CARBONYL-AMINE REACTION PRODUCTS AS POSSIBLE NITROSAMINE PRECURSORS

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ABSTRACT

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By

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Imines can be found in foods as a result of condensation of aldehydes with amino groups during non-enzymatic browning. The purposes of this investigation were to determine (1) if such imines would react directly with nitrite to form N-nitrosamines; (2) if N-nitrosamines would form from a reaction between sodium nitrite and an intermediate in the synthesis of imines; or (3) if nitrosamines could be formed in a model dry food system, designed to enhance non-enzymatic browning in the presence of aldehyde and/or sodium nitrite.

Aliphatic imines, synthesized from aldehydes and primary amines, were exposed to sodium nitrite or nitric oxide. Sodium nitrite was also added directly to the aldehyde-amine reaction mixture. Reaction products were tested for nitrosamine content with Griess and ninhydrin reagents; and, whenever possible, products

were characterized by boiling point and by infrared spectrophotometry and gas chromatography-mass spectrometry (GC-MS). Nitrosamines were not found in any of the reaction products.

Sodium nitrite was incorporated into emulsified ground ham muscle. Half of the samples were freeze-dried to remove water prior to a fifteen-hour heat treatment; the other half were given only the heat treatment. Colorimetric evidence of nitrosamines was found in samples to which an excessive quantity of sodium nitrite had been added. Non-freeze-dried samples consistently tested positive with Griess and ninhydrin reagents, while the freeze-dried meat contained only traces of apparent nitrosamine. Samples which gave positive colorimetric tests were analyzed by GC-MS but the apparent nitrosamine compounds were not identified when compared to mass spectra of known nitrosamines and pyrazines.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Inclusion of sodium nitrite in meat-curing mixtures was approved by the United States Department of Agriculture in 1925. Sodium nitrite stabilizes the color of cured meat, contributes flavor, and aids in preservation. The preservative function is especially important in semi-perishable cured meats and in smoked fish.

The addition of sodium nitrite to foods has been challenged, because of the participation of nitrite in formation of N-nitrosamines. The N-nitrosamines are toxic and carcinogenic for a wide range of animal species, and may be readily synthesized from secondary or tertiary amines and sodium nitrite under acidic conditions.

In order to establish whether nitrosamines are formed in foods which contain nitrite and/or nitrate, many investigators have analyzed food products directly for the volatile nitrosamines. Since non-volatile nitrosamines are difficult to detect, due to interference by other food constituents, their occurrence in foods has not been established. The concentrations

of volatile nitrosamines found have seldom exceeded a few parts per billion. Detection of a given nitrosamine does not indicate the source of the precursor; therefore, the conditions or events resulting in an increased occurrence cannot be controlled.

The likely occurrence of both volatile and non-volatile nitrosamines could be inferred if the necessary precursors are present in a food product. For example, imines can be found in foods as a result of condensation of aldehydes with amino groups during non-enzymatic browning. The purposes of this investigation were to determine (1) if such imines would react directly with nitrite to form N-nitrosamines; (2) if N-nitrosamines would form from a reaction of sodium nitrite with an intermediate in the synthesis of imines; or (3) if nitrosamines could be formed in a dry model food system, designed to enhance non-enzymatic browning in the presence of aldehyde and/or sodium nitrite.

REVIEW OF LITERATURE

The organic compounds R-N=O may be nitrites, -O-N=O; C-nitroso compounds, -C-N=O; or N-nitroso compounds, -N-N=O. The latter group of compounds has been investigated extensively in recent years, and several members of this class have been demonstrated to be toxic, carcinogenic, mutagenic, or teratogenic (12, 49, 80).

The hazard posed by these N-nitroso compounds was not well known prior to a report in 1954 of two men suffering with liver cirrhosis after having worked with dimethylnitrosamine (NDMA) in a research laboratory (3). Also reported were the results of NDMA toxicity studies using rats, rabbits, mice, guinea pigs, and dogs. With these animals, doses of 20-40 mg/kg of body weight resulted in severe liver necrosis leading to death. This early report of the hazardous properties of nitrosamines did not signal an instantaneous curtailment of their use by the chemical industry. As late as 1963, patents were issued for various nitrosamines for use as gasoline and lubricant additives, antioxidants, stabilizers, rubber additives, bacteriocides, etc. (81).

Another indication of N-nitrosamine toxicity resulted from a report in 1964 describing the circumstances surrounding the illness and death of several cattle, sheep, and goats in Norway (37). An investigation into the cause of an unusual liver disease which began to appear in these animals in 1961, established that all of the animals had been fed herring meal, and that most of the meal was produced by only one of approximately 100 herring meal factories in Norway. Koppang et al. described production of herring meal from herring preserved with nitrite and formaldehyde, and concluded that excessive levels of nitrite present in herring during processing can result in the formation of an unidentified toxic agent (38). The toxic compound was not nitrite itself, as addition of large amounts of nitrite to non-nitrite-treated meal just prior to feeding did not result in toxic hepatosis. The formaldehyde did not produce toxic hepatosis either, but excessively high concentrations did produce a meal which had some harmful effects of a different nature. The toxic substance was subsequently isolated and identified as dimethylnitrosamine; and its formation from methyl amines present in nitrite-treated herring meal was demonstrated (16, 17, 18, 58).

The acute oral LD_{50} in rats has been determined for several of the N-nitroso compounds (49); few have

been found to be more toxic than N-nitrosodimethylamine, the first to be tested. The manifestation of toxicity seems to be linked with the chemical structure of the various N-nitroso compounds. The two basic classes for N-nitroso compounds, by structure, are nitrosamines and nitrosamides.

The nitrosamines are fairly stable and appear to remain intact under physiological conditions (46). Nitrosamines may not be toxic as such; but if broken down by enzymes, an active metabolite, such as a diazoalkane or a carbonium ion, may be released (13, 49). Since the liver metabolizes foreign compounds at a high rate, more of the toxic component is released, and thus more damage results in the liver than in less enzymatically active organs (46).

The nitrosamides are much less stable and will decompose spontaneously in neutral to alkaline pH ranges (12, 47, 80). Thus, the nitrosamides do not exhibit a marked selective toxicity towards the liver as do nitrosamines.

Magee and Barnes, in 1956, reported the induction of malignant liver tumors in the rat by NDMA (48). Discovery of the strongly carcinogenic nature of the N-nitroso compounds led to studies by a number of scientists working in the field of cancer research, particularly those interested in experimental tumor

production (45, 49, 80). Approximately 100 N-nitroso compounds have been tested for carcinogenicity, and about 75% were active carcinogens for several animal species. Druckrey et al. reviewed data concerning the carcinogenic action of 65 different nitroso compounds in the rat (12). The nitrosamines appear to affect primarily those organs capable of enzymatic alteration or breakdown of the parent nitrosamine into a more active metabolite which Magee refers to as a "proximate carcinogen" (45). However, nitrosamines have induced cancer in the esophagus, kidney, bladder, lung, and nasal cavity (49). In addition, a wide range of species including the rat, mouse, guinea pig, hamster, pig, rabbit, trout, dog, and monkey have proven susceptible to nitrosamines (46, 49). Thus, while cancer in man has not been directly connected with nitrosamines, it would seem probable that man is susceptible.

Nitrosamides produce local sarcomas when injected subcutaneously, and if administered systemically, may be carcinogenic in virtually every organ of the rat, including the nervous system (49, 80). Tumors of the nervous system are especially pronounced in the case of transplacental induction of malignant tumors. For example, ethylnitrosourea has been shown to induce tumors of the nervous system in over 90% of the offspring when given as a single low dose to the pregnant adult (13, 80).

Initially, the toxic and carcinogenic properties of the N-nitroso compounds were considered a hazard primarily for workers in industrial and laboratory situations. However, the discovery of NDMA in nitrite-treated herring meal raised the specter of carcinogen formation occurring in foodstuffs containing nitrate and/or nitrite. Nitrates and/or nitrites are added to cured meats to enhance flavor, develop color, and aid in preservation. In countries other than the United States, these salts may also be added to certain types of cheese, as a preservative (2). Nitrates are also commonly found in plants, especially forage crops and vegetables, water supplies, and soil (2, 84). Thus, a source of nitrous acid, one of the major reactants necessary for nitroso synthesis, is common to many foods. If, in addition, a food contained amines or other compounds suitable for N-nitrosation, and if processing or storage conditions were favorable, the potential for N-nitrosamine formation would seem very real. It is not surprising, therefore, that a number of food products have been analyzed for N-nitrosamine content. A partial list of the commodities examined includes fish (7, 19, 21, 34, 39, 63, 65, 66), cured meats (6, 7, 22, 25, 26, 43, 52, 64, 68, 69, 74), cheese (7, 29, 30, 33, 65), milk (33), wheat flour (33, 65), mushrooms (19), ryebread (30), tobacco (35, 70),

spinach (7, 65), alcoholic beverages (67, 82), cereal grains (57), and fruit (15). It should be emphasized that (1) not all of these products were found to contain N-nitrosamines; (2) when N-nitrosamines were found, they were seldom present in concentrations more than 100 ppb (100 µg/kg) and usually less than 10 ppb; and (3) some of these reports are of questionable value, especially those published prior to about 1969, because the methods used to confirm the presence of nitroso compounds lacked the required specificity.

Fish was one of the first food products to be analyzed for nitrosamine content. If nitrosamines could form in fish meal, it seemed logical to suspect a similar occurrence in smoked fish intended for human consumption, as the processing entails immersion in a brine containing sodium nitrite, followed by a heat treatment. For example, smoke processing of chub, a freshwater fish, includes a brining treatment resulting in no less than 3.5% salt (NaCl) and 100-200 ppm sodium nitrite in the edible portion of the loin muscle. Thermal processing must be sufficient to assure a temperature of 160 F throughout the fish for a minimum of 30 minutes (23).

Nitrosamine quantities reported in several fish species and the methodology used to confirm their presence are presented in Table 1. Relying solely on

TABLE 1.--Levels of N-nitrosamines reported in several species of fish as confirmed by thin-layer chromatography (TLC), gas chromatography (GC), or mass spectrometry (MS)

Reference	N-nitrosamines, ppb										Method
	cod	chub	haddock	hake	herring	mackerel	sable	salmon	shad	skate	
Ender (19)			15		≤ 9.5	0.6					TLC
Koprowski (39)		< 500									TLC
Sen (65)					< 150						GC
Howard (34)		< 10									GC
Sen (66)	≤ 12.5		5	0-45		0-40		< 2.5			GC
Fazio (21)							4-26	0-17	0-12		GC-MS
Crosby (7)	1-9		1-9	1-9	< 1			< 1		< 1	GC-MS

thin-layer chromatography (TLC), Ender and Čeh found in smoked haddock as much as 15 ppb of a nitroso compound which appeared to be NDMA (19). Koprowski investigated the effect of two smoking temperatures and three nitrite concentrations on the formation of NDMA in smoked chub (39). He did not find NDMA; however, the minimum detectable nitroso concentration with his TLC method was 500 ppb. Using TLC, Sen et al. occasionally found Griess-positive compounds in the extract from herring, but the quantities were too small to confirm (65). The sensitivity of their TLC and gas chromatography (GC) methods was 150 ppb for NDMA and 50 ppb for diethyl- and di-n-propylnitrosamines (NDEA and NDPA).

The first investigators of nitrosamine formation in foods relied primarily upon TLC and/or GC data for the final identification of nitrosamines. However, beginning in about 1971, mass spectrometry (MS) began to find application in this area, and has increasingly been the preferred method for final nitrosamine identification. Fazio et al. confirmed by mass spectrometry (MS) levels of NDMA ranging from 4 to 26 ppb in samples of raw, smoked, and smoked nitrite- and/or nitrate-treated sable, salmon, and shad (21). The highest value, 26 ppb, was found in a smoked, nitrite- and nitrate-treated sable. Raw sable contained 4 ppb and smoked, uncured sable contained 9 ppb. Crosby et al. analyzed several

fish species and found from 1 to 9 ppb, which they confirmed with MS (7). It is interesting to note that the only fish containing more than 4 ppb were those which did not contain nitrite. Moreover, these fish had been cooked, either fried or baked, and the authors mention problems with quantitation in some of their cooked samples due to interference from nitrogenous compounds.

Maximum nitrosamine concentrations reported by several research groups which have analyzed cured meat products are presented in Table 2. Some authors reported finding no nitrosamines at or above the minimum concentration detectable with their methods; in these cases, the minimum detectable level is listed in Table 2 preceded by the symbol "<."

Fazio et al. analyzed 51 samples of cured meats, including (1) cold cuts, (2) sausages, (3) baby foods, (4) canned meats, (5) bacon, hams, other pork products, and (6) miscellaneous beef products (22). The maximum nitroso concentration they found was 5 ppb in a sample of smoked ham. This substance was identified by MS as NDMA. The other samples were considered to contain NDMA also, on the basis of GC retention times; but since these concentrations were too low to confirm by MS, the authors referred to these values as "apparent NDMA." This serves to illustrate the problem encountered

TABLE 2.--Maximum levels of N-nitrosamines reported in various meat products as confirmed by gas chromatography (GC) or mass spectrometry (MS)

Reference	N-nitrosamines, ppb											Method
	cold cuts	sausages	baby foods	canned meats	bacon	ham	other pork products	misc. beef products	fresh pork	fresh beef	seasonings	
Fazio (22)	4	3	3	3	2	5	2	2				MS
Fiddler (25, 26)		84				<10						GC
Telling (74)				< 25	<25	< 25			<25	< 25		MS
Crosby (7)	4	4			40							MS
Logten (43)				50								MS
Sen (64, 68, 69)	80	20			30						25,000	MS
Christiansen (6)				<10								MS
Panalaks (52)	10	3		3	3	3		12				GC

by many persons attempting to isolate and identify such low nitrosamine concentrations. Very often, an indication of possible nitrosamine presence is seen, such as a positive color appearing on a thin-layer plate, or a GC retention time that is identical to the nitrosamine standard; but unless the suspected compound is present in a concentration of 10 ppb or greater, it is usually impossible to isolate enough of the unknown to obtain a mass spectrum.

Crosby et al. examined extracts of bacon, fish, cheese, spinach, frying oil, and an assortment of cured meats (7). If a sample showed retention times corresponding to one of seven nitrosamine standards they used, it was further analyzed by high-resolution mass spectrometry. While most of the bacon samples analyzed had no detectable nitrosamines, a few had 1 to 4 ppb, and one sample had 40 ppb.

Fiddler et al. reported finding 11 to 84 ppb NDMA in three of forty commercial frankfurter samples (26). As 16 billion frankfurters were consumed in the United States in 1971, the authors emphasized the significance of finding even a few μg/kg. They also investigated the relationship between NDMA formation and the addition of excessive levels of sodium nitrite to frankfurter formulations. Even when 750 mg nitrite were added per kg of meat (5 times the legal limit of

156 mg/kg), no significant NDMA formation occurred. With 1500 mg nitrite/kg or more, NDMA concentrations of 10 ppb or more were found in the franks. A reduction in NDMA formation occurred when sodium ascorbate was added to the frankfurter formulation, which the authors attributed to the reduction of nitrite to nitric oxide during curing. The blocking of nitroso formation with ascorbate was also demonstrated by Mirvish et al. (50).

In a study which illustrates the importance of adding sodium nitrite to cured meat, Christiansen et al. varied the nitrite levels in samples of canned comminuted pork between 0 and 500 μg/kg (6). The cans of pork were inoculated with types A and B Clostridium botulinum, given a normal thermal process, and then placed in storage for as long as six months, at 7 C or 27 C. Since this is a product which is labeled "keep under refrigeration," the latter temperature would test the keeping quality of the product and favor the growth of C. botulinum. This was intended to simulate the abuse which this product is believed to receive at the retail and consumer level. No nitrosamines were found in any of the cans. Toxin production was reduced or completely inhibited, depending upon the number of spores inoculated at a given level of nitrite. In addition to demonstrating the protective effect of nitrite against toxin production, this

experiment also illustrated the value of sodium nitrite in preventing non-toxic spoilage. Those cans to which no nitrite was added and which did not receive spores underwent non-toxic spoilage within one month, even when stored at 7 C.

Sen et al. reported a possible explanation for the occasional presence of nitrosamines in cured sausages (69). They confirmed the presence of 2500 ppb nitrosopyrrolidine (NPYR), 850 ppb NDMA, and 25,000 ppb nitrosopiperidine (NPIP) in a dry curing powder which consisted of black pepper, paprika, two secret spices, salt, sodium nitrate, and sodium nitrite (0.96%). In further tests they demonstrated that both black pepper and paprika would react with nitrite to produce the NPYR and NPIP, while the paprika was apparently responsible for the NDMA formation. The two secret ingredients did not react to form either NPYR or NPIP. The authors suggest that pre-mixing of the dry spices with nitrite, followed by prolonged storage before use, could be a hazardous practice.

Methods for the recovery, quantitation, and confirmation of N-nitrosamines in foods were reviewed in 1971 by Walters (76) and in 1972 by Wasserman (79). Many researchers have analyzed foods for N-nitrosamine content, but the methods they have used were usually developed independently and for a specific application.

