Nitrosation of the Alkaloids Hordenine and Gramine, Potential Precursors of *N*-Nitrosodimethylamine in Barley Malt

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Hordenine and gramine, two tertiary amine alkaloids found in barley malt, were nitrosated in aqueous acid to determine their potential to form *N*-nitrosodimethylamine (NDMA). At 24 °C and pH 3.4, the initial rate of gramine nitrosation to NDMA was equivalent to that seen for dimethylamine. Under the same conditions, the rate of hordenine nitrosation to NDMA was no faster than that observed for conversion of trimethylamine to NDMA. The rate of gramine nitrosation and the nature of the nitrosation products indicated that gramine did not undergo nitrosation by the expected mechanism of nitrosative dealkylation. A mechanistic hypothesis is offered to explain the labile nature of the dimethylamino group found in gramine. The experimental results suggest that both gramine and hordenine must be considered potential precursors of NDMA in barley malt dried by direct-fired kilning.

INTRODUCTION

The occurrence of trace levels of the carcinogen *N*-nitrosodimethylamine (NDMA) in beer and brewing malt is now well documented (Spiegelhalder et al., 1979; Goff and Fine, 1979; Havery et al., 1981). For example, in the original analytical survey of West German beers (Speigelhalder et al., 1979), approximately 70% of all beer samples analyzed contained NDMA at a mean concentration of 2.7 μg/L. In an early survey of domestic U.S. beer brands, Scanlan et al. (1980) found that 23 of 25 samples were positive (0.1 μg/L) and the mean level of NDMA was 5.9 μg/L. Since mid-1980, levels of NDMA in beer have been significantly reduced as a result of measures taken by the malting and brewing industries to inhibit nitrosamine formation. The employment of sulfuring has been especially effective in this regard (Hardwick et al., 1981; O'Brien et al., 1980).

Investigation of raw materials used in the brewing process showed that only malt dried by direct-fired kilning could account for the levels of NDMA seen in the final beer (Hardwick et al., 1981; Spiegelhalder et al., 1980). During the malt kilning process, nitrogen oxides (NO_x) formed by combustion of ambient nitrogen come into direct contact with the malt being dried. Nitrogen oxides are implicated in the nitrosation of amines in malt since Challis and Kyrtopoulos (1978) showed that N_2O_3 and N_2O_4 are both extremely effective nitrosating agents when formed in neutral or alkaline solution from their component gases. Nitrous anhydride (N_2O_3) is also known as the principal nitrosating agent present in dilute aqueous acid during the nitrosation of amines in vitro (Hughes et al., 1958).

Our interest in the nitrosation of malt amines led us to an investigation of the nitrosation of the tertiary amine alkaloids hordenine and gramine (Figure 1). Hordenine is formed biosynthetically from tyrosine and constitutes the major alkaloid found in malt roots (Frank and Marion, 1956). Gramine is formed biosynthetically from tryptophan and is found in malt acrospires (shoots) after germination (Gower and Leete, 1963).

We have presented preliminary evidence showing that the alkaloid gramine is highly susceptible to nitrosation to liberate NDMA in vitro under conditions normally used to study the potential of tertiary amines to undergo nitrosation (Mangino et al., 1981). In the current study, we present kinetic data confirming the unusual reactivity of gramine, and we describe model experiments which suggest a mechanistic hypothesis to explain the facile nitrosation reaction which gramine undergoes.

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EXPERIMENTAL SECTION

Materials. Dichloromethane (DCM), chloroform (CF), and methanol were "distilled in glass" solvents obtained from the Burdick and Jackson Co. All other solvents and common reagents were the best analytical grades available.

The following compounds or reagents were obtained from the Aldrich Chem Co. (Milwaukee, WI): dimethylamine hydrochloride, trimethylamine hydrochloride, aminopyrine, *N*,*N*-dimethyl-5-methoxytryptamine, 5-methoxygramine, NDMA, sodium cyanoborohydride, and deuteriochloroform. The following were obtained from the Sigma Chemical Co. (St. Louis. MO): gramine, hordenine hemisulfate, indole-3-carboxaldehyde, and 3Å molecular sieves.

Reaction Conditions. In the first series of experiments, dimethylamine, trimethylamine, hordenine, and gramine were nitrosated for prolonged reaction times. Reactions were carried out in the following buffer solutions: a pH 4.4 solution made by adding anhydrous sodium acetate to 60% acetic acid; a pH 6.4 solution made by mixing 6.9 parts of dibasic sodium phosphate (0.2 M) and 3.1 parts of citric acid (0.1 M). Solutions of the amines (0.1 M, 10 mL) were pipetted into 25-mL KIMAX glass tubes. Sodium nitrite (0.35 g) was added and the tubes were sealed with Teflon-lined screw caps and placed in a water bath at 65 ± 1 °C for 16 h. The tubes were cooled to room temperature and reaction mixtures extracted with two 10-mL portions of DCM. Combined DCM extracts were dried over sodium sulfate and the volume was made up to 25 mL with DCM. The NDMA concentrations were determined by gas chromatography-thermal energy analysis (GC-TEA). The instrument was a Varian 3700 GC (Walnut Creek, CA) interfaced to a Thermal Energy Analyzer (Thermo Electron Corp., Waltham, MA). The GC column was 10 ft × $^1/_8$ in. stainless steel packed with 20% Carbowax 20 M plus 2% NaOH coated on Chromasorb W-AW. The column was operated at 140 or 170 °C with helium flow rate of 25 mL/min. NDMA solutions of known concentration were injected as external standards; quantitation was done by comparison of peak height measurement of NDMA in samples and external standards.

