

## The Interaction of Formaldehyde, Isothiazolone and Copper

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### ABSTRACT

*Evidence has been presented for the synergism between copper (Cu) and formaldehyde (FA) and isothiazolone (IT) and also for the equivalence of many FA adducts with FA. A distinction as well as a dual site for Cu-IT interaction has been made with both protection from environmental nucleophiles as well as enhancement of antimicrobial activity. Since the major FA dehydrogenase associated with FA resistance is glutathione dependent, the prospect of Cu-glutathione interaction is a probable cause of Cu-FA synergism.*

*Although there is no clear explanation for the IT-FA synergism, the combination offers many avenues for future exploration.*

### INTRODUCTION

According to the most recent Kline and Co. report on industrial biocide sales, more than 50% of the total dollar value are from isothiazolones (IT) and so-called formaldehyde (FA) condensate adducts. This popularity is undoubtedly due to their broad-spectrum efficacy and cost effectiveness. In spite of this extensive use in a variety of applications, they do exhibit shortcomings. The FA products are less effective against fungi and the IT are very sensitive to selected nucleophiles (Law & Lashen, 1990; Riha *et al.*, 1990).

The use of FA adducts as antimicrobial agents has been in vogue for

more than 100 years and most were recognized as being frank FA releasers (Rossmore & Sondossi, 1988). However, recent concerns with the potential hazards of FA have caused not only a reluctance to claim FA as the mode of action by the producers but also a hesitancy to apply by the user population.

The reactivity of FA, especially with nucleophiles, is directly related to its microbiological efficacy as well as its biological toxicity.

Paulus (1988), in his most recent review, refers to 'methylol groups capable of being cleaved off to give formaldehyde or methylene groups capable of being hydrolyzed to give formaldehyde'. He emphasizes the electrophilic nature of FA and therefore its reactivity with microbial cell nucleophiles (e.g. amino acids).

### FORMALDEHYDE AND FORMALDEHYDE ADDUCTS

Although FA adducts can and do act as depots for FA, the question of availability and release has not always been satisfactorily answered. For example, the most prominent adduct in clinical use, hexamethylene-tetramine, only releases FA in an acidic environment and to treat urinary tract infections must be dosed with an organic acid. The condensate of FA and ethylamine appears very stable at neutral and alkaline pH with no FA detected by NMR (Rossmore & Sondossi, 1988). However, it was obvious that FA must be involved in some way with the mode of action of the FA adducts.

De Mare *et al.* (1972) were unable to show a positive dimethone reaction with several hexahydrotriazine derivatives until the pH was lowered to 5. This finding left unanswered how FA got to the cell. It was speculated that the anionic microenvironment around the cell caused hydrolysis. Holtzman & Rossmore (1977) showed that homocysteine neutralized hexahydro-1,3,5-(2-hydroxyethyl)-s-triazine (HEAT). This was similar to the findings of Neely (1963) with FA who postulated the synthesis of a stable 6-membered thiazane ring. It appeared then that triazines might biologically release FA.

In an extensive series of studies (Sondossi *et al.*, 1986a), 15 biocides were compared to FA with regard to resistance induction. The results indicated that resistance and cross-resistance to FA and each biocide was a function of the amount of FA available from each molecule at the pH of the study. These data permitted a *post facto* grouping of the 15 biocides based on biological responsiveness in which the dose applied was determined by the number of molecules of FA used in synthesis. The relationship between FA/molecule and FA available was confirmed

TABLE 1  
Relationship Between Available Formaldehyde (FA) and Cross-Resistance Induction in Selected Biocides<sup>a</sup>

