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# Quantitation and Identification of Organic *N*-Chloramines Formed in Stomach Fluid on Ingestion of Aqueous Hypochlorite

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**The chemical reactions that hypochlorite undergoes in the body when chlorinated water is ingested have received very little attention. Because amino nitrogen compounds are important components of the average diet, the reactions of hypochlorite with amino compounds in the stomach were investigated.**

**Stomach fluid was recovered from Sprague-Dawley rats that had been fasted for 48 hr and administered 4 mL deionized water. The chlorine demand of the stomach fluid was determined. An average volume-independent demand of 2.7 mg chlorine was measured. At doses below 40 mg/L chlorine reducing reactions appeared to account for reduction of all oxidizing species within 15 min as measured by the FAS-DPD titrimetric method.**

**At least part of the chlorine demand is associated with amino acids present in the stomach fluid. Amino acids were identified and quantified in the stomach fluid by precolumn derivatization with *o*-phthalaldehyde and high-pressure liquid chromatography (HPLC).**

**When stomach fluid is chlorinated to concentrations of chlorine between 200 and 1000 mg/L, organic *N*-chloramines are formed. After derivatization of chlorinated stomach fluid with dansyl sulfinic acid, fluorescent derivatives of chloramines were separated by HPLC. Three chloramino acid derivatives, *N*-chloroalanine, *N*-chloroglycine, and *N*-chlorophenylalanine, were identified by cochromatography with known standards using two chromatographic methods.**

**The yield of a chloramine that would form in stomach fluid on administration of hypochlorite to animals was determined using tritiated piperidine and doses of 200 and 1000 mg/L chlorine. Yields of tritiated *N*-chloropiperidine in recovered stomach fluid were 70% and 42%, respectively, of the theoretical amount expected.**

## Introduction

In the United States the average person drinks between 2 and 3 L of water each day, which may typically contain between 1 and 2 mg/L of a residual chlorine oxidant such as aqueous chlorine ($Cl_2$). In recent years, concern over the need to evaluate the potential health effects of water disinfection has focused on the identification of the products formed by aqueous chlorine when it reacts with trace organic compounds present in natural waters. As a result, health effect projections have had to rely on the dose-response data from toxicological studies of these by-products and the concentrations of these chemicals typically ingested by the population at large. Naturally, water treatment policies have been primarily concerned with minimizing the concentrations of these trace contaminants.

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However, by comparison with the U.S. Environmental Protection Agency's (EPA) Maximum Contaminant Level for chloroform, the molar concentration of chlorine in a drinking water containing 2 mg/L $Cl_2$ is 30 times higher. Furthermore, when drinking water is ingested, it enters a medium with a total organic carbon (TOC) content that is several orders of magnitude higher than that found in natural waters. Therefore, we have been concerned with the health implications of the reactions of aqueous chlorine in the stomach.

Recently, studies on the toxicity and pharmacokinetics of hypochlorous acid and monochloramine were reported (*1–4*). These studies showed that the $^{36}Cl$ used in these studies is retained in animals much longer than $^{36}Cl$-enriched chloride. Therefore, it is important to determine what chemistry that can take place in the stomach can account for this greater degree of retention.

Since stomach fluid contains high concentrations of organic amino nitrogen compounds and since hypochlorite reacts rapidly with these types of compounds to form chloramines, we have investigated the *in vivo* and *in vitro* formation of organic N-chloramines.

Recently, we reported that *N*-chloropiperidine and N-chloroglycine are formed in the stomachs of laboratory rats after successive administration of the parent amines and aqueous hypochlorite (*5*). In addition, we showed that *N*-chloropiperidine can be absorbed into the blood of laboratory animals. In this paper we have measured the amount of an organic chloramine that forms in a stomach fluid on administration of aqueous hypochlorite and have determined the percentage yield of that chloramine considering that it must compete with many other amino nitrogen compounds in the stomach for chlorination. Lastly, we have chlorinated stomach fluid and identified several organic *N*-chloramino acids.

# Materials and Methods

## General

The method used for the derivatization of organic *N*-chloramines and the liquid chromatographic equipment used in these studies has been described elsewhere (*6*). [G-$^3$H]piperidine hydrochloride was purchased from Amersham Corporation. Radiochemicals were greater than 98% pure. Solutions of sodium hypochlorite were prepared and standardized as described in EPA Method 510.1 (*7*).

