Potent Bacterial Mutagens Produced by Chlorination of Simulated Poultry Chiller Water

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Hypochlorite treatment of a simulated food-processing mixture produces 3,4-dichloromaleimide and 3,3-dichloro-4-(dichloromethylene)-2,5-pyrrolidinedione ($C_5HCl_4NO_2$). The tetrachloro compound and two analogs, which can be synthesized from citraconic anhydride and itaconic anhydride, are direct-acting Ames mutagens in *Salmonella typhimurium* TA100 tester strain. These novel five-carbon cyclic imides are structurally similar to 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), the principal mutagenic compound present in paper pulp bleaching liquors. Molecular structure analysis of the mutagens was based on X-ray crystallography, ^{13}C NMR, and mass spectrometry of synthetic chlorinated imides with identical mass spectra and gas chromatographic retention indices. The tetrachloroimide accounts for much of the mutagenicity of the dichloromethane-extractable pH 2 fraction from chlorination of a simulated food-processing system consisting of chicken frankfurters. In the Ames TA100 tester strain it has a molecular mutagenicity of 1450 revertants/nmol without microsomal activation, making it the second most potent mutagen reported from a chlorination process.

Keywords: *Poultry chiller water; bacterial mutagens; chlorine disinfection byproducts; chlorinated imides*

INTRODUCTION

The chlorinated organic compounds that are produced during the chlorination of drinking water have been extensively studied for over 15 years, and hundreds of disinfection byproducts of chlorination have been identified. From a toxicology standpoint, one of the more interesting aspects of these studies was the surprising discovery that a single compound, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX, **5**) can account for 30−50% of the bacterial mutagenicity of drinking water, as measured in the Ames/*Salmonella* assay, when chlorination is carried out in the presence of humic material (Holmbom et al., 1981; Maier et al., 1987; Holmbom, 1990). In food processing, chlorination is also widely employed. Chlorination has special importance as a food safety measure in the processing of the several billion chickens produced yearly in the United States. The chlorination of poultry chiller water is especially effective in minimizing the levels of *Salmonella typhimurium*, *Campylobacter jejunei*, and other bacterial pathogens (National Research Council, 1988).

Previous studies have established a dose-dependent Ames-mutagenic response for chlorination of poultry chiller water in several bacterial test strains (Masri, 1986). Although the levels of mutagenic activity are very low under actual plant conditions, identification of the specific bacterial mutagens in poultry chiller water would be desirable, both to aid in the risk assessment of the chlorination process and to evaluate more specifically the effect of processing variables, including the extent of water reuse. Alternatives to chlorine disinfection procedures for poultry have been described (Kim et al., 1994), but use of Cl_2, where the active chlorinating agent is hypochlorous acid, is currently the most widely used method.

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In poultry processing, carcass temperatures are reduced to a mandated value of 40 °F in a chiller bath to which chlorine is added as a disinfectant. This paper reports the results of a study to identify, at elevated chlorination levels and in a simulated processing mixture, compounds that may contribute to the observed bacterial mutagenicity of chlorinated poultry chiller water.

MATERIALS AND METHODS

Chlorination. Two types of chlorinated samples were investigated. Initially, we chlorinated water suspensions of homogenized chicken frankfurters because this mixture could be prepared easily and reproducibly. Once the mutagenic compounds had been identified, their presence was confirmed in chlorinated homogenates prepared from white and dark meats and skin of whole chicken. In both cases, artificially high levels of chlorine were employed to enhance the production of mutagens.

Stock hypochlorite solutions with about 4% available chlorine as hypochlorous acid were prepared by bubbling ultrapure Cl_2 (g) into 1.2 N NaOH until a rapid drop to pH 11 occurred. The solutions were stored until needed, at which time chlorine concentrations (free, combined available and total available chlorine) were determined by titration (Schade et al., 1990).

Chlorination of Chicken Frankfurter Homogenates. Commercial chicken frankfurters containing approximately 50% solids were purchased locally and frozen until used. Two frankfurters were slightly thawed and diced into approximately 1 cm cubes. The diced cubes and an equal weight of high-purity water (prepared with Milli-Q UV-*Plus*, Millipore S.A., Molsheim, France) were homogenized in a Model 17105 Omni mixer (OCI Instruments, Waterbury, CT) to yield a suspension consisting of about 25% solids. Chlorine stock solution was added to this suspension until the amount of chlorine was equal, in weight, to the frankfurter solids. The pH was then adjusted to 7.2. The mixture was allowed to stand overnight at room temperature with gentle shaking; after 12 h, the homogenate was filtered and adjusted to pH 11.

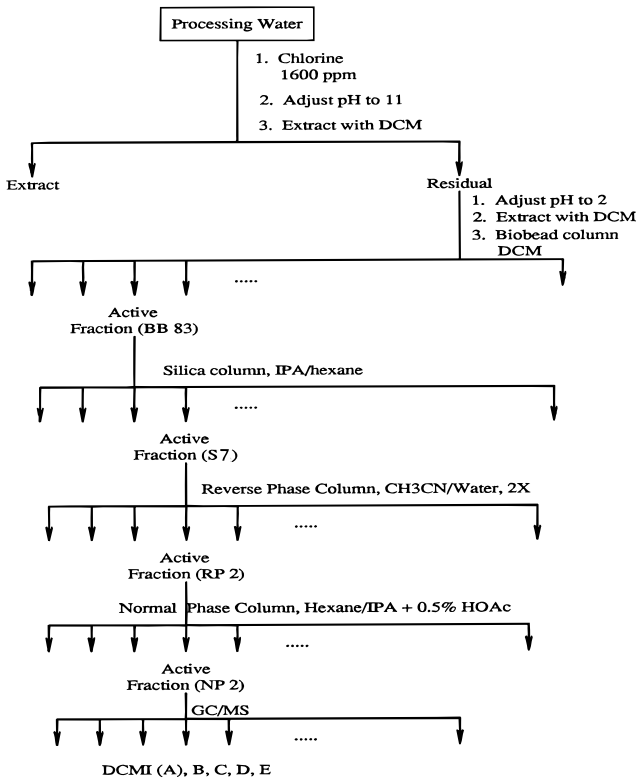

Figure 1. Procedure for extraction and fractionation of simulated poultry chiller water derived from chicken frankfurters or homogenized chicken. The designated fractions were active in TA100(−S9). DCM, dichloromethane; IPA, isopropyl alcohol; CH_3CN, acetonitrile; HOAC, acetic acid.

