Inactivation of a Tetrachloroimide Mutagen from Simulated Processing Water

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The tetrachloroimide 3-(dichloromethylene)-4,4-dichloro-2,5-pyrrolidinedione (compound **1**), a potent, direct-acting mutagen inducing more than 1500 revertants/nmol in *Salmonella typhimurium* strain TA100 (Ames assay) without metabolic activation, is formed during chlorination of simulated poultry chiller water. The mutagenicity of this compound decreased rapidly upon exposure to various nucleophilic compounds such as L-cysteine, *N*-acetyl-L-cysteine, cysteine ethyl ester, reduced glutathione, sodium sulfite, sodium bisulfite, and L-lysine. The amino acids aspartic acid and glycine were ineffective under the test condition of pH 7 at room temperature. Loss of mutagenicity was directly related to time of exposure and concentration of nucleophile as measured with *N*-acetyl-L-cysteine and L-lysine. The pH of the reaction medium in the range of 6–8 did not influence the inactivation. Although the reaction products resulting from the exposure of the imide to the nucleophilic compounds have not been identified, ultraviolet spectral studies and mechanistic considerations suggest that they may result from displacement of a chloride on the imide. The possible significance of these findings to food safety and to the reduction of the mutagenic potential of poultry chiller water is discussed.

Keywords: *N-Acetyl-L-cysteine; L-cysteine; food safety; glutathione; L-lysine; mutagen inactivation; poultry chiller water; sodium bisulfite; sodium sulfite; tetrachloroimide mutagen*

INTRODUCTION

The use of chlorine to control pathogenic bacteria in poultry processing water is widespread and is currently the only method approved by regulatory agencies. To cool the poultry carcasses to the required 40 °F within 2 h of slaughter, the eviscerated and defeathered poultry are immersed in chilled water baths which are chlorinated to prevent buildup of potentially harmful bacteria. The chiller water contains substantial quantities of both soluble and insoluble organic material which can readily react with chlorine directly or with hypochlorous acid formed on hydrolysis of chlorine by water (Tsai et al., 1992). For example, Tsai et al. (1987) identified a large number of volatile compounds that are produced by such reactions. Robinson et al. (1981) identified chloroform in chlorinated water containing chicken carcasses. In a relevant study, Masri (1986) demonstrated that chlorination is directly correlated with the degree of mutagenicity as determined by the Ames assay. In that study, the mutagen(s) were not identified, nor were their specific mutagenicities determined.

To better control the production of these mutagens while a sanitary chiller bath is maintained, an investigation was initiated to determine the makeup and potency of the specific mutagens formed in simulated chiller water (Haddon et al., 1995). The "poultry chiller water" was prepared by homogenizing either chicken franks or whole chicken in water as described by Tsai et al. (1992). Haddon et al. found that the tetrachloroimide 3-dichloromethylene-4,4-dichloro-2,5-pyrrolidinedione (compound **1**) accounts for much of the mutagenicity of the pH 2 fraction of chlorinated poultry chiller water. Compound **1** is one of the most potent mutagens known, with an activity of more than 1500 revertants/nmol (0.24 μg) in the *Salmonella typhimurium* TA100 bacterial strain (Table 1).

Table 1. Effect of Concentration of *N*-Acetyl-L-cysteine on the Mutagenicity of the Tetrachloroimide 1 in *S. typhimurium* Strain TA100 without Microsomal Activation[a]

test sample	TA100 activity (revertants/plate)
tetrachloroimide 1 only (μg/plate)	
0.025[b]	359 ± 25[c]
0.05	513 ± 62
0.1	867 ± 57
0.15	>1200 ± 0
0.25	>1200 ± 0
dioxane control	191 ± 32
N-acetyl-L-cysteine (NAC) + 1	
0.05 M NAC + 1	519, 525[d]
0.10 M NAC + 1	268, 273
0.15 M NAC + 1	163, 166
0.20 M NAC + 1	156, 176
0.20 M NAC only (negative control)	141, 149
methyl methanesulfonate (0.4 μL) (positive control)	>1200, >1200

[a] Incubation of imide 1 with *N*-acetyl-L-cysteine was carried out in 2 M acetate buffer at pH 5 and 25 °C for 32.5 min. The initial concentration of tetrachloroimide 1 in all solutions was 2.4 μg/mL. For the Ames test, 0.1 mL of each solution was added to each plate. [b] Dose response of tetrachloroimide 1. [c] Average of three plates ± standard deviation. [d] Each assay was carried out in duplicate.