## Animals

Adult Sprague-Dawley rats of mixed sex weighing between 200 and 350 g were starved for 48 hr before each experiment. The animals were allowed to drink water *ad libitum*. Prior to each administration by gavage, the animals were anesthetized with ether. All solutions were administered by gavage using a syringe fitted with a 3-in. curved intubation needle or using a polyethylene tube introduced directly into the stomach.

## Treatment of Animals

An aqueous solution of piperidine (1.0 mL of a 0.2 M solution in deionized water) containing 35 μCi of [$^3$H]piperidine was administered to test animals by gavage. This procedure was followed within 5 min by administration of 3.0 mL of standardized aqueous hypochlorite (220 mg/L or 1016 mg/L as $Cl_2$). To obtain fluid used to measure the chlorine demand of stomach fluid, animals were administered 4 mL deionized water. For all other *in vitro* experiments requiring stomach fluid, deionized water (3 mL) was administered to animals before stomach fluid was recovered.

## Collection of Stomach Fluid

In all cases, the contents of the stomach were recovered within 10 min by removal of the whole stomach. Animals were anesthetized with ether, shaved, and opened. The stomach was tied off with hemostats before it was excised. The stomach contents were recovered by snipping the wall of the stomach and draining the contents into a beaker. The fluid was chilled immediately on ice. Centrifuging was occasionally necessary to remove particulate matter, and all samples were passed through a glass fiber filter before further handling. When stomach fluid was required for identifying amines and chloramines, the fluid that was recovered from several animals was pooled to provide a single sample.

## Chlorine Demand of Stomach Fluid

Stomach fluid (2.0 mL) was diluted to 200 mL with deionized water. Seven aliquots of this diluted sample were chlorinated to 1, 2, 4, 6, 8, 10, and 12 mg/L $Cl_2$ with standardized hypochlorite and stored in the dark for either 15 min or 1 hr. These chlorine concentrations corresponded to 100, 200, 400, 600, 800, 1000, and 1200 mg/L $Cl_2$ in the undiluted stomach fluid. Each aliquot was then diluted to 100 mL, and the chlorine residual was determined by the FAS-DPD method (*8*). The measured residual chlorine concentrations were corrected for the dilutions of the stomach fluid to determine the residual chlorine concentration that would have been observed if the undiluted stomach fluid had been chlorinated.

## Identification of Organic *N*-Chloramines in Stomach Fluid

While being stirred at room temperature, stomach fluid (5 mL) which had been recovered from rats as described above was chlorinated with standardized sodium hypochlorite (3.4 mL of a solution, 1011 mg/L as $Cl_2$). The solution was incubated at room temperature in the dark for 15 min. The solution was then passed through an equilibrated Waters Associates octadecylsilica cartridge (SEP-PAK) and chloramines recovered by passing 5 mL of 10% methanol in water through the cartridge. Samples were then derivatized with dansyl sulfinic acid. The eluate (5 mL) was derivatized by adding 100 mg sodium bicarbonate ($NaHCO_3$), 5 mL acetonitrile, 1 mL of $10^{-2}$ M dansyl sulfinic acid reagent solution (*6*), and 2 drops 10 N sodium hydroxide (NaOH). The resulting solution was stirred overnight at room temperature in the dark, concentrated to remove acetonitrile, and acidified to pH 3–5. The solution was then passed through an octadecylsilica cartridge. The cartridge was washed with 2 mL deionized water. Dansylated amino acids were recovered from the cartridge by washing it with 6 mL of Solvent B (see below). The dansylated amino acids were chromatographed as described below.

## Yield of [$^3$H]N-Chloropiperidine Formed *in Vivo*

After administration of [$^3$H]piperidine and hypochlorite to an animal and recovery of its stomach contents, an aliquot of the stomach fluid was assayed by liquid scintillation counting. A known volume was ap-

plied to an equilibrated SEP-PAK. A 2.0 mL wash of water was followed by elution of the chloramine with 1.0 mL of acetonitrile. The wash and the eluant were assayed by liquid scintillation counting and analyzed by high pressure liquid chromatography (HPLC) as described below.