After gramine was found to be highly reactive with nitrous acid, a third buffer solution was prepared from 15% acetic acid adjusted to pH 3.4 with anhydrous sodium

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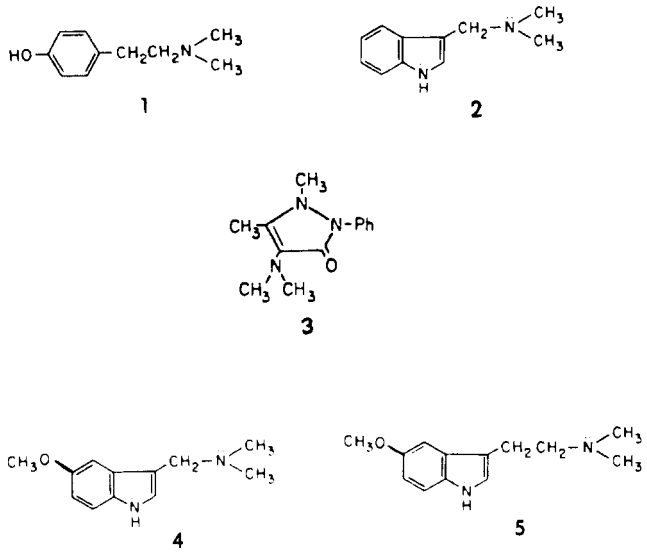

Figure 1. Structures of amines employed in nitrosation rate study: 1, hordenine; 2, gramine; 3, aminopyrine; 4, 5-methoxygramine; 5, *N,N*-dimethyl-5-methoxytryptamine.

acetate. This buffer was used to carry out the nitrosation of gramine and dimethylamine at ambient temperature (23–25 °C). Extraction and quantitation of NDMA were similar to that described previously.

In a second set of experiments, the formation of NDMA from dimethylamine and a series of tertiary amines was measured. For these experiments, amine solutions of pH 3.4 and 0.1 M were desired. A solution of 15% acetic acid was raised to pH 3.15 by addition of anhydrous sodium acetate. This buffer was used to make 0.1 M solutions of gramine, 5-methoxygramine, *N,N*-dimethyl-5-methoxytryptamine, and aminopyrine; the final pH of each solution was pH 3.4. A second buffer was made by adjusting the pH of 15% acetic acid solution to pH 3.45 by addition of anhydrous sodium acetate. This buffer was used to make 0.1 M solutions of dimethylamine HCl, trimethylamine·HCl, and hordenine hemisulfate; the final pH of each solution was pH 3.4.

Samples to be nitrosated were prepared by pipetting 10 mL of the amine solution into 20-mL KIMAX glass tubes. The ambient temperature reactions (23–24 °C) were initiated by addition of 0.69 g of sodium nitrite and the tubes were sealed. When the desired reaction time was reached, mixtures were poured into ice cold solutions of 6.2 M ammonium sulfamate (5 mL, pH 1.5). When foaming subsided, quenched reaction mixtures were extracted with three 5-mL portions of CF and extracts combined and made up to 25 mL. The NDMA concentrations were determined by GC-TEA as outlined previously. For reactions at elevated temperature, each amine solution was preincubated in a water bath to 37 ± 0.5 °C or 65 ± 0.5 °C before addition of sodium nitrite. Heated tubes were cooled in ice–salt baths before opening and quenching.

Synthesis. The synthesis of *N*-methyl-3-(aminomethyl)indole was accomplished by a modification of the general method of Borch et al. (1971). Indole-3-carboxaldehyde (8.7 g, 0.06 mol) was dissolved in absolute methanol and made alkaline to pH 11 with 5% KOH in methanol. Molecular sieves (3Å) were added, followed by methylamine hydrochloride (0.3 mol) and sodium cyanoborohydride (0.064 mol). The pH was lowered to 6.5 by addition of glacial acetic acid and the mixture warmed to 55 °C for 2.5 h. After cooling and acidification (aqueous HCl) to pH 1.5, methanol was removed in vacuo and the residual oil taken up in water and extracted with three 35-mL volumes of ethyl acetate. The remaining aqueous layer was slowly made alkaline to liberate an insoluble oil, which was extracted with ethyl acetate, dried over Na_2SO_4, and concentrated in vacuo to yield a nearly colorless oil (7.3 g, 76%) which crystallized completely on standing at room temperature. The material was homogenous by TLC (acetone–CF–28% NH_4OH [12:6:1]) and could be recrystallized with difficulty from benzene to give biege-colored prisms (mp 78–80 °C). A picrate derivative recrystallized from 95% ethanol: mp 173–175 °C dec (lit. mp 176–176.5 °C dec (Gower and Leete, 1963)); mass spectrum 160 (25) M^+, 159 (23), 130 (100), 129 (42), 102 (31), 77 (31), 42 (25); high-resolution mass spectrum for $C_{10}H_{12}N_2$, calculated mass 160.1000, found 160.100; ^1H NMR ($CDCl_3$) 2.46 (s, 3 H), 3.89 (s, 2 H), 6.94–7.58 (m, 6 H); IR (KBr) 3305 (m, NH), 3125 (b, indole NH).

The nitrosation of *N*-methyl-3-(aminomethyl)indole was described in detail previously (Mangino et al., 1982) and will be summarized here. The amine (4 g, 0.025 mol) was dissolved in 30% acetic acid and the pH adjusted to 3.4. Sodium nitrite was added, and the mixture stirred for 4 h. The mixture was slowly made alkaline to pH 10 and extracted with three 30-mL volumes of DCM and the solvent removed in vacuo to give an amber colored oil (4.55 g). TLC showed two components. A portion of the oil (1.15 g) was taken up in 2 mL of CF and applied to a glass column containing 115 g of silica gel packed by slurry in neat CF. Elution was begun with neat CF and continued until a bright yellow band was completely eluted. Removal of solvent left an homogeneous oil (0.69 g) which turned to a crystalline mass after storage in the dark for several days at −20 °C. Recrystallization of this material from warm methanol yielded 0.19 g of yellow prisms (mp 47–48 °C). This crystalline material showed a mass spectrum corresponding to N^1,*N*-dinitroso-*N*-methyl-3-(aminomethyl)indole. Continued elution of the chromatography column with 2% ethyl acetate in CF yielded a second homogeneous material which gave 0.08 g of crystalline product (mp 122–123 °C) after recrystallization from methanol. This product showed spectroscopic properties consistent with *N*-nitroso-*N*-methyl-3-(aminomethyl)-indole.