Earlier studies revealed that sulfhydryl compounds inhibited the mutagenicity of aflatoxin B_1 (DeFlora et al., 1989; Friedman et al., 1982) and of compound MX [3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone] formed in chlorinated drinking water (Watanabe et al., 1994). Since the structure of the tetrachloroimide **1** suggests that it might also act as an electrophile, it was of interest to determine whether nucleophiles would transform it to derivatives that would exhibit less mutagenic activity.

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Our objective was to compare the effectiveness of three classes of nucleophiles in reducing the mutagenicity of compound **1**. These are (1) SH compounds such as L-cysteine, *N*-acetyl-L-cysteine, and the natural tripeptide reduced glutathione; (2) inorganic sulfites such as sodium bisulfite and sodium sulfite; and (3) NH_2 groups present in three types of amino acids, i.e., L-aspartic acid (acidic), glycine (neutral), and L-lysine (basic).

The design of this study was based on previous observations on the kinetics and mechanisms of nucleophilic addition reactions of SH and NH_2 groups of amino acids and peptides to conjugated compounds such as methyl vinyl ketone (Friedman and Wall, 1964; Friedman et al., 1964, 1966). Because this ketone is a very reactive electrophile, we expected that the conjugated vinyl ketone moiety present in the tetrachloromide **1** would readily participate in analogous reactions. The results of this study show that our expectations were realized.

We selected strain TA100 for the inactivation studies because Masri (1985) and Schade et al. (1990) previously found that this strain was most sensitive to mutagens from chlorinated poultry chiller water.

MATERIALS AND METHODS

Materials. The tetrachloroimide **1** was synthesized according to the method described by Haddon et al. (1995) and was >99% pure, as confirmed by nuclear magnetic resonance (NMR) and mass spectrometry. *N*-Acetyl-L-cysteine, L-cysteine, sodium sulfite, reduced glutathione, sodium bisulfite, L-lysine, glycine, and L-aspartic acid were obtained from Sigma Chemical Co. (St. Louis, MO) and were not further purified. Dioxane, reagents for buffers, and methyl methanesulfonate were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Inactivation Studies. Reactions of **1** with a series of nucleophilic compounds were carried out in volumetric flasks in the dark at room temperature. The nucleophile was dissolved in 2 M acetate buffer to a concentration of 0.2 M. Before *N*-acetyl-L-cysteine was added, but not for other compounds, nitrogen was bubbled into the buffer for 3 min to displace the oxygen. Compound **1** in dioxane solution was next added to the flasks, which were then brought to volume with buffer to give a final concentration of 2.4 μg/mL of compound **1**. The reaction mixtures along with suitable solvent controls were allowed to react for various time periods. Additional experimental details are given in the footnotes to Tables 1–4.

Mutagenicity Tests. The Ames *Salmonella* mutagenicity assay was performed using *S. typhimurium* tester strain TA100 provided by Dr. Bruce Ames (University of California, Berkeley, CA). The standard top-agar incorporation method without preincubation was employed. Procedures for growth, storage, and verification of the genetic markers were carried out according to the method of Maron and Ames (1983). The positive control methyl methanesulfonate (MMS), a liquid, gave >1200 revertants at 0.4 μL per plate.

Plate counts were made manually or with an Artek automatic colony counter, Model 980 (Artek Systems Corp., Farmingdale, NY), and adjusted for accuracy with an experimentally derived correlation graph. Representative manual counts are made with each experiment to calibrate the counter. Numbers >1200 are not routinely determined, as the automatic counter is not accurate in that range and manual counting is necessary if a precise number is desired. For example, the 1486 and 1561 revertants for the tetrachloroimide **1** in Table 2 were counted manually, whereas the >1200 revertants for methyl methanesulfonate listed in the same table were obtained with the automatic counter.

The effectiveness of 0.15 M *N*-acetyl-L-cysteine to inactivate compound **1** was verified in duplicate experiments, shown in Tables 1 and 3. Standard deviations for the dose–response study of **1** shown in Table 1 are similar to those previously reported for other mutagens (Friedman et al., 1990a,b).

Ultraviolet Spectral Studies. Reaction of compound **1** with *N*-acetyl-L-cysteine was monitored by UV using a Hewlett-Packard diode array spectrophotometer (Model 8451A). Solutions of *N*-acetyl-l-cysteine (0.2 M) in acetate buffer, with and without compound **1**, were added to 1 cm cuvettes and the spectra taken at approximately 1 min intervals. Intensities at 250 nm were monitored. The control intensity (buffer + *N*-acetyl-L-cysteine) at 250 nm was subtracted from the reaction product and plotted as a function of time. Stability of compound **1** was also monitored by periodically checking the spectrum of **1** in buffer without *N*-acetyl-L-cysteine. The determined extinction coefficient of **1** at 250 nm in ethanol was 11 990.

