Chloramines VII: Chlorination of Alanylphenylalanine in Model Solutions and in a Wastewater

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Products of the 30-min chlorination of the dipeptide alanylphenylalanine were determined at pH 7.0 in model solutions. At Cl_2/peptide mole ratios ≤1, *N*-chloroalanylphenylalanine (**I**) is the only product. **I** is very stable at 23 °C ($t_{1/2}$ = 111 ± 8.8 h). At mole ratios ≥2, *N,N*-dichloroalanylphenylalanine (**II**) is the only product. **II** decomposes in model solutions ($t_{1/2}$ = 4.1 ± 0.2 h) at pH 7.0 to form a compound identified as the *N*-chloroketimine *N*-[2-(*N*′-chloroimino)propanoyl]-phenylalanine (**III**). The structure of **III** was identified by converting it to *N*-pyruvylphenylalanine *tert*-butyl ester by reduction, hydrolysis, and esterification and correlating the mass spectrum and GC retention time of this derivative with those of an authentic sample. **III** is unusually stable and decomposes slowly ($t_{1/2}$ = 125 ± 6.5 h) to phenylalanine. In order to monitor the reactions of the dipeptide at low concentrations in a wastewater, alanyl-*p*-[^{3}H]-phenylalanine was synthesized. A primary wastewater (TKN = 19.82 mg/L; [NH_3] = 19.79 mg/L) was inoculated with the radiolabeled dipeptide and chlorinated to seven different chlorine concentrations spanning the breakpoint curve of the wastewater. Products identical to those observed in model solutions were formed. The stabilities of the tritiated analogs of **II** and **III** in the wastewater were similar to those determined in model solutions. Time studies of the decomposition of *N,N*-dichloroalanyl-*p*-[^{3}H]-phenylalanine revealed the formation of an intermediate (**A**) not previously recognized. Modeling of the reactions of **II** suggested that **A** was a decomposition product of **III** in the formation of phenylalanine and was probably either an isocyanate or a carbamic acid formed from hydrolysis of an isocyanate intermediate.

Introduction

Chloramines formed on disinfection of wastewaters are believed to be responsible for toxic responses of some fish to the discharge of chlorinated wastewaters and for stronger fish avoidance reactions than free residual chlorine (*1*−*7*). Organic chloramines in particular are believed to be responsible for disinfection interferences in certain types of wastewaters containing high concentrations of organic amino nitrogen compounds (*8*−*16*). Because organic chloramines in chlorinated wastewaters are poorly characterized, we have begun a systematic study of the chlorination reactions of organic amino nitrogen compounds found in wastewaters. We have reported studies of several amino acids and identified a new class of unusually stable chlorination products, *N*-chloroaldimines (*17*−*21*). However, dissolved free amino acids represent only a small percentage of the total amount of amino acids present in wastewaters (*8*) and, in fact, in most natural waters as well. For that reason, we began a systematic study of the reactions of polypeptides. In the preceding paper in this series (*22*), we reported a study of the dipeptide glycylphenylalanine, which reacts with 2 equiv of aqueous chlorine to form a product that is dichlorinated on the terminal amino nitrogen. This product loses 2 mol of HCl in successive elimination steps to form a cyanoacyl derivative of phenylalanine. However, compounds with *N*-terminal residues other than glycine cannot lose 2 equiv of HCl. Consequently, we studied the chlorination reactions of alanylphenylalanine in model solutions and in a primary wastewater and report the results of this work here.

Experimental Methods

General. All chemicals, buffers, standard solutions, and all syntheses and analyses, unless specifically described below, are described in the preceding paper (*22*). All instrumentation used in this work is also described in the preceding paper (*22*). Separation of chlorination products was carried out on a Whatman 5 μm Partisil ODS-3 analytical HPLC column with a dual-solvent system described previously (*17*). The solvent program (1 mL/min) consisted of a 5-min isocratic elution with 95% A/5% B, followed by a linear gradient to 55% A/45% B over 20 min, isocratic elution for 3 min, and a final linear gradient over 4 min to 10% A/90% B. Alanyl-*p*-bromophenylalanine was converted to alanyl-*p*-[^{3}H]-phenylalanine by Moravek Biochemicals, Inc. by metal-catalyzed halogen reduction in the presence of tritium gas. A solution of the tritiated dipeptide (250 μL; 2 μCi/μL) was obtained with a specific activity of 25 Ci/mmol.

Chlorination and Analysis of Model Solutions of Alanylphenylalanine. Determination of the breakpoint curve of model solutions of alanylphenylalanine as well as identification of chlorination products and studies of the decomposition of these products were carried out on 1.43 × 10^{-3} M solutions of the dipeptide in 0.025 M phosphate buffer (pH 7.0) at 23 °C in the same manner as that described for glycylphenylalanine (*22*).