The mass spectrum of N^1,*N*-dinitroso-*N*-methyl-3-(aminomethyl)indole: 218 (4) M^+, 188 (92), 158 (99), 143 (94), 129 (99), 117 (57), 102 (85), 89 (34), 76 (34), 43 (100); high-resolution mass spectrum for $C_{10}H_{10}N_4O_2$, calculated mass 218.0804, found 218.080; ^1H NMR ($CDCl_3$) 2.98 (*syn*-CH_2), 3.72 (*anti*-CH_3), 4.88 (*syn*-CH_2), 5.43 (*anti*-CH_3), 7.17–7.62 (m, 4 H), 8.12 (1 H, not exchangeable with D_2O).

The mass spectrum of *N*-nitroso-*N*-methyl-3-(aminomethyl)indole: 189 (39) M^+, 159 (18), 144 (68), 131 (100), 130 (92), 117 (32), 103 (54), 89 (35), 77 (85), 42 (100); ^1H NMR ($CDCl_3$) 2.89 (s, *syn*-CH_3), 3.48 (s, *anti*-CH_3), 4.90 (s, *syn*-CH_2), 5.41 (s, *anti*-CH_2), 6.94–7.49 (m, 5 H), 8.35 (^1H, slowly exchangeable with D_2O).

HPLC Analysis. In order to observe the formation of nonvolatile products of gramine nitrosation, the following experiments were performed. Sodium nitrite (0.35 g) was added to solutions of gramine (10 mL, 0.1 M) in the acetic acid–acetate buffer at pH 3.4 and 20 °C. Reaction was allowed to continue for a specified time, then 10 mL of acetonitrile was added to the reaction tube, and the contents were shaken to insure complete solution. Aliquots of the reaction mixture (10 μL) were immediately injected onto an SP8700 HPLC (Spectra-Physics, San Jose, CA) with a 4.6 mm × 250 mm Spherisorb C_{18} column with 10 μm packing. The flow rate was 2 mL/min with UV detection at 254 nm. The eluting solvent was a gradient

Table I. Yield (%) of NDMA after Nitrosation of Potential Amine Precursors[a]

amine	pH 4.4[b]	pH 6.4[c]
dimethylamine	78	65
trimethylamine	8	0.8
hordenine	11	2
gramine	76	5

[a] Conditions: 0.1 M amine in 0.5 M $NaNO_2$ at 65 °C for 16 h. [b] Acetate buffer. [c] Citrate–phosphate buffer.

mobile phase (75% water–25% methanol to 60% water–40% methanol). The results showed the progressive increase with time of peaks corresponding to NDMA as well as peaks with retention times of 18.4 and 19 min; the latter two peaks and NDMA appeared to be the major reaction products after a 60-min reaction period. Spiking experiments showed that neither peak eluting at 18.4 or 19 min coeluted with a standard of indole-3-carboxaldehyde. The aldehyde would have been detected if present at a level of 0.5% or greater in any reaction product (based on amount of starting amine). The yield of NDMA obtained after 60 min under these conditions was approximately 40%. The stability of indole-3-carboxaldehyde under vigorous reaction conditions was determined as follows. A solution of standard indole-3-carboxaldehyde (0.04 M, 10 mL) in acetic acid–acetate buffer (pH 3.4) was treated with 0.7 g of sodium nitrite and the mixture heated at 65 °C for 60 min. The mixture was cooled and analyzed by HPLC as described previously. The results showed essentially no loss of indole-3-carboxaldehyde. The same result was seen when indole-3-carboxaldehyde was heated in the presence of both gramine (0.1 M) and sodium nitrite (1 M) in the acetic acid–acetate buffer (pH 3.4).

In separate experiments under the same HPLC conditions described previously but with a different gradient mobile phase (60% water–40% methanol to 55% water–45% methanol), it was observed that neither of the indolic nitrosamines (*N*-nitroso-*N*-methyl-3-(aminomethyl)indole or N^1,*N*-dinitroso-*N*-methyl-3-(aminomethyl)indole) was present in the gramine nitrosation product after a 60-min reaction time. Both nitrosamines were stable under these reaction conditions, and either one could have been detected if present at a concentration representing as little as 1% of the reaction product (based on the amount of starting amine). **Caution!** Nitrosamines have been shown to induce cancer in laboratory animals. Proper care should be exercised in the handling and disposal of nitrosamines. In this study, nitrosamine solutions were decontaminated by nickel–aluminum alloy reduction in alkali as described by Lunn et al. (1981).