## Instrumentation and Analysis

The HPLC equipment used in this study has been described elsewhere (*6*). After clean-up of recovered stomach fluid samples on an octadecylsilica cartridge, the quantity of [$^3$H]N-chloropiperidine formed *in vivo* after administration of piperidine and hypochlorite was determined by direct chromatography on a Waters 10 μM μBondapak $C_{18}$ reversed-phase column. Samples were chromatographed under isocratic conditions: 65% water (containing 1% acetic acid)/35% acetonitrile. Under these conditions *N*-chloropiperidine had a retention time of approximately 7 min. Fractions were collected at 1-min intervals following injection and analyzed by liquid scintillation counting.

Amino acids present in stomach fluid were identified and quantified in the following manner. A 0.5-mL aliquot of stomach fluid was applied to a SEP-PAK and eluted with 2.0 mL of chlorine demand-free water and analyzed by HPLC using the Waters Associates AUTO*TAG automatic precolumn derivatization technique (*9*). This method involved automatic derivatization of the amino acids with *o*-phthalaldehyde (OPA), chromatography on a stainless steel (100 mm × 4.6 mm) Astec 5 μM, spherical $C_{18}$ reversed-phase column, and fluorescence detection. The OPA derivatizing solution was prepared by dissolving 250 mg OPA in 5 mL methanol and diluted to 50 mL with a saturated aqueous solution of sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$). A standard solution of amino acids ($2.5 \times 10^{-3}$ M in 0.1 N hydrochloric acid [HCl]) was obtained from Sigma Chemical Co. and diluted 250-fold to 10 μM. Amino acids were identified as their OPA derivatives by comparing their retention times with those of the standards. Their concentrations were determined by comparing the detector response of each amino acid derivative with the response of the standard solution of amino acids. A 5 μL aliquot of either stomach fluid or standard amino acid solution was derivatized with 50 μL of OPA solution.

Stomach fluid, which had been chlorinated and derivatized with dansyl sulfinic acid, was chromatographed on a Whatman Partisil 5 ODS-3 RAC column using two different chromatographic conditions. The first consisted of a linear solvent gradient (flow = 1.7 mL/min) from 80% water (1% acetic acid)/20% acetonitrile to 20% water (1% acetic acid)/80% acetonitrile over 60 min. The second elution program involved two solvents: 0.1 M potassium acetate adjusted to pH 4.4 with formic acid (Solvent A) and 45% Solvent A/15% 2-propanol/40% acetonitrile (Solvent B). The elution program (flow = 1.7 mL/min) consisted of a linear gradient from 80% Solvent A/20% Solvent B to 65% Solvent A/35% Solvent B in 8 min followed by a 4-min isocratic elution. This procedure was followed in turn by a linear gradient to 100% Solvent B over 50 minutes.

A Beckmann Model LS 100C Liquid Scintillation System was used to determine radioactivity. Samples were prepared by mixing an aliquot of test sample with 10 mL of Scintiverse LC scintillation fluor. Counting efficiency was 46%.

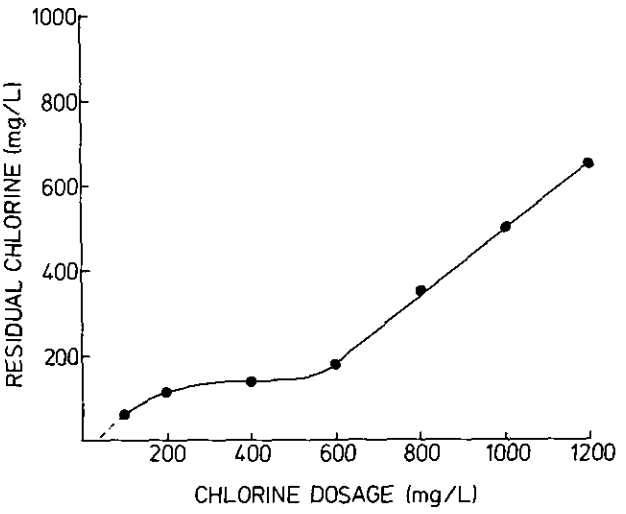


FIGURE 1. Total residual chlorine measured 15 min after chlorination of rat stomach fluid as a function of initial chlorine dosage.

