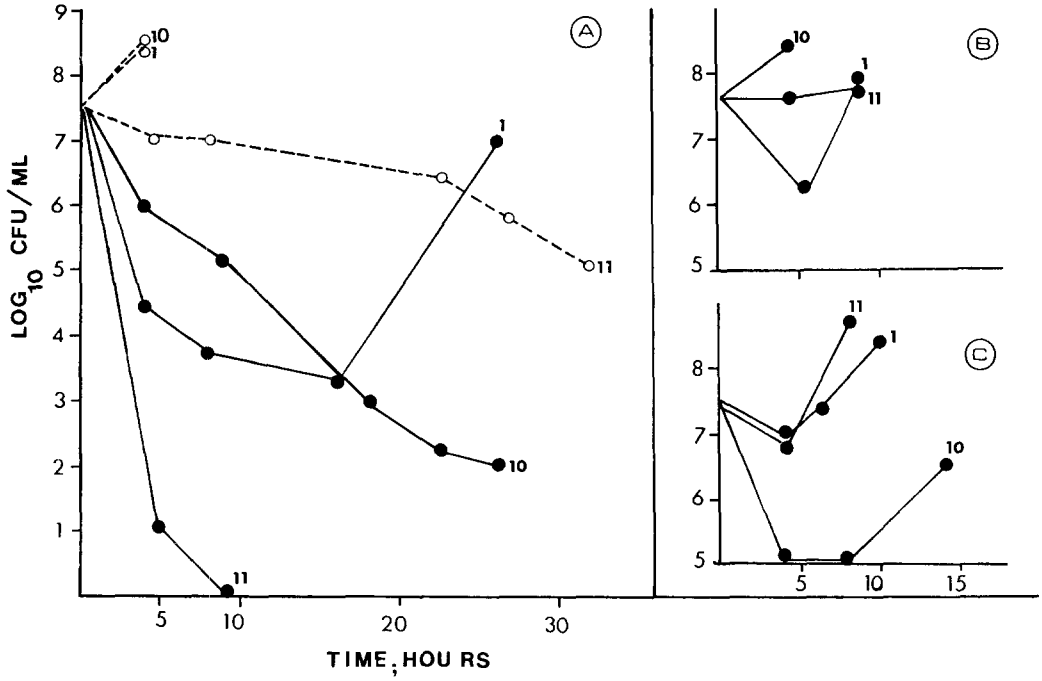


Fig. 3. (A) Exponentially growing sensitive (S) and resistant (R) strains of *Pseudomonas aeruginosa* were exposed to: 1, Formaldehyde 3 mM; 10, 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane, 4.64 mM (1000 ppm active); 11, *N*-methylolchloroacetamide, 8.16 mM (1000 ppm active); S, ●——●; R, O-----O. (B) The sensitive (S) strain was induced with 3 mM of formaldehyde. Then cells were exposed to the above concentrations of biocides. (C) The sensitive (S) strain was induced with 3.46 mM (750 ppm) of Biocide 10 and 6.12 mM (750 ppm) of Biocide 11. Then they were exposed to 3 mM of formaldehyde.