Chlorination of Chicken Homogenate. Whole chickens were obtained from a local commercial chicken processing plant, prior to immersion cooling (Tsai et al., 1987). Chickens were manually deboned, and the meats and skin, except the wings, tail, and abdominal fat, were mashed with a meat chopper (Model 84181D, Hobart Mfg. Co., Troy OH) until the mixture was homogeneous. Dry ice was added to maintain the mixture at 2−5 °C during chopping. About 20 g of the meat mixture and an equal weight of water were homogenized with the Omni mixer, and the homogenate was chlorinated in the same manner as for the frankfurter mixture. A portion of the homogenate was not chlorinated and served as a control.

Isolation of Mutagenic Fractions. Figure 1 shows the overall isolation scheme of the mutagenic material from chlorinated simulated poultry chiller water. Ames/*Salmonella* assays were carried out after each extraction and on all chromatographic fractions. The pH 11 filtrates from chlorination were extracted three times with 0.2 volume amounts of reagent grade dichloromethane (DCM); these were designated alkaline extracts and were saved. The aqueous phase was acidified to pH 2 and again extracted with DCM as above. The extracts were combined and designated as the DCM-extractable acidic fraction (Schade et al., 1990). A control sample was extracted in the same manner and found to be nonmutagenic by Ames assay.

Molecular Size Chromatography. The pH 2 fraction of the original chlorination mixture was chromatographed in eight equal portions on a molecular size exclusion column (Biobeads S-X1, 5 cm × 100 cm) using DCM at 4 mL/min. The effluent was monitored at 254 nm, and 20 mL fractions were collected. Mutagenic activity appeared in fractions designated BB81-86 eluting between 6 h 10 min and 7 h 30 min.

Silica Gel Chromatography. Fraction BB83 was subsequently chromatographed on a normal phase column (silica gel, 10 mm × 250 mm) with hexane/isopropyl alcohol (99:1) at 5 mL/min. The most Ames-active fraction, designated S7, eluted between 6 and 7.5 min. There was some additional mutagenicity seen in a relatively nonpolar fraction eluting at the beginning of the separation. This early eluting material has not yet been characterized.

Reversed Phase HPLC. Fraction S7 from silica chromatography was chromatographed on an HPLC column (Zorbax, C8, 250 mm × 21.2 mm) with 20 mL/min of H_2O/CH_3CN in a linear gradient from 70:30 to 10:90, over 20 min, and held at the final concentration for 10 min. The active fraction (7−9 min) was diluted with an equal volume of water and extracted seven times with DCM. After the extract was concentrated, the material was again chromatographed on the same column at 16 mL/min with a linear gradient of H_2O/CH_3CN from 80:20 to 10:90, over 30 min, and held at the final concentration for 10 min. The active fraction, designated RP2, eluted between 11 and 13 min and was again diluted with an equal volume of water and extracted with 7 portions of DCM. The extract was concentrated by removing the DCM with a stream of dry nitrogen, redissolved in 150 μL of DCM, and analyzed by combined gas chromatography−mass spectrometry (GC−MS).

Normal Phase HPLC. Fraction RP2 was chromatographed on a normal phase column (silica, 4.5 mm × 250 mm) with 1 mL/min of hexane/isopropyl alcohol in a linear gradient from 98:2 to 94:6, over 12 min, and held at the final concentration for 3 min. Each solvent reservoir contained 0.5% acetic acid. The active fraction NP1 eluted between 7.6 and 8.6 min. The solvent was removed by freeze-drying in a Savant Speed Vac concentrator until dry. A portion was taken for Ames testing, and the remainder was dissolved in DCM and a portion taken for GC−MS.

GC−MS. GC−MS analyses were carried out on a VG 7070/HS mass spectrometer (Fisons Ltd., Manchester, England) and HP 5830 gas chromatograph (Hewlett-Packard, Palo Alto, CA) using 0.25 mm i.d., 0.25 μm, 30 m DB-5 or DB-5MS (5% phenyl, 95% methyl silicone) capillary columns (J&W Scientific, Folsom, CA) with splitless injection. Electron ionization (EI) spectra were recorded at 70 eV ionizing voltage. For negative ion mass spectra we used electron capture negative ionization (ECNI) with methane reagent gas. Retention index values were assigned by interpolating between the retention times of *n*-alkane standards. All GC runs used linear temperature programs, usually 3 °C/min from 60 to 280 °C, and splitless injection at 220 °C. The ion source temperature was 180 °C, and transfer lines to the mass spectrometer were maintained at 230 °C. Samples were usually concentrated 10-fold prior to GC injection.

Preparation of Chlorinated Imides. Synthetic mixtures were examined by gas chromatography with a 30 m, 0.25 μm DB-5 column (J&W Scientific). The reported percent compositions for synthetic mixtures analyzed by GC are not precise as GC response factors were unknown and the response was often nonlinear with respect to the amount of sample injected. See Figure 2 for structures. UV spectra were determined in 1,4-dioxane using an HP diode array spectrometer (Model 8451A, Hewlett-Packard).

CAUTION: During the chlorination reactions, Cl_2 and HCl may vent from the reaction container.