RESULTS

In order to compare the relative capacity for NDMA formation, dimethylamine, trimethylamine, hordenine, and gramine were nitrosated in aqueous solution at the temperature and pH normally encountered during malt kilning. Consequently, the reactions were performed at pH 4.4 and pH 6.4 which represent the extremes of pH encountered during malt kilning as governed by the use of sulfuring techniques (O'Brien et al., 1980). The yields of NDMA obtained from each amine are shown in Table I. The results showed that dimethylamine was readily nitrosated at pH 4.4 as expected, and hordenine and gramine were also nitrosated to give NDMA. Gramine was found to be extremely susceptible to nitrosation at pH 4.4, since it gave a yield of NDMA an order of magnitude larger than the yield of NDMA obtained from trimethylamine. At pH 4.4, gramine appeared to be nitrosated to yield NDMA as readily as dimethylamine. In separate experiments, it was found that nitrosation of gramine to yield NDMA was a facile reaction at ambient temperature. For example, nitrosation of gramine at pH 3.4 in a 3-fold excess of nitrite at 23 °C for 6 h resulted in a 61% yield of NDMA. Nitrosation at pH 3.4 in a 3-fold excess of nitrite at 23 °C for 10 min resulted in a 24% yield of NDMA. Apparently, gramine is nitrosated to yield NDMA as readily as dimethylamine even at the optimum pH for conversion of dimethylamine to NDMA, which is reported to be pH 3.4 (Mirvish, 1975).

The nitrosation of gramine to liberate NDMA appeared to be an unusually facile reaction. In order to firmly establish the validity of this conclusion and to obtain initial evidence for a mechanistic explanation for the reactivity, a group of "comparative nitrosation" experiments was carried out. Among the objectives of the experiments were (1) to compare at ambient temperature and short time the initial rate of gramine nitrosation with that of aminopyrine (**3**) (Figure 1), the most reactive known tertiary amine which yields NDMA (Mirvish et al., 1974), (2) to compare the nitrosation of gramine with trimethylamine, from which NDMA is the only nitrosamine formed, (3) to establish the magnitude of difference in reactivity between gramine and hordenine, and (4) to determine if the N-substituted indole-3-methylene group of gramine is essential to the observed reactivity. In order to fulfill the fourth objective, it was desired to compare the nitrosation of gramine with that of *N*,*N*-dimethyltryptamine. Since the latter compound is a legally controlled drug, the comparison was made between the nitrosation reactions of 5-methoxygramine (**4**) and *N*,*N*-dimethyl-5-methoxytryptamine (**5**) (Figure 1). All nitrosation reactions were run at pH 3.4 with a 10-fold excess of nitrite to insure that measureable yields of NDMA would be obtained from each tertiary amine.

The comparison in rates of NDMA formation from aminopyrine, gramine, dimethylamine, and trimethylamine at 24 °C is shown in Figure 2. The initial reaction of aminopyrine with nitrous acid was so fast that accurate yield data could not be determined at times less than 2.5 min. Under the conditions used, no difference in the initial rates of gramine and dimethylamine nitrosation could be distinguished but the yield curves diverged after 5 min. After 2 h at 24 °C, nitrosation of gramine to NDMA did not go to completion but the reaction apparently could continue at a reduced rate (Figure 2). In contrast with the nitrosation of gramine, the nitrosation of trimethylamine to give NDMA was only 0.19% complete after 2 h at 24 °C.

The comparison in rates of NDMA formation from gramine and hordenine at 23, 37, and 65 °C is shown in Figure 3 parts A–C. The magnitude of difference in reactivity between gramine and hordenine is dramatically illustrated in Figure 3 part C. At 65 °C, there was a quantitative conversion of gramine to NDMA in 10 min, whereas the conversion of hordenine to NDMA was only 7.2% complete after 30 min.

Finally, we compared the rates of NDMA formation from gramine, 5-methoxygramine, and *N*,*N*-dimethyl-5-methoxytryptamine. Our rationale for employing the latter two compounds was that the 5-methoxy substituent should exert the same relative influence on the reactivity of each amine, but the methoxy group itself would be distant enough from the aliphatic nitrogen to have a limited effect (by induction or resonance) on direct nitrosation at the aliphatic amino group. As shown in Figure 4, gramine and 5-methoxygramine were clearly different kinetically from *N*,*N*-dimethyl-5-methoxytryptamine in their susceptibility

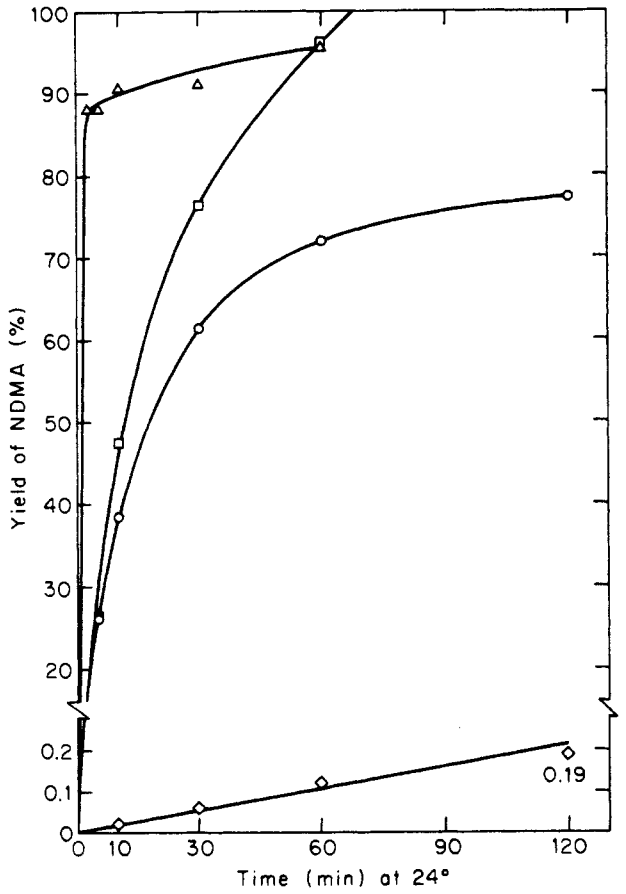

Figure 2. Rates of NDMA formation at 24 °C from (△) aminopyrine, (□) dimethylamine, (O) gramine, and (◇) trimethylamine. Reactions were performed with 0.1 M amine and 1.0 M nitrite at pH 3.4.

to undergo nitrosation to give NDMA. The same pattern of NDMA yield curves was seen at 23 and 37 °C. These results strongly suggested that the N-substituted indole-3-methylene moiety is an essential requirement for the observed rate of nitrosation of gramine and 5-methoxygramine. In contrast, elongation of the indole-3-methylene moiety by an additional methylene group caused complete loss of the enhanced reactivity.