# Results

## Characterization of Nitrogen Compounds in Stomach Fluid

Figure 1 is the 15-min chlorine demand curve for rat stomach fluid obtained from rats fasted 48 hr and administered 3 mL deionized water. The chlorine demand of the stomach fluid was measured as the breakpoint (600 ppm in Fig. 1) beyond which free residual chlorine could be detected. This measurement represents a volume-independent demand (600 mg/L × 0.003 L) of 1.8 mg $Cl_2$. The chlorine demand of stomach fluid recovered from 8 animals administered 4 mL deionized water was determined. The chlorine demand ranged from 1.6 mg to 5.0 mg $Cl_2$ with an average of 2.7 mg $Cl_2$. This value assumes that the chlorine demand of the stomach contents is independent of the volume of water administered or the fraction of fluid recovered.

An aliquot of a pooled sample of stomach fluid recovered from 10 animals was analyzed for amino acids by precolumn derivatization of the fluid with OPA followed by HPLC. The resulting chromatogram is shown in Figure 2. Most of the 20 essential amino acids can be identified in the sample in varying concentrations. The concentrations of amino acids in the sample are listed in Table 1.

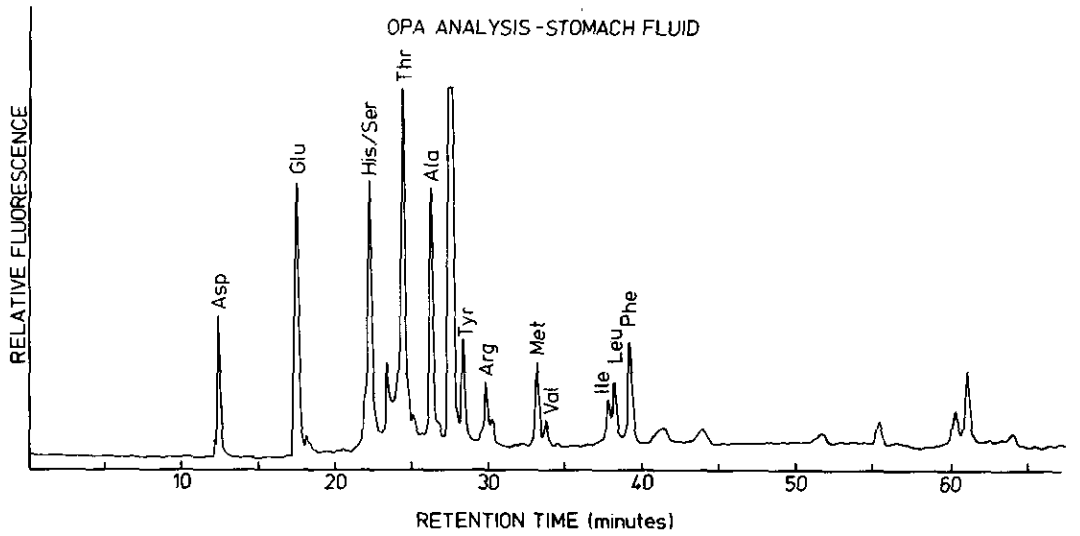


FIGURE 2. High pressure liquid chromatogram of amino acids found in rat stomach fluid. Amino acids were derivatized with *o*-phthalaldehyde (OPA) before chromatography.

**Table 1. Concentrations of amino acids in rat stomach fluid.**

| *Amino Acid* | *Concentration, μM*[a] |
|---|---|
| Aspartic acid | 3.4 |
| Glutamic acid | 10.0 |
| Histidine/serine | 8.0 |
| Threonine | 7.4 |
| Alanine | 8.0 |
| Tyrosine | 3.2 |
| Arginine | 3.2 |
| Methionine | 2.4 |
| Valine | 0.8 |
| Isoleucine | 1.2 |
| Leucine | 2.0 |
| Phenylalanine | 3.8 |
| Total | 53.4 μM |

[a] Concentration in a composite sample of stomach fluid recovered from 10 animals administered 3 mL deionized water.

