AN ABSTRACT OF THE THESIS OF

Mario M. Mangino for the degree of Doctor of Philosophy

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Title: Formation of N-Nitrosodimethylamine and Nonvolatile N-Nitrosamines From Barley Malt Alkaloids

Abstract Approved: R.A. Scanlan

Amines formed by biosynthesis in germinated barley have been suggested as precursors for N-nitrosodimethylamine (NDMA) in barley malt dried by direct-fired kilning. This hypothesis was verified by germinating raw barley and exposing the resulting malt roots and clean malt to nitrous acid. Quantitation of the NDMA formed indicated that malt roots contained relatively large amounts of NDMA precursor which could only have been formed as a result of germination. The clean malt also contained NDMA precursor, but the amount of precursor in clean malt was only slightly higher than the level of precursor already present in raw barley.

The two tertiary amine alkaloids, hordenine and gramine, which are biosynthesized in malt during germination were subjected to nitrosation. Nitrosation of both amines at 65° (pH 4.4 or pH 6.4) resulted in NDMA formation. Gramine was highly susceptible to nitrosation to yield NDMA. At 24° in dilute acetic acid (pH 3.4), the initial rate of nitrosation of gramine to yield NDMA was nearly equal to the initial rate of NDMA formation from dimethylamine. The ratio of initial rates of formation of NDMA from gramine and trimethylamine was

6250:1. At 23°, the ratio of initial rates of formation of NDMA from gramine and hordenine was 5200:1.

N-Methyltyramine and N-methyl-3-aminomethylindole, the immediate biosynthetic precursors of hordenine and gramine, were synthesized. Nitrosation of N-methyltyramine yielded p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine as the major reaction product and p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine as the minor product. Nitrosation of N-methyl-3-aminomethylindole yielded N-nitroso-N-methyl-3-aminomethylindole as the minor reaction product; the major product was a dinitroso compound identified as N^1-nitroso-N-nitroso-N-methyl-3-aminomethylindole.

Investigation of the products of hordenine nitrosation at 65° (pH 4.4) indicated the formation of NDMA and p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine; p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine was not observed as a product of hordenine nitrosation at 65°. NDMA appeared to be the major nitrosamine formed from hordenine.

The rapid reaction of gramine with nitrous acid and investigation of the gramine nitrosation reaction products both indicated that gramine did not undergo nitrosation by the expected mechanism of nitrosative dealkylation. A new mechanism is proposed to explain the rapid reaction of gramine with nitrous acid and to account for the fact that NDMA was the only N-nitrosamine formed during the nitrosation of gramine.

FORMATION OF N-NITROSODIMETHYLAMINE AND NONVOLATILE N-NITROSAMINES FROM BARLEY MALT ALKALOIDS

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I. INTRODUCTION

The Nitrosamine Problem

N-nitrosamines are derivatives of secondary amines and have been known in the chemical literature for over a hundred years. These compounds elicited little practical interest until the toxicological properties of N-nitrosodimethylamine (NDMA) were reported by Barnes and Magee (1954). Single exposures of rats to high doses of NDMA caused severe liver necrosis, and long-term, chronic feeding of NDMA to rats was subsequently shown to cause induction of liver tumors (Magee and Barnes, 1956). In the past 25 years, a large number of N-nitroso compounds have been synthesized and tested for carcinogenic potential by administration to the rat in small doses, usually by the oral route. The number of N-nitroso compounds tested by chronic administration to rodents now exceeds 120, and some three-fourths of these compounds have been found to be carcinogenic (Lijinsky and Taylor, 1977).

While most of these carcinogenicity tests have been carried out in the rat, two compounds, NDMA and N-nitrosodiethylamine (NDEA) have been tested in 20 species or more, and both have been shown to induce tumors in all species (Lijinsky and Taylor, 1977). It is likely, therefore, that no species is immune to the carcinogenic effect of N-nitrosamines. Although no direct epidemiologic evidence linking N-nitrosamines with human cancer is available, there is a multitude of indirect evidence that N-nitrosamines should be

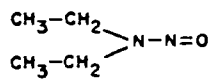
carcinogenic to man.

Three important factors involved in the structure-activity and biological activity of N-nitrosamines will be mentioned briefly:

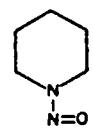
1) Carcinogenic nitrosamines comprise a wide range of structural types. N-nitrosamines may be aliphatic in nature, as represented by the dialkylnitrosamine,N-nitrosodiethylamine; or heterocyclic in nature, represented by N-nitrosopiperidine; or partly aliphatic and partly aromatic as represented by N-nitrosobenzylmethylamine. The structures of some representative N-nitrosamines are shown in Figure 1.
2) N-nitrosamines show an exceptional degree of organ specificity and usually induce tumor formation in a particular organ or tissue regardless of the route of administration. There appears to be a strong relationship between the chemical structure of a particular nitrosamine and its potency and site of tumor induction, a fact which is currently under intensive study.
3) A number of nitrosamines produce lesions which are effective models of certain types of human cancer. For example, N-nitrososarcosine ethyl ester was found to induce a series of pre-neoplastic and neoplastic changes in the rat esophagus which are similar to the sequence of neoplasm formation seen in esophageal cancer formation in the people of certain regions of Northern China (Yang, 1980). A second example is N-nitroso-2,6-dimethylmorpholine which induces ductal adenocarcinomas in the pancreas of Syrian hamsters. These

Figure 1. Structures of some representative N-Nitrosamines

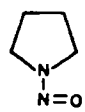
Figure 1



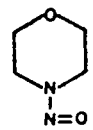
DIETHYLNITROSAMINE



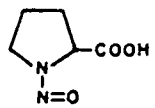
NITROSOPIPERIDINE



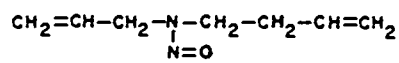
NITROSOPYRROLIDINE



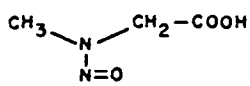
NITROSOMORPHOLINE



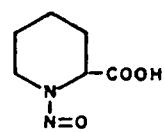
NITROSOPROLINE



γ-BUTENYL (β-PROPENYL) NITROSAMINE



NITROSOSARCOSINE



NITROSOPIPECOLIC ACID

lesions closely resemble the histological and morphological type of malignant neoplasm of pancreas seen most often in man (Reddy et al., 1979).

In view of the factors discussed above, the possibility that man might be exposed to nitrosamines in food and other environmental sources has become a question of considerable public interest. The suspicion that nitrosamines could form in food was first brought to light in Norway, where ruminant animals fed on herring meal preserved with high levels of sodium nitrite were found to suffer severe liver disorders. The toxic herring meal contained NDMA, and the reaction of nitrite with endogenous methylamines in the fish meal was proposed to explain the formation of the nitrosamine (Ender, et al., 1964).

Extensive investigations have shown that nitrosamines may be formed in low levels in certain foods used for human consumption. Since nitrite salts are added to cured meat to achieve color fixation, microbial inhibition, and flavor enhancement, the majority of reports on nitrosamines in food have focused on cured meat products. The most consistent source of nitrosamines among the cured foods is fried bacon, in which both NDMA and N-nitrosopyrrolidine are found after cooking (Scanlan, 1975). Other cured meats and some nitrate preserved cheeses have occasionally been found to contain volatile nitrosamines (Gray, 1981).

The presence of volatile nitrosamines in alcoholic beverages was first reported by Spiegelhalder et al. (1979). During a large scale analytical survey of West German foods, it was found that 70% of all beer samples analyzed contained NDMA at a mean concentration

of 2.7 μg/kg. Investigation of all 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 (Kann et al., 1980; Spiegelhalder et al., 1980; Hardwick et al., 1981). During the malt kilning process, nitrogen oxides (NO_x) formed by combustion of ambient nitrogen come into direct contact with the malt being dried. The reaction of NO_x with amines in the green malt has been proposed to explain the presence of NDMA in dried malt (Hardwick et al., 1981; Mangino et al., 1981).

The formation of nitrosamines in food is part of the larger problem of the exposure of the human population to nitrosamine in the environment. For example, tobacco and tobacco smoke contain at least four volatile nitrosamines and four nonvolatile nitrosamines (Hoffmann et al., 1981a). The major precursor of the nonvolatile nitrosamines in tobacco is the tertiary amine nicotine, which constitutes 0.5 to 2.6% of commercial tobaccos (Hecht et al., 1978).

N-nitrosodiethanolamine (NDELA) has been found in some cosmetics in which di-or triethanolamine was used as an emulsifying agent (Fan et al., 1977). Since the emulsions also contained nitrite but no preformed NDELA, the nitrosamine was assumed to arise by <u>in situ</u> formation during manufacture or storage (Kabacoff et al., 1981).

Some pesticides and herbicides can contain relatively large amounts of volatile nitrosamines (Ross et al., 1977). One of the best known examples was the discovery of NDMA at parts per million levels in formulations of 2,4-dichlorophenoxyacetic acid (2,4-D). The nitrosamine was assumed to form because 2,4-D was formulated as a salt

of dimethylamine and nitrite was used as a corrosion inhibitor in the containers holding the pesticide. Elimination of nitrite lowered the NDMA concentration to non-detectable levels (Keefer, 1981).

Preussmann et al. (1981) reported the finding of volatile nitrosamines in many samples of baby nipples and pacifiers. The nitrosamine levels ranged from 1 to 230 μg/kg and many samples contained more than one type of volatile nitrosamine. Incubation of some of the rubber samples with an artificial saliva solution showed that nitrosamines were capable of migrating into the solution. Nitrosamine formation in these rubber products is the result of the reaction of amine-containing accelerators and stabilizers with nitrosating agents present in the rubber vulcanization mixture, or by reaction of the amines with nitrogen oxides in the industrial atmosphere. It has been suggested that carcinogenic nitrosamine contamination could be reduced by replacing the usual amine-containing accelerators with "safe" secondary amines whose nitrosamine derivatives are non-carcinogenic or only weakly carcinogenic (Preussmann et al., 1981).

The presence of NDMA has been reported in the air and water near certain industrial environments (Fishbein, 1979); but comprehensive data on nitrosamine presence in ambient air and water does not appear to be available.

The final and perhaps the most significant exposure of man to nitrosamines may occur as a result of *in vivo* nitrosation in which nitrosamines are produced in the body from ingested or endogenous amines and nitrosating agents. Sander and Burkle (1969) originally showed that tumors could be induced in experimental animals by feeding

a secondary amine together with nitrite dissolved in drinking water. The carcinogenic agent was presumably a nitrosamine formed in the animal stomach at acidic pH by the reaction of ingested amine with nitrite. Taylor and Lijinsky (1975) extended these findings by carrying out large scale experiments in which rats were fed particular amines along with sodium nitrite in drinking water. For example, feeding of sodium nitrite to rats together with the cyclic secondary amine heptamethyleneimine resulted in a high incidence of esophageal tumors and squamous carcinomas of the lung. These were the same tumors induced by feeding the nitrosamine itself (N-nitrosoheptamethyleneimine).

Direct evidence that in vivo formation of nitrosamines can occur in the human was obtained from experiments in which human volunteers ingested nitrate and proline as constituents of vegetable juice. N-nitrosoproline (NPRO) was found in the urine of the test subjects (Ohshima and Bartsch, 1981). The amount of NPRO excreted was proportional to the proline dose and increased exponentially with the nitrate dose. Since the urinary excretion of NPRO is rapid and the compound is excreted almost completely in the unmetabolized form (Ohshima and Bartsch, 1981), monitoring of NPRO excreted in the urine has been suggested as a procedure for estimating daily human exposure to endogenously formed N-nitroso compounds. In view of its significance to human carcinogenicity, the search for biological evidence of in vivo N-nitroso compound formation is an area under intensive study.

Purpose of the Study

Preliminary investigations on the presence of NDMA in beer indicated that barley malt dried by direct-fired kilning was the source of the NDMA contamination found in the final product. The primary objective of this research was to investigate the formation of NDMA from amines which are biosynthesized during the manufacture of malt. Data is presented showing that one of these tertiary amine natural products is extremely susceptible to nitrosation to yield NDMA. A further objective of this research was to show that secondary amine natural products, also formed during biosynthesis, could be nitrosated to give nonvolatile N-nitrosamines. These nitrosamines would escape detection by the analytical methodology currently used to analyze foods for volatile nitrosamines.

II. LITERATURE REVIEW

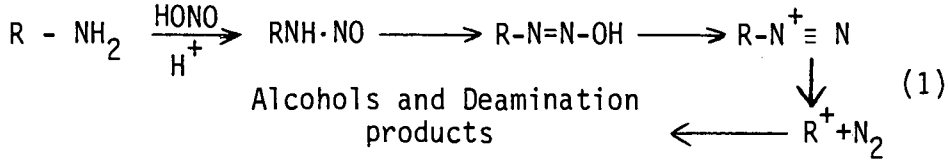
Introduction

In the view of Keefer (1981), the possible modes of N-nitrosamine formation are summarized as follows: 1) almost any derivative of ammonia can serve as the precursor of an N-nitrosamine and 2) any of the higher oxidation states of nitrogen can serve as nitrosating agents. According to this view, an N-nitrosamine could theoretically be formed whenever an amine encounters a nitrosating agent under the proper conditions. Fortunately, only a few of the possible combinations have been found to be environmentally important causes of N-nitrosamine formation. The most important are the interaction of secondary and tertiary amines with derivatives of nitrite ion under the influence of acid, and the interaction of amines with preformed nitrogen oxides (NO_x). The nitrite/acid and NO_x pathways will be the main focus of this review. A situation in which N-nitrosamine formation was discovered in direct-fired dried malt will also be discussed.

A. N-Nitrosation in Aqueous Acid

Nitrosation of Secondary Amines

N-nitrosation is the term used to include three different reactions of amines with nitrous acid: (1) the nitrosation of secondary amines to produce stable N-nitrosamines, (2) the diazotization of primary aromatic amines, and (3) the deamination of primary aliphatic amines to alcohols and a number of deamination products (Ridd, 1961). Each reaction is part of the "nitrosation pathway":



(1)

With secondary amines, the reaction stops at the nitrosamine stage; with aromatic amines, the reaction stops at the diazonium ion stage; with primary aliphatic amines, the reaction proceeds all the way to deamination products. Monoalkylnitrosamines are almost never isolated.

Nitrosation is carried out when nitrous acid (HONO) is formed in acidic solution by the protonation of the nitrite ion (NO_2^-). Nitrous acid is often regarded as the hydroxylated form of the powerful nitrosating agent, nitrosonium ion (NO^+). Replacement of the hydroxyl group with other anions gives compounds of the type NOX, which may also serve as nitrosating agents. The reaction for formation of the nitrosonium ion is:

$$HONO + H^+ \rightleftharpoons NO^+ + H_2O \qquad (2)$$

Formation of nitrosonium ion is favored by very high acidity and low water activity, which explains why NO^+ is detected only in concentrated acid.

Nitrous acidium ion (H_2ONO^+) is an unstable species which exists in low equilibrium concentration in acidic solution. The reaction for its formation is:

$$HONO + H^+ \rightleftharpoons H_2ONO^+ \qquad (3)$$

Despite its instability, nitrous acidium ion is an important nitrosating agent for diazotization of primary aromatic amines at high acidity.

Dinitrogen trioxide (N_2O_3) also called "nitrous anhydride" is the anhydride of nitrous acid and is formed by the equilibrium:

$$2HONO \rightleftharpoons N_2O_3 + H_2O \qquad (4)$$

Formation of nitrous anhydride is favored by low water activity and moderate acidity. From Equation (4), it is apparent that nitrous anhydride concentration is dependent on the square of the nitrous acid concentration, and this relationship is important to the understanding of kinetics and mechanism of secondary amine nitrosation.

Molecules of type NOX were X can be Cl^-, Br^- or other anions are also formed in aqueous solution by the reaction:

$$HONO + H^+ + X^- \rightleftharpoons NOX + H_2O \qquad (5)$$

Therefore, the concentration of NOX depends on acidity, water activity, and the concentration of X^-.

The reactions described above are all part of the complex equilibria which occur when nitrous acid is formed in acidic solution. Knowledge of this equilibrium is helpful for understanding of the kinetics and mechanism of secondary amine nitrosation.

Elucidation of the mechanism of secondary amine nitrosation evolved in parallel with studies on the kinetics of diazotization and deamination. The history of the mechanistic development was reviewed by Turney and Wright (1959) and Ridd (1961).

By 1930 it was recognized that the deamination of methylamine and the nitrosation of dimethylamine followed third order kinetics expressed in the form (Taylor and Price, 1929):

$$\text{Rate} = k_1[R_2NH][HONO]^2 \qquad (6)$$

In Equation (6), the amine concentration is that of the unprotonated amine. The correct interpretation of this rate law was provided by Hammett (1940), who suggested that the second order dependence on nitrous acid arose from nitrosation by nitrous anhydride (N_2O_3). This resulted in the mechanism outlined in Scheme 1:

$$2HONO \underset{}{\overset{\text{Fast}}{\rightleftharpoons}} N_2O_3 + H_2O \qquad (7)$$

$$R_2NH + N_2O_3 \xrightarrow{\text{Slow}} R_2N\text{-}NO + HONO \qquad (8)$$

Scheme 1

Independent evidence for the second order dependence on nitrous acid was based on two lines of experimentation. First, reactions run at low acidity (0.002M $HClO_4$) showed that the concentration of free amine was sufficient to react with nitrous anhydride before a significant proportion of N_2O_3 could be hydrolyzed to nitrous acid (Hughes et al., 1958). The rate-determining step then became the formation of nitrous anhydride and the kinetic form was:

$$\text{Rate} = k[HONO]^2 \qquad (9)$$

The reaction rate was independent of amine concentration and independent of the nature of the amine over a limited range of basicity. Second, at very low acidity, the equilibrium concentration of nitrous anhydride is low, and rapid hydrolysis to nitrous acid occurs. In the absence of amine, the ^{18}O-exchange between nitrous acid and water gives the maximum possible value for the rate of nitrous anhydride formation (Ridd, 1961). The rate of this oxygen exchange was found

to be second order with respect to nitrous acid and in good agreement with the rate of diazotization according to Equation (9) (Bunton et al., 1959).

The careful studies by Mirvish (1970) on nitrosation of dimethylamine, and Fan and Tannenbaum (1973) on nitrosation of morpholine have verified that secondary amines are nitrosated in aqueous acid according to Scheme 1. In order to explain the reaction rates expected for nitrosation of secondary amines, the following relationships are useful. Equation (6) uses the concentration of nonionized amine and free HONO, so k_1 is independent of pH, but $[R_2NH]$ and [HONO] must be calculated for each pH. Equation (10) is easier to apply since the total concentrations of amine and nitrite are used, irrespective of the species present:

$$\text{Rate} = k_2[\text{amine}][\text{nitrite}]^2 \qquad (10)$$

For Equation (10), the stoichiometric rate constant k_2 varies with pH. In actual practice, for most secondary amines, the reaction rate and k_2 show maximum values near pH 3.4 for the following reasons:

(1) The pk_a of nitrous acid is 3.36. Below pH 3.36, nitrite ion is almost completely coverted to HONO, and the main effect of a further drop in pH is a continuing decrease of $[R_2NH]$, causing reaction rate to drop.

(2) For any secondary amine with a pK_a greater than 5, nitrosation will occur at the fastest rate near pH 3.36. Fan and Tannenbaum (1973) determined this relationship mathematically based on use of the Henderson-Hasselbach equation to calculate concentrations of reactive species at any pH from knowledge of the pK_a.

Mirvish (1975) has calculated the k_2 values of 14 secondary amines from knowledge of their experimental optimum pH of nitrosation. The results showed a good relationship between k_2 and the amine pK_a: reaction rate increased as the basicity of the amine decreased. When basicity was low enough, as for N-methylaniline (pK_a 4.85), the formation of N_2O_3 became rate-limiting, and the nitrosation followed Equation (9). In this case, the pH optimum was pH 2, when almost all nitrite becomes converted to HONO.

The mechanism of secondary amine nitrosation is complicated somewhat by the fact that nitrosation can be catalyzed by nucleophiles, and nucleophiles may alter the nature of the rate-determining step. This effect was first noted in the diazotization of aniline in hydrochloric acid exceeding 0.1M (Schmid and Muhr, 1937). The kinetic effect was removal of the second order dependence on nitrite and addition of a term corresponding to the concentration of X^-, where X^- was chloride or bromide:

$$\text{Rate} = k[R_2NH][H^+][HONO][X^-] \qquad (11)$$

The kinetic expression of Equation (11) has been interpreted to require the mechanism of Scheme 2:

$$HONO + X^- + H^+ \underset{\text{Fast}}{\rightleftharpoons} NOX + H_2O \qquad (12)$$

$$NOX + R_2NH \xrightarrow{\text{Slow}} R_2N\text{-}NO + HX \qquad (13)$$

Scheme 2

Equation (12) is further interpreted as requiring the attack of X^- on nitrous acidium ion (H_2ONO^+), which would explain the appearance of $[H^+]$ in equation (11). Two experimental observations have provided

evidence that agents of the type NOX are more potent nitrosating agents than nitrous anhydride itself. First, the rate coefficients and equilibrium constants of equation (11) are known and were used to determine the true rate coefficients for the reaction of free amines with molecular nitrosyl halides, k_3 in Equation (14):

$$\text{Rate} = k_3[R_2NH][NOX] \qquad (14)$$

The values of k_3 for reaction of several primary aromatic amines with NOCl were found to be insensitive to the basicity of the amine. The values approached those expected for diffusion-controlled reactions in aqueous solution (Ridd, 1961). Secondly, addition of nucleophiles to reactions of secondary amines with nitrous acid resulted in a catalytic effect on the rate of N-nitrosamine formation.

The catalytic effect was most pronounced with thiocyanate and iodide ions. Boyland et al. (1971) found that addition of 1mM iodide or 1mM thiocyanate to a reaction of 0.1mM N-methylaniline and 0.1mM nitrite at pH 2 accelerated the rate of nitrosation by factors of 390 and 255, respectively. Added bromide enhanced the rate by a factor of only 3.5. The pH optimum for the uncatalyzed reaction was pH 3.4, and for the catalyzed reaction, the pH optimum was near pH 1.

Fan and Tannenbaum (1973) studied the nitrosation of morpholine with and without the presence of added nucleophiles. The order of nucleophilic catalysis was shown to be $SCN^- >>> Br^- >> Cl^- > SO_4^{-2}$. The sulfate, perchlorate, and phosphate ions were found to be approximately equal in catalytic effect. The pH optimum was shifted from pH 3.4 for the uncatalyzed reaction to pH 2.3 in the presence of thiocyanate. The rate of the catalyzed reaction was found to follow

Equation (15) from pH 0.5 to pH 4.0 (Fan and Tannenbaum, 1973):

$$\text{Rate} = k_4[R_2NH][HONO]^2 + k_5[R_2NH][H^+][HONO][X^-] \qquad (15)$$

Equation (15) means that conditions may exist when the overall reaction is between first and second order for nitrite and less than first order for X^-; such conditions are likely to exist at pH above 2.0. The effectiveness of various anions as nitrosation promotors depends on the relative nucleophilicity and the equilibrium constant for formation of NOX from HONO and HX. Fan and Tannenbaum (1973) found the experimental ratios of k_5 for thiocyanate, bromide, and chloride to be 15,000:30:1 over the pH range of 1 to 3.

Thiocyanate catalyzed nitrosation of secondary amines is important in the evaluation of in vivo nitrosation as a carcinogenic risk factor for man. Thiocyanate is naturally secreted in saliva, and reaches a concentration of approximately 50 mg/liter in nonsmokers, but can reach up to 300 mg/liter in cigarette smokers (Densen et al., 1967). Since saliva contains nitrite derived from food and nitrite from reduction of nitrate by oral microflora, the necessary reactants for nucleophile-catalyzed nitrosation are available and reach the gastric fluid (pH 1 to pH 3) whenever food or drugs containing nitrosatable amines are ingested. Evidence that the nucleophile-catalyzed nitrosation mechanism may operate in vivo was obtained by Lane and Bailey (1973). When a dilute solution of dimethylamine and nitrite was incubated in human gastric juice, the pH maximum for NDMA formation was 2.5 as expected if NCS^- and/or Cl^- ion were involved, and not pH 3.4, as expected if only nitrous anhydride (N_2O_3) were involved.

Finally, one acid catalyzed mechanism of nitrosation which may

operate under special conditions should be mentioned. This mechanism has the kinetic form of Equation (16):

$$\text{Rate} = k[RNH_2][HONO][H^+] \tag{16}$$

This result was interpreted as a requirement for rate-determining reaction of the free amine with the nitrous acidium ion (H_2ONO^+) as shown in Scheme 3 (Hughes et al., 1958):

$$HONO + H^+ \overset{\text{Fast}}{\rightleftharpoons} H_2ONO^+ \tag{17}$$

$$ArNH_2 + H_2ONO^+ \xrightarrow{\text{Slow}} ArNH_2NO^+ + H_2O \tag{18}$$

$$ArNH_2NO^+ \xrightarrow{\text{Fast}} ArN_2^+ + H_2O \tag{19}$$

Scheme 3

Nitrous acidium ion is generally accepted to be a more reactive nitrosating agent than nitrous anhydride, because reactions that followed Equation (16) were much less sensitive to amine basicity than reactions which followed Equation (6). Consequently, Equation (16) is usually observed for the diazotization of weakly basic aromatic amines.

In conclusion, the important factors involved in secondary amine nitrosation are summarized as follows:

(1) Nitrosation of secondary amines may be important environmentally since these amines occur in food as a result of biosynthesis, fermentation, and cooking (Smith, 1981). Also, some drugs and pesticides are secondary amines.

(2) Most secondary amines are nitrosated by a third order reaction in which the rate of nitrosation is proportional to the amine concentration and the square of nitrous acid concentration. The nitrosating agent is nitrous anhydride

formed from two molecules of nitrous acid. When nitrous anhydride is the nitrosating agent, the pH optimum is near pH 3.4 for nitrosation of secondary amines with a pK_a greater than 5. The reaction rate is proportional to amine basicity as predicted by the amine pK_a.

(3) Thiocyanate and other anions such as chloride and bromide catalyze the nitrosation of secondary amines because the nitrosating agent NOX (X = SCN^-, Cl^-, Br^-) is formed in competition with N_2O_3. Reaction rates are increased because NOX is a stronger nitrosating agent than N_2O_3. The pH optimum for nitrosation by NOX is shifted to lower pH and shows a maximum of pH 2 for nitrosyl thiocyanate (NOSCN).

(4) The mechanisms discussed above can be applied to explain the formation of N-nitrosamines in food systems and in vivo.

Nitrosation of Tertiary Amines

The possibility that tertiary amines could be nitrosated to give N-nitrosamine derivatives was a point of uncertainty for decades, until the reaction was proven conclusively in 1959. The early history of the reaction was discussed by Hein (1963). A short review of the important points follows.

In 1864, Guether reported that a small amount of nitrosodiethylamine was formed when triethylamine was treated with nitrous acid. Heintz (1866) refuted this claim and stated that purified triethylamine remained unchanged in the presence of nitrous acid. Only a year later, Limpricht (1867) reported that nitrous acid reacted with tribenzylamine

and the primary products were benzaldehyde and a low melting soild later identified by Rohde (1869) as N-nitrosodibenzylamine. However, Heintz's result came into general acceptance and further experimentation was discouraged.

The reaction practically disappeared from the chemical literature until 1925 when chemists at Merck and Co. rediscovered the reaction during a search for methods to prepare N-nitroso compounds. Shortly thereafter, some natural products chemists began using the reaction to accomplish selective dealkylation of alkaloids as a method for determining the original position of alkyl substitution (Speyer and Walther, 1930; Cookson and Trevett, 1956).

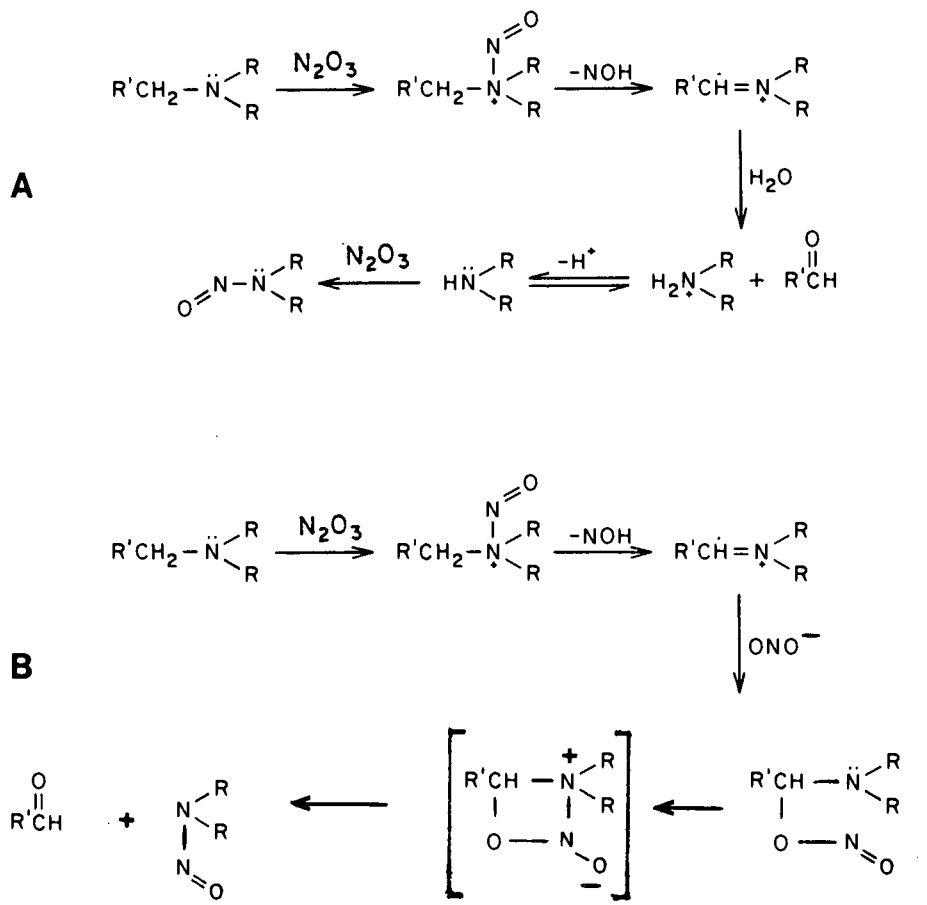
Wegler and Frank (1936) published the first paper in which successful reaction conditions were defined. Their original observation was that heating of dimethylbornylamine with nitrous acid led to degradation of the amine and formation of camphor as one of the products. They also showed that a group of tertiary amines could be converted into N-nitrosamines and aldehydes or ketones. The successful reactions were run at 40° to 60° in acetic acid rather than mineral acid. Smith and Pars (1959) discovered that tribenzylamine and tri-n-butylamine reacted with nitrous acid to give the secondary N-nitrosamine and either benzaldehyde or butraldehyde. The conditions were reaction of the amines at 70°-85° with excess nitrous acid in 60% acetic acid buffered to pH above 3. The same authors proposed that the "nitrosative dealkylation" reaction occurred by formation of an initial nitrosammonium ion followed by elimination of a proton from an α-carbon atom to give a dialkylimmonium ion (Figure 2A). The

Figure 2. The proposed mechanism for nitrosative dealkylation of tertiary amines

(A) The original mechanism of Smith and Pars (1959) and Smith and Leoppky (1967)

(B) The modified mechanism according to Keefer (1978)

Figure 2



immonium ion would be subject to hydrolysis to a secondary amine which could be nitrosated to give the observed N-nitrosamine.

Further support for the mechanism of Figure 2A was obtained from a more extensive study of tertiary amine nitrosation by Smith and Loeppky (1967). The structural requirements and product selectivity of tertiary amine nitrosation were explored using tribenzylamine and N,N-dibenzylaniline and some of their derivatives. The reactions were run in warm aqueous acetic acid, usually with a large excess of nitrous acid so that product yields could be maximized. The important experimental results are summarized as follows:

(1) The maximum yield of nitrosodibenzylamine obtained from an equimolar reaction of nitrous acid and tribenzylamine was 38%, and 50% of unreacted starting material was collected. When gases evolved from the reaction were collected, 0.5 mole of gas corresponding to nitrous oxide (N_2O) was obtained. The appearance of nitrous oxide was taken as evidence for formation of nitroxyl (NOH). Nitroxyl is unstable and undergoes dimerization and dehydration to nitrous oxide (Smith, 1965):

$$NOH \longrightarrow H_2N_2O_2 \longrightarrow N_2O + H_2O \qquad (20)$$

(2) The amine quinuclidine, which contains a bridgehead nitrogen atom, was not converted to an N-Nitroso derivative. This was taken as evidence that resistance to double bond formation interfered with nitrosative dealkylation.

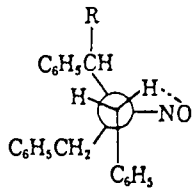
(3) To test the nature of electronic effects on product selection, a group of tribenzylamines each bearing a different

para substituent (CH_3O-, CH_3-, Cl-, or $-NO_2$) on one benzyl group was used; the ratio of aldehyde products C_6H_5CHO: RC_6H_4CHO was determined. The results were correlated by the Hammett equation $\log (RC_6H_4CHO/0.5C_6H_5CHO) = \rho\sigma$, but ρ showed a value of only -0.17.

(4) The steric effect of α-substitution on product selection was tested using α-methyl, α-ethyl, and α-carboethoxy derivatives of tribenzylamine: $C_6H_5CHRN(CH_2C_6H_5)_2$; the ratio of aldehyde: ketone products was determined. The ratios were found to be extremely susceptible to α-substitution. For example, nitrosation of α-carboethoxytribenzylamine gave a benzaldehyde: $C_6H_5COCOOEt$ ratio of 98:2. This was exactly opposite to the result expected if the presence of an acidic proton at the α-carbon atom were an important factor in product determination.

(5) The authors correlated their results with a mechanism requiring an intramolecular cyclic transition state resulting in syn-elimination of nitroxyl (NOH). The α-substituent effects were correlated with a cyclic transition state on the basis of the nonbonded interactions shown by Newman projections I and II. Rotation of an unsubstituted benzyl group into position for syn-elimination of NOH (Projection I) produces one unfavorable eclipsing interaction. Rotatation of a substituted benzyl group into position for syn-elimination of NOH (Projection II) produces two unfavorable eclipsing interactions. Thus, unsubstituted benzyl groups

are predicted to be preferentially cleaved from the α-substituted tribenzylamines, in agreement with the experimental observations.



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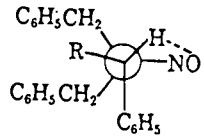


II

An alternative pathway for product formation during nitrosative dealkylation was offered by Keefer (1978). Since nitrosation reaction mixtures at pH 4-6 still contain unprotonated nitrite ion, the remaining nitrite could act as a nucleophile to attack the dialkylammonium ion (Figure 2B). Assumption of a four-center transition state and nucleophilic attack by the amino nitrogen on the neighboring nitroso group would lead to the same N-nitrosamine and carbonyl products predicted by the mechanism of Smith and Loeppky (Figure 2A). The mechanism of Keefer should have no effect on product ratios, but removes the requirement for secondary amines as intermediates in tertiary amine nitrosation.

More recent mechanistic studies have tended to confirm the nitrosative dealkylation mechanism proposed by Smith and Leoppky (1967). Lijinsky et al. (1972b) conducted a study on the nitrosation of 14 aliphatic or heterocyclic tertiary amines under reaction conditions

similar to those used by Smith and Loeppky. In a study of the effect of steric factors, the product distribution from nitrosation of methyl-ethyl-n-propylamine was compared to the product distribution from methylethyl-iso-propylamine. The results showed that the n-propyl group was cleaved 5 to 6 times as readily as the iso-propyl group. The steric factor was further demonstrated in the nitrosation of N,N-dimethylcyclohexylamine. The ratio of N-nitrosocyclohexylmethylamine to N-nitrosodimethylamine in the product was 12:1, again indicating the importance of substitution at the α-carbon atom for determining the product ratios. The results were explained on the basis of non-bonded interaction expected in the transition state required for syn-elimination of NOH.

A small number of kinetic studies have been carried out as a means for investigating the mechanism of tertiary amine nitrosation. Ohshima and Kawabata (1978) attempted to determine the kinetics of NDMA formation from trimethylamine (TMA). At 90° or 100°, the optimum pH for conversion of TMA to NDMA was pH 3. At pH 3 and 100°, the initial rate of NDMA formation from TMA was proportional to the TMA concentration and the square of the nitrite concentration, in analogy with the nitrosation of secondary amines. The authors concluded that the nitrosating agent was N_2O_3 and the mechanism for NDMA formation was nitrosammonium ion formation followed by dealkylation to an immonium ion, and either hydrolysis or attack by nitrite ion. The kinetics would indicate rate-limiting formation of a nitrosammonium ion, but the authors offered no comment on this point.

Singer (1980) studied the kinetics and product formation from

nitrosation of tri-n-butylamine. At pH 3 and 50°, the initial rate of nitrosodibutylamine formation was proportional to amine concentration and the square of nitrite concentration. The expected nitrosamine and butraldehyde were formed in almost equal yield. The author concluded that the nitrosating agent was N_2O_3 and the first reaction intermediate was a nitrosammonium ion as proposed by Smith and Loeppky (1967).

The most comprehensive study of the kinetics of tertiary amine nitrosation was carried out by Gowenlock et al. (1979). They studied the rates of nitrosative dealkylation of symmetrical tertiary amines, R_3N(R = CH_3-, CH_3CH_2-, n-Pro, n-Bu, benzyl, $HOCH_2CH_2$-, and HO_2CCH_2-), in acetic acid-acetate buffers. For the reaction of triethylamine with nitrous acid at 90°, the products were nitrous oxide, N-nitrosodiethylamine (NDEA) and acetaldehyde. At 75°, the pH optimum for reaction with triethylamine was pH 3. At pH 3.8 and a temperature of 75°, the rate of NDEA formation was proportional to amine concentration and nitrous acid concentration. In the pH range from 3.1-3.9, the rate coefficient was linearly proportional to the pH, so the rate expression was described by Equation (21), where k_2 is a pH independent rate constant.

$$d[NDEA]/dt = k_2[H^+][Et_3N][HONO] \qquad (21)$$

The rate of nitrosation of tri-n-butylamine also followed Equation (21), and the value of k_2 for all amines was calculated on the basis of Equation (21). Addition of chloride and thiocyanate ions caused no change in the initial rate for triethylamine. Furthermore,

there was no correlation between the rate constant k_2 and the pK_a of the amines. For example, triethylamine (pK_a 10.7) was 60 times more reactive than tribenzylamine (pK_a 8.7), even though the latter is the weaker base. This was exactly opposite to the basicity effect for secondary amine nitrosation (Mirvish, 1975).

Based on the evidence described above, the authors concluded that the initial nitrosation step was not rate-limiting. The proposed mechanism was rapid, reversible nitrosation followed by slower steps leading to product formation (Scheme 4):

$$HONO + HX \rightleftharpoons NOX + H_2O \quad (22)$$

$$R_3N + NOX \rightleftharpoons R_3NNO^+ + X^- \quad (23)$$

$$R_3NNO^+ \longrightarrow R_2N^+ = CHR' + NOH \quad (24)$$

$$R_2N^+ = CHR' \longrightarrow \text{Products} \quad (25)$$

Scheme 4

As an explanation for the observed first order dependence on nitrous acid, it was concluded that the active nitrosating species was nitrosyl acetate (AcONO). However, since the rate expression (Equation 21) was consistent with nitrosation by nitrous acidium ion (H_2ONO^+), the acetate ion may be acting only as a catalyst for N_2O_3 formation as explained by Hughes et al. (1958):

$$AcO^- + H_2ONO^+ \rightleftharpoons AcONO + H_2O \quad (26)$$

$$NO_2^- + AcONO \longrightarrow N_2O_3 + AcO^- \quad (27)$$

Consequently, N_2O_3 could still be the nitrosating agent, and its rate of formation effected by the high level of acetate ion used.

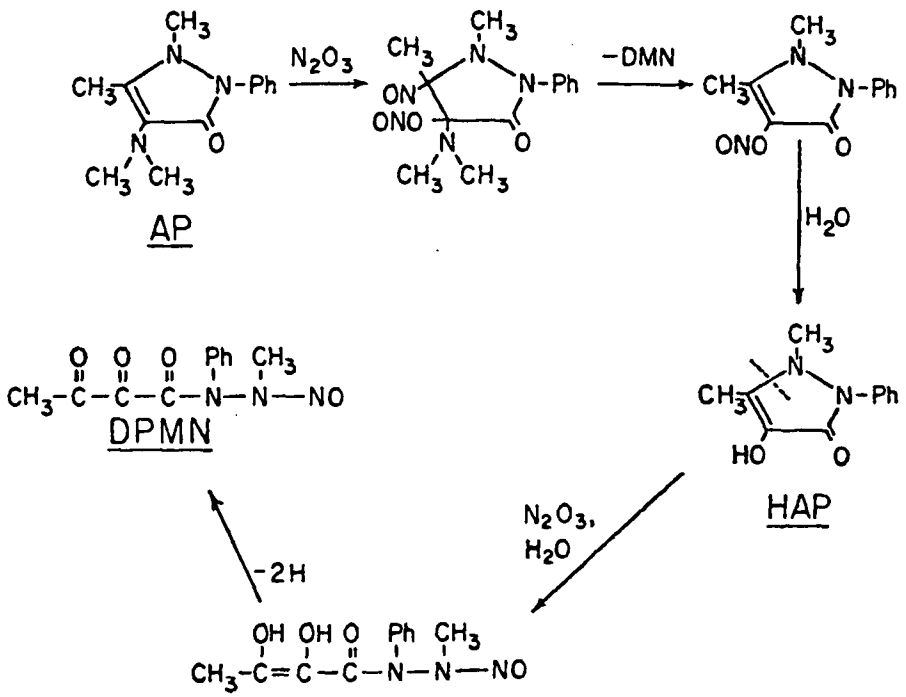
Nitrosation of tertiary amines usually gives lower yields of

N-nitrosamines than nitrosation of secondary amines, and the nitrosation of tertiary amines is more temperature dependent. Nevertheless, tertiary amine nitrosation remains a point of interest because of its biological implications. Many natural products and food constituents are tertiary amines, and many commonly used drugs are tertiary amines. Lijinsky et al. (1972a) found that several commonly used drugs including oxytetracycline (antibiotic) and aminopyrine (analgesic) gave considerable amounts of NDMA when reacted with nitrous acid at 37°. Many such drugs are ingested chronically by people who are also exposed to nitrites in food. A later study (Lijinsky, 1974), showed that twelve commonly used tertiary amine drugs all liberated measurable amounts of NDMA, NDEA, or a nonvolatile nitrosamine after treatment with nitrous acid at 37°. The reaction conditions were similar to those used to predict in vivo formation of NDMA from dimethylamine nitrosation in the gastric fluid of man (Mirvish, 1970). Since it is not easy to correlate the potential for nitrosamine formation from a given tertiary amine based on structure or pK_a, tertiary amines could be a more insidious source of N-nitrosamines than secondary amines.

The nitrosation of aminopyrine (Figure 3) illustrates the difficulty of predicting the reactivity of tertiary amines toward nitrous acid. Aminopyrine is an analgesic drug which was widely used in Europe in the mid-1970's. Lijinsky et al. (1972a) originally showed that aminopyrine could be nitrosated under mild conditions (37°,pH 4) to form NDMA in yields exceeding 70% of theoretical. Aminopyrine also undergoes the reaction readily in vivo. A group of rats given a combination by gavage of aminopyrine and nitrite developed the

Figure 3. The proposed mechanism for nitrosation of aminopyrine; Mirvish et al., (1974)

Figure 3



severe liver necrosis characteristic of exposure to NDMA (Lijinsky and Greenblatt, 1972).

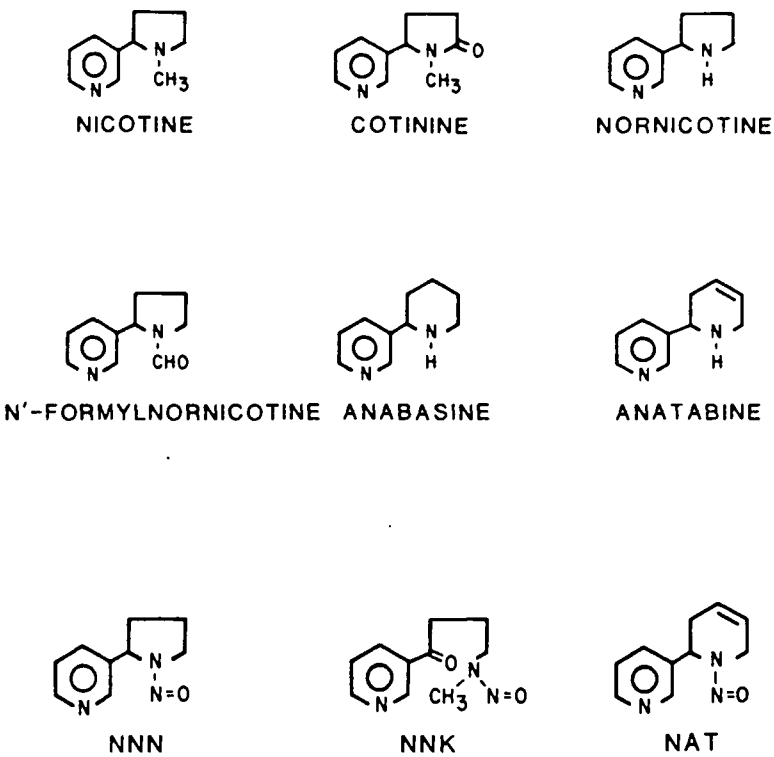
Mirvish et al. (1974) studied the kinetics of aminopyrine nitrosation. At 0°, the pH optimum was pH 2 and the initial rate was independent of amine concentration but proportional to the square of nitrous acid concentration. This result was consistent with rate-limiting formation of N_2O_3. The pH independent rate constant at pH 2 was higher than the rate constants at optimum pH for all secondary amines previously studied by Mirvish (1975), except N-methylaniline. Product analysis showed that NDMA was the only nitrosamine formed in the initial reaction, and the other major product was 1-diketo-butryl-1-phenyl-2-methyl-2-nitrosohydrazide (DPMN, Figure 3). The proposed mechanism is addition of N_2O_3 to aminopyrine to form a nitroso-nitrite ester which loses NDMA, presumably by attack of the amino nitrogen on the neighboring nitroso group. The high reactivity of aminopyrine is attributed to its enamine structure and low basicity (pK_a 5.04).

A clear case of a tertiary amine natural product as a source of N-nitrosamines is the tobacco alkaloid nicotine. Tobacco used for commercial production in the U.S.A. contains between 0.5 and 2.7% alkaloids; nicotine constitutes 85-95% of the total alkaloids (Hoffmann et al., 1981a). Important minor alkaloids are nornicotine, anatabine, anabasine, cotinine, and N^1-formylnornicotine (Figure 4). Several of these alkaloids have been found in tobacco or tobacco smoke in the N-nitrosated form (Figure 4). These include N'-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosoanatabine (NAT).