The fast reaction of gramine with nitrous acid to give high yields of NDMA indicated that gramine might not undergo nitrosation by the expected mechanism of nitrosative dealkylation (Smith and Loeppky, 1967). According to the mechanism of nitrosative dealkylation (Figure 5), *N*,*N*-dimethyl-substituted tertiary amines such as gramine should undergo extensive demethylation as a result of steric factors encountered in the transition state required for syn cyclic elimination of NOH from a preformed nitrosammonium ion (Figure 5). If demethylation was occurring during the nitrosation of gramine, then two of the expected products would include the nitrosamines *N*-nitroso-*N*-methyl-3-(aminomethyl)inole (**6**) and N^1,*N*-dinitroso-*N*-methyl-3-(aminomethyl)indole (**7**) (Figure 6). We previously reported the synthesis and characterization of these two nitrosamines from the secondary amine *N*-methyl-3-(aminomethyl)indole (Mangino et al., 1982). Another product expected from nitrosative dealkylation of gramine is the carbonyl compound indole-3-carboxaldehyde (Figure 6).

In order to investigate the possible formation of these nitrosative dealkylation products during nitrosation of gramine, an HPLC system was devised which allowed the retention times and chromatographic characteristics of these compounds to be compared with the HPLC profile of the actual nitrosation reaction of gramine in dilute acetic acid at 20 °C and pH 3.4. The HPLC analysis indicated the formation of at least two major initial products in addition to NDMA after a 60-min reaction period at 20 °C (Figure 7). When the nitrosation reaction was conducted in phosphate buffer (20 °C, pH 3.4), the same major initial reaction products were seen after a 60-min reaction time. The identity of the major reaction products from gramine nitrosation is now under investigation in our laboratory.

Extensive coelution experiments indicated that nitrosamines **6** and **7** (Figure 6) were not formed after 60 min of gramine nitrosation at 20 °C in a 5-fold excess of nitrite

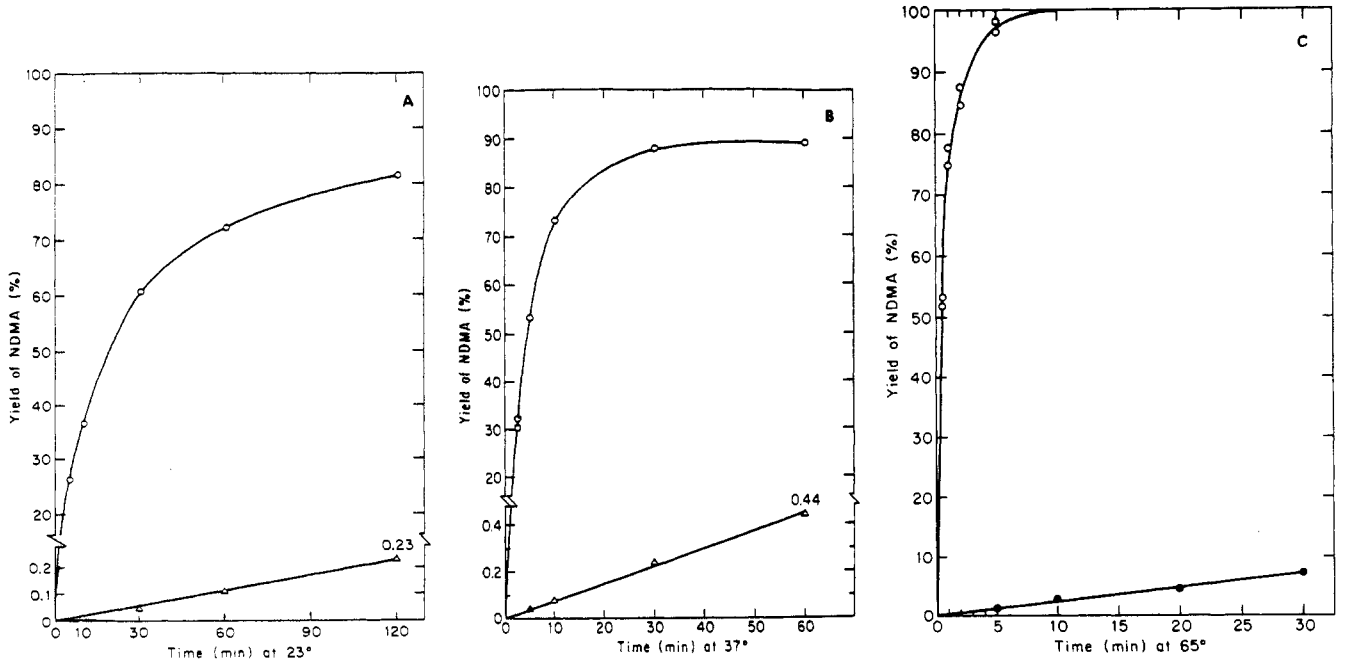

Figure 3. Rates of NDMA formation with 0.1 M amine and 1.0 M nitrite at pH 3.4. (A) Rates of NDMA formation at 23 °C from (O) gramine and (◇) hordenine. (B) Rates of NDMA formation at 37 °C from (O) gramine and (△) hordenine. (C) Rates of NDMA formation at 65 °C from (O) gramine and (•) hordenine.