Table 2. Quantitative Plate Tests of Tetrachloroimide 1 plus Different Concentrations of L-Lysine in *S. typhimurium* Strain TA100 without Microsomal Activation[a]

test sample	TA100 activity[b] (revertants/plate)
L-lysine (Lys) + **1**	
1 (0.24 μg)	1486, 1561
0.05 M Lys	192, 200
0.05 M Lys + **1**	848, 853
0.10 M Lys	195, 208
0.10 M Lys + **1**	262, 295
0.20 M Lys	183, 187
0.20 M Lys + **1**	205, 236
acetate buffer control	171, 187
methyl methanesulfonate (0.4 μL) (positive control)	>1200, >1200

[a] Samples were incubated in 2 M acetate buffer at pH 7 and 25 °C for 2 h in the dark. The initial concentration of tetrachloroimide **1** in all solutions was 2.4 μg/mL. For the Ames test, 0.1 mL of each solution was added to each plate. [b] Each assay was carried out in duplicate.

Table 3. Quantitative Plate Tests of Compound 1 plus Different Thiols in *S. typhimurium* Strain TA100 without Microsomal Activation[a]

sample	TA100 activity[b] (revertants/plate)
N-acetyl-L-cysteine (0.15 M)	148, 149
N-acetyl-L-cysteine (0.15 M) + **1**	153, 181
L-cysteine (0.15 M)	118, 126
L-cysteine (0.15 M) + **1**	130, 134
L-cysteine ethyl ester (0.15 M)	117, 139
L-cysteine ethyl ester (0.15 M) + **1**	102, 104
reduced glutathione (0.15 M)	113, 118
reduced glutathione (0.15 M) + **1**	107, 108
acetate buffer plus dioxane	121, 169
compound **1** (0.24 μg) + acetate buffer	>1200, >1200
methyl methanesulfonate (0.4 μL) (positive control)	>1200, >1200

[a] Samples were incubated in 2 M acetate buffer at pH 5 and 25 °C for 30 min in the dark. The initial concentration of tetrachloroimide **1** in all solutions was 2.4 μg/mL. For the Ames test, 0.1 mL of each solution was added to each plate. [b] Each assay was carried out in duplicate.

RESULTS AND DISCUSSION

Effectiveness of Structurally Different Nucleophiles. Figure 1 shows that the mutagenicity of compound **1** decreased rapidly in the presence of *N*-acetyl-L-cysteine (0.2 M), reaching the level of spontaneous reversion after about 30 min. The effect was similar to that observed in the change in the ultraviolet absorbance of compound **1** at 250 nm in the presence of *N*-acetyl-L-cysteine (Figures 2 and 3). The change in UV absorbance is associated with the reaction of the nucleophile with **1** to yield a nonmutagenic compound having a UV spectrum different from that of **1**. The time required to reduce mutagenic activity by 50% was about 5 min compared to 160 s to reach 50% reduction in UV absorbance, with a lower concentration of **1**. The effect of pH in the range 5–8 on the deactivation of compound **1** was minor (data not shown). A time study revealed that inactivation of the tetrachloroimide at 0.2

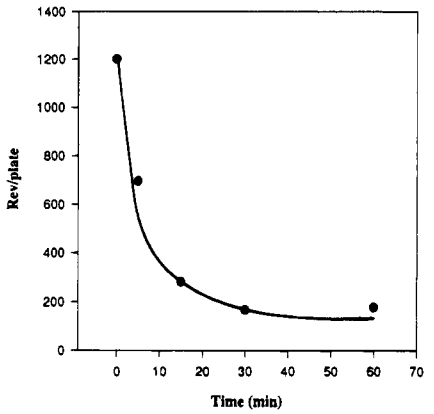


Figure 1. Effect of *N*-acetyl-L-cysteine on the mutagenic activity of the tetrachloroimide **1** as a function of time.

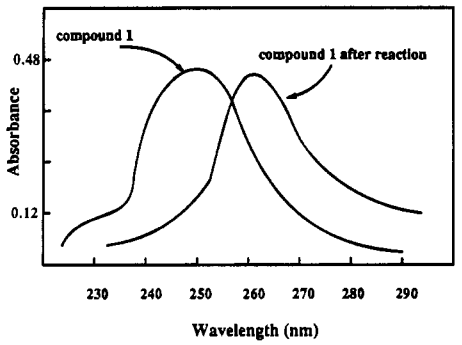


Figure 2. UV spectra of **1** in the absence and presence of *N*-acetyl-L-cysteine.