In a study to determine the precursor of NNN, individual Burley

Figure 4. The common tobacco alkaloids and some nitrosamines derived from the tobacco alkaloids; Hoffman et al., (1981a)

Figure 4


NICOTINE
COTININE
NORNICOTINE
N'-FORMYLNORNICOTINE
ANABASINE
ANATABINE
NNN
NNK
NAT

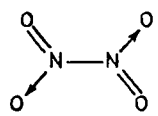
tobacco leaves were stem-fed with nicotine-2'-^{14}C or nornicotine-2'-^{14}C (Hecht et al., 1978). Air curing, drying, and analysis for NNN-^{14}C showed that 0.009% of the NNN-^{14}C activity originated from nicotine-^{14}C and 0.007% of the activity originated from nornicotine-^{14}C. This result demonstrated that nicotine was the major precursor for NNN since the secondary amine nornicotine constitutes only 0.005 to 0.06% of the tobacco.

Tobacco-specific nitrosamines are formed during the air-curing or flue-curing of tobacco, and in tobacco smoke. Nitrosonornicotine has been determined in unburned commercial tobacco in levels ranging from 2 to 90 μg/g (Hoffmann, et al., 1974). These are the highest known levels of a preformed N-nitrosamine from an environmental source. Both NNN and NNK are carcinogens which induce lung adenomas in mice and malignant carcinomas of the nasal cavity in rats. For this reason, tobacco-specific nitrosamines are being studied as factors in the etiology of lung cancer and oral cavity cancer in man (Hoffmann et al., 1981b).

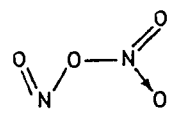
B. N-Nitrosation and N-Nitration in Aqueous Solution by Dissolved Gaseous Nitrogen Oxides (NO_x)

The nitrosation of amines in aqueous acid was previously described to take place because nitrous acid is in equilibrium with nitrous anhydride (N_2O_3). A well-documented body of experimentation has shown that for amines with a pK_a greater than 5, N_2O_3 is the only significant nitrosating agent. For weakly basic amines at lower pH, other nitrosating agents may be important (Ridd, 1961).

The possibility of performing N-nitrosation without acid catalysis was first illustrated by White and Feldman (1957). Gaseous dinitrogen-tetroxide (N_2O_4) was used to nitrosate diethylamine in dichloromethane or ether solution. Almost instantaneous reaction of amine and N_2O_4 occurred. At 0° in dichloromethane, the resulting organic product was N-nitrosodiethylamine. At -80° in dichloromethane, the resulting product was N-nitrodiethylamine (Et_2NNO_2). At -80° in ether, both products were obtained. The results were interpreted in terms of an equilibrium between isomeric forms of dinitrogen tetroxide (III and IV).



III



IV

Nucleophilic displacement of NO_2^+ from III resulted in N-nitration and nucleophilic displacement of NO^+ from IV resulted in N-nitrosation. The same kind of isomerism was proposed earlier to explain the hydrolysis reactions of N_2O_4 (Anbar and Taube, 1955).

Studies on the reaction of nitrogen oxides with secondary amines were discontinued and not taken up again until the carcinogenic potential of N-nitrosamines was recognized. An extensive study of the reactions between secondary amines and gaseous N_2O_3 and N_2O_4 has been conducted by B.C. Challis and co-workers. The primary objective of the study was to determine if preformed gaseous nitrogen oxides reacted with amines in aqueous solution. The results would have significance for the evaluation of NO_x as a causative agent for

nitrosamine formation in the environment. The principle parameters and experimental results are summarized below:

(1) The relevant equilibria for formation of N_2O_3 and N_2O_4 from their constituent gases are:

$$N_2O_3 \overset{K_1}{\rightleftharpoons} NO + NO_2 \qquad (28)$$

$$N_2O_4 \overset{K_2}{\rightleftharpoons} 2NO_2 \qquad (29)$$

In the gas phase, N_2O_3 and N_2O_4 are extensively dissociated into NO and NO_2. Upon dissolving in the aqueous phase, the dissociation is rapidly diminished. At 20° in water, the rate of recombination of NO_2 is 4.5×10^8 and K_2 is 1.53×10^{-5} mol^{-1} (Grätzel et al., 1969). The rate of recombination of NO with NO_2 is 1.1×10^9 liter $mol^{-1} sec^{-1}$ and K_1 is 7.3×10^{-5} (Grätzel et al., 1970). The equilibrium between N_2O_3 and N_2O_4 in Equation (30) lies well to the left in water at 20° ($K_3 = 3.5 \times 10^{-4}$ mol $liter^{-1}$).

$$2N_2O_3 \overset{K_3}{\rightleftharpoons} N_2O_4 + 2NO \qquad (30)$$

(2) Nitric oxide (NO) in an oxygen free atmosphere was a poor nitrosating agent in acetonitrile or ethanol solvent since the half-life for reaction of NO with piperidine or morpholine was 8 days. Introduction of air or oxygen resulted in quantitative N-nitrosamine formation in 4 minutes, presumably because some NO was oxidized to NO_2 (Challis and Kyrtopoulos, 1976).

(3) In acetonitrile at 25°, reaction of piperidine or diphenylamine with excess N_2O_3 or N_2O_4 gave quantitative yields of

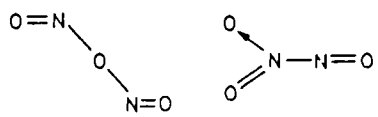
N-nitrosamines in 3 minutes. N-nitropiperidine was obtained from reaction of piperidine with N_2O_4 only when an excess of amine was present (Challis and Kyrtopoulos, 1979).

(4) At 25° in 0.1 M NaOH, piperdine was reacted with excess N_2O_4 to give 39% N-nitrosamine and 1% N-nitramine. N-methylpiperazine reacted with excess N_2O_4 gave 33% N-nitrosamine and 8% N-nitramine. So the principle product of secondary amine reaction with N_2O_4 was an N-nitrosamine. Reaction of secondary amines with excess N_2O_3 gave high yields only of N-nitrosamines. All reactions were too fast to allow initial rate measurements, but N_2O_3 was found to be a better nitrosating agent than N_2O_4 on a molar basis (Challis and Kyrtopoulos, 1979).

(5) Reaction of NO_x with amines ranging in pK_a from 1.0 to 11.1 showed that the amount of nitrosation was insensitive to amine basicity in 0.1 M NaOH. Even the diazotization of p-nitroaniline (pK_a 1.0) was rapid in dissolved N_2O_3 and N_2O_4, whereas the same reaction in aqueous nitrous acid requires a strong nitrosating agent, probably nitrous acidium ion (H_2ONO^+). Conducting the reactions in pH 6.8 phosphate buffer effected the product yield only for the strongest base (piperidine). It was concluded that reaction of OH^- with N_2O_3 and N_2O_4 was not an important competing reaction. The only important competing reaction was hydrolysis of N_2O_3 and N_2O_4 by water. On a molar basis, N_2O_3 was calculated to be 3000 times more reactive with piperidine than with water, and

N_2O_4 was 2000 times more reactive with piperidine than with water (Challis and Kyrtopoulos, 1978).

(6) It was concluded that nitrosation by dissolved nitrogen oxides in neutral or basic solution is much faster than conventional N-nitrosation in aqueous acid. The faster reaction rate with NO_x was related to the inability of dissolved N_2O_4 and N_2O_3 to discriminate differences in base strength and also to the existence of multiple isomers of N_2O_3 and N_2O_4. To explain the enhanced reactivity of dissolved N_2O_3 toward weakly basic amines, the authors postulate that the less stable symmetrical isomer (V) of N_2O_3 is formed when gaseous mixtures of NO· and NO_2· are dissolved in aqueous solution (Challis and Kyrtopoulos, 1978). The more stable unsymmetrical isomer (VI) is the one formed in solutions of nitrous acid (Ingold and Ingold, 1947).



V **VI**

To explain the N-nitrosation and N-nitration of piperidine by N_2O_4, the symmetrical (III) and unsymmetrical (IV) isomers of N_2O_4 were proposed to form in slow equilibrium. Faster hydrolysis of III by water would

explain why N-nitrosamine formation from IV is the more dominant reaction (Challis and Kyrtopoulos, 1978).

Despite the implications of the results obtained by Challis and co-workers, very few experiments have been conducted to assess the importance of the reactions between amines and NO_x in environmental situations. The lack of experimental studies is due to the difficulty of directly measuring the interaction of NO_x gases with environmental substrates, since the ambient level of NO_x is rarely greater than 1 ppm, except in some industrial areas.

Two experiments will be described which illustrate the role that NO_x could play in the formation of N-nitrosamines in ambient air, especially in polluted or heavily industrial environments.

The first experiment which indicated that NO_x could effect nitrosation in the gas phase was conducted by Hanst et al. (1977). The reaction between dimethylamine (1 ppm), NO_2 (1 ppm), and NO (4 ppm) in dry nitrogen was monitored in a long-path fourier-infrared spectrometer. The rate of nitrosation of dimethylamine to NDMA was only 1%/min. When dimethylamine was mixed with NO, NO_2, and 13000 ppm water in room air, the nitrosation rate rose to 4%/min. The increase in yield was attributed to the formation of nitrous acid:

$$H_2O_{(g)} + NO_{(g)} + NO_{2(g)} \underset{}{\overset{K_{eq}}{\rightleftharpoons}} 2HONO_{(g)} \qquad (31)$$

At 23°, the value of K_{eq} is 1.5×10^{-6} ppm^{-1}. In a moderately polluted urban environment, the concentration of HONO could reach 0.0035 ppm in the dark, and the resulting rate of dimethylamine nitrosation would be approximately 2%/hr. The calculations applied only in the dark. On exposure to sunlight, nitrous acid was quickly destroyed by

photolysis; the half-life of NDMA in full daylight was 30 min. The authors concluded that atmospheric formation of NDMA was not a general problem since dimethylamine concentrations are only appreciable in the vicinity of manufacturing plants which release the amine. Photolysis of HONO and NDMA in sunlight would prevent day-to-day accumulation of NDMA.

The reactions of diethylamine and triethylamine with NO_x under simulated atmospheric conditions were studied by Pitts, et al. (1978). In the dark, the reaction of diethylamine with ambient levels of NO and NO_2 in air with 20-50% relative humidity gave a 3% yield of N-nitrosodiethylamine (NDEA). The yield of NDMA depended on the amount of HONO initially present. Triethylamine reacted with ambient levels of NO_x to give a 1% yield of NDEA. In sunlight, photochemical oxidation of the amines occurred. Diethylamine and triethylamine both yielded NDEA and diethylnitramine. Sunlight causes photolysis of HONO to hydroxyl radical. The proposed mechanism was hydroxyl radical initiated oxidation of the amines to dialkylamino radicals which react directly with $\cdot NO$ and $\cdot NO_2$ (Equations 32 and 33).

$$(C_2H_5)_2N\cdot + \cdot NO \rightleftharpoons (C_2H_5)_2NNO \qquad (32)$$

$$(C_2H_5)_2N\cdot + \cdot NO_2 \rightleftharpoons (C_2H_5)_2NNO_2 \qquad (33)$$

Diethylnitramine was stable in sunlight and accumulated in the reaction chamber. The experiments of Pitts et al. (1978) are the only examples available of the reaction of tertiary amines with NO_x.

C. Nitrosamine Formation in Beer and Malted Barley

Introduction

The presence of trace levels of N-nitrosamines in foods for human consumption was mentioned in Chapter I. A comprehensive review published in 1975 described nitrite cured meat, fish, and cheese as the only known sources of N-nitrosamines in food (Scanlan, 1975). This situation was changed when the presence of NDMA was reported in a significant proportion of samples of German beer (Spiegelhalder et al., 1979). A large scale survey of West German brewery products for the presence of volatile N-nitrosamines showed that 70% of 158 tested samples were contaminated with NDMA at a mean concentration of 2.7 μg/liter. The highest concentration for any individual sample was 68 μg/liter, and samples with a level below 0.5 μg/liter were taken as negative. Samples were analyzed by the most nitrosamine-specific method available: combined gas chromatography-thermal energy analysis (GC-TEA).

This report was followed quickly by a number of analytical surveys whose collective results indicated that NDMA contamination in beer was a worldwide problem. In all of the surveys, NDMA was analyzed by GC-TEA, and in six of the eight surveys, the presence of NDMA in several samples was confirmed by combined gas chromatography-mass spectrometry (GC-MS) (Mangino et al., 1981). Two of the surveys will be mentioned as representative examples. In a sampling of mostly U.S. beers, Fazio et al. (1980) found that 62 of 64 samples were positive (> 0.2 μg/kg) for NDMA and the mean level was 2.8 μg/kg. In a survey

of 25 U.S. beer brands, Scanlan et al. (1980) found that 23 samples were positive (> 0.1 μg/kg) and the mean level of NDMA was 5.9 μg/kg.

The analytical data described above was obtained three years ago, and those data do not reflect the current levels of NDMA in beer. Since mid-1980, levels of NDMA in beer have been reduced as a direct result of measures taken by the malting and brewing industries to inhibit nitrosamine formation. The methods for reducing NDMA formation will be discussed later in this section.

Another significant result of the original analytical surveys is that NDMA was by far the most predominant volatile nitrosamine found in beer. Nitrosodiethylamine was found in a small number of samples in two surveys (Spiegelhalder et al., 1979; Walker et al., 1979), and N-nitrosopyrrolidine was reported in only two samples of Japanese beer (Kawabata et al., 1980). It is very likely that nitrosopyrrolidine is formed by heat-induced decarboxylation of nitrosoproline; proline is the most abundant amino acid in malted barley. Pollack (1981) reported the presence of nitrosoproline in some samples of malt which had been dried by direct-fired kilning.

Sources of NDMA in Beer

Analysis of the raw materials of brewing as well as analysis of the NDMA content at different stages of beer production have been reported by several groups (Kann et al., 1980; Spiegelhalder et al., 1980; Hardwick et al., 1981). With the exception of a very few samples of hops, the other raw materials of brewing and the brewing additives (filter aids, adjuncts, salts, and enzymes) were found to be essentially

free from NDMA contamination. When attention was turned to the different stages of the brewing process, it was found that in all steps following the production of malt there was no increase in the NDMA content of beer (Spiegelhalder et al., 1980; Hardwick et al., 1981).

Invariably, the malting process was shown to be the source of NDMA in beer. Malting is the process in which barley grain is chemically and physically "modified" to a product from which the brewer obtains the extract or "wort" used for the fermentation stage in beer production. During malting the following operations are performed:

(1) Raw barley is steeped in water to soften the outer hull so that the kernal will become permeable to water and air. Steeping raises the moisture content from approximately 10% to 45%.

(2) The steeped barley is germinated for approximately four days during which time enzymes are biosynthesized. The α-and-β-amylase activities increase, and some breakdown of protein and non-starchy polysaccharides also occurs. A number of nitrogen-containing secondary metabolites are biosynthesized.

(3) The germinated malt ("green malt") is dried or "kilned" for a two day period during which time growth is stopped, enzyme activities are arrested, and desirable color and flavor changes are induced. The dried malt has a 4-6% moisture content and is stable against microbial attack.

(4) The final product, called "clean malt" is obtained by passing dried malt through a screw conveyor to remove the rootlets.

Clean malt contains the barley plant shoot, called the "acrospire".

Analytical work carried out by the malting industry showed that raw barley and green malt contained negligible amounts of NDMA, but dried malt did contain NDMA, often at a level in excess of 50 μg/kg (Hardwick, et al., 1981). Therefore, NDMA formation occurred during the kilning operation.

The type of heating system used during kilning was found to drastically effect the level of NDMA in the finished malt. The "direct-firing" process in which hot air is generated from gas-fueled or oil-fueled combustion produced the highest levels of NDMA (Spiegelhalder et al., 1980; Hardwick et al., 1981). "Indirect-firing" in which hot air is generated from steam or electric coils produced much lower levels of NDMA. Typically, dried-firing resulted in 40 to 70 times greater NDMA formation in finished malt than did the indirect-firing process depending on the type of malt used (Spiegelhalder et al., 1980).

During the direct-firing process, the products of combustion from a flame operated at 1800-2000°C are directly incorporated into the drying air, and therefore come into direct contact with the product being dried. The combusion causes nitrogen oxides (NO_x) to be formed. Some of the important thermodynamic relationships in this process are:

(1) The activation energy for the formation of NO from N_2 has a a value of 129 kcal/mole, so the reaction is very temperature dependent (USHEW, 1970):

$$N_2 + O_2 \rightleftharpoons 2NO \qquad (34)$$

(2) NO is oxidized by air after the NO moves away from the flame; the reaction rate increases with decreasing temperature (USHEW, 1970):

$$2NO + O_2 \rightleftharpoons 2NO_2 \qquad (35)$$

(3) NO_x forms much more readily from nitrogen contained in fossil fuel rather than from molecular nitrogen. In natural gas, the amount of fuel-bound nitrogen is usually negligible; so oxidation of atmospheric nitrogen is the source of NO_x formation in natural gas combustion.

The relationship between NO_x generation and NDMA formation in malt has been substantiated by use of a pilot kiln in which heat is produced indirectly from electric coils and nitrogen oxides are added to the drying air from an external source. With this system, malts can be produced which contain NDMA at levels comparable to those found in production malt kilned by direct-firing.[1]

Another set of equilibria which may be present in moisture-laden air is shown in Equations (36) and (37):

$$NO(g) + NO_2(g) + H_2O(g) \rightleftharpoons 2HONO(g) \qquad (36)$$

$$2HONO(liq.) \overset{K_4}{\rightleftharpoons} N_2O_3(liq.) + H_2O \qquad (37)$$

The value of K_4 is 9×10^{-3} at 25° (Turney and Wright, 1959). Thus, NO_x may enter the condensed phase and become a nitrosating agent by three different routes: by direct recombination of dissolved NO and

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[1] R.A. Scanlan and T.J. O'Brien, unpublished results.

and NO_2, recombination of two molecules of dissolved NO_2, and by condensation of nitrous acid.

It is not known if there is enough moisture on the surface of malt during the latter stages of kilning to effect a condensed phase nitrosation reaction. Even if the moisture content were too low, the possibility exists that NO and NO_2 form more N_2O_3 and N_2O_4 than would be predicted from their gas phase equilibrium constants due to "third body" collisions on the surface of malt. In a third body collision, the malt surface would serve as a medium for dissipating or absorbing the excess kinetic energy of NO and NO_2 radicals so that the possibility of forming a covalent bond between NO and NO_2 or two molecules of NO_2 would be increased.[2]

The relationship between NDMA in direct-fired malt and the moisture content and kilning temperature of malt have been followed in tracking experiments (O'Brien et al., 1980). For example, a study of NDMA accumulation during a 42 hr. kilning cycle showed that significant NDMA accumulation did not occur until an air temperature of 140°F was reached at a moisture content slightly below 10%. Approximately half of the NDMA accumulation took place in the final six hours of drying when the moisture content was being lowered from 7.4% to 4.3%. Dehydration could promote nitrosamine formation by at least three modes of action: (1) dehydration eventually allows for an increase in temperature at the malt surface, (2) dehydration promotes the physical migration of NDMA precursors to the malt surface, and (3) dehydration causes conversion of residual dissloved nitrous acid to nitrous anhydride.

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[2]Private communication with R.N. Leoppky; October, 1980.

Amine Precursors in Green Malt

It would be helpful to know the identity of the amine precursor or group of precursors to NDMA in green malt. Such information could be used to devise a program for inhibiting NDMA formation by removing or reducing the amount of precursor.

A group of amines which have the potential to be NDMA precursors in malt and an estimate of the relative amount of each amine in green malt is listed in Table 1. Dimethylamine (DMA) and trimethylamine (TMA) are both reported to be present in beer (Drews et al., 1957; Hrdlicka et al., 1964; Singer and Lijinsky, 1976). Malt is the most likely source for these volatile amines, since they were reported not to be formed during fermentation (Drews et al., 1957). In Table 1, the estimate for DMA was obtained by multiplying the average literature value for DMA in beer by a factor of ten. The factor of ten accounts for the maximum dilution of soluble substances which occurs when malt is mashed to make wort.

The brewing and malting industries have concluded that DMA is not an important precursor to NDMA in kilned malt (Hardwick et al., 1981). It was known that small amounts of DMA could be present in raw barley as a result of the use of herbicides formulated as salts of DMA. But direct-fired kilning of raw barley samples seldom resulted in detectable levels of NDMA. Furthermore, when raw barley samples were dried in an electric pilot kiln in the presence of NO_x, there was no difference in NDMA formation between herbicide treated samples and untreated samples (Hardwick et al., 1981).

Two secondary metabolites formed in malt by biosynthetic activity

TABLE 1. Potential Precursors of NDMA in Green Malt

Amine	Estimate of Amount in Green Malt (ppm)
Dimethylamine	1-5 (estimated from beer)
Trimethylamine	"presence probable"
Hordenine	67
Gramine	15-20 (estimated from acrospires)

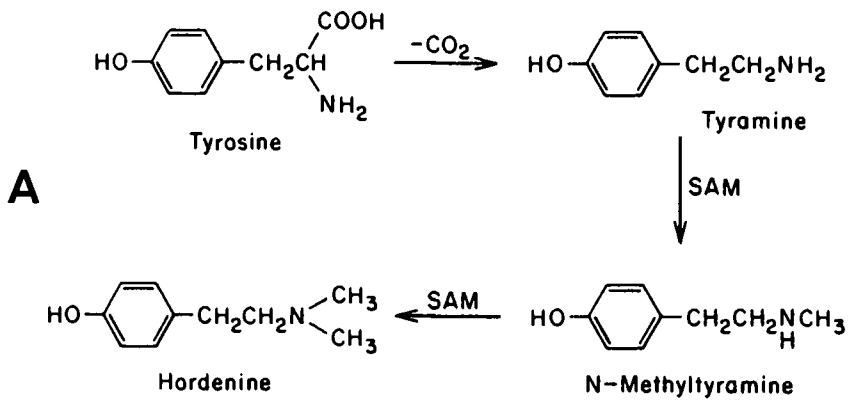
are both potential precursors to NDMA. Hordenine is formed biosynthetically from tyrosine (Figure 5A). Hordenine and its immediate precursor, N-methyltyramine, are the principle alkaloids formed in malt roots during germination. During biosynthesis, tyrosine is enzymatically decarboxylated to tyramine which is methylated in two successive steps using the methyl-donor-S-adenosylmethionine (SAM). Leete and Marion (1953) showed that ^{14}C-labelled tyrosine was a direct precursor to both N-methyltyramine and hordenine in malt roots. Hordenine biosynthesis begins on the first day of germination and would reach a maximum by the ninth day if the germinated malt were not kilned (Frank and Marion, 1956).

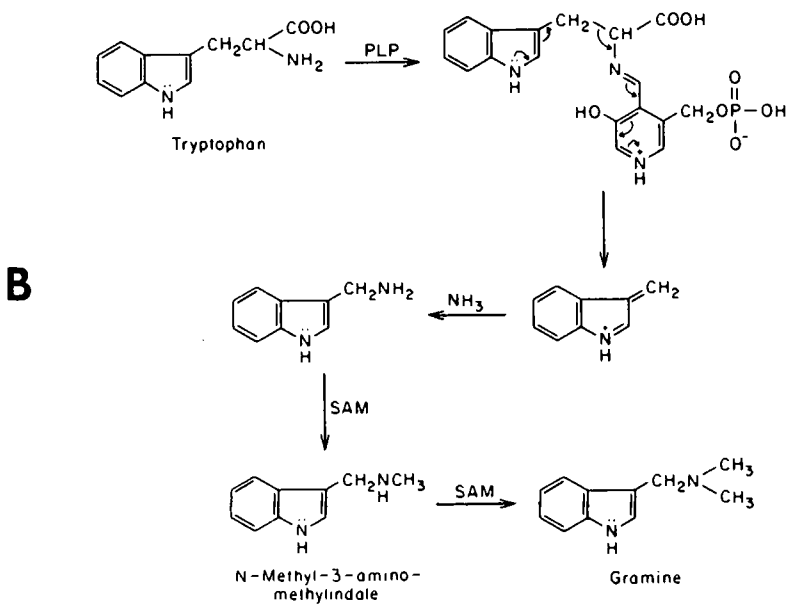
McFarlane (1965) carried out the only available quantitative study on hordenine in malt and malt fractions. The alkaloid was removed by methanol extraction of kilned malt or roots, isolated by column chromatography, and determined colorimetrically. As exptected, malt roots contained a high level of hordenine (1600 ppm). But the malt acrospires also contained hordenine (217 ppm); acrospires are the

Figure 5. A (top): The biosynthesis of hordenine

B (bottom): The biosynthesis of gramine

Figure 5


A
HO
CH2CH
COOH
NH2
-CO2
Tyrosine
HO
CH2CH2NH2
Tyramine
SAM
HO
CH2CH2N
CH3
CH3
SAM
Hordenine
HO
CH2CH2NCH3
H
N-Methyltyramine


B
CH2CH
COOH
NH2
PLP
Tryptophan
CH2
CH
COOH
HO
CH2OP
OH
O
O-
CH2NH2
NH3
CH2
SAM
CH2NCH3
H
SAM
CH2N
CH3
CH3
N-Methyl-3-amino-methylindole
Gramine

shoots of the barley plant, and would eventually become leaves if germination were continued. Clean malt also contained hordenine (67 ppm). Since the biosynthetic pathway leading to hordenine is reported to operate only in rootlets (Schneider and Wightman, 1974), the finding of hordenine in acrospires and in clean malt was unexpected. One explanation for this finding is the possibility that hordenine is "rubbed-off" or physically transferred from malt roots to malt husk during handling and derooting. However, it is hard to rationalize how this could explain the high level of hordenine found in malt acrospires. Another explanation is that some hordenine formed in rootlets could be transported back to the embryo and eventually deposited in the acrospires.

The other major tertiary amine alkaloid found in malt is gramine (Figure 5B). This alkaloid is found in malt acrospires after germination. The biosynthesis of gramine is initiated by the condensation of tryptophan with pyridoxal phosphate (PLP) followed by loss of the α-carbon of tryptophan as a glycine unit. The resulting indole moiety is aminated to a primary amine and methylated in two successive steps to yield gramine. The primary evidence for this pathway was obtained by administering 3-amino methylindole-[α-$^{14}CH_2$] and N-methyl-3-aminomethylindole-[α-$^{14}CH_2$] to germinating barley shoots. Each amine produced gramine having a high incorporation of tracer solely at the α-CH_2 position (Gower and Leete, 1963).

Few quantitative studies of gramine levels in germinating malt have been carried out. Schneider and Wightman (1974) isolated and measured the level of gramine in the shoots of growing barley

seedlings at different intervals over a 26 day period. After four days, the gramine level in shoots was 490 ppm as determined by densitometry of a colored derivative of gramine. The gramine level reached a maximum on the ninth day, then went into a steady decline. Since this level for gramine was obtained from only one barley variety by a colorimetric method, more reliable quantitative values for gramine in malt are needed. Therefore, a very conservative estimate for gramine concentration in green malt is given in Table 1.[3]

Only one experiment has been reported in which an attempt was made to find a correlation between NDMA formation in green malt and the presence of hordenine and gramine. Slack and Wainwright (1981) prepared methanol extracts of derooted malt which had undergone indirect-drying. The alkaloidal fraction of the extract was obtained and chromatographed on paper. The resulting chromatograms were cut into equally-sized zones ranging from R_f zero to R_f 1.0. Each paper zone was incubated with sodium nitrite at pH 5.5 for 16 hr. at 90°. When the reaction extracts were analyzed for NDMA by GC-TEA, it was found that the precursor to NDMA in the original chromatogram had the same R_f values as were seen for standards of hordenine and gramine. So both amines were found to be precursors of NDMA. Nevertheless, the authors concluded that hordenine was the precursor to NDMA in kilned malt because hordenine was present at sufficient concentration (20-30 ppm) to account for all NDMA found during direct-fired kilning.

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[3]Gramine levels in green malt from several different varieties of barley are now being determined by B. Poocharoen and R.A. Scanlan (Dept. of Food Science, Oregon State University). An analytical method using HPLC has been developed.

However, no values for the gramine level in malt were given, and no indication of the relative recovery of hordenine and gramine by the extraction method were stated.

Obviously, a more systematic approach is needed to determine the relative susceptibility of hordenine and gramine to undergo nitrosation to give NDMA. Experiments designed to compare the susceptibility of hordenine and gramine to nitrosation are the subject of this thesis.

Reduction of NDMA Formation in Malted Barley

It was stated earlier that measures were being taken by the brewing industry to reduce NDMA formation in beer. Among the methods suggested, the most effective are changes in the technology of the malting process at the kilning stage. The conversion to indirect-fired kilning causes a dramatic reduction in the level of NDMA in malt (Spiegelhalder et al., 1980). In the indirect-firing process, combustion products are not mixed with the drying air. The conversion to indirect-firing is costly both in capital investment and in increased fuel costs. In spite of these factors, part of the U.S. malting industry is converting to indirect-firing because it ultimately may be the most effective method for reducing nitrosamine levels in malt and beer.

The use of sulfur dioxide or the burning of elemental sulfur during direct-fired kilning is currently being employed with considerable success to retard NDMA formation during kilning. Historically, sulfuring of green malt at the start of kilning was done to increase the solubility of proteins in malt (Pomeranz, 1974). Sulfur

application is done either by burning elemental sulfur to produce SO_2 or by direct injection of SO_2 into the drying air. Injection of SO_2 can be accurately controlled by mechanical metering (O'Brien et al., 1980). Detailed experiments showed that the most effective inhibition of NDMA formation occurred when SO_2 was applied during the first 8 hours of the kilning cycle. Any time delay between the start of kilning and the application of SO_2 resulted in much less effective inhibition of NDMA formation (O'Brien et al., 1980).

The use of SO_2 during malt kilning can retard nitrosamine formation by at least two modes of action. First, SO_2 dissolves in the aqueous phase on the surface of green malt thereby forming acid which lowers the surface pH of the malt by as much as two pH units (O'Brien et al., 1980). The lower pH leads to an increase in the level of protonated amines; only amines in the unprotonated form can be nitrosated. Secondly, SO_2 in solution is in equilibrium with the bisulfite ion (HSO_3^-). The bisulfite ion is a reducing agent in food systems (Roberts and McWeeny, 1972), and may chemically reduce nitrosating agents on the surface of malt. In this respect, the action of bisulfite would be analogous to the inhibition of nitrosamine formation by ascorbate (Gray and Dugan, 1975).

The effect of SO_2 application to malt on levels of NDMA in beer is reflected in recent data. Havery et al. (1981) recently conducted a large scale survey of domestic (U.S.) and imported beers. A mean value of less than 1μg/kg of NDMA was found for 180 samples of domestic beer; a mean value of 1 μg/kg of NDMA was found for 80 samples of imported beer. Both values represented a reduction in NDMA levels

compared to the original survey (Fazio et al., 1980). In a survey completed in 1979, Scanlan et al. (1980) found a mean NDMA level of 5.9 μg/kg in 25 brands of beer representing 18 different labels. In a more recent survey of the same brands, Mangino and Scanlan (1982) reported a new mean NDMA level of 0.2 μg/kg. Sulfur application during kilning was responsible for the reduction in NDMA levels.

A similar reduction in NDMA levels in beer was seen when SO_2 application to green malt was initiated by European malting plants (Preussman et al., 1981). The level of NDMA in kilned malt was reduced to a maximum of 5 μg/kg; this would result in an NDMA level of less than 1 μg/kg in beer.

III. EXPERIMENTAL

A. Reagents and Equipment

Raw barley and some samples of green malt and dried malt were obtained from the Great Western Malting Co., Vancouver, Washington. Freeze-dehydration of malt samples was carried out in a Hull pilot freeze-dehydration unit. Moisture content (MC) of malt samples is reported on the wet basis.

Melting points were obtained on a Fisher hot stage and are uncorrected. Infrared spectra were taken on a Beckman IR-18A. Proton nuclear magnetic resonance spectra were obtained on a Varian HA-100 or a Varian FT-80. Carbon-13 NMR spectra were obtained by full proton decoupling on a Varian FT-80 using a sweep width of 4000 Hz. High resolution mass spectral peak match data were obtained on a CEC-21-110B located at the University of Oregon. Low resolution mass spectra were obtained as direct probe samples on a Finnigan 1015C spectrometer. GC-MS was performed on a Varian 1400 gas chromatograph interfaced with a Finnigan 1015C spectrometer; the 1015C spectrometer was interfaced to a Digital Equipment Corp. PDP8/E computer, a Diablo 31 Disk System, and a Tektronix 4010-1 Display Terminal. The column for GC-MS was a 500 ft. x 0.03 in. Carbowax 20M wall coated open tubular Column; injection temperature was 200°C.

GC-TEA was performed on a Varian 3700 GC interfaced to a Thermal Energy Analyzer (Thermo Electron Corp.); the GC column was a 10 ft. x 1/8 in. SS column packed with 20% Carbowax 20M plus 2% NaOH coated on Chromosorb W-AW. The column oven was operated at 140°

or 170° and helium flow rate was 25 ml/min.

Dichloromethane (DCM), chloroform (CF), and methanol (MeOH) were distilled in glass solvents obtained from the Burdick and Jackson Co. All other solvents were of the best analytical grade available. Deionized water and MeOH used for High Performance Liquid Chromatography (HPLC) were filtered through 0.45 μ Millipore filters before use. Thin Layer Chromatography (TLC) was done using the following solvent systems: A, Acetone: chloroform: 28% NH_4OH (12:6:1): B, ethyl acetate; MeOH: 28% NH_4OH (17:3:1); C, ethyl acetate: CF (3:2); D, Hexane: ethylether: DCM (3:7:10).

HPLC was carried out on a Spectra-Physics 8000 or a Spectra-Physics 8700. The columns used were 4.6 mm x 250 mm Spherisorb C_{18} reverse phase columns with 10 micron packing. Column A was an underivatized C_{18} reverse phase column. Column B was a "derivatized" C_{18} reverse phase column commercially treated to display reduced peak tailing. The Mobile Phases used for the HPLC are listed in Table 2.

The following compounds or reagents were obtained from the Aldrich Chemical Co: benzaldehyde, dimethylamine hydrochloride, trimethylamine hydrochloride, 2,4-dimethylaminoantipyrine (Aminopyrine), dimethylsulfate, N,N-dimethyl-5-methoxytryptamine, 5-methoxygramine, indole-3-carbinol, NDMA, N-nitrosopyrrolidine, sodium cyanoborohydride, deuteriochloroform, acetone-d_6, and deuterium oxide.

The following compounds or reagents were obtained from the Sigma Chemical Co: gramine, hordenine hemisulfate, indole-3-carboxaldehyde, tyramine, α-naphthylamine, sulfanilic acid, and 3A molecular sieves.

TABLE 2. Mobile Phases Used for HPLC

Mobile Phase 1:	Time (Min.)	% H_2O	% MeOH
	0	75	25
	8	70	30
	13	60	40
	22.5	60	40
	29.5	75	25
Mobile Phase 2:	Time (Min.)	% H_2O	% MeOH
	0	60	40
	10	60	40
	12.5	55	45
	27	55	45
	29	60	40
Mobile Phase 3:	Time (Min.)	.05 M NaH_2PO_4[a]	MeOH
	0	100	0
	2	95	5
	4	90	10
	8	80	20
	10	75	25
Mobile Phase 4:	Time (Min.)	0.1 M TCA[b]	MeOH
	0	75	25
	4	70	30
	6	65	35
	8	60	40
	10	55	45

[a]Phosphate buffer adjusted to pH 3

[b]Trichloroacetic acid adjusted to pH 3

B. Formation of NDMA in Malts Nitrosated Under Laboratory Conditions

PART 1: Nitrosation of Raw Barley, Freeze-Dried Clean Malt, and Freeze-Dried Malt Roots

The starting material for germination was Montana Larker raw barley. To initiate germination, 58 g of raw barley kernels were spread over 16 metal trays each lined with paper towels. Kernels were spaced at intervals of approximately 3 in. to prevent physical contact during germination. Germination was started by wetting the paper towels thoroughly with water. All trays were stored at 65-70°F for four days, and fresh distilled water was sprayed on each tray at least once per day. After four days, the trays were stored at 34°F for two days. The resulting green malt was separated into roots and clean malt by cutting the roots free from each malt kernel using a surgical scissors. The collected roots and clean malt were placed in separate beakers and freeze-dried for 18 hr. Samples of the freeze-dried roots and freeze-dried clean malt were nitrosated in duplicate by the following procedure. To 1 g of the appropriate fraction contained in a 250 ml beaker was added 50 ml of 15% acetic acid (AcOH) raised to pH 3.2 with 6 N NaOH. Then 5 ml of a 1 g/ml solution of $NaNO_2$ was added, and the beakers placed under a fume hood for 18 hr. The mixtures were acidified to pH 1.5 with concentration H_2SO_4 and 10 ml of an 0.8 g/ml solution of ammonium sulfamate were added to quench the reaction. Each mixture was filtered through glass wool and the marc pressed with a glass stopper and washed with fresh distilled water. Each filtrate was made up to a volume of 100 ml and extracted once with 25 ml of DCM, then twice more with 20 ml DCM. The combined

extracts were added to 20 ml of 2.5 N NaOH contained in a 250 ml separatory funnel, and the mixture was shaken. The DCM layer was passed through a sintered glass funnel containing anhydrous sodium sulfate, and the DCM was collected into a Kuderna-Danish apparatus which was connected to a 10 ml concentrator tube. The DCM was concentrated to a final volume of 4 ml for the root extracts and 1 ml for the clean malt extracts. Then 5 µl volumes of the final extracts were injected on the GC-TEA. At least two injections were made for each sample. NDMA solutions of known concentration were injected as external standards. Quantitation was done by peak height measurement of the samples and standards. Values of NDMA obtained for the experimental samples were corrected by a factor corresponding to the moisture content so that all results could be compared on a dry weight basis.

Subsequently, it was desired to determine if the physical structure of the clean malt kernel had a significant effect on the nitrosation and extraction steps from which the NDMA values were obtained. For this reason, a portion of the clean malt was frozen in liquid N_2 and pulverized in a high speed blender. Then 1 g samples of the powder were nitrosated and quantitated as previously described.

The Montana Larker raw barley from which the roots and clean malt were obtained was also nitrosated. Two 1 g samples of the whole raw barley were nitrosated and quantitated for NDMA as described previously. Then two 1 g samples of pulverized raw barley were nitrosated and quantitated for NDMA.

Since the roots obtained by germination were a thin, stringy material of high surface area, no pulverized root samples were used

in these experiments. On a dry-weight basis, the roots accounted for 6.2% of the weight of the germinated malt.

PART 2: Nitrosation of Clean Malt Dried by Heat or by Freeze Dehydration

In order to determine if heat used during malt kilning could generate immediate precursors of NDMA, the following experiment was designed. Two different varieties of raw barley (Mid-Larker and Idaho Klages) were carried through the commercial steeping and germination procedure at Great Western Malting Co. Approximately 10 kg of each variety of green malt were collected, and one portion of each variety was dried by direct heat in a Seeger electric pilot malt kiln until a moisture content below 4% was obtained. This drying procedure approximates commercial kilning, but the malt is not exposed to NO_x. A second portion of each green malt variety was cooled in ice, transferred to Oregon State University, and blast frozen as quickly as possible. The frozen malts were freeze dehydrated until the moisture contents were below 3%.

Samples of both varieties of the "electric-dried" and "freeze-dried" malt were cleaned manually on a precision sieve (slotted, 4 7/8| 64 in. x 3/4 in.) to remove rootlets. Three 25 g samples of both varieties of electric-dried and freeze-dried malt were weighed into separate 250 ml beakers to which were added 50 ml of 15% AcOH at pH 3.2. Each sample was nitrosated at room temperature for 18 hr. as described in Part 1. The work-up of reaction mixtures was the same as in Part 1 except each filtrate was made up to 150 ml with distilled water before DCM extraction. DCM extracts were concentrated

to 4 ml and quantitated for NDMA as in Part 1. The values of NDMA obtained for the experimental samples were corrected for moisture content so that all results could be compared on a dry weight basis.

C. Determination of NDMA Obtained By Nitrosation of Potential Amine Precursors Under Simulated Kilning Conditions

The amines used for preliminary nitrosation experiments were gramine, hordenine, trimethylamine, and diemthylamine: the four amines suggested as the most likely precursors to NDMA in malt. The amines were obtained from commercial sources and the purities checked by TLC (Solvent A or Solvent B).

The nitrosation reactions were carried out in the following buffer solutions. The first buffer was 60% AcOH raised to pH 4.4 by addition of anhydrous sodium acetate. The second buffer was a pH 6.4 solution made by mixing 69 ml of 0.2 M Na_2HPO_4 and 31 ml of 0.1 M citric acid (CRC, 1976). Reactions were carried out by pipetting 10 ml of 0.1 M solutions of each amine into 25 ml Kimax glass tubes sealed with TEFLON-lined screw caps. Sodium nitrite (0.35 g, 0.005 mol) was added and each tube sealed and placed in a water bath at 65°±1° for 16 hr. The tubes were cooled to room temperature and 10 ml DCM added to each with shaking. The contents were transferred to a 60 ml separatory funnel and the DCM removed. The aqueous layer was extracted again with 10 ml of DCM. The combined DCM extracts were dried over sodium sulfate and the volume of DCM made up to 25 ml. Appropriate aliquots were removed and diluted further with DCM if necessary. Quantitation of NDMA was carried out as in Part A. The value of NDMA

obtained for each sample was converted to % yield by calculating the theoretical maximum yield of NDMA which could be obtained at the appropriate sample dilution. The sensitivity limit of the GC-TEA for NDMA was at least 0.01 ng/μl under the conditions used.

Since gramine was found to be highly reactive with nitrite, a third buffer solution made from 15% AcOH raised to pH 3.4 with anhydrous sodium acetate was prepared. This buffer was used to carry out the nitrosation of gramine at room temperature (23-25°) for 10 min. and 6 hr. in separate experiments. Work-up and quantitation were similar to that described above. Gramine nitrosation was later investigated more extensively under a different set of reaction conditions from those described here (Part E).

D. Confirmation of NDMA Formation From Hordenine and Gramine by Combined Gas Chromatography-Mass Spectrometry (GC-MS)

Hordenine nitrosation was carried out by adding 0.69 g of $NaNO_2$ to 10 ml of a 0.1 M solution of hordenine hemisulfate at pH 4.4 After reaction in a sealed tube for 4 hr. at 65°, the mixture was acidified to pH 1, extracted with DCM, and the DCM was back-extracted with 2 M NaOH to remove phenolic material. The residual DCM extract was concentrated to dryness slowly under a stream of N_2. Hexane (5 ml) was added to the brown residue; the NDMA concentration in this hexane extract was 88ng/μl as determined by GC-TEA. The sample used for GC-MS was obtained by concentrating 1 ml of the hexane extract to 0.1 ml under a stream of N_2.

Nitrosation of gramine was performed by adding 0.69 g of $NaNO_2$

to 10 ml of a 0.1 M solution of gramine at pH 3.4 and allowing the reaction tube to stand at room temperature for 10 min. The reaction product was treated as above for hordenine. The final extract was a 10 ml hexane solution with an NDMA concentration of 462 ng/μl. The sample for GC-MS was obtained by diluting 1 ml of the hexane extract to 10 ml with fresh hexane.

To perform the GC-MS analysis, the column oven temperature was set at 120° and the helium flow rate adjusted to 15 ml/min. The retention time of NDMA in the system was determined by injecting 1 μl of a 50 ng/μl NDMA standard. The spectrum of the eluted NDMA was obtained by reconstructing the Total Ion Current data and determining the spectrum number at which the ion m/e 74 was maximized. Then 1 μl of acetone was injected to purge the column. After this, the extracts of gramine and hordenine nitrosation were injected, and the mass spectrum of the eluted NDMA determined as above. The reconstructed Total Ion Current data of both of the reaction products showed that the m/e 74 ion was maximized at the retention time corresponding to NDMA.

E. Comparison of the Formation of NDMA From Gramine and Selected Secondary and Tertiary Amines As A Function of Time and Temperature

PART 1: Formation of NDMA from Aminopyrine, Dimethylamine, Gramine, and Trimethylamine at 24°±1°

Aminopyrine and gramine were found to be pure by TLC (Solvent A) and by melting point. Dimethylamine hydrochloride was obtained from

a freshly opened bottle. A sample of trimethylamine hydrochloride was suspended in acetone, and the suspension filtered on a Buchner funnel in vacuo to collect the amine hydrochloride. This was placed in a dessicator containing P_2O_5 and residual acetone removed in vacuo until the weight remained constant.

For these experiments, amine solutions of pH 3.4 and 0.1 M concentration were desired. To achieve this, buffered amine solutions were made up as follows. A solution of 15% AcOH was raised to pH 3.15 by addition of anhydrous sodium acetate. This buffer was used to make a 0.10 M solution of gramine (final pH of 3.44) and 0.10 M solution of aminopyrine (final pH of 3.47). A second buffer was made by raising the pH of a 15% AcOH solution to pH 3.45. This buffer was used to make a 0.10 M solution of dimethylamine hydrochloride (final pH of 3.43) and a 0.10 M solution of trimethylamine hydrochloride (final pH of 3.41). Samples to be nitrosated were prepared by pipetting 10 ml of the appropriate amine solution into 20 ml Kimax glass tubes sealed by TEFLON-lined screw caps. Reactions were initiated by addition of 0.69 g (0.01 mol) of $NaNO_2$; 10 sec of mixing time were allowed, then the elapsed time of each reaction was recorded. When the desired reaction time was complete, the tube was opened quickly, and the reaction mixture quenched by pouring as quickly as possible into a 50 ml beaker containing an ice-cold solution of 5 ml of 6.2 M ammonium sulfamate plus 2 ml of concentration HCl. When foaming subsided, the mixture was poured into a 60 ml separatory funnel. The quenched reaction was extracted with three 5 ml portions of CF. One of the 5 ml portions of CF was used to rinse the reaction tube and

one CF portion was used to rinse the quench beaker. All the CF extracts were combined and made up to 25 ml in a volumetric flask using additional CF. The NDMA concentration was determined by GC-TEA using known NDMA solutions as external standards. All reactions were run at an ambient temperature of 24±1°. Reaction times chosen for each amine were as follows; aminopyrine: 2.5 min., 5 min., 10 min., 30 min., and 60 min.; dimethylamine: 5 min., 10 min., 30 min., 60 min., and 120 min.; gramine: 5 min., 10 min., 30 min., 60 min., and 120 min.; trimethylamine: 10 min., 30 min., 60 min., and 120 min.

PART 2: Formation of NDMA from Gramine, 5-Methoxygramine, N,N-Dimethyl-5-methoxytryptamine, and Hordenine at 23±1°

Commercial samples of hordenine hemisulfate, 5-methoxygramine, and N,N-dimethyl-5-methoxytrytamine were found to be pure by TLC (Solvent A) and by their melting points. Amine solutions of 0.1 M in gramine, 5-methoxygramine, and N,N-dimethyl-5-methoxytryptamine were made up using the pH 3.15 AcOH-AcO^- buffer described in Part 1. A solution 0.1 M in hordenine hemisulfate was made up using the pH 3.45 AcOH-AcO^- buffer described in Part 1. Nitrosation reactions were initiated, quenched, and worked-up as described in Part 1. All reactions were run at ambient temperature of 23±1°. Reaction times chosen for each amine were as follows; gramine: 5 min., 10 min., 30 min., 60 min., and 120 min.; 5-methoxygramine: 10 min., 30 min., 60 min., and 120 min.; N,N-dimethyl-5-methoxytryptamine: 10 min., 30 min., 60 min., and 120 min.; hordenine hemisulfate: 10 min., 30 min., 60 min., and 120 min.

