

CHAPTER 70

Evaluation of Action of a Formaldehyde Condensate Germicide

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Germicides that are condensation products of formaldehyde (F) and monalkylamines act by presumptively releasing F under use conditions in metal-working fluids. The ability of homocysteine to counteract the affects of F and hexahydro 1,3,5-(2-hydroxyethyl)-s-triazine on *Pseudomonas aeruginosa* was employed to demonstrate potential antagonism and a mode of action for the formaldehyde condensates. Initial results indicate that at use levels and conditions associated with metal-working fluids homocysteine does block the action of formaldehyde condensates.

INTRODUCTION

Formaldehyde and alkylamine condensates find application in the metal-working industry as germicides to prolong cutting fluid longevity. These compounds, the substituted hexahydro-triazines, are presumed to be at least partially effective due to the release of formaldehyde under use conditions (Bennett 1973) although this possibility had been previously rejected (Smith 1969). Studies to show formaldehyde release under pH conditions associated with metal-working fluids (8.0-9.5) were not positive (DeMare et al. 1972). However, some of these compounds yielded positive results at acid pH. Indeed, it is a well-established clinical practice in treatment of urinary tract infections with hexamethylenetetramine (a condensate of ammonia and formaldehyde) that the urine be lowered therapeutically to pH 6 in order to facilitate formaldehyde release. These data leave unanswered the question of the mode of action of these compounds under alkaline conditions. An earlier report (Neely 1963b) indicated that homocysteine equimolar with formaldehyde had neutralized its lethal effects against a gram-negative bacterial population. *Pseudomonas aeruginosa*, a prime cause of deterioration in metal-working fluids was selected as the test organism for evaluating formaldehyde release as a factor in assessing the effectiveness of substituted hexahydrotriazines at the pH associated with metal-working fluids.

MATERIALS AND METHODS

The premise for these experiments is related to work by Neely (1963a,b,c) in which he demonstrated that homocysteine neutralized the inhibitory action of formaldehyde. He postulated that its antimetabolic action results from the blocking of methionine biosynthesis by the formation of a thiazane ring with the homocysteine precursor. In the study presented here, viability rather than metabolic function is used as a measure of the interaction between homocysteine and formaldehyde.

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Reagents. Homocysteine thiolactone HCl (Sigma Chemical Company, St. Louis, MO) was used as a source of homocysteine throughout these experiments. The thiolactone in solution is quite acid (pH 2.3) and very stable. Initially, it was used unmodified. Then the thiolactone was hydrolyzed with 1 N NaOH to pH 9 and readjusted to the various use pH values with 1 N HCl. The hexahydro 1,3,5-(2-hydroxyethyl)-s-triazine was used from a commercially available 78% w/v solution (Grotan, TM Lehn & Fink, Co., Montvale, NJ) and the source of the formaldehyde was reagent grade formalin. The concn of all reagents is given in Table 1.

TABLE 1. Concentration of reactants

Reactant	Molar Concn
Formaldehyde ^a	3.3×10^{-3}
Homocysteine thiolactone ^a	3.8×10^{-3}
Hexahydro 1,3,5-(2-hydroxyethyl)-s-triazine ^b	5.3×10^{-3}

^aNeely (1963b).

^bOptimal use level in metal-working fluid.

Culture preparation. A culture of *Ps. aeruginosa* (Wayne State University culture collection) was maintained on Trypticase Soy Agar (TSA, Baltimore Biological Lab) slants at 30 C and transferred every 48 h until ready for use. Bacterial suspensions were prepared from the TSA slants by washing the cells with 0.9% NaCl. An aliquot of this suspension was then diluted to yield a test inoculum of about 10^7 cells/ml.

Experimental procedures. All tests were carried out in 30-ml screw cap vials with 20 ml of test solution and all solutions were adjusted to appropriate pH by the addition of NaOH (0.1 - 1.0 N) or HCl (0.1 - 1.0 N) prior to use. However, in the case of homocysteine thiolactone, it was necessary to hydrolyze the thiolactone with NaOH for all except the first experiment. In experiments involving homocysteine, the solutions were allowed 30 to 45 min together prior to adding the inoculum. Samples were removed for estimations of viability at indicated times. Viability determinations were done by standard plate-count method using TSA as retrieval medium.