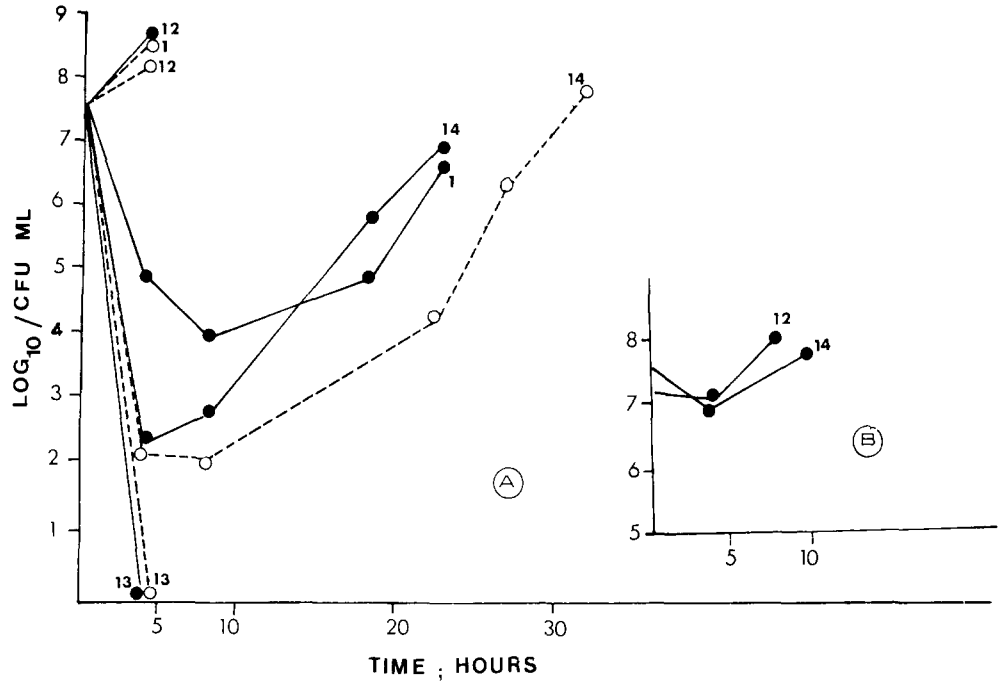


Fig. 4. (A) Exponentially growing cultures of sensitive (S) and resistant (R) strains of *Pseudomonas aeruginosa* were exposed to: 1, Formaldehyde, 3 mM; 12, Tris-(hydroxymethyl)nitromethane, 13.24 mM (2000 ppm active); 13, 2-bromo-2-nitro-1,3-propanediol, 1.5 mM (300 ppm active); 14, 4-(2-nitrobutyl) morpholine and 4,4-(2-ethyl-2-nitrotrimethylene)-dimorpholine (585 ppm active), a commercial mixture; S, ●——●; R, O-----O. (B) Sensitive (S) strain was induced with 16.55 mM (2500 ppm active) of Biocide 12 and 585 ppm of Biocide 14, then exposed to 3 mM of formaldehyde.

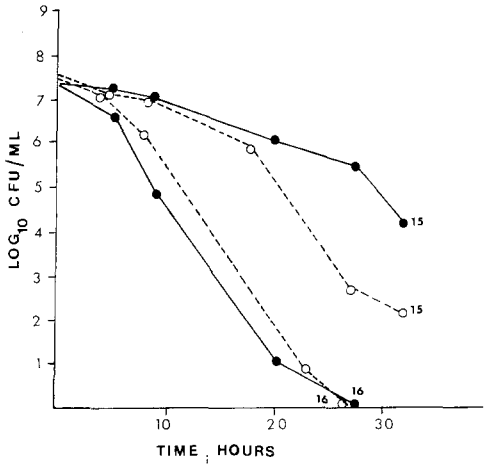


Fig. 5. Exponentially growing cultures of sensitive (S) and resistant (R) strains of *Pseudomonas aeruginosa* were exposed to: 15, 2-acetoxyl-2,4-dimethyl-*m*-dioxane, 8.77 mM (1000 ppm active); 16, sodium 2-pyridinethiol-1-oxide, 6.71 mM (1000 ppm active); S, ●——●; R, O-----O.

DISCUSSION

In this study, induction of resistance in a formaldehyde-sensitive strain [17] and a formaldehyde-resistant strain [18] were used to evaluate formaldehyde release from a variety of formaldehyde condensate biocides.

MICs and dose ranges of industrial biocides are usually expressed as ppm. This could be misleading when the effectiveness of formaldehyde condensate biocides are compared. This is also true if comparisons are based on their molar concentrations. If formaldehyde is the toxic moiety, the only reliable comparable data would be derived from equimolar concentrations of potentially available formaldehyde.