PART 3: Formation of NDMA from Gramine, 5-Methoxygramine, N,N-Dimethyl-5-methoxytryptamine, and Hordenine at 37°±0.5°

Solutions of 0.1 M of each amine were made as described in Part 2. Reactions were carried out as described in Part 1, except that each amine solution was pre-incubated in a water bath to 37°±0.5° before the addition of $NaNO_2$. When the reaction times were complete, each tube was cooled quickly in an ice-salt bath before opening. Reaction times chosen for each amine were as follows; gramine: 2.5 min., 5 min., 10 min., 30 min., and 60 min.; 5-methoxygramine: 2.5 min., 5 min., 10 min., 30 min., and 60 min.; N,N-dimethyl-5-methoxytryptamine: 5 min., 10 min., 30 min., and 60 min.; hordenine hemisulfate: 5 min., 10 min., 30 min., and 60 min.

PART 4: Formation of NDMA From Gramine and Hordenine at 65°±0.5°

Solutions of 0.1 M in gramine and hordenine hemisulfate were made as described in Part 1 and Part 2. Reactions were carried out as described in Part 1 except that each amine was pre-incubated in a water bath at 65°±0.5° before the addition of sodium nitrite. Reaction tubes were cooled before opening as described in Part 3. The gramine nitrosation reactions were run in duplicate at the following times: 30 sec., 1 min., 2 min., 5 min., and 10 min. The hordenine hemisulfate reactions were run for 5 min., 10 min., 20 min., and 30 min.

PART 5: Recovery Determination From NDMA Solutions of Known Concentration

Since it was suspected that NDMA would not be extracted and recovered with the same efficiency from each reaction mixture, the

following recovery determinations were made from known solutions of NDMA dissolved in the same type of buffer used for the reactions in parts 1 through 4. One ml of a stock solution containing 0.074 g/ml of NDMA dissolved in pH 3.45 AcOH-AcO^- buffer was added to 9 ml of the blank buffer contained in each of two reaction tubes. Then 0.69 g of $NaNO_2$ was added to each tube; the tubes were capped and allowed to stand at room temperature for 30 min. The tubes were quenched, extracted, and NDMA determined as in Part 1. The NDMA stock solution was used to prepare duplicate tubes containing 0.037 g NDMA or 0.0074 g of NDMA, and the quenching, extraction, and quantitation procedure was done on the contents of each of these tubes. The value of NDMA from the six determinations was converted to an actual % recovery for each determination. The % recovery was graphed as a function of the % of the theoretical yield of NDMA which each standard solution represented. The results (Figure 6) described a line which was adopted as the recovery curve for the correction of the NDMA determinations performed in Part 1 through Part 4.

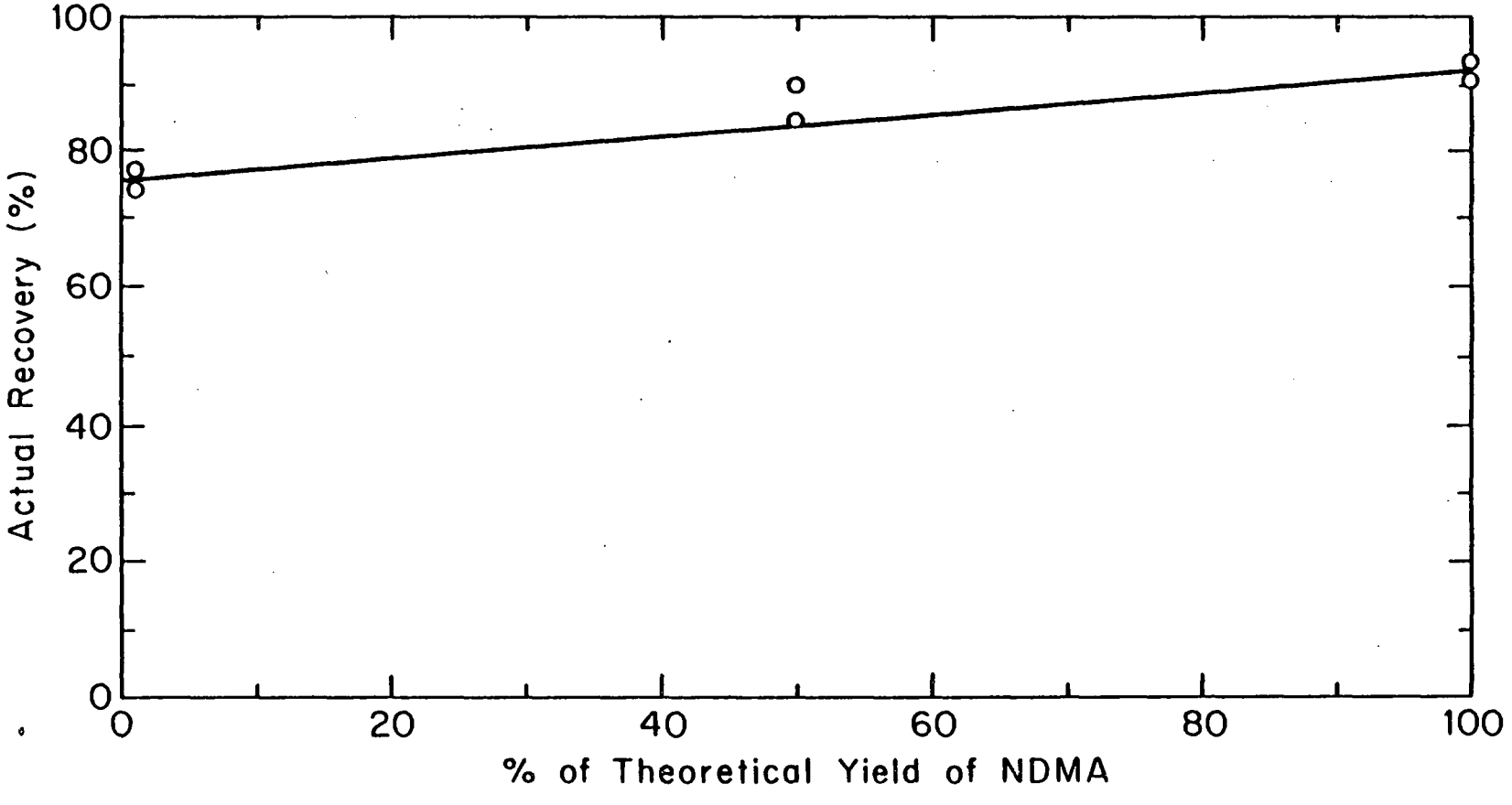
F. Synthesis of N-Methyltyramine [p-Hydroxy-N-methyl-2-phenylethylamine] and N-Methyl-3-aminomothylindole [3-(N-methylaminomethyl)indole]

PART 1: Synthesis of N-Methyltyramine

Tyramine (10.3 g, 0.075 mol) was placed in a 250 ml round-bottom flask and 100 ml of absolute ethanol were added. The mixture was heated slowly until tyramine dissolved, then benzaldehyde (8.75 g, 0.082 mol) was added in portions and the mixture heated at reflux for 5 hr. Heating was stopped, and the flask allowed to stand overnight

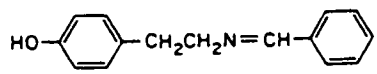
Figure 6. Correction curve for the recovery of NDMA from nitrosation reaction mixtures

Figure 6


100
80
60
40
20
0
Actual Recovery (%)
0
20
40
60
80
100
% of Theoretical Yield of NDMA

in a refrigerator at 34°F. The nearly colorless crystalline product which formed was collected on a Buchner funnel in vacuo, washed with cold ethanol, and dried in vacuo to yield 14.8 g of short, white needles (m.p. 152-152.5°). This product represented 88% yield of p-hydroxy-2-phenylethylbenzaldimine. The compound was characterized as the imine on the basis of the high resolution mass spectral peak match of its molecular ion ($C_{15}H_{15}NO$ requires 225.1154; found, M^+ = 225.115); the mass spectral fragmentation data are shown in Table 3. The imine (14.7 g, 0.065 mol) was refluxed for 1 hr. with 50 ml of benzene containing dimethylsulfate (6.6 ml, 0.07 mol). After cooling to near room temperature, the upper benzene layer was decanted and 125 ml of 95% ethanol was added. This new mixture was refluxed for 1 hr., cooled to room temperature and the ethanol removed in vacuo. The residual material was made strongly acidic by addition of 6N HCl then diluted with an equal volume of distilled water. The aqueous solution was extracted 6 times with 35 ml volumes of CF to remove the benzaldehyde, then transferred to a 250 ml beaker. The pH was raised to 10.1 by addition of 6N NaOH and the mixture was saturated with NaCl to release an insoluble oil. The oil was extracted with ethyl-acetate, and the extract dried over Na_2SO_4 and solvent removed in vacuo to leave a crude crystalline material (5.5 g). Two recrystal-lizations from ethyl acetate yielded a nearly colorless crystalline material (4.5 g, 40%) which was dried in vacuo and gave a melting point of 129-131°; lit. m.p. 130-131° (Kirkwood and Marion, 1950). TLC (Solvent A) showed that the product contained a small amount of tyramine impurity. The impurity could be removed using the method

TABLE 3. Mass Spectral Fragmentation Data For p-Hydroxy-2-phenylethylbenzaldimine



m/e	Relative[a] Intensity
225 (M^+)	23
135	7
130	10
119	16
118	100
107	14
91	39
77	6

[a]Data obtained on CEC-21-110B instrument located at the University of Oregon

developed by Poocharoen.[3] Activated Aluminum oxide (50 g) was made into a slurry with CF and this was packed onto a chromatography column. One gram of the impure N-methyltyramine was dissolved in 10 ml of MeOH and this solution applied to the column. Elution was begun with MeOH and continued until 200 ml of MeOH had been collected. Removal of the solvent in vacuo left a white crystalline powder (0.91 g) which was dried in vacuo to constant weight. This material had m.p. 130° and showed no tyramine impurity by TLC. HPLC (Mobile Phase 3) on Column A also showed this product to be free of tyramine impurity. The material which was not chromatographed on aluminum oxide had a 2% impurity of tyramine as determined by HPLC. The mass spectrum of N-methyltyramine showed: m/e (relative intensity): 151 (30) M^+, 121(13), 108(47), 107(100), 77(92), 42(25). The high resolution mass spectral peak match for M^+ showed: for $C_9H_{13}NO$, calculated mass: 151.0997; found: 151.100. The ^1H NMR spectrum (D_2O) showed: 2.49δ (s,3H), 2.84δ(q,4H), 6.54δ(d,2H), 6.97δ(d,2H) (See Appendix).

PART 2: Synthesis of N-Methyl-3-aminomethylindole

Indole-3-carboxaldehyde (8.7 g, 0.06 mol) was placed in a 400 ml beaker and 200 ml of MeOH added with stirring and mild heating to obtain a complete solution. The beaker was transferred to a pH meter and the solution raised to pH 11 by addition of 5% KOH in MeOH. Molecular sieves (3A, 5 g) were added, followed by methylamine hydrochloride (20.4 g, 0.3 mol). The solution was readjusted to pH 8.8 using

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[3]Private Communication with B. Poocharoen; Dept. of Food Science, Oregon State University.

KOH/MeOH and allowed to stir for 15 min. Sodium cyanoborohydride (4 g, 0.064 mol) was added in one batch and the mixture adjusted to pH 6.7 by dropwise addition of glacial AcOH. The beaker was immediately transferred to a fume hood and the reaction mixture heated to 55-60° for 2 hr. TLC (Solvent C) indicated the disappearance of starting material. The reaction was cooled to near room temperature and acidified to pH 1 by slow addition of 6N HCl. The mixture was filtered through glass wool and MeOH removed in vacuo. The residual oil was taken up in 100 ml distilled water and extracted 3 times with 35 ml volumes of ethyl acetate. The remaining aqueous layer was filtered through glass wool, and made alkaline to pH 10, which released an insoluble oil. Extraction of the oil by ethyl acetate and removal of solvent in vacuo left a beige-colored oil (7.3 g, 76%) which crystallized completely on standing at room temperature. The material could be recrystallized with difficulty by dissolving slowly in the minimum amount of warm benzene followed by slow cooling. The recrystallized product had beige-colored prisms (m.p. 78-80°). TLC (Solvents A and B) and HPLC (Mobile Phase 3) on Column A showed this product to be homogenous and free of starting compound. A picrate derivative recrystallized from 95% ethanol gave m.p. 173-175° dec; lit. m.p. 176-176.5° dec (Gower and Leete, 1963). The mass spectrum showed: 160(25)M^+, 159(23), 130(100), 129(42), 102(31), 77(31), 42(25). The high resolution mass spectral peak match for M^+ showed: for $C_{10}H_{12}N_2$, calculated mass: 160.1000; found: 160.100. IR(KBr): 3305(m,-NH), 3125(b,indole-NH). The ^{1}H NMR spectrum showed: 2.46δ(s,3H), 3.89δ(s,2H), 6.94-7.58δ(m,6H) (See Appendix).

For C-13 NMR, see Appendix and Discussion.

G. Nitrosation of N-Methyltyramine: Isolation of p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine and p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine

A sample of N-methyltyramine (3.77 g, 0.025 mol) which showed m.p. 129-131° and contained no more than 2% impurity of tyramine was dissolved in 40 ml of 30% AcOH. The pH was adjusted to 3.4 with 2N NaOH. Sodium nitrite (4.3 g, 0.06 mol) was added and the mixture stirred for 3 hr. Then the reaction mixture was acidified to pH 1.6 by addition of 3M HCl and extracted 4 times with 25 volumes of 10% NaOH, and this alkaline extract was reacidified slowly to pH 3.5. The new acidic fraction was extracted 4 times with 35 ml volumes of CF and the CF filtered through a bed of Celite and the solvent removed in vacuo to leave a dark amber oil (2.5 g). TLC (Solvents A and B) of the oil showed two major components and some material which remained at the origin of the plate. Chromatography was carried out as follows. A large sample of Silica Gel was activated at 120° for 90 min.; 3% by weight of distilled water was added and the flask containing the gel was turned on a rotary evaporator for 2 hr. to achieve equilibration. The gel (190 g) was packed onto a chromatography column as a slurry in neat CF. The amber oil (2.4 g) dissolved in CF (5 ml) was applied to the column and elution begun with neat CF and continued until a bright yellow band of material reached the bottom of the column. The eluant was changed to 1% ethyl acetate/CF and elution continued until 200 ml of the new solvent had been used. Elution was continued with successive 200 ml volumes of 2%, 4%, and 8% ethyl acetate/CF mixtures.

Fractions of the eluant were collected into tubes at 10 min. intervals. The bright yellow band was collected and solvent removed *in vacuo* to give a bright yellow oil (0.52 g) which crystallized on standing. This material was recrystallized from ethanol to give 0.30 g of yellow prisms (m.p. 89-92°), which co-chromatographed on TLC with the major reaction product having R_f 0.41 in Solvent B. This material had spectroscopic properties consistent with *p*-hydroxy-*m*-nitro-N-nitroso-N-methyl-2-phenylethylamine.

Continued elution of the silica gel column yielded a second homogenous product which crystallized after solvent removal. This material was recrystallized from benzene to yield 0.43 g of light green flakes (m.p. 103.5-104.5°). This product co-chromatographed on TLC with the major reaction product having R_f 0.67 in Solvent B, and showed spectroscopic properties consistent with *p*-hydroxy-N-nitroso-N-methyl-2-phenylethylamine.

The mass spectrum of *p*-hydroxy-*m*-nitro-N-nitroso-N-methyl-2-phenyl-ethylamine showed: 225(3)M^+, 195(5), 179(5), 165(70), 152(97), 106(74), 73(73), 43(100), 42(99). The high resolution spectral peak match for M^+ showed: for $C_9H_{11}N_3O_4$, calculated mass: 225.0749; found: 225.075. The ^1H NMR($CDCl_3$) showed: 2.76δ(t,β-CH_2-), 3.00δ(s,*syn*-CH_3), 3.65δ(s,*anti*-CH_3), 3.75δ(t,*syn*-CH_2-), 4.35δ(t,*anti*-CH_2-), 6.98-7.38δ (m,arom.), 10.43δ(s,-OH). For C-13 NMR, see Appendix and Results.

The mass spectrum of *p*-hydroxy-N-nitroso-N-methyl-2-phenylethyl-amine showed: 180(14), 150(4), 134(21), 120(82), 108(36), 107(100), 77(74), 44(73), 42(96). The high resolution mass spectral peak match for M^+ showed: for $C_9H_{12}N_2O_2$, calculated mass: 180.0899; found: 180.088.

The ^{1}H NMR (Acetone-d_6) showed: 2.75δ(t,β-CH_2-), 2.95δ(s,syn-CH_3), 3.58δ(s,anti-CH_3), 3.71δ(t,syn-CH_2-), 4.31δ(t,anti-CH_2-), 6.64-7.05δ (m,4H,arom.). For C-13 NMR, see Appendix and Results.

H. Nitrosation of N-Methyl-3-aminomethylindole: Isolation of N^1-Nitroso-N-nitroso-N-methyl-3-aminomethylindole and N-Nitroso-N-methyl-3-aminomethylindole

N-methyl-3-aminomethylindole (4 g, 0.025 mol) was dissolved in 30% AcOH (75 ml) and the pH adjusted to 3.4. Sodium nitrite (4.3 g, 0.06 mol) was added and the mixture stirred for 4 hr. The mixture was made alkaline to pH 10, extracted 3 times with 30 ml DCM and the solvent removed in vacuo to give an amber colored oil (4.55 g). TLC (Solvent C) showed two components. Chromatography of part of this oil was carried out by packing a chromatography column with 115 g of activated silica gel to which 3% water had been added. The gel was packed by slurry in neat CF and 1.15 g of the oil dissolved in 2 ml of neat CF was applied. The column was wrapped with a loose fitting piece of aluminum foil to reduce light exposure. Elution was begun with neat CF and continued until a bright yellow band was completely eluted from the column. Removal of solvent left a homogeneous oil (0.69 g) which was stored under-cover in the dark at -20°. After three weeks, the oil had turned to a crystalline mass. Recrystallization of this material from warm MeOH yielded 0.19 g of yellow prisms (m.p. 47-48°) which co-chromatographed on TLC with the reaction product having R_f 0.57 (Solvent C). Both the original oil and the yellow crystalline material showed a mass spectrum corresponding to N^1-nitroso-N-nitroso-N-methyl-3-aminomethylindole.

Continued elution of the chromatography column with 2% ethyl acetate/CF yielded a second homogenous material which gave 0.08 g of crystalline product (m.p. 122-123°) after recrystallization from MeOH. This product showed spectroscopic properties consistent with N-nitroso-N-methyl-3-aminomethylindole and co-chromatographed on TLC with the reaction product having R_f 0.43 (Solvent C).

The mass spectrum of N^1-nitroso-N-nitroso-N-methyl-3-aminomethylindole showed: 218(4)M^+, 188(92), 158(99), 143(94), 129(99), 117(57), 102(85), 89(34), 76(34), 42(100). The high resolution mass spectral peak match for M^+ showed: for $C_{10}H_{10}N_4O_2$, calculated mass 218.0804; found: 218.080. The ^1H NMR($CDCl_3$) spectrum showed: 2.98δ(Syn-CH_3), 3.72δ(anti-CH_3), 4.88δ(syn,CH_2-), 5.43δ(anti-CH_2-), 7.17-7.62δ(m,4H), 8.12δ(d,1H, not exchangeable with D_2O). For C-13 NMR, see Appendix and Results.

The mass spectrum of N-nitroso-N-methyl-3-aminomethylindole showed: 189(39)M^+, 159(18), 144(68), 131(100), 130(92), 117(32), 103(54), 89(35), 77(85), 42(100). The high resolution mass spectral peak match for M^+ showed: for $C_{10}H_{11}N_3O$, calculated mass: 189.0902; found: 189.088. The ^1H NMR($CDCl_3$) showed: 2.89δ(s,syn -CH_3), 3.48δ-(s,anti-CH_3), 4.90δ(s,syn -CH_2-), 5.41δ(s,anti-CH_2-), 6.94-7.49δ(m,5H), 8.35δ(1H, slowly exchangeable with D_2O). For C-13 NMR, see Appendix and Results.

It was discovered that solutions of N^1-nitroso-N-nitroso-N-methyl-3-aminomethylindole were unstable when exposed to light. The half-life of the compound after exposure to white light was determined as follows. The SP8700 HPLC instrument was set up using Column B and

a Mobile Phase of 50% MeOH: 50% water with a flow rate of 2 ml/min. A solution of the compound was made up in acetonitrile, and the solution was immediately injected onto the SP8700 and the peak height determined. The solution was then placed inside a laboratory cabinet and exposed to a 60 watt lamp placed in the cabinet. The solution vial was positioned at a distance 10 cm from the bulb. Aliquots of the solution were removed at recorded time intervals, injected on the SP8700, and the peak height measured. The decomposition product was N-nitroso-N-methyl-3-aminomethylindole. The half-life for the transformation was calculated to be 165 min.

When the solution of N-nitrosopyrrolidine was made up in acetonitrile and exposed to the same white light treatment as above, no decomposition had occurred after 11 hr.

I. Collection and Re-equilibration of Syn and Anti Conformers of p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine

Preliminary HPLC experiments using Mobile Phase 1 and Column A showed that a freshly prepared solution of p-hydroxy-N-nitroso-N-methyl-2 phenylethylamine chromatographed as two peaks which could be separated by unit resolution. Storage of the sample for an extended time period did not alter this behavior. The same behavior was seen for p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine and the two indole N-nitroso compounds described in Part H.

In order to determine that the two chromatographic peaks observed for p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine represented syn and anti conformers, the following experiment was performed.

An 8 mg/ml solution of the N-nitrosamine was allowed to stand at

room temperature for 24 hr. in the dark. The SP8700 HPLC instrument was set up using Mobile Phase 1 and Column A with a flow rate of 1.8 ml/min. At this flow rate, the delay time between detector flow cell and detector outlet tube was 18 sec. A 10 µl sample loop was used to inject the nitrosamine solution and each peak was collected into a separate vial after detection at 254 nm. The two collected peaks were re-injected at recorded time intervals using a higher detector sensitivity; peak height ratios were measured on each of the resulting chromatograms. The re-injection process was continued until both of the collected peaks had fully re-equilibrated to give a syn: anti peak height ratio of 4.6:1.

J. Determination by HPLC of Product Yields From the Nitrosation of N-Methyltyramine and N-Methyl-3-aminomethylindole

Because of the difficulty of determining reliable product yields from the nitrosation of N-methyltyramine and N-methyl-3-aminomethylindole by the isolation methods used in Parts G and H, a more suitable analytical method was required.

To examine the products of nitrosation of N-methyltyramine, the SP8700 HPLC instrument was set-up using Column A with Mobile Phase 1. The UV-vis detector was set at 254 nm and a 10 µl sample loop was installed. To determine that detector response was linear with concentration over the range of interest, standard solutions of p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine and p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine were injected at a flow rate of 1.8 ml/min. Peak areas were determined by multiplying peak height by

the width at half-height using a chart speed of 2 cm/min. Areas of the syn and anti conformers or each nitrosamine were summed together to obtain a total area for each nitrosamine peak. A plot of peak area vs. amount injected for each nitrosamine is shown in Figure 7. Detector response was found to be linear with concentration over the range required. The reaction was performed using N-methyltyramine which had been freed of tyramine impurity by elution through an aluminum oxide column as described in Part F. To initiate the reaction, 0.22 g(0.003 mol) of $NaNO_2$ was added to 0.19 g(0.0012 mol) of N-methyltyramine dissolved in 2 ml of 30% AcOH solution at pH 3.15. After 3 hr. at room temperature, the reaction was diluted to 50 ml with MeOH and 10 µl injections were made and peak areas determined as described previously. Concentrations were determined using peak areas of external standards of the nitrosamines. Since unreacted amine would not elute from the column under the conditions used, unreacted amine could not be quantitated.

For the nitrosation reaction of N-methyl-3-aminomethylindole, the HPLC conditions were similar except that a flow rate of 2 ml/min. was used with Mobile Phase 2. Standard solutions of N^1-nitroso-N-nitroso-N-methyl-3-aminomethylindole and N-nitroso-N-methyl-3-aminomethylindole were injected and peak areas determined as described previously. For the mononitroso compound, only the syn conformer peak area could be determined, since the anti peak was a shoulder on the syn peak. A plot of peak area vs. amount injected for each nitrosoamine is shown in Figure 8. To initiate the reaction, $NaNO_2$ (0.22 g, 0.003 mol) was added to N-methyl-3-aminomethylindole (0.20 g,

Figure 7. Linearity curves of detector response vs. concentration for p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine (A) and p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine (B)

Figure 7

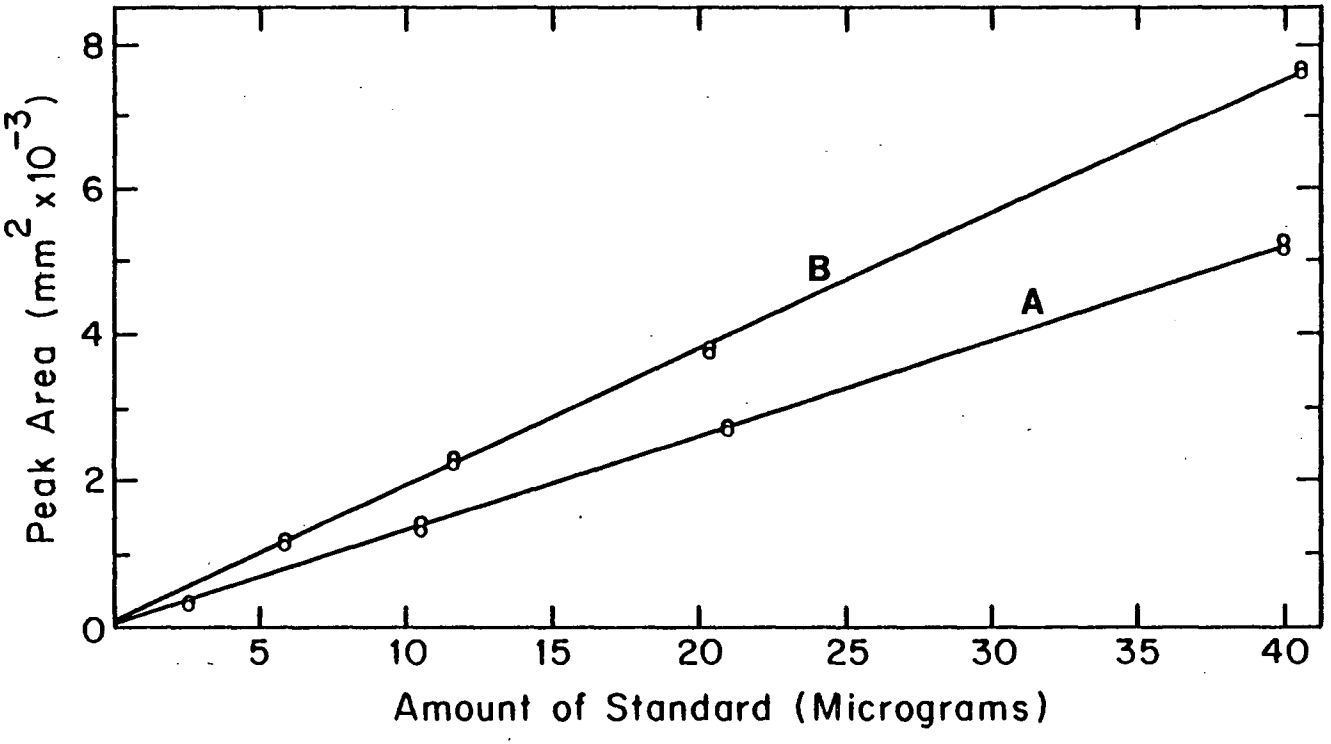
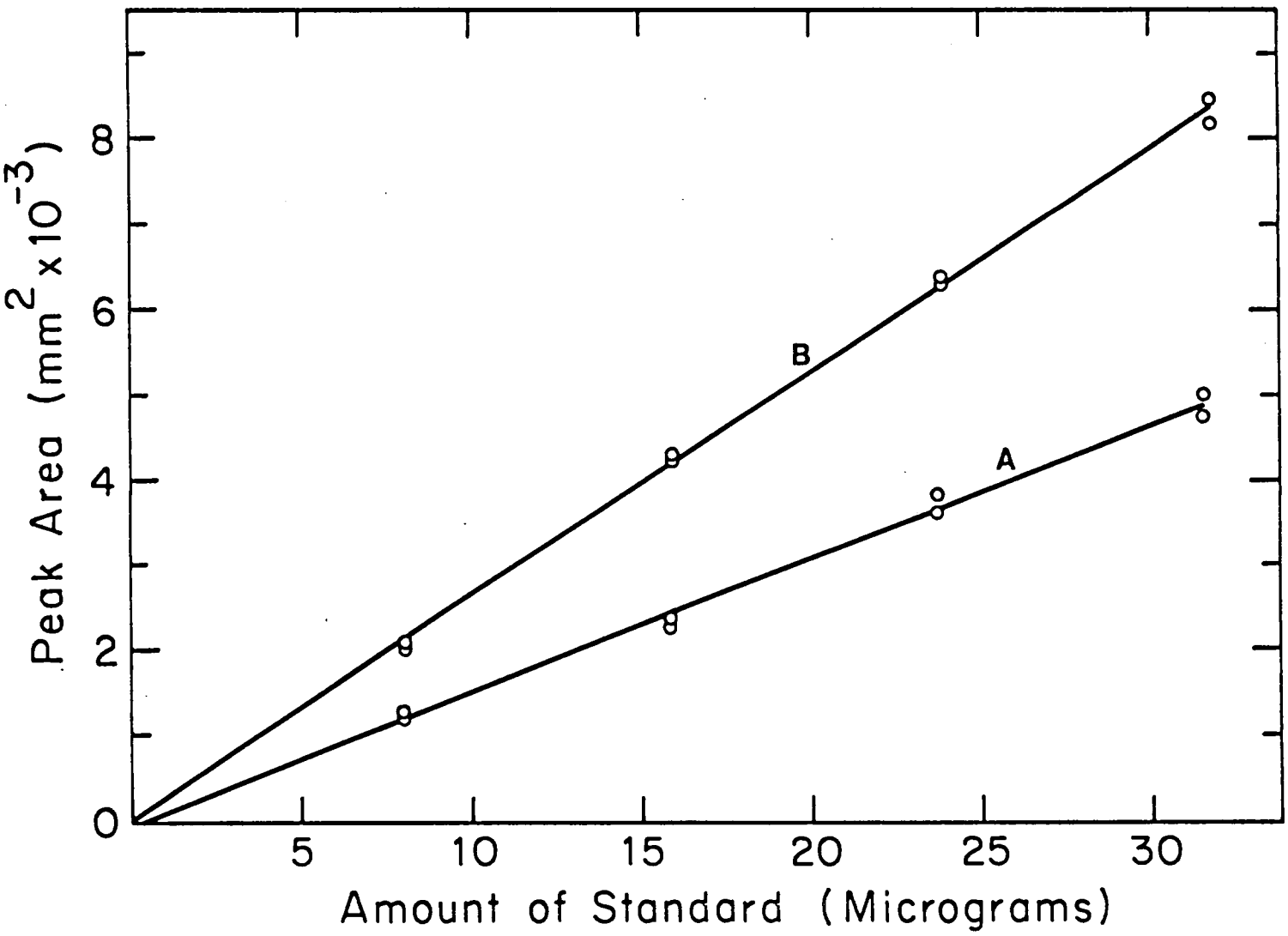

Peak Area ($mm^2 \times 10^{-3}$)
0
2
4
6
8
5
10
15
20
25
30
35
40
Amount of Standard (Micrograms)
B
A

Figure 8. Linearity curves of detector response vs. concentration for N-Nitroso-N-methyl-3-aminomethylindole (A) and N^1-Nitroso-N-nitroso-N-methyl-3-aminomethylindole (B)

Figure 8


Peak Area ($mm^2 \times 10^{-3}$)
0
2
4
6
8
5
10
15
20
25
30
Amount of Standard (Micrograms)
A
B

0.0012 mol) dissolved in 4 ml of 30% AcOH at pH 3.15. After 4 hr. at room temperature, the reaction was diluted to 100 ml with acetonitrile and 10 µl injections were made. Sample concentrations were determined on the basis of peak areas of external standards of the nitrosamines.

K. Isolation and Quantitative Determination of p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine As a Product of Hordenine Nitrosation

In order to determine NDMA and identifiable nonvolatile products of hordenine nitrosation at elevated temperature, the following experiments were carried out. A solution of 15% AcOH was raised to pH 4.5 by addition of anhydrous sodium acetate. This buffer was used to make 0.1 M Hordenine hemisulfate solution having a final pH of 4.46. Ten ml aliquots of this solution were placed in Kimax reaction tubes sealed with TEFLON-lined screw caps and pre-heated to 65±0.5°. Sodium nitrite (0.69 g) was added and each tube returned to the water bath for a period of 2 hr., 4 hr., 8 hr., or 12 hr. At the end of the specified time, tubes were cooled in ice, quenched, and extracted with CF as described in Part E. After the CF extract was diluted to 25 ml, 1 ml was removed to determine the NDMA concentration by GC-TEA. For HPLC analysis, 20 ml of the remaining CF extract were concentrated to near dryness under a stream of N_2. The remaining oil was made up to 1 ml with MeOH. The SP8700 instrument was set up with Column A using Mobile Phase 1 at a flow rate of 1.7 ml/min, with detector at 254 nm. Injection of the MeOH extract of the 12 hr. reaction product showed peaks at 14.2 min. and 16.2 min. which matched closely with the retention time seen previously for the syn and anti peaks

of p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine. Addition of a small amount of the pure nitrosamine to the MeOH extract showed that the pure nitrosamine co-eluted with the two unknown peaks having retention times of 14.2 and 16.2 min. To obtain verification by mass spectrometry, the following was done. Seven-10 µl injections of an 8 µg/µl solution of the pure nitrosamine were injected on the SP8700 using the conditions already described. The syn and anti conformer peaks were collected and combined. MeOH was removed under a stream of N_2, and the remaining aqueous solution was saturated with salt and extracted with two-1 ml portions of DCM. The DCM was concentrated to dryness to leave a yellow oil, the mass spectrum of which was determined by direct probe on the Finnigan 1015C. This material gave the mass spectrum expected for p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine. The 12 hr. reaction product was then injected on the SP8700 a total of 8 times, and the peaks corresponding to the nitrosamine were collected, combined, and extracted with DCM as described above. Removal of solvent left an amber oil which gave a mass spectrum that agreed well with the mass spectrum of the nitrosamine standard.

HPLC analysis showed that this nitrosamine was present in all four of the hordenine nitrosation samples. So the nitrosamine was quantitated as follows. The linearity of the detector was previously determined for the applicable concentration range. The peak areas of injected samples were measured using a chart speed of 2 cm/min. Concentrations were determined by reference to known external standards of p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine. The

recovery of the nitrosamine under the conditions used was determined by dissolving 2.6 mg of the standard nitrosamine in 10 ml of the blank buffer. To this was added 0.69 g $NaNO_2$; quenching, extraction, and concentration were carried out as previously described. The recovery of the nitrosamine after HPLC analysis was found to be 93.8%.

It was suspected that p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine might also be present in the reaction mixtures since two peaks eluting at retention times of 7 min. and 8.7 min. closely approximated the observed retention times of the syn and anti conformers of the pure nitrosamine. But addition of some of the pure nitrosamine to the 2 hr. reaction product showed that the pure nitrosamine did not co-elute with peaks at 7 min. and 8.7 min. Under the analytical conditions used, it was determined that p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine could not have been present at a level of more than 0.3% (based on starting amine) in any reaction product. At a level below 0.3%, the nitrosamine would not have been observed because of overlap from the peaks eluting at 7 min. and 8.7 min.

L. Investigation of Gramine Nitrosation Reaction by HPLC

In order to observe the formation of nonvolatile products of gramine nitrosation, the following experiments were performed. To 10 ml of 0.1 M solutions of gramine in AcOH-AcO^- buffer at pH 3.4 and 20° was added 0.35 g of $NaNO_2$. Reaction was allowed to continue for a specified time, then 10 ml of acetonitrile was added to the reaction tube and the contents shaken to insure complete solution. Ten µl volumes were immediately injected on the SP8700 using Column B with

Mobile Phase 1 at a flow rate of 2 ml/min. with detection at 254 nm. Reaction times chosen were 5 min., 10 min., 30 min., and 60 min. The results showed the progressive increase with time of peaks corresponding to NDMA as well as peaks with retention times of 18.4 min. and 19 min.; the latter two peaks and NDMA were the major reaction products after 60 min. Unreacted gramine would not elute from the column under these conditions. Spiking experiments showed that neither of the peaks eluting at 18.5 min. and 19 min. co-eluted with standards of indole-3-carboxaldehyde or indole-3-carbinol. A minor peak eluting at 16.5 min in the reaction products did co-elute with standard indole-3-carbinol. No peaks in the reaction products co-eluted with indole-3-carboxaldehyde. The aldehyde could have been detected easily if present at a level of 0.5% or greater in any reaction product.

In separate experiments under the same HPLC conditions as above or using Column A with Mobile Phase 2, it was observed that neither product eluting at 18.4 min. or 19 min. co-eluted with standard samples of N-nitroso-N-methyl-3-amino-methylindole or N^1-nitroso-N-nitroso-N-nitroso-N-methyl-3-aminomethylindole. Further spiking experiments showed that the two indolic nitrosamines were not present in the gramine nitrosation product after a 60 min. reaction time. Under the analytical conditions used, either nitrosamine would have been detected if present at a concentration representing as little as 1% of the reaction product (based on the starting amine).

A final experiment was performed to determine that gramine was stable in the AcOH-AcO^- buffer. A gramine solution of approximately 2 μg/μl was made up in the 15% AcOH-AcO^- buffer (pH 3.4). The

solution was immediately injected on the SP8700 using Column B and Mobile Phase 4 at a flow rate of 2 ml/min.; the peak height was measured. The solution was kept at room temperature; re-injection of the solution after 23 min. and after 15 hr. showed no change in the peak height.

IV. RESULTS

A. Formation of NDMA in Barley Malt Nitrosated Under Laboratory Conditions

PART 1: Nitrosation of Raw Barley, Freeze-Dried Clean Malt, and Freeze-Dried and Malt Roots

The purposes of this experiment were two-fold. The primary objective was to verify that NDMA precursors are generated in malted barley during the germination step. The second purpose was to determine if the potential precursors are localized in a particular fraction of the malt kernel. The experimental criterion adopted was the following: if nitrosation of a malt fraction in aqueous acid resulted in NDMA formation, then the fraction must contain NDMA precursor. To add validity to the experiment, the following precautions were taken. First, the raw barley was germinated in a way which prevented the physical transfer of amine precursors from one malt kernel to another, or from malt roots to malt husk. Second, no direct heat was used to dry the malt, a process which might also be responsible for precursor generation.

Figure 9 and Figure 10 show diagrammatically the yields of NDMA obtained after nitrosation of malt roots, clean malt, and raw barley. The results show that malt roots were an overwhelming source of NDMA precursor when compared to clean malt and raw barley. Pulverized clean malt and pulverized raw barley were also nitrosated in an attempt to compensate for possible differences in the surface areas of intact raw barley and intact clean malt. The differences in NDMA yields between raw barley and clean malt were not significant enough

Figure 9. Production of freeze-dried roots and freeze-dried clean malt; NDMA values (μg/kg) from nitrosation of freeze-dried roots and freeze-dried clean malt

Figure 9

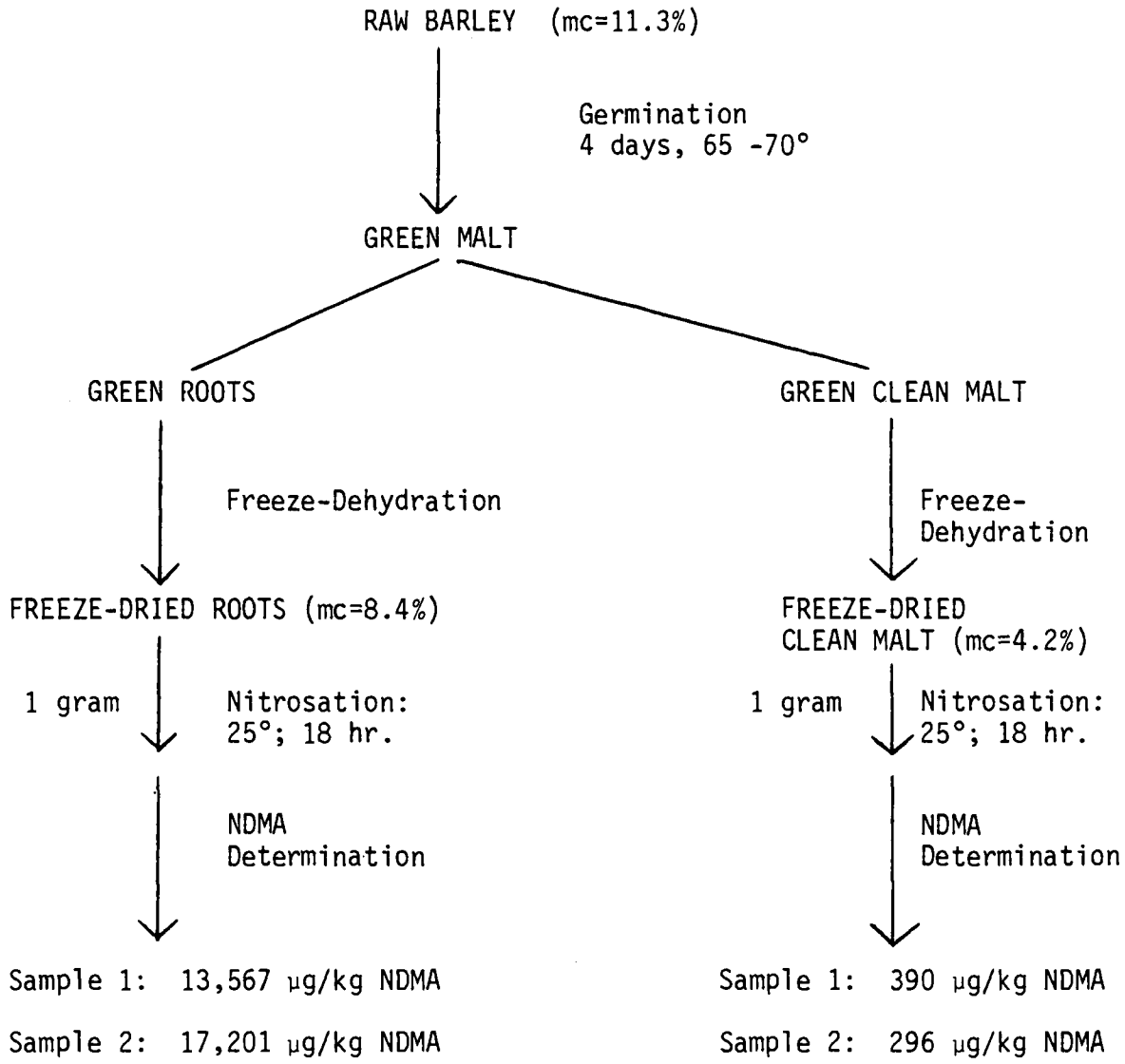
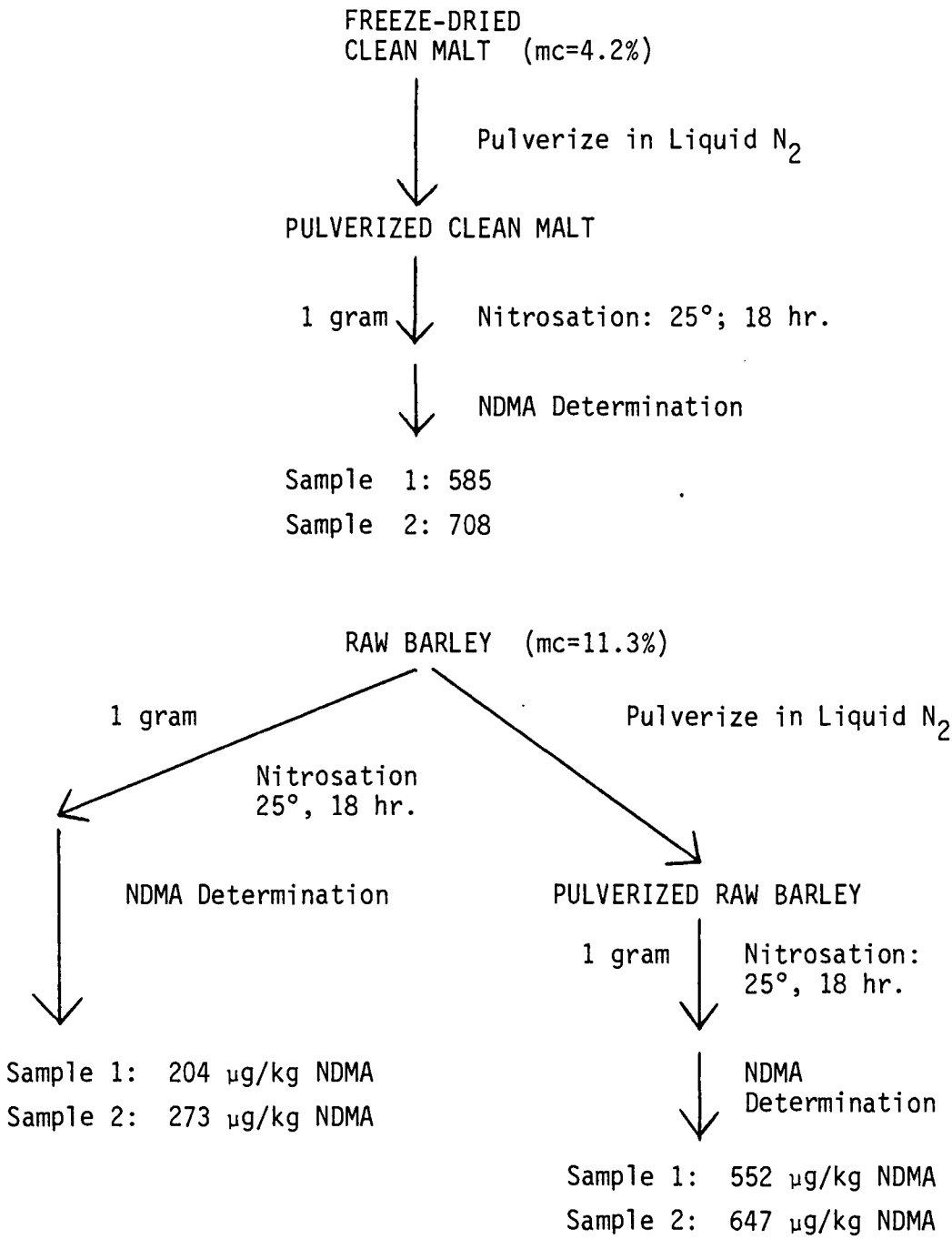

RAW BARLEY (mc=11.3%)
Germination
4 days, 65 -70°
GREEN MALT
GREEN ROOTS
GREEN CLEAN MALT
Freeze-Dehydration
Freeze-
Dehydration
FREEZE-DRIED ROOTS (mc=8.4%)
FREEZE-DRIED
CLEAN MALT (mc=4.2%)
1 gram
Nitrosation:
25°; 18 hr.
1 gram
Nitrosation:
25°; 18 hr.
NDMA
Determination
NDMA
Determination
Sample 1: 13,567 µg/kg NDMA
Sample 2: 17,201 µg/kg NDMA
Sample 1: 390 µg/kg NDMA
Sample 2: 296 µg/kg NDMA

Figure 10. Production of pulverized clean malt and pulverized raw barley; NDMA values (μg/kg) from nitrosation of raw barley, pulverized raw barley, and pulverized clean malt

Figure 10


FREEZE-DRIED
CLEAN MALT (mc=4.2%)
Pulverize in Liquid N_2
PULVERIZED CLEAN MALT
1 gram
Nitrosation: 25°; 18 hr.
NDMA Determination
Sample 1: 585
Sample 2: 708
RAW BARLEY (mc=11.3%)
1 gram
Nitrosation
25°, 18 hr.
Pulverize in Liquid N_2
NDMA Determination
PULVERIZED RAW BARLEY
1 gram
Nitrosation:
25°, 18 hr.
Sample 1: 204 µg/kg NDMA
Sample 2: 273 µg/kg NDMA
NDMA
Determination
Sample 1: 552 µg/kg NDMA
Sample 2: 647 µg/kg NDMA

to allow for the conclusion that germination causes precursor formation in the clean malt. When assessing the relative contribution of clean malt and malt roots to the total amount of NDMA precursor, it must be kept in mind that the malt roots represented only 6.2% of the dry weight of the malted barley, but the NDMA values reported in Figures 9 and 10 are based on equal weights of each fraction.