## Identification of Organic *N*-Chloramines Formed in Chlorinated Stomach Fluid

When stomach fluid is chlorinated, derivatized with dansyl sulfinic acid, and chromatographed, the high pressure liquid chromatogram shown in Figure 3 is obtained. Several major peaks appear in the chromatogram. The largest peak, which elutes between 10 and 20 min after injection, is caused by unreacted dansyl sulfinic acid, the reagent used to derivatize the chloramines. The large peak, which elutes with a retention time of approximately 32 min, is caused by the dansyl derivative of inorganic chloramine. The dansyl derivatives of the major amino acids elute between 18 and 45 min with the gradient used in Figure 3. The retention times of several of the peaks in the chromatogram have been correlated with the retention times of known dansyl amino acids to identify chloramine derivatives present. The derivatized stomach fluid sample was also chromatographed using a second gradient, which gave a different elution order for the dansyl amines and dansyl amino acids. Dansyl amino acids, tentatively identified by their retention times using the first gradient program, gave retention times with the second gradient consistent with their proposed structures. In this way the chloramine derivatives that were present were identified.

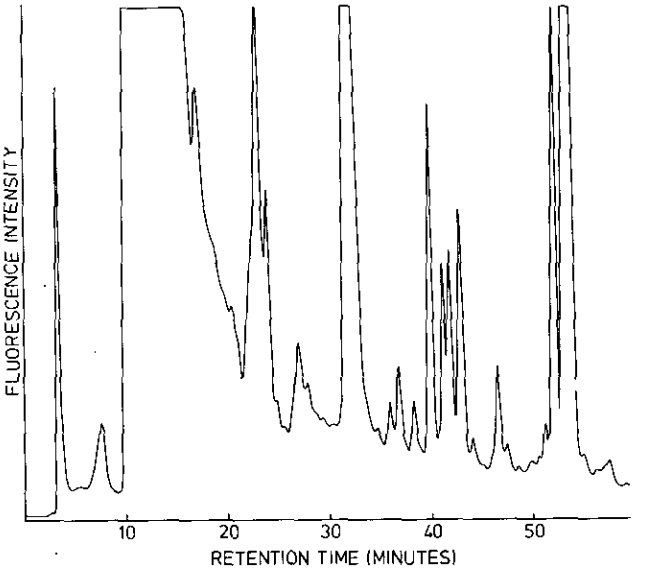


FIGURE 3. *High pressure liquid chromatogram of dansyl derivatives* of chloramines formed after rat stomach fluid was chlorinated to 400 mg/L and derivatized overnight with dansyl sulfinic acid. The second elution program described in the materials and methods section was used.

### Quantification of Organic *N*-Chloramines Formed on Chlorination of Stomach Fluid *in Vivo*

An aqueous solution of piperidine (1.0 mL of a 0.2 M solution) containing 35 μCi of [$^3$H]piperidine was administered to test animals shortly before dosing with hypochlorite. The quantity of *N*-chloropiperidine formed was determined by direct liquid chromatography of the compound. When the stomach fluid was isolated, only 30% of the radiolabel could be recovered. Approximately 30–40% of the recovered label was lost in the clean-up of the fluid with an octadecylsilica cartridge (SEP-PAK). The radiochromatogram of the labeled material, which was recovered when a 1016 mg/L solution was used, showed that 8% was *N*-chloropiperidine. When 220 mg/L hypochlorite was used, 3% of the material recovered after clean-up was *N*-chloropiperidine. The remaining labeled material had the same retention time as [$^3$H]piperidine.

## Discussion

The shape of the chlorine demand curve for stomach fluid is similar to demand curves of water containing high concentrations of ammonia or amino nitrogen. The breakpoint (600 ppm) exhibits a significant irreducible minimum, which is typical of solutions containing high concentrations of proteins. There is evidence in the low chlorine dosage end of the curve of a minimum chlorine dosage (approximately 40 to 50 mg/L) below which no residual oxidant is measurable after 15 min. This may be caused by very rapid reducing reactions that supersede chloramine formation or to reactions that reduce chloramines after they are formed. In this case, ingestion of active chlorine compounds at concentrations below this minimum chlorine concentration would result in deactivation of the oxidant. Although the FAS-DPD titrimetric method can give false negative results in the presence of some reducing agents (*10*), Abdel-Rahman has shown that there is a rapid decrease in blood glutathione following oral administration of hypochlorous acid to rats (*3*). Since all cells contain glutathione, this finding would support the theory that rapid reducing reactions are operative both in the stomach and in the blood.