The maximum available formaldehyde should not exceed a theoretical level based on the number

of moles of formaldehyde involved in the biocide synthesis. However, several factors control the total amount of formaldehyde available as well as the rate of availability. The intrinsic factor controlling release and/or availability is a function of the biocide structure. Cyclic, condensed formaldehyde compounds behave differently from acyclic; in general, the latter are more stable at acid pH, while the former have a greater alkaline stability. In addition, rates of release and the total yield of formaldehyde increase when the potential formaldehyde is released from a C-N bond, as compared to a C-C bond. Monomethylol derivatives are very labile [6], and most cyclic formaldehyde condensates will produce methylol derivatives (hydroxymethylamines) upon hydrolysis.

The Group 1 biocides are all based on N-bound formaldehyde and predictably more related to formaldehyde than compounds in Group 3. Compounds in Group 2 should be more active, based on their structure, than observed. However, both biocides contain a large percentage of alkaline stabilizers (25–40%) in the commercial product, which conceivably affects activity.

In addition, the adamantane (Group 2, Biocide 10) was already shown to yield only low levels of formaldehyde at neutral or alkaline pH [14]. This is in contrast to hexamethylenetetramine which is completely stable and releases no formaldehyde at these pHs [7]. The basis for even a partial formaldehyde release from an alkaline *s*-triazine was shown by Graymore [8] to be related to quaternization. This also proved to be true for quaternized hexamethylenetetramine [14]. In both cases, approximately 30% formaldehyde equivalent was available at pH 7–8.

An early report stated that resistance to formaldehyde condensate biocides does not develop [4,13]; however, these studies used much higher concentrations of biocide, and since then, studies showing the contrary have been cited [3,9,17,18].

Tris-(hydroxymethyl)nitromethane has been considered as a slow formaldehyde releaser and one of the most effective antimicrobials in metalworking fluids. This compound results from formaldehyde alkylation of the central C atom. The observed lack of efficacy in TSB most likely is the result of the stability at pH 7 with only minimal formaldehyde release (Fig. 4A). Conversely, its analog, 2-bromo-2-nitro-1,3-propanediol, was extremly effective (Fig. 4A). The reactivity of this biocide is much higher than formaldehyde (3 mM formaldehyde equivalents result in 100% kill in less than 5 h) (Fig. 4A). There were no detectable differences when used against the (S) and (R) strains. There was a striking difference between the efficacy of tris-(hydroxymethyl)nitromethane and the bromonitro analog. The substitution of bromine for CH_2OH increases activity significantly, and this activity appears to be unrelated to formaldehyde. The positioning of a bromine on the same carbon as with a nitro (NO_2) group increases the electronegativity of the group and subsequently also its reactivity [5]. Whatever activity can be attributable to formaldehyde is probably masked by the NO_2-C(-Br)- contribution.

Results with the morpholine derivative (Compound 14) offer another contrast with Groups 1 and 2. The development of cross-resistance appears to be unidirectional, with morpholine survivors being resistant to formaldehyde but without the converse being true. This anomaly could result from the presence of residual formaldehyde in the morpholine mixture, since formaldehyde is involved in its synthesis. Although the synthesis is carried out with the other reactants (nitropropane and morpholine) in excess, quality assurance tests by the manufacturer reveal no detectable formaldehyde.

The Group 4 compounds were used as negative controls, and it appears that, at least in the case of one of them, sodium pyridinethiol, resistance to formaldehyde has no bearing on the response to that compound. The dioxane derivative is unexpectedly more effective against the resistant strain. This compound hydrolyses to acetaldehyde and crotonaldehyde [19] and potentially could induce resistance based on the reported oxidation of acetaldehyde and other aldehydes by formaldehyde dehydrogenase [1].

This study provides an insight into at least three areas of interest related to the practical applications of biocides. First, structural designations have min-

imal value when selecting biocides; at least a presumptive knowledge of the mode of action is required. Second, without this information, sequential use of seemingly different chemical types might meet with previously developed resistance. Third, the selection of appropriate doses should more accurately reflect the portion of the total molecule equivalent to the active moiety.

A number of questions remain unanswered. The environmental conditions optimal for each formaldehyde-adduct have not been determined, nor has the nature of the contribution of other active parts of the biocide molecule. However, this system of resistance determination will permit more rational selection and application of biocides.

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