PART 2: Nitrosation of Clean Malt Dried by Heat or by Freeze-Drying

The samples for this experiment were two varieties of clean malt obtained by commercial germination. Before the nitrosation step, each sample was divided into a portion dried by electric-heat and a portion dried by freeze-dehydration. If heat-drying could generate immediate precursors of NDMA, it was assumed that, after nitrosation, the NDMA yield from heat-dried malt would be larger than the NDMA yield from freeze-dried malt. As the results in Table 4 indicate, for both malt varieties, there was no significant difference in NDMA yield between the two methods of drying. These results indicate that heat did not play an important role in NDMA precursor formation. One of the malt varieties actually showed a higher mean NDMA yield from the freeze-dried sample. This could be the result of volatilization or decomposition of NDMA precursor in the heat-dried sample. The possibility of dimethylamine formation during kilning from tertiary amines such as hordenine and gramine has been suggested by Smith (1981), but there is no experimental evidence available to support the suggestion.

TABLE 4. NDMA Values (μg/kg) From Nitrosation of Freeze-Dried Clean Malt[a] and Heat-Dried Clean Malt[a]

Variety	Freeze-Dried	Direct-Dried
Klages	996 ± 220[b]	719 ± 25[b]
Laker	847 ± 201[b]	892 ± 187[b]

[a]Conditions: 25 g nitrosated for 18 hr. in 0.26 M $NaNO_2$ (pH 3.2)
[b]N=3 repetitions

B. Nitrosation of Potential NDMA Precursors Under Simulated Kilning Conditions

A group of four amines, each of which is indigenous to malt, have been proposed as possible precursors of NDMA in direct-fired malt. A set of initial conditions was chosen to determine the relative susceptibility of each amine to yield NDMA after nitrosation. The conditions chosen were pH 4.4 and pH 6.4, a temperature of 65°, and a time of 16 hr. These conditions represent the extremes of pH and intermediates of temperature and time which are encountered during kilning. Reactions were run using 0.1 M concentrations of the amines under conditions such that nitrous anhydride (N_2O_3) would be the nitrosating agent. The yields of NDMA obtained from each amine are shown in Table 5. The results showed that dimethylamine was readily nitrosated as expected, and hordenine and gramine were also nitrosated to give NDMA at pH 4.4. Gramine was found to be extremely susceptible to nitrosation at pH 4.4, giving a yield of NDMA an order of magnitude larger than the yield of NDMA obtained from trimethylamine. Gramine

was nitrosated as readily as dimethylamine at pH 4.4. In separate experiments, nitrosation of gramine at pH 3.4 in a three-fold excess of nitrite at room temperature for 6 hr. resulted in a 61% yield of NDMA; nitrosation at pH 3.4 in a threefold excess of nitrite at room temperature for 10 min. resulted in a 24% yield of NDMA.

TABLE 5. 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° for 16 hr.
[b]Acetate buffer
[c]Citrate-phosphate buffer

These results for gramine are not in agreement with the reactivity normally observed for tertiary amines containing an N,N-dimethylamino group. For example, Lijinsky et al. (1972b) found that nitrosation of N,N-dimethylcyclohexylamine, N,N-dimethyldodecylamine, and 2-dimethylamino-1,2,3,4-tetrahydronaphthalene at 90° for 16 hr. in aqueous acid (pH 4) gave NDMA yields of 5.9%, 4.5%, and 2.5%, respectively. The predominant N-nitrosamine obtained from each tertiary amine was the one corresponding to preferential loss of a methyl group rather than the more highly substituted alkyl group. For example, nitrosation of N,N-dimethyl-dodecylamine yielded 21% of nitrosomethyldodecylamine and 4.5% of NDMA. In a second study of tertiary amine

nitrosation at lower temperature, Lijinsky and Singer (1974) found that nitrosation of trimethylamine at 37° for 4 hr. yielded only 0.8% NDMA using a four-fold excess of nitrite at pH 4.

In response to these observations, a more extensive study of gramine nitrosation was undertaken and is described in Section D.

C. Confirmation of NDMA Formation From Hordenine and Gramine by Combined Gas Chromatography-Mass Spectrometry (GC-MS)

The initial evidence that nitrosation of hordenine and gramine resulted in the formation of NDMA was based on two criteria: (1) An extract of both reaction products showed a positive response when introduced into a Thermal Energy Analyzer (a detector which is highly sensitive and very specific for N-nitroso compounds), and (2) The peak showing a positive TEA response co-eluted with the peak of the NDMA standard.

To obtain confirmatory evidence that NDMA is a product of nitrosation of hordenine and gramine, the nitrosation of both compounds was carried out under the conditions outlined previously. The GC-MS analysis of the products of both reactions indicated that NDMA was present. Figure 11 shows the mass spectrum obtained from the GC-MS of a standard solution of NDMA in hexane. Figure 12 shows a spectrum obtained from the GC-MS of the hordenine nitrosation product; Figure 13 shows a spectrum obtained from the GC-MS of the gramine nitrosation product. These mass spectra are taken as confirmatory evidence of NDMA since, (1) the observed retention times of the products illustrated in Figure 12 and Figure 13 were the same as that of

Figure 11. Mass spectrum from the GC-MS of a standard solution of NDMA in hexane

Figure 11

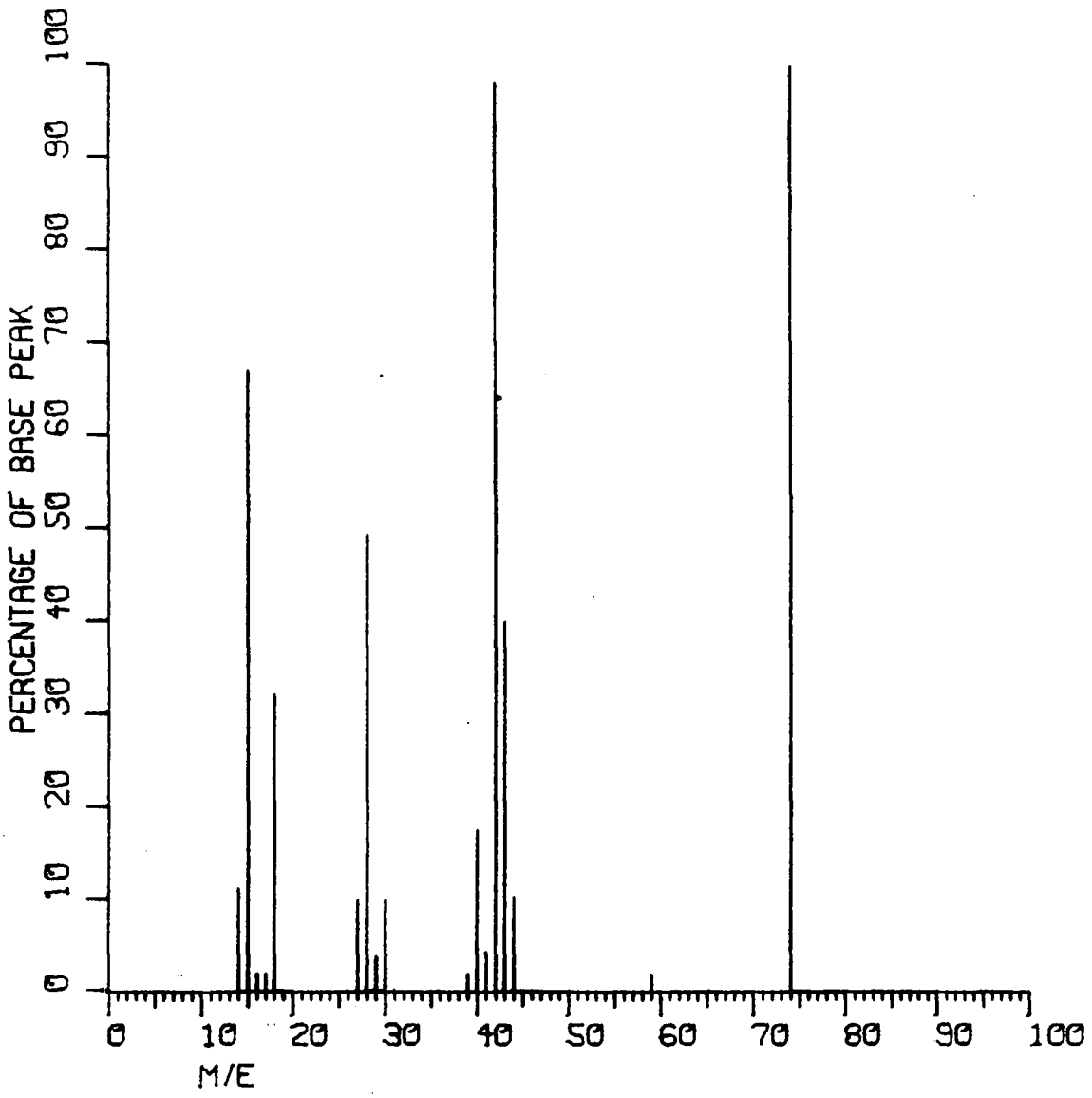

PERCENTAGE OF BASE PEAK
0
10
20
30
40
50
60
70
80
90
100
M/E
0
10
20
30
40
50
60
70
80
90
100

Figure 12. A mass spectrum of NDMA obtained from the GC-MS of a hordenine nitrosation reaction product

Figure 12

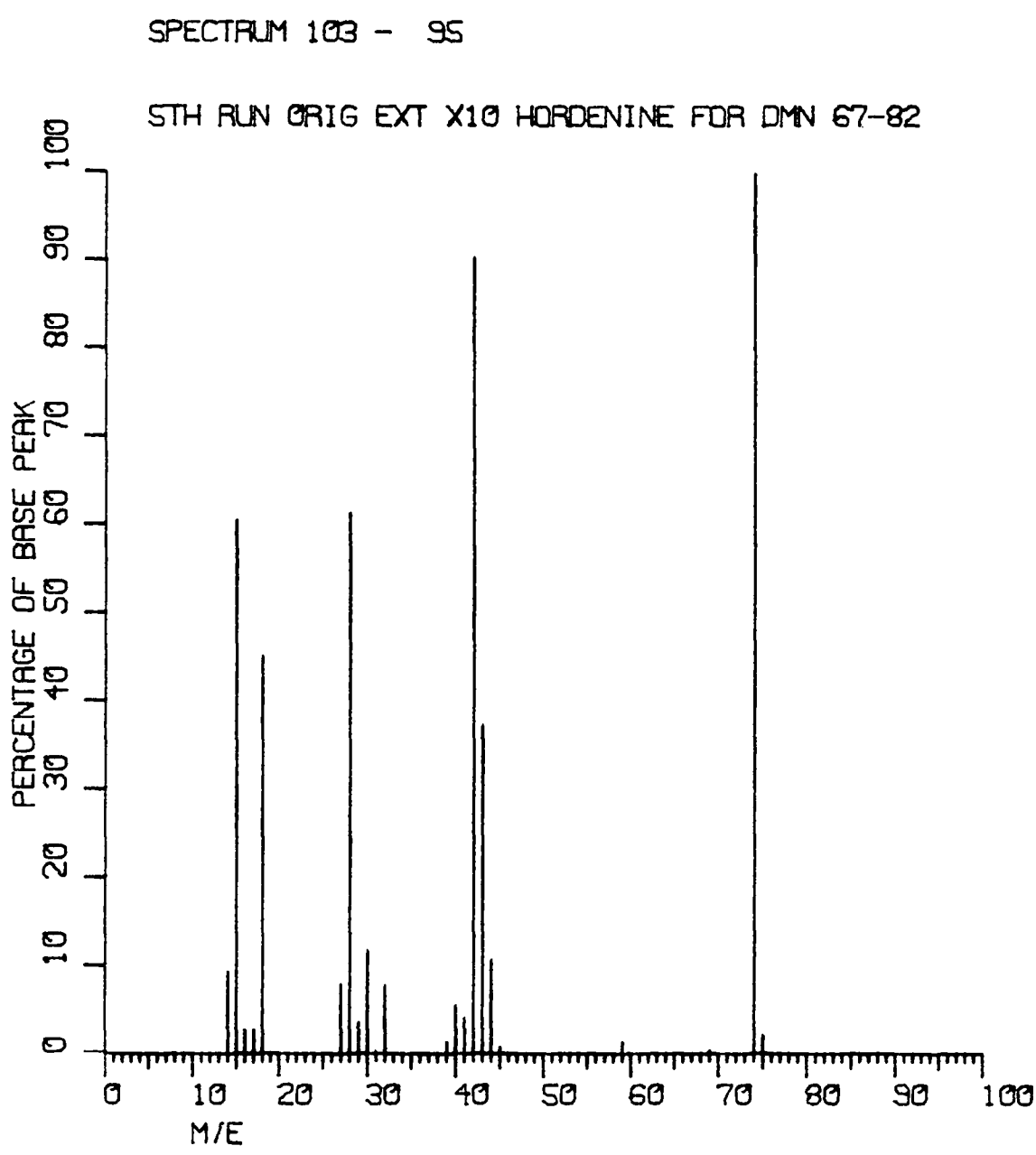
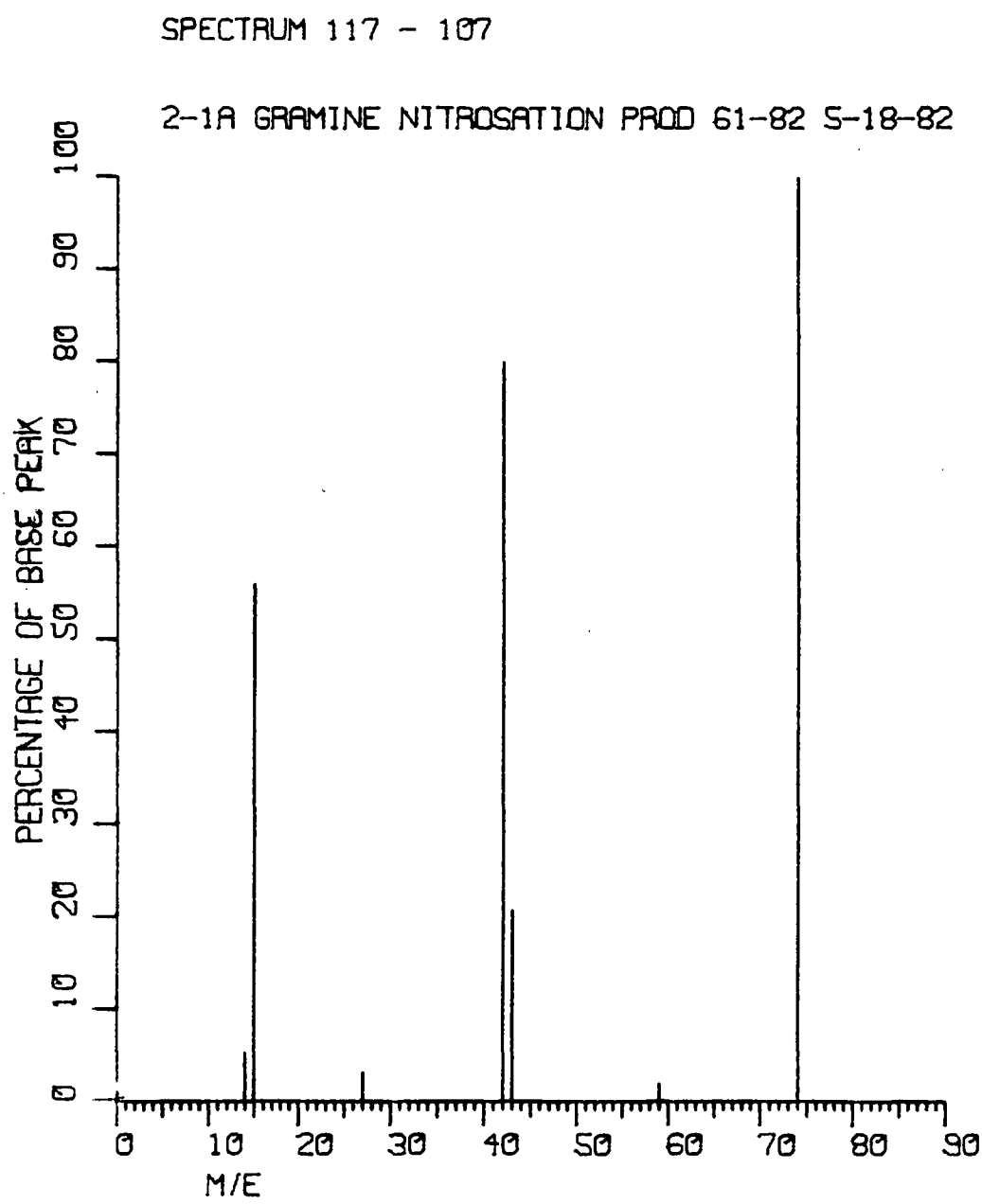

SPECTRUM 103 - 95
STH RUN ORIG EXT X10 HORDENINE FOR DMN 67-82
PERCENTAGE OF BASE PEAK
0
10
20
30
40
50
60
70
80
90
100
M/E

Figure 13. A mass spectrum of NDMA obtained from the GC-MS of a gramine nitrosation reaction product

Figure 13


SPECTRUM 117 - 107
2-1A GRAMINE NITROSATION PROD 61-82 5-18-82
PERCENTAGE OF BASE PEAK
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70
80
90
M/E

the NDMA standard; (2) the ion corresponding to m/e 74 (M^+) was maximized at the observed retention time; and (3) the spectra obtained displayed the same molecular ion and principal fragment ions observed for the NDMA standard.

D. Comparison of the Formation of NDMA from Gramine and Selected Secondary and Tertiary Amines As a Function of Time and Temperature

As a result of the preliminary nitrosation experiments outlined in Section B, it was concluded that production of NDMA from gramine was an unexpectedly facile reaction. In order to firmly establish the validity of this conclusion and to obtain initial evidence for a mechanistic explanation of the reactivity, a group of "comparative nitrosation" experiments was carried out. There were four experimental objectives in mind:

(1) To compare at ambient temperature and short time the nitrosation of gramine with dimethylamine, the most reactive secondary amine which is likely to be a precursor to NDMA in malted barley.

(2) To compare at ambient temperature and short time the nitrosation of gramine with aminopyrine, the most reactive known tertiary amine which yields NDMA; to compare the nitrosation of gramine with trimethylamine, from which NDMA is the only nitrosamine obtainable.

(3) To establish the magnitude of the difference in reactivity between gramine and hordenine.

(4) To determine if the indole-3-carbinyl group of gramine is essential to the observed reactivity.

The structures of the amines used are shown in Figure 14. In order to fulfill the last goal listed, it was desired to compare the nitrosation of gramine with N,N-dimethyltryptamine. Since N,N-dimethyltryptamine is a controlled drug, the comparison was made between the nitrosations of 5-methoxygramine and N,N-dimethyl-5-methoxytryptamine.

The reaction conditions were similar to those employed in Section B except that a ten-fold excess of nitrite was used with dilute acetic acid as the reaction medium. The increase in nitrite: amine ratio was employed to insure that measureable yields of NDMA would be obtained from nitrosation of each tertiary amine. A pH of 3.4 was used for all reactions since 3.4 was determined to be the optimum pH for nitrosation of dimethylamine (Mirvish, 1970).

Figure 15 shows the comparison in NDMA yields from aminopyrine, gramine, dimethylamine, and trimethylamine at 24°. The initial reaction of aminopyrine with nitrite was so fast that accurate yield data could not be determined at times less than 2.5 min. Mirvish et al. (1974) conducted the reaction of aminopyrine with nitrite at 0° in order to determine the initial rate. Under the conditions used, no difference in the initial rate of gramine and dimethylamine nitrosation could be distinguished, but the yield curves diverged after 5 min. Nitrosation of dimethylamine went to completion within 70 min. The nitrosation of trimethylamine was only 0.19% complete in 2 hr.

Figure 16 shows the comparison in NDMA yields from gramine, 5-methoxygramine, N,N-dimethyl-5-methoxytryptamine, and hordenine at

23°. The two amines possessing an N-substituted indole-3-carbinyl group (gramine and 5-methoxygramine) are clearly different kinectly from hordenine and N,N-dimethyl-5-methoxytryptamine. Figure 17 shows the same comparison at 37°. The same pairs of kinetic curves are seen as were seen in Figure 16.

Figure 18 shows that the nitrosation of gramine reached completion in 10 min. at 65° while hordenine was still reacting under initial rate conditions after 30 min.

These data clearly establish the difference in reactivity between gramine and other N,N-dimethyl-substituted tertiary amines. Gramine is nitrosated as readily to give NDMA as is dimethylamine in which alkyl group cleavage is not required before nitrosamine formation can take place. The N-substituted indole-3-carbinyl group of gramine is an essential requirement for the observed reactivity, since elongation of the indole-3-carbinyl group by an additional methylene group caused complete loss of the enhanced reactivity (5-methoxygramine vs. N,N-dimethyl-5-methoxytryptamine).

The data in Figures 16, 17, and 18 for the nitrosation of gramine and hordenine were used to construct Arrhenius plots for the temperature range 23-65°. For gramine, the initial rates of reaction were approximated by taking tangents to the yield curves at 23°, 37°, and 65°. For hordenine, the initial rate data could be obtained directly from the yield curves at 23°, 37°, and 65°. The Arrhenius plots for the production of NDMA from gramine and hordenine at pH 3.4 are shown in Figure 19. From these plots, the activation energies for the nitrosation to obtain NDMA from gramine and hordenine were calculated

Figure 14. Structures of the amines used in the comparative nitrosation reaction study

A: Aminopyrine

B: Gramine

C: Hordenine

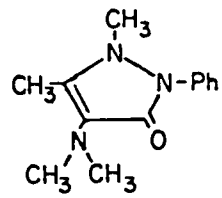
D: 5-Methoxygramine

E: N,N-Dimethyl-5-methoxytryptamine

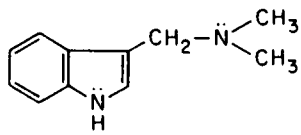
F: Dimethylamine

G: Trimethylamine

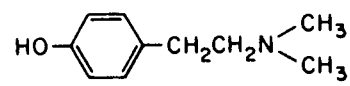
Figure 14



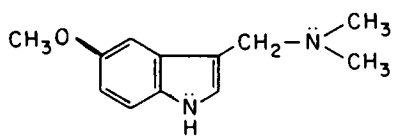
A



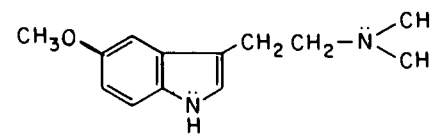
B



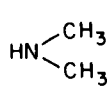
C



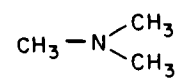
D



E



F



G

Figure 15. NDMA yields from nitrosation of aminopyrine, dimethylamine, gramine, and trimethylamine at 24°±1°

Curve A: NDMA from aminopyrine

Curve B: NDMA from dimethylamine

Curve C: NDMA from gramine

Curve D: NDMA from trimethylamine

Figure 15

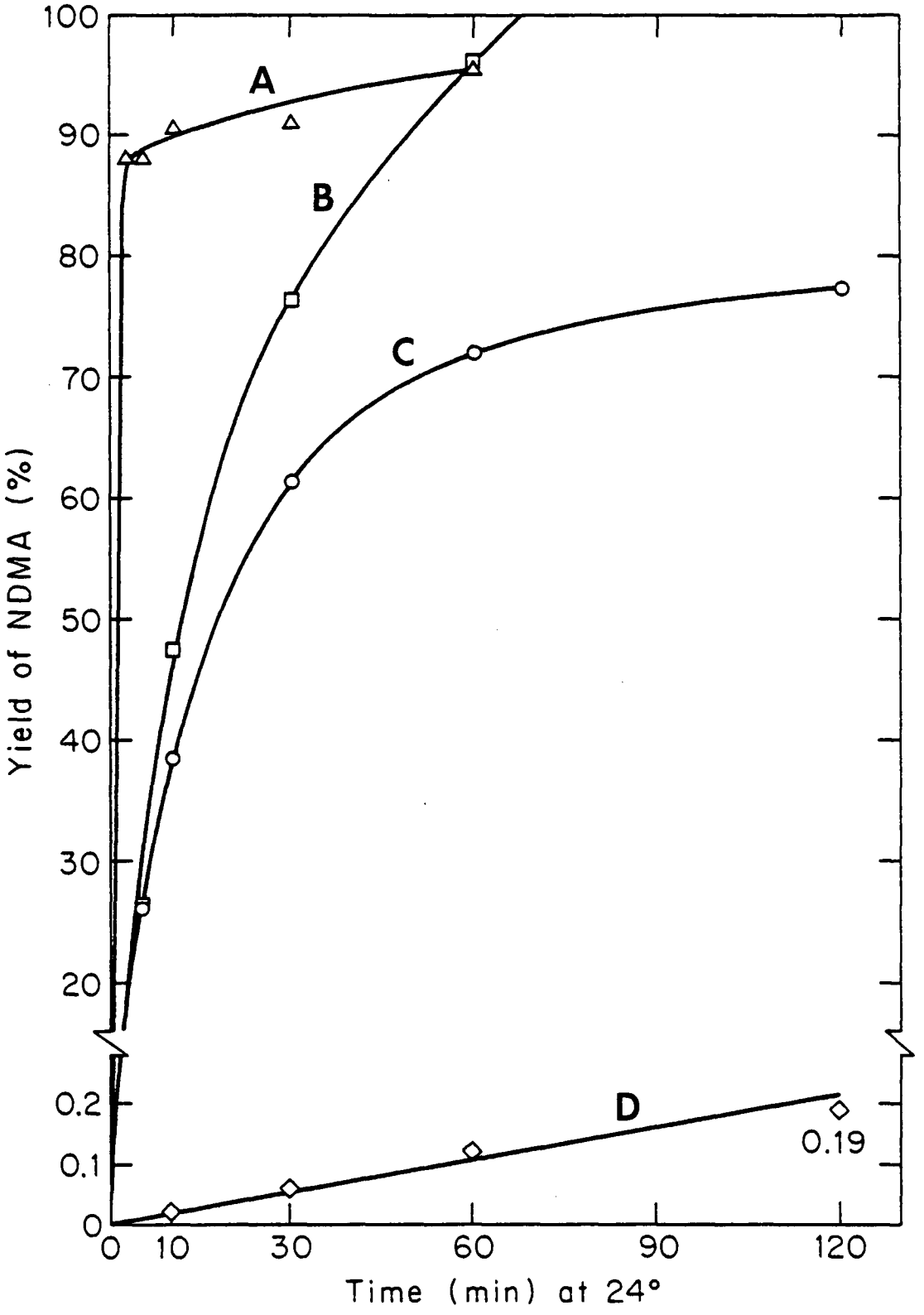

100
90
80
70
60
50
40
30
20
0.2
0.1
0
A
B
C
D
0.19
Yield of NDMA (%)
0
10
30
60
90
120
Time (min) at 24°

Figure 16. NDMA yields from nitrosation of gramine, 5-methoxygramine, hordenine, and N,N-dimethyl-5-methoxytryptamine at 23°±1°

Curve A: NDMA from gramine

Curve B: NDMA from 5-methoxygramine

Curve C: NDMA from hordenine

Curve D: NDMA from N,N-Dimethyl-5-Methoxytryptamine

Figure 16

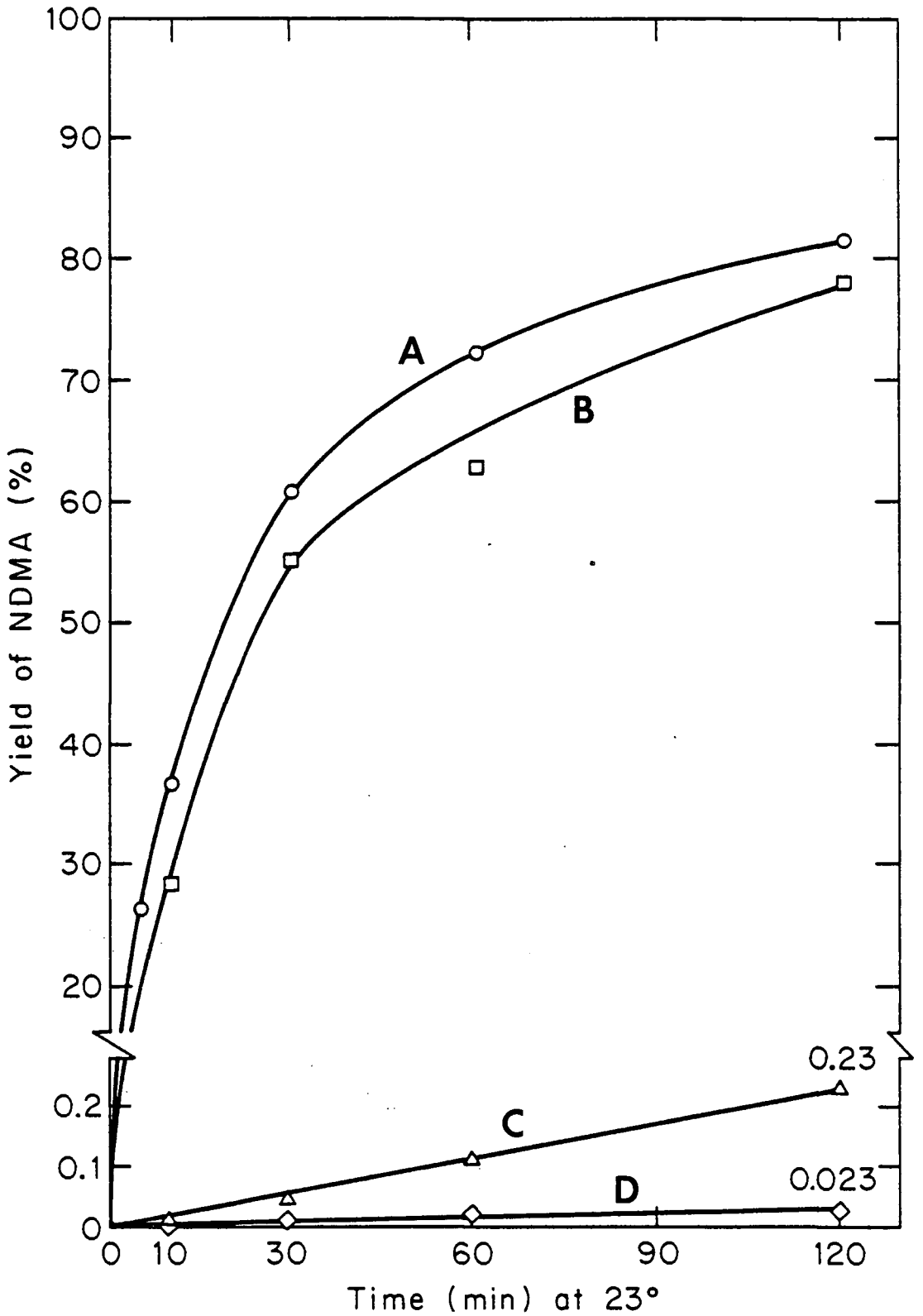

100
90
80
70
60
50
40
30
20
0.2
0.1
0
Yield of NDMA (%)
A
B
C
D
0.23
0.023
0
10
30
60
90
120
Time (min) at 23°

Figure 17. NDMA yields from nitrosation of gramine, 5-methoxygramine, hordenine, and N,N-dimethyl-5-methoxytryptamine at 37°±0.5°

Curve A: NDMA from gramine

Curve B: NDMA from 5-methoxygramine

Curve C: NDMA from hordenine

Curve D: NDMA from N,N-dimethyl-5-methoxytryptamine

Figure 17

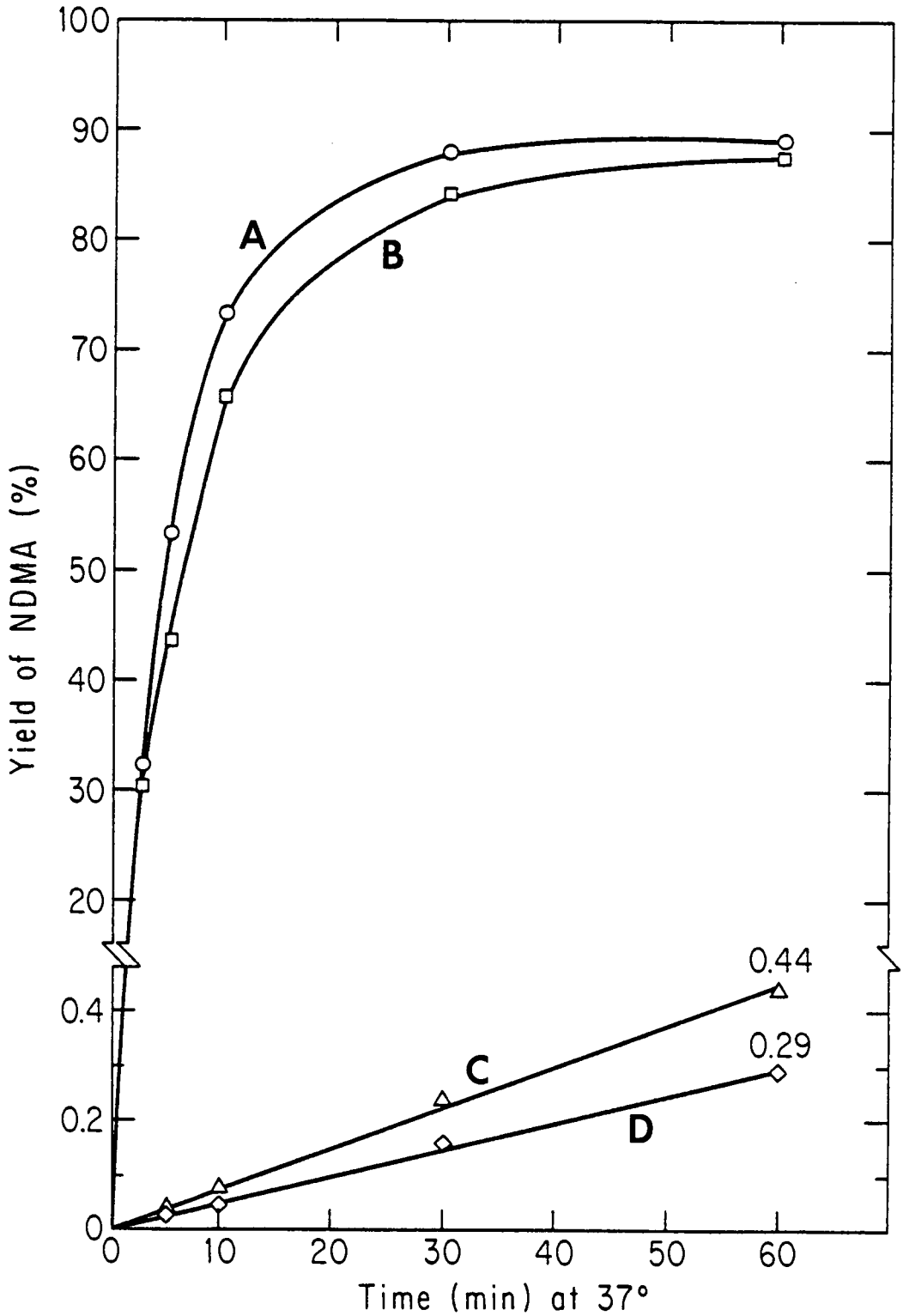

100
90
80
70
60
50
40
30
20
0.4
0.2
0
Yield of NDMA (%)
A
B
C
D
0.44
0.29
0
10
20
30
40
50
60
Time (min) at 37°

Figure 18. NDMA yields from nitrosation of gramine and hordenine at 65°±0.5°

Curve A: NDMA from gramine

Curve B: NDMA from hordenine

Figure 18

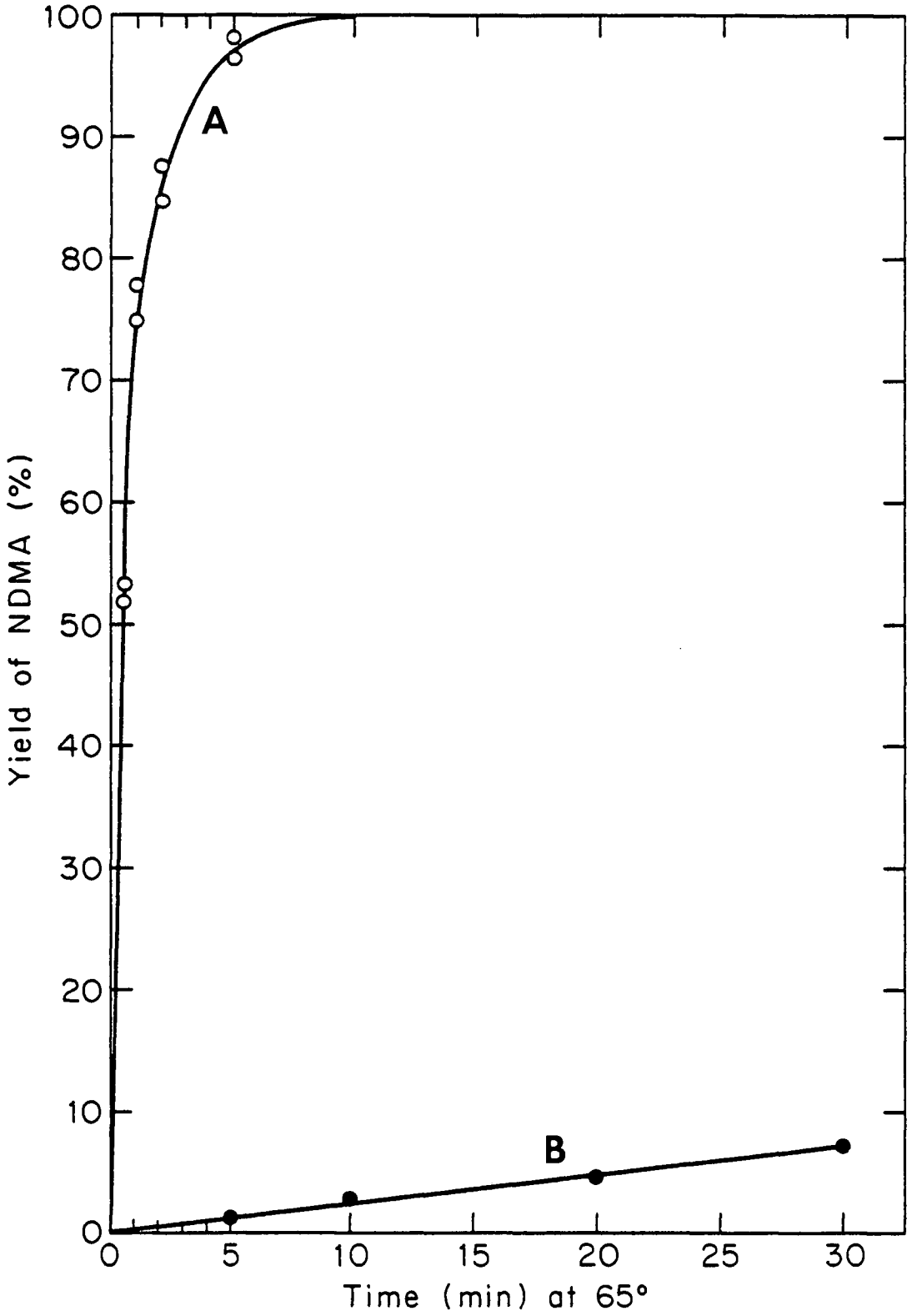
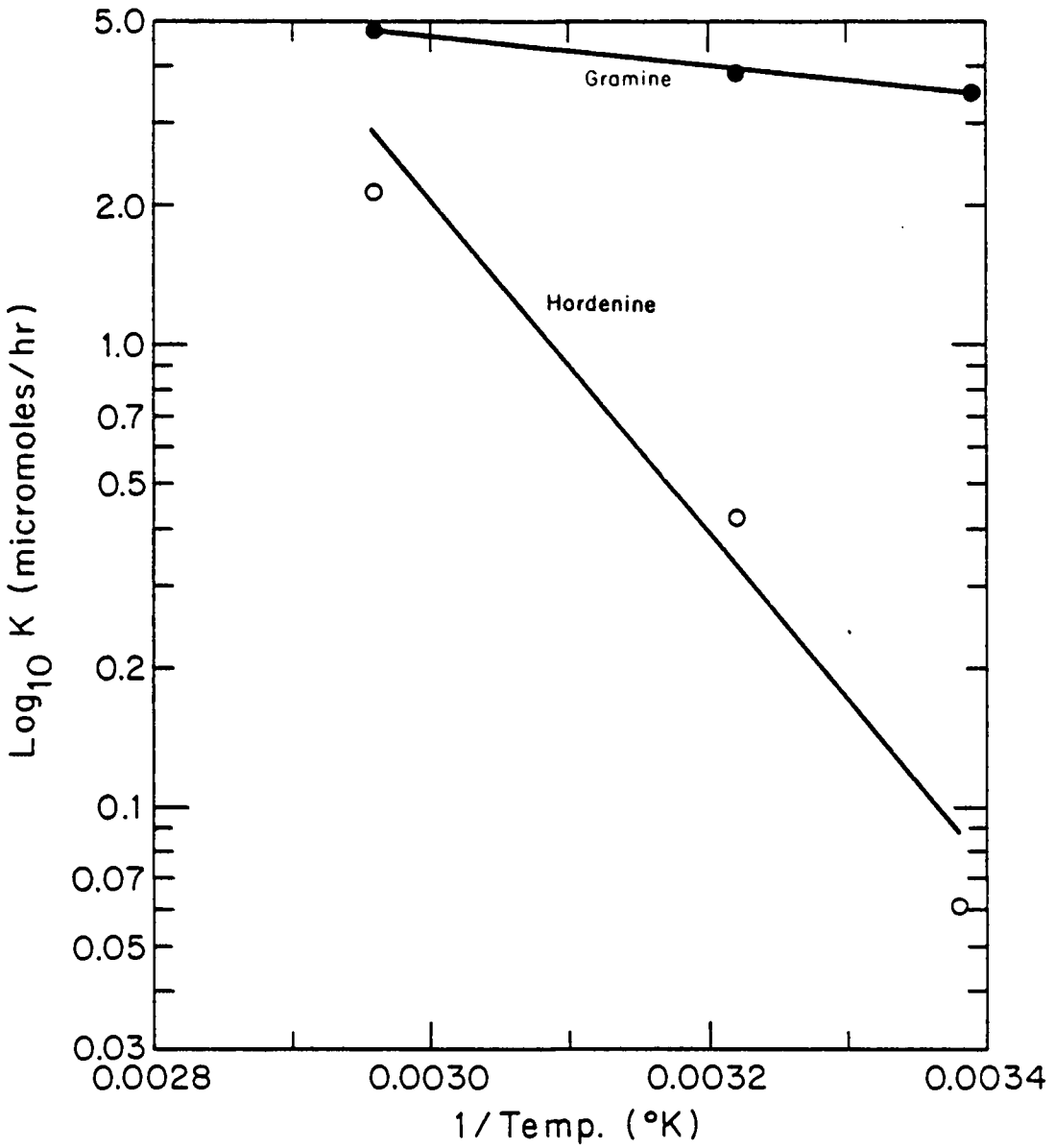

100
90
80
70
60
50
40
30
20
10
0
Yield of NDMA (%)
A
B
0
5
10
15
20
25
30
Time (min) at 65°

Figure 19. Arrhenius plots for the formation of NDMA from the nitrosation of gramine and hordenine at pH 3.4 over the temperature range 23°-65°

Figure 19


5.0
2.0
1.0
0.7
0.5
0.2
0.1
0.07
0.05
0.03
Log10 K (micromoles/hr)
Gramine
Hordenine
0.0028
0.0030
0.0032
0.0034
1/Temp. (°K)

based on the slope of each regression line. For gramine, the value of E_a was 14.2 Kcal/mol; for hordenine, the value of E_a was 23.6 Kcal/mol.