Determining the amount of each chlorination product formed was made possible using alanyl-*p*-[^{3}H]-phenylalanine (0.7 μCi/mL) in the same manner used for determination of the products of the tritiated analog of glycylphenylalanine (*22*.).

Synthesis and Identification of *N*-[2-(*N*′-Chloroimino)-propanoyl]phenylalanine (III). With constant stirring, 5.0 mmol of aqueous chlorine was added to 50 mL of buffered (0.55 g of NaH_2PO_4, pH 7.0) Milli-Q water containing 2.5 mmol (0.59 g) of alanylphenylalanine. The solution was incubated in the dark with periodic removal of 10-μL aliquots for HPLC analysis. After all of the dichlorinated alanylphenylalanine had decomposed to the chloroketimine, the solution was acidified to pH 2.0 with concentrated HCl. The oily product was extracted with diethyl ether and dried over anhydrous Na_2SO_4, and the solvent was removed *in vacuo*. ^{13}C-NMR (acetone-d_6) (ppm): 176 (s, *C*═O), 173 (s, *C*═O), 161 (s, *C*═N), 127−136 (aromatic), 54 (d, NH−*C*H), 38 (t, C_6H_5−*C*H_2), 18 (q, *C*H_3−C); ^{1}H-NMR (acetone-d_6) (ppm): 7.4 (1, m, NH), 7.3 (5, d, aromatic), 4.8 (1, m, NH−C*H*), 3.32−3.15 (2, m, C*H*$_2$−C_6H_5), 2.3 (3, s, C*H*$_3$−C═N); IR (thin film): 2500−3500 (m, O−H), 1750 (s, C═O), 1690 (s, C═O) cm^{-1}.

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The structure of **III** was confirmed by converting it to *N*-pyruvylphenylalanine *tert*-butyl ester and comparing its GC/MS retention time and spectrum with an authentic sample. Alanylphenylalanine (1.0 mmol, 0.24 g) was dissolved in 50 mL of buffered (0.41 g of NaH_2PO_4, pH 7.0) Milli-Q water. Standardized aqueous chlorine (2.0 mmol) was added to the solution with continuous stirring to form the dichlorinated dipeptide. This solution was incubated in the dark, and 10-μL aliquots were periodically removed for analysis by HPLC. When all of the dichlorinated dipeptide had decomposed to the chloroketimine, portions of Na_2S were added to reduce the chloroketimine. The progress of this reaction was also followed by HPLC. When reduction was complete, the pH of the solution was reduced to approximately 2.0 with concentrated HCl and extracted with diethyl ether. The extract was dried over Na_2SO_4 and the solvent removed *in vacuo*. ^{13}C-NMR ($CDCl_3$) (ppm): 197 (s, *C*═O); 172 (s, *C*═O); 161 (s, *C*═O); 127−136 (aromatic); 54 (d, NH−*C*H); 38 (t, $C_6H_5CH_2$); 24 (q, CH_3−C═O).

An ether extract of the reaction mixture was concentrated, and the residue (0.18 g) was dissolved in 15 mL of *tert*-butyl acetate. To this solution 0.18 mL of $HClO_4$ (60% aqueous solution) was added. The reaction was stirred for 10 min and allowed to react for 4 days (*23*). After the incubation time, the solution was cooled in an ice bath and extracted with 0.5 M HCl (3 × 10 mL). The volume of *tert*-butyl acetate was reduced *in vacuo*, and the remaining solution was analyzed by GC/MS, *m/z* (relative abundance): 235 (47, M − C_4H_8), 190 (47, M − C_4H_8 − COOH), 148 (87, 190 − H_2C═C═O), 120 (100, C_6H_5−CH_2−CH═NH_2^+), 91 (37, $C_7H_7^+$), 57 (83, $C_4H_9^+$).

Synthesis of *N*-Pyruvylphenylalanine *tert*-Butyl Ester. Dicyclohexylcarbodiimide was added to a solution of 40 mg (0.45 mmol) of freshly distilled pyruvic acid in 1 mL of dry THF under argon at 0−5 °C. After 1−2 min, a solution of 100 mg of phenylalanine *tert*-butyl ester in 0.5 mL of dry THF was added dropwise. After stirring 1 h at room temperature, the solution was filtered to remove dicyclohexylurea. The solvent was evaporated, and the residue was purified by flash chromatography on 10−15 g of silica gel using an ethyl acetate: hexane gradient (1−50%) to yield 46 mg (35% yield) of product. Exact mass measurements on the parent ion found *m/z* 291.14848 (calcd mass 291.14706). ^{1}H NMR (DMSO-d_6) (ppm): 7.35 (1, m, N*H*), 7.2 (5, d of d, aromatic), 4.67 (1, m, C*H*−CH_2−C_6H_5), 3.1 (2, d, CH_2−C_6H_5), 2.45 (3, s, CH_3), 1.4 (9, s, C(CH_3)$_3$).

