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**ECBC-TR-419**

**DEVELOPMENT AND TESTING  
OF A COLORIMETRIC 96 WELL PLATE ASSAY  
FOR THE DETERMINATION  
OF HD HYDROLYSIS RATE IN VARIOUS FORMULATIONS**

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**January 2005**

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| <b>14. ABSTRACT</b><br>Previous aqueous studies using HD pre-dissolved in isopropanol have shown that the effectiveness of HD enzymatic degradation is a function of the homogeneity of the HD-water system. In this study, a microtiter plate assay was developed for the purpose of screening a series of compounds with the potential to increase the solubility or dispersion of HD in an aqueous matrix. The assay used meta-cresol purple dye as a pH indicator in a series of buffer concentrations to monitor the acid produced from HD hydrolysis. The extent of hydrolysis could be observed colorimetrically in a time-controlled series of reactions that allowed simultaneous comparison of numerous compounds or conditions on a single microtiter plate. Sixty-seven different detergents, surfactants, or different concentrations thereof were screened to determine their effect on HD hydrolysis rate. All were observed to inhibit HD hydrolysis. The simplest explanation of the results observed was probably that the micelles effectively sequestered the HD molecules from water in a hydrophobic environment. |                         |                                |                                   |                                                                |                                                                    |
| <b>15. SUBJECT TERMS</b><br>HD hydrolysis<br>Detergents<br>Colorimetric assay<br>Surfactants                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                         |                                |                                   |                                                                |                                                                    |
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## PREFACE

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## CONTENTS

|    |                              |    |
|----|------------------------------|----|
| 1. | INTRODUCTION .....           | 7  |
| 2. | MATERIALS AND METHODS.....   | 8  |
| 3. | RESULTS AND DISCUSSION ..... | 8  |
| 4. | CONCLUSIONS.....             | 14 |
|    | LITERATURE CITED .....       | 15 |

## FIGURES

|    |                                                                                                                                                     |    |
|----|-----------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 1. | Rates of Chloride Release in Spontaneous Versus Enzyme-Catalyzed Reactions of HD Added as a 10% Solution in Isopropanol to the Reaction Buffer..... | 8  |
| 2. | Observed Differences in HD Hydrolysis Rate Between Neat HD and 10% Solution in IPA .....                                                            | 10 |
| 3. | Typical Microtiter Plate Assay Results .....                                                                                                        | 11 |

## TABLE

|                                                                        |    |
|------------------------------------------------------------------------|----|
| List of Compounds Tested Against HD in the Microtiter Plate Assay..... | 12 |
|------------------------------------------------------------------------|----|



DEVELOPMENT AND TESTING  
OF A COLORIMETRIC 96 WELL PLATE ASSAY  
FOR THE DETERMINATION  
OF HD HYDROLYSIS RATE IN VARIOUS FORMULATIONS

1. INTRODUCTION

Chemical agents can be decontaminated via oxidation reactions such as with aqueous bleach or via substitution reactions using either aqueous alkali, organic alkali, basic hydrogen peroxide, or monoethanolamine. Typically, these approaches offer the advantages of rapid rates and broad range reactivity but can cause material compatibility problems, and require large amounts of reactants that impose a logistical burden. Oxidation offers a particularly broad-spectrum approach but reactant stability is variable and the logistics are problematic due to the disadvantageous stoichiometry of the reactions. Some oxidation/substitution reactions have also been shown to be effective against biological agents; others might be effective but remain untested.

Hydrolysis reactions can also be used for decontamination, and offer their own unique advantages, such as favorable stoichiometry (one or two water molecules react with one agent molecule of about 10 times greater mass) and the almost universal availability of water. Although hydrolysis rates are typically slow as compared to oxidation reactions for instance, they can often be accelerated with catalysts. Enzymatic catalysts offer particularly dramatic rate enhancements, although they can have a relatively narrow specificity, showing significant activity differences even between two stereoisomers of the same compound.