Chlorine demand in stomach fluid can be exerted by amines, organic sulfides, and reducing metals. Since the reactions of amines with aqueous chlorine are so rapid (second-order rate constants $> 10^8$) (*11*), formation of organic *N*-chloramines is very likely to be one of the primary hypochlorite and hypochlorous acid reactions occurring in the stomach after ingestion of chlorinated water.

At least part of the chlorine demand of the stomach fluid results from the presence of amino acids (*12*). As the chromatogram in Figure 2 demonstrates, stomach fluid contains most of the 20 major amino acids. The amino acids in highest concentration are glutamic acid, alanine, threonine, and either histidine or serine or both (Table 1). Also present in appreciable concentrations are phenylalanine, aspartic acid, tyrosine, arginine, and leucine. The sum of their concentrations alone (see Table 1) would account for a chlorine demand of 0.11 mg $Cl_2$ or only about 4% of the average chlorine demand of stomach fluid. This percentage seems low and suggests that other important chemical factors account for the chlorine demand of stomach fluid. Among the species that could account for the remaining chlorine demand are the terminal amino functions on proteins and polypeptides, labile side chains of proteins that contain sulfhydryl, guanidine, indole, and imidazole moieties; the amide functions of proteins and polypeptides; and unsaturated lipids.

When solutions containing amino acids are chlorinated, the amino acids are rapidly converted to their *N*-chloramines. This conversion occurs in stomach fluid as well. Stomach fluid was recovered from laboratory rats, chlorinated, and derivatized with dansyl sulfinic acid. Dansyl sulfinic acid reacts with chloramines to form fluorescent sulfonamide derivatives (*6*). HPLC of the derivatized stomach fluid (Fig. 3) revealed many suspected chloramine derivatives. Several of these derivatives have been isolated by preparative liquid chromatography and have been identified by comparing their retention times with known chloramino acid derivatives using two different chromatographic methods.

Since the dansyl derivatives of amino acids are themselves amino acids, their ionic forms vary with pH. Therefore, the primary gradient used for preparative isolation of the dansyl derivatives involved chromatography of the dansyl compounds at a pH close to their $pK_a$. A simple aqueous/organic gradient was used for comparison of retention times. Chloramines identified in this fashion included *N*-chloroalanine, *N*-chloroglycine, and *N*-chlorophenylalanine.

Theory suggests that the more long-lived a compound is, the greater opportunity it has to exert any toxic effects. *N*-Chloroglycine is a comparatively long-lived chloramine with a half-life of 9000 min at 22°C (*13*). Consequently, toxic effects associated with chloramines are very likely to be more significant for this compound than for shorter-lived chloramines. In contrast, the significance of the presence of *N*-chloroalanine and *N*-chlorophenylalanine in chlorinated stomach fluid is diminished by the fact that they have comparatively short half-lives (96 min at 21°C for *N*-chloroalanine and 52 min at 25°C for *N*-chlorophenylalanine) (*13*). Decomposition of these and other short-lived chloramines is very likely to account for dissipation of some of the oxidant burden to which the body is subjected following ingestion of hypochlorite. On the other hand, it is possible that toxicological effects associated with ingestion of hypochlorite may be attributed not only to long-lived chloramino acids but also to the decomposition products of short-lived chloramino acids.

Süssman has recently reported that chlorinated solutions of some of the common amino acids found in the

body are mutagenic (*14*). However, no chemical identification of the active mutagens in these solutions was made, whether they are the *N*-chlorinated amino acids themselves or their decomposition products.

A number of other chromatographic peaks that do not coincide with dansyl derivatives of amino acids also appear in Figure 3. These peaks are believed to represent dansyl derivatives of small polypeptides. Terminal *N*-chlorinated analogs of dipeptides, such as *N*-chloroglycylglycine, are far more stable than most of the simple *N*-chloramino acids (*13*). Their identification, however, will require further study.