Unfortunately, there is no standard method which can be used for all N-nitrosamines and all foods.

Thin-layer chromatography has been used extensively for detection of nitrosamines (28). Some of the earlier reports of nitrosamine occurrence in food extracts relied solely on TLC for detection and conformation. A primary application of TLC in more recent investigations has been the screening of food extracts. Those samples which TLC indicates as containing nitrosamines are then subjected to more extensive clean-up and analytical procedures. Quite often, TLC is used to isolate nitrosamines from contaminants so that a "clean" mass spectrum may be obtained.

The TLC visualization method most often used is the photolytic cleavage by ultraviolet light of the -N=O group, which then combines with Griess reagent (sulfanilic acid and 1-naphthylamine, or one of several similar diazotizing reagents) to produce a colored spot on the plate (9, 54). Compounds other than nitrosamines, such as esters of nitrous acid, C-nitro- compounds, inorganic nitrate, inorganic nitrite, and pyrazines can cause development of a positive Griess color (77). It is, therefore, a good practice to confirm a positive Griess test by using some other visualizing reagent. Since the photolytic cleavage of nitrosamines produces

the parent amine (24), the ninhydrin test is often used in conjunction with the Griess test (65, 67).

Infrared spectrophotometry (IR) has found little application in the analysis of foods for nitrosamine content, as the quantities extracted are insufficient to obtain IR spectra. However, IR is used in confirming the identity of nitrosamine standards. Aliphatic nitrosamines show N=O stretching absorption in the 1425-1460 cm^{-1} region; N-N stretching absorbs around 1030-1150 cm^{-1} (83).

Polarography has found a very limited use in nitrosamine analysis. Devik used TLC, GC, and polarography to analyze products of a Maillard reaction between glucose and amino acids (10). He reported the formation of nitrosamines in this model system. However, this statement was retracted in a subsequent paper by Kadar and Devik (36). Re-examination of the products of the amino acid-glucose reaction by MS confirmed the apparent nitrosamines were actually pyrazines. This agrees with the results found by Havre and Ender (32). Walters et al. state that unsaturated aldehydes and ketones also give polarographic results similar to nitrosamines (77).

The nitrosamine concentrations in foods reported to date have been quite low, often less than 10 ppb (μg/kg). To isolate, identify, and quantitate such low

levels of nitrosamine is a difficult task, made more so by the complexity and variety of potentially interfering substances present in various food products. The minimum nitrosamine quantity which can be visualized on a thin-layer plate when using Griess or ninhydrin reagents is, at best, 0.2 to 0.5 μg (65, 76). A 1 μg sample is a reasonable quantity to spot on a thin-layer plate if consistent sample visualization is to be obtained. Thus, to test an extract with Griess and ninhydrin, in duplicate, would require 4 μg of nitrosamine. If the original food contained 4 ppb (μg/kg) nitrosamine, the sample size prior to extraction would have to be at least 1000 g and the nitrosamine recovery 100%. It is apparent that examination of a food containing 10 ppb nitrosamine entails very large samples if the method of determination is thin-layer chromatography.

One of the major advantages of gas chromatography as compared to TLC is its greater sensitivity. Howard et al. described a GC method capable of detecting 10 ppb NDMA in smoked fish (34). The quantity of NDMA required to produce a clearly pronounced peak was 4.5 ng. This is about 1000 times less than the quantity needed for TLC visualization. Sen obtained another 1000-fold increase in sensitivity by oxidizing NDMA to dimethyl-nitramine ($C_2H_6NNO_2$), which is extremely sensitive to electron capture detection (63). However, the conversion to the nitramine is not quantitative and may be variable.

Gas chromatography is clearly more sensitive than TLC, but it does have certain limitations. When using TLC, nitrosamines are distinguished from other compounds which may be present in a food extract by means of a fairly specific colorimetric reaction. With gas chromatography, one must compare the retention time of a peak in question to the retention time of a standard nitrosamine. This immediately limits GC analysis to those nitrosamines for which standards are available. In addition, there is always the chance that some other compound present in the extract may have the same retention time. For example, pyrazines commonly found in cooked foods often have retention times similar to nitrosamines (59, 61).

Williams et al. used a gas chromatograph coupled with a mass spectrometer (GC-MS) to demonstrate that the aldehyde furfural was the compound previously mistaken for a nitrosamine in an extract from an alcoholic beverage (82). GC-MS combines the sensitivity and resolving power of gas chromatography with the ability of a mass spectrometer to unambiguously characterize the sample. Mass spectra of several N-nitrosamines have been deposited with the ACS Microfilm Depository Service (53). Saxby investigated the fragmentation by electron impact of 24 dialkyl-N-nitrosamines, and proposed decomposition mechanisms (60).

N-nitrosamines are generally yellow, neutral to weakly basic compounds, and are insoluble in dilute aqueous mineral acids (51, 71). The stability of four representative N-nitrosamine structures has been reported by Fan and Tannenbaum (20). Secondary amines, whether aliphatic or aromatic, readily combine with nitrous acid to yield N-nitrosamines. Aliphatic tertiary amines will also form N-nitrosamines, but aromatic tertiary amines undergo ring substitution to yield C-nitroso-compounds (51). When primary amines react with nitrous acid, the product is a diazonium salt. The diazonium salts of primary aliphatic amines are very unstable and break down to yield a mixture of alcohols and alkenes (51). However, Ender et al. reported a 9% yield of NDMA from monomethylamine and nitrous acid (18).

The presence in foods of substantial quantities of nitrogen-containing compounds which could serve as precursors to N-nitrosamine formation has been questioned. Abundant quantities of volatile secondary amines are not common in most biological systems, although marine fish contain more of these amines than do fresh water fish (39). Fiddler et al. have demonstrated formation of NDMA from naturally occurring quarternary ammonium compounds (27). Archer et al. were able to show formation of N-nitrososarcosine from creatine, a normal constituent of meat (1). Various amino acids have

served as precursors of N-nitrosamines (41, 78). Lijinsky et al. obtained measurable yields of nitrosamine from each of six drugs which had tertiary nitrogen structures (42).

Still other sources of nitrogen compounds which have not received attention as nitrosamine precursors are the reaction intermediate and end-product resulting from the interaction of primary amines and aldehydes. Dugan and Rao have investigated the Maillard browning reaction in dry model systems containing phospholipids and aldehydes (14). They were primarily interested in the effect of such Maillard reactions on the flavor of dry foods in prolonged storage situations. However, they demonstrated the ready formation of tertiary nitrogen Schiff bases which may provide another source of N-nitrosamine precursors. The proposed mechanism for the reaction between phosphatidylethanolamine (PE) and nonanal is shown in Figure 1. The intermediate compound contains a secondary amino group which could possibly react with nitrous acid, provided the intermediate exists as a secondary amine for a finite time span. The end-product is a tertiary amine, and it could also prove to be a site for attack by nitrous acid.

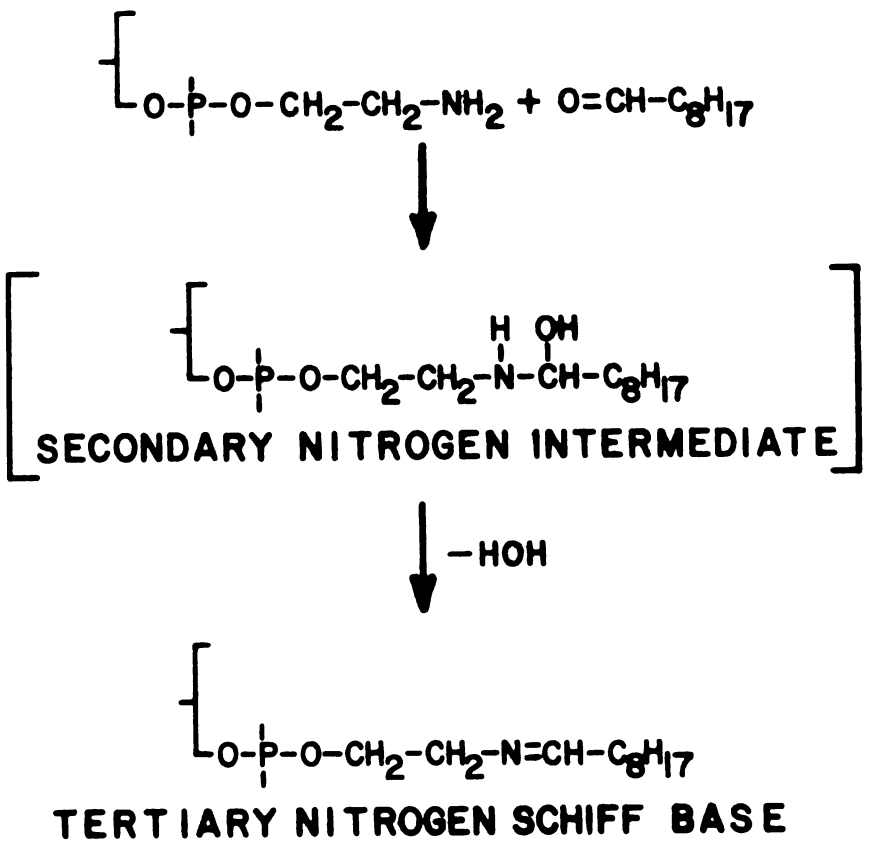

Figure 1.--Mechanism for the reaction between phosphatidylethanolamine and nonanal

EXPERIMENTAL PROCEDURES

Safety Precautions

N-nitroso compounds are both toxic and carcinogenic; therefore, rubber gloves were always used when working with them. All experiments involving these compounds or nitric oxide gas were conducted under an exhaust hood.

Synthesis of Nitrosamine Standards from Secondary Amines

N-nitrosamine standards not purchased from commercial sources were prepared from secondary amines by a method similar to that described by Hatt (31). The synthesis of N-nitrosoethylbutylamine (NEBA) is a typical example.

In a 500-ml round-bottom flask provided with an oval-shaped magnetic stirring bar were placed 101 g (1 mole) of N-ethyl-n-butylamine and sufficient 2N HCl to obtain a pH of 6.0 to 6.5. This solution was stirred vigorously and maintained at 70 to 75 C by heating on a water bath, while 100 g (1.4 moles) of 97%-pure $NaNO_2$ suspended in 100 ml water were added from a dropping funnel over a period of an hour. The reaction mixture

was tested frequently and maintained at pH 6.0 to 6.5 by further addition of 2N HCl when necessary. Stirring and heating were continued for two hours after all the sodium nitrite had been added. The flask was arranged for distillation, and the reaction mixture distilled under vacuum until the residue was practically dry. To the residue 140 ml water were added and the process of distillation to dryness repeated. The distillates were combined and saturated with potassium carbonate (approximately 1 lb was required). The organic layer was removed, dried over anhydrous potassium carbonate, then purified by another vacuum distillation.

The identity of the nitrosamine standards was confirmed by Griess and ninhydrin tests, and by comparison of boiling points and infrared and mass spectra with data reported in the literature (53, 55, 60, 83).

Synthesis of Imines

Imines were prepared from aliphatic aldehydes and primary amines using a modification of the procedure used by Campbell et al. (5). A typical example is the synthesis of N-n-butylidene-ethylamine ($C_3H_7CH{:}NC_2H_5$).

In a 500-ml round-bottom flask provided with an oval-shaped magnetic stirring bar, 81.5 g (1 mole) ethylamine hydrochloride and 100 ml distilled water were stirred into solution and chilled in an ice bath.

A solution of 56 g (1 mole) KOH in 100 ml water, previously prepared and cooled to 5 C, was added and the mixture stirred for thirty minutes to ensure chilling. By means of a dropping funnel, 72 g (1 mole) butanol, previously chilled to -26 C, were added gradually over a period of one hour. Stirring was continued for fifteen minutes after addition was completed; then the organic layer was removed and stored over KOH pellets overnight. The following morning, the reaction products were filtered into a 250-ml round-bottom flask, fresh KOH pellets were added, and the mixture was distilled at atmospheric pressure.

The imines prepared in this manner were characterized by boiling points and by infrared and mass spectra.

Attempted Syntheses of Nitrosamines

Imine and Sodium Nitrite

Either N-n-hexylidenemethylamine (HMI) or N-n-butylidene-ethylamine (BEI) were mixed with an excess of acidified sodium nitrite. In a typical case, 69 g (1 mole) $NaNO_2$ and 200 ml water were placed in a 500-ml two-necked round-bottom flask. The solution was acidified with 6N HCl to pH 6.0 to 6.5 and maintained in this range during the dropwise addition of 25 ml N-n-butylidene-ethylamine. Stirring was continued for

one hour after all the imine had been added. The aqueous layer was removed and washed with three 50-ml aliquots of ether. The ether extracts and the organic layer were combined and fractionally distilled in vacuo. The fractions, separated according to boiling point, were further characterized by infrared spectra and by the Griess test. Based on these results, selected fractions were analyzed by gas chromatography-mass spectrometry.

Imine and Nitric Oxide

Ray and Ogg (56) have shown that a powdered mixture of sodium nitrite, sodium nitrate, chromic oxide, and ferric oxide (3:1:2:3 molar ratio) will evolve nitric oxide gas which is 99.78% pure. Nitric oxide generated by heating this powder was bubbled through either BEI or N-benzylidenemethylamine. The apparatus used for this treatment consisted of two small Pyrex test tubes (20 mm x 150 mm) and a cylinder of nitrogen. A 10-ml aliquot of the imine was placed in the first tube; 10 g of the dry powder in the second. The tubes and the nitrogen tank were connected in series, with the nitric oxide tube upstream from the tube containing imine. The tubes were flushed with nitrogen, and a small stream of nitric oxide was evolved from the powder by mild heating with a Bunsen burner. Heating was terminated when gas production was no longer evident, usually after two hours. The sample was purged with nitrogen

for fifteen minutes, then transferred to a storage vial. The infrared spectra and Griess reaction of each product was recorded.

Aldehyde, Primary Amine, and Sodium Nitrite

To a 500-ml two-necked round-bottom flask were added 32 ml of a 70% solution of ethylamine (approximately 0.5 mole). The amine solution was chilled in an ice bath, and the acidity adjusted to pH 3.0 with 6N HCl. A 100-ml aqueous solution containing 69 g (1 mole) sodium nitrite was then added, followed immediately by the dropwise addition of 36 g (0.5 mole) n-butyraldehyde. Stirring was continued for an hour after all the aldehyde had been added. The mixture was then extracted with three 50-ml aliquots of dichloromethane (CH_2Cl_2). The extracts were transferred to a 500-ml Kuderna-Danish evaporative concentrator, and the dichloromethane removed by warming over a steam table. The concentrated product was tested with Griess reagent and the infrared spectra recorded.

Ground Ham Model System

Preparation of Ham Samples

Fresh (uncured) ham muscle, trimmed free of visible fat prior to grinding, was obtained from the Michigan State University Food Stores. The previous

history of the meat was not known. The ham was divided into 100-g portions, and various additives were dispersed throughout the ham by a 60-sec blending in a 250-ml stainless steel Waring blender jar. Additives incorporated into the ham were

(1) 20 ml water (control);

(2) 21 ml water containing 2.76 g $NaNO_2$;

(3) 20 ml water containing 2.76 g $NaNO_2$ and 2.0 g NaCl;

(4) 20 ml water containing 2.76 g $NaNO_2$ and 2.0 g hexanal;

(5) 20 ml water plus 2.0 g hexanal;

(6) 20 ml water plus 2.0 g hexanal; 2.76 g $NaNO_2$ added just prior to extraction; and

(7) 20 ml water plus 2 ml ethanol containing 1 mg each dimethylnitrosamine and dibutylnitrosamine.

After blending, all samples were frozen at -26 C.

Two treatments were used in this model study: (1) Frozen ham samples were transferred directly into a forced air oven (Cenco No. 95396-16) and heated at 70 C for fifteen hours. (2) Ham samples were freeze-dried (Stokes Freeze Dryer, Model 2003-F2) and then heated as above.

Extraction

After the heat treatment, the ham samples were broken into small pieces and placed in a one-quart stainless steel Waring blender jar, covered with 200 ml dichloromethane, and blended for five minutes. The blender was powered by an explosion-proof motor (Waring EP-1).

The extract was filtered through Whatman Sharkskin filter paper into a 500-ml distillation flask. A few boiling chips and several KOH pellets were added, and the flask was arranged for distillation at atmospheric pressure. Distillation was terminated when the flask appeared dry and liquid no longer distilled.

The distillate was transferred to a 500-ml Kuderna-Danish evaporative concentrator fitted with a 5-ml calibrated concentrator tube and a three-section Snyder distilling column. Carborundum boiling chips were placed in the concentrator tube, and the solvent was carefully concentrated to about 4 ml in a hot water bath. The apparatus was removed from the bath and allowed to cool, draining any remaining solvent from the distilling column. The column was then removed and the solvent concentrated to 0.5 ml under a slow stream of nitrogen.

Analysis of Ham Extract

The concentrated ham extract was analyzed for nitroso content by spotting 40-µl aliquots on thin-layer plates (pre-coated with 0.25 mm silica gel G), developing the plates with hexane: diethyl ether: dichloromethane (4:3:2), and visualizing the sample with either Griess or ninhydrin reagents.