*3,4-Dichloro-1H-pyrrole-2,5-dione (3,4-Dichloromaleimide, DCMI) (**1**).* The method of Relles (1972) was used to prepare dichloromaleic anhydride. Initial crystallization from heptane gave product with mp 117.5−120.0 °C. Sublimation and resublimation provided pure product: mp 119.3−120.3 °C [lit. mp 118−120 °C (Relles, 1972)]; ^{13}C NMR ($CDCl_3$) δ 135.7 (C-3, 4), 157.9 (C-2, 5). Dichloromaleic anhydride was converted to dichloromaleimide by heating with urea and NaCl at 120 °C for 20 min (Chow and Naguib, 1984). The reaction mixture was partitioned between ether and water. The ether solution was dried with sodium sulfate, treated with decolorizing carbon, filtered, and concentrated. Addition of hexane and boiling out of some ether led to crystallization of dichloromaleimide: mp 175.0−177.0 °C [lit. mp 174−177 °C (Chow and Naguib, 1984)]; ^{13}C NMR ($CDCl_3$) δ 134.1 (C-3, 4), 162.0 (C-2, 5).

*3-(Dichloromethylene)-2,5-pyrrolidinedione (Compound **B**) (**2**).* Hydrogenation of 5.5 mg of compound **E** (4) (vide infra) in benzene solution with 7 mg of 5% rhodium on charcoal catalyst yielded 4.0 mg of white solid with RI of 1515 on DB-5: ^1H NMR ($CDCl_3$) δ 3.47 (s, 2H), (CH_2), 8.19 (s, 1H); ^{13}C NMR

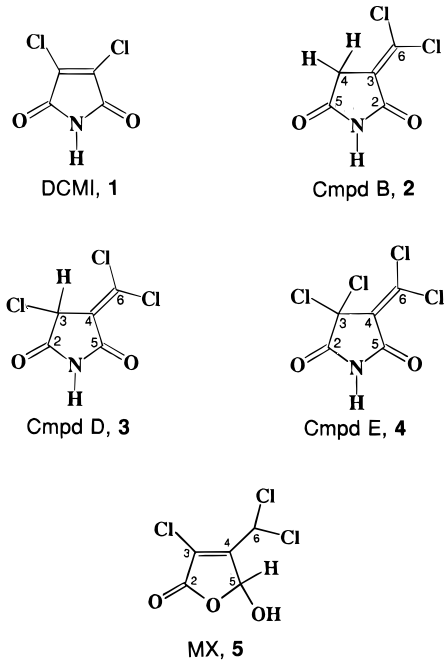

Figure 2. Chemical structures of Ames-active chlorinated imides **1**−**4** from poultry chiller water and of MX (**5**), previously isolated from paper bleach liquors and from water that has been chlorinated in the presence of humic material (Holmbom, 1981, 1990).

($CDCl_3$) δ 37.2, 124.2, 131.6, 164.4, 170.2. After preparation of a larger amount, the compound was dissolved in ether and diluted with either CCl_4 or benzene. After evaporation of the ether, 3-(dichloromethylene)-2,5-pyrrolidinedione crystallized; mp 192.0−192.5 °C; UV λ_{max} 242 nm (14 200).

*3-Chloro-4-(dichloromethylene)-2,5-pyrrolidinedione (Compound **D**) (**3**).* Itaconic anhydride was chlorinated as described by Schreiber et al. (1961). Vacuum distillation of the product gave a mixture containing chloromethylmaleic anhydride and, as indicated by GC−MS, an isomeric compound that is most likely 3-chloromethylenesuccinic anhydride. The mixture was refluxed at 150 °C under vacuum for an hour and then chlorinated as before. These procedures were repeated until a mixture containing anhydrides with three or four chlorine atoms was obtained.

Two grams of anhydride mixture, 5 g of urea, and 5 g of NaCl were stirred with a spatula for 15 min at 125 °C. Product was partitioned between ethyl acetate and water. Solvent was removed from the ethyl acetate extract, and the residue was taken up in ether and filtered through a 1 cm column of oven-dried silica gel H in a 15 mL filter funnel. Ether was evaporated, and the 0.9 g of yellowish liquid residue was dissolved in CCl_4 and applied to a 15 mm silica gel H column and eluted with 10 mL portions of CCl_4, benzene, and ether. After removal of benzene from the benzene eluate, there was 0.2 g of liquid in which crystals formed in a few days. The crystals were washed with CCl_4, dissolved in ether, and recrystallized from CCl_4 after evaporation of ether to yield 20 mg of white solid. A sample recrystallized from benzene had mp 153.0−154.5 °C, UV λ_{max} 246 nm (14 200), and RI of 1645 on DB-5: ^{1}H NMR ($CDCl_3$) δ 5.09 (s), δ 8.86 (bs); ^{13}C NMR ($CDCl_3$) δ 51.8, 125.7, 139.6, 162.6, 168.1.

*3,3-Dichloro-4-(dichloromethylene)-2,5-pyrrolidinedione (Compound **E**) (**4**).* Citraconimide was synthesized according to the procedure of Earl et al. (1978) from citraconic anhydride and ammonium acetate in acetic acid. Product recrystallized from CCl_4 was a white solid: mp 104.5−105.3 °C [lit. mp 103.5−105.5 °C (Earl et al., 1978)]; ^{1}H NMR ($CDCl_3$) δ 2.07 (d, 3H, J = 2 Hz), 6.35 (t, 1H, J = 2 Hz), 8.19 (bs, 1H); ^{13}C NMR ($CDCl_3$) δ 10.7, 128.3, 148.9, 171.1, 172.1; RI on DB-5, 1084.

Citraconimide was chlorinated (Earl et al., 1978) to provide 3,4-dichloro-3-methylsuccinimide, which reacted with hot water to yield 3-chlorocitraconimide. Pure compound was obtained via sublimation and crystallization from toluene, mp 149.0−149.8 °C [lit. mp 147−148.5 °C (Earl et al., 1978); 142−154 °C (Hine et al., 1988)]; ^{1}H NMR ($CDCl_3$) δ 2.08 (s, CH_3), 7.58 (bs, NH); ^{13}C NMR ($CDCl_3$) δ 9.0, 134.4, 138.8, 164.7, 168.4; RI on DB-5, 1228.