RESULTS

The initial experiment was run comparing the nonhydrolyzed and hydrolyzed thiolactone to validate the putative mode of action of formaldehyde (Neely 1963a,b,c). There is no indication that the lactone without free SH/NH₂ has any effect on formaldehyde activity. After

TABLE 2. Effect of hydrolysis on homocysteine thiolactone activity

Treatment ^b	Percent survival of inoculum ^a	
	pH 4 (before hydrolysis)	pH 9 (after hydrolysis)
0.9 % NaCl	100	100
Formaldehyde	0.07	7.0
Homocysteine thiolactone and formaldehyde	0.002	100

^aInoculum 4.5×10^7 /ml.

^bIncubation period, 1 h.

TABLE 3. Effect of pH on formaldehyde activity

Treatment	Percent survival of inoculum ^a		
	pH 7	pH 8	pH 9
0.9% NaCl	100	100	100
Homocysteine ^b	100	100	100
Formaldehyde	0.07	0.08	0.5
Homocysteine ^b and Formaldehyde	100	100	100

^aInoculum 5×10^7 /ml.

^bHomocysteine thiolactone hydrolyzed then adjusted.

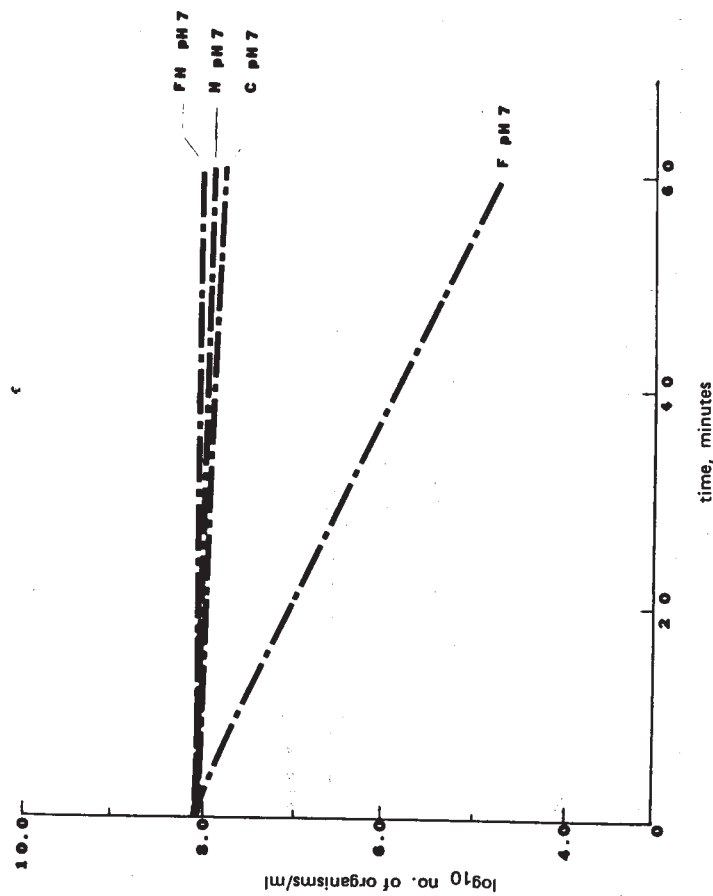


FIG. 1. Neutralization of formaldehyde activity by homocysteine. Inoculum was 5.6×10^7 *Pseudomonas aeruginosa*/ml; incubation 1 h. F = Formaldehyde 3.3×10^{-3} M; H = Homocysteine 3.8×10^{-3} M; FH = F & H; C = 0.9% NaCl.