Gas Chromatography

A Beckman GC-5 dual column gas chromatograph equipped with flame ionization detectors was used. Temperatures of the detector and injection port were 240 and 185 C, respectively. A 9 ft 8-in x 1/8-in o.d. stainless steel column containing 3% OV-210 on 100-120 mesh Supelcoport was used with a nitrogen flow of 26 cm^3 per minute. The air and hydrogen flows were 300 and 18 cm^3 per minute, respectively.

Gas Chromatography--Mass Spectrometry

Mass spectra were obtained using a combined GLC-mass spectrometer LKB 9000 equipped with a glass column (6 ft x 1/8 in) of 3% OV-210, with ionizing electron energies of 22.5 or 70 eV; the flash heater set 20 degrees above the GC column temperature, molecular separator at 230 C, and the ion source at 290 C. The spectra were recorded as bar graphs by means of an on-line data acquisition and processing program (73).

Infrared Spectrophotometry

A Beckman IR-12 double beam infrared spectrophotometer was used to record the spectra of neat samples (thin films on NaCl cells) with air as a reference.

pH

A Corning model 12 pH meter equipped with a Sargent-Welch (S-30070-10) miniature combination electrode was used to monitor the pH during the synthesis of nitrosamines.

Thin-Layer Chromatography

Glass plates, coated with 0.25 mm silica gel G or silica gel G-HR, were used for the separation and colorimetric detection of nitrosamines in synthesis end-products and in model system extracts. Large plates (20 cm x 20 cm), coated with silica gel G-HR, were activated for one hour at 100 C; smaller plates (5 cm x 20 cm), precoated with silica gel G, were used without activation. Standard nitrosamines were diluted to 0.1% (1 µg/µl) with dichloromethane, and 5-µg aliquots were spotted on each plate as a reference.

The plates were developed with one of two solvent systems, n-hexane: diethyl ether: dichloromethane (4:3:2) or chloroform: methanol: water (65:25:4); then visualized with Griess reagent and/or ninhydrin. To visualize with Griess reagent, the developed plate

was sprayed, observed briefly, then placed in a light-proof drawer. After five minutes, the plate was inspected for red spots and irradiated at 254 nm for five minutes using a Mineralight R-52. Red spots which developed prior to UV irradiation were considered false positive. When ninhydrin was the visualizing agent, the plate was sprayed with 30% acetic acid, irradiated for five minutes, sprayed with ninhydrin reagent, and then heated at 100 C. Red-purple spots which appeared within 10-15 minutes indicated the presence of a ninhydrin-positive compound. Occasionally, both colorimetric reagents were used on a single plate. This was achieved by covering one side of the plate while spraying the other with Griess reagent. Then the Griess-treated side was covered while 30% acetic acid was sprayed on the side originally covered. The plate was irradiated, checked for Griess-positive spots, then sprayed with ninhydrin and heated as before.

Griess Reagent

Solutions of sulfanilic acid (1% in 30% acetic acid) and α-naphthylamine (0.1% in 30% acetic acid) were stored at 4 C and mixed 1:1 just before using.

Ninhydrin Reagent

A 0.3% ninhydrin solution was prepared using ethanol containing 2% pyridine.

RESULTS AND DISCUSSION

Synthesis and Identification of Standard NEBA

Since there is only a limited selection of N-nitrosamines available through chemical supply companies, consisting mainly of short-chained, symmetrical, dialkyl-N-nitrosamines and a few cyclic compounds, N-nitrosoethylbutylamine (NEBA) was prepared by reacting ethylbutylamine with nitrous acid. Confirmation of the product as NEBA was based on its boiling point, Rf value, and IR and mass spectra as compared with values reported in the literature.

The product was a clear yellow liquid which boiled at 92 C/15 mm. The Rf value obtained for this compound using TLC plates precoated with silica gel G and the chloroform: methanol: water solvent system was 0.95; similar plates developed in the hexane: ether: dichloromethane solvent system showed a positive spot at an Rf of 0.51. The latter Rf increased to 0.65 when plates which had been coated with silica gel G-HR and activated at 100 C were used. The GC retention time of this compound was recorded as 3.8 minutes when an

oven temperature of 110 C was used. The IR spectrum, Figure 2, shows strong absorption bands at wavelengths 1450-1470 cm^{-1} and 1075 cm^{-1}, corresponding with stretching of the N=O and N-N groups, respectively (53, 55, 82). A mass spectrum for this compound is shown in Figure 3.

Pensabene et al. reported a boiling point of 95 C/14 mm for NEBA (53). A reported Rf value was not found for NEBA; however, Sen et al. reported Rf values of 0.57 and 0.77 for methyl-n-butylnitrosamine and di-n-propylnitrosamine, respectively (65). The Rf value of NEBA should fall within this range.

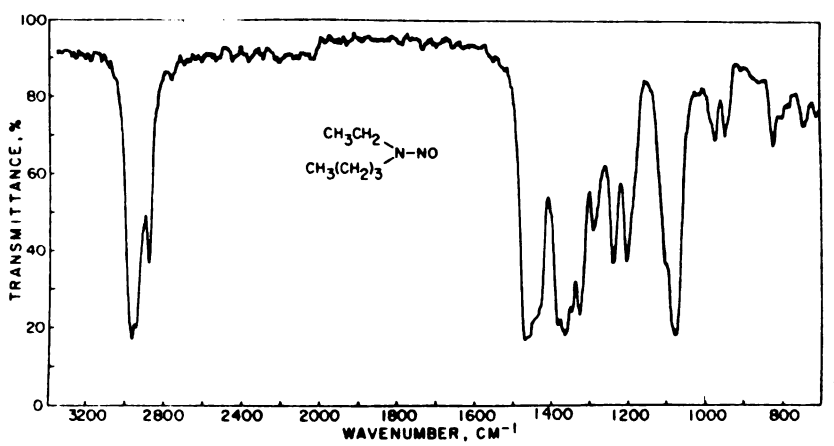

Figure 2.--Infrared spectrum of N-nitrosoethylbutylamine (NEBA) standard prepared in this laboratory

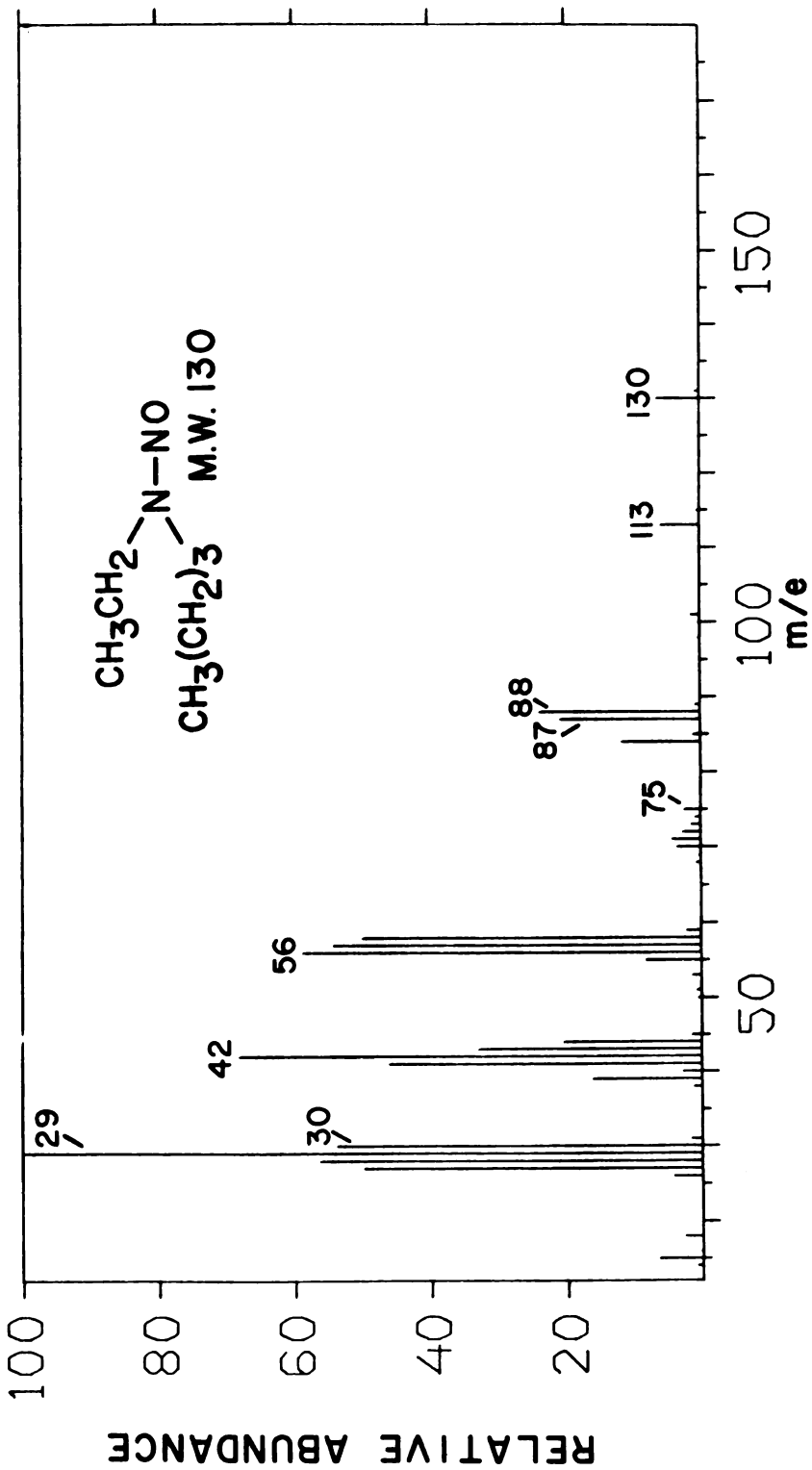

Figure 3.--Mass spectrum of N-nitrosoethylbutylamine (NEBA) standard prepared in this laboratory

Saxby has investigated the fragmentation pattern of twenty-four dialkyl-N-nitrosamines subjected to 75 eV electron impact, and tabulated the relative occurrence of eight important ions (60). All of these ions were present in the mass spectrum of the NEBA synthesis end-product, and their occurrence relative to the total ionization is compared in Table 3 with the values reported by Saxby. A reading of Σ_{29} 2.8% for the ion at m/e 130 is interpreted as meaning that ion m/e 130 contributed 2.8% of the total ionization, excluding ions smaller than m/e 29.

TABLE 3.--Relative contribution of eight ions to the total ionization produced by electron impact fragmentation in synthesized NEBA as compared with values previously reported (60)

Fragmentation ions	% Total ionization (Σ_{29})	
m/e	NEBA (60)	NEBA
130	2.8	3.1
113	1.7	1.7
87	4.5	5.4
56	9.8	9.1
42	9.4	9.6
88	5.5	7.9
75	0.6	0.6
30	7.2	5.8

As an additional check on the procedure used for the synthesis of NEBA, N-nitrosodimethylamine (NDMA) was prepared from dimethylamine and compared to NDMA purchased from Eastman Kodak Company, Rochester, New York, 14650. The IR and mass spectra of NDMA from both sources are in the Appendix (Figures 16, 17, 18), along with spectra of seven other nitrosamine standards (Figures 19-26), N-nitroso-diethylamine, -dipropylamine, -dibutylamine, -methylpropylamine, -methylbutylamine, -pyrrolidine, and -piperidine. The mass spectra of these standards are almost identical to the spectra published by Pensabene, et al. (53); however, the IR spectra obtained from a thin film of these standards do not contain the large solvent peaks present in the IR spectra reported by Pensabene, et al. (53).

Synthesis and Identification of Imines

Imines synthesized from aldehydes and primary amines were fractionally distilled, and their identity confirmed by infrared and mass spectra prior to their use as substrates in subsequent nitrosamine syntheses.

The boiling points of the two imines synthesized were 26-30 C/20 mm for N-hexylidenemethylamine (HMI), and 98 C/735 mm or 20 C/15 mm for N-butylidene-ethylamine (BEI). A boiling point of 100-108 C/760 mm has been reported for BEI (5, 40).

The IR spectra of HMI, Figure 4, and BEI, Figure 5, each contain a strong absorption band at 1675-1678 cm^{-1}. Absorption between 1666-1680 cm^{-1} is typical of C=N stretching (8). The absence of a C=O stretch in the 1700-1800 cm^{-1} range indicates that any excess aldehyde from the reaction mixture had been removed from the imines during fractional distillation.

A mass spectrometer equipped with a gas chromatographic inlet was used for the final identification of the distillation fractions which had tentatively been identified as imines on the basis of boiling point and IR spectrum. Both HMI and BEI were positively identified by inspection of their fragmentation patterns. The mass spectrum of HMI ($C_5H_{11}CH{:}NCH_3$), Figure 6, shows the molecular ion at m/e 113. A base peak at m/e 57 is the result of a McLafferty rearrangement (Figure 7) in which a C_4H_8 molecule is lost and the m/e 57 ion, $CH_2{:}CH\overset{+}{N}HCH_3$, is produced (4). The series of peaks separated by 14 m/e units, 42, 56, 70, 84, 98, represent the ions produced by the simple carbon-carbon fission at each of the five bonds of this type. The ion at m/e 112 results from loss of a hydrogen atom from the methyl group attached to nitrogen.

The molecular ion (M^+) for BEI ($C_3H_7CH{:}NC_2H_5$) is not present in the mass spectrum, Figure 8. Ions are present for M^+-H (m/e 98), M^+-CH_3 (m/e 84), $M^+-C_2H_5$

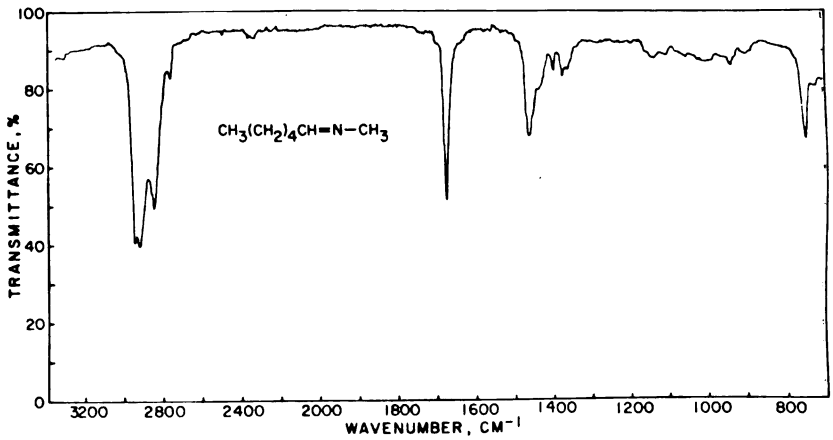

Figure 4.--Infrared spectrum of N-hexylidenemethylamine (HMI)

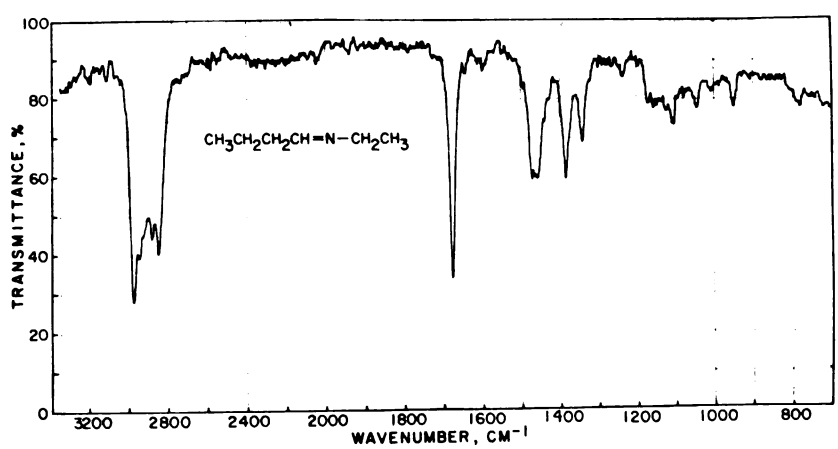

Figure 5.--Infrared spectrum of N-butylidene-ethylamine (BEI)