Description of Wastewater. A primary wastewater was obtained from the Army Base Treatment Plant located in Norfolk, VA, at a point following settling. The facility has been described previously as Plant 2 (*24*). Handling and storage of the wastewater has been described previously (*24*). The total Kjeldahl nitrogen (TKN) and ammonia concentrations were determined by the Hampton Roads Sanitation District according to published procedures (*25*). Amino acid concentrations in the wastewater were determined before and after hydrolysis (*26*) by precolumn derivatization with *o*-phthalaldehyde (OPA) and HPLC analysis (*18*, *19*).

Analysis of Chlorination Products in Wastewater. Wastewater was buffered at pH 7.0 as described previously. The breakpoint curve was determined by chlorinating 100-mL aliquots to concentrations of 40, 80, 120, 160, 200, 240, and 280 mg of Cl_2/L and treating them as described previously (*17*).

Aliquots (15.0 mL) of wastewater were inoculated with 1.0 mL of a stock solution of alanyl-*p*-[^{3}H]-phenylalanine (7.0 μCi/10 mL; specific activity 25 Ci/mmol; 2.8 × 10^{-8} M), chlorinated to each of the seven levels used in determining the breakpoint curve. The volumes of each solution were normalized to 17.0 mL, and the reaction mixtures were incubated, fractionated, and assayed as described previously (*17*). The rates of decomposition of *N*,*N*-dichloroalanylphenylalanine (**II**) and *N*-[2-(*N*′-chloroimino)propanoyl]phenylalanine (**III**) were determined in a similar manner over a 65-h time period by HPLC analysis of buffered wastewater chlorinated to 240 mg/L.

Results and Discussion

Because wastewaters are not typically discharged until 30 min after they are chlorinated, the reactions that take place within the first 30 min are more likely to have the greatest initial environmental impact on receiving waters. However, depending on the byproducts formed, decomposition of the primary chlorination products over longer time periods may have even greater long-term environmental impact. Consequently, in this study the chlorination products of alanylphenylalanine formed in 30 min were identified, and their subsequent decomposition reactions were studied to estimate the potential environmental impact of the chlorination reactions of polypeptides on the discharge of chlorinated wastewater.

Identification of Primary Alanylphenylalanine Chlorination Products in Model Solutions. The 30-min chlorine demand curve of an alanylphenylalanine model solution is similar to that of glycylphenylalanine (*22*). Neither a noticeable chloramine maximum nor a breakpoint is observed. At Cl_2/peptide mole ratios ≤1.0, the 30-min residual chlorine concentration equals the amount of chlorine added to the solution. A slight inflection point is seen at a mole ratio of approximately 1.5, which indicates some decomposition of the oxidant formed at the higher concentration levels. At mole ratios >2.0, free residual chlorine is measured, and the total residual chlorine concentration increases linearly with increases in the amount of aqueous chlorine added.

When model solutions of alanylphenylalanine (Ala-Phe) were chlorinated to Cl_2/peptide mole ratios ≤1 and analyzed by HPLC, only one chlorination product was observed in the chromatogram. Alanylphenylalanine itself elutes in two peaks because it exists as a mixture of diastereomers. The one chlorination product elutes as a single peak and is believed to be *N*-chloroalanylphenylalanine (**I**) for the same reasons *N*-chloroglycylphenylalanine was identified as such (*22*). First, as it eluted from the column, it oxidized iodide to iodine. Second, as the molar ratio of Cl_2/Ala-Phe increased up to 1.0, the peak area increased to a maximum and began to decrease at mole ratios >1.0.

As the Cl_2/Ala-Phe mole ratio was increased between 1 and 2, three new chromatographic peaks appeared within 30 min with HPLC retention times of 22.3, 22.6, and 22.9 min (Figure 1). The latter two peaks formed immediately after chlorination, and their intensity increased in proportion to increases in the Cl_2/Ala-Phe mole ratio and also in proportion to decreases in the amount of **I** present. The peak that eluted at 22.3 min increased over time as the other two peaks decreased (Figure 1). All three compounds oxidized iodide as they eluted from the column, suggesting that they contained oxidizing moieties like *N*-chlorinated amines.

In the preceding study of glycylphenylalanine (*22*), the addition of 2 equiv of aqueous chlorine produced *N*,*N*-dichloroglycylphenylalanine, a crystalline solid that could be precipitated from solution by the addition of acid, purified by recrystallization, and characterized by elemental and spectral analysis. When a solution of alanylphenylalanine was chlorinated with 2 equiv and acidified, an oil was formed that did not lend itself to further purification and elemental analysis. However, based on the study of glycylphenylalanine, the peaks that eluted at 22.6 and 22.9 min were believed to be diastereomers of *N*,*N*-dichloroalanylphenylalanine (**II**).