A saturated solution of Cl_2 in 55 mL of CCl_4 was added to a solution of 8.3 g of 3-chlorocitraconimide in 115 mL of CH_2Cl_2, and the mixture was put in indirect sunlight on the laboratory roof for 1.5 h. At hourly intervals thereafter, 3.5, 4.5, and 3.7 g of Cl_2 was added and the mixture was put in direct sunlight. Evaporation of solvent left 14.2 g of white semisolid, seemingly 88% pure by GC (RI of 1470 on DB-5). A portion dissolved in hot heptane upon cooling deposited 93% pure 3-methyl-3,4,4-trichloro-2,5-pyrrolidinedione: ^{1}H NMR ($CDCl_3$) δ 2.02, 8.23; ^{13}C NMR ($CDCl_3$) δ 20.4, 71.1, 84.3, 165.6, 168.8.

A solution of 88% 3-methyl-3,4,4-trichloro-2,5-pyrrolidinedione in CCl_4 along with 35% molar excess of Cl_2 was exposed to direct sunlight (HCl evolution). Over the course of 5 days, more Cl_2 was added to the mixture and the chlorination reaction was continued until less than 20% of the original compound remained unreacted. After CCl_4 was removed, the reaction product was dissolved in ethyl acetate and extracted with dilute NaOH solution. The alkaline solution was acidified with HCl and extracted to provide a mixture containing compound **E** (**4**), DB-5 RI 1717. Multiple chromatographies on a 2 cm column of silica gel H in a 15 mL filter funnel with eluting solvent sequence CCl_4, benzene, and ether gave a 80−85% concentrate of **E**, which was dissolved in ether and diluted with CCl_4. After evaporation of ether, crystals formed. Several recrystallizations yielded 3,3-dichloro-4-(dichloromethylene)-2,5-pyrrolidinedione: mp 174.0−175.0 °C; UV λ_{max} 250 nm (13 500); ^{1}H NMR ($CDCl_3$) δ 8.41; ^{13}C NMR ($CDCl_3$) δ 73.7, 127.9, 144.1, 159.6, 164.9.

X-ray Crystallographic Analysis. Tables of anisotropic thermal parameters with their estimated standard deviations for the non-hydrogen atoms and observed−calculated structure factors have been deposited at the Cambridge Crystallographic Data Center.

*Crystal Data of **D** (**3**):* $C_5H_2Cl_3NO_2$, M = 214.4, monoclinic, space group $P2_1/c$, a = 6.929 (1), b = 5.837 (4), c = 19.911 (2) Å, β = 98.77 (2)°, V = 795.9 Å^3, Z = 4, $F(000)$ = 424, μ(Cu Kα) = 102.8 cm^{-1}, and D_{calc} = 1.79 g cm^{-3}; final R = 0.056 (100 parameters), R_w = 0.067 for 978 unique reflections, the final average parameter shift is $\pm 0.01\sigma$, and the difference Fourier synthesis excursions are within $\pm$0.4 Å^{-3}.

*Crystal Data of **E** (**4**):* $C_5HCl_4NO_2$, M = 248.9, monoclinic, space group $P2_1/c$, a = 6.455(1), b = 17.754(4), c = 7.697(2) Å, β = 103.30(2)°, V = 858.4 Å^3, Z = 4, $F(000)$ = 488, μ(Cu Kα) = 125.3 cm^{-1} and D_{calc} = 1.93 g cm^{-3}; final R = 0.054 (109 parameters), R_w = 0.062 for 1039 unique reflections, the final average parameter shift is $\pm 0.01\sigma$, and the difference Fourier synthesis excursions are within $\pm$0.3 Å^{-3}.

*Data Collection and Structure Refinement for **3** and **4**.* Single crystals of both compounds suitable for X-ray investigation were obtained by slow evaporation from a mixture of ether and CCl_4. Intensity data were measured in the range of (3° ≤ 2θ ≤ 114°) on a Nicolet R3 diffractometer with graphite monochromatized Cu Kα radiation (λ = 1.5418 Å) by the θ−2θ scan technique with variable scan speed (4−30° min^{-1}) at room temperature. The lattice constants were refined by least-squares fit to setting angles of 20 independent reflections (10° ≤ 2θ ≤ 25°) measured on the diffractometer. Two standard reflections were monitored periodically for crystal and instrument stability; no significant change in their intensities was noted during the course of the experiment. The intensity data were corrected for background Lorentz polarization effects, and absorption by the empirical method, but not for secondary extinction. The crystal structure was solved by direct methods and refined by a blocked-cascade full-matrix least-squares procedure. The function minimized was $[\sum(\omega|F(\text{obs})| - |F(\text{calcd})|)^2]$, where $\omega = [\sigma^2|F(\text{obs})| + 0.001|F(\text{obs})|^2]^{-1}$. Unique reflections with the criteria of ($|F(\text{obs})| \geq 3\sigma|F(\text{obs})|$) were included in the structure refinement calculation. Scattering factors were from the *International Tables for X-ray Crystallography* (1974); those of nitrogen, oxygen, and chlorine were

corrected for anomalous dispersion. Atomic parameters of all non-hydrogen atoms were refined anisotropically, and the hydrogen position was located in subsequent difference Fourier maps and included with invariant idealized values in the respective structure-factor calculation. All structure computations were accomplished with the SHELXTL (Sheldrick, 1981) program package.

Nuclear Magnetic Resonance Spectroscopy. NMR spectra were run in $CDCl_3$ with TMS as an internal standard on a Bruker ARX400 spectrometer operating at 100.62 MHz for carbon and 400.13 MHz for proton. One pulse experiments were run for both nuclei at a 22° pulse for carbon at a 2.3 s repetition rate and at a 90° pulse for proton at a 7−8 s repetition rate. Carbon−carbon connectivity studies of **4** were conducted using a solution of **4** (23 mg in $CDCl_3$) with 1 mg (0.0029 mmol) of chromium acetylacetone. A one-pulse experiment was run with a 59° pulse at a 1.57 s repetition rate for 572 217 scans. The one-pulse experiment was utilized rather than an INADEQUATE experiment because of its increased sensitivity. A relaxation reagent, Gd[fod]$_3$, was added to decrease the relaxation times of the carbons, so that the one-pulse experiment could be repeated with no relaxation delay.