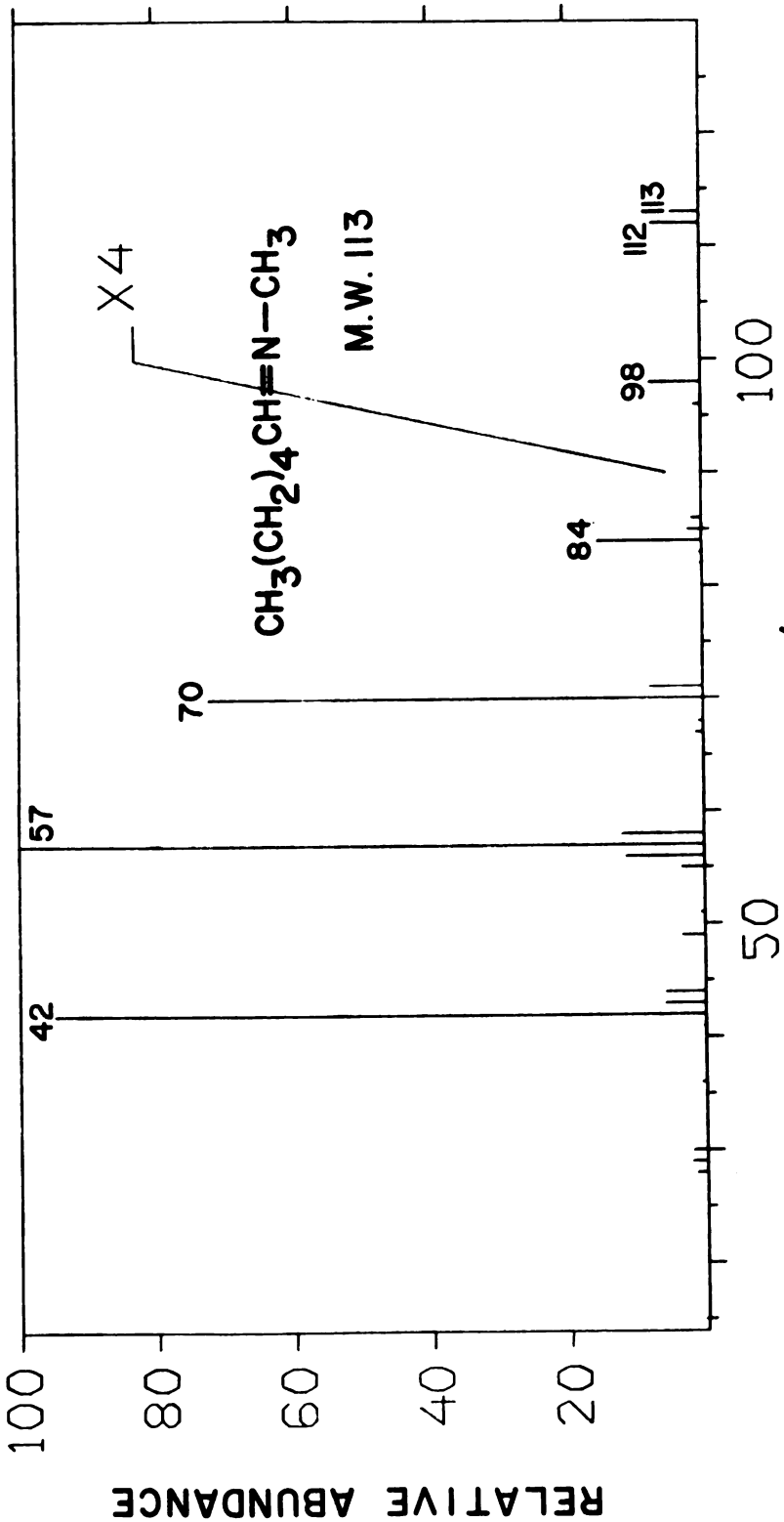

Figure 6.--Mass spectrum of N-hexylidenemethylamine (HMI)

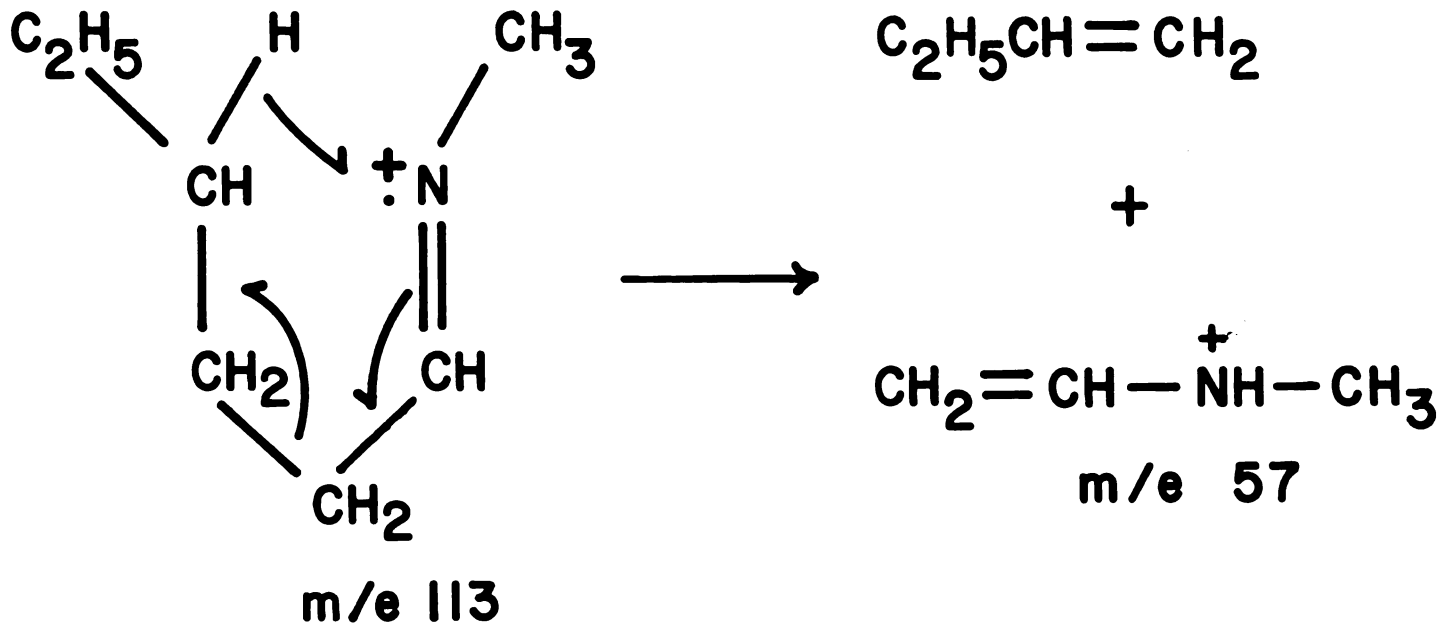

Figure 7.--McLafferty rearrangement of HMI to form an m/e 57 fragment ion

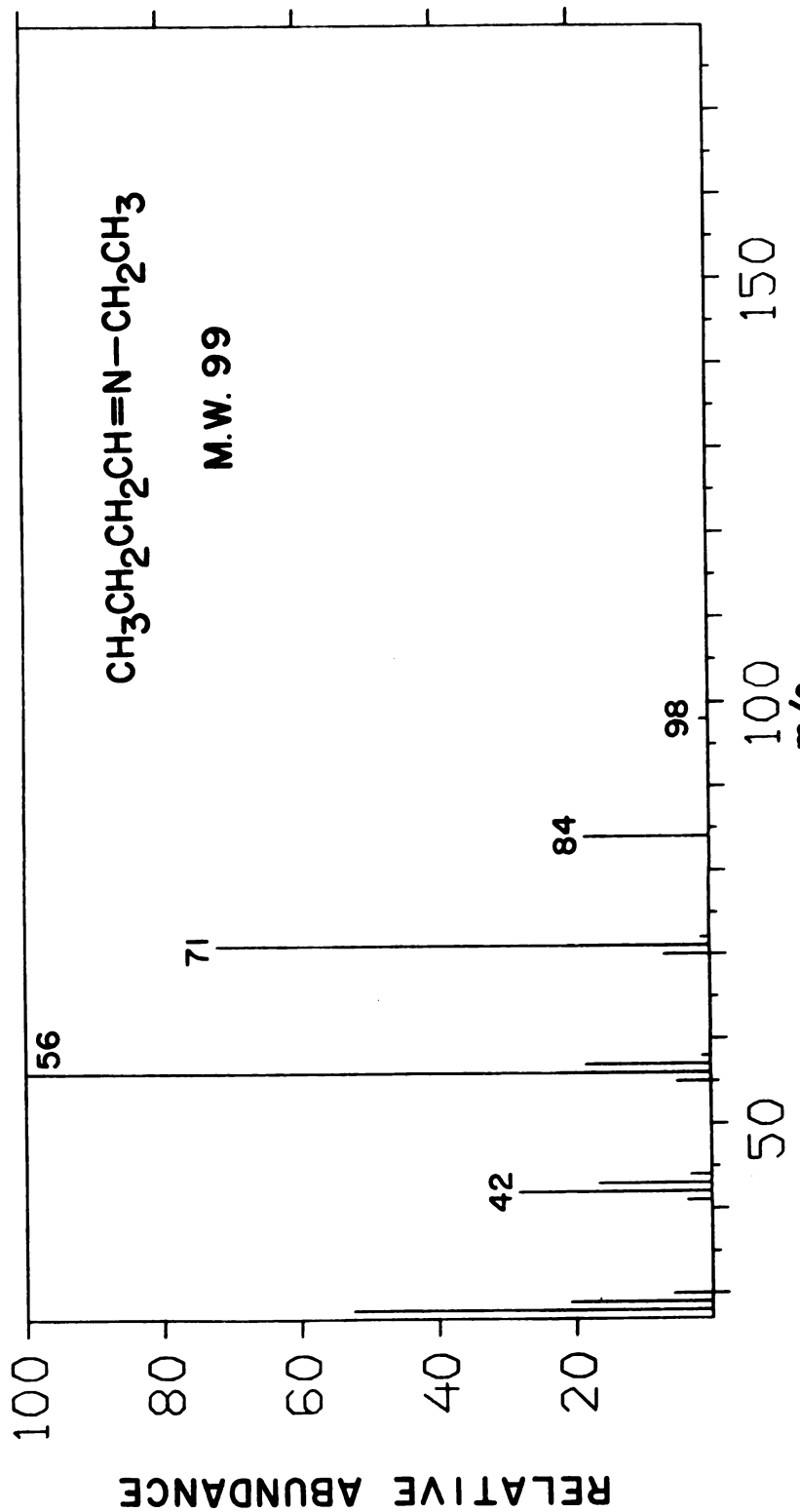

Figure 8.--Mass spectrum of N-butylidene-ethylamine (BEI)

(m/e 70), and $M^+-C_3H_7$ (m/e 56). The large ion at m/e 71 is accounted for by a McLafferty rearrangement which produces $CH_2{:}\overset{+}{C}HNHC_2H_5$.

Aldehyde molecules are prone to interact by aldol condensation to form β-hydroxyaldehydes. These β-hydroxyaldehydes easily dehydrate to form stable aldehydes in which a carbon-carbon double bond between the α- and β-carbon atoms is conjugated with the carbonyl group (51). Imines with an α-methylene group can also undergo an aldol-type carbon-carbon condensation (72). Analysis of the various fractions obtained from the distillation of HMI and BEI confirmed the presence of aldol-imine compounds in the higher boiling fractions. For example, the aldol "dimer" of HMI ($C_4H_9-\underset{\underset{CHC_5H_{11}}{\|}}{C}-CH{:}NCH_3$ M.W. 195) was found in a distillation fraction collected at 100-115 C/20 mm. The mass spectrum of this imine, Figure 9, has a molecular ion at m/e 195. The large peaks at m/e 42 and 152 represent the favored carbon-carbon fission at the alpha and gamma bonds, respectively (4). The series of peaks at m/e 110, 124, 138, 152, 166, and 180 represent ions formed by the successive elimination of methylene units from the alkyl chains. A similar fragmentation pattern was obtained for the dimer of BEI, Figure 10. These imine dimers could have resulted from (1) the

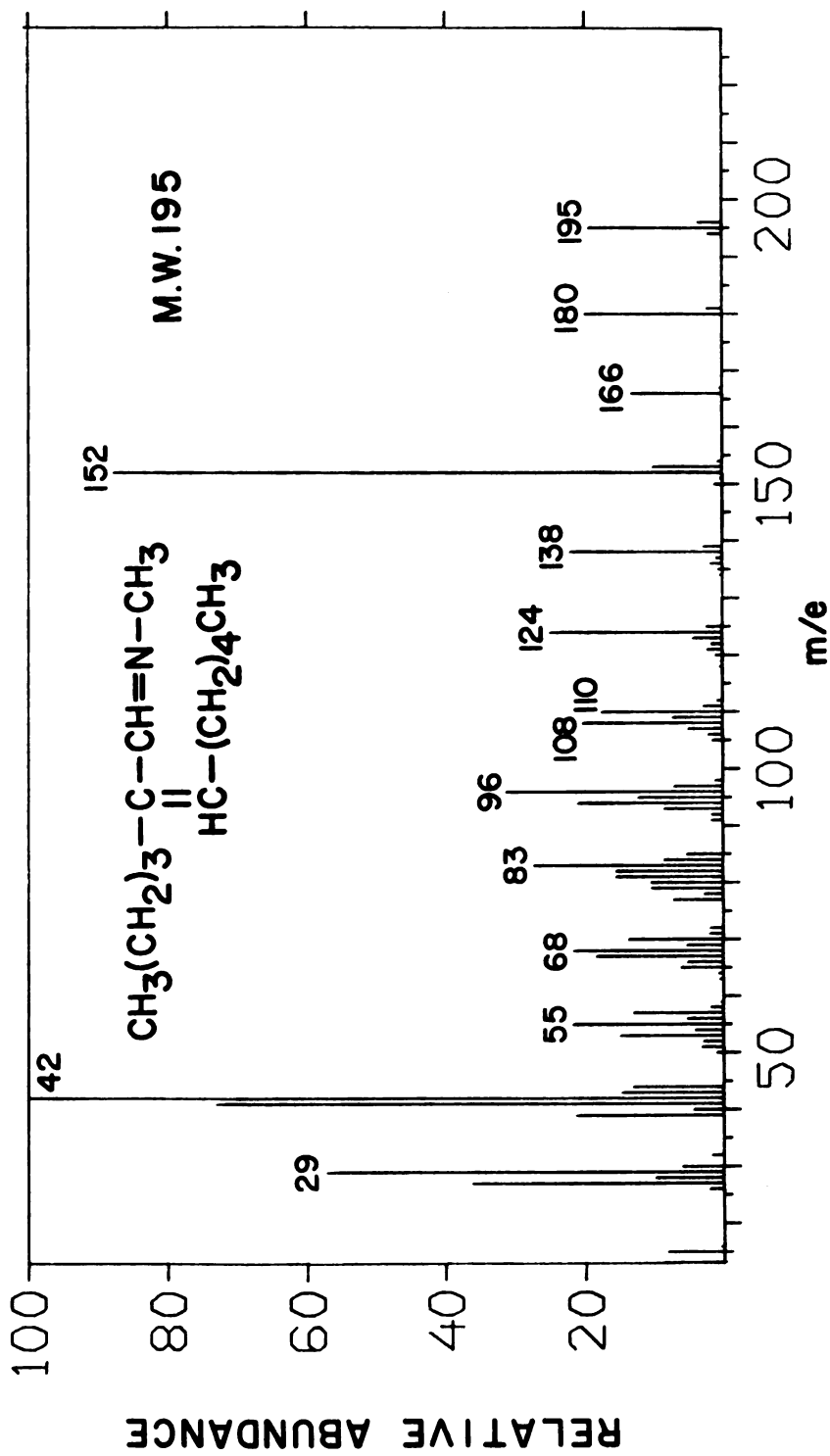

Figure 9.--Mass spectrum of HMI "dimer"

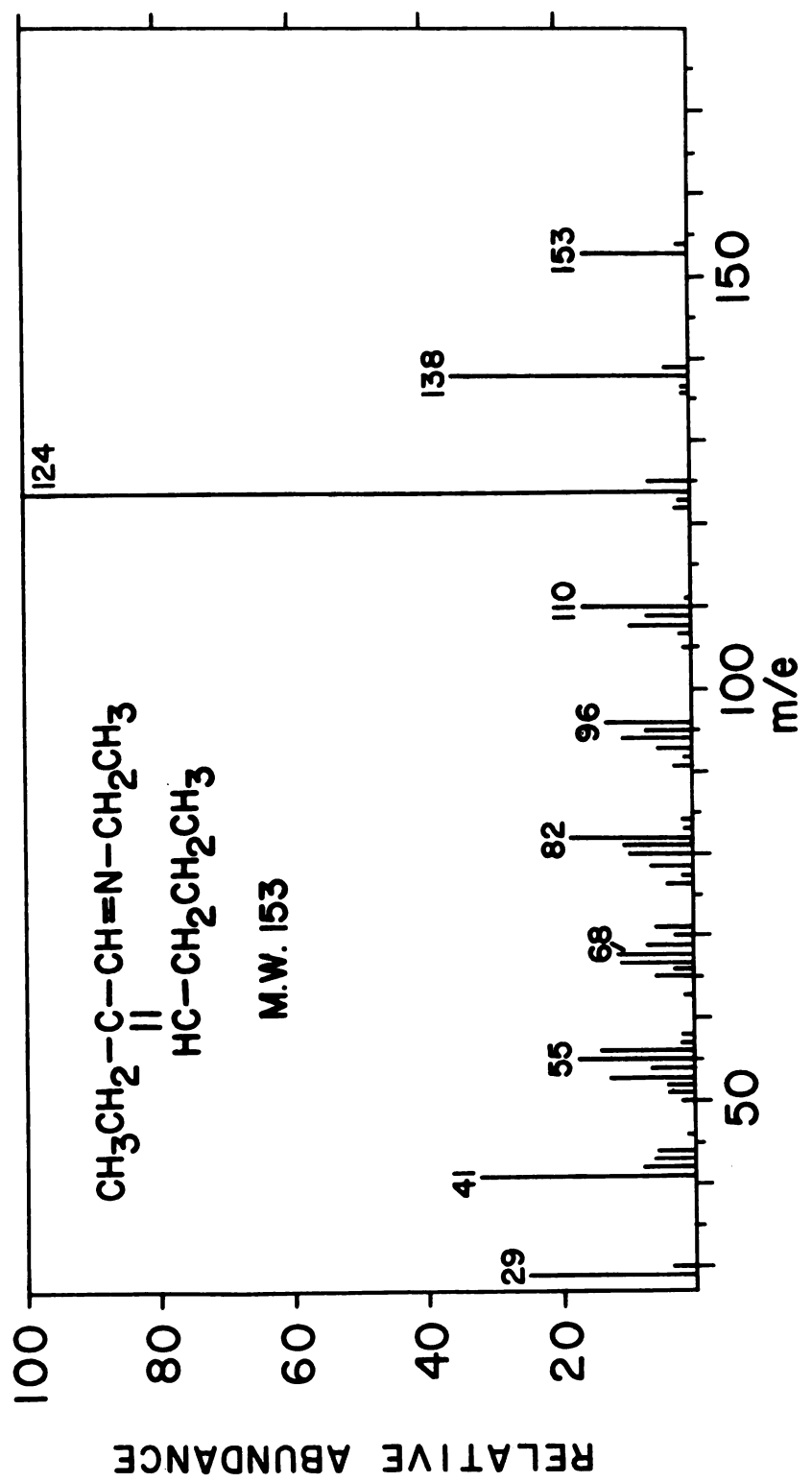

Figure 10.--Mass spectrum of BEI "dimer"

condensation of an aldehyde "dimer" with an amine group or (2) an aldol-type condensation of two imine monomers.