Spectral Identification of *N*-[2-(*N*′-Chloroimino)propanoyl]phenylalanine (III). Based on its spectral properties, the product that eluted at 22.3 min was believed to be the *N*-chloroketimine *N*-[2-(*N*′-chloroimino)propanoyl]phenylalanine (**III**). **III** was isolated by extraction after an aqueous solution of *N*,*N*-dichloroalanylphenylalanine had been al-

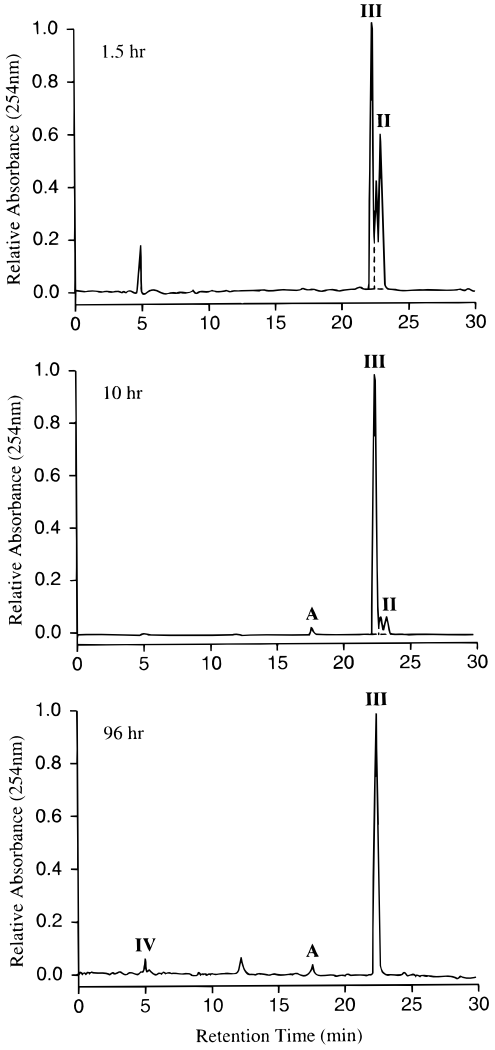

FIGURE 1. HPLC chromatograms of an alanylphenylalanine model solution (1.43×10^{-3} M, pH 7.0) chlorinated with 2 equiv and analyzed after 1.5, 10, and 96 h.

lowed to decompose by >90% and acidified. All NMR and IR spectra were consistent with the proposed structure. Distinctive spectral characteristics that supported the assignment of structure included the presence of three carbonyl carbons (161, 173, and 176 ppm) in the decoupled ^{13}C-NMR, a proper off-resonance ^{13}C spectrum, and the fact that the ^{1}H-NMR spectrum demonstrated that the carbon adjacent to the alanyl methyl protons in the parent compound had lost a proton to form the C*H*$_3$−C═N group.

The *N*-chloroketimine was converted to *N*-pyruvylphenylalanine *tert*-butyl ester, and its GC/MS retention time and mass spectrum were compared to those of a sample independently synthesized. The retention times of the two were identical. The fragmentation patterns for both samples were also identical and consistent with the proposed structure.

Identification of Secondary Alanylphenylalanine Chlorination Products in Model Solutions. Over time in aqueous solution (23 °C, pH 7.0) three new decomposition products of *N*,*N*-dichloroalanylphenylalanine (**II**) appeared in the chromatograms (Figure 1) with retention times of 5.1, 12.3, and 17.6 min. The peak eluting at 5.1 min was identified as phenylalanine both by its retention time and by derivatizing the solution with *o*-phthalaldehyde (OPA) and comparing the retention time of the OPA derivative with that of an authentic sample. In radiotracer studies discussed below, the compound eluting at 12.3 min was found to represent less than 1% of the decomposition products formed and could not be identified. The compound eluting at 17.6 min was found to make up approximately 8% of the total decomposition products and has been labeled unknown A.

The quantitation of both the primary and secondary chlorination products was made possible by inoculating model solutions with the radiotracer alanyl-*p*-[^{3}H]-phenylalanine. The solution was chlorinated to a Cl_2/Ala-Phe mole ratio of 2.0/1 and analyzed periodically by HPLC and liquid scintillation counting of collected fractions. The retention times of all peaks in the radiochromatograms corresponded to those observed in the UV chromatograms. Although the structure of the compound eluting at 17.6 min was not confirmed by spectral analysis, modeling studies discussed below strongly suggested it is an isocyanate (or its hydrolysis product, a carbamic acid) formed by loss of HCl and acetonitrile from **III**.