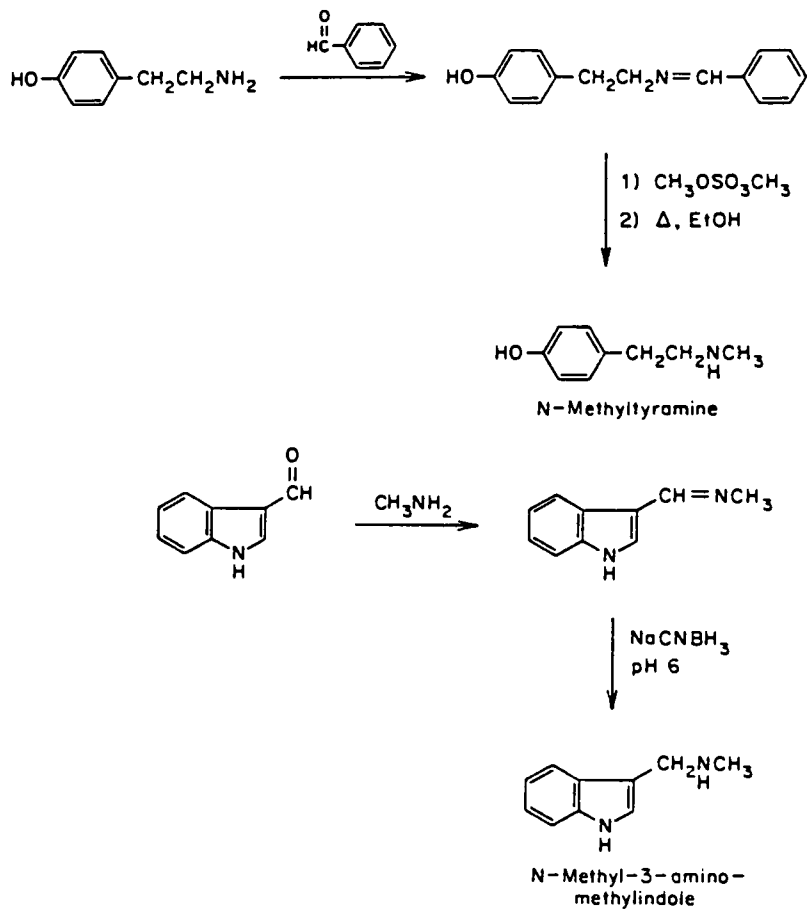
E. Synthesis of N-Methyltyramine and N-Methyl-3-aminomethylindole

PART 1: Synthesis of N-Methyltyramine

N-Methyltyramine was originally synthesized by Kirkwood and Marion (1950) starting from condensation of p-methoxy-2-phenylethylamine with benzaldehyde. The resulting imine was not isolated, but was immediately methylated followed by mild hydrolysis to give p-methoxy-N-methyl-2-phenylethylamine. Strong hydrolysis in HBr gave N-methyltyramine in 57% overall yield. Since the starting compound used by Kirkwood and Marion could only be obtained from the four step synthesis of Kindler and Peschke (1932), the synthesis of N-methyltyramine from commercially available tyramine was proposed in this study. Condensation of tyramine with benzaldehyde gave a crystalline material which could be isolated and characterized as p-hydroxy-2-phenylethylbenzaldimine on the basis of accurate mass measurement of its parent ion and its fragmentation pattern. Isolation of the imine was required to insure the success of the following steps. The imine was methylated by dimethylsulfate followed by mild hydrolysis and work-up which gave the free amine in 40% overall yield (Figure 20). Chromatography on alumina gave N-methyltyramine containing no tyramine impurity. The amine was characterized on the basis of melting point, accurate mass measurement of the parent ion,

Figure 20. The synthesis of N-Methyltyramine and N-Methyl-3-aminomethylindole

Figure 20


HO
CH2CH2NH2
HC
O
HO
CH2CH2N=CH
1) CH3OSO3CH3
2) Δ, EtOH
HO
CH2CH2NCH3
H
N-Methyltyramine
O
CH
N
H
CH3NH2
CH=NCH3
N
H
NaCNBH3
pH 6
CH2NCH3
H
N
H
N-Methyl-3-amino-
methylindole

fragmentation pattern, and proton NMR specturm. The mass spectrum showed a parent ion at m/e 151 and important fragment ions at m/e 107 and m/e 44 which were consistent with the expected ring-assisted α-cleavage fragmentation mechanism (McLafferty, 1973).

PART 2: Synthesis of N-Methyl-3-aminomethylindole

N-methyl-3-aminomethylindole was originally synthesized by Gower and Leete (1963) by condensation of indole-3-carboxaldehyde with methylamine. The isolated imine was reduced with sodium borohydride to give a colorless oil which was characterized as the title compound on the basis of elemental analysis. The same synthesis was used later by Alemany et al. (1975) and the amine was isolated as its hydrobromide.

The work of Borch et al. (1971) indicated that reductive amination was a useful method for making a secondary amine by condensation of an aldehyde and a primary amine without isolation of the intermediate imine. For this study, the title compound was synthesized using indole-3-carboxaldehyde, methylamine, and cyanoborohydride anion (Figure 20) in a modification of the general method of Borch et al. (1971). The two modifications: were (1) only anhydrous acids and bases were used to make pH adjustments and (2) the reaction mixture was warmed to reduce the reaction time.

The product was obtained as a free amine which was isolated as a crystalline solid with sharp melting point. The product was characterized on the basis of its mass spectrum, accurate mass measurement of the parent ion, and proton and C-13 NMR spectra. The mass

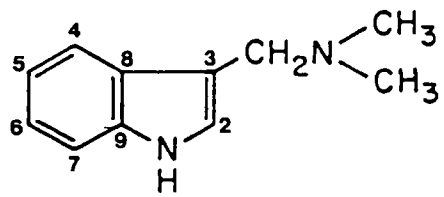
spectrum showed a parent ion of m/e 160 and a base peak at m/e 130 characteristic of the indole-3-carbinyl cation (Budzikiewicz et al., 1964). The C-13 chemical shift assignments (Table 6) were based on the data of Wenkert et al. (1974) for gramine, and the assignments actually made by obtaining the C-13 NMR spectrum of gramine (Table 6). The proton NMR showed a 3 proton singlet at 2.46δ($N-CH_3$) and a 2 proton singlet at 3.89δ($N-CH_2-$).

F. Nitrosation of N-Methyltyramine

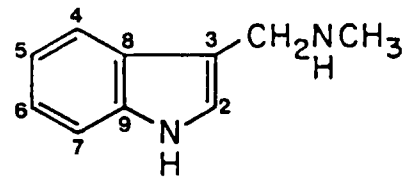
Nitrosation of N-methyltyramine was carried out for two reasons: (1) to characterize the products formed, since N-methyltyramine is a secondary metabolite of malted barley, and (2) the N-nitrosamines derived from N-methyltyramine would be products expected from the nitrosative dealkylation of hordenine.

Examination of the product of N-methyltyramine nitrosation showed the formation of two N-nitrosamines. The major product was characterized as p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine (I) (Figure 21). The mass spectrum showed m/e 180 (M^+) and m/e 150 (M-30), and accurate measurement of the parent in agreed with the formula $C_9H_{12}N_2O_2$. The proton NMR spectrum showed the characteristic resonances expected for syn (δ2.95) and anti (δ3.58) methyl groups and syn (δ3.71) and anti (δ4.31) methylene groups in the α-positions adjacent to an N-nitroso function (Brown and Hollis, 1964). The C-13 NMR shift assignments are shown in Table 7. Assignments were made on the basis of the values given by Levy et al. (1980) for substituted benzenes, and the values given by Pregosin and Randall (1971) for

TABLE 6. ^{13}C-NMR Chemical Shift Data[a] For N-Methyl-3-aminomethyl-indole and Gramine



Gramine



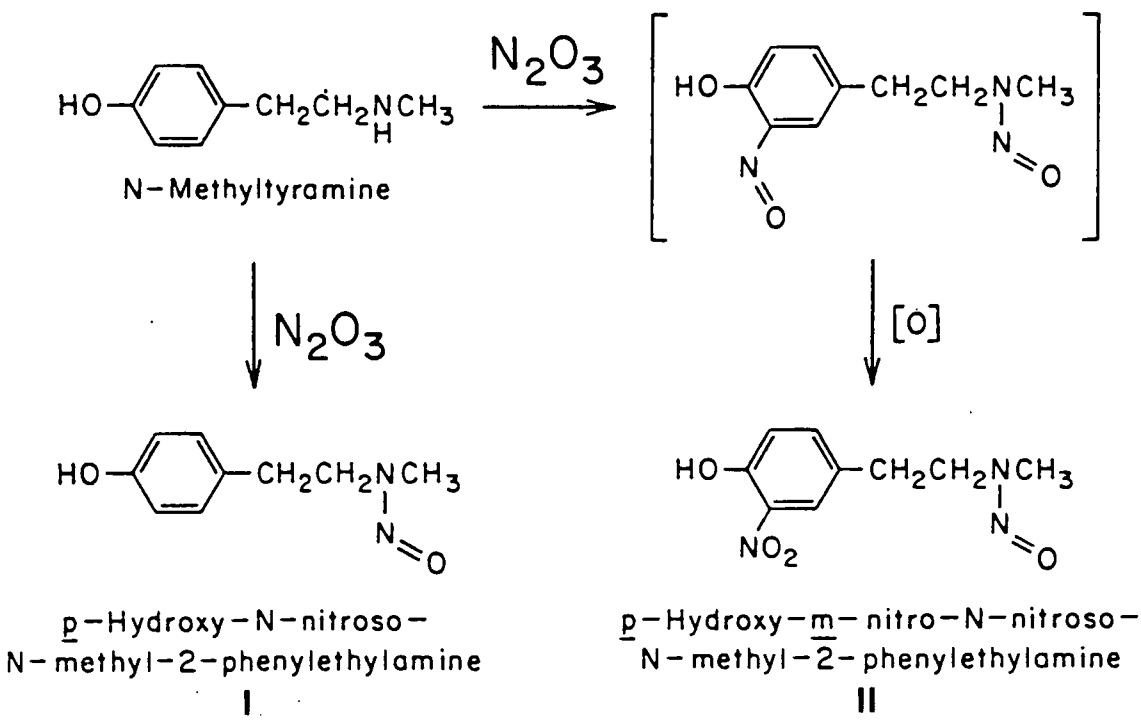
N-Methyl-3-aminomethylindole (NMAMI)

Position	Gramine	NMAMI
α -CH_3	45.1	36.0
α -CH_2	54.3	46.6
C-2	124.0	122.8
C-3	112.5	114.2
C-4	119.0	118.5
C-5	121.7	121.8
C-6	119.3	119.2
C-7	111.1	111.2
C-8	127.8	126.9
C-9	136.2	136.3

[a]Solvent and internal standard: deuteriochloroform; chemical shifts, δ, in ppm downfield from tetramethylsilane.

Figure 21. Nitrosation of N-Methyltyramine

Figure 21



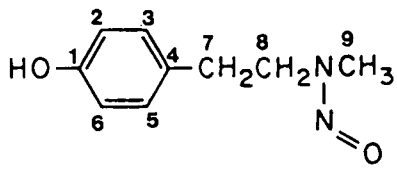
p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine

I

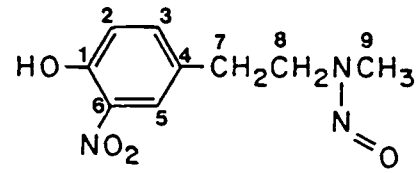
p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine

II

TABLE 7. ^{13}C-NMR Chemical Shift Data[a] For p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine (I) and p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine (II)



p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine



p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine

	I		II	
Position	Compound I		Compound II	
1	155.0		154.1	
2	116.0		120.6	
3	129.8		137.9	
4	128.5		129.5	
5	129.8		124.6	
6	116.0		137.8	
	SYN	ANTI	SYN	ANTI
7	30.9	32.3	30.5	31.8
8	47.6	55.4	46.2	54.3
9	34.1	39.9	33.6	39.5

[a]Solvent and internal standard: deuteriochloroform; chemical shifts, δ, in ppm downfield from tetramethylsilane.

the chemical shifts of carbon atoms in the positions α- and β- to an N-nitroso group.

The minor product obtained by nitrosation of N-methyltyramine was characterized as p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine (II) (Figure 21). The mass spectrum showed m/e 225 (M^+) and m/e 195 (M-30), and m/e 179 (M-60). Accurate measurement of the parent ion agreed with the formula $C_9H_{11}N_3O_4$. The proton NMR spectrum showed the resonances for syn (δ3.00) and anti (δ3.65) methyl and syn (δ3.75) and anti (δ4.35) methylene groups in the α-positions adjacent to an N-nitroso function. A singlet at 10.43δ was consistent with a phenolic proton shifted downfield by hydrogen bonding with an electron withdrawing substituent in the o-position. The C-13 NMR chemical shift assignments for II are shown in Table 7. The resonance at 137.8 ppm was assigned to the carbon bearing an electron withdrawing group at the position ortho to the phenolic hydroxyl group.

The formation of a nitrophenol from nitrosation of N-methyltyramine is expected on the basis of experiments by Challis and Higgins (1973). They were able to isolate only ortho-nitrophenols from the nitrosation of para-substituted phenols. This result was explained via initial nitrosation followed by in situ oxidation as in Figure 21. The same authors determined that o-nitrosophenols made independently by the method of Cronheim (1947) were oxidized by distilled water or dilute acid to o-nitrophenols.

G. Nitrosation of N-Methyl-3-aminomethylindole

Nitrosation of N-methyl-3-aminomethylindole was carried out for the

same reasons as the nitrosation of N-methyltyramine. Nitrosation of N-methyl-3-aminomethylindole also yielded two products. The minor product was characterized as N-nitroso-N-methyl-3-aminomethylindole (III) (Figure 22). The mass spectrum showed m/e 189(M^+), m/e 159(M-30), m/e 144(m-45), and m/e 130. Accurate measurement of the parent ion agreed with the formula $C_{10}H_{11}N_3O$. The proton NMR spectrum showed resonances expected for syn (δ2.99) and anti (δ3.48) methyl and syn (δ4.90) and anti (δ5.41) methylene groups. The C-13 NMR chemical shift assignments are shown in Table 8. The carbons of the indole ring were assigned with reference to Table 6; a chemical shift for the syn-α-CH_2 carbon was not observed, so no assignment was possible.

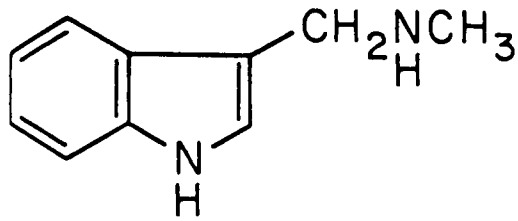
The major product from nitrosation of N-methyl-3-aminomethylindole had the characteristics of a di-nitrosated derivative of N-methyl-3-aminomethylindole, with one of the nitroso groups located on the aliphatic-NH. Initially, it seemed possible that the second nitroso function could be on either C-3 of the indole moiety or on the indolic nitrogen atom. The conclusion that the second nitroso group was on the indolic nitrogen was made on the following evidence:

(1) Infrared data showed the absence of both the aliphatic and indolic-NH stretch. A strong band corresponding to the N-NO function was seen at 1450 cm^{-1}.

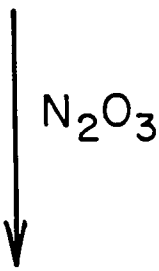
(2) The proton NMR spectrum showed the characteristic resonances for syn and anti α-methyl and α-methylene groups, but the expected singlets were split into asymmetric doublets. The resonances for the syn and anti α-methylene groups were

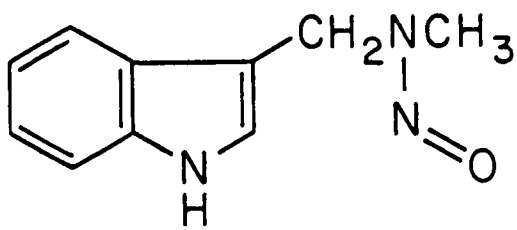
Figure 22. Nitrosation of N-Methyl-3-aminomethylindole

Figure 22



N-Methyl-3-amino-methylindole

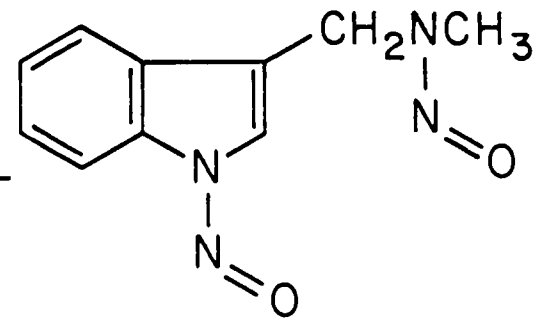




N-Nitroso-N-methyl-3-aminomethylindole

III

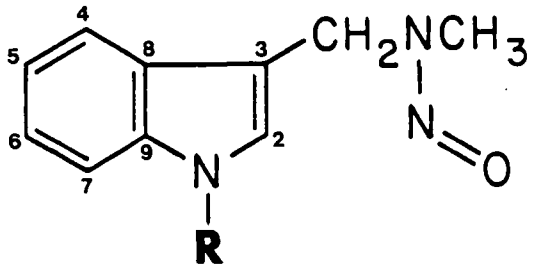
+



N^1-Nitroso-N-nitroso-N-methyl-3-amino-methylindole

IV

TABLE 8. ^{13}C-NMR Chemical Shift Data[a] For N-Nitroso-N-methyl-3-amino-methylindole (III) and N'-Nitroso-N-nitroso-N-methyl-3-aminomethyl-indole (IV)



R=H(III)

R=NO(IV)

Position	Compound III		Compound IV	
	SYN	ANTI	SYN	ANTI
α-CH_3	30.4	39.1	30.7	38.2
α-CH_3	-	49.6	-	49.0
C-2	124.2		127.4,126.4[b]	
C-3	108.8		108.6[b]	
C-4	118.7		114.0,113.7[b]	
C-5	122.3		120.6,119.3[b]	
C-6	120.3		115.8[b]	
C-7	111.5		112.0[b]	
C-8	124.9		122.8[b]	
C-9	126.4		124.2[b]	

[a]Solvent and internal standard: deuteriochloroform; chemical shift, δ, in ppm downfield from tetramethylsilane

[b]Tentative assignment

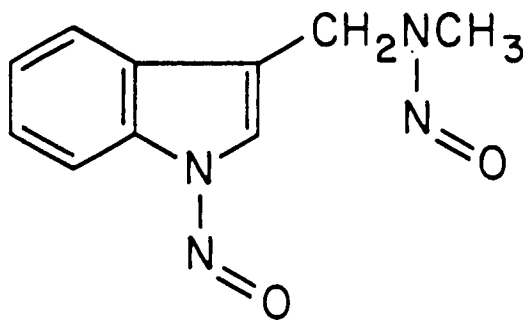
centered at δ4.86 and δ5.41, respectively; the same resonances in N-nitroso-N-methyl-3-aminomethylindole were found at δ4.90 and δ5.41, respectively. The aromatic region of the dinitroso derivative integrated for five protons, with a one proton multiplet at δ8.12 which did not exchange with D_2O. This indicated the absence of a free indolic-NH hydrogen.

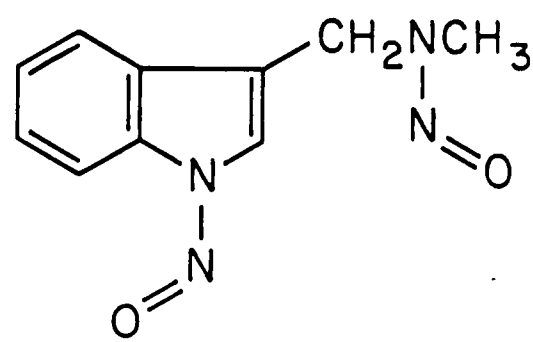
(3) The C-13 NMR data (Table 8) showed that all carbon chemical shifts were seen in the same narrow region of the spectrum (108.6 ppm to 127.4 ppm) as they had appeared in III. For the dinitroso derivative, the <u>syn</u> α-CH_3 and <u>anti</u> α-CH_3 resonances were seen at 30.7 ppm and 38.2 ppm, respectively. The <u>anti</u> α-CH_2-resonance was seen at 49.0 ppm. For III, the <u>anti</u> α-CH_2-resonance appeared at 49.6 ppm. If a nitroso group were located on C-3 of the dinitroso compound, a significant shift (β effect) in the resonance of α-CH_2- would be expected because of conversion of C-3 from Sp^2 to Sp^3 hybridization and because of introduction of an electron with drawing group (Levy, 1980). Since no significant shift was observed, it was concluded that the second nitroso group was located on the indolic nitrogen atom, consistent with the structure N^1-nitroso-N-nitroso-N-methyl-3-aminomethylindole (IV) (Figure 22).

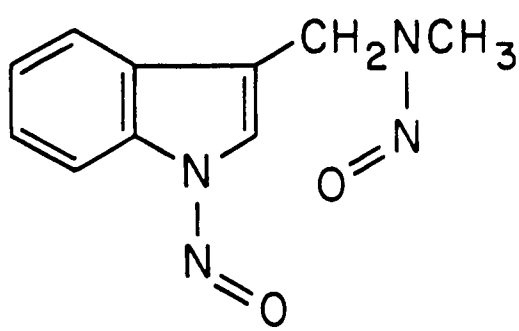
Introduction of nitroso group on the N^1-indole nitrogen atom should create a second set of conformational isomers in which the indolic N-nitroso group can be either <u>syn</u> or <u>anti</u> to C-2 and C-9.

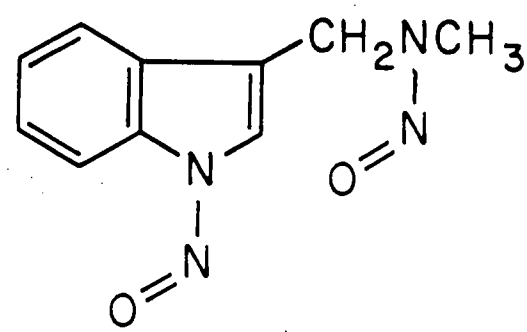
Figure 23. Proposed conformers of N^1-Nitroso-N-Nitroso-N-methyl-3-aminomethylindole

Figure 23


CH2NCH3
N
N=O
N
N=O


CH2NCH3
N
N=O
N
O=N


CH2NCH3
N
O=N
N
N=O

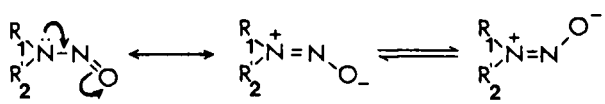

CH2NCH3
N
O=N
N
O=N

Therefore, the conformers postulated for IV are shown in Figure 23. The introduction of the second nitroso group in IV is apparently responsible for the multiplicity seen for the syn and anti α-methyl and α-methylene resonance in the proton NMR spectrum of IV. The instability of compound IV in solution, especially on exposure to normal daylight, precluded the opportunity to perform extensive NMR experiments.

H. Collection and Re-equilibration of Syn and Anti Conformers p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine

Preliminary HPLC experiments showed that samples of nitrosamines I, II, III, and IV each chromatographed as two peaks. Storage of the samples did not change the observed behavior. This behavior suggested that the two peaks observed for each nitrosamine were the syn and anti conformers.

Conformational isomerism occurs in N-nitroso compounds because electron delocalization in the hetero-atomic π electron system leads to multiple resonance forms which represent the distribution of electron density over the π system. In one of these resonance forms there is partial double bond character of the N-N bond which leads to a barrier to free rotation of the nitroso group. This rotational barrier, which amounts to 23 Kcal/mole in NDMA (Looney et al., 1957), causes the nitroso group to assume a planar conformation in which the O-atom is syn to one α-carbon and anti to the other:



The conformational isomerism produces a magnetic anisotropy (Harris and Spragg, 1967) which is large enough to allow the two conformers to be distinguished on the NMR time scale when the N-nitroso compound is asymmetric ($R_1 \neq R_2$).

Iwaoka et al. (1975) showed that the syn and anti conformers of N-nitroso proline could each be collected by HPLC and re-equilibrated to give a mixture containing both the syn and anti conformers.

To determine if the two chromatographic peaks observed for p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine represented syn and anti conformers, a similar approach to that of Iwaoka et al. was taken.

Figure 24 shows the separation of the two chromatographic peaks of p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine obtained from a methanol solution which had been kept at room temperature for 24 hr. Each peak was collected separately and re-injected at known time intervals after the initial collection. Chromatograms of the re-injected peaks are shown in Figure 25 and Figure 26. Measurements of peak height ratio showed that peak 1 reverted to a mixture with a peak 1: peak 2 height ratio of 4.6:1; likewise, peak 2 reverted to a mixture with a peak 1: peak 2 height ratio 4.6:1. These results were taken as evidence that the original chromatographic peaks were conformational isomers; peak 1 is the syn conformer (NO syn to $-CH_3$) on the basis of the NMR analysis of Section F. Figure 27 shows the time course for the re-equilibration of the two conformers. Full re-equilibration was obtained in 33 hr., and the half-life of the transformation was 4.4 hr.

This experiment was taken as adequate evidence that the two

Figure 24. HPLC separation of an equilibrium mixture of the Syn and Anti conformers of p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine

Figure 24

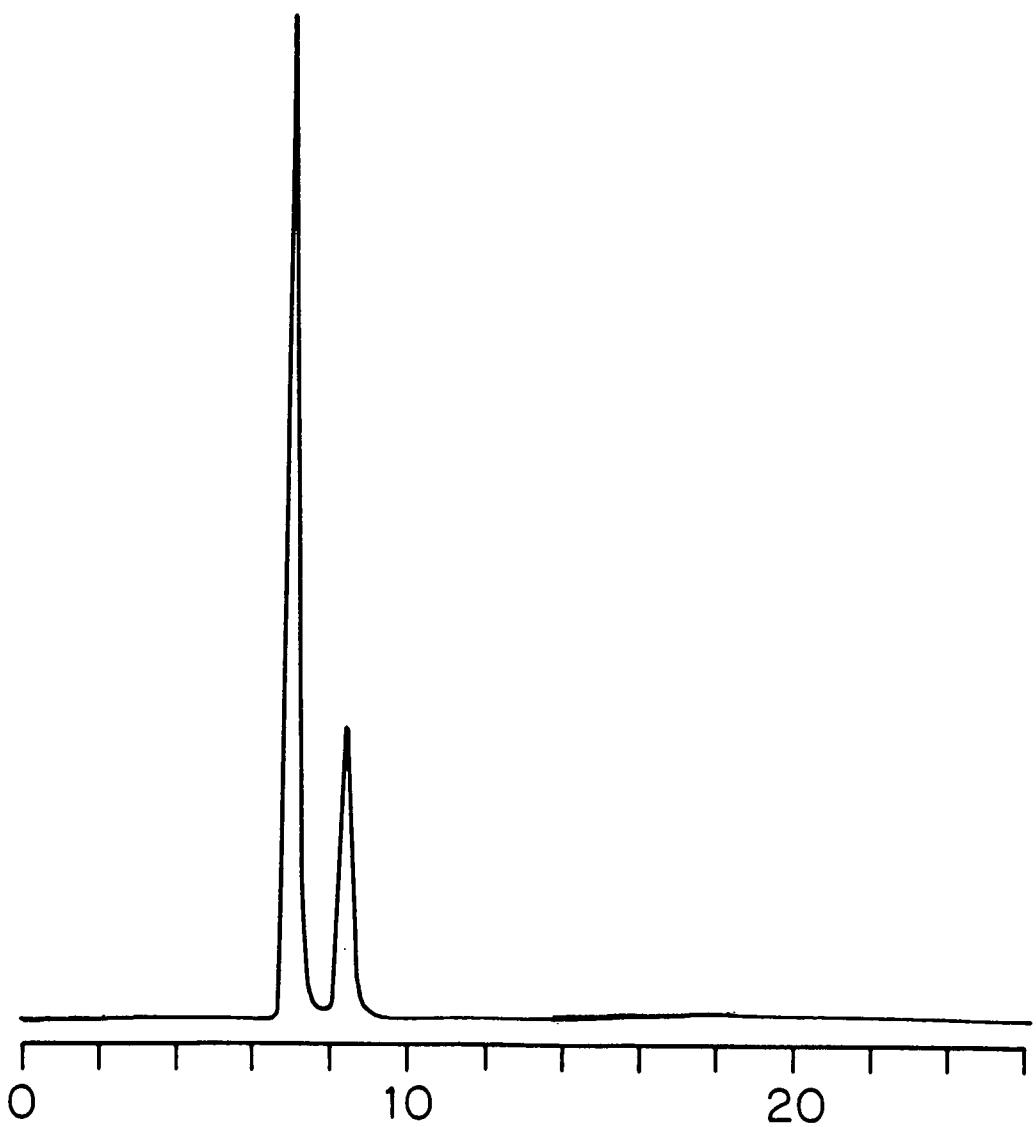


Figure 25. Re-equilibration of the two chromatographic peaks of p-Hydroxy-N-nitroso-N-methyl-2-phenylethyl-amine (Part 1)

A: Peak 2 re-injected 42 min. after collection

B: Peak 2 re-injected 240 min. after collection

C: Peak 1 re-injected 25 min. after collection

D: Peak 1 re-injected 225 min. after initial collection

Figure 25

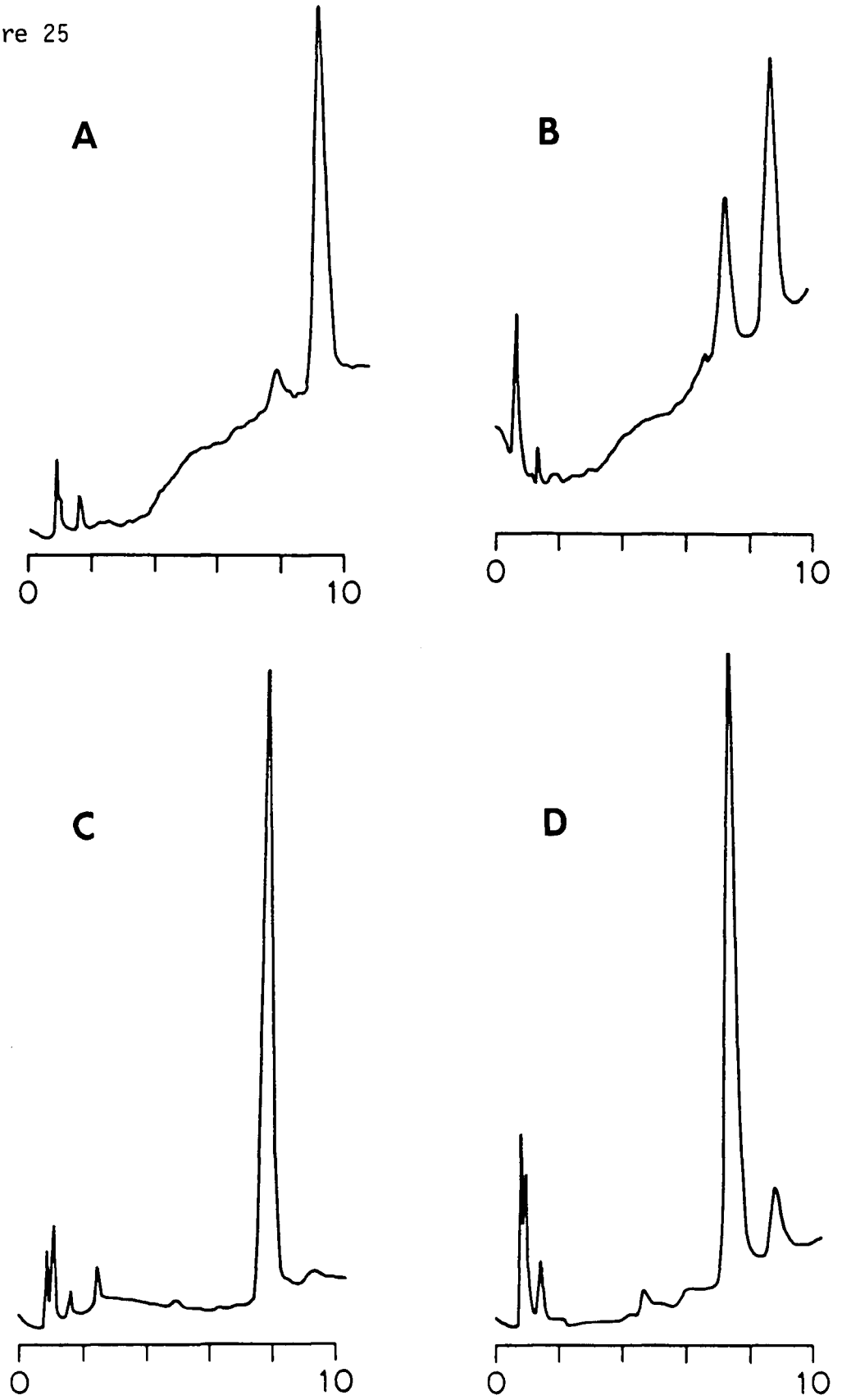


Figure 26. Re-equilibration of the two chromatographic peaks of p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine (Part 2)

A: Peak 2 re-injected 510 min after collection

B: Peak 2 re-injected 2000 min after collection

C: Peak 1 re-injected 494 min after collection

D: Peak 1 re-injected 1985 min after collection

Figure 26

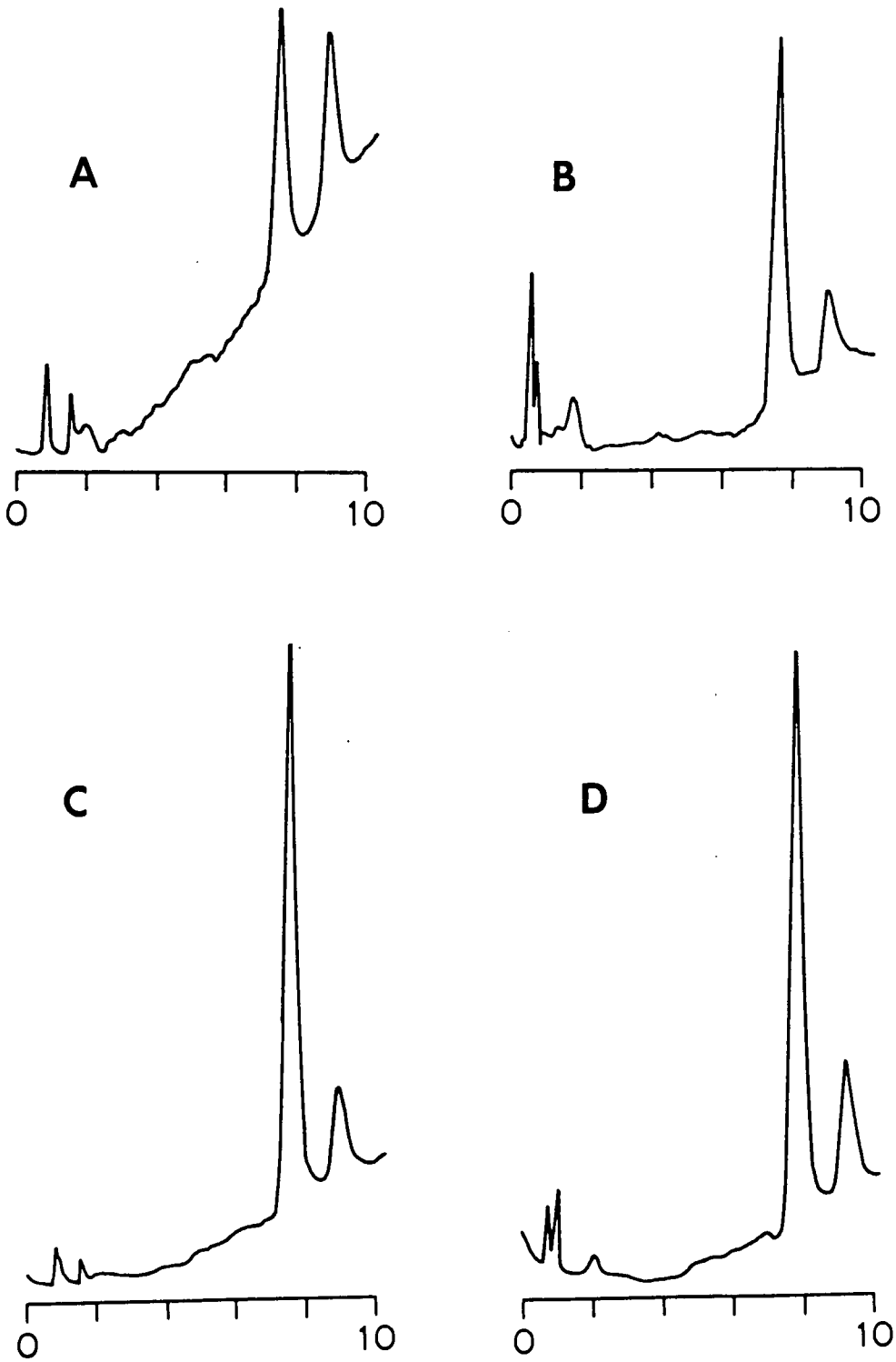
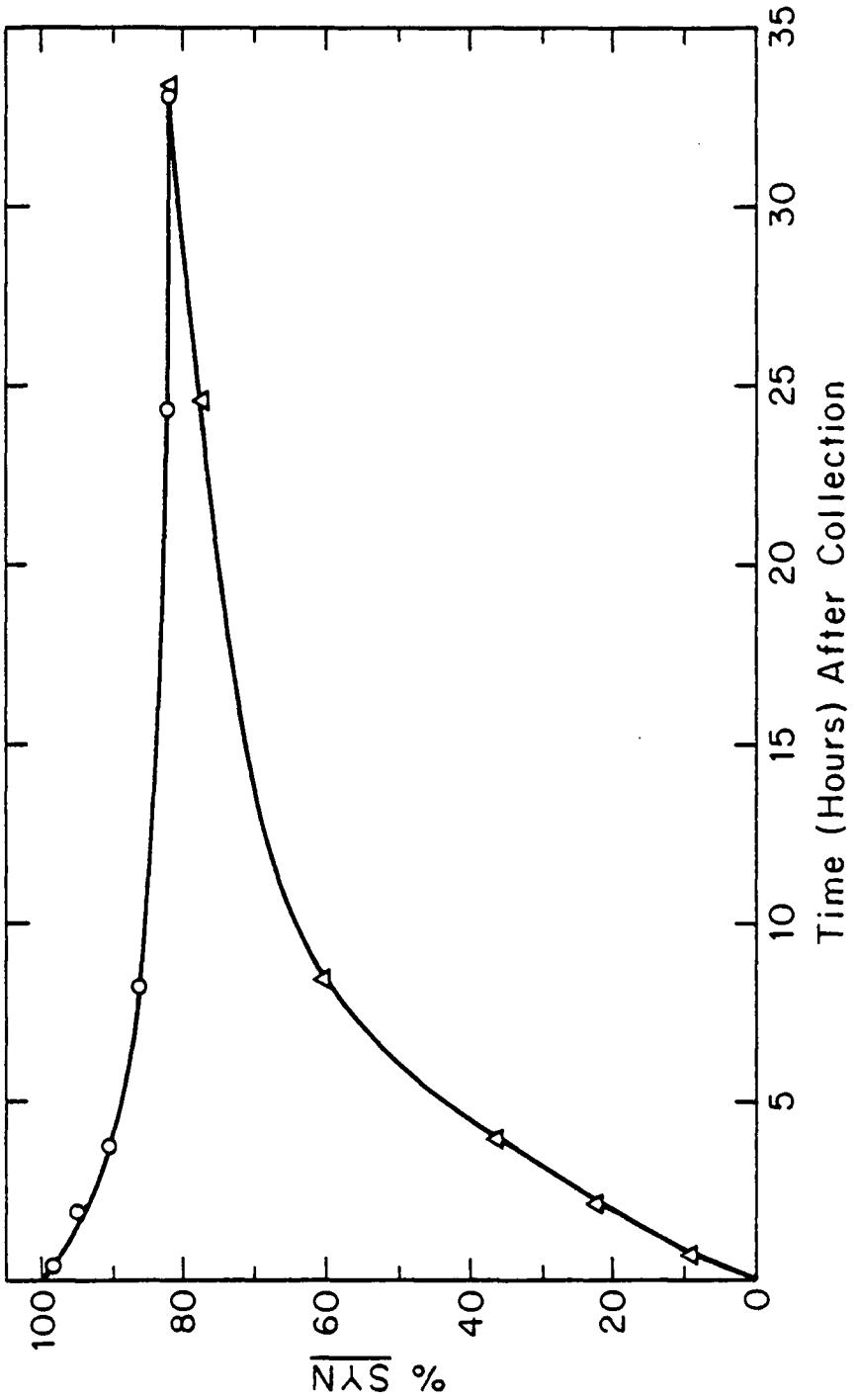


Figure 27. Time course for the re-equilibration of the Syn and Anti conformers of p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine

Top Curve: Re-equilibration of peak 1 (syn conformer) after collection and re-injection of peak 1

Bottom Curve: Re-equilibration of peak 1 (syn conformer) after collection and re-injection of peak 2

Figure 27


100
80
60
40
20
0
NAS %
5
10
15
20
25
30
35
Time (Hours) After Collection

chromatographic peaks observed for each N-nitrosoamine in this study represent conformational isomers. The difficulty in analysis and quantitation created by two conformer compounds will be seen in the upcoming sections.

I. Determination by HPLC of Product Yields From the Nitrosation of N-Methyltyramine and N-Methyl-3-aminomethylindole

Since HPLC in the reverse phase mode uses an aqueous mobile phase, it seemed possible to directly analyze the products of nitrosation without the necessity for extraction or concentration steps. This approach was used to quantitate the N-nitrosamines formed during the nitrosation of N-methyltyramine and N-Methyl-3-aminomethylindole.

The reactions were run on a small scale using the same substrate concentration, amine: nitrite ratio, and reaction time that was used in Sections F and G. Product yields were determined with the aid of external standards after determining that the standards were within the linear range of the detector. The results of duplicate reactions on each amine are shown in Table 9. The average material balance for nitrosation of N-methyltyramine was 86.4%. The average material balance for nitrosation of N-methyl-3-aminomethylindole was 92.2%. TLC of the product from N-methyltryamine nitrosation indicated that some unreacted amine or amine by-product was present which would not have eluted from the HPLC column under the conditions used. Chromatograms of standard mixtures of the nitrosamines are shown in Figure 28. No HPLC conditions were found in which the products could be

Figure 28. HPLC separation of N-nitrosamines obtained from the Nitrosation of N-Methyltyramine and N-Methyl-3-aminomethylindole

A: Standard mixture of p-Hydroxy-N-nitroso-N-methyl-2-phenylethylamine and p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine

B: Standard mixture of N-Nitroso-N-methyl-3-aminomethylindole and N^1-Nitroso-N-nitroso-N-methyl-3-aminomethylindole

Figure 28

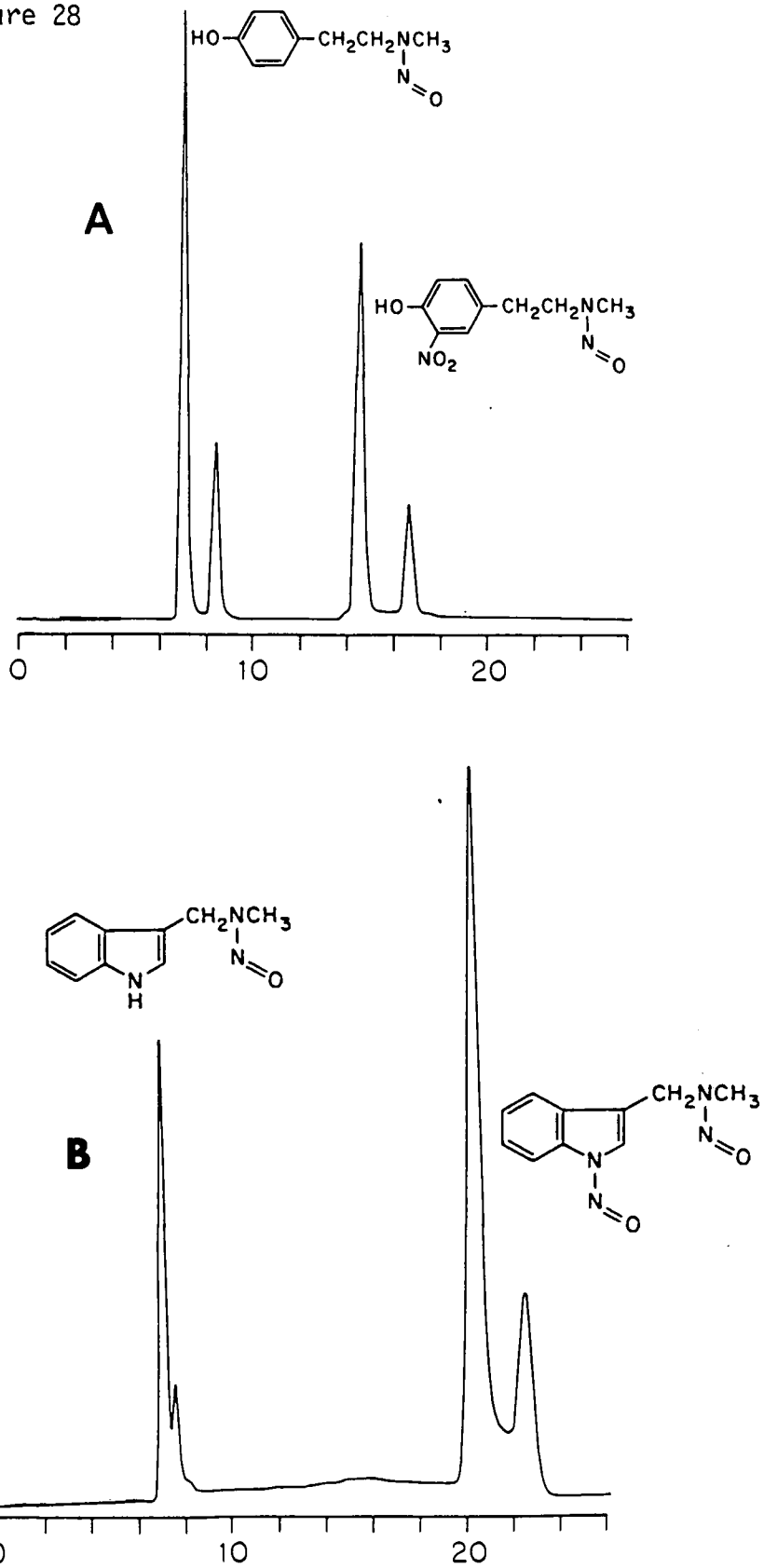

HO
CH2CH2NCH3
N
O
A
HO
NO2
CH2CH2NCH3
N
O
0
10
20
CH2NCH3
N
O
H
B
CH2NCH3
N
O
N
N
O
0
10
20

TABLE 9. Product Yields (%) From Nitrosation of N-Methyltyramine and N-Methyl-3-aminomethylindole

Amine	Product	Yield[a]
N-Methyltyramine	Nitrosamine I	75 ± 1
	Nitrosamine II	11.5 ± 0.5
N-Methyl-3-amino-methylindole	Nitrosamine III	26 ± 1
	Nitrosamine IV	66 ± 1

[a]Average of two reactions on each amine

separated and the individual N-nitrosamines also eluted as single peaks.