The presence of aldol-imine dimers and/or polymers in the imine to be used in subsequent nitrosamine syntheses was deemed undesirable, as this would complicate identification of the end-products. Fortunately, the presence of aldol-imines may be easily detected by a shift in the C=N absorption. The peak at 1675 cm^{-1} in the IR spectrum of the HMI monomer (M.W. 113), Figure 4, is shifted to 1650 cm^{-1} in the HMI dimer (M.W. 195), Figure 11. Conjugation with an ethylenic double bond has been reported to cause such a shift in absorbance (8).

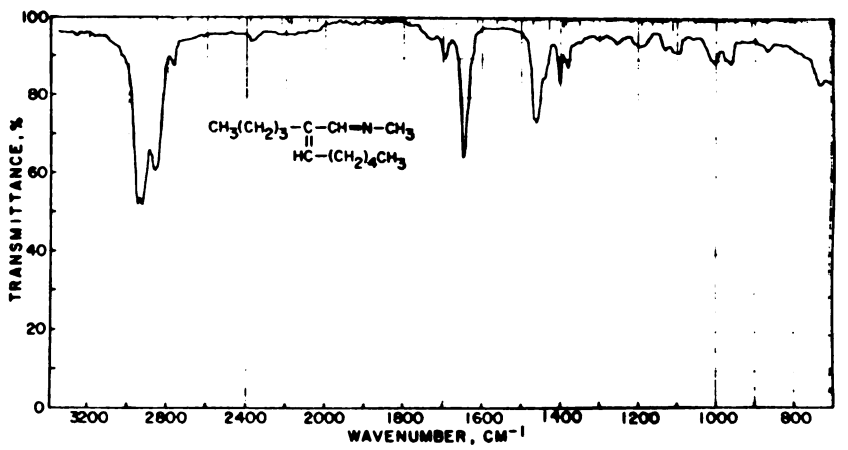

Figure 11.--Infrared spectrum of HMI "dimer"

Attempted Syntheses of Nitrosamines

HMI and Sodium Nitrite

The end-products of the reaction between HMI and sodium nitrite were collected over four temperature ranges during vacuum distillation at 17 mm: less than 30 C, 30-80 C, 80-115 C, and 115-150 C. Each of these four fractions and the distillation residue were tested with Griess reagent for nitrosamine content. Negative results were obtained in every instance.

IR spectra for each distillation fraction above 30 C and the spectrum of HMI prior to addition of sodium nitrite are presented in Figure 12. Recalling that IR spectra of aliphatic N-nitrosamines are distinguished by absorption bands at 1425-1460 cm^{-1} for N=O and at 1030-1150 cm^{-1} for N-N (83), it is interesting to note that all of the spectra in Figure 12 show absorption between 1450-1470 cm^{-1}, but none at 1075 cm^{-1}. Since the HMI absorbs at 1460 cm^{-1}, it is difficult to place much significance on a similar absorption by these "nitrosated" imines. The IR spectra of the standard dialkyl-N-nitrosamines in the Appendix all show a strong absorbance at or near 1075 cm^{-1}. Since none of the nitrosated HMI distillation fractions demonstrated this absorbance, it would appear that nitrosamines were not formed by reacting HMI with sodium nitrite. Of further

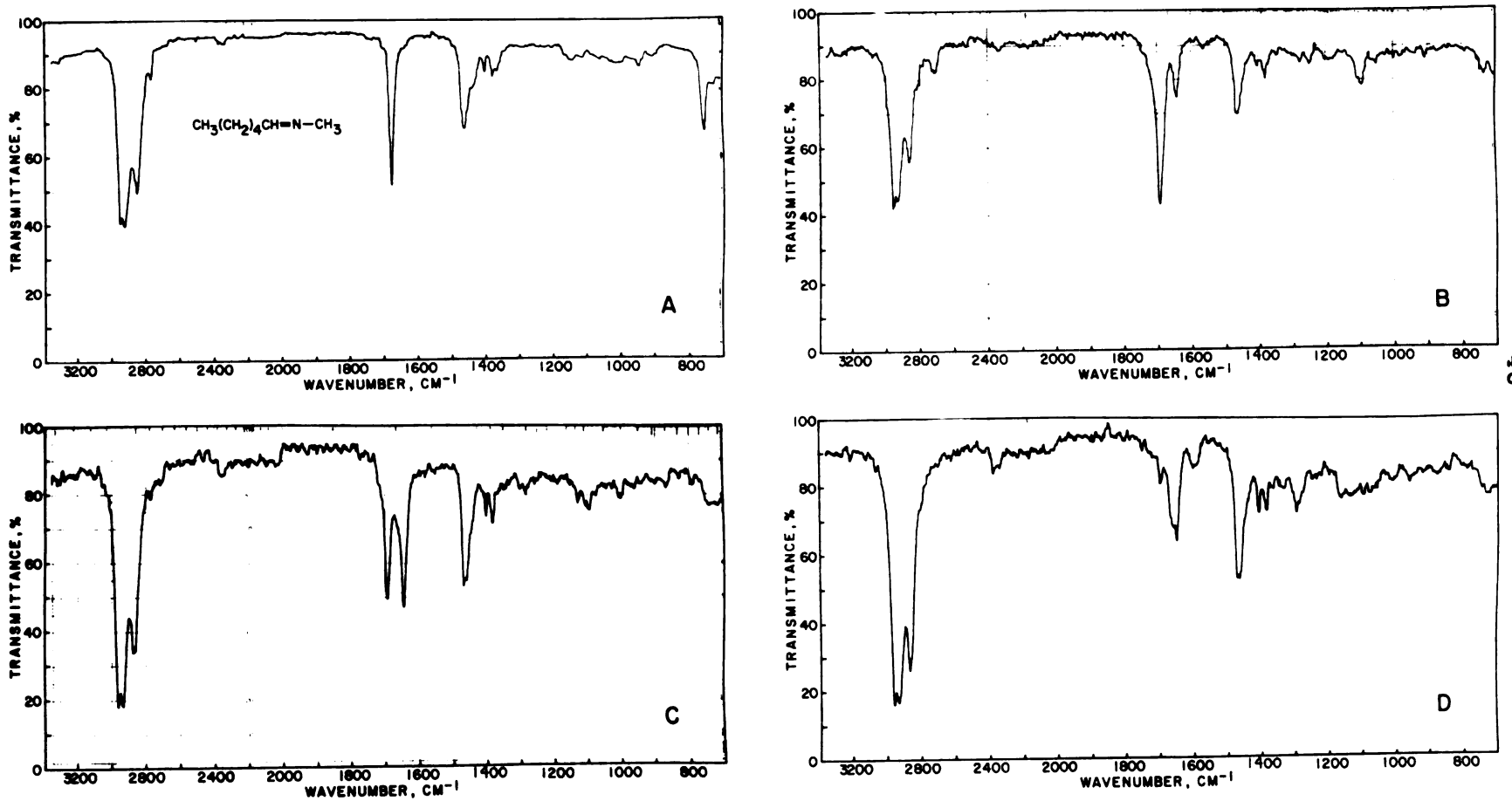

Figure 12.--Infrared spectra of HMI and the 30, 113, and 125 C/17 mm fractions (A, B, C, and D, respectively) distilled from a mixture of HMI and sodium nitrite

interest concerning the IR spectra in Figure 12 are the changes which occur between 1600 and 1700 cm^{-1}. The HMI has a strong peak at 1675 cm^{-1} which is replaced by peaks at 1645 and 1695 cm^{-1} in the nitrosated fractions. The peak at 1695 cm^{-1} predominates in the lower boiling fractions, but decreases and almost disappears in the higher boiling fractions.

Mass spectra of nitrosated HMI fractions which boiled at 113 C and 125 C/20 mm are presented in Figures 13A and 13B. The spectra are similar, but the 125 C-fraction apparently has a higher molecular weight. The compounds responsible for these spectra were not identified.

BEI and Sodium Nitrite

A second imine, N-n-butylidene-ethylamine (BEI), was allowed to react with aqueous solutions of sodium nitrite, fractionally distilled, and the distillate tested for nitrosamine content. Two fractions were collected, the first boiling at 62-72 C/12 mm, and the second at 108-112 C/12 mm. Both fractions gave negative Griess tests.

The IR spectrum of BEI prior to the addition of sodium nitrite and the spectra of both distillation fractions of the "nitrosated" BEI are shown in Figure 14. As with HMI, the large C=N peak at 1675 cm^{-1} is replaced

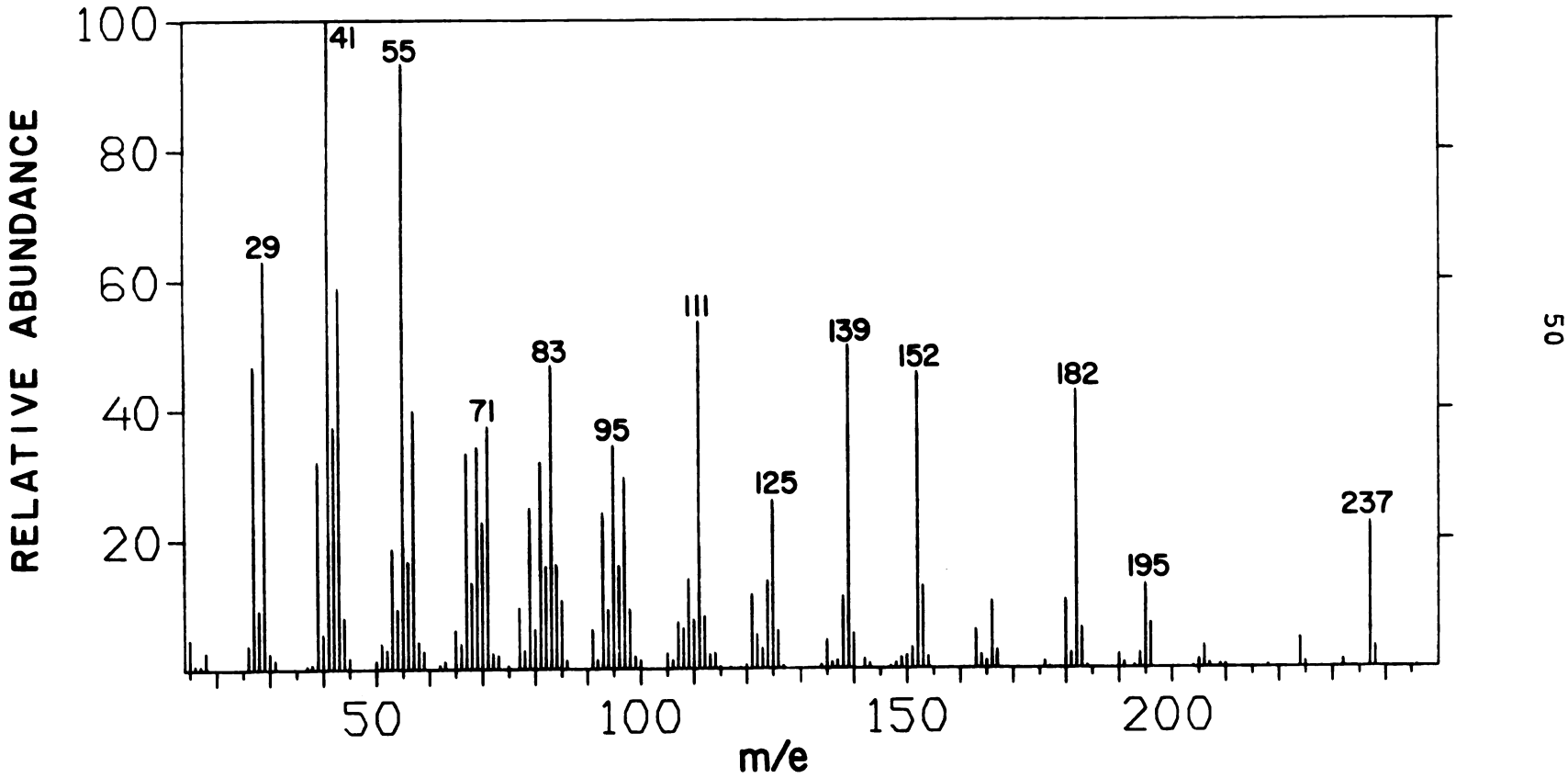

Figure 13A.--Mass spectrum of the 113 C/20 mm fraction distilled from a mixture of HMI and sodium nitrite

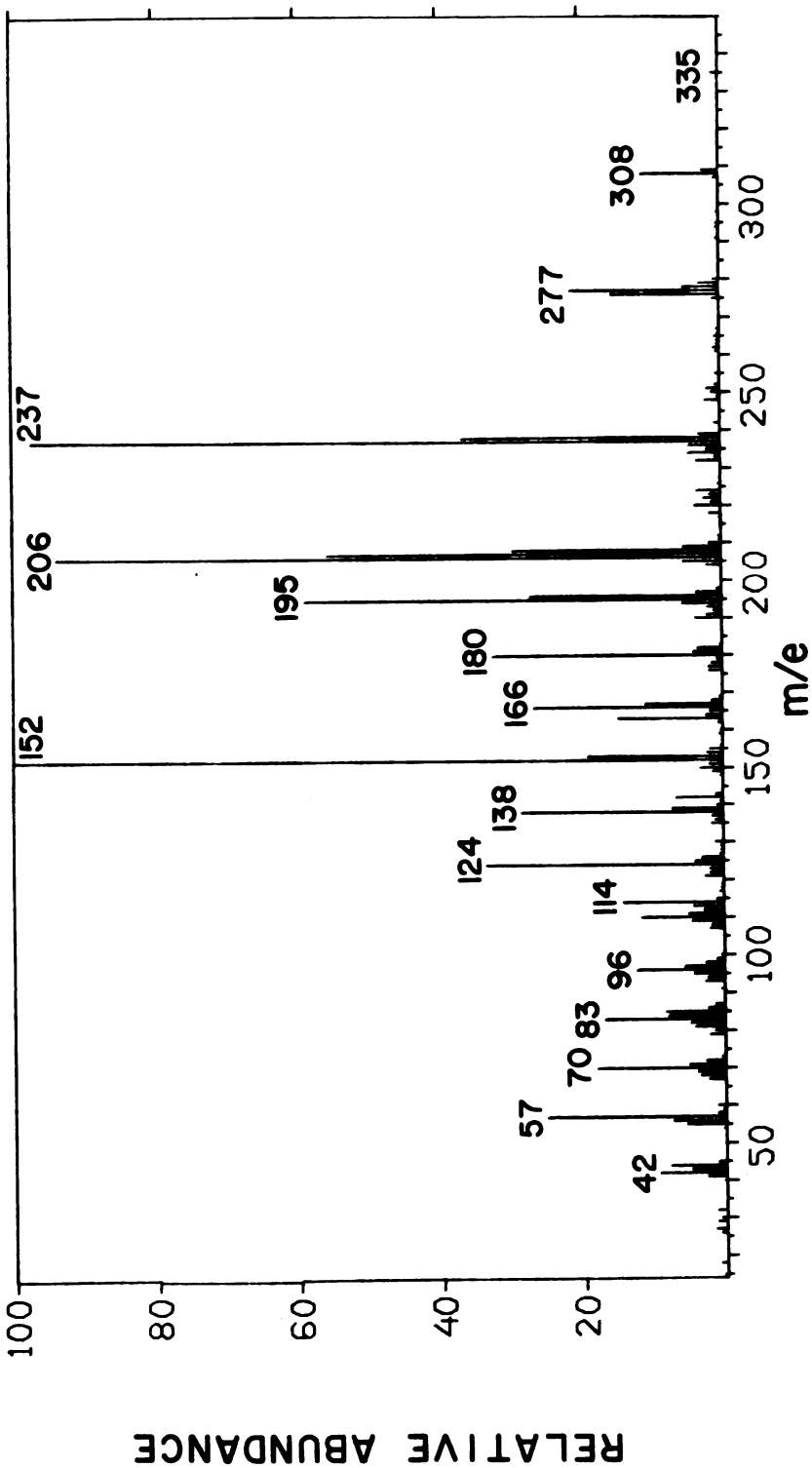

Figure 13B.--Mass spectrum of the 125 C/20 mm fraction distilled from a mixture of HMI and sodium nitrite