In the case of G-type organophosphate agents, these enzymatic reactions are well characterized<sup>1-6</sup> and are feasible for a wide range of G-type substrates. For V-type phosphonothiolate agents, the organophosphate hydrolase (OPH) enzyme has been shown to catalyze the hydrolysis of the P-S bond.<sup>7</sup>

In the case of HD (sulfur mustard, 2,2'-dichlorodiethyl sulfide), enzymes have been shown to possess dehalogenase activity when the HD is first dissolved in alcohol prior to addition to the aqueous reaction matrix.<sup>8</sup> However, neat HD has essentially no solubility in water so enzymes have little access to the HD molecules in the absence of alcohol. Hydrolytic dehalogenase enzymes are particularly interesting for HD decontamination because the hydrolysis reaction yields thiodiglycol (TDG) from HD. The difference in toxicity between HD and TDG is a factor of 4,200 to 5,700 (the oral LD<sub>50</sub> of HD is 0.7 mg/kg whereas that of TDG is 3000 - 4000 mg/kg).<sup>9,10</sup> This large reduction in toxicity offers the potential to seriously reduce the damage caused by HD if it can be decontaminated quickly enough. For this reason, we sought to address the issues of HD solubility and contact in an aqueous matrix. Our initial objective was to develop a plate assay that would allow rapid testing of materials that might facilitate HD aqueous solubility. Subsequent to that, a series of materials were evaluated for their actual effects on HD solubility as a function of its hydrolysis rate.

## 2. MATERIALS AND METHODS

The HD was obtained from a 1-ton storage container (Aberdeen Proving Ground, MD). The HD was stabilized with tributylamine and was approximately 90% pure.

Quantitative assays were conducted with a chloride electrode attached to a Fisher Accumet 925 meter. Reactions were conducted in a temperature-controlled vessel in a total volume of 5 mL. Buffering was provided by a 50 mM solution of MOPS at pH 7.2. Data logging was automated through an RS-232 connection to a computer.

The microtiter plate assays were performed solely with polypropylene plates to prevent direct HD reaction with the plate material as was observed with polyethylene plates. All assays were performed at room temperature and the buffer used was ammonium carbonate. Indicators were purchased from Aldrich Chemical Company, St. Louis, MO.

## 3. RESULTS AND DISCUSSION

Enzyme-catalyzed hydrolysis of HD has been demonstrated in reactions where HD dissolved in alcohol is added to aqueous buffer. Figure 1 shows the corresponding increase in the chloride release rate in a sample reaction.