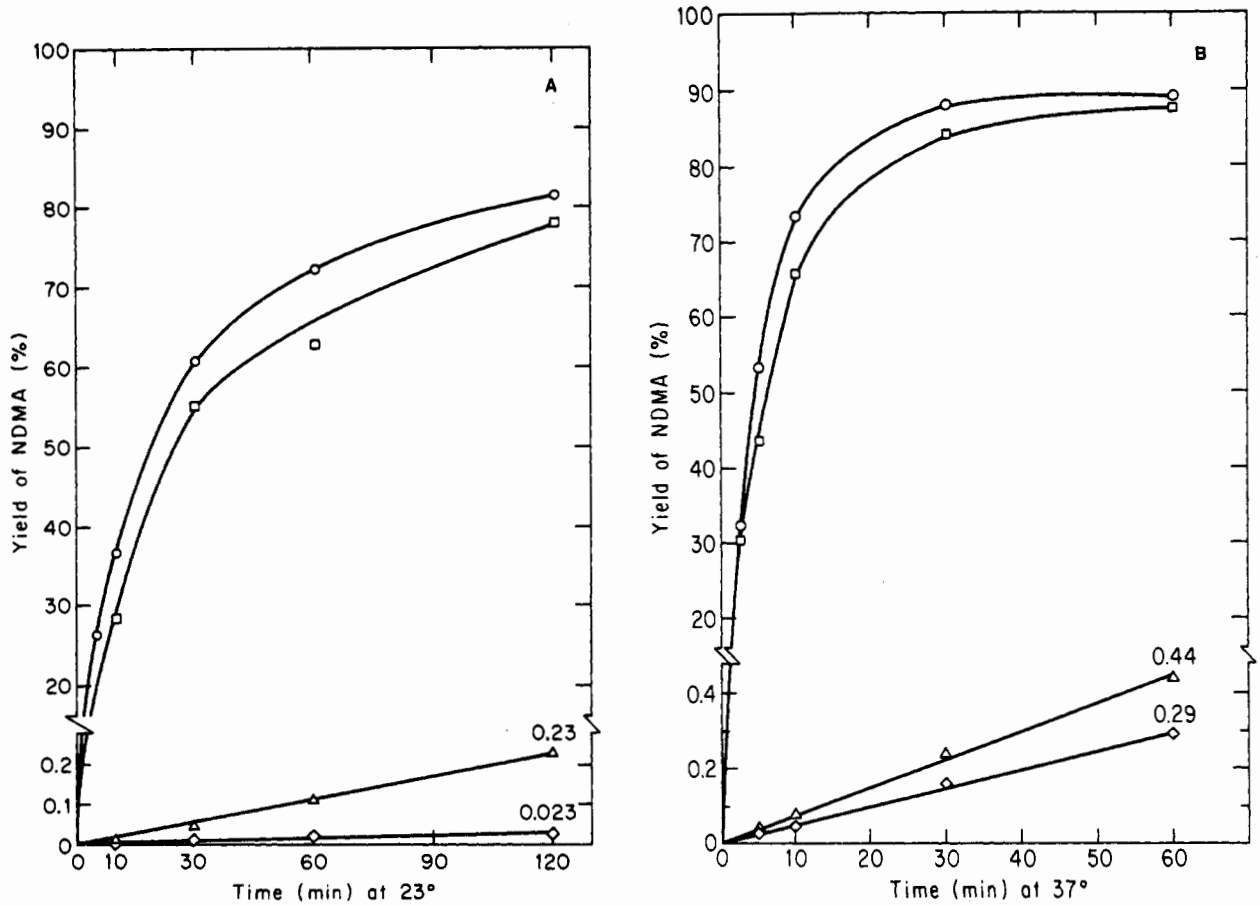

Figure 4. Rates of NDMA formation with 0.1 M amine and 1.0 M nitrite at pH 3.4. (A) Rates of NDMA formation at 23 °C from (O) gramine, (□) 5-methoxygramine, (△) hordenine, and (◇) *N,N*-dimethyl-5-methoxytryptamine. (B) Rates of NDMA formation at 37 °C from (O) gramine, (□) 5-methoxygramine, (△) hordenine, and (◇) *N,N*-dimethyl-5-methoxytryptamine.

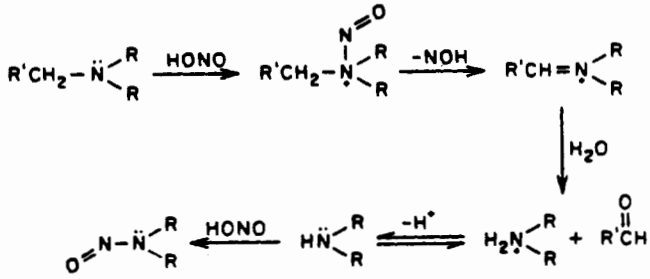

Figure 5. Proposed mechanistic pathway for nitrosative dealkylation of tertiary amines (Smith and Loeppky, 1967).

at pH 3.4. Under these conditions, compounds **6** and **7** are sufficiently stable to be detectable and either would have been observed if present at concentrations representing as little as 1% of the reaction product (based on the amount of starting amine). The yield of NDMA under these conditions was approximately 40%.

In corresponding fashion, it was observed that indole-3-carboxaldehyde was not formed from gramine at ambient temperature or at 65 °C in a large excess of nitrite. The aldehyde was stable under these conditions as determined by heating authentic samples of the aldehyde in aqueous acetic acid at 65 °C (pH 3.4) in the presence of excess nitrite.

DISCUSSION

Alkaloidal tertiary amines which are products of germination in malted barley have the potential to serve as precursors of *N*-nitrosodimethylamine (NDMA) during malt kilning. Two amines which are likely candidates for precursors of NDMA are the phenolic alkaloid hordenine and the indole alkaloid gramine.