When a complex mixture of amines is partially chlorinated, the yield of the chlorinated analog of any one amine is determined by the concentration of that amine (in its unprotonated form) and the rate of its reaction with hypochlorite relative to the concentration and reaction rates of the deprotonated form of other amines in solution. The average chlorine demand of the stomach contents of the animals examined in this study was 2.7 mg $Cl_2$. If it is assumed that the entire chlorine demand is created by primary organic amino nitrogen compounds, then it requires half this amount (or 2.7/2 = 1.4 mg $Cl_2$) to form monochloramino analogues of these amines. Piperidine (1.0 mL of a 0.2 M solution) would require an additional 14 mg $Cl_2$ to become monochlorinated. Therefore, when 3.0 mL of a solution of hypochlorite (0.003 L × 220 mg/L as $Cl_2$ = 0.66 mg $Cl_2$) is added to a stomach fluid that requires a total of 15.4 (1.4 + 14) mg $Cl_2$ to reach a chloramine maximum, then 0.66/15.4 × 100% = 4.3% of the piperidine can react to form its chloramine, assuming that all amino nitrogens react at equal rates. When 3.0 mL of hypochlorite with a concentration of 1016 ppm $Cl_2$ is used, 3.0/15.4 × 100% = 19% of the piperidine can react to form N-chloropiperidine.

The actual yield of chloramine formed was determined by administration of tritium-labeled piperidine to fasted animals followed by administration of aqueous hypochlorite. Fasted animals were used to minimize the added chlorine demand of food present in the stomach. After clean-up of the stomach fluid, the percent recovery of the administered label was low. Two factors appeared to be responsible for this finding. First, some of the unrecovered label was believed to be absorbed into stomach tissue, and, secondly, much of the label was believed to be lost because some of the solution of tritiated piperidine administered to the fasted rats passed rapidly through the stomach and into the intestinal tract before the hypochlorite was administered. Radiolabeled material could also have been associated with the small amount of particulate matter that was filtered from the stomach fluid before analysis.

Nevertheless, the fraction of radiolabel present as N-chloropiperidine in the recovered fluid (8% and 3%) represents 42% and 70%, respectively, of the theoretical amount expected for chlorine doses of 1016 and 220 mg/L $Cl_2$, respectively. Determinations of yields at lower doses were not feasible because of the low levels of conversion, requiring large corrections for chlorine demand by other components of the stomach fluid. However, this experiment does confirm that a chloramine can form in the stomach and that its low yield is caused by the reaction of other species that exert a chlorine demand in the stomach.

## Conclusions

This paper demonstrates that amino acids account for a portion of the chlorine demand of stomach fluid and that short-lived *N*-chloramino acids may be partially responsible for the destruction of ingested hypochlorite in the body. Admittedly, the concentrations of hypochlorite used in these studies are considerably higher than concentrations encountered in drinking water. There are two reasons for using these higher concentrations. First, it was not feasible to measure concentrations of *N*-chloropiperidine formed *in vivo* using concentrations of hypochlorite lower than 200 ppm, partly because of losses of the administered radiolabel during attempted recovery and partly because of the considerable corrections required for chlorine demand. Secondly, the higher concentrations of hypochlorite were required to optimize the recovery and facilitate identification of individual chloramino acid derivatives from such a complex mixture as stomach fluid.

It is quite possible that under normal conditions the stomach contains reducing agents that would either block formation of organic *N*-chloramines altogether or subsequently reduce the chloramines at a rate sufficient to prevent them from being absorbed into the blood. However, many toxicological studies are conducted using concentrations of the toxic agent that far exceed normal levels of exposure so that a toxicological response can be observed within a reasonable time. For instance, a concentration of 200 ppm (as $Cl_2$) is two log units higher than the concentration of inorganic chloramine ($NH_2Cl$) used in drinking waters in the United States. It is also the highest concentration of $NH_2Cl$ chosen for investigation by the National Toxicology Program's (NTP) study of the possible carcinogenicity of $NH_2Cl$. At this concentration of hypochlorite, organic *N*-chloramines are formed in stomach fluid and may account for toxicological effects observed in animals administered hypochlorite at these high concentrations.

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