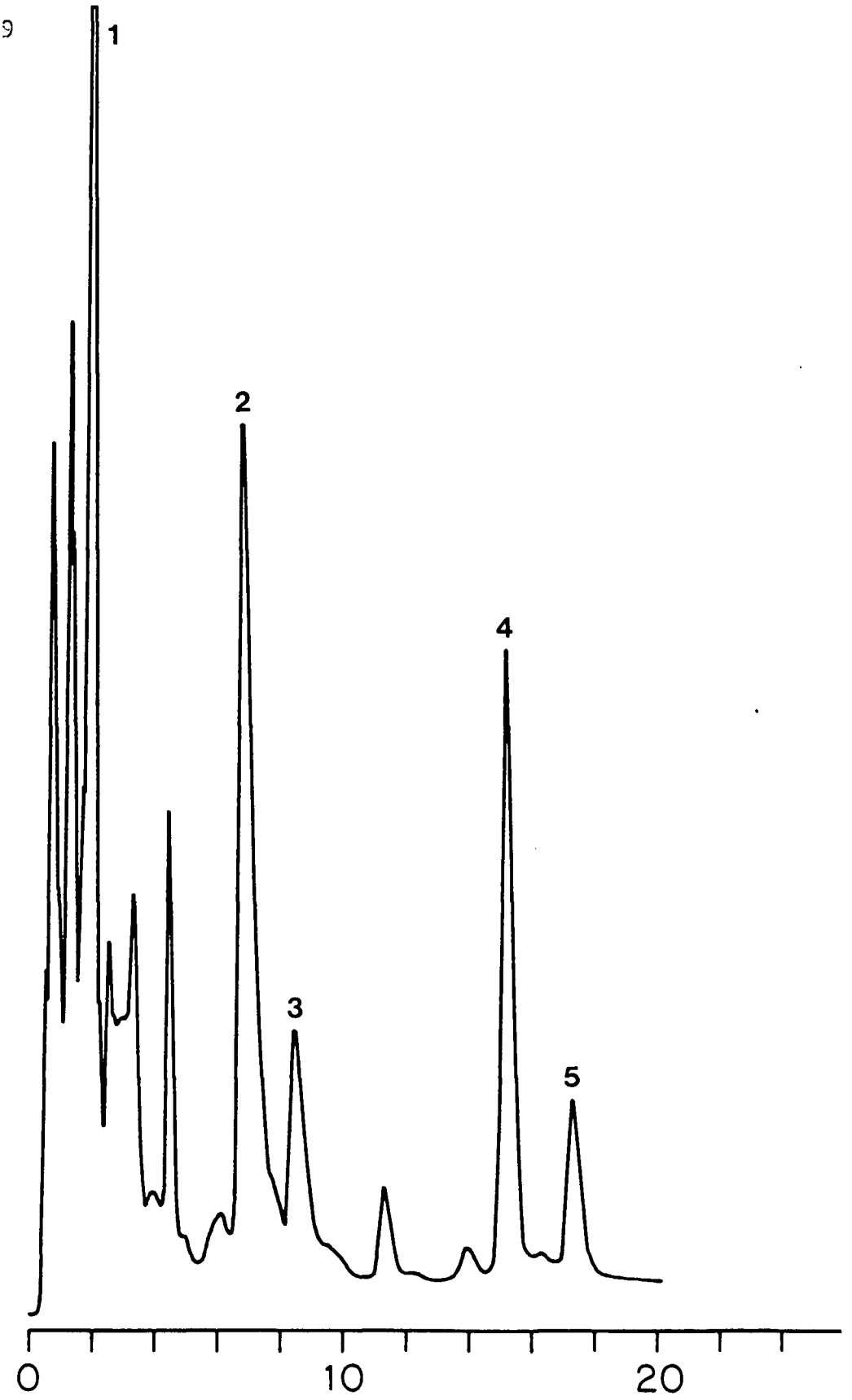
J. Isolation and Quantitative Determination of p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine as a Product of Hordenine Nitrosation

The data on the yield of NDMA from the nitrosation of hordenine at elevated temperature (65°) and in dilute acid (pH 3.4 or pH 4.4) indicated that the susceptibility of hordenine to be nitrosated to give NDMA was well within the expected limits based on previous work on tertiary amine nitrosation (Lijinsky et al., 1972b; Linjinsky and Singer, 1974; Smith and Loeppky, 1967). If hordenine undergoes nitrosative dealkylation, then two of the predicted products in addition to NDMA would be nitrosamine I and nitrosamine II. When hordenine was nitrosated in a 10-fold excess of nitrite for 2 hr. at 65° and pH 4.4, the yield of NDMA obtained was 11.9% as determined by GC-TEA. The HPLC of the product from the same reaction is shown in Figure 29. Five peaks are numbered as reference peaks. Peak 1 is NDMA. Peaks 2, 3,

Figure 29. HPLC trace of the product from nitrosation of hordenine for 2 hr at 65°

Conditions: Column A with Mobile Phase 1

Figure 29



4, and 5 had retention times closely approximating the retention times expected for the syn and anti conformers of nitrosamine I and nitrosamine II. Spiking experiments with a standard solution of nitrosamine I (Figure 30) showed that nitrosamine I did not co-elute with any components of the original reaction product. Spiking experiments with a standard solution of nitrosamine II (Figure 31) showed that the syn and anti conformers of the standard co-eluted with peak 4 and peak 5 of the original reaction product.

To obtain material for mass spectral analysis, a standard of nitrosamine II was injected several times and collected under the same HPLC conditions used for the original reaction. The mass spectrum of the collected material is shown in Figure 32. Then the two peaks corresponding to peak 4 and peak 5 were collected from the product of a hordenine sample nitrosated for 12 hr. at 65°. The mass spectrum of the collected product is shown in Figure 33. The two mass spectra show the same molecular ion and principle fragment ions. These results were taken as confirmatory evidence that nitrosamine II is a product of hordenine nitrosation at elevated temperature.

Nitrosation of hordenine at 65° (pH 4.4) was carried out for times of 2 hr., 4 hr., 8 hr., and 12 hr.; the NDMA formed was quantitated by GC-TEA. Nitrosamine II was quantitated by HPLC. The yields of NDMA and nitrosamine II are shown in Table 10. The low yield of nitrosamine II is not due to the method of work-up or analysis. If nitrosamine I were present as a reaction product, The yield would not be greater than 0.3% under the analytical conditions used.

Figure 30. HPLC trace of a mixture of nitrosamine I and the product from nitrosation of hordenine for 2 hr at 65°

Conditions: Column A with mobile phase 1

Figure 30

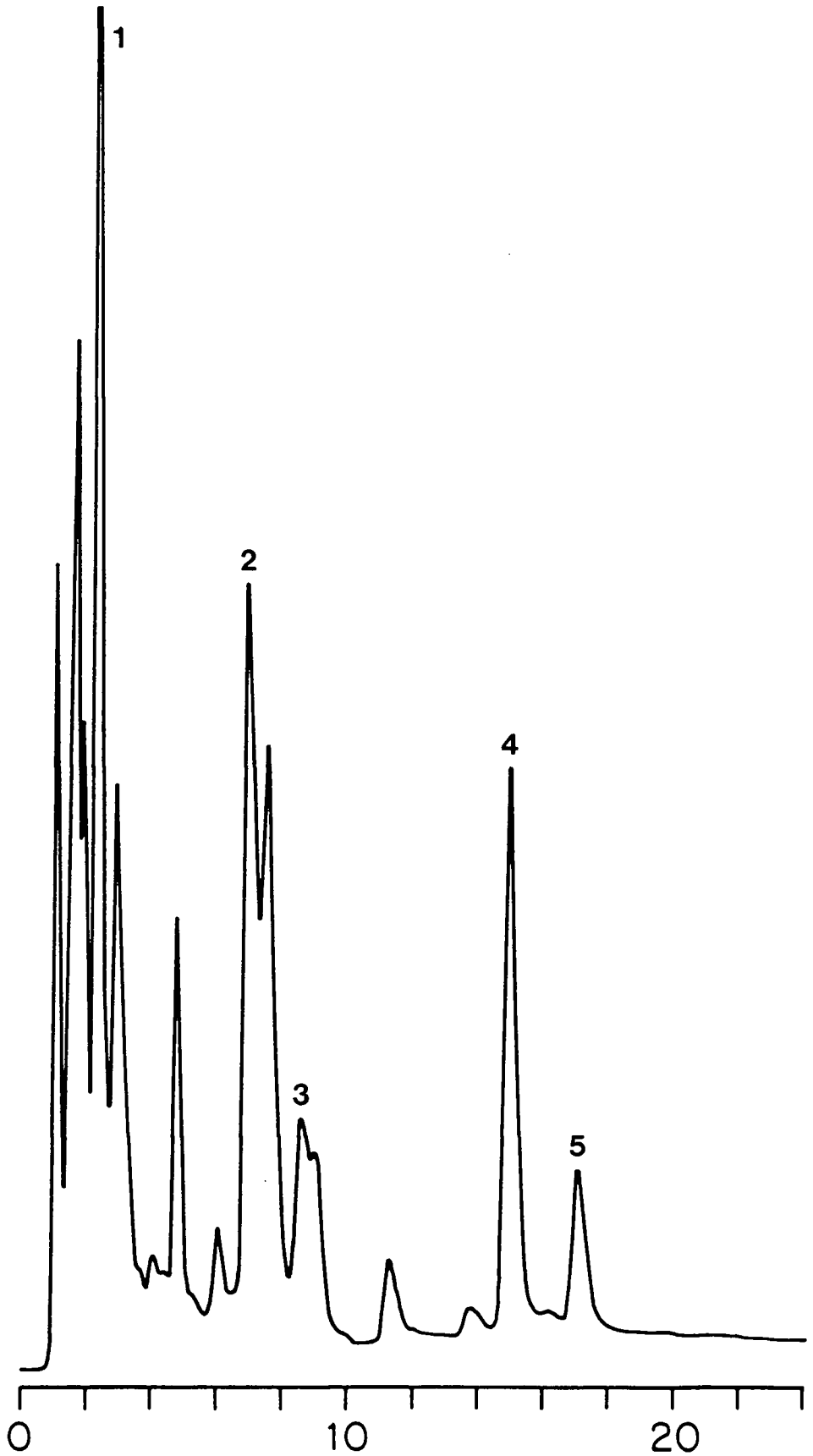


Figure 31. HPLC trace of a mixture of nitrosamine II and the product from nitrosation of hordenine for 2 hr at 65°

Conditions: Column A with mobile phase 1

Figure 31

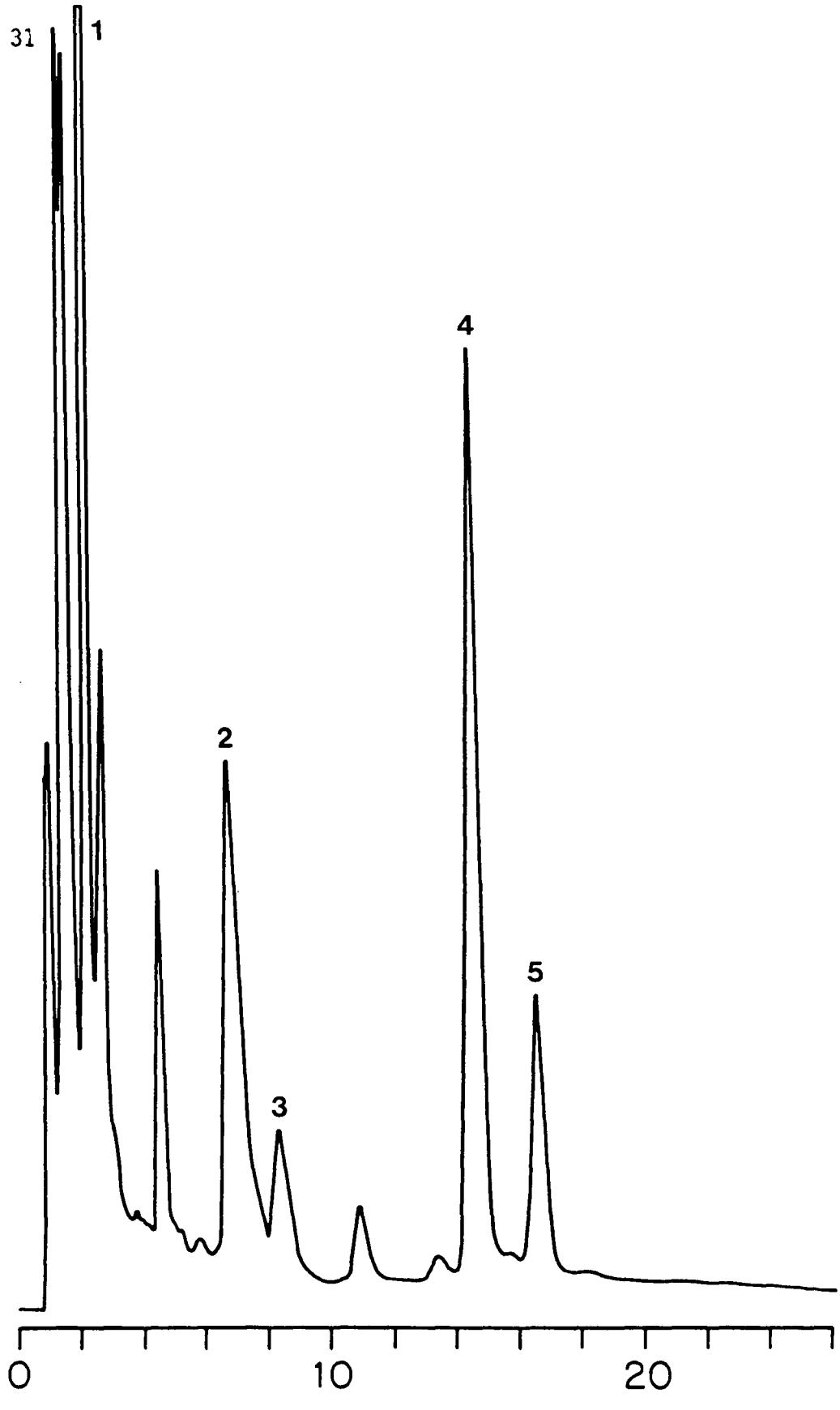


Figure 32. Mass Spectrum of a sample of p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine collected by HPLC of a standard solution of the compound

Figure 32

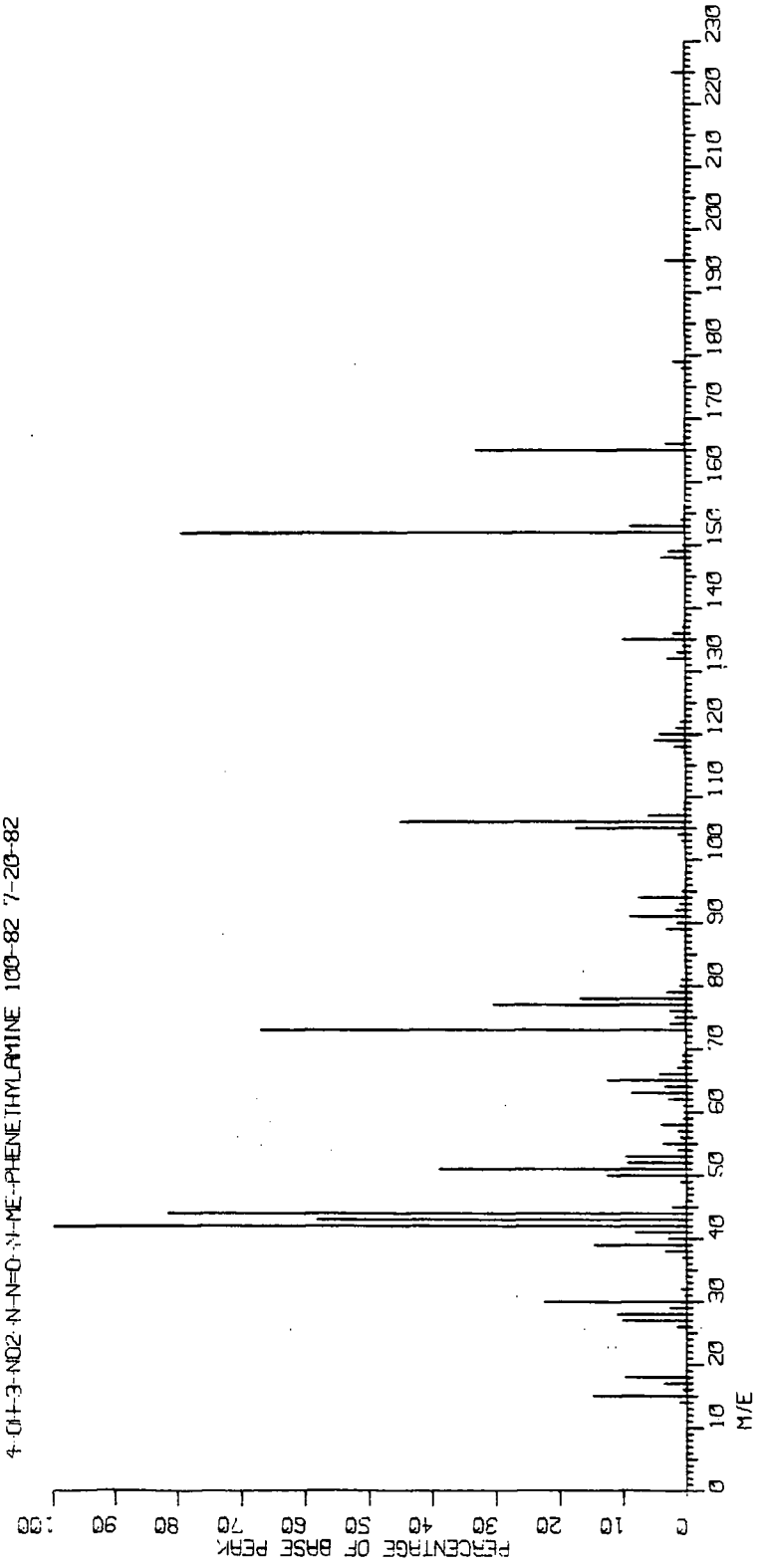

4-CH-3-NO2-N-N=O-N-ME-PHENETHYLAMINE 100-82 7-20-82
PERCENTAGE OF BASE PEAK
M/E
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230

Figure 33. Mass spectrum of a sample of p-Hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine collected by HPLC of the product from nitrosation of hordenine for 12 hr. at 65°

Figure 33

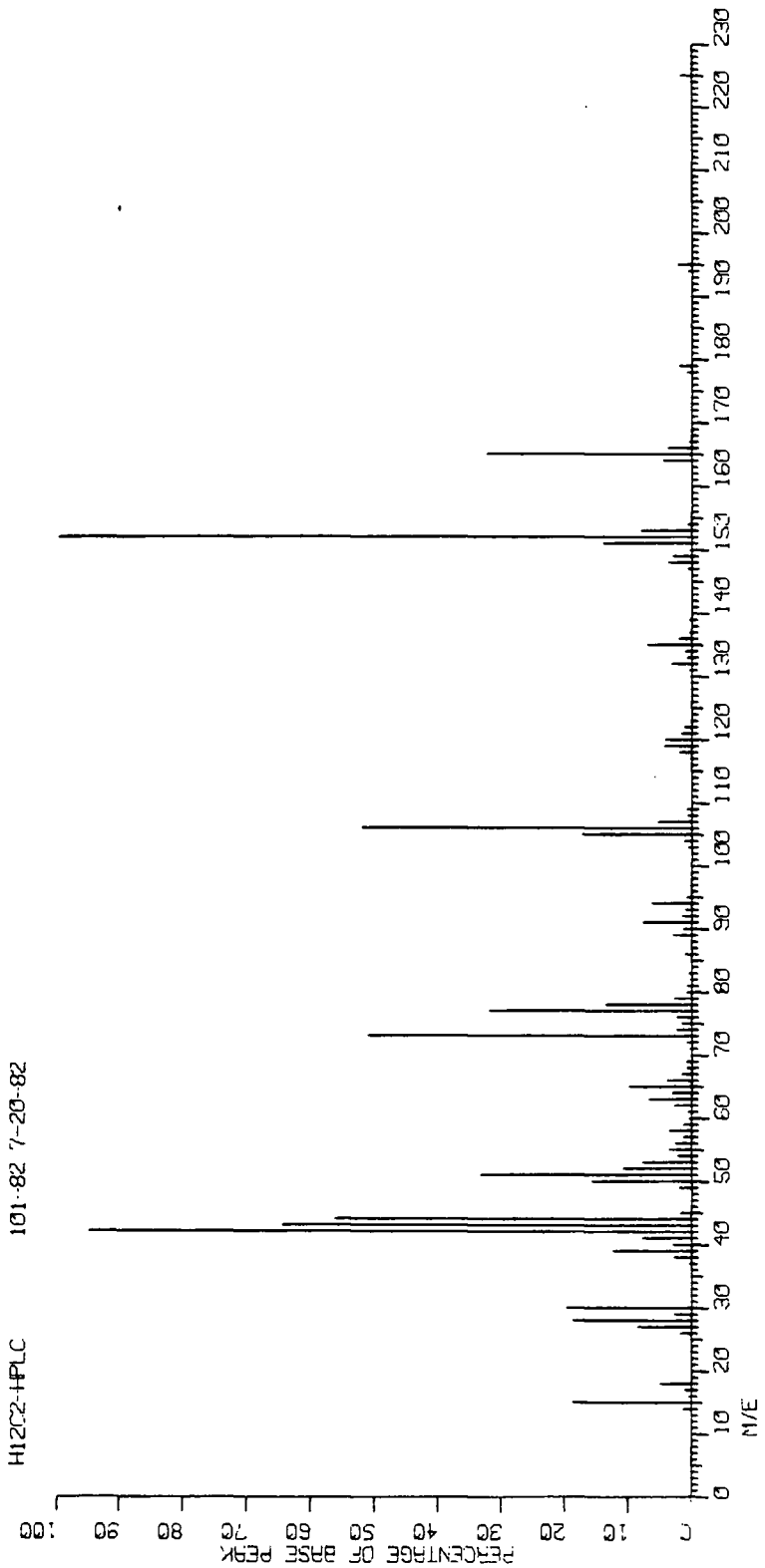

H12C2+HPLC
101-82 7-20-82
PERCENTAGE OF BASE PEAK
M/E
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230

Figure 34. HPLC trace of the product from nitrosation of gramine for 5 min. at 20°

Conditions: Column B with mobile phase 1

Figure 34

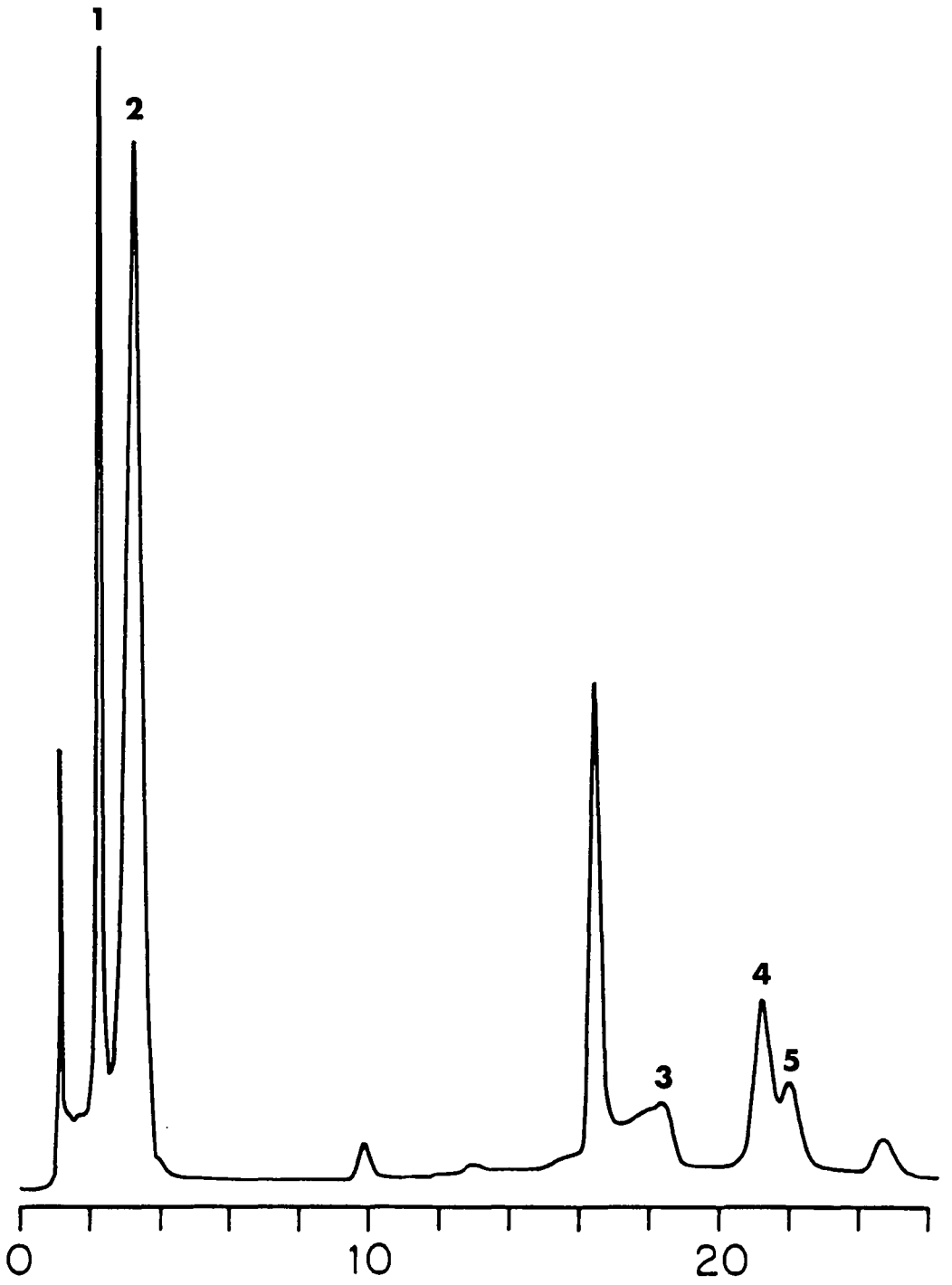


Figure 35. HPLC trace of the product from nitrosation of gramine for 10 min. at 20°

Conditions: Column B with mobile phase 1

Figure 35

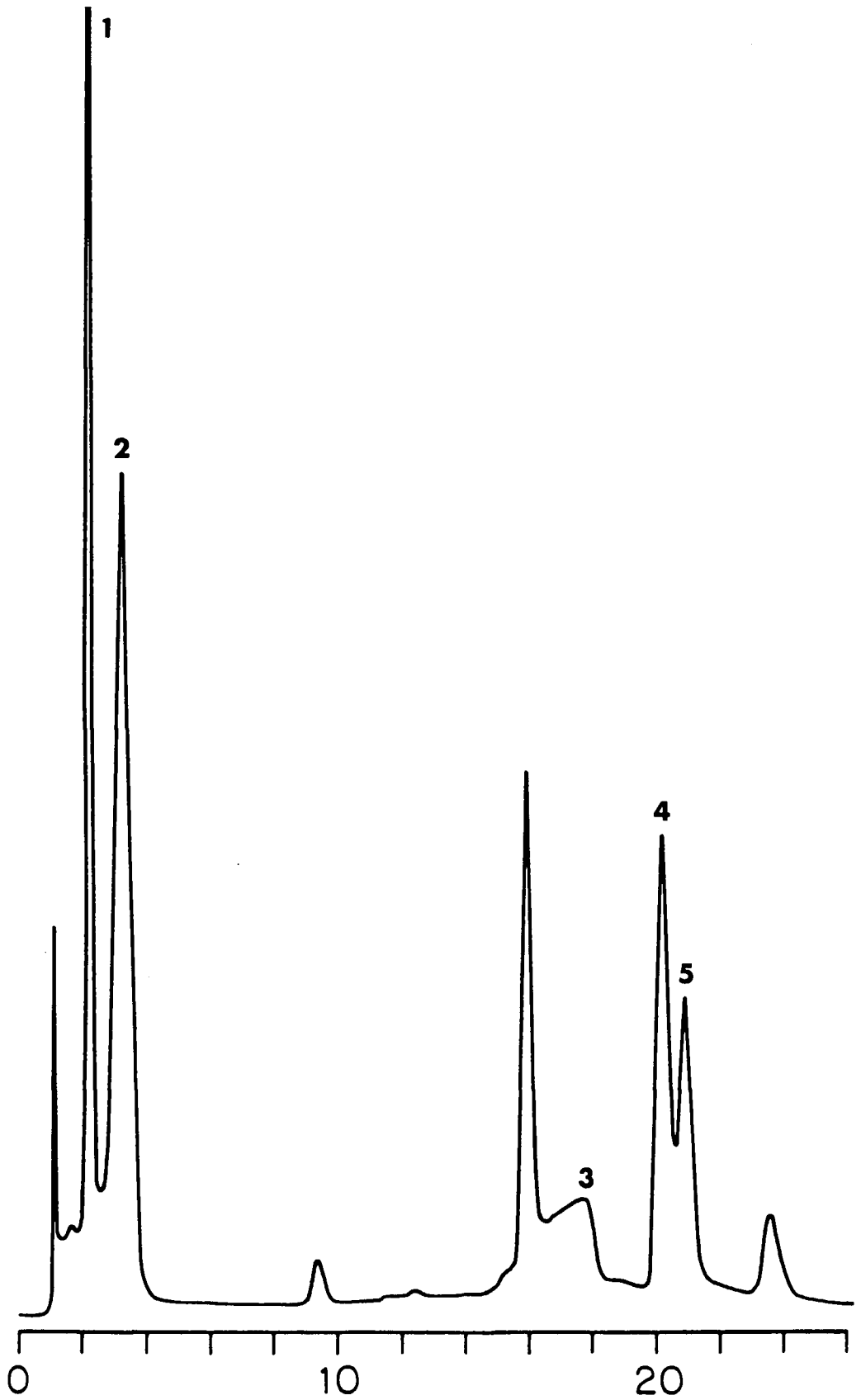


Figure 36. HPLC trace of the product from nitrosation of gramine for 30 min. at 20°

Conditions: Column B with mobile phase 1

Figure 36

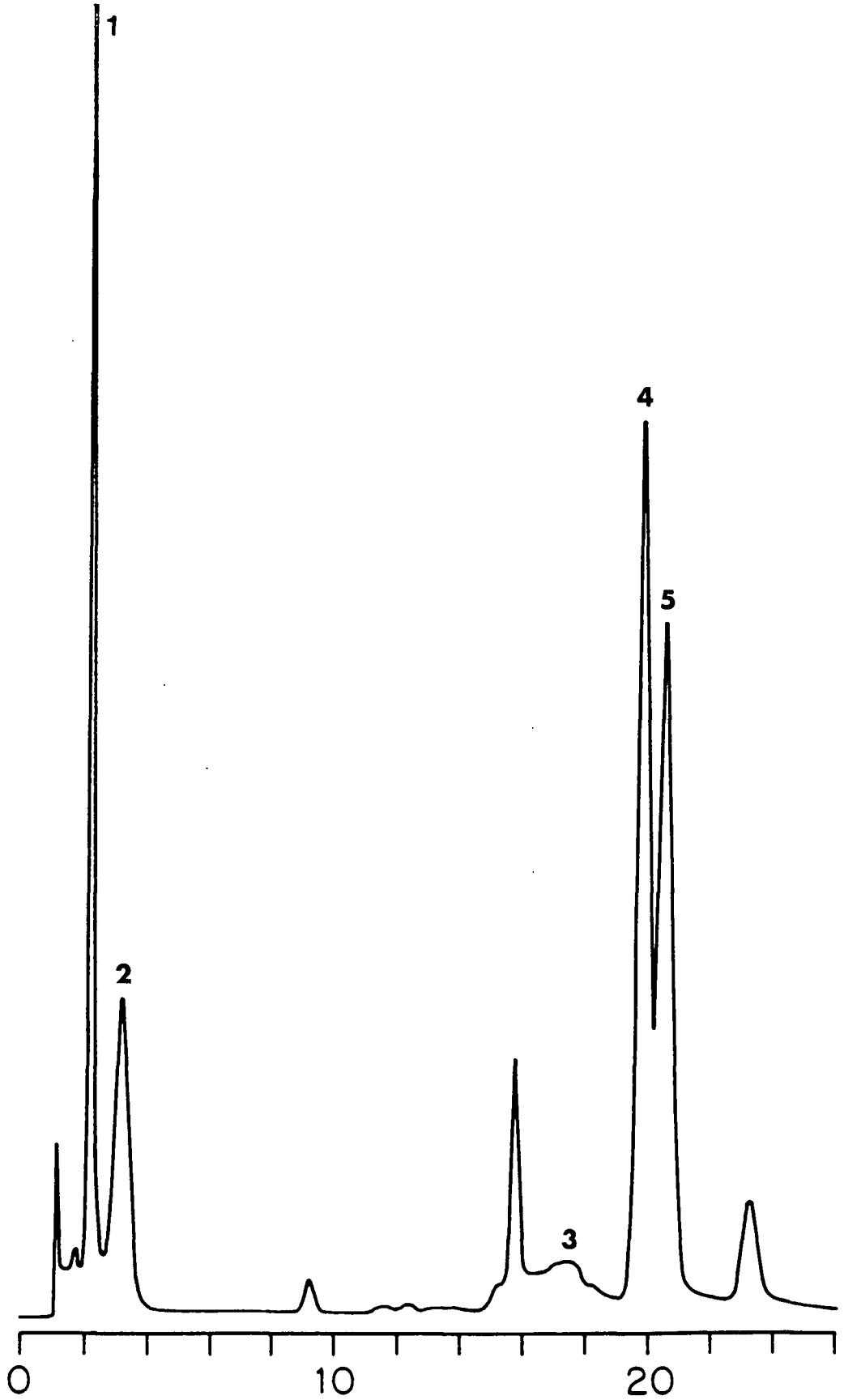


Figure 37. HPLC trace of the product from nitrosation of gramine for 60 min. at 20°

Conditions: Column B with mobile phase 1

Figure 37

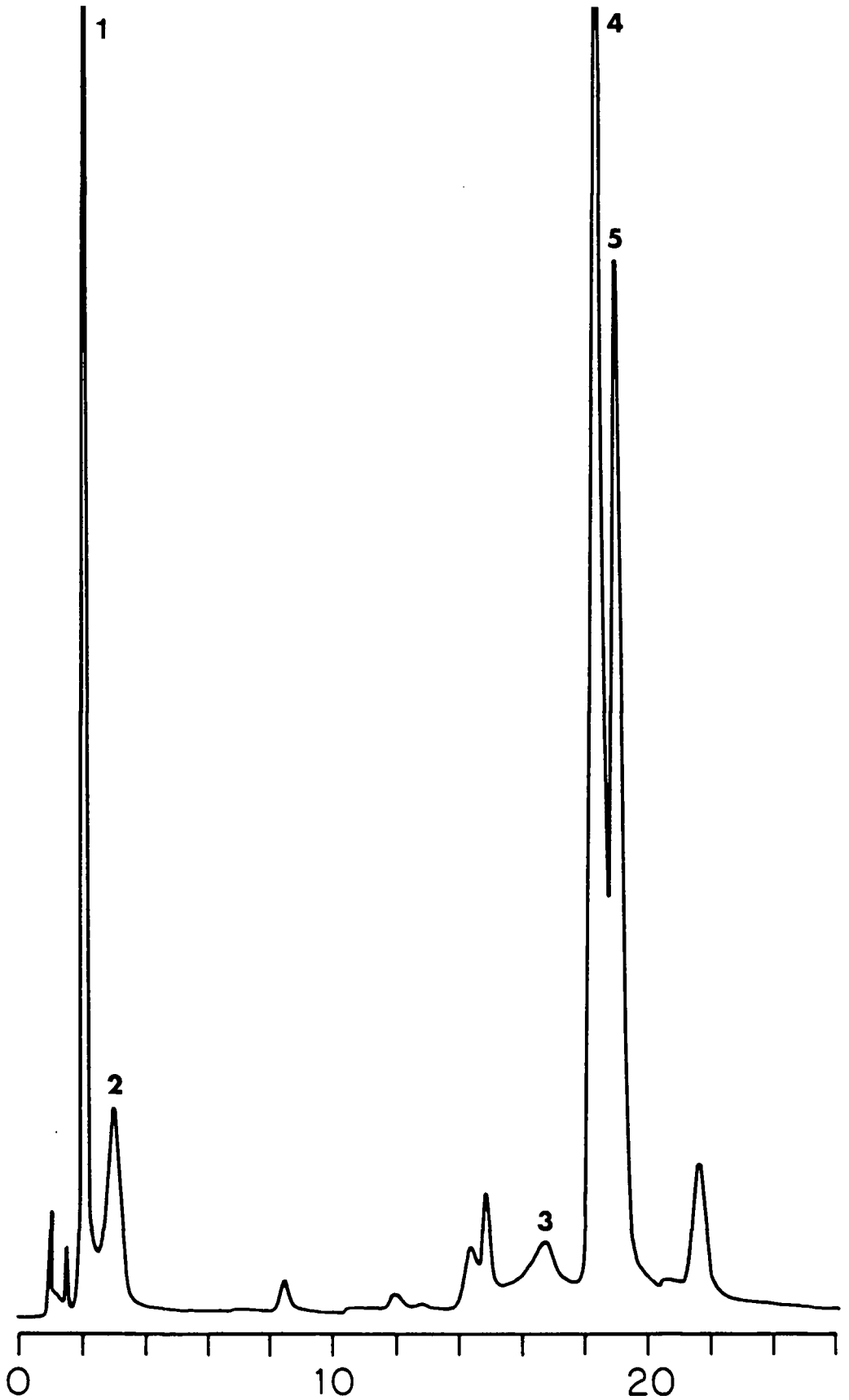


Figure 38. HPLC trace of (A): The product from nitrosation of gramine for 60 min. at 28° and (B): A mixture of (A) with standard N-Nitroso-N-methyl-3-aminomethyl-indole

Figure 38

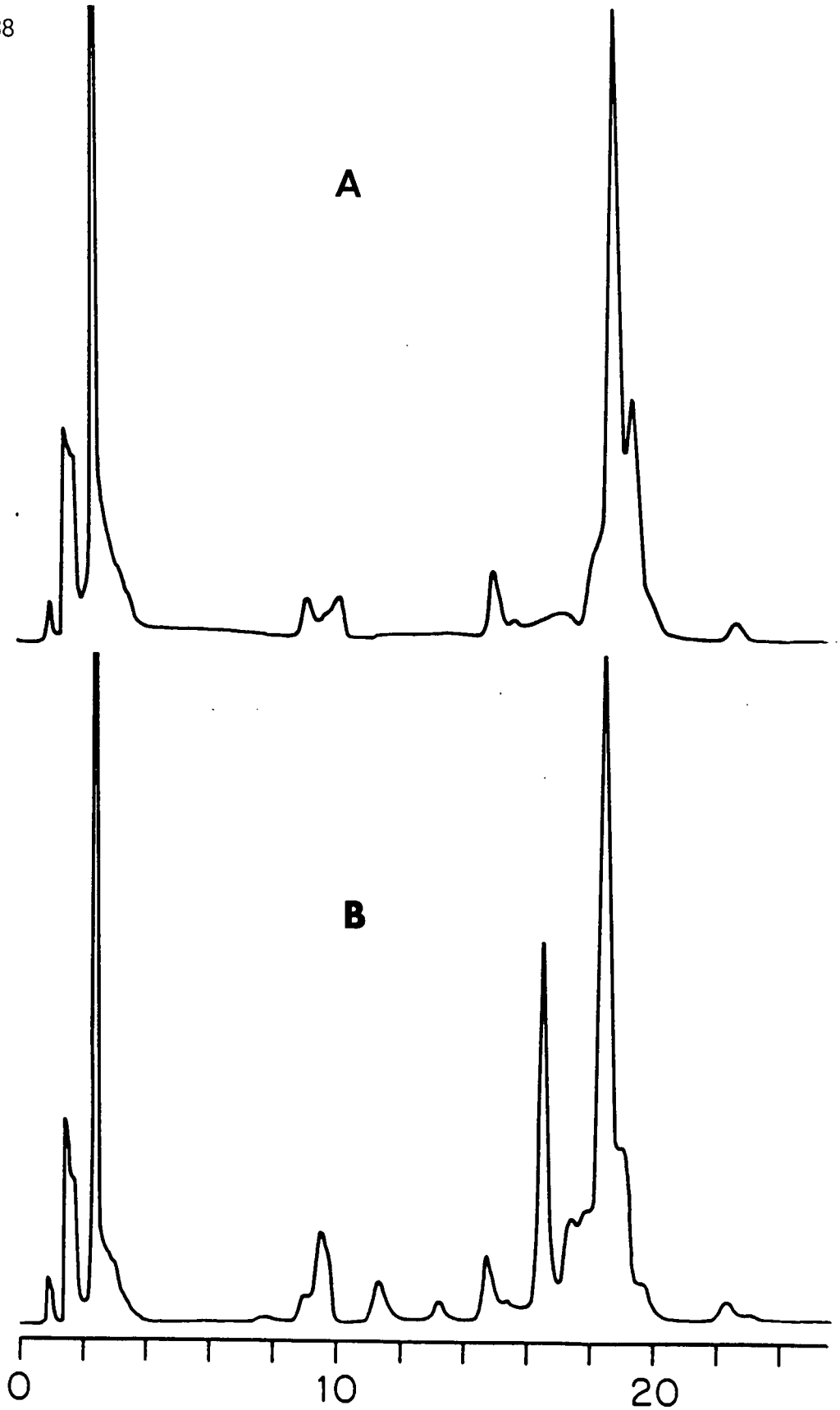
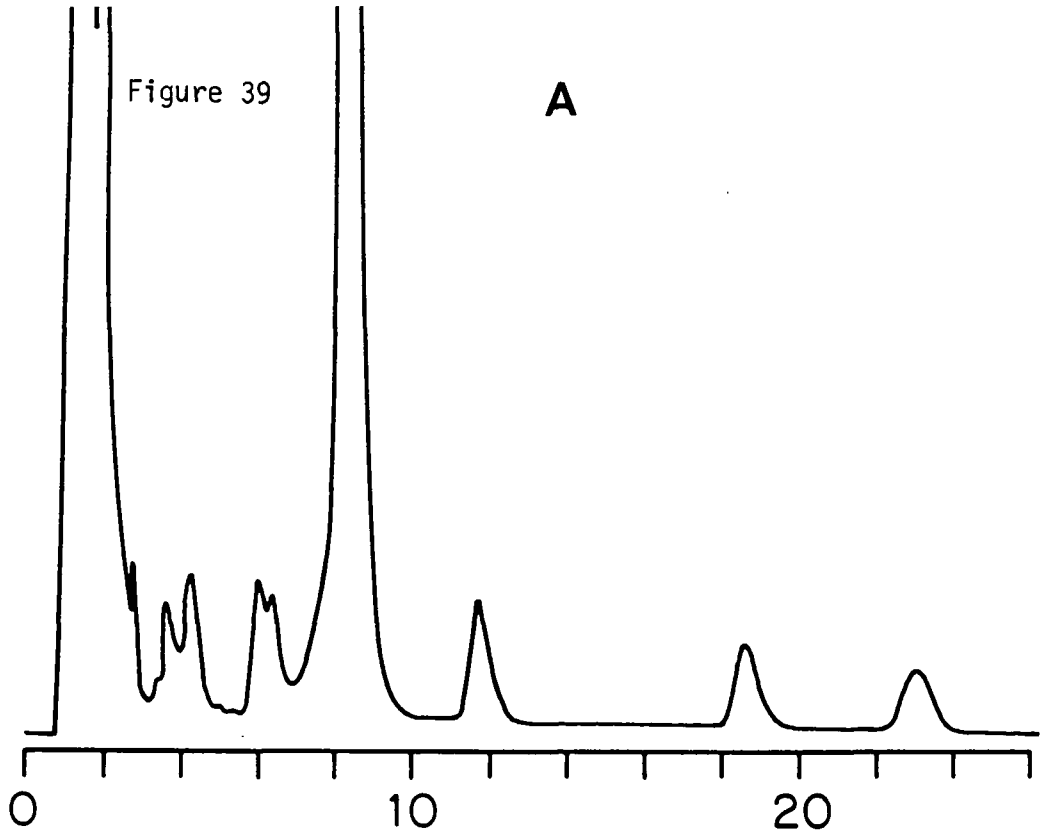


Figure 39. HPLC traces of (A): the product from Nitrosation of gramine for 60 min at 28° and (B): a mixture of (A) with standard N^1-Nitroso-N-nitroso-N-methyl-3-amino-methylindole

Conditions: Column A with mobile phase 2

Figure 39



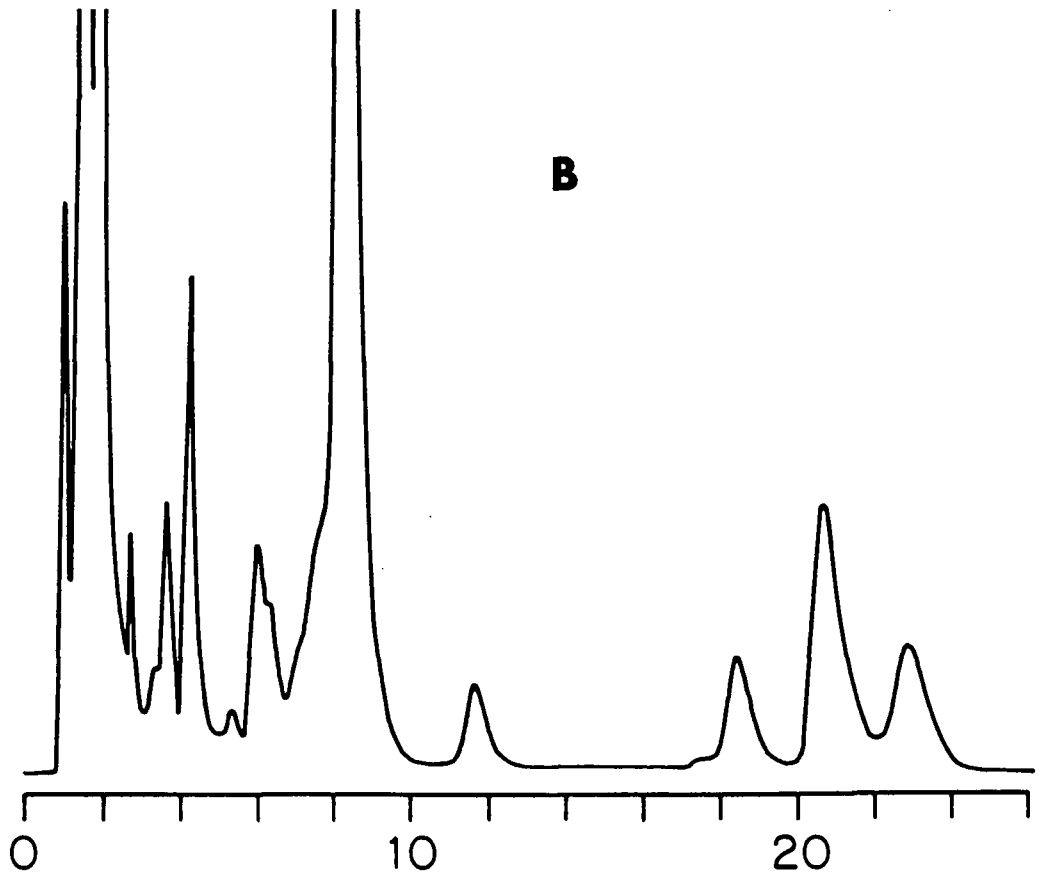


TABLE 10. Yields (%) of NDMA and p-Hydroxy-m-nitro-N-Nitroso-N-methyl-2-phenyl-ethylamine (II) After Nitrosation of Hordenine at 65°

Reaction Time (Hr.)[a]	NDMA[b,c]	Nitrosamine II[b,d]
2	11.9	0.5
4	16.3	0.8
8	20.4	1.0
12	21.4	1.2

[a]Conditions: 0.1 M Hordenine Hemisulfate in 1.0 M $NaNO_2$ (pH 4.4) at 65°

[b]Values corrected for recovery

[c]Determined by GC-TEA

[d]Determined by HPLC

K. Investigation of Gramine Nitrosation Reaction by HPLC

The yields of NDMA obtained from gramine nitrosation (Sections B and D) were far above the expected limits based on reactivity considerations (Lijinsky et al., 1972b) and stereochemical factors (Smith and Leoppky, 1967). If gramine were nitrosated via the nitrosative dealkylation mechanism, then two of the predicted products in addition to NDMA would be nitrosamines III and IV, the two nitrosamines obtained from nitrosation of N-methyl-3-aminomethylindole.