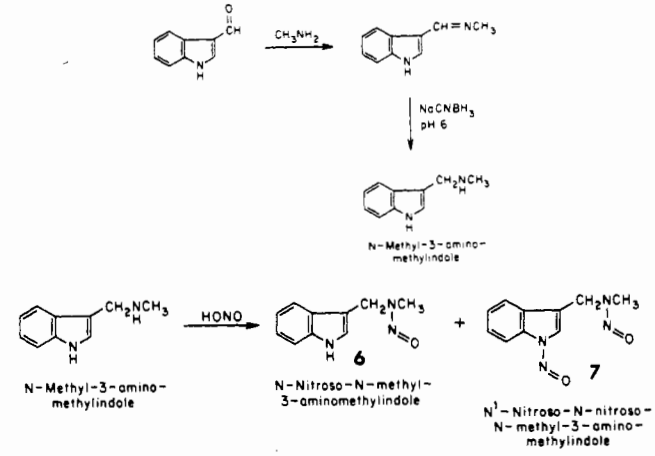

Figure 6. Outline for the synthesis of *N*-methyl-3-(aminomethyl)indole starting from indole-3-carboxaldehyde and products from the nitrosation of *N*-methyl-3-(aminomethyl)indole.

The presence of these two alkaloids in germinated malt provides a rationale for two important observations: (1) The relatively high levels of NDMA found in direct-fired, unsulfured malt. (2) The discovery that NDMA is the predominant volatile *N*-nitrosamine detected in direct-fired malt. The second observation is supported by analysis of hundreds of different malt samples representing many different barley cultivars.

An assessment of the relative contribution of hordenine and gramine to the NDMA level in direct-fired malt requires a knowledge of the relative reactivity of each alkaloid toward nitrosation to yield NDMA. In this report, the nitrosation of both alkaloids was studied by using

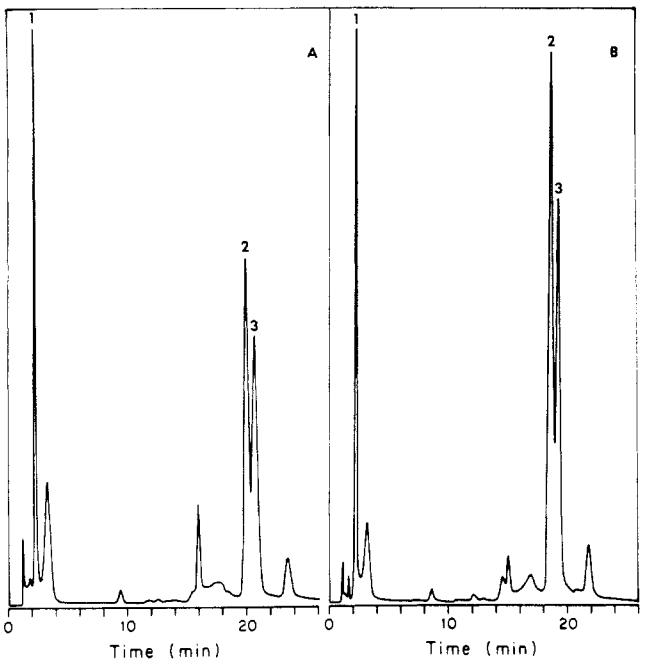

Figure 7. HPLC chromatograms of products from the nitrosation of gramine at 20 °C with 0.1 M amine and 0.5 M nitrite at pH 3.4: (A) 30-min reaction; (B) 60-min reaction. Peak 1 is NDMA. Peak 2 and 3 are unidentified major reaction products. Chromatography was performed on a 4.6 mm × 250 mm C_{18} reverse-phase column with methanol–water eluant at a flow rate of 2 mL/min with UV detection at 254 nm.

aqueous acid as the model system since nitrosation mechanisms in this system are well understood. The nitrosating agent in aqueous acid is nitrous anhydride (N_2O_3), the same species implicated as a nitrosating agent in the gases resulting from the combustion process used in direct-fired kilning.

Nitrosation of gramine and hordenine under various conditions of pH and temperature showed that gramine was much more highly susceptible to nitrosation to give NDMA. The rapid reaction of nitrous acid with gramine was surprising based on the usual reactivity observed for tertiary amines (Lijinsky et al., 1972b). Furthermore, the preponderance for NDMA formation from gramine was not predicted on the basis of the usual steric preferences seen for the nitrosative dealkylation of tertiary amines (Smith and Loeppky, 1967). For example, Smith and Loeppky found that nitrosation of *N*,*N*-diethylbenzylamine resulted mainly in loss of an ethyl group so that the most predominant nitrosamine formed (in a ratio of 4:1) was *N*-nitrosobenzylethylamine rather than *N*-nitrosodiethylamine. By way of analogy with these results, it was expected that loss of a methyl group rather than loss of the indole-3-methylene group would be the most favored pathway during nitrosation of gramine if the usual nitrosative dealkylation mechanism were operating.

The experimental results which indicated that gramine does not undergo nitrosative dealkylation in nitrous acid can be summarized as follows: (*1*) The reaction of gramine with nitrous acid to yield NDMA was fast even at room temperature. The initial rate of gramine nitrosation to yield NDMA appeared to be as fast as the rate of nitrosation of the secondary amine dimethylamine. (2) At elevated temperature under conditions normally used to study tertiary amine nitrosation, the reaction of nitrous acid with gramine gave a quantitative yield of NDMA. Therefore, the expected loss of a methyl group from gramine by nitrosative cleavage could not have occurred. (3) Investigation of the reaction products from nitrosation of