hydrolysis of the homocysteine thiolactone at pH 9, neutralization of formaldehyde is quite evident (Table 2). Also evident is the lowered mortality at pH 9 from formaldehyde. This is probably due to some polymerization of formaldehyde at alkaline pH's. In another experiment homocysteine thiolactone was first hydrolyzed at pH 9 and then readjusted to pH 7 and pH 8. The same results were obtained as in the previous experiment. Again, formaldehyde at pH 9 was effective but in all instances homocysteine completely neutralized its action (Table 3). These results were obtained after a 60 min contact time and time course of data at pH 7 is

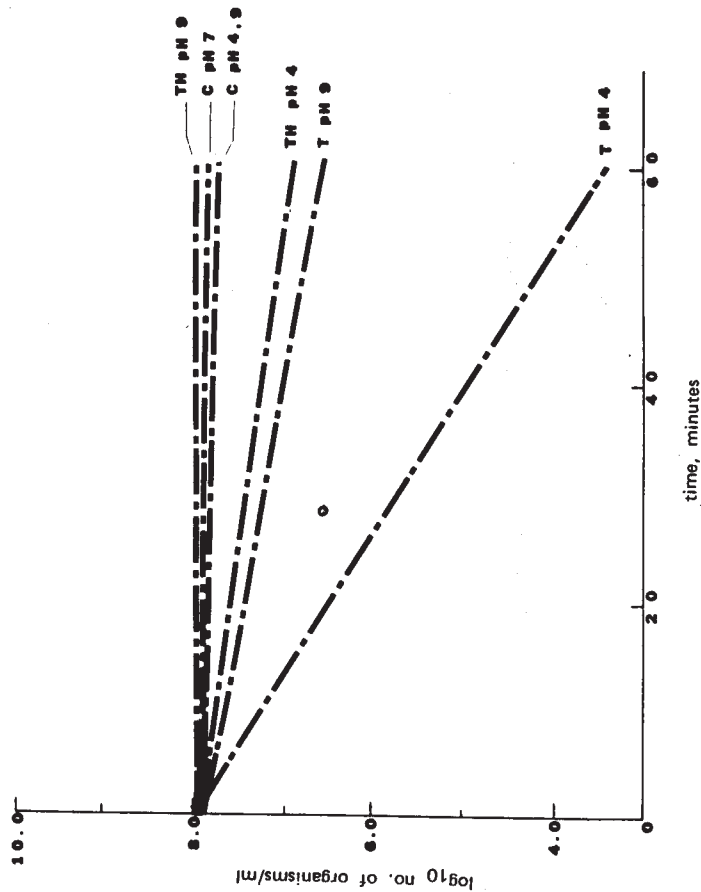


FIG. 2. Neutralization of hexahydro 1,3,5-(2-hydroxyethyl)-s-triazine by homocysteine. Inoculum was 8.7×10^7 *Pseudomonas aeruginosa*/ml. T = Hexahydro 1,3,5-(2-hydroxyethyl)-s-triazine; H = Homocysteine; TH = T & H; C = 0.9% NaCl.

presented in Fig. 1. Exponential death of the culture is demonstrated clearly from survivor samples taken every 20 min.

In a second series of experiments, hydrolyzed homocysteine thiolactone was utilized with the hexahydrotriazine. Here, activity of the triazine over a broad pH range is a critical part of the experiment. We reported earlier (DeMare et al. 1971) that no formaldehyde was detectable at alkaline pH, using dimethone complex formation as a qualitative indicator. In Fig. 2, several points should be noted; the extent of lethality of the triazine is greater at pH 4 than pH 9 and homocysteine neutralized the triazine completely at the alkaline pH but not under acid conditions. Both of these facts could be attributed to overt formaldehyde release from hydrolyzed triazine. In this experiment, there is insufficient homocysteine to neutralize all the potentially available formaldehyde. Doubling the amount of homocysteine to 7.7×10^{-3} molar produced no reduction in triazine-produced mortality at pH 4 in a subsequent experiment.