Adduct	FA molecules in chemical structure detected (Nash reagent) based on synthesis	Number of FA molecules detected (Nash reagent) (range of different experiments)	Biological response group <sup>b</sup>
Hexahydro-1,3,5-triethyl-s-triazine	3	2.65-3.09	1a
Hexahydro-1,3,5-(2-hydroxyethyl)-s-triazine	3	2.75-2.97	1a
A commercial mixture containing hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine	3	(2.916)	1a
1,3-(Dihydroxymethyl)-5,5-dimethyl-hydantoin	2	1.10-1.6	1b
5-Hydroxymethyl-1-aza-3,7-dioxabicyclo[3.3.0]octane (one of three cyclooctanes in commercial mixture)	2	1.26-1.29	1b
4,4-Dimethyloxazolidine (one of two oxazolidines in commercial mixture)	1	0.939-1.04	1a
2-(Hydroxymethyl)aminoethanol	1	0.824-0.979	1a
2-(Hydroxymethyl)amino-2-methylpropanol	1	0.832-0.857	1a
Tris(hydroxymethyl)nitromethane	?	0.063-0.281	3
2-Bromo-2-nitro-1,3-propanediol	?	0.2-0.5	3
N-Methylolchloroacetamide	1	0.16-0.18	2
1-(3-Chloroallyl)-3,5,7-triaz-1-azoniaadamantane	6	4-4.5	2
Sodium 2-pyridinethiol-1-oxide	0	(0.008)	4
4-(2-Nitrobutyl)morpholine (70%) + 4,4'-(2-ethyl-2-nitrotrimethylene)-dimorpholine (20%)	1	0.05-0.08	3

<sup>a</sup> Modified from Rossmore & Sondossi (1988). Assays were conducted at pH 7 in water by the method of Nash (1953).

<sup>b</sup> Group 1a = stoichiometric equivalence inducing resistance and cross-resistance; 1b = almost stoichiometric equivalence in inducing resistance and cross-resistance;

2 = increased dose needed to show cross-resistance;

3 = cross-resistance to FA induced with biocide but not conversely;

4 = no cross-resistance.

chemically (Rossmore & Sondossi, 1988) and is shown in Table 1.

It should be noted that for seven of the biocides, there is good agreement between FA content and cross-resistance and fair agreement between FA and two others. It is certainly possible that lack of quantitative agreement between FA content and resistance induction could be related to the conditions of the assay as well as to non-biocidal stabilizers in the commercial product. Nevertheless, the general case has been made that FA adducts with biocidal activity owe that activity wholly or in part to FA.

Subsequent studies determined that a major mechanism of resistance development involved increase in levels of formaldehyde dehydrogenase (FADH) to both FA and to hexahydro-1,3,5-triethyl-s-triazine (ET) (Eagon & Barnes, 1986; Sondossi *et al.*, 1986b). Eagon & Barnes (1986) reported on enzyme activity of a resistant isolate of *Pseudomonas putida* while Sondossi *et al.* (1986b) induced resistance in a sensitive industrial isolate of *Pseudomonas aeruginosa*. Nevertheless, both groups confirmed an earlier report (Sondossi *et al.*, 1985) that demonstrated two facts: (1) purified FADH from *P. putida* oxidized FA and ET with stoichiometric equivalence (i.e. one ET = 3 FA); and (2) crude extract from cells induced with ET oxidized FA and appeared to be glutathione dependent. This was verified (Sondossi *et al.*, 1986b) and, furthermore, seemed to be the major form of the enzyme associated with induction of resistance, increasing almost 20-fold after induction.

One well known deficiency of FA is the relative resistance of fungi to both FA and FA adducts. Several reports (Schutte *et al.*, 1976; Kato *et al.*, 1982, 1983) have described this resistance to constitutive presence of high levels of glutathione-dependent FADH. In addition, Sondossi *et al.*, (1990) were unable to increase resistance of several fungi to FA biocides with induction techniques. A method for potential increase in fungicidal activity of FA biocides will be discussed later in this paper.

## ISOTHIAZOLONES

The 3-isothiazolones are one of the more recent biocide groups to appear on the industrial scene. They are active at extremely low levels, especially in the absence of certain nucleophiles. In fact, nucleophilic attack makes them readily degradable in the environment (Krzeminski *et al.*, 1975a, b). In addition, their high reactivity is related in part to aqueous instability, and metal salts of nitrates and nitrites must be used to stabilize the molecules (Miller & Weiler, 1978). One major commercial product

contains 14.5% IT (10.9% 5-chloro-2-methyl-4-isothiazolin-3-one and 3.6% 2-methyl-4-isothiazolin-3-one) and 1.5% Mg(NO<sub>3</sub>)<sub>2</sub>. In the mixture, it is the chloro derivative that is considered the active component and is most easily degraded by nucleophiles (Fig. 1).

Although this IT mixture is perhaps more effective as a bactericide, it is considered more of a broad spectrum biocide than FA adducts. Nevertheless, its deficiency in the presence of some nucleophiles, sulphydryls especially and to a lesser extent secondary amines, suggested the need for an additive that would protect and/or enhance the IT.