To test this hypothesis, gramine nitrosation was carried out at room temperature under conditions previously determined to result in rapid yields of NDMA. The product was examined by HPLC without quenching or extraction to insure that initial products would be observed. Chromatograms obtained from reaction times of 5 min., 10 min.,

30 min., and 60 min. are shown in Figures 34, 35, 36, and 37. Five peaks are numbered as reference peaks. Peak 1 is NDMA; peak 2 was identified as nitrosylacetate, since a peak having the same retention time and peak shape was found when a solution of sodium nitrite in 15% acetic acid was injected. Peak 2 did not correspond to nitrite ion, and no peak corresponding to Peak 2 was formed with nitrite was dissolved in a pH 3.4 phosphate buffer.

The results of the sequential reactions indicated the progressive increase in products corresponding to NDMA as well as two components labeled as peak 4 and peak 5. The latter two peaks and NDMA were the major reaction products after 60 min. Previous experiments on gramine nitrosation indicated an NDMA yield of 40% would be expected after a 60 min. reaction time. When the nitrosation reaction was conducted in phosphate buffer (pH 3.4), the same major reaction products were seen after a 60 min. reaction time.

Spiking experiments with known standard solutions and freshly prepared 60 min. reaction products showed the following:

(1) Neither peak 4 nor peak 5 co-eluted with indole-3-carboxaldehyde, the carbonyl compound expected to form if gramine underwent nitrosative dealkylation to yield NDMA. No peak in the chromatograms of Figures 34-37 corresponded to indole-3-carboxaldehyde.

(2) Peak 3 co-eluted with a standard of indole-3-carbinol.

(3) Neither peak 4 nor peak 5 co-eluted with nitrosamine III, and nitrosamine III was not present as a reaction product after 60 min. of reaction time (Figure 38).

(4) Neither peak 4 or peak 5 co-eluted with nitrosamine IV, and nitrosamine IV was not present as a reaction product after 60 min. of reaction time (Figure 39).

Nitrosamines III and IV could both survive under the reaction conditions used, and either could have been detected if present in as little as 1% yield (based on starting amine).

From the above results, the following conclusions were drawn:

(1) Indole-3-carboxaldehyde is not a by-product from the initial reaction which leads to NDMA formation.

(2) Nitrosamines III and IV were not formed as reaction products within the time that the reaction is 40% complete.

(3) The formation of NDMA and peak 4 and peak 5 were not the result of substrate reactions with acetic acid or acetate ion, since the same products were formed in phosphate buffer containing no organic acid.

(4) Peak 4 and peak 5 were direct by-products from the reaction which produced NDMA. Identification of these products would allow a more definitive statement on the mechanism for NDMA production from gramine.

V. DISCUSSION

In malt which is produced commercially, the identification of the amine precursors of NDMA is complicated by the fact that the rootlet portion of the germinated product is removed immediately after kilning. This creates difficulty in assessing the role that roots might play in contributing to the amount of NDMA precursors, and consequently, to the level of NDMA in clean malt after kilning. It has been suggested that hordenine formed in malt roots during germination can be physically transferred to the malt husk as a result of agitation and mixing (Hardwick et al., 1981). One piece of evidence used to support this idea was an experiment which showed that samples of husk from direct-fired malt contained an average of 815 μg/kg of NDMA, and a sample of rootlets from the same malt contained 890 μg/kg of NDMA (O'Brien et al., 1980). The argument was advanced that NDMA could be found at such a level in husk only if the precursors were translocated from the roots. This argument discounts the possibility that the acrospire may also contain precursors which move toward the husk during the drying stage. A satisfactory answer to this dilemma cannot be achieved until reliable data on the nitrosatable alkaloid content of malt acrospires is available.

McFarlane (1965) reported the presence of a considerable level of hordenine in the acrospires of kilned malt. The details of McFarlane's paper indicate an awareness of the high level of hordenine in roots, and he took care to separate the acrospires from the husk. McFarlane concluded that acrospires were the main source of the hordenine found in brewery wort. If malt acrospires are a source of

nitrosatable alkaloids, as McFarlane's findings would suggest, then an interest in the gramine concentration in malt and the gramine nitrosation reaction is justified.

Formation of NDMA in Barley Malt Nitrosated Under Laboratory Conditions

The purpose for germinating malt under controlled conditions was to obtain a malt sample in which the usual handling steps were eliminated. Clean malt that had not been in physical contact with malt roots was obtained. Nitrosation of the individual malt fractions (raw barley, clean malt, and malt roots) in aqueous acid showed that malt roots contained a huge amount of NDMA precursor(s) compared to clean malt; raw barley served as the "control". The experiment represented results on only one barley cultivar (variety). The following conclusions were drawn from the results:

(1) Malt roots do not exist until germination is initiated; therefore, the germination step must be considered as a source of NDMA precursor in malted barley.

(2) Clean malt is composed predominantly of starchy endosperm, whereas the products of biosynthesis in clean malt are expected to be concentrated in the acrospires, which were not isolated in this experiment. Consequently, the nitrosation of malt roots and malt acrospires were not compared on an equal weight basis. Furthermore, the dry weight of malt roots constituted only 6.2% of the total dry weight of the malt product. On this basis, the precursor amines

of the clean malt fraction cannot be ignored as a significant factor in explaining the observed NDMA levels in direct-fired malt.

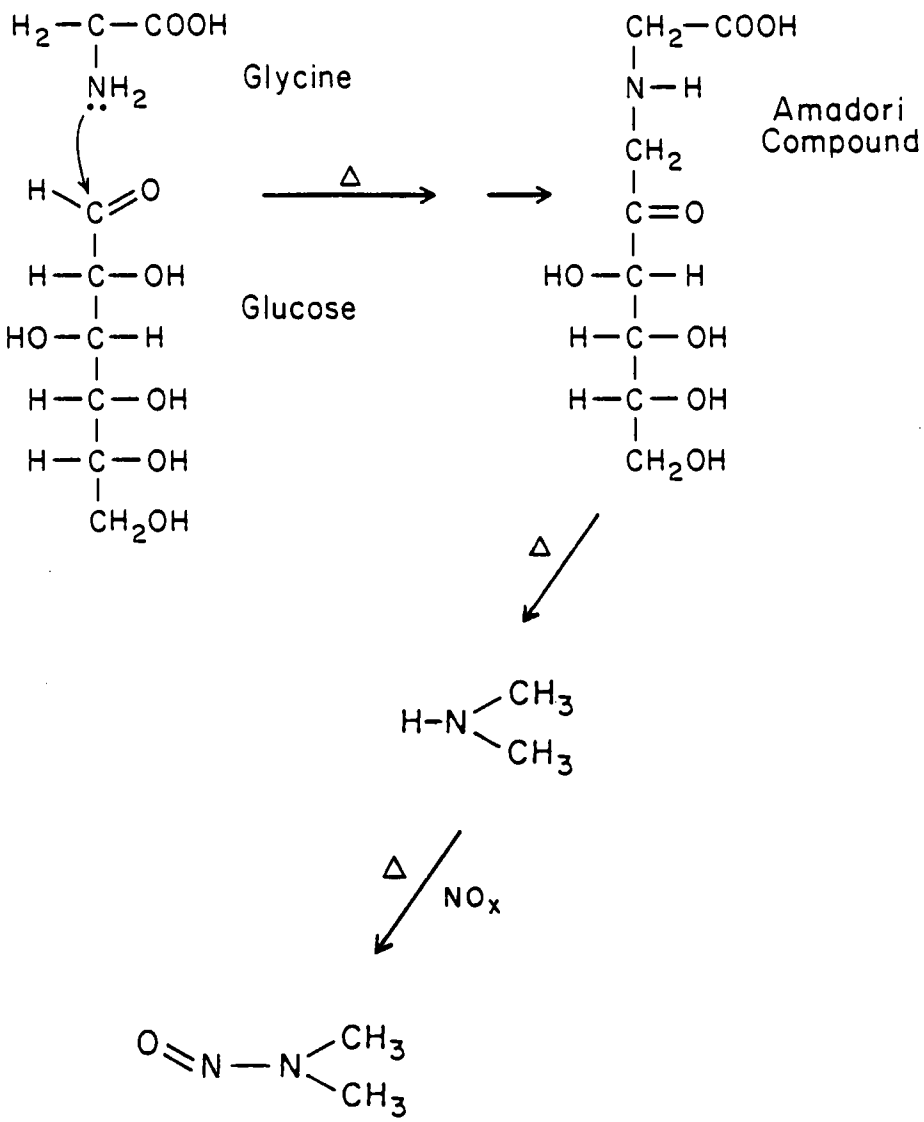
A second experiment was designed to determine if heat-drying played an important role in generation of immediate precursors to NDMA. For this experiment, two commercial malt varieties were dried with or without heat under conditions that would not result in NDMA formation. Nitrosation of the dried samples indicated that heat-drying did not cause formation of immediate precursors to NDMA. However, volatile amine precursors of NDMA that might have formed and escaped during heating were not accounted for since the electric pilot kiln could not be fitted with a suitable trap.

Heat could conceivably promote NDMA precursor formation as a result of Maillard browning reactions. For example, heat induced Maillard browning reactions between glycine and glucose would lead to the Amadori compound shown in Figure 40. Decarboxylation and fragmentation of the Amadori compound under the influence of heat could lead to formation of dimethylamine. Alternatively, Smith (1981) proposed that heat induced fragmentation of hordenine and gramine could lead to formation of dimethylamine. Unfortunately, the experiment used here was not designed for the detection of NDMA formation; dimethylamine lost by volatilization would not have contributed to the level of NDMA determined for the heat-dried samples.

In their investigation of NDMA formation in malt, brewing industry experts assumed that dimethylamine on the surface of green malt would be rapidly volatilized, since the boiling point of

Figure 40. Potential formation of an NDMA precursor as a result of Maillard browning reaction and thermally induced decomposition of an Amadori intermediate

Figure 40



dimethylamine is 7°C. But most dimethylamine would be present in the salt form $[(CH_3)_2NH_2^+X^-]$ in green malt which has a pH of approximately 5.6. So it is likely that a considerable proportion of any dimethylamine present would be in contact with NO_x nitrosating agent before the dimethylamine could be removed by volatilization. More specific analytical experiments and tracking experiments will have to be performed before a definitive statement can be made as to whether dimethylamine is an important precursor to NDMA in direct-fired malt.

Nitrosation of N-Methyltyramine and N-Methyl-3-aminomethylindole

The synthesis and nitrosation of N-methyltyramine and N-methyl-3-aminomethylindole were carried out for two reasons: (1) These secondary amines are immediate biosynthetic precursors to hordenine and gramine, respectively; consequently, both amines should be present in germinated malt. McFarlane (1965) reported an N-methyltyramine concentration of 61 ppm in the acrospires of dried malt. Schneider and Wightman (1974) reported an N-methyl-3-aminomethylindole level of 141 ppm in the four day old shoots from germinated barley seeds. (2) The secondary amines could be formed from hordenine and gramine, respectively, if nitrosation of hordenine and gramine occurred by the nitrosative dealkylation mechanism. This mode of formation for the secondary amines would be a very minor pathway compared to direct biosynthesis.

Nitrosation of N-methyltyramine in aqueous acid resulted in formation of two nitrosamines: p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine and p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine.

The formation of the second compound deserves some comment. It is well known from previous studies that the nitrosation of phenol is a rapid reaction with a mechanism independent of pH between pH 1 and pH 5 (Challis and Lawson, 1971). The reactive species for accomplishing aromatic C-nitrosation is the nitrosonium ion (Turney and Wright, 1959). In activated aromatic molecules such as phenols, the nitrosating agent can be nitrous acidium ion or nitrous anhydride (March, 1968). Phenolic compounds with no substituent in the para position form stable para-nitrosophenols; but phenolic compounds already possessing a para substituent do not form stable ortho-nitroso phenols, except under special conditions. The special conditions required to produce stable ortho-nitroso phenols were discovered by Baudisch (1940). In the Baudisch reaction, an aromatic compound is treated with a mixture of oxidized hydroxylamine in the presence of cupric-ions. A complex of the type $[Cu^{1}NO]$ is formed and attacks the aromatic ring to produce an o-quinonemonoxime. (An o-quinonemonoxime is the tautomeric form of an o-nitroso phenol.) The quinonemonoxime rearranges to a stable inner complex cupric salt. Cronheim (1947) showed that the same reaction was possible if the aromatic ring already contained the oxygen function in the form of a phenolic derivative.

Without such stabilization, o-nitroso phenols are subject to rapid oxidation. Thus, Challis and Higgins (1973) obtained only o-nitrophenols during the nitrosation of para-substituted phenols in perchloric acid. It is known that one of the decomposition products of nitrous acid is nitric acid, and Turney and Wright (1959) proposed

that nitric acid is responsible for oxidation of intermediate o-nitrosophenols to o-nitrophenols. Direct nitration by nitronium ion (NO_2^+) was ruled out because formation of NO_2^+ is not observed in the low acidity solutions used for N-nitrosation. Challis and Higgins (1973) found that o-nitroso phenols made independently by Cronheim's method were oxidized to o-nitrophenols even in distilled water. This implies that the o-quinonemonoxime may be the species subject to facile oxidation. At present, no definitive mechanism for o-nitrosophenol oxidation is available.

Nitrosation of N-methyl-3-aminomethylindole in aqueous acid also resulted in the formation of two N-nitrosamines: N-nitroso-N-methyl-3-aminomethylindole (III) and N^1-nitroso-N-nitroso-N-methyl-3-aminomethylindole (IV). The major product was nitrosamine IV which underwent denitrosation at the N^1-indolic position in solution after exposure to white light. Standard solutions of IV had to be kept in the dark at 0°C or lower. Denitrosation of most N-nitrosamines can be carried out only by photolysis in the ultraviolet (Chow, 1973) or in strong acids under the influence of nucleophilic catalysis (Biggs and Williams, 1975). However, nitrosocarbazole and 3-nitronitrosocarbazole (both of which have a nitroso group at the indolic nitrogen) lose the N-nitroso group readily in solution, making them good reagents for "transnitrosation" reactions (Smith, 1966).

Nitrosation of the indolic-NH function was also reported by Bonnett and Holleyhead (1974) as a result of nitrosation of tryptophan derivatives in mild acid solution. Evidence for nitrosation of the N^1-indolic position of N-acetyl-tryptophan methyl ester included

infrared spectroscopy, ultraviolet spectroscopy, and ^{15}N-NMR measurements (Bonnett and Holleyhead, 1975).

Nitrosation of Hordenine in Aqueous Acid

The production of NDMA from the nitrosation of hordenine was studied under a number of reaction conditions and at three different temperatures. Both the yield of NDMA at elevated temperature (65°) for prolonged time (16 hr.) and the reaction rate as a function of temperature were consistent with the results seen for the nitrosation of other N,N-dimethyl-substituted tertiary amines. For example, Lijinsky et al. (1972b) found that nitrosation of N,N-diemthyldodecylamine at 90° for 16 hr. with a four-fold excess of nitrite yielded 4.5% NDMA. Nitrosation of hordenine at 65° for 16 hr. under very similar conditions yielded 11% NDMA. The activation energy for the production of NDMA from hordenine was found to be 23.6 Kcal/mol for the temperature range 23° to 65°. Gowenlock et al. (1979) found the activation energy for the production of N-nitrosodiethylamine from triethylamine to be 20.3 kcal/mole over the temperature range 58°-81°. Triethylamine was found to undergo nitrosative dealkylation, since the products of its reaction with nitrite in acetic acid were N-nitrosodiethylamine, acetaldehyde, and nitrous oxide.

An investigation of the reaction products from nitrosation of hordenine was undertaken in order to obtain further evidence that hordenine undergoes nitrosative dealkylation. The product from nitrosation of hordenine at 65° in a ten-fold excess of nitrite was used for the analysis, since these conditions gave reasonable yields of

NDMA. HPLC of the reaction products showed that p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine (II) was a product of hordenine nitrosation after two hr. and its yield increased steadily up to a 12 hr. reaction time. Analysis of the same reaction products showed that p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine (I) did not appear to be a product of hordenine nitrosation. Nitrosamine I could not have been present at more than 0.3% in any reaction product for reaction times of 2 hr., 4 hr., 8 hr., and 12 hr.

It was expected that nitrosation of hordenine by nitrosative dealkylation should give a higher combined yield of nitrosamine I and nitrosamine II than the yield seen for NDMA. At the present time, it appears that NDMA is the major N-nitrosamine formed from the nitrosation of hordenine. The reaction is certainly more complicated than that predicted by the original nitrosative dealkylation mechanism. The presence of the phenolic moiety in hordenine opens up some new reaction routes which could be operating in addition to the expected nitrosative cleavage reaction. For example, in a study of the reaction of bovine serum albumin with nitrous acid at pH 4 and 37°, Knowles et al. (1974) found that hydrolysis of the reaction product gave two abnormal derivatives of tyrosine in addition to the expected amino acids. The two products were identified as o-nitrotyrosine and o-hydroxytyrosine (3,4-dihydroxyphenylamine:DOPA). The 3,4-dihydroxyphenylaline was postulated to arise via thermal decomposition of nitrous acid, which occurs by the following overall reaction (Turney and Wright, 1959):

$$3HOHO \rightleftharpoons HNO_3 + 2NO + H_2O \quad (35)$$

The nitric oxide produced could react with an intermediate o-nitroso-phenol to give o-diazotyrosine which would be hydrolyzed in acid to DOPA. Philpot and Small (1938) previously showed that a similar sequence of reactions occurred when p-cresol reacted with nitrous acid. The diazo-derivative was verified by coupling with β-naphthol. In a related reaction, Tedder and Theaker (1959) found that nitric oxide converted nitrosobenzene into a dizaonium derivative which could be coupled to β-naphthol.

In light of the above reports, a more comprehensive reaction sequence is postulated to explain the reaction of hordenine with nitrous acid at elevated temperature (Figure 41). In this sequence, the ring nitrosation and oxidation products are shown to from before the products of nitrosative dealkylation in keeping with the rapid rate expected for C-nitrosation of phenols compared with the rate of nitrosative cleavage (Challis, 1973).

Further investigation of the hordenine nitrosation reaction should include the following studies:

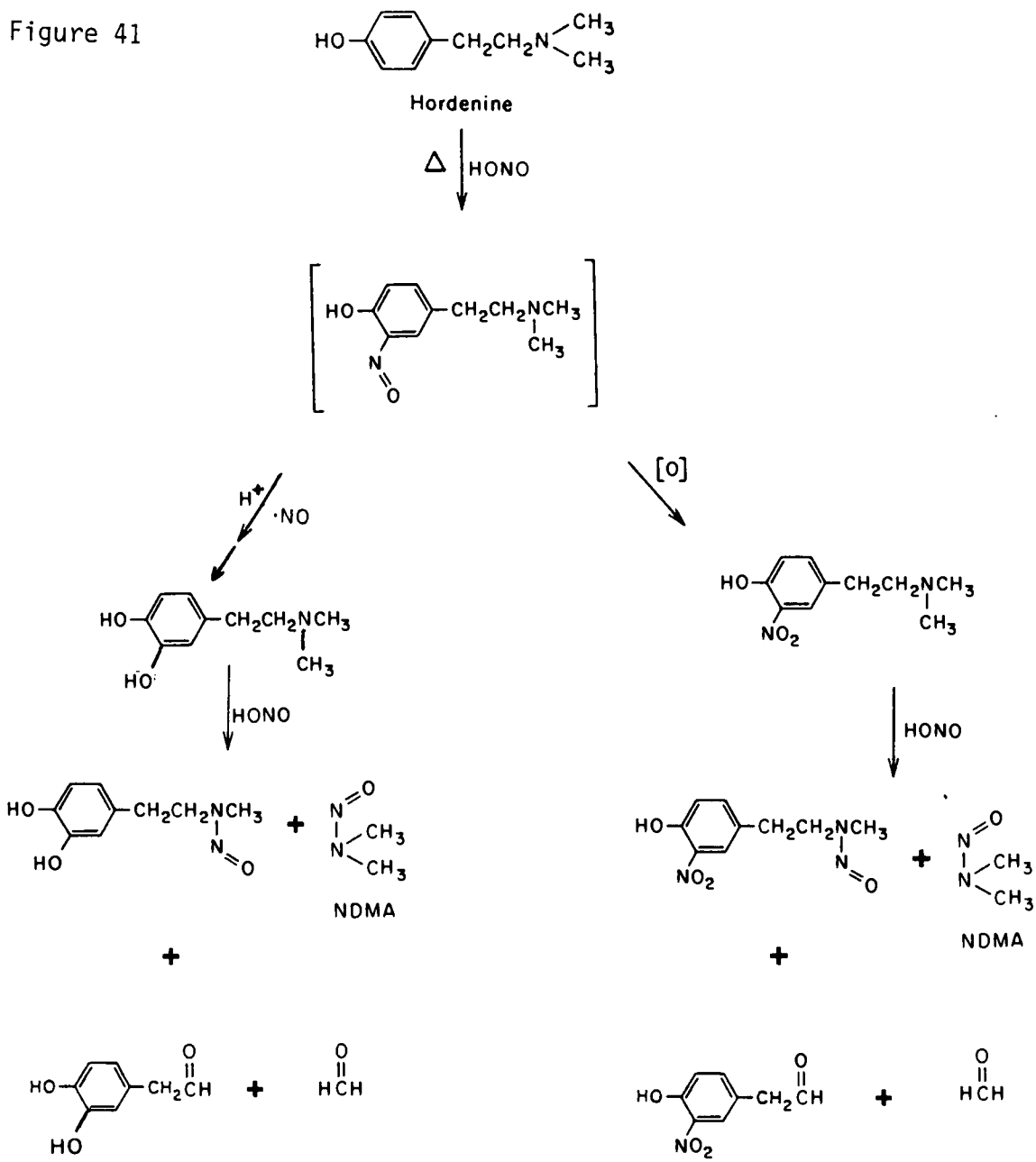
(1) Determination of the identity and total yield of "residual" amines: products which have undergone C-nitrosation and oxidation or hydrolysis but still retain the N,N-dimethyl-substituted tertiary amino-group.

(2) Identification of other nitrosaminesin addition to NDMA and nitrosamine II.

(3) Determination of the presence of at least one of the

Figure 41. Proposed reaction products from the nitrosation of hordenine at elevated temperature

Figure 41



carboxyl compounds expected to form when NDMA is produced by nitrosative dealkylation.

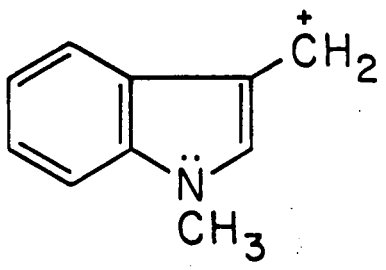
Nitrosation of Gramine in Aqueous Acid

The history of gramine reaction chemistry dates to the original isolation of the compound from natural sources. Madinaveitia (1937) isolated gramine from the leaves of the reed plant Arundo donax. When an attempt was made to prepare the methiodide in methanolic KOH, only tetramethylammonium iodide and a compound identified as 3-methoxymethylindole were obtained. When gramine was treated with methyl iodide in methanol, trimethylamine was liberated.

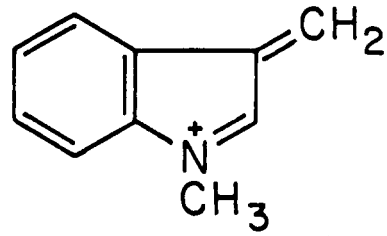
Shortly after the above result was obtained, it was discovered that gramine and N^1-methylgramine were good alkylating agents for making new carbon-carbon bonds. Salts of Mannich bases like gramine were found to be much more reactive toward alkylation than the salts of N,N-dimethylbenzylamine. Snyder et al. (1944) found that gramine methiodide (prepared by treating gramine with neat methyl iodide) reacted with the sodium salt of diethylmalonate to make a susbstituted malonic ester in high yield at room temperature. The same reaction with quaternary salts of benzylamine required heat and rarely gave greater than 50% yield of the alkylated product. The reactivity of gramine as an alkylating agent became the basis of a patented synthesis of dl-tryptophan (Snyder and Smith, 1944). Gramine methiodide was found to react smoothly with the sodium salt of acetoaminomalonic ester to give the 3-methylindole derivative of the ester. Saponification, decarboxylation, and alkaline hydrolisis gave

dl-tryptophan in high yield. The same synthesis was possible when gramine itself was heated with ethyl acetoaminomalonate in refluxing toluene or xylene (Howe et al., 1945).

The first mechanistic proposal to explain facile alkylation with gramine was offered by Snyder and Eliel (1948). Reaction of N^1-methylgramine methiodide with aqueous sodium cyanide gave mostly N^1-methyl-3-indoleacetonitrile and a small amount of N^1-methyl-2-cyano-3-methylindole. To explain the results, the carbonium ion (V) or the imminium ion (VI) were proposed as intermediates in the formation of the two cyano derivatives. N^1-Methyl-2-cyano-3-methyl-indole would arrise by attack of CN- on the C-2 position of VI, followed by rearrangement to give a methyl group at C-3.



V



VI

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 large performance for NDMA formation from gramine was not predicted on the basis of the usual steric preferances 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-nitroso benzylethylamine 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-carbinyl group would be the most favored pathway during nitrosation of gramine if the usual nitrosative dealkylation mechanism were operating.

The experimental results which indicate that gramine does not undergo nitrosative dealkylation in nitrous acid can be summarized as follows:

(1) The reaction with nitrous acid to yield NDMA is very fast even at room temperature. The initial rate of gramine nitrosation to yield NDMA appears to be nearly as fast as the rate of nitrosation of the secondary amine dimethylamine. The activation energy (E_a=14.2 Kcal/mol) for the transformation of gramine to NDMA at pH 3.4 was reasonably close to that expected for the nitrosation of a secondary amine. For example, Fan and Tannenbaum (1973) found an activation energy of 10 Kcal/mol for the nitrosation of morpholine, and Mirvish et al. (1973) found an activation energy of 13 Kcal/mol for the nitrosation of proline. In contrast, the activation energy observed for the nitrosative dealkylation of triethylamine at pH 3.8 was 20.3 Kcal/mol (Gowenlock et al., 1979).

(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.

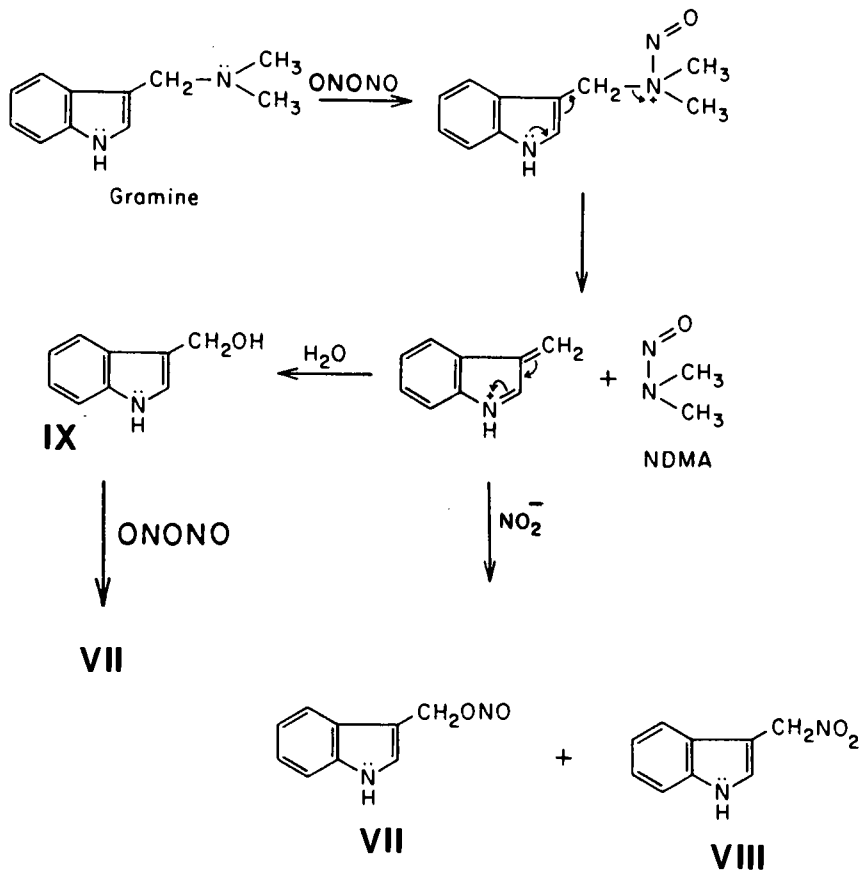
(3) Investigation of the reaction products from nitrosation at room temperature showed that indole-3-carboxaldehyde was not formed after a time sufficient for obtaining a 40% yield of NDMA. Indole-3-carboxaldehyde is the expected carbonyl by-product if NDMA were formed from gramine as a result of nitrosative dealkylation. A product was observed which co-eluted on HPLC with indole-3-carbinol.

(4) Further investigation of the reaction products at room temperature showed the absence of N-nitroso-N-methyl-3-amionomethylindole and N^1-nitroso-N-nitroso-N-methyl-3-aminomethylindole, the two nitrosamines expected to form in addition to NDMA if gramine were subject to nitrosative dealkylation.

In response to these experimental results, the mechanism illustrated in Figure 42 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 could result in formation of the nitrite ester (VII) and the nitro compound (VIII). 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 (IX), which could be nitrosated to give the nitrite ester (VII). The nucleophilic attack of alcohols on inorganic acid halides (e.g. NOCl) or

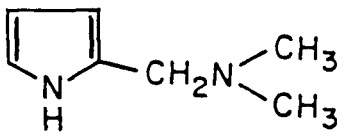
Figure 42. Proposed mechanism for the nitrosation of gramine

Figure 42

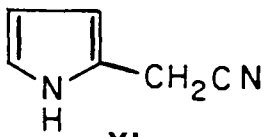


inorganic acid anhydrides (e.g. N_2O_3) is the classic reaction for production of nitrite esters (March, 1968).

Shortly after the initial results on the nitrosation of gramine were reported (Mangino et al., 1981), Leoppky and Outram (1982) prepared the tertiary amine 2-(N,N-dimethylaminomethyl) pyrrole (X). Compound X is a Mannich base analog of gramine. Nitrosation of X in



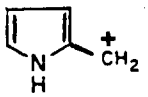
X



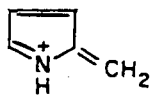
XI

aqueous nitrous acid at 25° resulted in NDMA formation. Reaction of X with a ten-fold excess of nitrite in glacial acetic acid at 65° resulted in an 80% yield of NDMA in five minutes. NDMA was the only N-nitrosamine detected. The remaining reaction product was amorphous material which apparently was the result of degradation of the pyrrole ring. Nevertheless, one mechanistic experiment was attempted. A cold solution of nitrosyltetrafluorborate: pyridine in dichloromethane was added slowly to a solution of X in dichloromethane at -78°. The 18-crown-6-ether complex of KCN in dichloromethane was added and the mixture warmed to room temperature. Chromatography on silica gel gave NDMA and pyrrole-2-acetonitrile (XI).

The results were consistent with attack of CN^- on the carbonium ion (XII) or the imminium ion (XIII).



XII



XIII

Further experimentation on the nitrosation of gramine should focus on the following points:

(1) The major initial reaction products from gramine nitrosation should be isolated and characterized. Identification of these products will have a direct bearing on establishing the validity of the mechanism illustrated in Figure 42.

(2) The presence of indole-3-carbinol in the initial reaction product should be verified.

(3) The nitrosation of gramine should be conducted in the presence of other nucleophiles, especailly CN^-, to determine if indole-3-acetonitrile is formed. Indole-3-acetonitrile is a commercially available compound and should be a more stable reaction product than the nitrite ester VII.

(4) The true optimum pH for conversion of gramine to NDMA in-dilute acid should be determined in the absence of acetic acid or nucleophiles. In many of the experiments described in this study. A pH of 3.4 was used so that formation of NDMA from gramine could be directly compared with the formation of NDMA from dimethylamine, for which the pH optimum of nitrosation is 3.4. No assumption was made that

pH 3.4 was also the optimum pH for conversion of gramine to NDMA.

VI. SUMMARY

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. The two amines which are likely candidates as precursors to NDMA are the phenolic alkaloid hordenine and the indole alkaloid gramine.

The presence of these two alkaloids in germinated malt provides a rationale for two important observations: (1) the relatively high level of NDMA found in direct-fired, unsulfured malt, and (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. The nitrosation of both alkaloids was studied using aqueous acid as the model system since nitrosation mechanisms in this system are reasonably 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 highly susceptible to nitrosation to give NDMA. The nitrosation reaction of gramine showed none of the characteristics usually observed for the

nitrosative dealkylation of a tertiary amine. The reactivity of gramine was determined to be a result of the indole-3-carbinyl group substitution on the amino nitrogen atom. Nitrosation of gramine apparently represents a new type of nitrosation mechanism in which the product-determining steps are governed by electronic factors. In contrast, for the nitrosation of most other tertiary amines, the product-determining steps are governed primarily by steric factors, and electronic factors are of minor importance.

Hordenine was less susceptible than gramine toward nitrosation to give NDMA. Significant yields of NDMA were obtained from hordenine only at elevated temperature for prolonged times in a considerable excess of nitrous acid. The nitrosation of hordenine showed some of the characteristics expected for nitrosative dealkylation of a tertiary amine. However, NDMA appeared to be the major N-nitrosamine formed. This was not the expected result based on the proposed mechanism for nitrosative dealkylation of other tertiary amines. Two other N-nitrosamines expected to be formed from hordenine were p-hydroxy-m-nitro-N-nitroso-N-methyl-2-phenylethylamine and p-hydroxy-N-nitroso-N-methyl-2-phenylethylamine. The nitro-compound was formed, but in considerably lower yield than NDMA during the nitrosation of hordenine. The latter compound was not detected as a product of hordenine nitrosation at 65°. The nitrosation of hordenine is complicated by the fact that alternative pathways leading to C-nitrosation are available since hordenine is a phenolic compound activated toward electrophilic substitution. The potential for aromatic substitution is probably more important kinetically than the potential for nitrosative dealkylation.

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 prime candidates for precursors to NDMA. Hordenine may be present at higher average levels than gramine in germinated malt, but gramine may be the more significant precursor to NDMA from the standpoint of kinetics and reactivity.

The importance of nitrogen oxides (NO_x) as environmental nitrosating agents was introduced through the studies of Challis and coworkers on the reactions of NO_x with secondary amines. The formation of NDMA in malt indicates that the reaction of NO_x with tertiary amines is also a likely possibility. The reaction has been studied only in the gas phase (Pitts et al., 1978), and clearly deserves further investigation. The direct reaction of NO_x with tertiary amines would be the most realistic model system for studying N-nitrosamine formation in direct-fired malt and other direct-fired dried food.