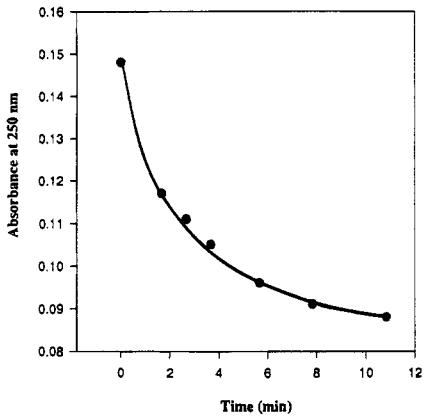


Figure 3. Effect of *N*-acetyl-L-cysteine on the UV absorbance of the tetrachloroimide **1**.

M concentration was essentially complete after about 1.5 h at pH 7. Inactivation increased with concentration in the range of 0.05–0.2 M (Tables 1 and 2). These results suggest that the reduction in mutagenic activity approximately parallels the extent of chemical modification of **1**.

These promising results prompted us to evaluate several other nucleophilic compounds for ability to suppress the mutagenic activity of the imide. Tables 3 and 4 show (a) L-cysteine, *N*-acetyl-L-cysteine, cysteine ethyl ester, reduced glutathione, and L-lysine were all effective in reducing the mutagenic potency of the imide to control levels and (b) glycine and aspartic acid were ineffective under the same conditions. A concentration study with the amino acid L-lysine (Table 2) shows that at pH 7 and 2 h of incubation time, substantial reduction in mutagenic activity is observed with 0.05 M L-lysine, with almost total elimination of mutagenicity at 0.1 M L-lysine concentration.

The observed effectiveness of aspartic acid, glycine, and lysine deserve further comment. The α-NH_2 group

Table 4. Effect of Sodium Sulfite, Sodium Bisulfite, L-Lysine, Glycine, and L-Aspartic Acid on the Mutagenic Activity of the Tetrachloroimide 1 in *S. typhimurium* Strain TA100 without Microsomal Activation[a]

test sample	TA100 activity[b] (revertants/plate)
sodium sulfite	123, 142
sodium sulfite + **1**	136, 141
sodium bisulfite	149, 152
sodium bisulfite + 1	162, 186
L-lysine	166, 181
L-lysine + **1**	178[c]
glycine	138, 173
glycine + 1	1468, 1597
L-aspartic acid	137, 154
L-aspartic acid + **1**	1485, 1532
acetate buffer control	159, 164
1 in acetate buffer	1778, 1920
methyl methanesulfonate (0.4 μL) (positive control)	>1200, >1200

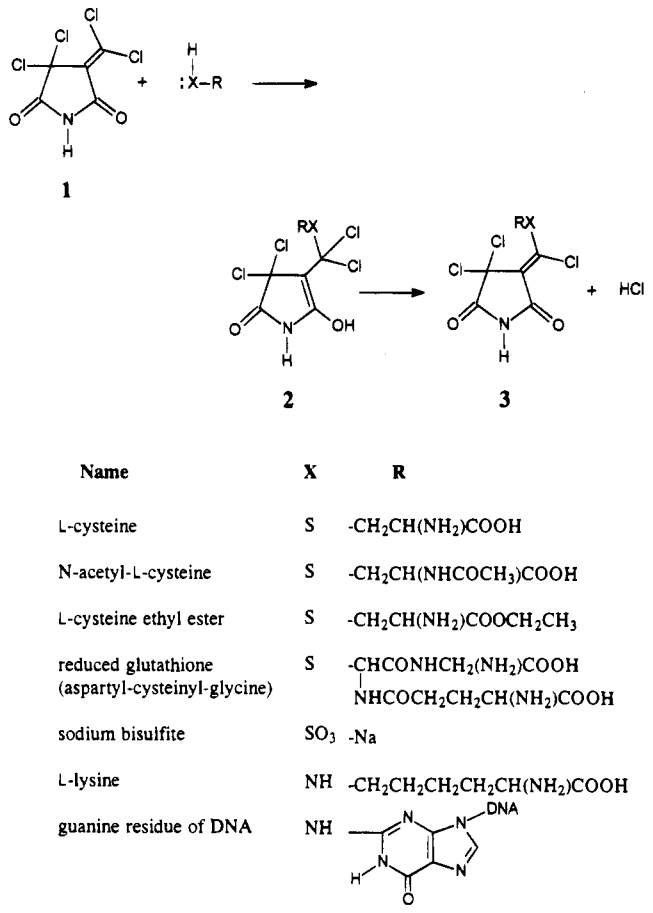
[a] Samples (0.2 M), with and without compound 1, were incubated in 2 M acetate buffer at pH 7 and 25 °C for 2 h in the dark. The initial concentration of tetrachloroimide **1** in all solutions was 2.4 μg/mL. For the Ames test, 0.1 mL of each solution was added to each plate. [b] Each assay was carried out in duplicate. [c] Second plate was contaminated.