## ISOTHIAZOLONES AND COPPER

A report that disodium monocopper citrate (CC), originally patented as an antimicrobial agent (Maurer & Shringapuray, 1977) and later at reduced levels as an oil/water stabilizer (Maurer & Shringapuray, 1978) reduced 'putrefactive' odors in metalworking fluids (MWF), led to its evaluation as an additive for IT (Piet & Rossmore, 1985; Rossmore, 1986). The use of CC with IT in a severely spotted MWF (2 × 10<sup>8</sup> cfu ml<sup>-1</sup>) reduced the count in 48 h to <10<sup>2</sup> cfu ml<sup>-1</sup> while IT alone and the untreated control rose to 7 × 10<sup>8</sup> cfu ml<sup>-1</sup>. A subsequent report (Law & Lashen, 1990) utilized CuSO<sub>4</sub> and IT in equal amounts in a synthetic MWF incompatible with IT. They not only showed the maintenance of antibacterial activity with the CuSO<sub>4</sub> added to the MWF, but they also showed the concomitant stability of the IT by HPLC in both the working solution and the concentrate, the latter after six months of storage at 31°C.

Copper (Cu<sup>2+</sup>) undoubtedly prevents nucleophiles from degrading IT. However, since all vital molecules in the cell are also nucleophilic, it would appear that Cu<sup>2+</sup> might also interact with cellular components in the same way. By blocking non-vital nucleophiles, Cu<sup>2+</sup> would save the IT for the lethal targets. This possibility was investigated (Riha *et al.*, 1990) in two ways. An incompatible MWF concentrate was treated with Cu<sup>2+</sup> at day 1 or day 28, diluted and challenged with bacterial inoculum at day 28, and these results were compared with those in a compatible MWF. It was found that Cu<sup>2+</sup> protected IT in the incompatible fluid and enhanced the activity of IT in the compatible fluid (i.e. synergism). The other approach involved sequential treatment of the bacterial population with Cu<sup>2+</sup> and IT, washing in between treatments. These results indicated that pretreatment with Cu<sup>2+</sup> enhanced the activity of IT. This was in the absence of external nucleophiles.