Stabilities of Chlorination Products in Model Solutions. The shape of the breakpoint curve of alanylphenylalanine can be explained by the unusual stability of the chloramines formed. When alanylphenylalanine is chlorinated to Cl_2/Ala-Phe molar ratios ≤1.0, *N*-chloroalanylphenylalanine is the only product formed and, because it is so stable ($t_{1/2}$ = 111 ± 8.8 h in model solutions), a linear relationship exists in this range between the amount of chlorine applied and the total residual chlorine measured 30 min later. The stabilities of monochlorinated dipeptides can vary greatly. At 22 °C, *N*-chloroglycylphenylalanine has a half-life of 127 h (*22*). At 37 °C, the half-lives of the monochlorinated dipeptides examined by Stelmaszynska *et al.* (*27*) ranged from 6.6 h for monochlorinated Gly-Leu to 25 h for monochlorinated Leu-Gly (pH 6.6). At temperatures 15 °C lower, these half-lives would be much longer.

As the Cl_2/Ala-Phe molar ratio increases to ≥1.0, *N*,*N*-dichloroalanylphenylalanine (**II**) begins to form and an inflection point is observed in the breakpoint curve because of its decomposition ($t_{1/2}$ = 4.1 ± 0.2 h, $k_{1m} = 2.85 \times 10^{-3}$ min^{-1}; $r^2 = 0.997$, where k_{1m} is the first-order decomposition rate constant in the model solution at 23 °C). However, within 30 min at 23 °C only about 9% of the dichlorinated dipeptide decomposes. The decomposition product itself contains oxidizing chlorine and, consequently, the decrease in the oxidizing power of the solution is not large. Keefe *et al.* (*22*) reported that the half-life of *N*,*N*-dichloroglycylphenylalanine is slightly longer ($t_{1/2}$ = 6.4 h at 22 °C) than the dichlorinated dipeptide described here.

Decomposition of **II** produces the *N*-chloroketimine **III**, which is even more stable ($t_{1/2}$ = 125 ± 6.5 h, $k_{2m} = 9.25 \times 10^{-5}$ min^{-1}; $r^2 = 0.989$, where k_{2m} is the first-order decomposition rate constant in the model solution at 23 °C) than **I** or **II**. It is also significantly more stable than the corresponding *N*-chloroaldimine ($t_{1/2}$ = 36 h at 22 °C) formed from chlorination of Gly-Phe (*22*). At molar ratios ≥2.0, all of the dipeptide is dichlorinated, and free residual chlorine is detected.

The product distribution of a tritiated alanylphenylalanine model solution chlorinated with 2 equiv of aqueous chlorine and analyzed periodically over 164 h is shown in Figure 2. The major decomposition product of *N*,*N*-dichloroalanylphenylalanine (**II**) is the corresponding *N*-chloroketimine (**III**). After the concentration of *N*-chloroketimine (**III**) reaches a maximum within 24 h, the concentration of phenylalanine (**IV**) appears to rise. However, *N*-chloroketimine (**III**) and phenylalanine (**IV**) do not account for 100% of the decomposition products of *N*,*N*-dichloroalanylphenylalanine (**II**). Unknown A accounts for the difference. Its concentration rises within the first 24 h and appears to approach a steady-state concentration at the same time the concentration of *N*-chloroketimine reaches a maximum. Modeling studies described below suggest that this compound is an intermediate such as an isocyanate or a carbamic acid in the formation of phenylalanine (**IV**) from the *N*-chloroketimine (**III**).

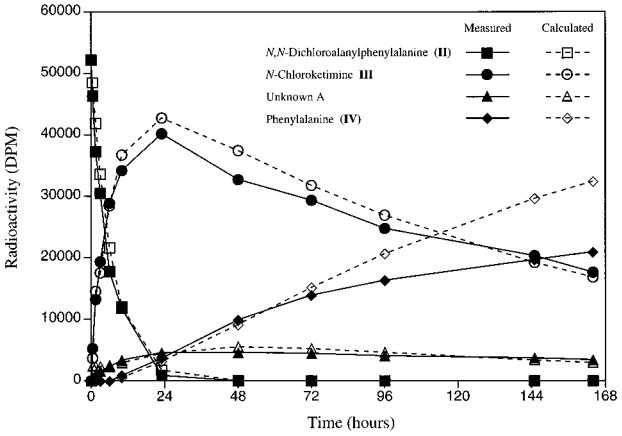

FIGURE 2. Measured and calculated chlorination product distributions of an alanyl-phenylalanine model solution (1.43×10^{-3} M, pH 7.0) chlorinated with 2 equiv and analyzed over 164 h.