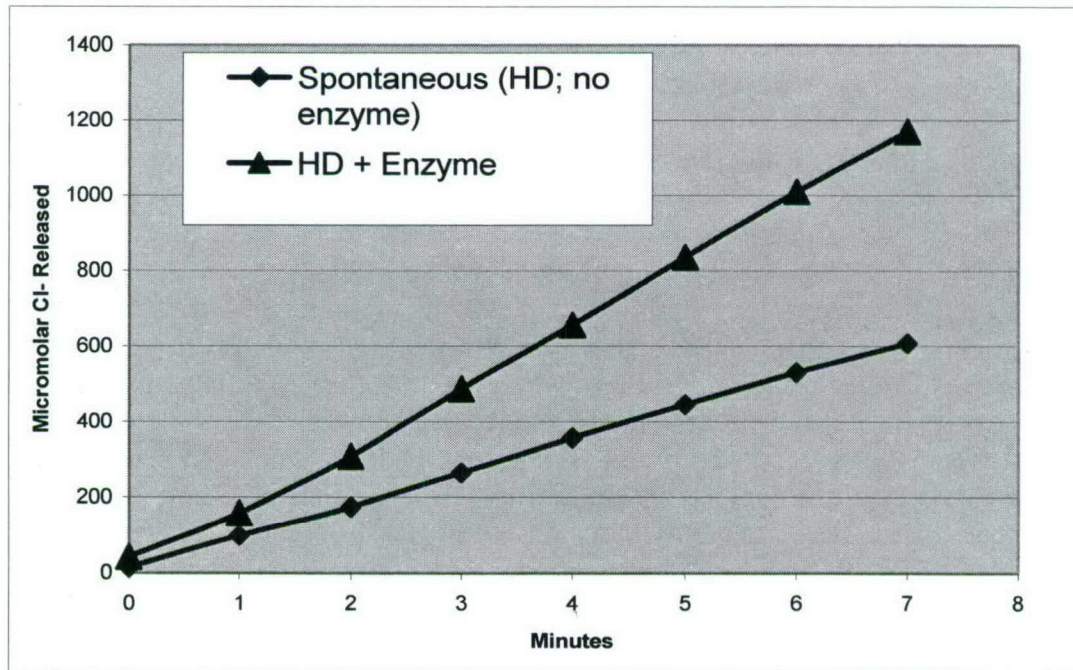


Figure 1. Rates of Chloride Release in Spontaneous Versus Enzyme-Catalyzed Reactions of HD Added as a 10% Solution in Isopropanol to the Reaction Buffer (50 mM MOPS buffer, pH 7.2)



However, when the HD is added neat to the same reaction above, there is no detectable difference in rates between the spontaneous and enzyme-catalyzed reactions. The spontaneous rate is also much slower in the absence of the alcohol solvent. The simplest explanation of these results is that the insoluble neat HD is poorly distributed in the aqueous buffer and is presumably relatively inaccessible to the aqueous-dissolved enzyme. This explanation is consistent with the visual observation of a relatively homogeneous reaction matrix with HD/isopropanol versus a heterogeneous matrix observed when neat HD is added to aqueous buffer.

While the chloride electrode assay is quantitative and reproducible as an assay of the HD hydrolysis rate, it permits evaluation of only a single set of conditions at a time. A plate assay, on the other hand, would offer the potential to evaluate several compounds plus controls simultaneously in a semi-quantitative manner. Such an assay could provide a powerful screening tool for the effect of various materials on the hydrolysis rate of HD.

The complete HD hydrolysis reaction proceeds through a series of sulfonium ion intermediates and yields two equivalents of HCl.<sup>11</sup> The overall balanced hydrolysis reaction is illustrated in the following Equation.



Because HD hydrolysis is a mass-transfer limited reaction, increases in solubility or dispersion are reflected in a corresponding increase in hydrolysis rate and acid production.

Many materials are known to facilitate the dissolution or dispersion of one compound in another for the purpose of facilitating chemical reactions. A few examples include phase transfer catalysts such as quaternary ammonium compounds and quaternary phosphonium compounds, or detergents. Because there were no reports known to us of compounds that facilitated the dissolution of HD in an aqueous matrix, we needed to test a number of materials that might dissolve or disperse HD in aqueous buffer. Toward that end we sought to develop a plate assay based on change in pH resulting from the corresponding increase in hydrolysis rate.

The decrease in pH can be followed by incorporating pH indicator dye into a series of buffer concentrations. The rate at which the hydrolysis reaction produces HCl can then potentially be tracked by observing the number of wells with color changes in a given time period.

The assay was set up as follows: Ammonium carbonate buffer was prepared in a series of concentrations between 0.5 and 5.0 mg/mL in 0.5 mg/mL increments. To these dilutions, meta-cresol purple dye was added at a final concentration of 1 mg/mL (thymol blue dye was also used, with essentially similar results). The dye changes color from purple (pH 9.0) to yellow (pH 7.4) to red (pH 1.2) as pH decreases due to HCl production from HD hydrolysis. Dye-containing buffer dilutions were then added to the plate at 140  $\mu\text{L}$  per well in the order of buffer concentration.