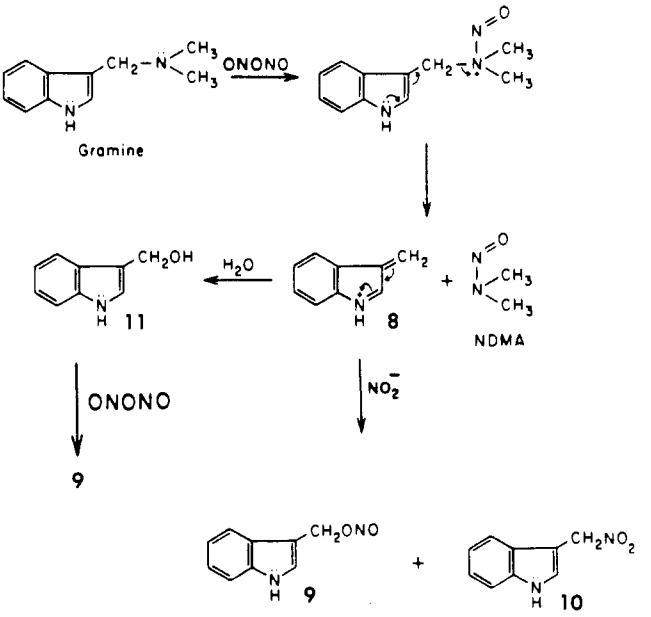

Figure 8. Suggested mechanism for NDMA formation during nitrosation of gramine.

gramine at room temperature showed that indole-3-carboxaldehyde was not formed after a time sufficient for obtaining a 40% yield of NDMA. Indole-3-carboxaldehyde was the expected carbonyl byproduct if NDMA were formed from gramine as a result of nitrosative dealkylation. (4) Further investigation of the reaction products at room temperature showed the absence of both of the indolic nitrosamines **6** and **7** (Figure 6), the two nitrosamines expected to be formed in addition to NDMA if gramine were subject to nitrosative dealkylation.

In response to these experimental results, the mechanism illustrated in Figure 8 is proposed to explain the facile nitrosation of gramine. This mechanism is a type of S_N1 reaction in which breakdown of an initial nitrosammonium ion results in direct elimination of NDMA. Attack of unprotonated nitrite ion on the imminium cation 8 could result in formation of the nitrite ester **9** and the nitro compound **10**. Nitrite ion is an ambident nucleophile which attacks electrophilic species to give nitrite esters and nitro compounds (March, 1968). Alternatively, hydration of the carbonium ion or the imminium cation would lead to indole-3-carbinol **11**, which could be nitrosated to give the nitrite ester **9**. The nucleophilic attack of alcohols on inorganic acid halides (e.g., NOCl) or inorganic acid anhydrides (e.g., N_2O_3) is the classic reaction for production of nitrite esters (March, 1968).

We are currently working on the further characterization of reaction products from the nitrosation of gramine in dilute aqueous acid. Obviously, determination of the fate of the indole-3-methylene moiety is essential for verification of the mechanism proposed in Figure 8.

The nitrosation of gramine and the analgesic drug aminopyrine (**3**) illustrate the difficulty of predicting the reactivity of tertiary amines to nitrosating conditions. Lijinsky et al. (1972a) originally showed that aminopyrine could be nitrosated under mild conditions (37 °C, pH 4) to form NDMA in yields exceeding 70% of theoretical. Mirvish et al. (1974) studied the kinetics of aminopyrine nitrosation. The initial rate of nitrosation was found to be independent of amine concentration but proportional to the square of nitrous acid concentration. This result was consistent with rate-limiting formation of N_2O_3. Product analysis of the nitrosation of aminopyrine showed that NDMA was the only nitrosamine formed in the initial

reaction. These results indicated that aminopyrine did not undergo nitrosative dealkylation, a phenomenon which we have now also observed in the nitrosation of gramine.

Gramine and 5-methoxygramine are Mannich base derivatives of the indole nucleus. Both of these alkaloids possess a single methylene group positioned between the aromatic nucleus and the aliphatic nitrogen atom. Our experimental results indicate that this structural feature is crucial to the reactivity observed when these compounds are subjected to nitrosating conditions.

Shortly following our initial report on the facile nitrosation of gramine, Loeppky et al. (1982) reported on the rapid nitrosation of 2-[*N*,*N*-(dimethylamino)methyl]-pyrrole. This compound is a Mannich base derivative of the pyrrole nucleus and also possesses a single methylene group positioned between the aromatic ring and the aliphatic nitrogen atom. This amine underwent nitrosation in acetic acid at 65 °C to give NDMA in 80% yield in 5 min in a 10-fold excess of nitrite. At 25 °C, the same compound gave a 75% yield of NDMA in 5 min; NDMA was the only nitrosamine detected under all reaction conditions employed. These results were consistent with a mechanism not involving nitrosative dealkylation. Since 2-[*N*,*N*-(dimethylamino)methyl]pyrrole is a Mannich base analogue of gramine, there may be a relationship between the mechanism of nitrosation of the two compounds (Loeppky et al., 1983).

A firm conclusion concerning the relative importance of hordenine or gramine as a precursor to NDMA in direct-fired malt cannot be made without reliable analytical data on the level of each alkaloid in green malt. At this point, both alkaloids must be considered as candidates for precursors to NDMA. We have developed a method for isolation of malt alkaloids which employs solvent extraction and separation by HPLC. Preliminary results (unpublished) indicate that the mean level of hordenine in some cultivars of barley malt is higher than the mean level of gramine. The difference in mean levels of the two alkaloids appears to depend on the type of barley cultivar employed.

With regard to the results presented here, it now seems reasonable that gramine could be a precursor of NDMA in barley malt. Other workers have concluded that hordenine must be the principal precursor of NDMA in barley malt based on the idea that hordenine is present in green malt at higher concentration than gramine (Slack and Wainwright, 1981). These workers, however, did not take into account the large difference in ease of NDMA formation from the two compounds. Additional work is underway in our laboratory to determine the relative contribution of the different amines in green malt to the formation of NDMA in barley malt.

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