The last experiment in this study extended the time of observation from 1 to 5 h for the triazine, homocysteine interaction. One result is worth noting (Fig. 3): at pH 4, the triazine survivor plot has an inflection point at 1 h and the extent of mortality thereafter is not appreciable, while at pH 9 death continues exponentially; however, the pattern of homocysteine neutralization remained the same as previously noted.

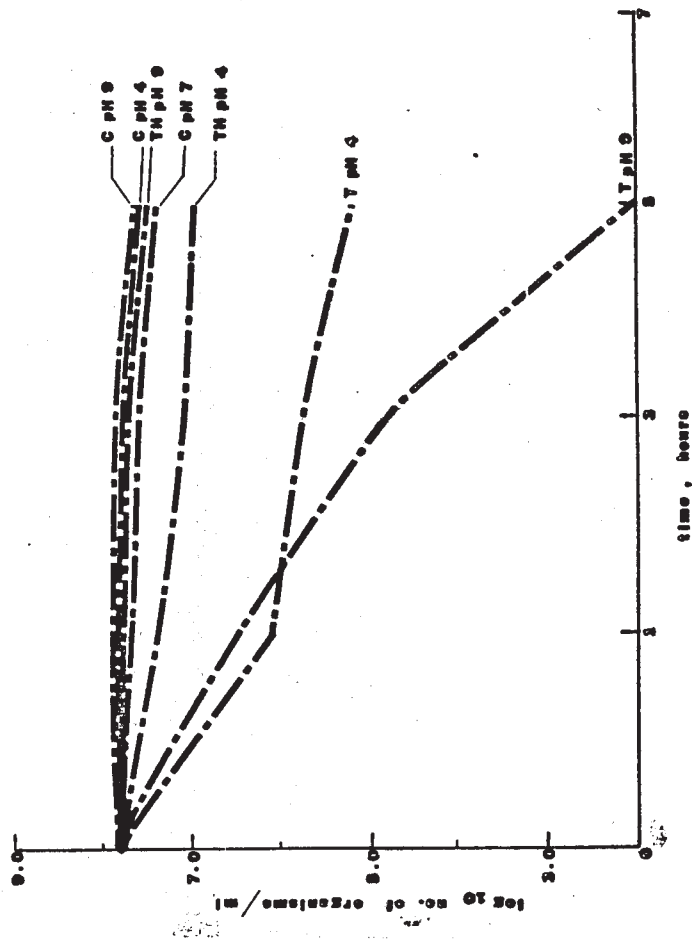


FIG. 3. Neutralization of hexahydro 1,3,5-(2-hydroxyethyl)-s-triazine by homocysteine during an extended contact time. Inoculum was 8.7×10^7 *Pseudomonas aeruginosa*/ml. T = Hexahydro 1,3,5-(2-hydroxyethyl)-s-triazine; H = Homocysteine; TH = T & H; C = 0.9% NaCl.

DISCUSSION

What is apparent from these results is that homocysteine blocks the action of the triazine at the same levels as proved effective for formaldehyde. This fact intimates that formaldehyde release may be a prime mode of action of the formaldehyde-alkylamine condensates. However, this conclusion is contradictory to what is considered a high degree of stability of the hexahydro-s-triazines at alkaline pH. Slow release not detectable by dimethone complexing cannot explain the lethality of triazines since they are far more effective than equivalent levels of formaldehyde. In a speculative vein, it is conceivable that the cell microenvironment proximal to the cell envelope is sufficiently acid to facilitate formaldehyde release. Also a possibility is the hydrolysis of the triazine within the cell or the periplasmic space. This latter hypothesis is testable using appropriate radioisotope tagged triazine and homocysteine. Based on the longevity of the triazines in use and their dose effectiveness, a mechanism involving intracellular release would be an attractive one.

The data presented are limited to only one formaldehyde condensate. Studies are presently under way with other available compounds in this class to assure that homocysteine complexing is a general phenomenon and not limited to hexahydro 1,3,5-(2-hydroxyethyl)-s-triazine.

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