Controls and experimental wells were as follows:

- a. A row of control wells containing only buffer dilutions and dye to establish the background color in the absence of either HD or the compound of interest (i.e., the compound to be assessed for its effect on HD solubility).
- b. A row of control wells containing only buffer dilutions, dye and HD to establish the spontaneous rate of HD hydrolysis in the absence of the compound of interest.
- c. A row of control wells containing buffer, dye and the compound of interest, but no HD. The purpose of this control was to observe and pH effects from the compound of interest in the absence of HD.
- d. A row of experimental wells the same as in (c.) above, plus 1% HD by weight. The difference in color change observed between (c.) above and the experimental wells could only be due to the effects resulting from the reaction of HD in the aqueous system.

Once set up, the plates were placed on a microtiter plate shaker to provide equivalent agitation to all the wells. As the HD hydrolyzed, acid was released and wells, in order of increasing buffer concentration, turned color from purple to yellow to red. Figure 2 shows a comparison of HD added neat and HD added as a 10% solution in isopropanol (the final effective concentration of HD was 1% in both cases). Clearly, the HD added as an isopropanol (IPA) solution hydrolyzed much more rapidly, as seen by the number of wells with color change in 30 min.

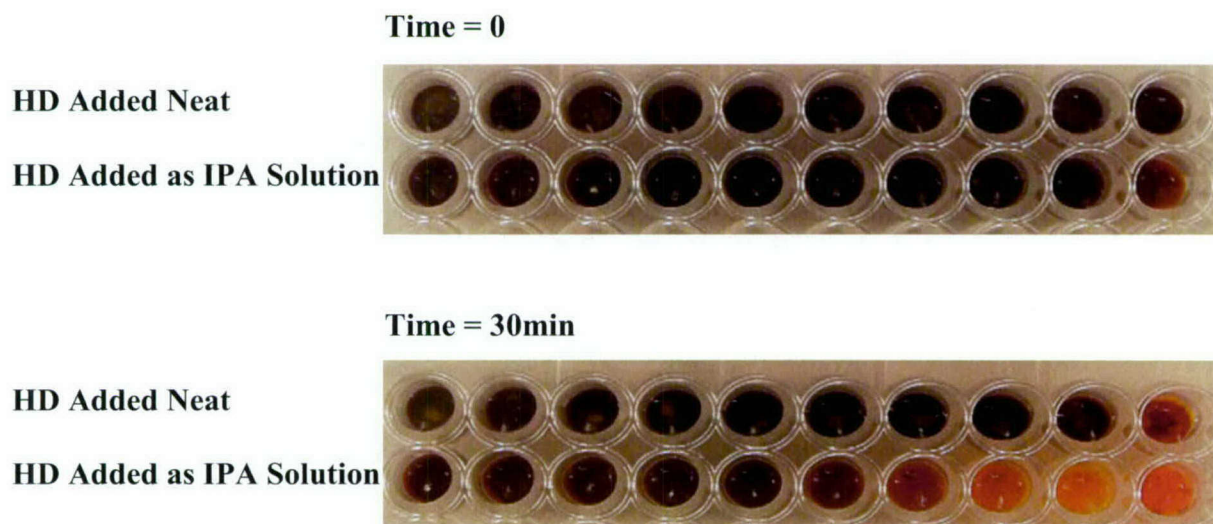


Figure 2. Observed Differences in HD Hydrolysis Rate Between Neat HD and 10% Solution in IPA. The HD/IPA Solution Effectively Served as a Positive Control for an Increase in HD Hydrolysis Rate.



The results shown in Figure 2 confirm what was already known from chloride electrode assays. The HD/IPA hydrolysis proceeds significantly more rapidly than that of neat HD under the same conditions. Therefore, differential rates can be semi-quantitatively visualized in the colorimetric assay based on the number of wells exhibiting color change in a given time period.

A series of detergents and other surfactants were tested in a similar manner. In all cases, their hydrolysis rates were compared directly to the rate of HD alone with no surfactant. Although some test compounds initially produced greater color change than the wells with HD alone, when control C (above) was observed (i.e., buffer, dye, compound of interest, but no HD), it was clear that the pH change was caused primarily by the acidity of the test compound itself, not by HD hydrolysis. Of all the compounds tested (see the Table), none were seen to clearly enhance the rate of HD hydrolysis, and most compounds significantly inhibited the rate of hydrolysis. This was essentially consistent with results reported from a previous study when a series of detergents were tested by chloride electrode assays.<sup>12</sup> Figure 3 shows the results of a typical assay.