Modeling the Decomposition of Chlorination Products. To demonstrate the relationship between the different chlorination products **II**, **III**, unknown A, and **IV**, a model based on three consecutive first-order reactions was developed. It was assumed that unknown A is an intermediate in the decomposition of the *N*-chloroketimine (**III**) to phenylalanine (**IV**). Initially, the rate constants k_{1m} and k_{2m} were used to model the decomposition of **II** and corresponding formation of **III** and the decomposition of **III** and corresponding formation of unknown A. The rate constant for the decomposition of unknown A (k_{3m}), which corresponded to the formation of phenylalanine (**IV**) was initially estimated. Once the model was developed, all three rate constants were manipulated so that calculated concentrations mirrored experimental values. The measured and calculated (from model) rate constants along with the corresponding half-lives are shown in Table 1. While the calculated and measured rate constants for the decomposition of the dichlorinated dipeptide (k_{1m}) are similar, the calculated rate constant for the decomposition of *N*-chloroketimine (**III**) appears to be significantly lower than the measured value. The measured value of k_{2m} is probably more accurate than the calculated value because k_{2m} had to be manipulated to model the appearance of unknown A. However, since the concentration of unknown A remains small throughout the 164 h of the study, its measurement (and consequently the calculated value of k_{2m}) contains a much greater error than the measurement of **III**. Figure 2 shows the measured and calculated concentrations of chlorination products over a period of 164 h. The correlation between the measured and calculated concentrations of **II**, **III**, and unknown A strongly suggests that unknown A is an intermediate in the formation of phenylalanine, which supports the model. Measured changes in the concentration of phenylalanine appear to deviate from calculated concentrations. This appears to be caused by degradation of phenylalanine in the solution or its adsorption on the walls of the reaction vessel.

Mechanisms. When alanylphenylalanine is chlorinated with 2 equiv, *N*,*N*-dichloroalanylphenylalanine (**II**) is formed, which then dehydrohalogenates to form an *N*-chloroketimine (**III**). The *N*-chloroketimine is then believed to undergo β-elimination to form an unstable isocyanate intermediate. Hydrolysis of the isocyanate leads to the formation of a carbamic acid which, in turn, would lose CO_2 to form phenylalanine (**IV**). Figure 3 shows the proposed mechanism for the decomposition of *N*,*N*-dichloroalanylphenylalanine (**II**).

Scully and Keefe (*22*) showed that *N*,*N*-dichloroglycylphenylalanine decomposes by two successive dehydrohalogenation steps to form an *N*-chloroaldimine and an *N*-cyanoacylphenylalanine intermediate. *N*-Cyanoacylphenylalanine then hydrolyzes to phenylalanine and HCN through possible isocyanate or carbamic acid intermediates. It is conceivable that the *N*-chloroaldimine also decomposes more directly to an isocyanate or carbamic acid in the same way the *N*-chloroketimine does in Figure 3. However, this pathway, if it exists, is minor and no evidence for it was apparent.

Stelmaszynska *et al.* (*27*) also suggested that an *N*-chloroketimine and an *N*-chloroaldimine would decompose to form a nitrile, carbon dioxide, and the *C*-terminal amino acid. This has been supported, as both dichlorinated Ala-Phe and dichlorinated Gly-Phe decompose to form phenylalanine. The overall mechanism for the decomposition appears to be dependent on the *N*-terminal residue.

Synthesis of Alanyl-*p*-[^{3}H]-phenylalanine. In order to observe the reactions of alanylphenylalanine in a wastewater and to measure the quantities of each chlorination product formed, alanyl-*p*-[^{3}H]-phenylalanine was synthesized. ^{1}H- and ^{13}C-NMR and IR spectra for all intermediates were consistent with proposed structures. Alanyl-*p*-bromophenylalanine also gave correct elemental analysis. It was converted to alanyl-*p*-[^{3}H]-phenylalanine by metal-catalyzed halogen reduction with tritium gas.

Chlorination Products and Their Rates of Decomposition in a Wastewater. The primary wastewater used in this work had an ammonia concentration of 19.79 mg/L and a TKN concentration of 19.82 mg/L. The breakpoint curve for the wastewater exhibited a chloramine maximum at approximately 120 mg/L and a breakpoint at approximately 210 mg/L. The concentrations of the dissolved free amino acids alanine and phenylalanine were found to be 5.87×10^{-7} and 1.04×10^{-7} M, respectively. The concentration of a hydrolyzable amino acid is a measure of the concentration of that amino acid which is bound in proteins. The concentrations of hydrolyzable alanine and phenylalanine in the wastewater were found to be 1.04×10^{-5} and 2.92×10^{-6} M, respectively. Thus the concentrations of these amino acids bound to proteins are between 20 and 30 times greater than their free dissolved concentrations.