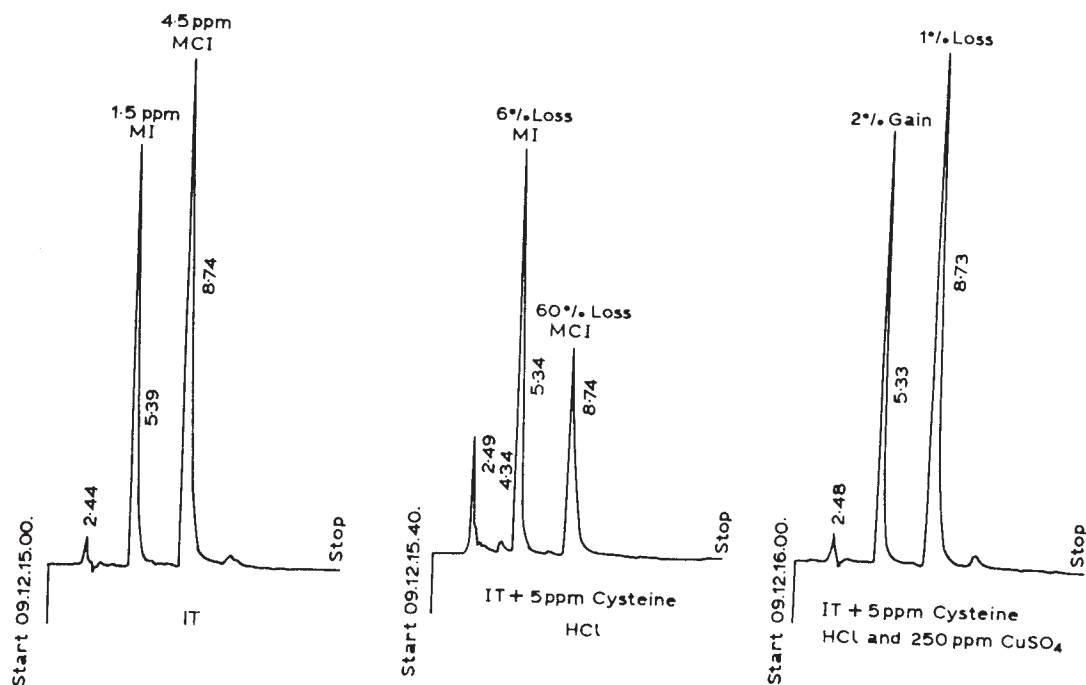


Fig. 1. HPLC of isothiazolone-nucleophile interaction with Cu protection. (MI = 2-methyl-4-isothiazolin-3-one; MCI = 5-chloro-2-methyl-4-isothiazolin-3-one; IT = isothiazolone). The experiment was performed on a Beckman 334 Gradient Liquid Chromatograph. The mobile phase was 80% and 20% methanol. The flow rate was 1 ml/min. Column pressure was approximately 1200 PSI. An Alltech 25 cm, 4.6 mm C-18 (Octadecylsilane) column was used. This column has particles of 5  $\mu\text{m}$  size, with a Latex-O-Si-O-(CH<sub>2</sub>)<sub>17</sub>-CH<sub>3</sub> configuration. Samples were injected with an Altex Model 500 Autosampler, equipped with a 100  $\mu\text{m}$  sample loop. Detection occurred at 275 nm with a Beckman 165 Variable Wavelength Detector. Data was recorded on an Altex C-Ria Recorder using Peak Area Integration.

## FORMALDEHYDE AND COPPER

Subsequent to the findings with CC and IT, a similar study was conducted on 20 industrial biocides, all registered with the US Environmental Protection Agency for use in MWF. Only those with an isothiazolone structure or involving FA in their syntheses showed enhanced activity with CC (Rossmore, 1987). An explanation is required for the preferential use of CC in this experiment; in contrast to most Cu<sup>2+</sup> salts, CC is soluble at pH 8.5, the operating pH of MWF emulsions. Thus it appeared that Cu<sup>2+</sup> also was in some way synergistic with FA adducts. This was confirmed for CuSO<sub>4</sub> and FA (Rossmore & Sondossi, 1988) where 2 mM CuSO<sub>4</sub> plus 3 mM FA results in 100% kill (8 logs) in 8 h while CuSO<sub>4</sub> alone did nothing and FA alone permitted regrowth to original levels in 30 h after a maximum drop of 4 logs.

A series of experiments involving sequential pretreatment of inoculum with Cu<sup>2+</sup> or FA showed that successful enhancement of FA activity only occurred with Cu<sup>2+</sup> exposure first and in mineral salts-glucose medium or NaCl solution and not in tryptic soy broth (TSB). This is indicative of the reactivity of Cu<sup>2+</sup> with nucleophiles in the broth, lessening its availability to enhance FA activity (Sondossi *et al.*, 1990).

## FORMALDEHYDE AND ISOTHIAZOLONES

In considering the similarities in reactivities of IT and FA with nucleophiles and enhancement by Cu<sup>2+</sup>, the possibility that IT and FA might interact favorably was examined (Table 2). In a very extensive listing of industrial biocides (Allsopp & Allsopp, 1983), there are nine

TABLE 2  
Summary of Interactions Between Copper, Formaldehyde (FA), and Isothiazolone

	Biocidal activity		Reactions with Isothiazolone	
	Antidonor	Antitender	FA	FA adduct
FA	Yes	Yes	—	CR <sup>a</sup>
FA adducts	Yes	Yes	CR	—
Isothiazolones	Yes	No	SYN/NCR	SYN/NCR
Copper	Yes	Yes	SYN	SYN

<sup>a</sup> Cross-resistant

<sup>b</sup> Synergistic.

<sup>c</sup> No cross-resistance.