Mutagenesis Assays. Samples were tested for mutagenicity by the Ames/*Salmonella* assay, using the standard plate incorporation method (Maron and Ames, 1983). *S. typhimurium* strain TA100, without S-9 mix (−S9), was chosen for these assays on the basis of its reported sensitivity to mutagens in poultry chiller water (Masri, 1986). Dose−response curves for the test compounds were determined from four concentrations in the linear range, selected by preliminary range-finding experiments. Positive control plates contained 0.4 μL/plate methyl methanesulfonate. Because of apparent increased stability of test solutions, 1,4-dioxane was used as solvent for final measurements on synthesized compounds; ethanol was used as solvent to follow mutagenicity during chromatographic purification. Positive mutagenic response was confirmed by duplicate plating in the case of DCMI, and triplicate plating for the other imides. Mean molar mutagenicities, expressed as revertants per nanomole, were calculated from the slopes of the dose−response curves as determined by linear regression.

CAUTION: Several of the compounds described in this work, particularly compound E (4), have been shown to be highly mutagenic in TA100 without microsomal activation. Until these compounds have been evaluated for possible carcinogenicity and other biological activity, we recommend that precautions be exercised in their handling and disposal. Compounds D (3) and E (4) may be deactivated by treatment with aqueous solutions of sodium bisulfite.

Chemicals. Dichloromethane, acetonitrile, isopropyl alcohol, and hexane used in the chromatographic separations were all of spectroscopic grade. The water was purified by reverse osmosis and filtered through a biofilter before use.

RESULTS

Identification of 3,4-Dichloromaleimide (DCMI, 1). GC−MS analysis of the reversed phase HPLC mutagenic fraction, RP2, revealed a complex mixture, represented by the partial TIC chromatogram of Figure 3A. Several chlorinated components could be identified from their mass spectra, including 2-chlorocyclohexanol (scan 411, RI 1019) and pentachlorophenol (RI 1747). Authentic 2-chlorocyclohexanol having the same mass spectrum and RI was nonmutagenic in TA100 and was suspected to be an impurity in the solvents (Iwaoka et al., 1994). The occurrence of a compound identified from its mass spectrum (Figure 3B) as 3,4-dichloromaleimide at scan 818, RI 1290, was notable. Its identity was confirmed by comparison with synthetic DCMI, which gave the same mass spectrum and RI. Although the mutagenicity of **1** was too low to account for the observed mutagenicity of the simulated chiller water,

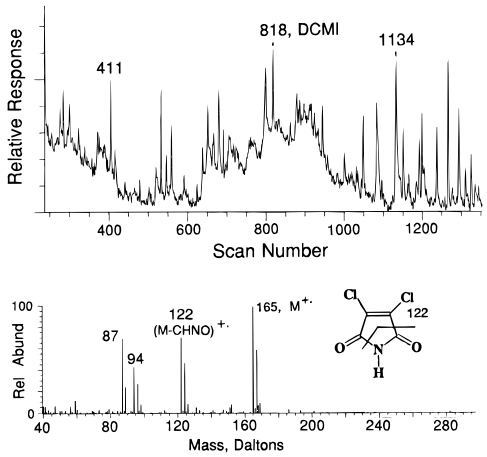

Figure 3. (A, top) Total ion current (TIC) chromatogram from GC−MS of Ames-active fraction RP2 from reversed phase chromatography of simulated poultry chiller water from chicken frankfurters (see Figure 1). (B, bottom) Electron ionization spectrum from peak at scan 818, RI 1299, identified as 3,4-dichloromaleimide (**1**). GC retention time is scan number × 1.957 s/scan.

there were several compounds, of possibly related structure, present at higher retention index, including a compound with four chlorines at scan 1134. The known electrophilic behavior of **1** (Lynch and Crovetti, 1972), its use as a reagent for determination of protein thiol and amino groups (Smith, 1987), and its patented use as an insecticide and acaracide in Japan increased our interest in these related compounds and suggested that additional purification of fraction RP2 should be carried out.

Presence of Five-Carbon Chlorinated Imides. *Normal Phase HPLC Separation.* Normal phase chromatography of RP2 on silica (hexane/isopropyl alcohol) gave a broad peak at 7.20 min with mutagenic activity. Acidifying the hexane/IPA with 0.5% acetic acid improved the chromatographic separation, and ultimately we obtained several well-defined chromatographic peaks, as shown in Figure 4A. The Ames-active fraction was under the peak at 7.90 min (λ_{max} 249 nm). Our collected fraction NP1 (7.6−8.6 min) contained all of the Ames activity of the mixture; it and the adjacent nonmutagenic fraction NP2 (8.6−9.7 min) were subjected to GC−MS, yielding the TIC chromatograms of Figure 4B,C. DCMI was present in NP1 at scan 561 (RI 1299).

Mass Spectra of Five-Carbon Chlorinated Imides. In fraction NP1 three peaks had mass spectra resembling the spectrum of DCMI, and these components were designated compounds **B** (scan 802, RI 1511), **D** (scan 940, RI 1640), and **E** (scan 1019, RI 1717). The corresponding mass spectra are shown in Figure 5A−C. Isotope patterns around *m/z* 136 (**B**), 170 (**C**), and 204 (**E**) suggested the presence of two, three, and four chlorine atoms, respectively. For **E**, we assigned the 4-Cl isotopic cluster at *m/z* 204 to the M − CHNO ion, analogous to the *m/z* 122 peak of DCMI. Loss of CHNO is characteristic in the mass spectra of unsaturated cyclic imides (Mitchell and Waller, 1970). These compounds were completely absent in the adjacent inactive HPLC fraction NP2 shown as the TIC trace in Figure 4C. Compound **C** (spectrum not shown) appeared as a shoulder on the trailing edge of **D** and gave a mass spectrum similar to that of compound **B**. We believe that **B** and **C** arose from decomposition of **D** in the injection port and transfer line into the mass spectrometer. When NP1 was introduced into the mass spec-