Wastewater was inoculated with alanyl-*p*-[^{3}H]-phenylalanine, chlorinated for 30 min to seven points along the breakpoint curve, and analyzed. Product distributions in the wastewater 30 min after chlorination were similar to those in model solutions except for a large amount of radioactivity (almost 20% of the total after 167 h), which eluted in the void volume. Throughout the reaction period, the radioactivity eluting in the void volume was equivalent to about half of the amount of phenylalanine expected to form in the wastewater at any given time as predicted by the model studies. Phenylalanine is a nonpolar amino acid and may bind reversibly to wastewater organic compounds and elute with them. On the other hand, it is possible that a radioactive intermediate, such as the proposed isocyanate precursor of phenylalanine discussed above, may react with -OH or -NH_2 moieties in polar wastewater humic substances to bind irreversibly.

The stability of *N*,*N*-dichloroalanylphenylalanine (**II**) was slightly less in wastewater than in model solutions with $t_{1/2} = 3.6 \pm 0.1$ h ($k_{1w} = 3.19 \times 10^{-3}$ min^{-1}, $r^2 = 0.999$, where k_{1w} is the first-order decomposition rate constant in the wastewater at 23 °C). The stability of the *N*-chloroketimine was similar in both model solutions and in the wastewater with $t_{1/2} = 138 \pm 6.8$ h ($k_{2w} = 8.36 \times 10^{-5}$ min^{-1}, $r^2 = 0.990$, where k_{2w} is the first-order decomposition rate constant in the wastewater at 23 °C).

Implications for Wastewater Treatment. Figure 4 is a plot of the chlorination products found after 30 min in a wastewater that had been chlorinated to various points along the breakpoint curve. The tritiated dipeptide was used as a tracer to follow the product formation. *N*-Chloroalanylphenylalanine (**I**), *N*,*N*-dichloroalanylphenylalanine (**II**), and the *N*-chloroketimine (**III**) are all observed at various chlorination

TABLE 1. Measured and Calculated Rate Constants and Half-Lives of Alanylphenylalanine Chlorination Products in a Model Solution

		rate constants (min^{-1})[a]		half-lives (h)[a]	
compound	constant	measured	calculated	measured	calculated
N,N-dichloroalanylphenylalanine (**II**)	k_{1m}	2.85×10^{-3}	2.6×10^{-3}	4.1 ± 0.2	4.4
N-chloroketimine (**III**)	k_{2m}	9.25×10^{-5}	1.2×10^{-4}	125 ± 6.5	100
unknown A	k_{3m}	N/A	8.0×10^{-4}	N/A	14.4

[a] At 23 °C.

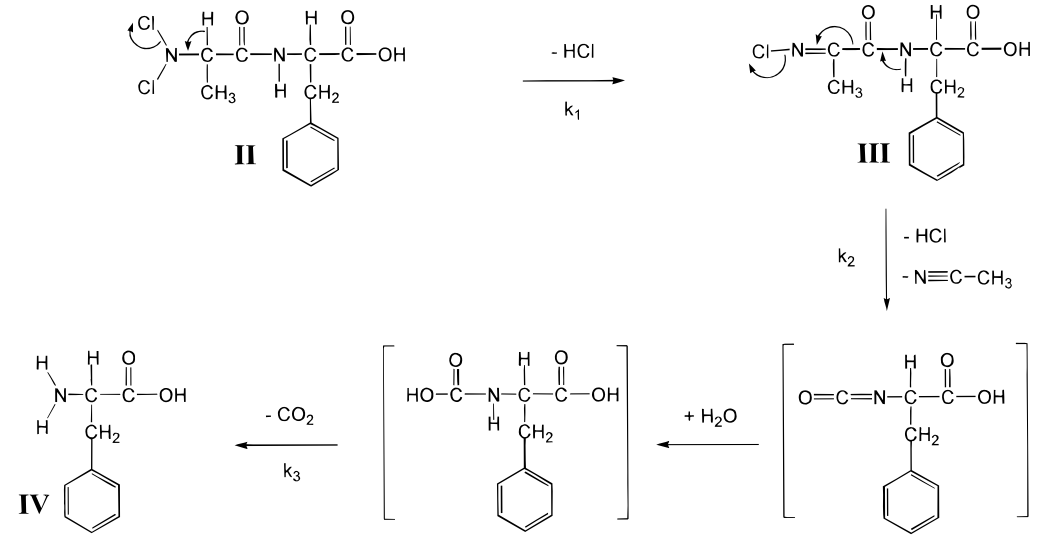

FIGURE 3. Proposed mechanism for the decomposition of *N,N*-dichloroalanylphenylalanine (II).