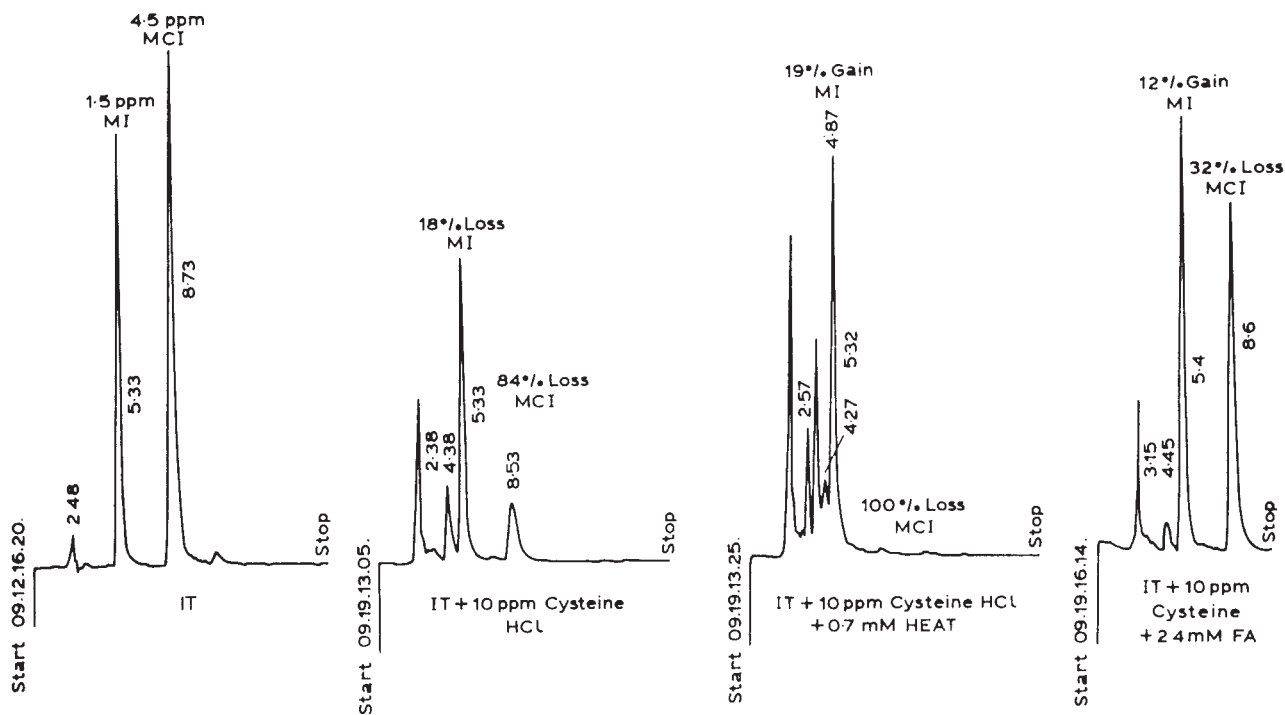


Fig. 2. HPLC of isothiazolone-nucleophile interaction with formaldehyde protection. (MI = 2-methyl-4-isothiazolin-3-one; MCI = 5-chloro-2-methyl-4-isothiazolin-3-one; IT = isothiazolone; HEAT = hexahydro-1,3,5-(2-hydroxyethyl)-s-triazine; FA = formaldehyde). For experiment detail see Fig. 1 caption.

commercial mixtures containing IT and some FA adduct. In addition, two patents (Hahn, 1979, 1984) deal with IT and dimethylolurea and tris(hydroxymethyl)nitromethane, respectively, and one with IT and glutaraldehyde (Clifford & Birchall, 1985). In my laboratory, the efficacy of the former mixture, but not the latter, was verified. This discrepancy may be due to conditions of the test since we earlier reported that trisnitro behaved neither biologically (Sondossi *et al.*, 1986a) nor chemically (Rossmore & Sondossi, 1988) with FA equivalence.

In another series of studies (Sondossi *et al.*, unpublished data), IT and FA were evaluated in a variety of solutions (TSB, MWF, and NaCl). In doses as low as 5  $\mu$ M IT with 2 mM FA or equivalent HEAT, enhanced activity was noted. The pretreatment sequence mentioned earlier also proved synergistic, although treatment with FA first followed by IT was not. No cross-resistance was seen in spite of what might appear to be similar targets to both agents. In an attempt to chemically protect the IT molecule from a biological nucleophile (cysteine), FA and HEAT in equivalents were used. The results are shown in Fig. 2. In this instance, FA and its adduct do not appear to be equal. This could explain the inconsistencies reported from the field when IT and FA adducts have been used as mixtures.

Although no critical supporting data are available to make a definitive assessment of the mode of action of IT-FA mixtures, it may be possible to offer a speculative view. Formaldehyde and FA adducts have been shown to react with Gram-negative lipopolysaccharide (LPS), probably nucleophilic components (e.g. aminoethanol), (Pfirrmann & Leslie, 1979; Douglas *et al.*, 1990, unpublished data). This potential elimination of outer membrane nucleophiles might allow the free passage of IT to more vital targets (e.g. cell membrane-bound ATPase, a sulfhydryl-based enzyme necessary for operating the energy-generating proton pump).

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