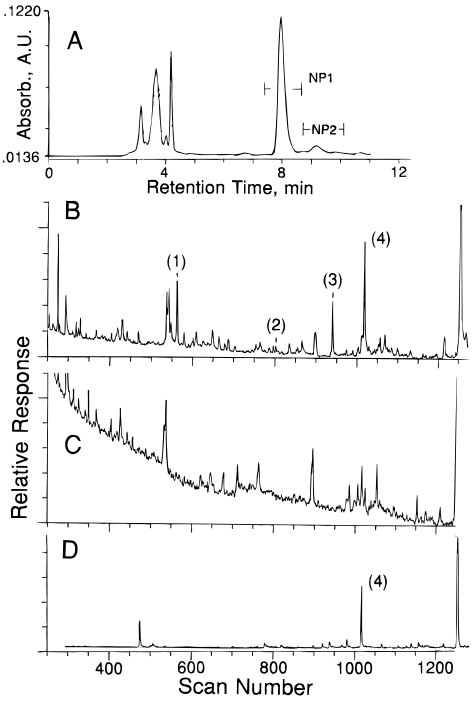

Figure 4. (A) UV chromatogram (254 nm) from normal phase HPLC separation of TA100-active fraction RP2 from simulated chiller water from chicken frankfurters. (B) TIC chromatogram from GC−MS of TA100-active fraction NP1 from normal phase HPLC. (C) TIC chromatogram for adjacent inactive fraction NP2. (D) TIC for equivalent active fraction NP1 from simulated chiller water based on homogenized chicken. The peak at scan 1254 is dibutyl phthlate. GC retention time is scan number × 2.162 s/scan.

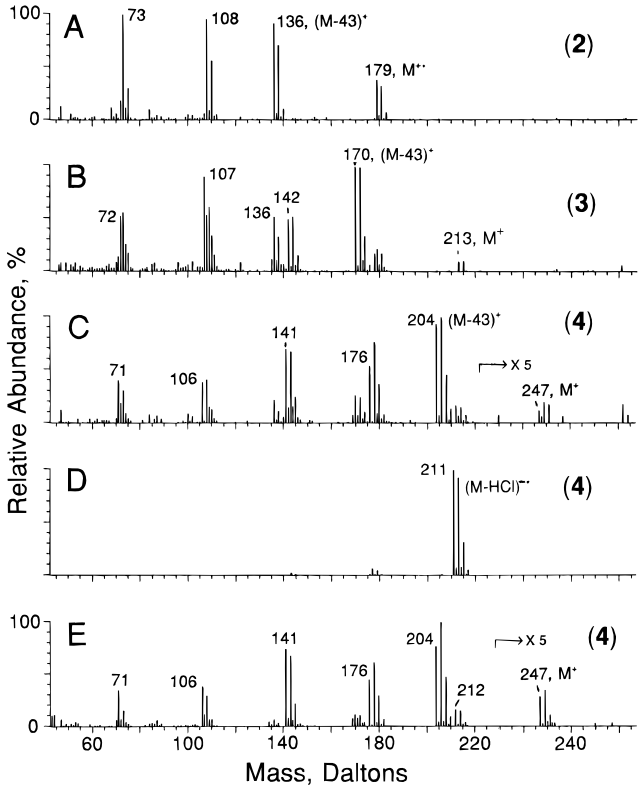

Figure 5. (A−C) Positive EI mass spectra (70 eV) of five-carbon chlorinated imides from GC−MS of fraction NP1; compound **B** (scan 802 of Figure 4B, RI 15.11); compound **D** (scan 940, RI 16.40); and compound **E** (scan 1019, RI 1717). (D) ECNI mass spectrum of compound **E** from fraction NP1. (E) Reference EI mass spectrum of synthetic 3-dichloro-4-(dichloromethylene)-2,5-pyrrolidinedione (**4**), RI 1715. All retention index values are for a DB5-MS GC column (see Materials and Methods).

trometer without GC separation via the direct insertion probe, compound **E** appeared to be the only chlorinated imide, thus indicating that **D** may also be an artifact; DCMI may not have been detected in the direct probe experiment because of its greater volatility.

For **E**, accurate mass measurement of the weak (0.5% relative abundance) ions at *m/z* 247 (± 10 ppm accuracy) was consistent with $C_5HCl_4NO_2$ for the composition of M^+, but we could not unambiguously assign the molecular formula at this level of accuracy. Negative ion mass spectra provided additional confirmation for the putative molecular weight of 247 for **E**; thus, in its ECNI spectrum, Figure 5D, we assigned the 3-Cl isotopic cluster at *m/z* 211 to $(M - HCl)^-$. Similar arguments suggested that **B** and **D** were 2- and 3-Cl imides with monoisotopic molecular weights of 179 and 213, respectively.

Chlorinated Imides in Chicken Homogenate, Simulated Processing Water. Chlorination of the simulated processing water from homogenized chicken showed the same mutagenicity profile during the extraction and purification. The TIC chromatogram of Figure 4D represents the mutagenic fraction from the normal phase HPLC separation, equivalent to fraction NP1. Compound **E** was easily detected in the sample, but compounds **B** and **D** were less prominent. These results provided further evidence that **B** and **D** may be artifacts of the analytical procedures used to isolate them.

Synthesis and Structure Analysis of Compounds B, D, and E. The mass spectra did not allow assignment of the double-bond position and thus failed to distinguish between the various chlorine-containing imides that could be derived from itaconic, citraconic, or glucaconic imides. Therefore, we prepared synthetic samples of the suspected mutagens. After several reactions starting from citraconic or itaconic anhydrides, we obtained chlorinated imides with identical mass spectra and retention indices for **B**, **D**, and **E** of 1506, 1642, and 1711, respectively. The mass spectrum of synthetic **E** is shown in Figure 5E. Synthetic samples of compounds **D** and **E** were highly mutagenic in TA100.