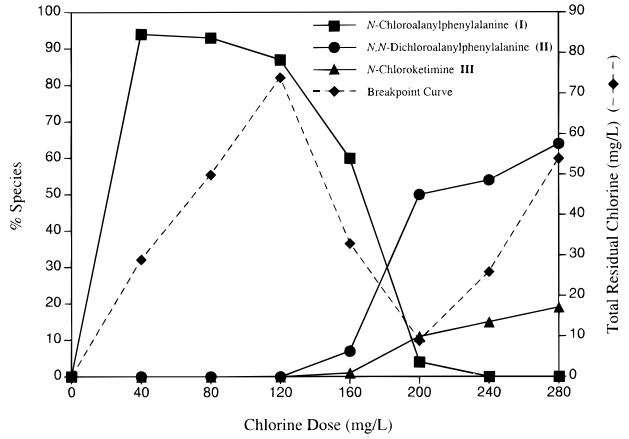

FIGURE 4. Product distribution in a wastewater (pH 7.0) inoculated with alanyl-*p*-[^{3}H]-phenylalanine and chlorinated along the breakpoint curve (40−280 mg/L).

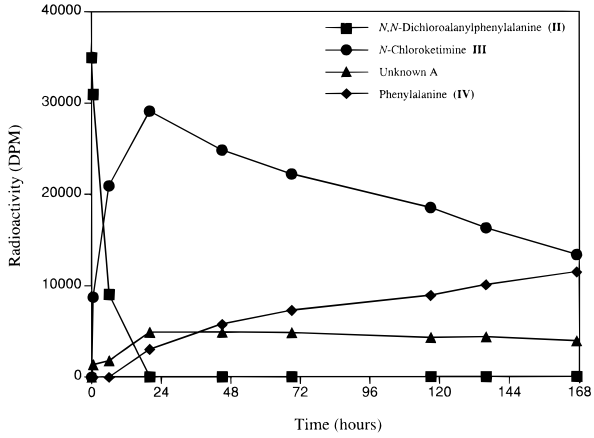

FIGURE 5. Product distribution in a wastewater (pH 7.0) inoculated with alanyl-*p*-[^{3}H]-phenylalanine that was chlorinated to 240 mg/L and analyzed over 168 h.

levels. The breakpoint curve for the wastewater is also shown in the figure.

At a chlorine dose of 40 mg/L all of the dipeptide had been converted to *N*-chloroalanylphenylalanine. This may seem unusual because the residual chlorine concentration suggests that all amino nitrogens were not fully chlorinated until a dose of 120 mg/L (chloramine maximum) had been reached. However, this type of observation has been observed in a previous study involving chlorination of the amino acid phenylalanine (*19*). Two possible factors could account for this: the more rapid direct chlorination of the dipeptide by free chlorine (hypochlorous acid) (*28*) and chlorine transfer to the organic nitrogen compound from inorganic chloramines (*29*) within 30 min. Beyond the chloramine maximum (120 mg/L), the amount of *N*-chloroalanylphenylalanine (**I**) decreased as *N,N*-dichloroalanylphenylalanine (**II**) and its decomposition product, an *N*-chloroketimine (**III**), began to appear. At the breakpoint (210 mg/L chlorine dose) and beyond, all of the dipeptide had been converted to *N,N*-dichloroalanylphenylalanine (**II**). This was to be expected as free chlorine was now present in the wastewater.

Figure 5 shows the product distributions in a wastewater inoculated with the tritiated dipeptide and chlorinated to 240 mg/L. At this chlorine level all of the dipeptide is converted to the dichlorinated analog. The plot traces the decomposition of the dichlorinated dipeptide and its decomposition products over a period of 168 h. The decomposition rates of *N,N*-dichloroalanylphenylalanine ($t_{1/2} = 3.6 \pm 0.1$ h) and the *N*-chloroketimine ($t_{1/2} = 138 \pm 6.8$ h) are very similar to those found in the model solutions. However, there are some differences in the product distribution between the two solutions. In the wastewater there appeared to be significant binding of the chlorinated dipeptide analogs to organic matter in the solution incubated at room temperature.

As a result, a large amount of radioactivity eluted in the void volume. Chlorination products of phenylalanine have also been found to bind to organic matter in wastewater (*19*).

Of more importance to current wastewater disinfection practices are the reactions occurring at the higher chlorination levels. In order to minimize nutrient loading into rivers and bays, many treatment plants have begun to nitrify their wastewaters. As a result, ammonia concentrations in these effluents are generally low as compared to organic nitrogen compounds. Consequently, compounds such as dipeptides and proteins comprise a larger fraction of the total amino nitrogen. This can lead to difficulty in determining if a wastewater has been adequately disinfected, by interfering with the measurement of the bactericidal potential in the wastewater. As discussed earlier, the organic chloramines formed have little or no bactericidal activity, and current techniques used to measure the oxidant level of the wastewater cannot distinguish between organic and inorganic chloramines (*8*−*16*). The toxicological impact of an increase of these chlorination products being released to a receiving body is also unknown.

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