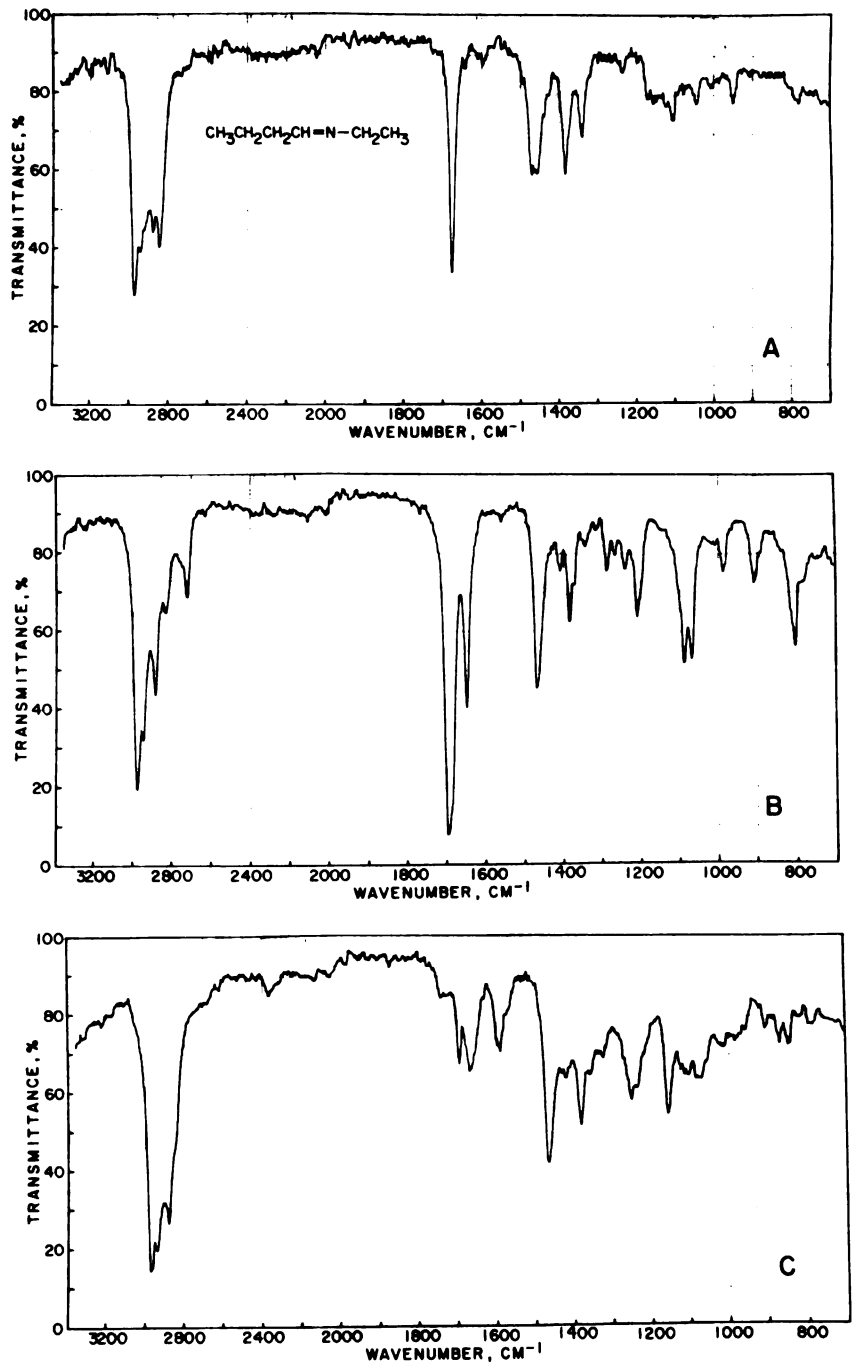

Figure 14.--Infrared spectra of BEI (A) and the 62-72 (B) and 108-112 (C) C/12 mm fractions distilled from a mixture of BEI and sodium nitrite

with peaks at 1695 and 1645 cm^{-1} in the "nitrosated" samples; and, again, the peak at 1695 cm^{-1} is very large in the lower boiling fraction, but small in the high boiling fraction. All three spectra in Figure 14 show absorbance in the 1450-1470 cm^{-1} N=O stretching region, but only the low-boiling "nitrosated" BEI fraction has appreciable absorbance between 1030 and 1150 cm^{-1}, the region of N-N stretching. The latter spectrum contains the peaks expected for a nitrosamine; however, their presence does not dominate the spectrum, as in the case of the standard nitrosamines.

The mass spectrum of the low-boiling fraction is shown in Figure 15. This compound was not identified.

Imine and Nitric Oxide

An attempt was made to synthesize nitrosamines by bubbling nitric oxide gas through the imine. This system was simple, and since no water came in contact with the reactants, the possibility of hydrolytic decomposition of the intermediates and/or end-products was eliminated (72).

Despite these advantages, this approach was unsuccessful with both imines used, BEI and N-benzylidenemethylamine. The products always gave a false-positive Griess test, i.e., spontaneous color development on a TLC plate without UV irradiation. Spontaneous color development also occurred in samples which had

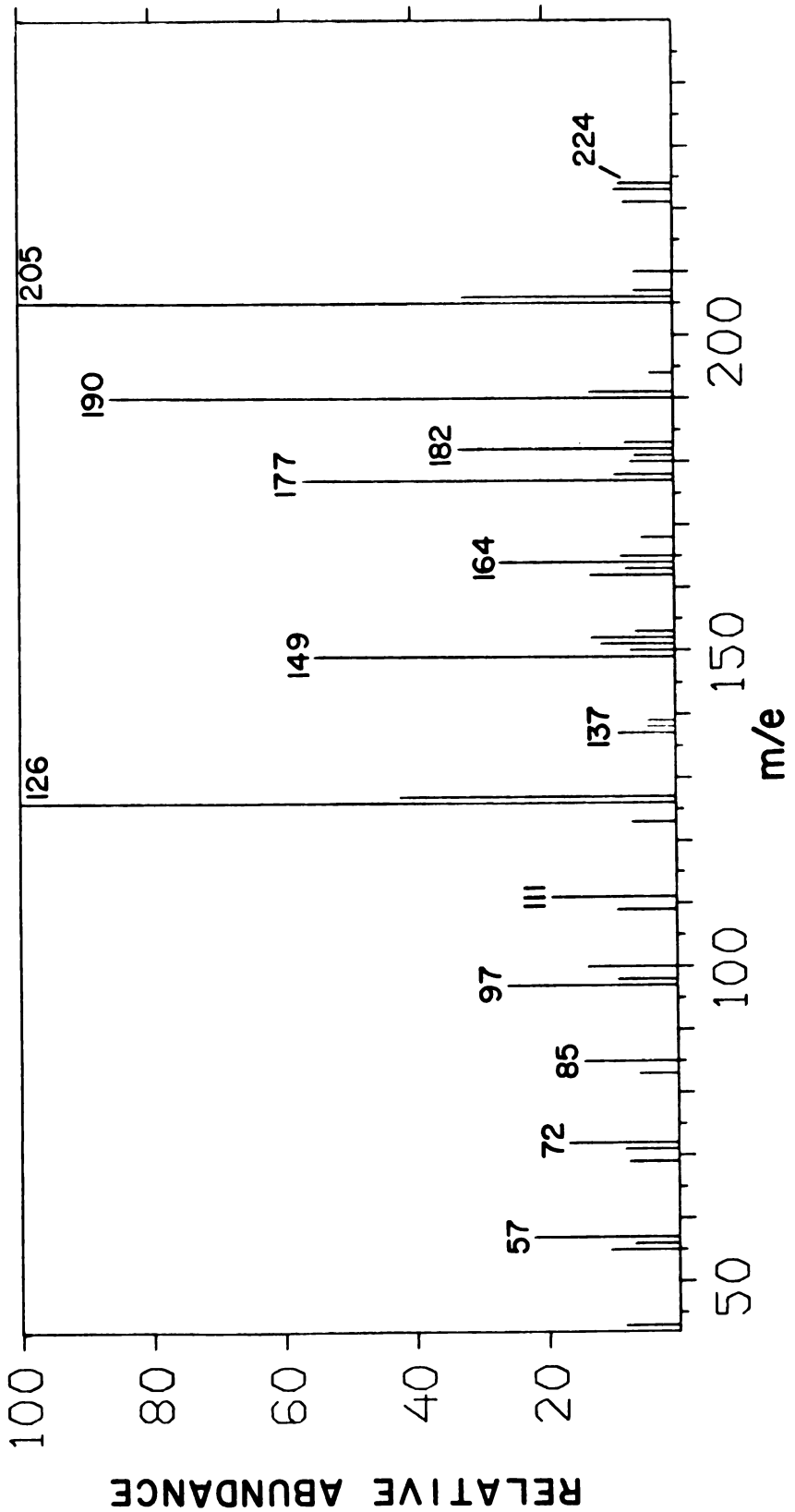

Figure 15.--Mass spectrum of the 62-72 C/12 mm fraction distilled from a mixture of BEI and sodium nitrite

been extensively purged with nitrogen prior to analysis. These false-positive results were probably due to residual oxides of nitrogen. A comparison was made of the IR spectra of BEI before and after exposure to nitric oxide, and the spectrum of the nitric oxide-treated imine following a ten-minute irradiation with UV light. No significant differences were noted in the three spectra.

Aldehyde, Primary Amine, and Sodium Nitrite

An aldehyde, n-butyraldehyde, was added to a mixture of ethylamine and sodium nitrite. The purpose of this procedure was to determine if the presence of sodium nitrite during the synthesis of imine would result in nitrosamine formation, where addition of nitrite to the previously synthesized imine had failed. In other words, would an intermediate in the formation of imines be easier to nitrosate than the imine itself?

Concentrated ether extracts consistently gave false-positive Griess tests. IR spectra from these products were almost identical to the spectra previously shown in Figure 14 for BEI following the addition of sodium nitrite. Apparently, the same product is obtained regardless of whether nitrite is added before or after the imine is formed. The shift of the major absorption peak from 1675 cm^{-1} to

1695 cm^{-1} could be due to a nitrosated intermediate such as $[R\text{-}CH{=}\overset{+}{N}\!<^{NO}_{R'}]$. A positively charged imine group has been reported to absorb at 1690 cm^{-1} (72).

Ground Ham Model System

Although aldehydes and sodium nitrite were not shown to form nitrosamines in the preceding experimental syntheses, an attempt was made to form nitrosamines by combining these reactants in a model dry food system. Rather than use a relatively inert material such as carboxy-methyl-cellulose (CMC), fresh (uncured) ground ham muscle was chosen as the matrix upon which the aldehyde and sodium nitrite were to be distributed. Ham was chosen because it is a common food normally consumed after being cured with sodium nitrite; and because ham muscle, unlike CMC, contains proteins which would supply free amino groups for reaction with aldehyde added to the model system. Aldehydes, from the oxidative decomposition of fatty acids, have been shown to react with the amino group of phospholipids to form imines (14).

Various combinations of sodium nitrite, sodium chloride, and hexanal were included in both the "wet" (non-freeze-dried) and "dry" (freeze-dried) systems. Freeze-drying the meat emulsions prior to heating was intended to reduce the possibility of decomposition of nitrosamines or nitrosamine-intermediates as a result of hydrolysis (Dr. W. H. Reusch, Michigan State

University, personal communication). The amounts of aldehyde and nitrite added to the model system were based on an estimate of the millimoles of free amino groups in 100 g ham muscle. Lean trimmed ham muscle contains 20% protein (75); and twenty grams of ham protein contain approximately 3.2 g of the amino acids lysine, arginine, asparagine, and glutamine (62). Based on these values, the free amino groups in 100 g ham muscle were estimated as 20 mM. Enough hexanal for a 1:1 stoichiometric reaction with the amino groups, a two-fold excess of sodium nitrite, and 2% sodium chloride were added to the appropriate model system variables. To insure that nitrosamines were not forming during the extraction procedure, sodium nitrite was incorporated into the baked emulsion of some of the samples at the beginning of the extraction procedure. The negative result obtained for this variable (see Table 4) indicates that nitrosamine formation did not occur during the extraction procedure. Recovery control samples were spiked with dimethyl- and dibutylnitrosamine standards at a concentration of 10 ppm (1 mg nitrosamine/100 g meat).

The minimum level of nitrosamines detectable with the Griess and ninhydrin reagents was 1 μg; therefore, a negative result in Table 4 means that

TABLE 4.--Presence of nitrosamine-positive compounds in the extract from "dry" and "wet" model food systems as indicated by Griess and ninhydrin reactions on thin-layer plates

Additives	Treatments	
	Freeze-dried	Non-freeze Dried
Control	-	-
Nitrite	-	+
Nitrite + NaCl	-	+
Nitrite + aldehyde	-	+
Aldehyde	-	-
Aldehyde + nitrite added just before extraction	-	-
Nitrosamine	+	+

less than 1 µg of nitrosamine was present in the 40 µl concentrated extract used for each determination.

All of the "wet" treatment samples which contained nitrite, alone or in combination with other variables, gave positive Griess and ninhydrin tests. Some of the nitrite-containing "dry"-system samples also gave positive Griess or ninhydrin tests, but negative results were recorded in Table 4 unless both tests were positive. In both wet and dry systems, the Rf values of the positive spots were the same, 0.30 and 0.65. Samples which gave a positive Griess test were analyzed by GC-MS. The spectra obtained were compared with the spectra of 25 standard nitrosamines (53) and 101 pyrazines (44), but could not be identified.

It seems probable that the same nitroso-positive compounds were formed in each system, but were present in larger quantities in or were more easily extracted from the "wet" (non-freeze-dried) system. Also, the surface of the "wet" samples developed extreme case-hardening during the 15-hour heating in circulating dry air, and this may have helped retain the nitroso-positive compounds during the heat treatment. Formation of nitrosamines in the non-freeze-dried ham muscle would seem reasonable in view of the excessive nitrite level used, 138-times the legal maximum. Fiddler et al. (26)

have shown nitrosamine formation will occur in meat emulsions, provided an excessive nitrite concentration is present.

The low levels of nitrosamine-positive compounds found in the freeze-dried ham emulsion could represent compounds which formed prior to freeze-drying, or low residual levels resulting from losses of volatile nitrosamines during the freeze-drying and/or heating steps.

SUMMARY AND CONCLUSIONS

Attempts were made to synthesize N-nitrosamines by the reaction of imines with sodium nitrite or with nitric oxide gas, and by the reaction of nitrite with aldehyde and primary amines. Under the reaction conditions imposed, nitrosamines either were not formed, were non-volatile, or were not identified by the detection methods used. Acid hydrolysis of imines and/or "nitrosated" imines probably accounts for the failure to form nitrosamines from carbonyl-amine precursors in aqueous systems. Any nitrosamines which may have been formed in the "dry" imine-nitric oxide system were masked by the spontaneous red color which formed when these samples were tested with Griess reagent.

In an attempt to react sodium nitrite with carbonyl-amine compounds in a dry food system, various combinations of sodium nitrite, sodium chloride, and hexanal were distributed on a ground ham muscle matrix prior to freeze-drying. The freeze-dried samples were exposed to heat over a prolonged time in an attempt

to enhance formation of carbonyl-amine condensation products. Freeze-dried meat, to which 138-times the maximum legal concentration of sodium nitrite had been added, contained traces of Griess-positive compounds. However, non-freeze-dried controls contained considerably more of these apparent nitrosamines. There was no evidence that the addition to the meat of components which would enhance the probability of non-enzymatic browning in the system contributed to the presence of nitroso-positive reactants in the extracts. Samples containing the apparent nitrosamines were analyzed by gas chromatography-mass spectrometry, but were not identified when compared to mass spectra of known nitrosamines and pyrazines.