*X-ray Crystallography of Compounds **D** and **E***. Single-crystal analyses unequivocally established that compounds **D** (**3**) and **E** (**4**) have the 4-(dichloromethylene)-2,5 pyrrolidinedione structures indicated in Figures 2 and 6. These two compounds, and compound **B** (**2**), whose structure was assigned from NMR, retain the exocyclic dichloromethylene group and are therefore chlorinated imides of itaconic acid. The five-membered ring for both **3** and **4** is planar with a maximum deviation of ±0.01 Å, suggesting that the ring nitrogen may have sp^2 hybridization. Preliminary molecular orbital calculations for **4** (data not shown) also indicated a planar ring for the minimum energy structure. These results are in agreement with force field calculations and X-ray crystallographic analysis of the structurally similar α-chloro and α,α-dichloro-3-(chloromethyl)-2-pyrrolidinones (as their *N*-tosylates) (Rachita and Slough, 1993). Although the crystal structure of **5** has not been determined, single-crystal analysis of the MX dimer showed that it was nearly planar (LaLonde et al., 1990).

^{13}C NMR of Chlorinated Imides. Attempts to ascertain the structure of **3** and **4** based on NMR correlation data proved to be difficult since it was apparent from an examination of published ^{13}C NMR spectra that the chemical shifts of the vinyl carbons would not indicate the extent of chlorine substitution. However, for chlorine-

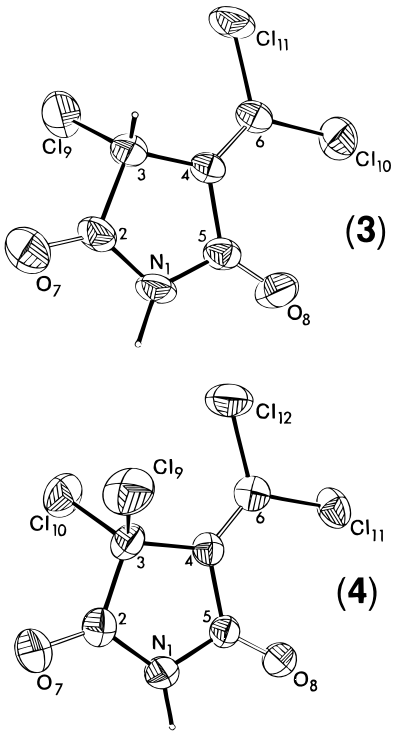

Figure 6. Molecular structures for 3-chloro-4-(dichloromethylene)-2,5-pyrrolidinedione (**3**) and 3,3-dichloro-4-(dichloromethylene)-2,5-pyrrolidinedione (**4**) determined from X-ray crystallographic analysis.

substituted aliphatic carbons, the chemical shifts of mono-, di-, and trisubstituted carbons fall within distinct ranges. The substituted carbon in monochloroalkanes has a shift in the range δ 40−50 (δ 40.2 for chloroethane, δ 47.0 for 1-chloropropane, δ 44.6 for 1-chlorobutane), in dichloroalkanes in the range δ 70−80 (δ 69.3 for 1,1-dichloroethane, δ 75.0 for 1,1-dichloropropane, δ 71.0 for 1,1-dichloro-3,3-dimethylbutane), and in trichloroalkanes in the proximity of δ 95 (δ 95.0 for 1,1,1-trichloroethane). From the position of the chemical shift of the aliphatic carbon (δ 73.7) for **4**, it is apparent that the compound has a dichloro aliphatic carbon rather than a trichloro substituted carbon. These data thus favor an exocyclic double bond for compound **4**.

To confirm the ^{13}C NMR assignments for the chlorinated carbons, an attempt was made to establish carbon−carbon connectivity. However, the relaxation times of the nonprotonated carbons in these compounds were exceedingly long, making it difficult to obtain carbon NMR spectra. Because of this, a one-pulse experiment to look at the one-bond C−C couplings was chosen for its increased sensitivity, rather than an INADEQUATE experiment to establish the carbon−carbon connectivity for the tetrachloroimide **4**. From the observed couplings (Table 1) it was possible to establish, unambiguously, the connectivity in agreement with structure **4** of Figure 2 and hence the shift assignments.

Mutagenicity in TA100. Compound **E** (**4**) was the most potent mutagen of the synthetic chlorinated imides, giving positive response in Ames/*Salmonella* strain TA100 without microsomal activation. This activity was substantially decreased by S9 microsomes (data not shown). Table 2 compares the mutagenic response of the four Ames-active chloroimides. Under the criteria of Prival and Dunkel (1989) **3** and **4** are direct-acting mutagens with molar mutagenicities of 7.7 and 1450 revertants/nmol, respectively. Assessment of 1 as mutagenic was problematic, as the dose−response was nonlinear above 1 μg/plate and definitely toxic at 2.5 μg/plate. Under the more liberal criteria of Maron and Ames (twice the spontaneous revertants in the linear range), all four chloroimides are mutagenic. The assigned molar mutagenicities (Table 2) for **1** and **2** were 28 and 0.24 revertants/nmol, respectively. Above 200 μg/plate, **2** was toxic in TA100. For compound **E** the mutagenic response in 1,4-dioxane was about twice that observed when ethanol was used as solvent. In ethanol, **4** gave 800 revertants/nmol on the basis of two independent dose−response assays (data not shown).