of glycine (NH_2CH_2COOH) has a p*K* value near 8, as does the corresponding group of aspartic acid [HOOCCH-(NH_2)CH_2COOH]. In contrast, the ε-NH_2 group of lysine [$NH_2CH_2CH_2CH_2CH_2CH(NH_2)COOH$] has a pK value of about 10.5 (Eggum and Sorensen, 1989). If basicity of a functional group, as determined by its p*K* value, governs nucleophilic reactivity, then the ε-NH_2 of lysine probably participates with compound **1** in reactions resulting in loss of mutagenic activity. The α-NH_2 groups of aspartic acid, glycine, and lysine are probably not sufficiently basic or nucleophilic under the limited reaction conditions used to transform **1** to an inactive derivative.

Mechanism of Inactivation. At this time we can only speculate about possible mechanisms of reaction of the nucleophiles with compound **1**. Since the tetrachloroimide contains a conjugated carbonyl group with two chlorine atoms which increase its electrophilic character, one likely possibility involves an initial 1,4-addition of the nucleophile to the conjugated system of **1** to form **2** (Figure 4), with subsequent expulsion of a chloride ion to generate the postulated product **3**. The conjugated product **3** is consistent with the shift in λ_{max} in the UV spectrum after addition of nucleophiles to **1** (Figure 2). A more conventional 1,4-addition (Michael addition) would result in a nonconjugated system. Isolation and characterization of the postulated reaction product would confirm the proposed mechanism.

Since the two maxima in Figure 2 have the same absorbance, the extinction coefficient of the postulated derivative **3** is probably similar to that of the tetrachloroimide **1**. This observation also implies that the postulated product **3** also has a conjugated system of double bonds. However, the cited findings do not rule out addition, displacement, and reduction reactions of **1** other than those shown in Figure 4 as described elsewhere for chloranil, trichloroacetic acid, and trichloroethylene (Friedman, 1973; Friedman and Bautista, 1995; Weisleder and Friedman, 1968).

Implications for Safety and Health. Since our data suggest that the inactivation is not affected by the pH of solvent in the range 5–8, adjustment of the pH of chiller water may not be necessary to inactivate the mutagen. Since L-lysine is more stable to oxidation than are the SH-containing compounds, our findings suggest that the natural amino acid L-lysine deserves further



Name	X	R
L-cysteine	S	$-CH_2CH(NH_2)COOH$
N-acetyl-L-cysteine	S	$-CH_2CH(NHCOCH_3)COOH$
L-cysteine ethyl ester	S	$-CH_2CH(NH_2)COOCH_2CH_3$
reduced glutathione (aspartyl-cysteinyl-glycine)	S	$-CHCONHCH_2(NH_2)COOH$ $NHCOCH_2CH_2CH(NH_2)COOH$
sodium bisulfite	SO_3	-Na
L-lysine	NH	$-CH_2CH_2CH_2CH_2CH(NH_2)COOH$
guanine residue of DNA	NH	

Figure 4. Postulated mechanism of reaction between the nucleophile R–X–H and compound **1** leading to the displacement of a chloride ion and formation of compound **3**.

study to establish whether it or related compounds can effectively and economically reduce the mutagenic potential of small amounts of the tetrachloroimide and other mutagens that may be present in chiller water and other processing waters.

Although the inorganic sulfites are also effective in suppressing the mutagenicity of the imide, their use could be restricted unless it can be shown that sulfite is not absorbed by the poultry carcasses, since some individuals are sensitive to sulfites. However, passing chiller water through a column containing immobilized substrates with amino or sulfite side chains would preclude the possibility of these nucleophiles entering the finished product.

Our results also suggest the possibility of *in vivo* deactivation through cysteine- and lysine-containing dietary proteins and the tripeptide reduced glutathione, which circulates widely in the bloodstream of animals and humans (Friedman, 1994; Smolin and Benevenga, 1989). In addition, lysine-rich histones located next to DNA in chromatin fibers of cell nuclei (Darnell et al., 1989) may competitively bind the tetrachloroimide **1** and thus block its reaction with DNA and the possible consequent genotoxic effects. Finally, since this compound is essentially deactivated by S9 liver microsomes (results not shown), it is also likely that it may be enzymatically deactivated after consumption.

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