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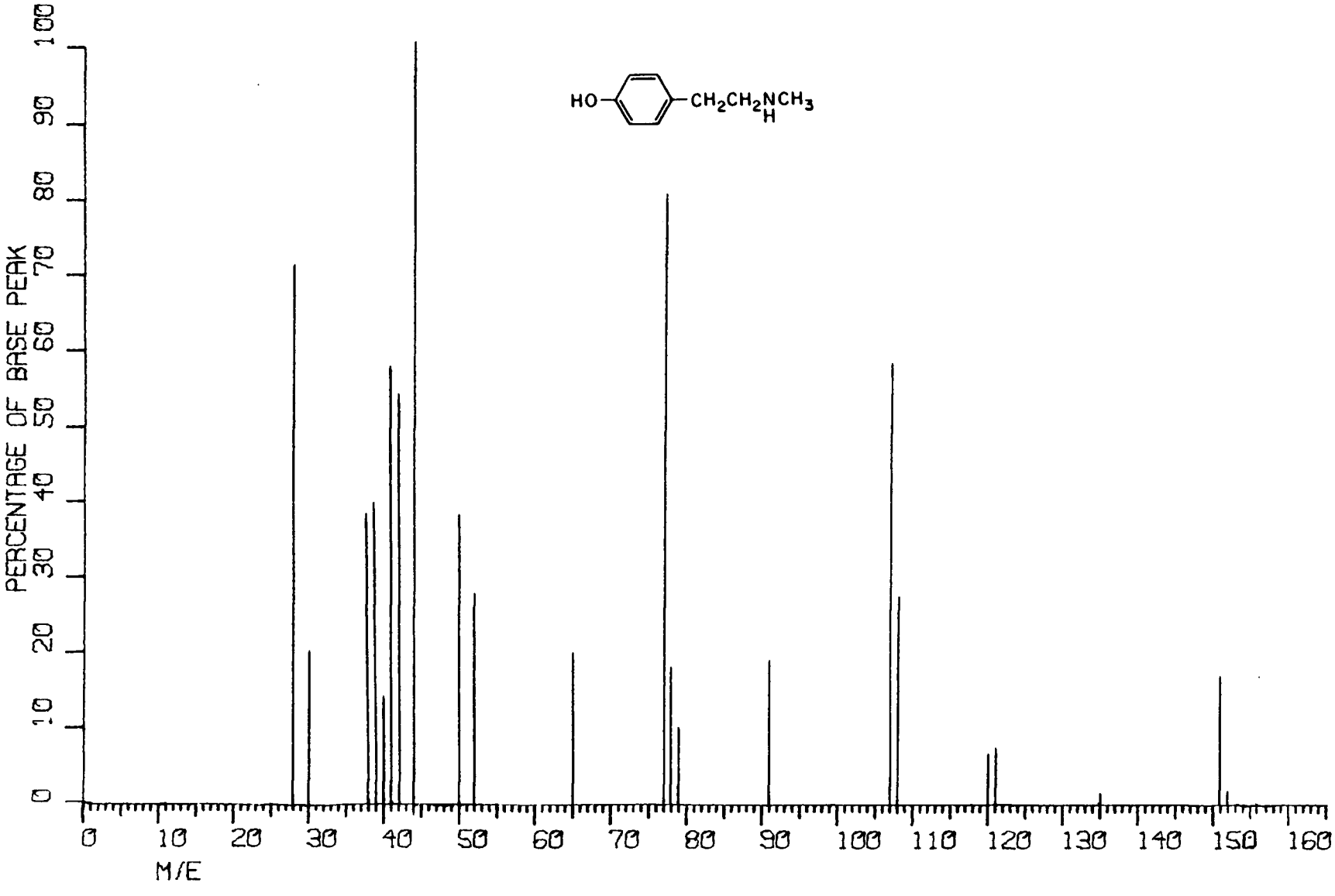
APPENDIX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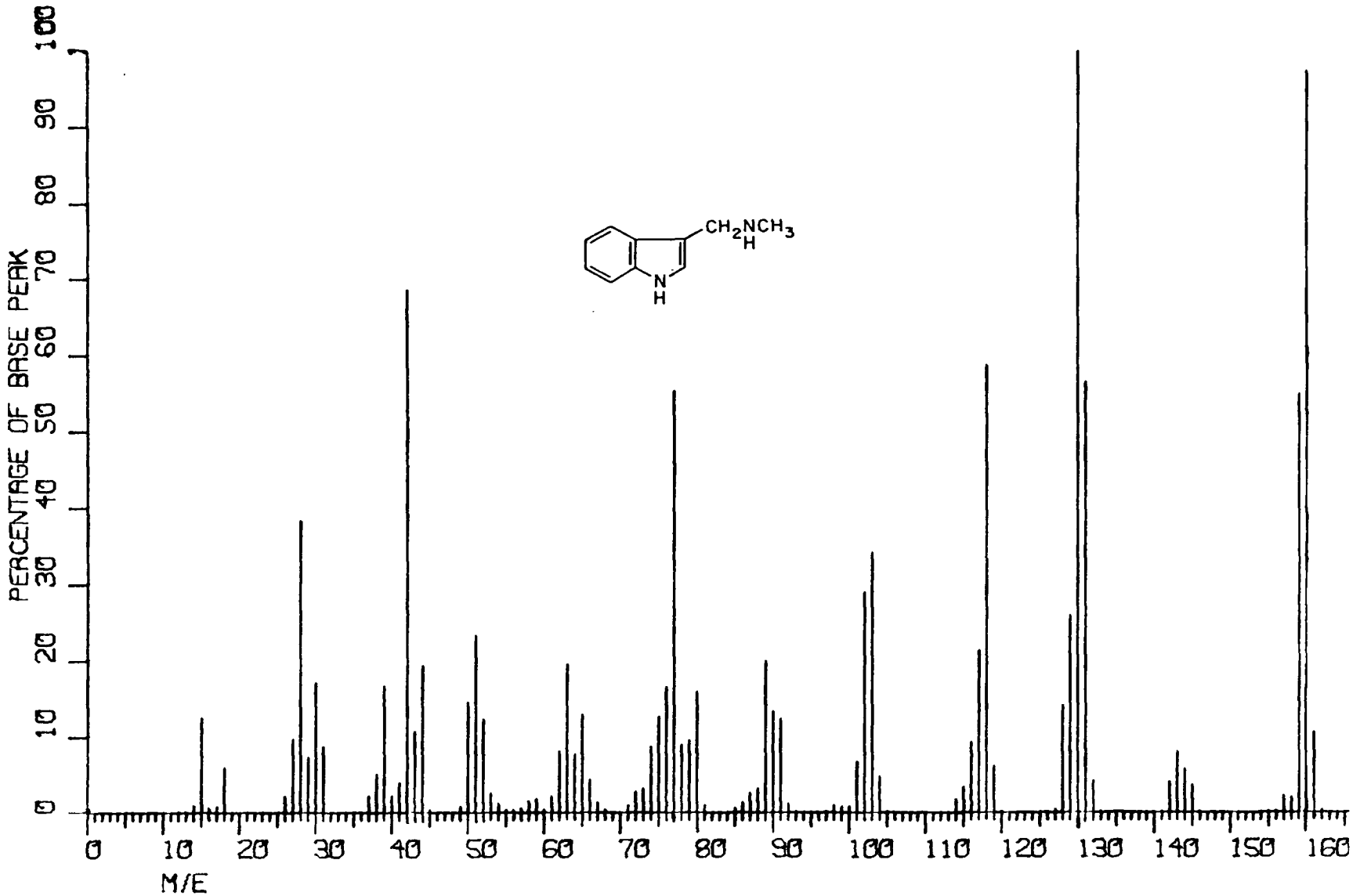
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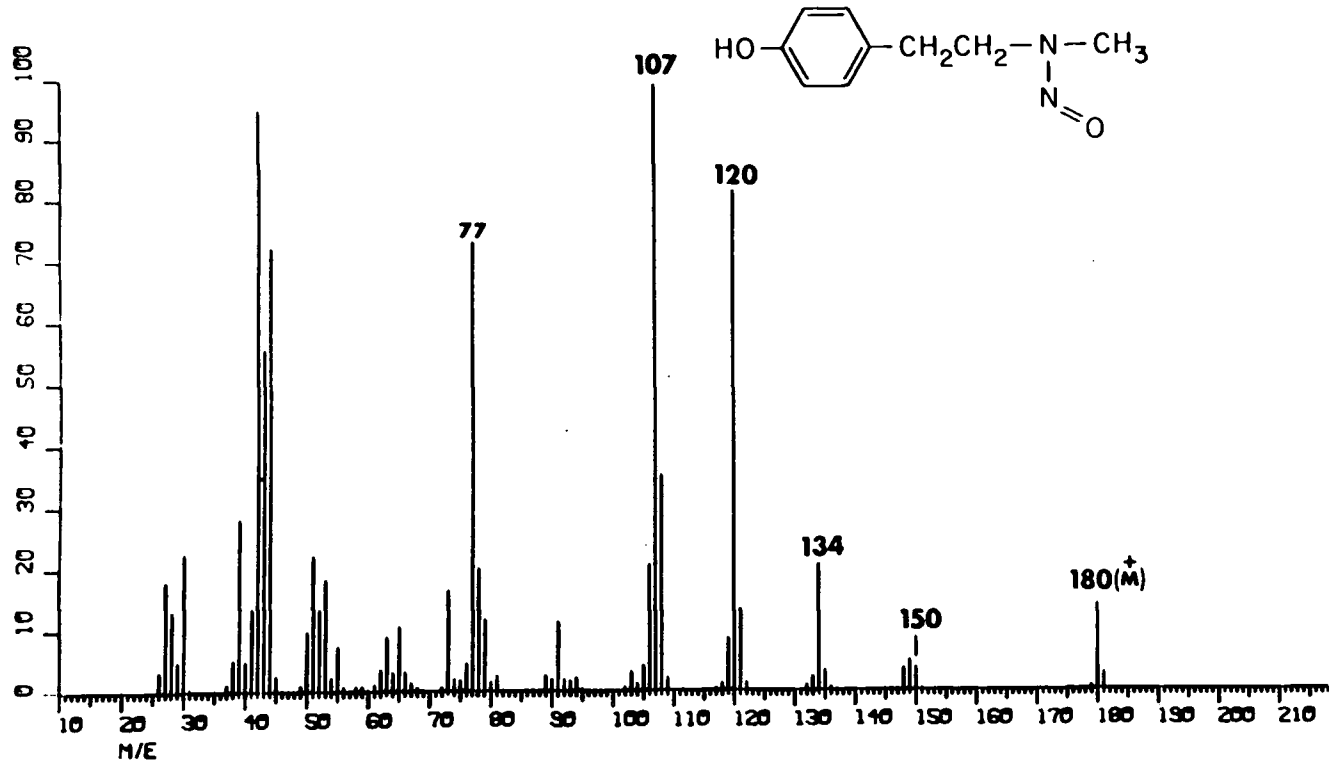
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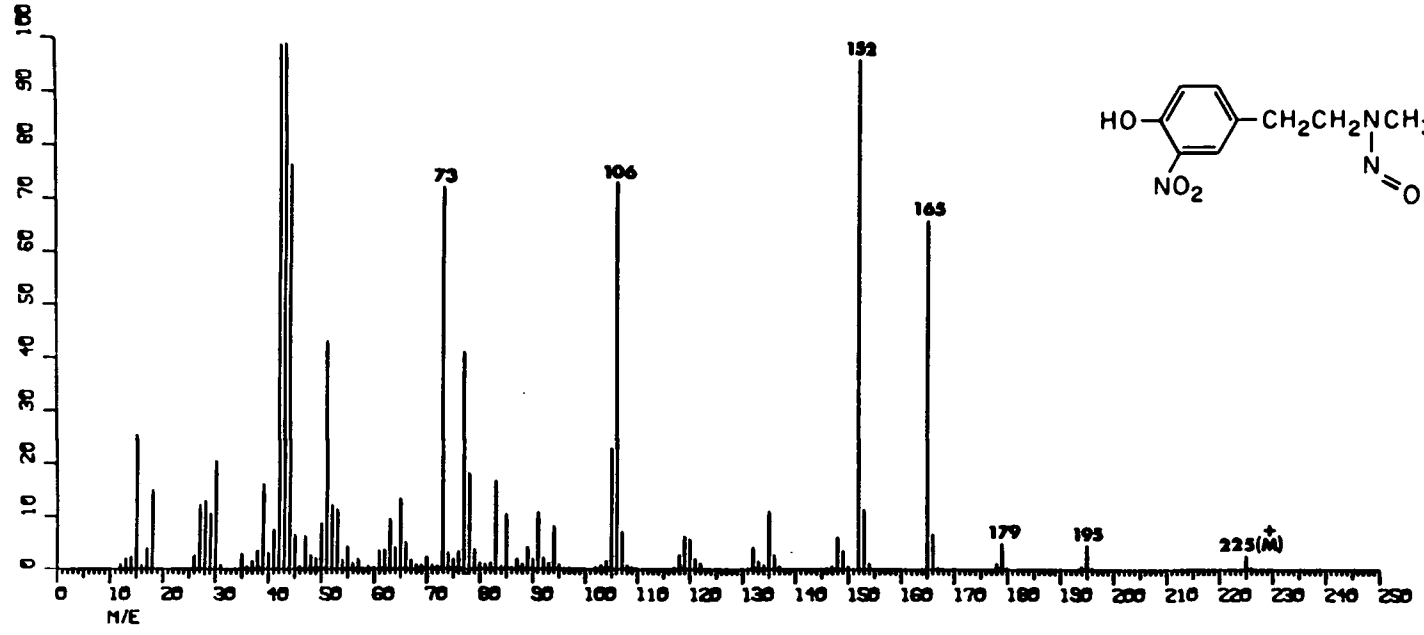
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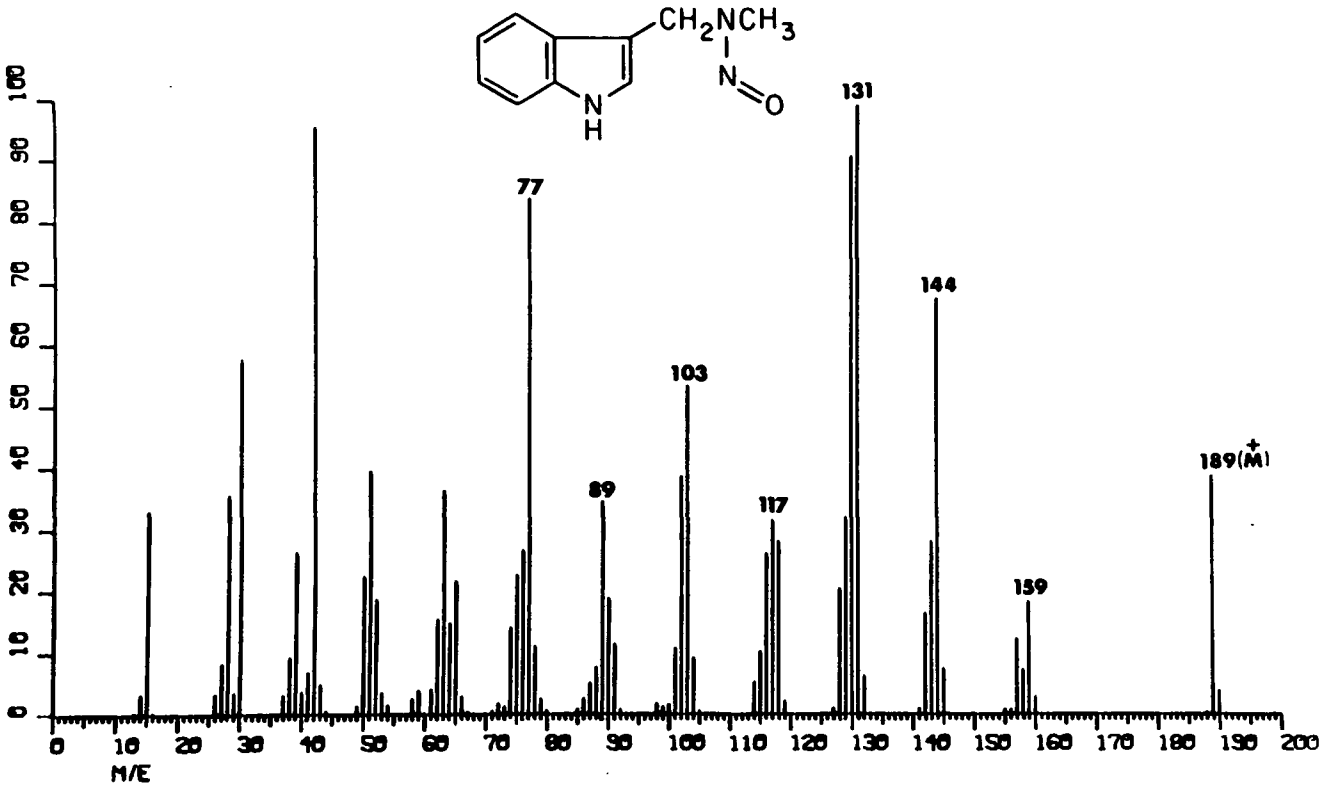
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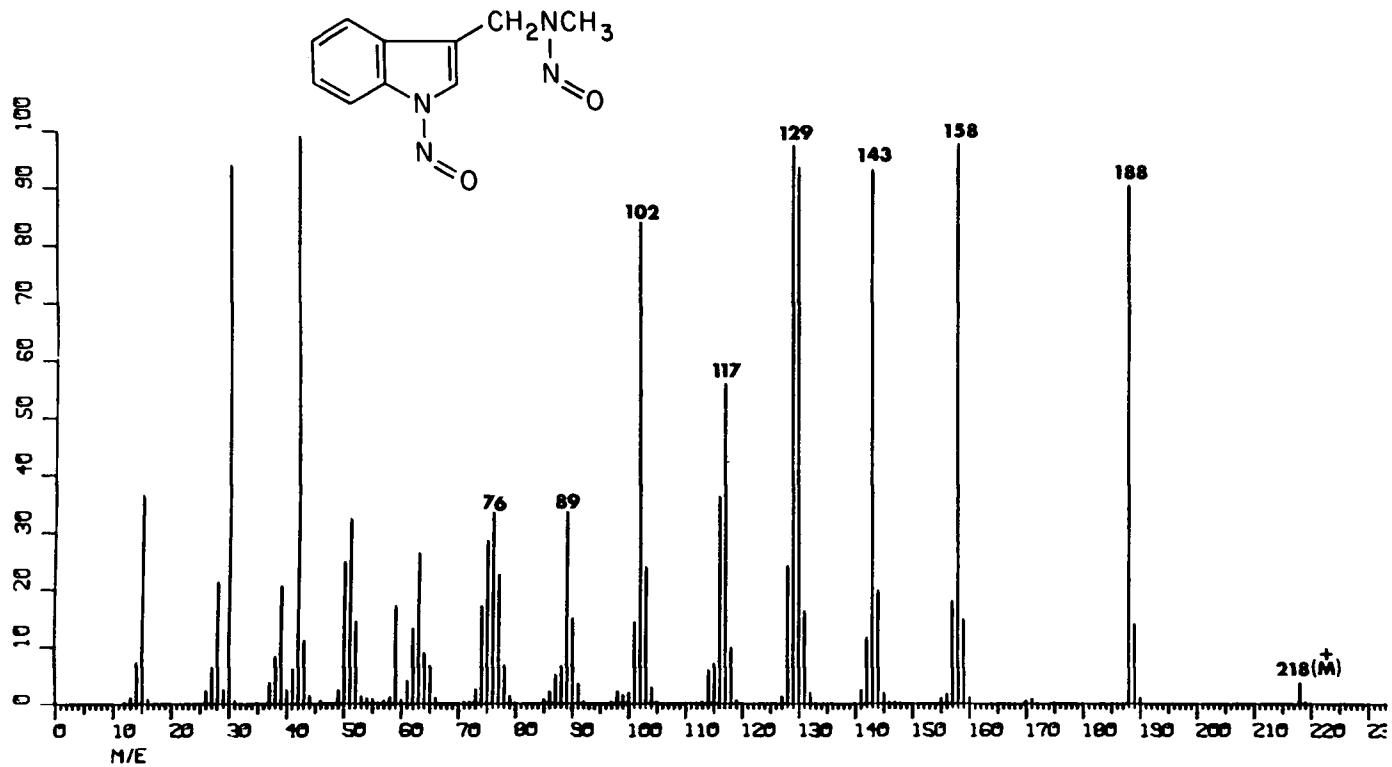
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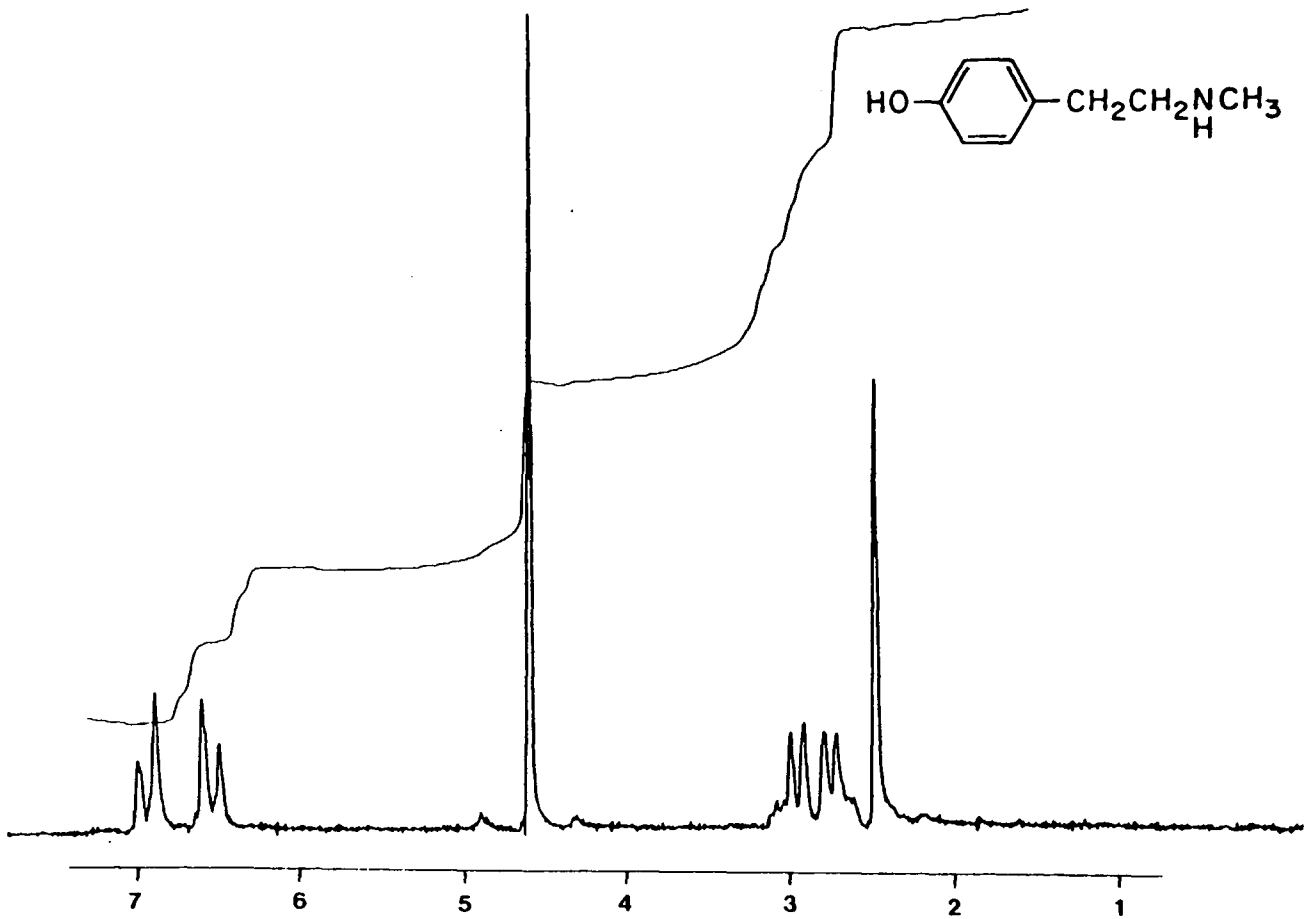
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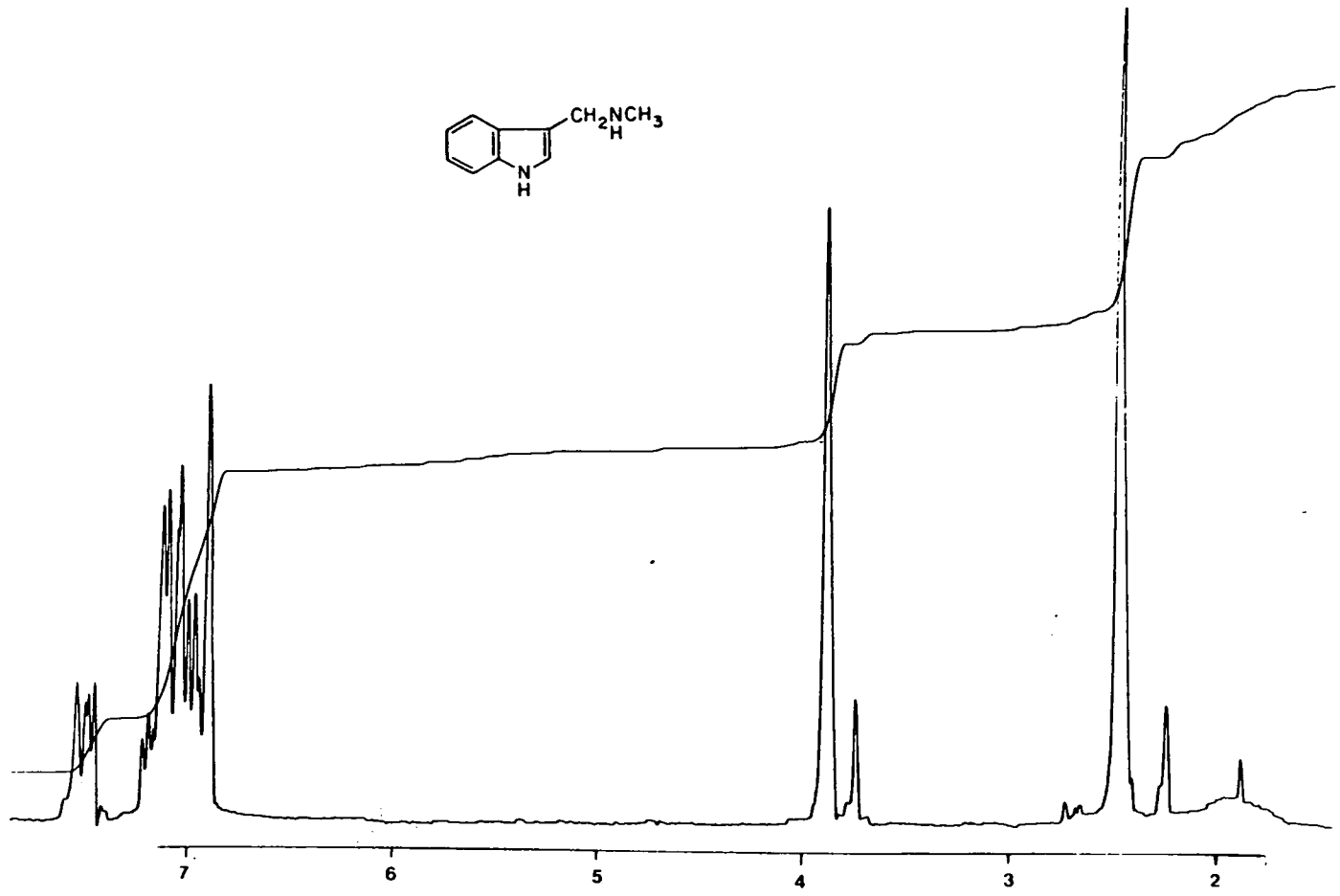
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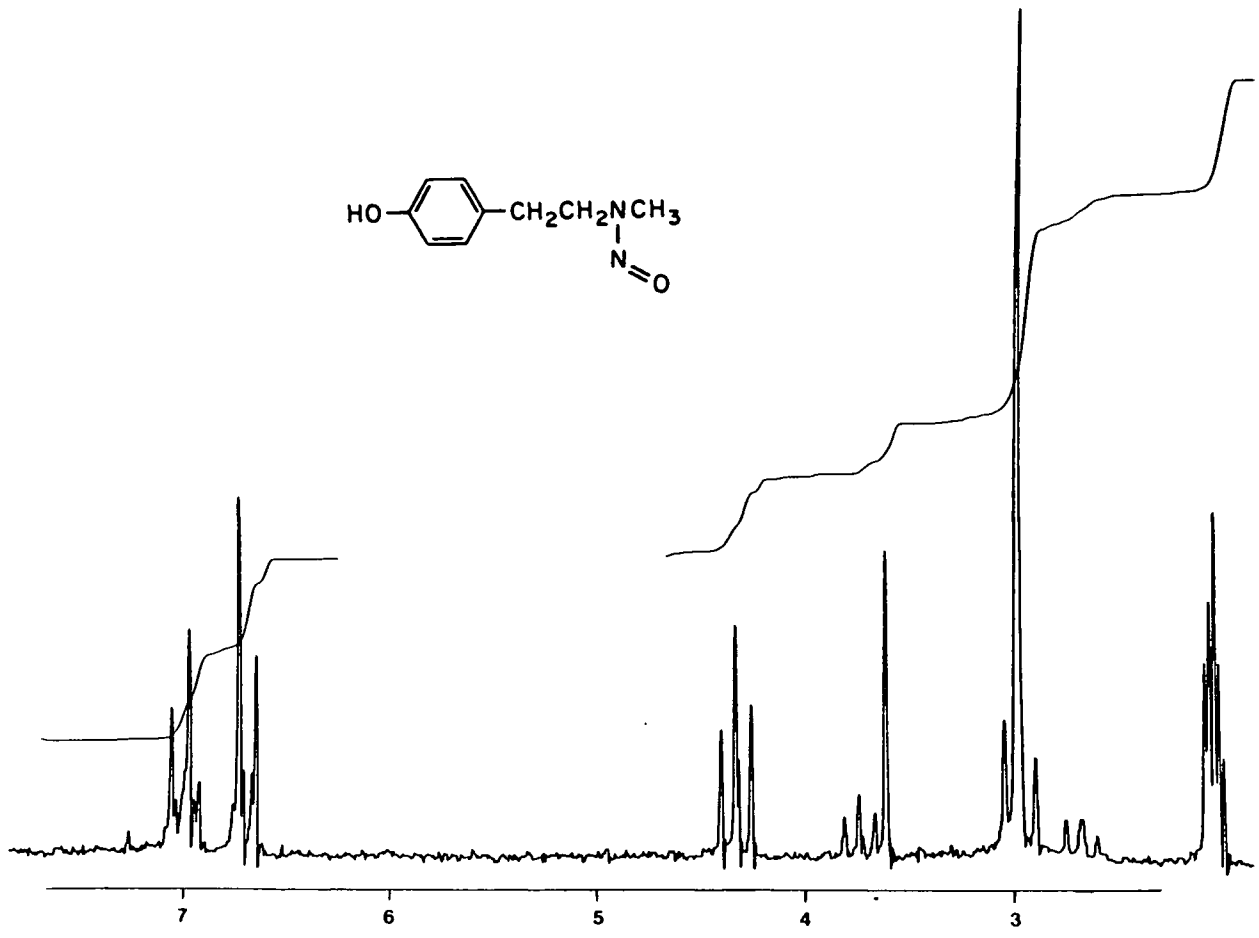
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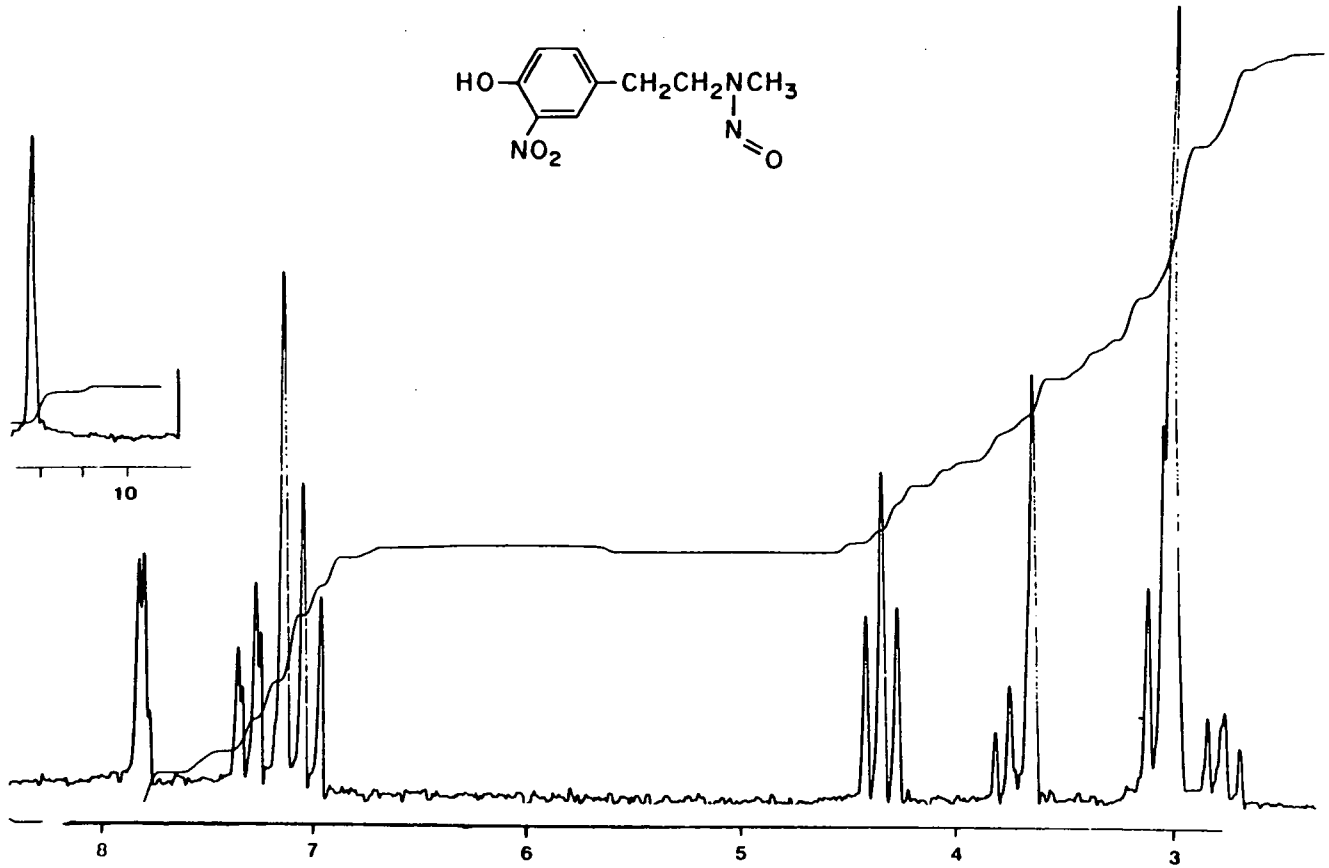
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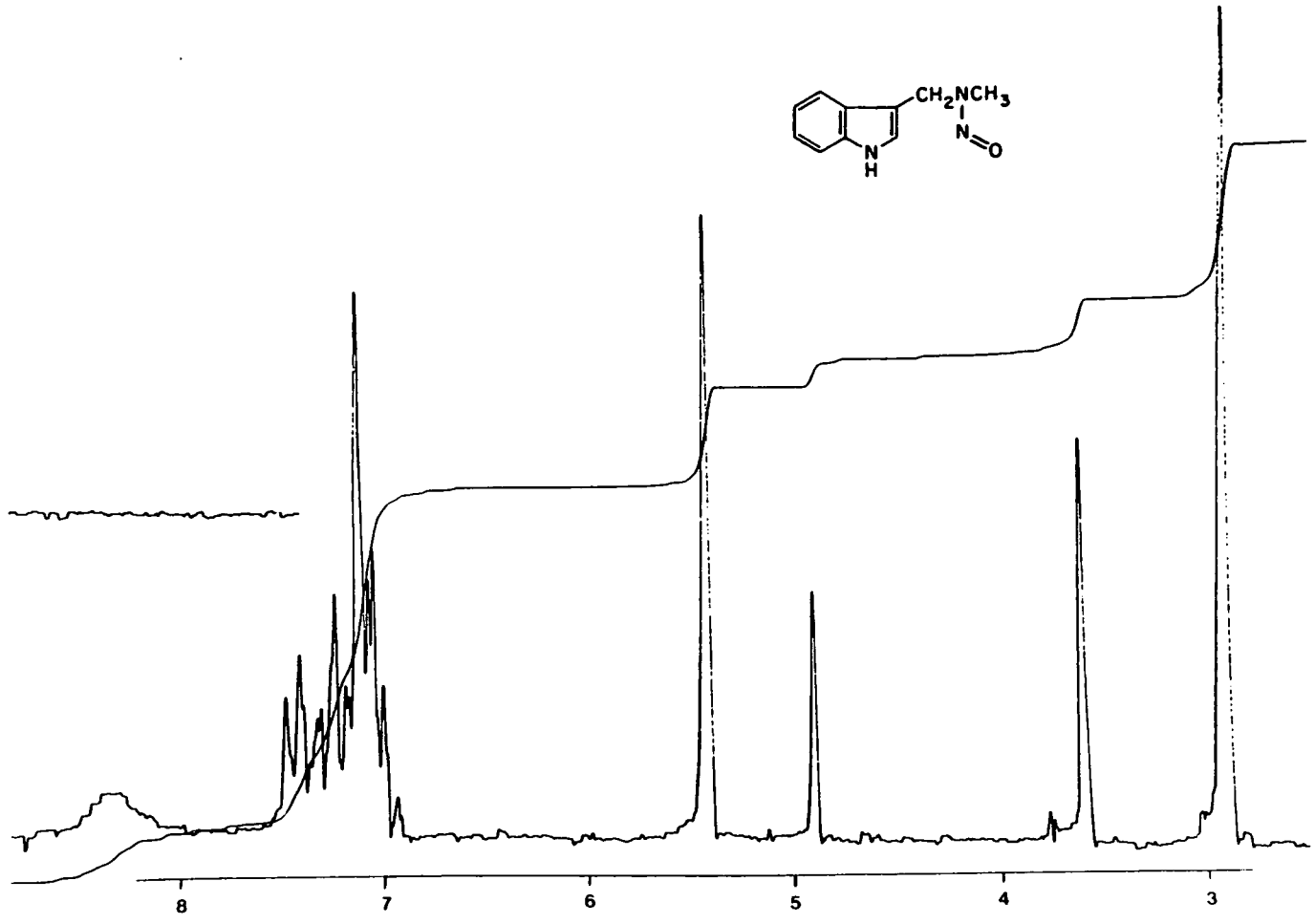
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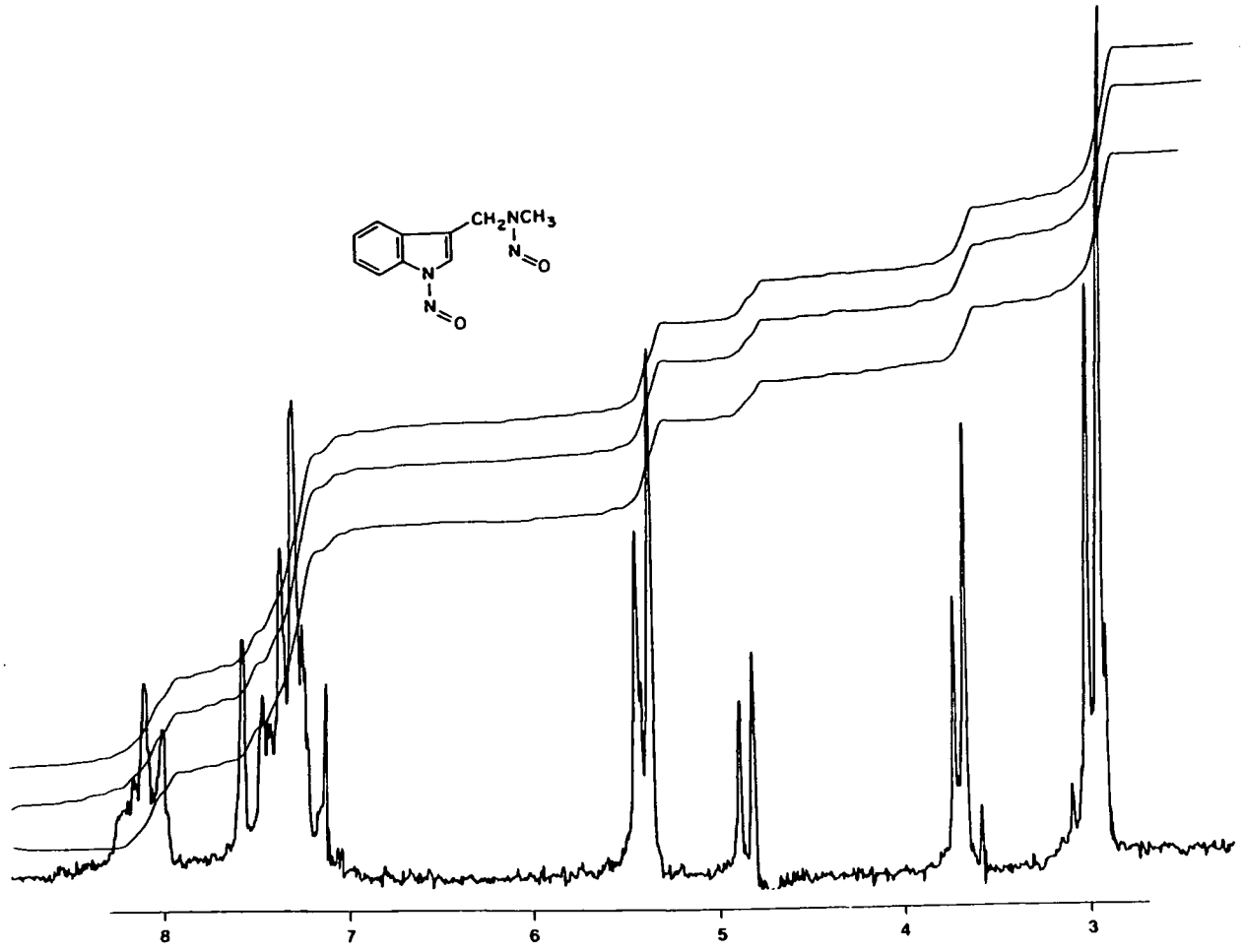
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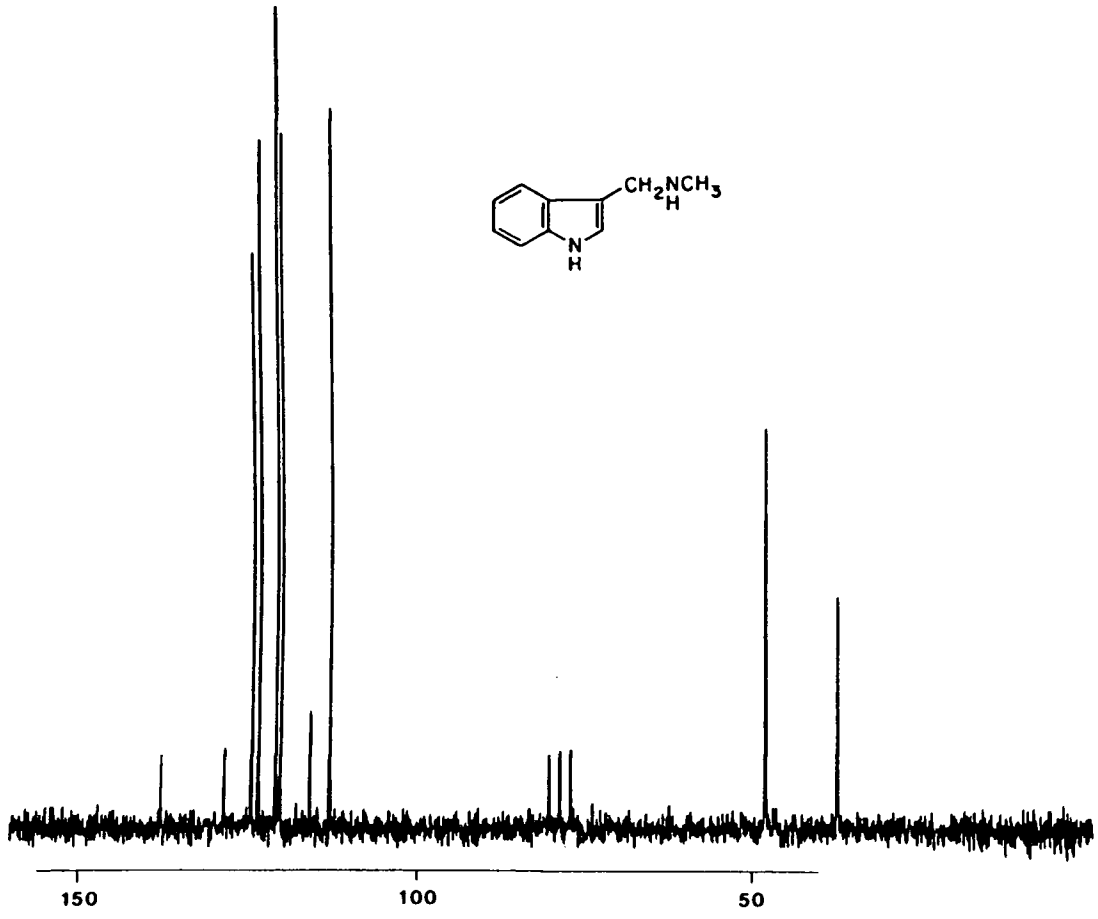
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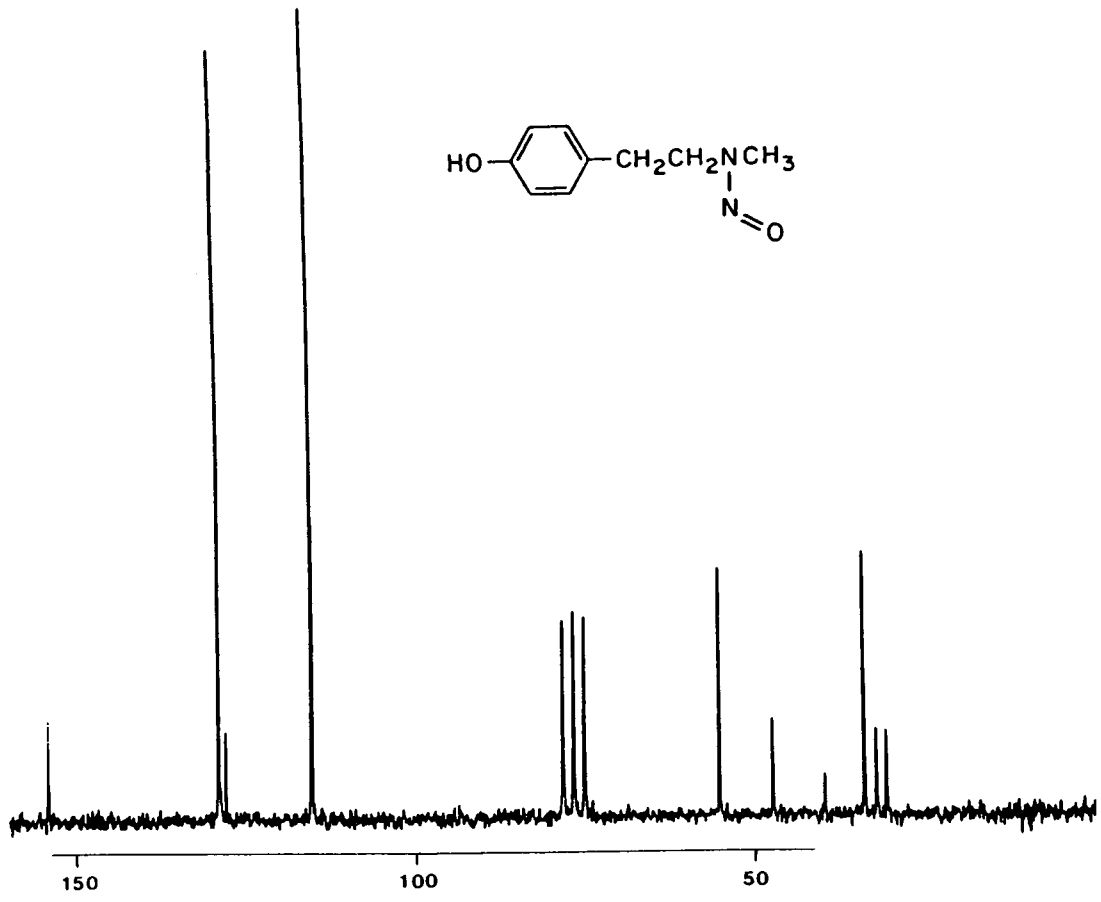
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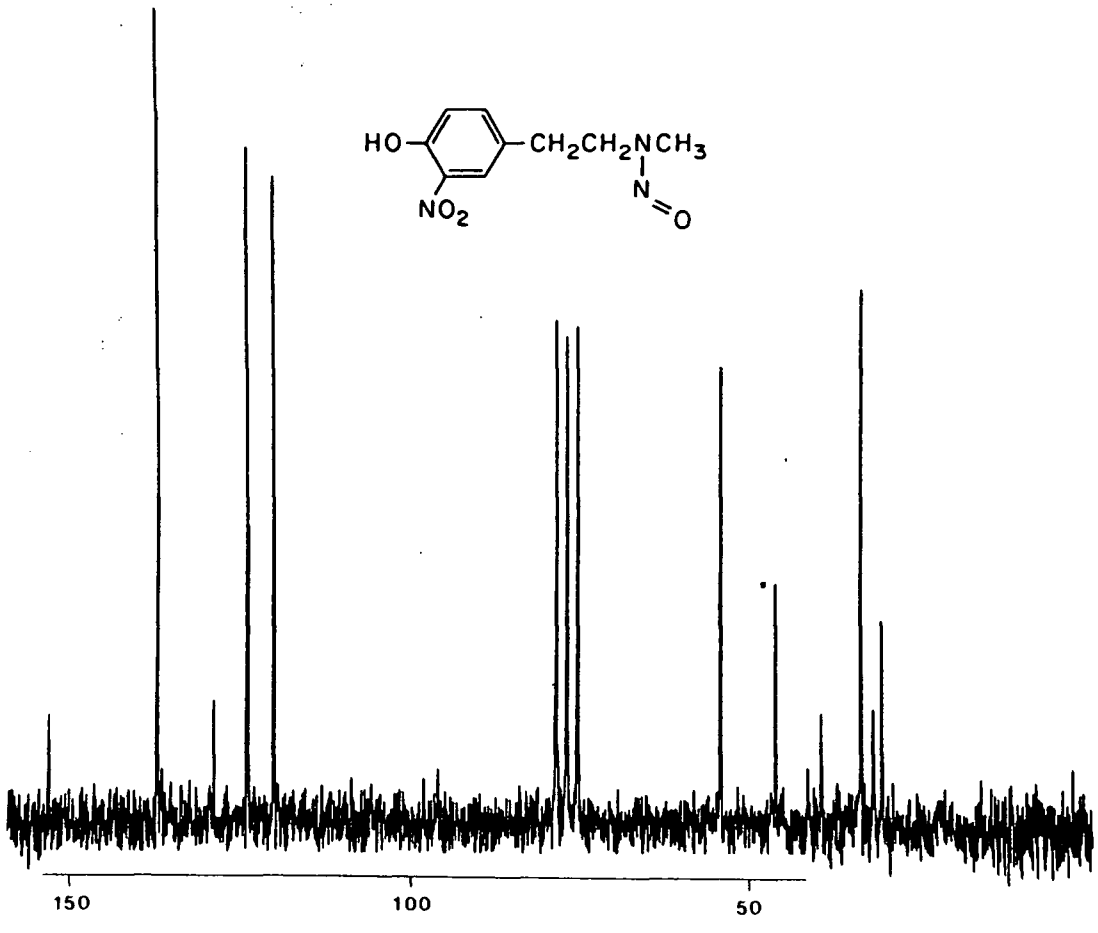
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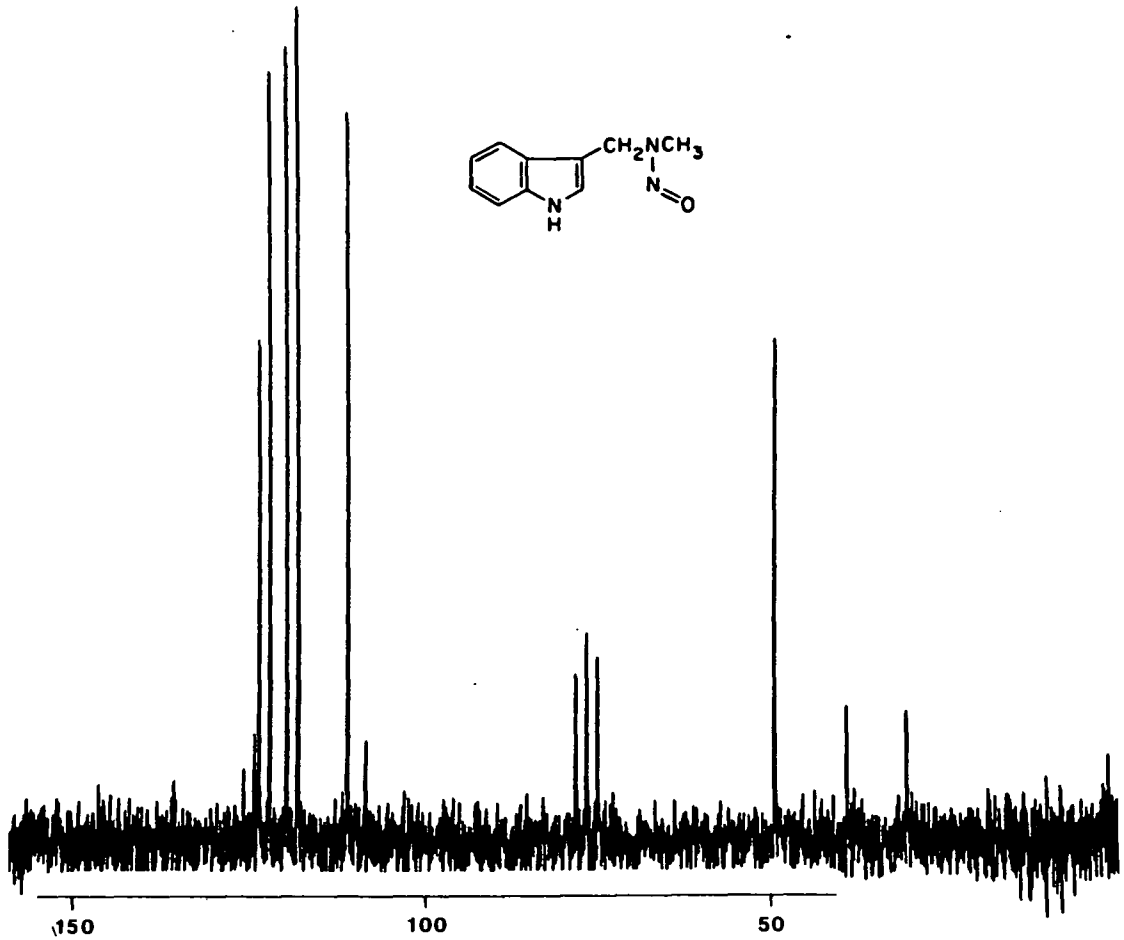
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N
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H
150
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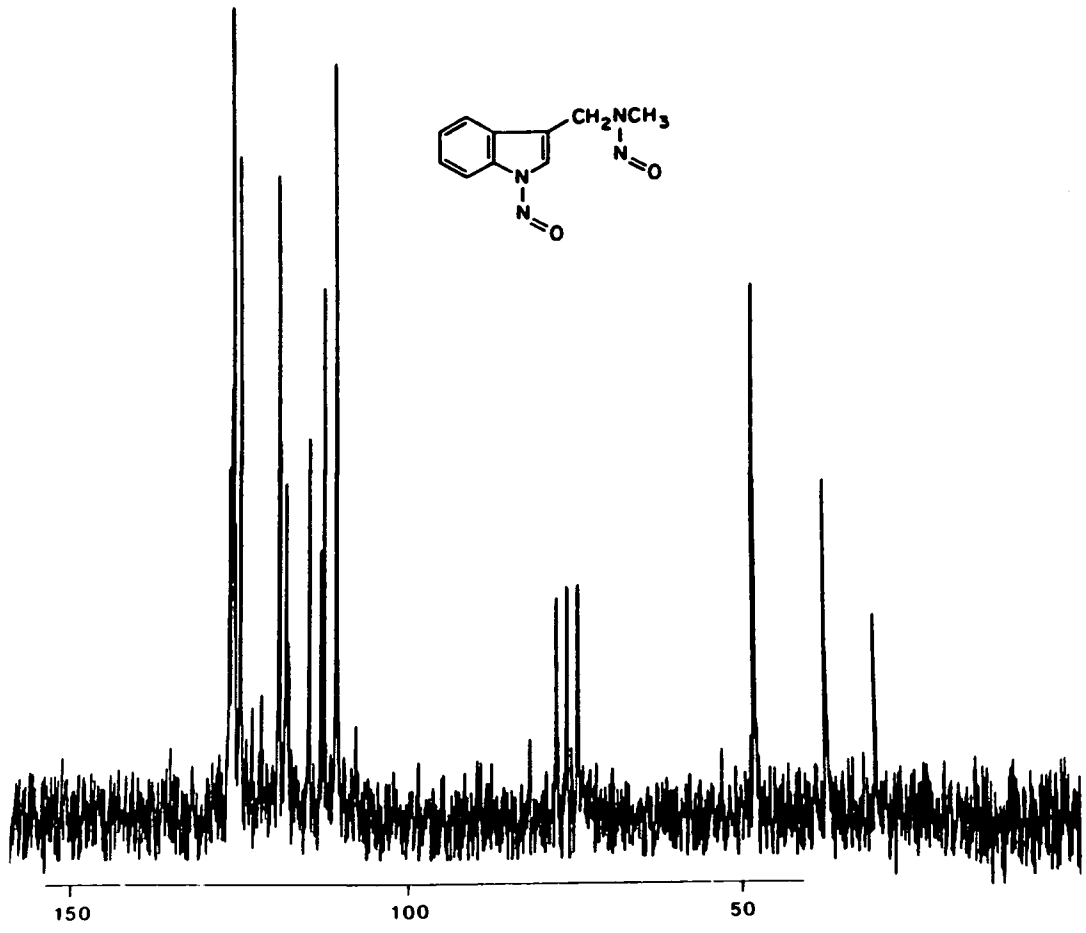
CH2NCH3
N
N=O
N
N=O
150
100
50