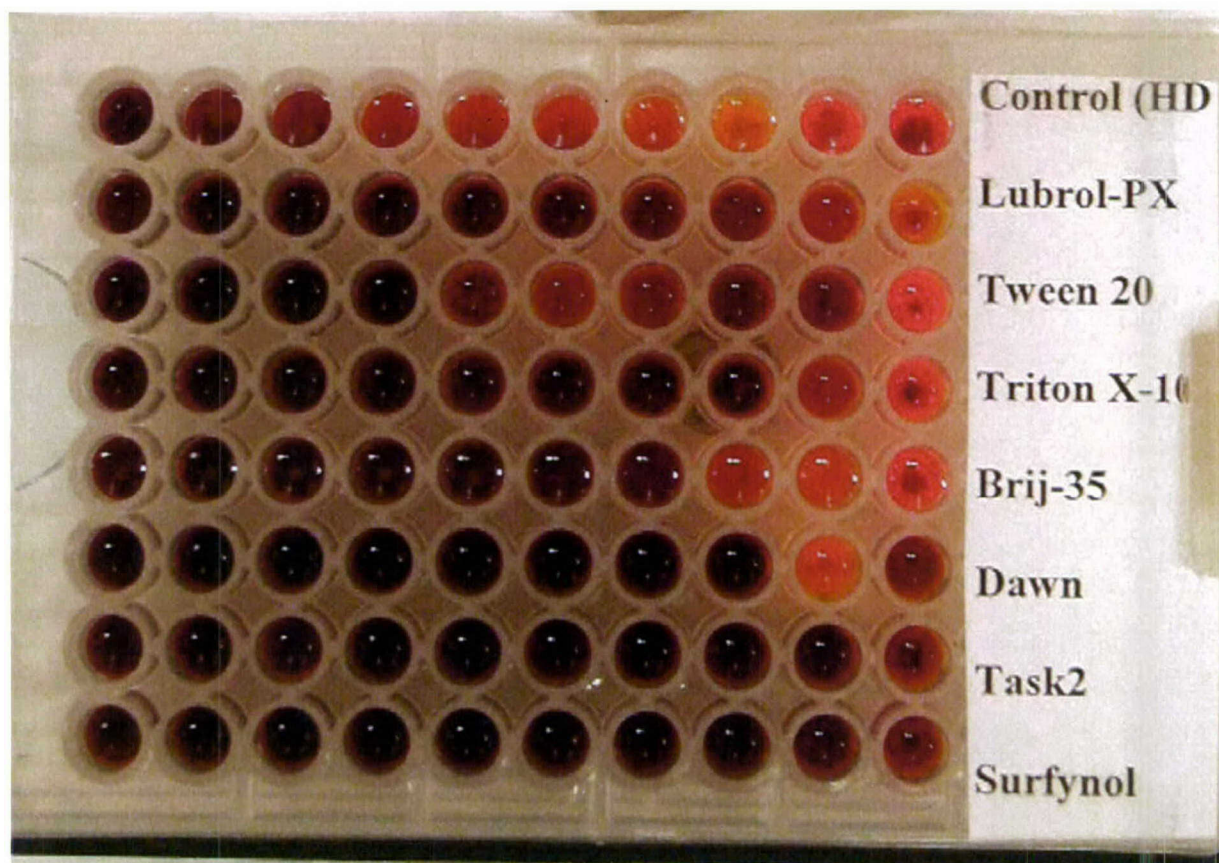


Figure 3. Typical Microtiter Plate Assay Results. All test compounds were added to a final concentration of 1%, and the plate was covered, and placed on the shaker for a total of two hours prior to photography. Results show that HD alone (control, top row) hydrolyzed faster than HD in the presence of any of the test compounds.