APPENDIX

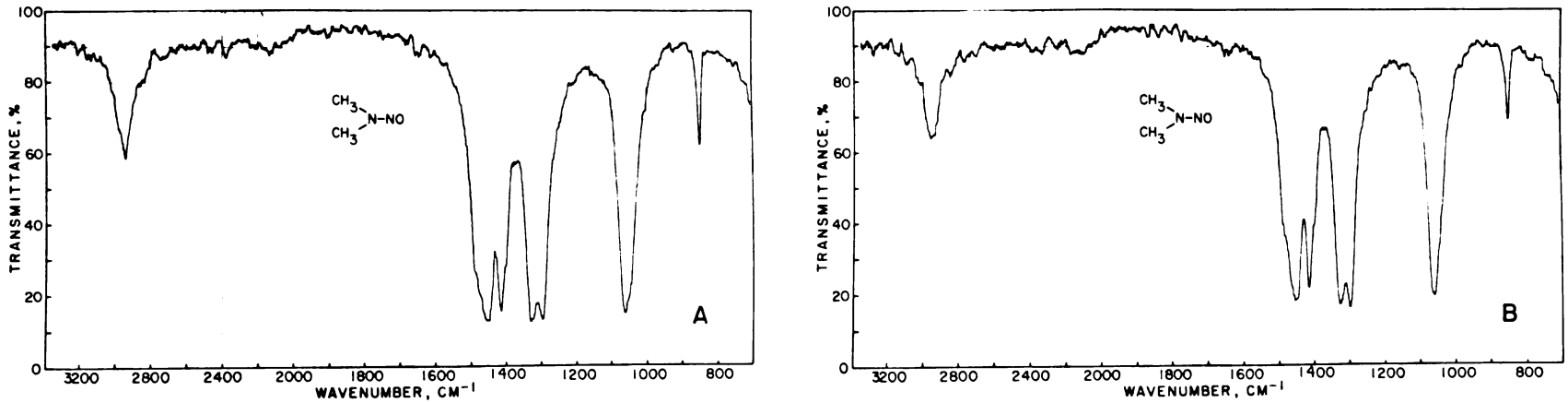

Figure 16.--Infrared spectra of N-nitrosodimethylamine (A) purchased from Eastman Kodak Company and (B) synthesized in this laboratory

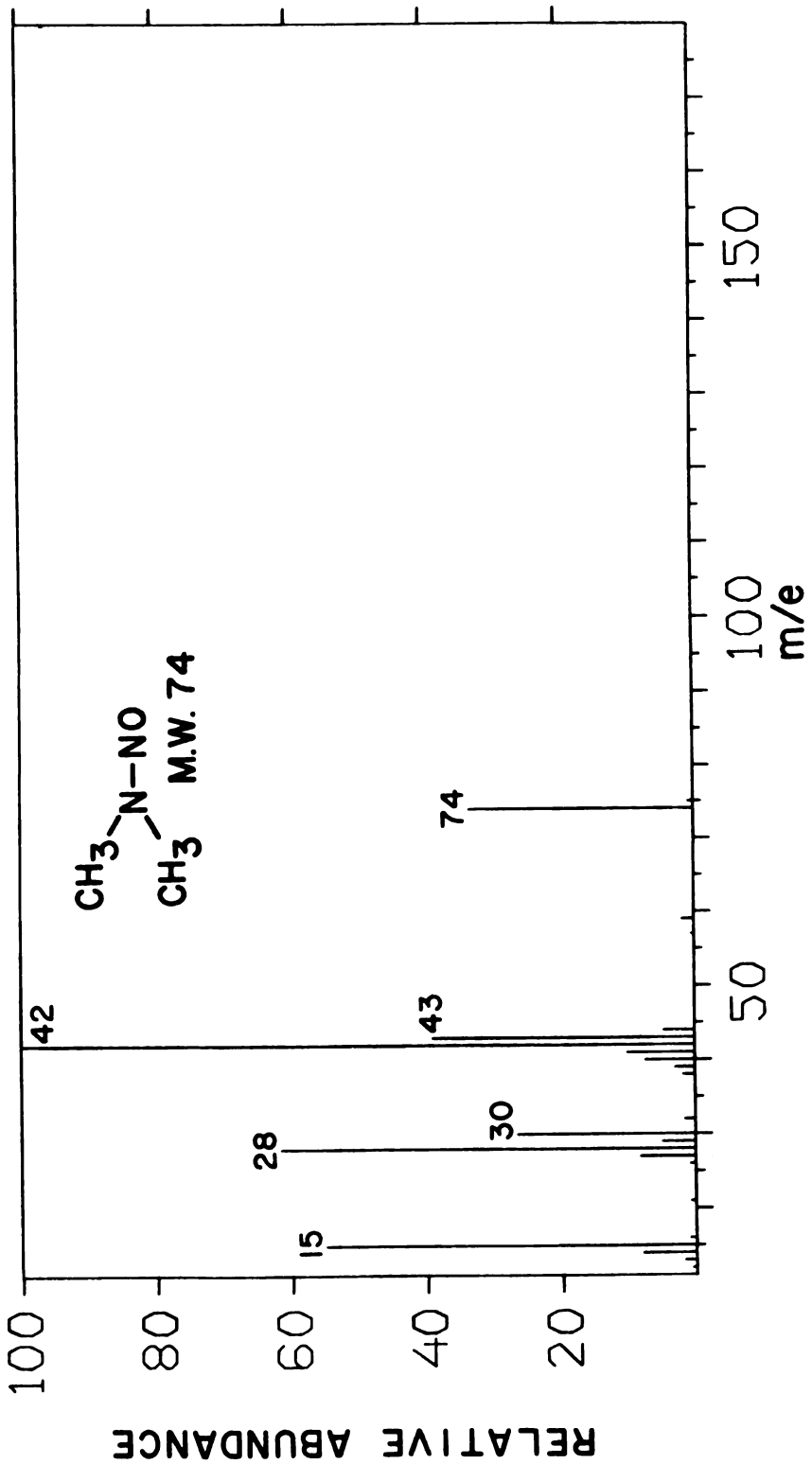

Figure 17.--Mass spectrum of N-nitrosodimethylamine purchased from Eastman Kodak Company

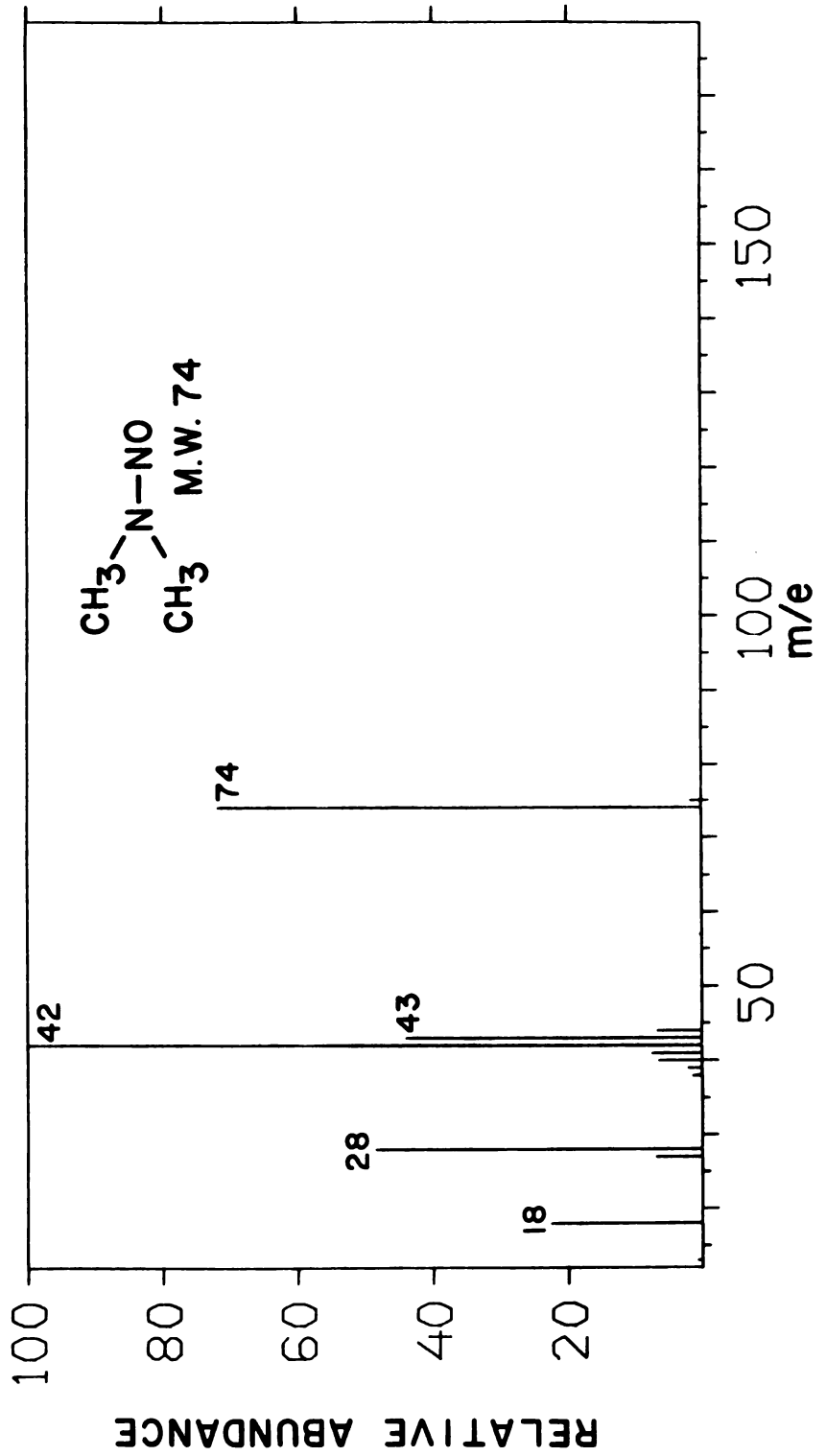

Figure 18.--Mass spectrum of N-nitrosodimethylamine synthesized in this laboratory

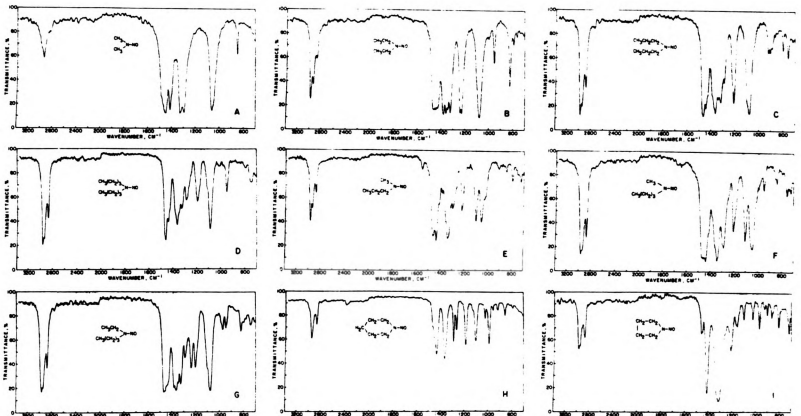

Figure 19.--Infrared spectra of N-nitrosodimethylamine (A), -diethylamine (B), -dipropylamine (C), -dibutylamine (D), -methylpropylamine (E), -methylbutylamine (F), -ethylbutylamine (G), -piperidine (H), and -pyrrolidine (I)

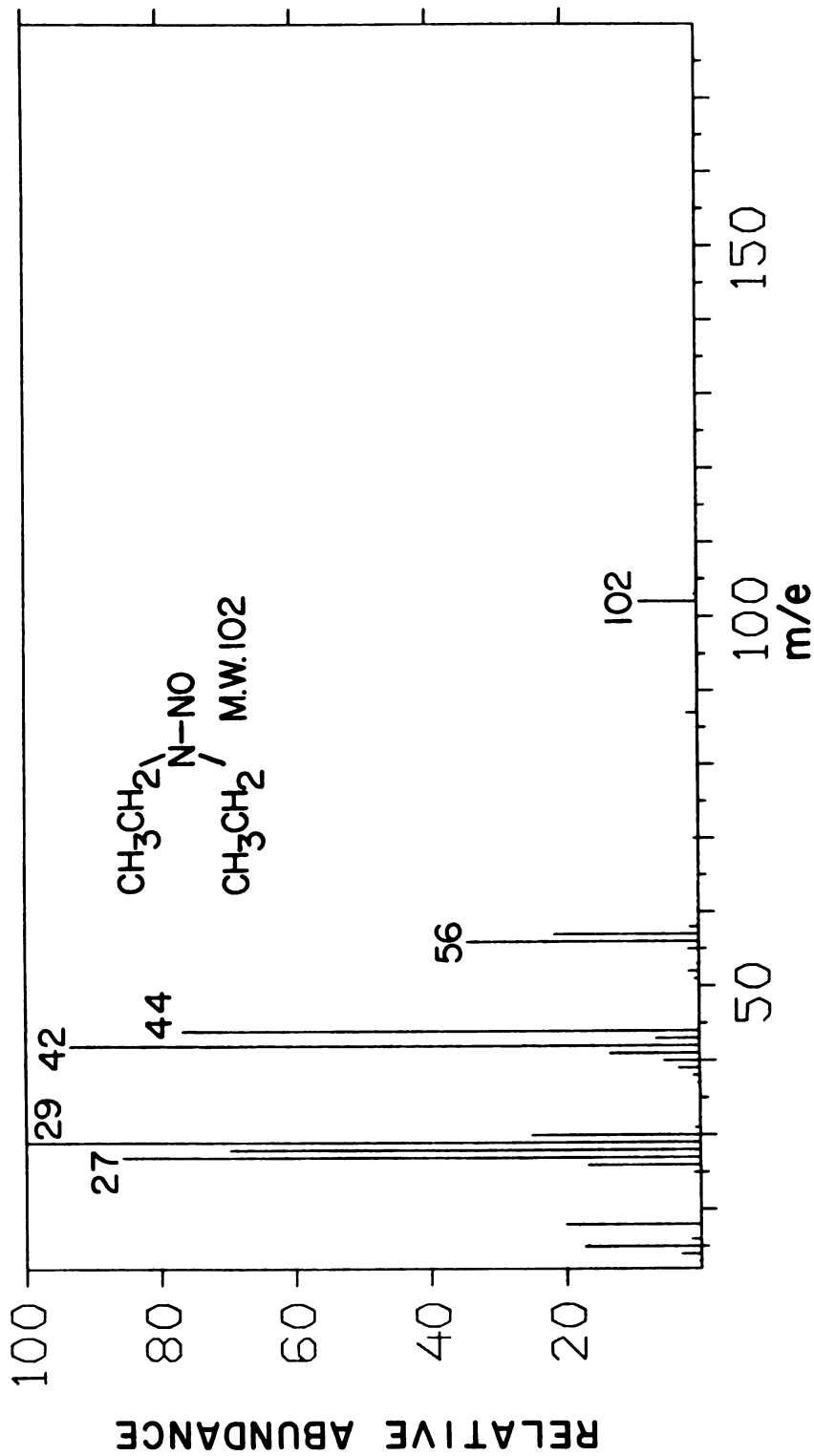

Figure 20.--Mass spectrum of N-nitrosodiethylamine purchased from Eastman Kodak Company

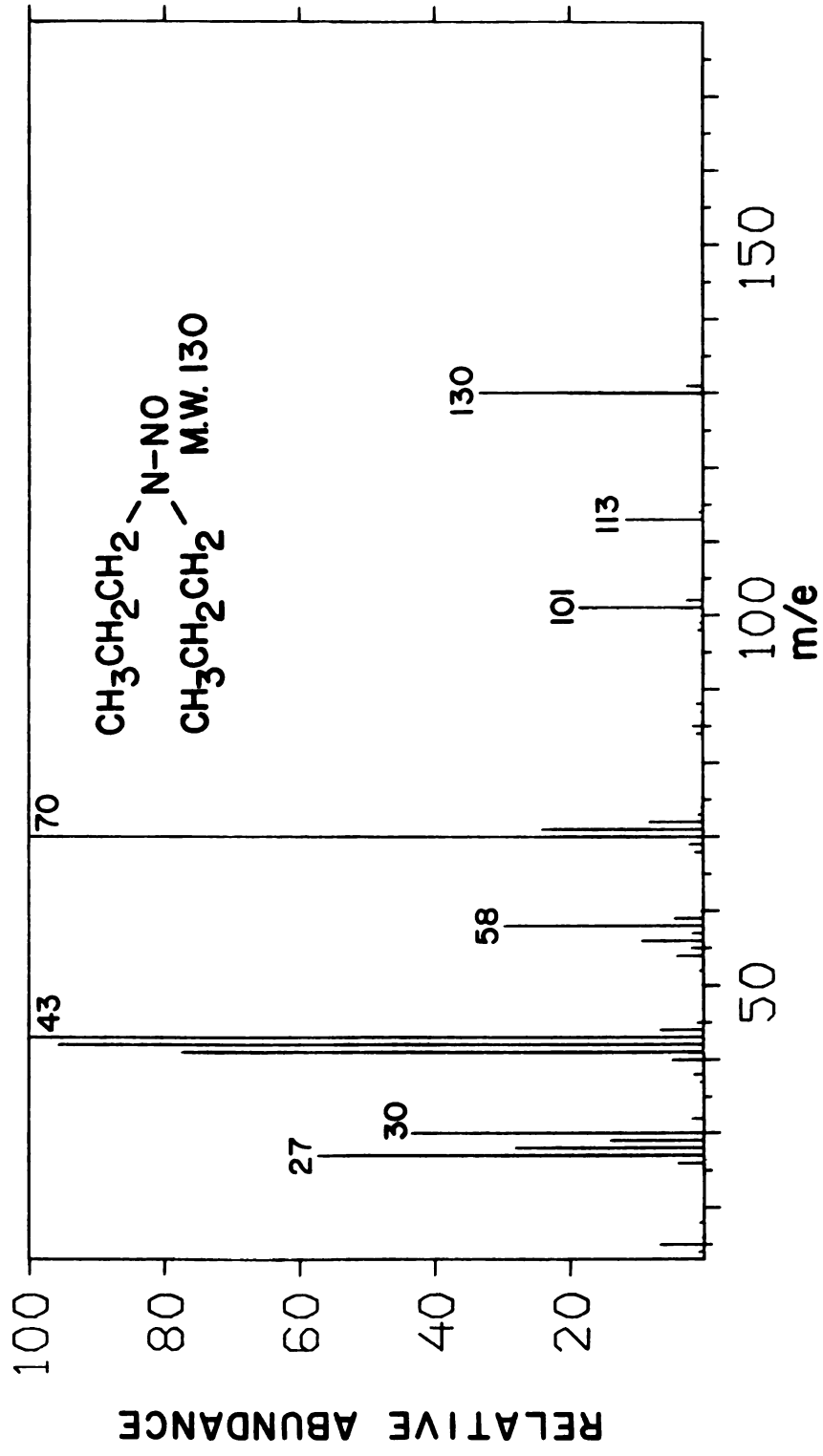

Figure 21.--Mass spectrum of N-nitrosodipropylamine purchased from Eastman Kodak Company