Table 1. ^{13}C CMR Shifts ($CDCl_3$) and $^1J_{cc}$ Observed for 4

C	shift (ppm)	$^1J_{cc}$ (Hz)	connectivity
3	73.7	53	3−4
		56	3−2
4	127.8	52	3−4
		66	4−5
		102	4−6
6	144.2	102	4−6
5	160.1	66	4−5
2	165.3	56	3−2

DISCUSSION

The purpose of this work was to identify chemical structures of mutagens that occur when chlorination is carried out in poultry chiller water at levels higher than those which are usually employed in poultry processing plants. Previous work showed mutagenic activity was generated in poultry chiller water, containing 1160 ppm of total solids, treated with chlorine at 1000 ppm (Masri, 1986). Similarly, this study utilized a 1:1 ratio of total solids to chlorine, but, to facilitate the isolation of active compounds, the total solids content was increased to 25%. The use of homogenized chicken frankfurters allowed us to carry out the chlorination reaction on a mixture that simulates the physical and chemical conditions in food processing plants but has defined and reproducible composition and history. The subsequent experiment with unchlorinated chickens provided an important confirmation of the occurrence of **4**. However, evidence suggests that other mutagens are produced by chlorination, and one cannot assume that the relative amounts of different chlorine disinfection byproducts would be independent of the extent of chlorination (Masri, 1986). While the identification of the highly mutagenic compound **4** is significant, it will be important to reduce the chlorination level to assess its possible contribution to mutagenic activity under more realistic simulated processing conditions.

Compound **E** (**4**) is a second example, the first being MX (**5**), in which a significant amount of mutagenic activity of a very complex mixture of chlorination byproducts can be attributed to a single compound (Holmbom et al., 1990). At 1450 revertants/nmol in TA100, the mutagenic potency of **4** in TA100 approaches that of MX (5000 revertants/nmol), making it the second most potent bacterial mutagen from chlorination so far reported in the literature (LaLonde et al., 1991b). For MX and its structural analogs, the propensity to undergo electrophilic reactions appears to be the best predictor of mutagenic potency (LaLonde et al., 1992; Tuppurainen et al., 1992). The activity of **4** is therefore in accord with the known electrophilic reactivity of **1**

Table 2. Mutagenicity of Synthesized Chlorinated Imides in *S. typhimurium* TA100[a]

compound	dose, μg/plate (nmol/plate)	revertants/plate	molar mutagenicity [slope (rev/nmol)]	r^b
DCMI[c]	1 (6)	351; 365	28	0.975
	0.5 (3)	244; 254		
	0.125 (0.75)	233; 201		
solvent control	(0)	179; 178		
B[d]	125 (694)	280; 318; 325	0.24	0.971
	50 (278)	190; 230; 218		
	10 (56)	142; 151; 128		
D	10 (47)	451; 533; 513	7.7	0.987
	5 (23)	337; 344; 347		
	1 (4.7)	188; 153; 165		
E	0.1 (0.4)	687; 590; 623	1450[e]	0.95[e]
	0.025 (0.1)	260; 295; 278		
solvent control	(0)	152; 148; 148		
E[f]	0.1 (0.4)	806; 875; 920		
	0.05 (0.2)	451; 514; 515		
	0.025 (0.1)	383; 333; 361		
solvent control	(0)	193; 221; 158		

[a] The positive control, methyl methanesulfonate, gave >1200 revertants/plate in all assays at 0.4 μL/plate. [b] Correlation coefficient from linear regression. [c] Assay 1. [d] Assay 2. [e] Data combined from assays 2 and 3. [f] Assay 3.

toward thiol and amino groups (Lynch and Crovetti, 1972; Smith, 1987). The structural similarity between **4** and **5** is noteworthy.

In view of the very low occurrence of **4**, it was not possible to isolate sufficient quantities of the mutagen for structure analysis beyond mass spectrometry, which could not determine the location of the double bond or distinguish between imides having a five- or six-membered ring. The crucial structural question was the choice between structures having an endo and exo double bond in **2**−**4**. These alternative structures would be related to itaconic acid (exocyclic) or citraconic acid (endocyclic double bond). Since there are no known exocyclic analogs of the MX family of compounds, existing structure−activity relationships established for the chlorinated hydroxyfuranones did not provide guidelines for choosing between the various alternative endo- and exocyclic structures or the six-membered ring structures that would be consistent with the elemental formulas determined from mass spectrometry. The X-ray crystallographic data on synthesized compounds with identical mass spectra and retention index values established unequivocally that the tetrachloroimide, **4**, and the trichloroimide, **3**, both had an exocyclic double bond and are thus derivatives of itaconic rather than citraconic acid. The assignment of structure for **2** could be made directly from ^{13}C NMR of the synthesized compound.

In our analyses for compound **E** in simulated processing water, the amounts of **2** and **3** were variable and were somewhat dependent on GC−MS injector and transfer line temperatures, suggesting that they may be artifacts of the analysis.

The approximately 200-fold reduction in mutagenicity for **3** compared to **4** is consistent with the data for analogs of MX, in which substitution of H for the Cl bonded to the exocyclic carbon or the OH in **5** reduces the mutagenic activity by 8 and 20 times, respectively (LaLonde et al., 1991a,b). As a direct-acting mutagen in TA100, **4** is about one-third as potent as MX. The lower activity for **4** compared to MX may reflect a reduced ability of C(6) in **4** to undergo 1,4 Michael addition because of the resonance effect of directly bonded Cl on the stability of the positive charge at the exocyclic carbon, the expected reaction center for 1,4 Michael addition in **4**. However, the resonance versus inductive effect may have reduced importance because, as with MX (LaLonde et al., 1992; Tuppurainen et al., 1992), the lowest unoccupied molecular orbital (LUMO) that appears to be associated with the electrophilicity of these molecules is considerably delocalized (data not shown). In addition, for **4**, the ring nitrogen appears to contribute to the LUMO via a hybridized sp^2 orbital, as reflected in the nearly planar structure of the molecule. These findings are in accord with frontier orbital MO calculations on maleimide (Mendez, 1992), which indicate that all four of the maleimide ring carbons can participate in nucleophilic addition, in agreement with the known chemistry of maleimide (Joseph-Nation et al., 1972). From these perspectives, a study of the reactivity of **4** and its analogs and their computed electronic properties should be of considerable interest. Some of the reaction byproducts observed in the course of attempts to synthesize **3** and **4** appeared, from their mass spectra, to be isomeric with **3** (two compounds) and, in one case, with **4**. These compounds, when isolated, should provide additional insight into the correlation of structure with mutagenic activity for this interesting new class of bacterial mutagens.

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