Table. List of Compounds Tested Against HD in the Microtiter Plate Assay. Initial concentrations of test compounds were generally 1%. In cases where the results of the 1% tests were ambiguous, other concentrations were tested and/or compounds were tested in the chloride electrode assay. None of the compounds in this table were clearly observed to enhance the hydrolysis of HD under the conditions tested.

---

1% AFFF Class B Foam  
 1% Class A 4 Knockdown  
 1% Uni A Class A Foam  
 1% XL-3 Fluoroprotein  
 1% dodecylbenzene SA  
 1% A4P 3/6 AFFF Alcohol-res  
 1% PEG 400  
 3% PEG400  
 5% PEG400  
 10% PEG400  
 1% PEG200  
 5% PEG200  
 10% PEG200  
 1% PEG dimethyl ether  
 5% PEG dimethyl ether  
 10% PEG dimethyl ether  
 1% tetramethylene glycol  
 5% tetramethylene glycol  
 10% tetramethylene glycol  
 1% thiodiglycol  
 5% thiodiglycol  
 10% thiodiglycol  
 1% 1,4 butanediol  
 1% Hexadecyl trimethylammonium bromide  
 1% PEG 6000  
 1% Bare Ground  
 1% Dextran sulfate  
 1% polyvinylpolypyrrolidone  
 1% Tide Free  
 2, 2 azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt  
 1% dioctyl sulfosuccinate sodium salt  
 1% Dimethyl-dioctadecylammonium bromide  
 1% Brij 56  
 1% 1-dodecane sulfonic acid  
 1% Dodecyl-beta-D-maltoside  
 1% Thiodiglycolic acid  
 1% Cetyltrimethyl ammonium chloride  
 1% Lauric acid



Table. List of Compounds Tested Against HD in the Microtiter Plate Assay. Initial concentrations of test compounds were generally 1%. In cases where the results of the 1% tests were ambiguous, other concentrations were tested and/or compounds were tested in the chloride electrode assay. None of the compounds in this table were clearly observed to enhance the hydrolysis of HD under the conditions tested. (Continued)

---

1% N-lauroylsarcosine sodium salt  
 1% Triton type CF-54  
 1% Glycine P.A.  
 1% Hexanediol  
 1% TAPSO  
 1% Petrogen surfactant  
 5% Petrogen surfactant  
 5% 1,4 butanediol  
 10% 1,4 butanediol  
 1% Universal Plus 3/6%  
 1% Universal Gold 3%  
 1% Aero-lite 3%  
 1% Tween 20  
 1% DDSAH  
 1% lactic acid  
 1% propylene glycol  
 1% Aero-lite 3% cold foam  
 1% Tween 80  
 1% Triton N101  
 1% 0.3% AFFF concentrate  
 1% Glutathione, reduced  
 acid water 3em  
 1% Triton X100, reduced  
 1% Aero-foam Cold Foam  
 1% ethylene glycol  
 1% benzalkonium chloride  
 1% polyvinylpyrrolidone  
 1% Brij 58  
 1% Benzyldimethyl tetradecyl ammonium chloride dihydrate

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#### 4. CONCLUSIONS

A colorimetric microtiter plate assay was developed based on the pH differences resulting from HD hydrolysis at varying rates. The assay provides a convenient,

semi-quantitative means by which to compare HD hydrolysis rates under a series of different conditions. Results observed with the plate assay were consistent with those from chloride electrode assays with the same materials.

Using the plate assay developed in this work, a series of detergents and other surfactants were screened to determine their effect on the HD hydrolysis rate. None of the compounds tested were observed to enhance HD hydrolysis and most of the compounds significantly inhibited the reaction. The simplest explanation for the decreased hydrolysis rates observed in the presence of the detergents is probably that the detergent micelles sequestered the HD in a hydrophobic environment where they are not as susceptible to hydrolysis.

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