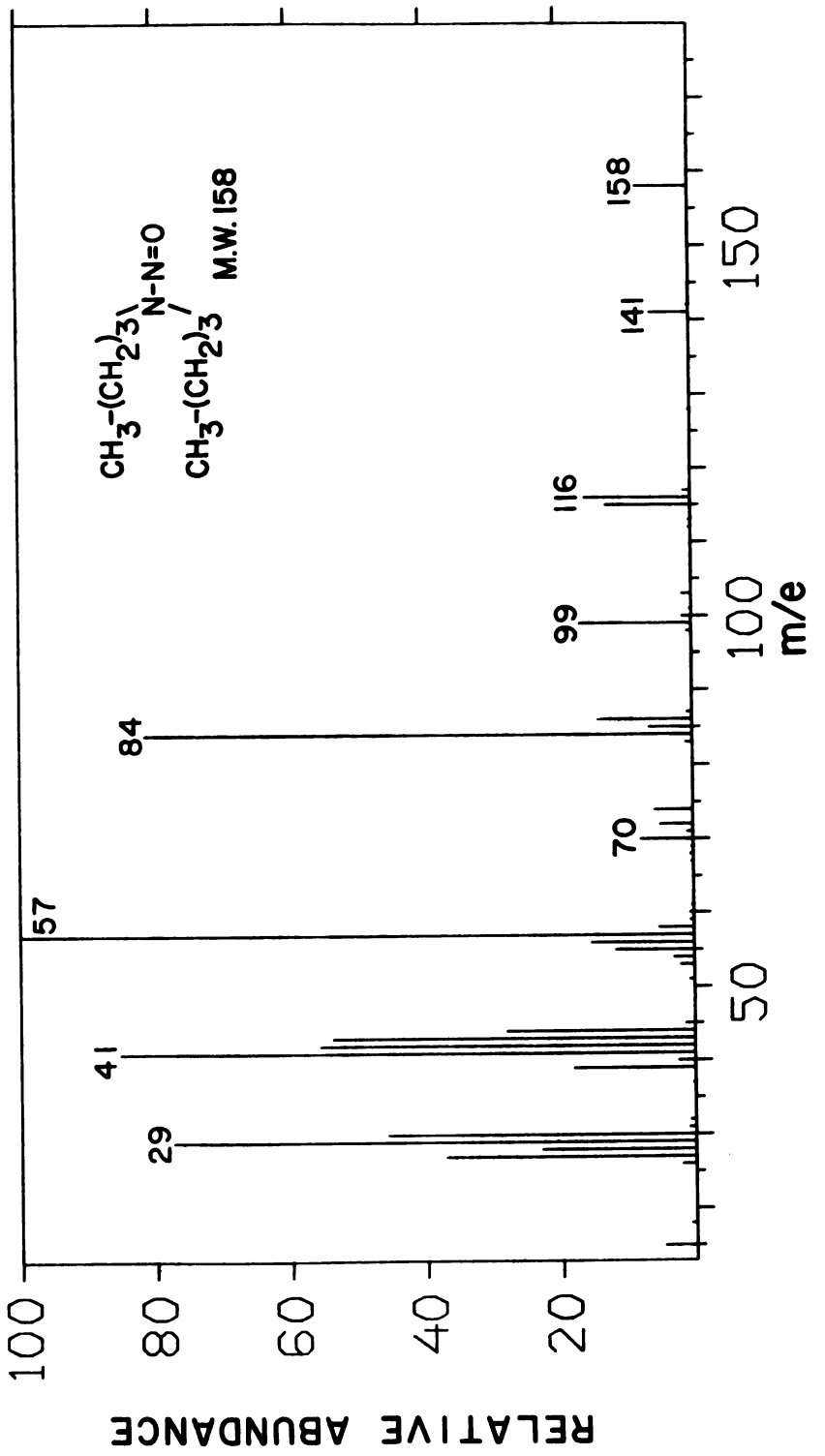

Figure 22.--Mass spectrum of N-nitrosodibutylamine purchased from Eastman Kodak Company

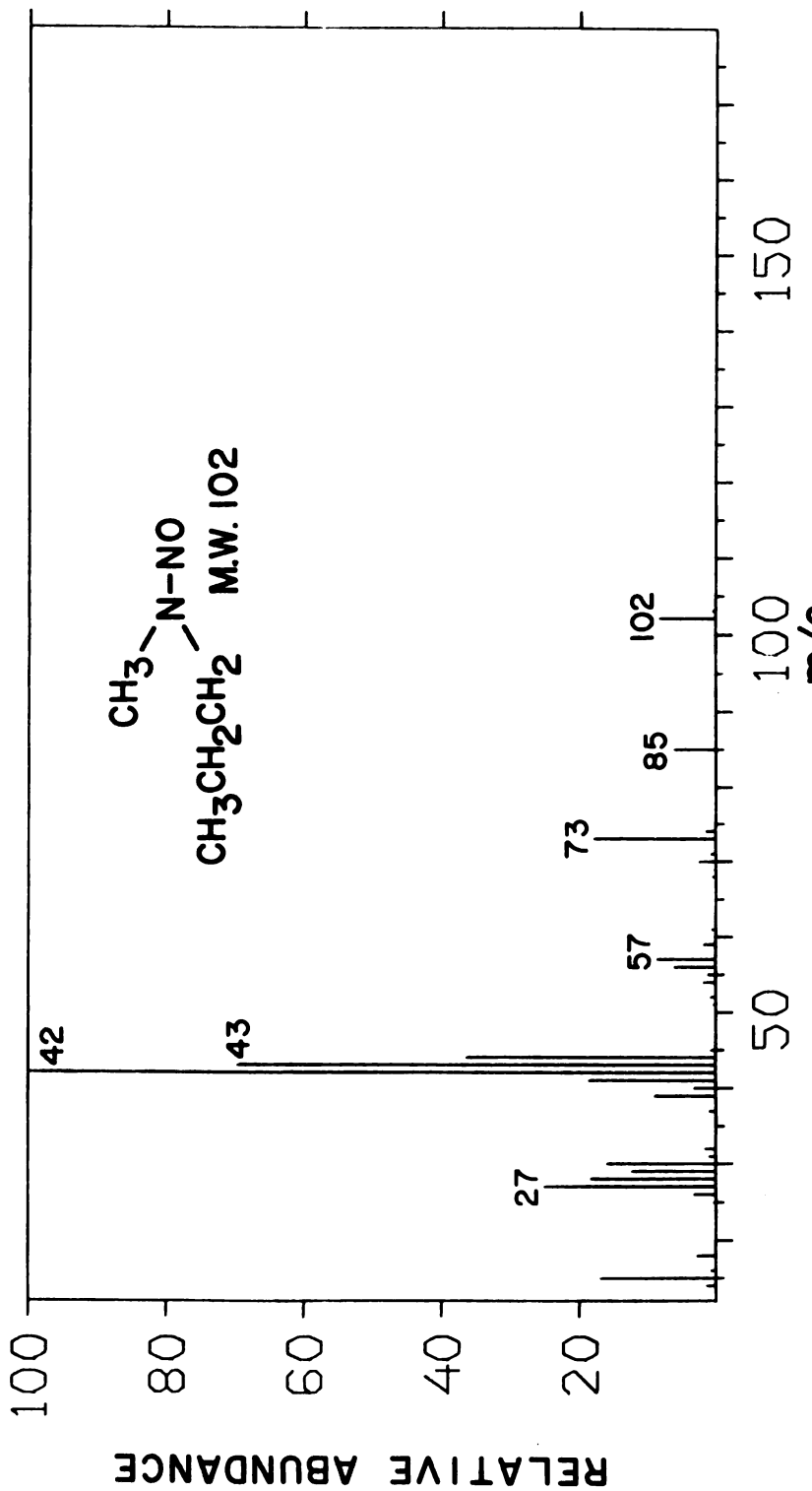

Figure 23.--Mass spectrum of N-nitrosomethylpropylamine obtained from Dr. H. Roper, Institut für Organische Chemie und Biochemie, 2 Hamburg 13, Papendamm

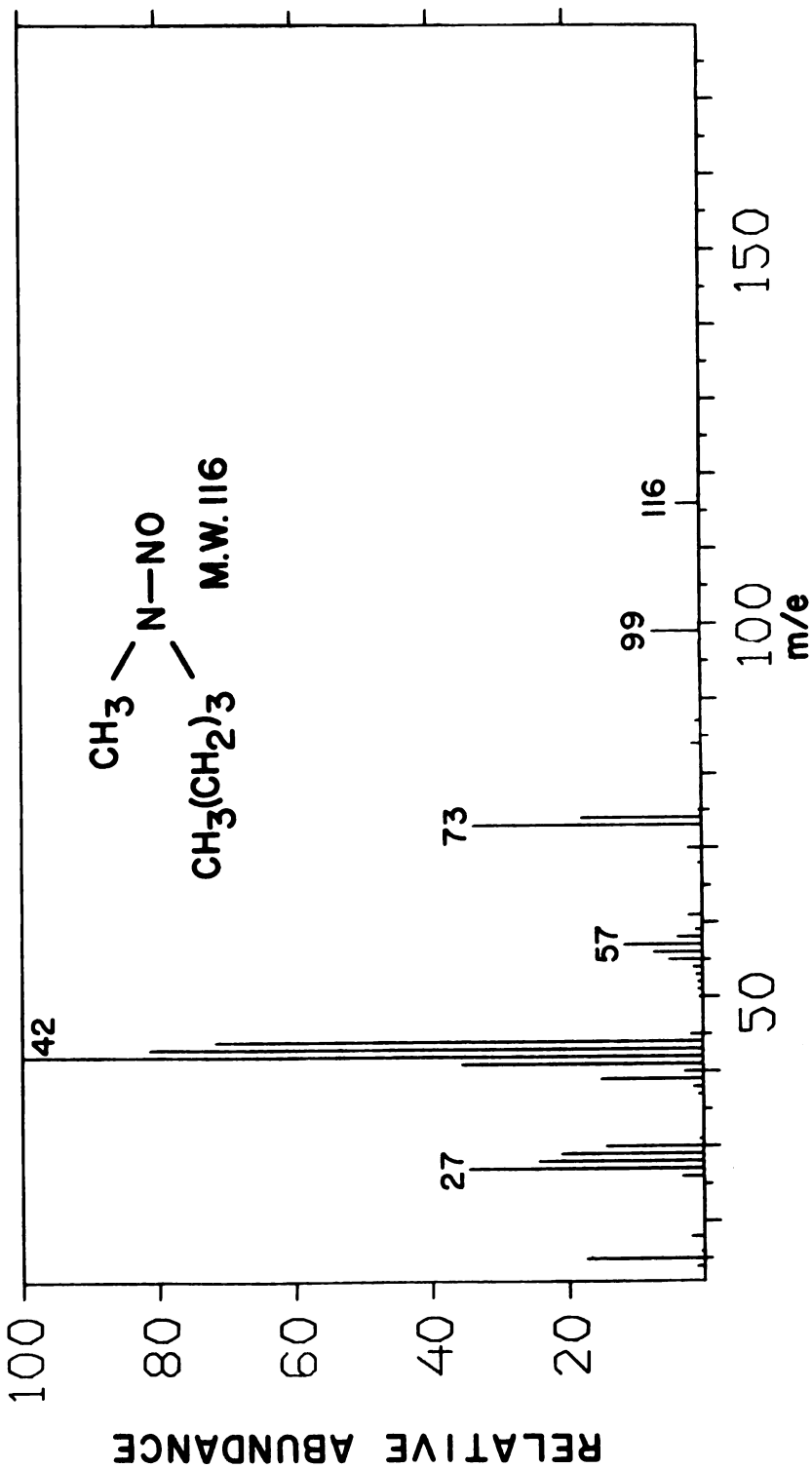

Figure 24.--Mass spectrum of N-nitrosomethylbutylamine obtained from Dr. D. Osborne, Unilever Research Laboratories, Colworth House, Sharnbrook, Bedfordshire, UK

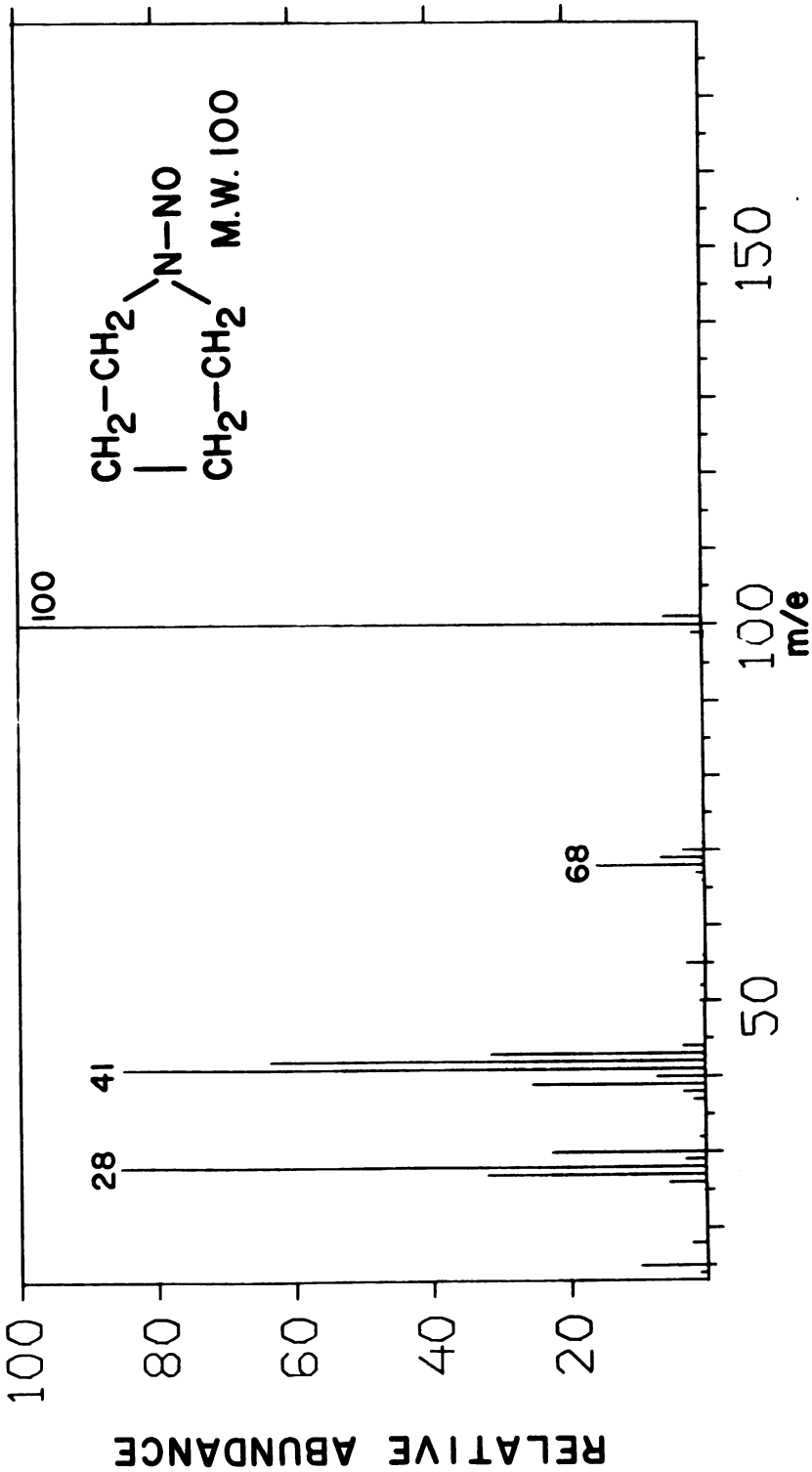

Figure 25.--Mass spectrum of N-nitrosopyrrolidine purchased from Eastman Kodak Company

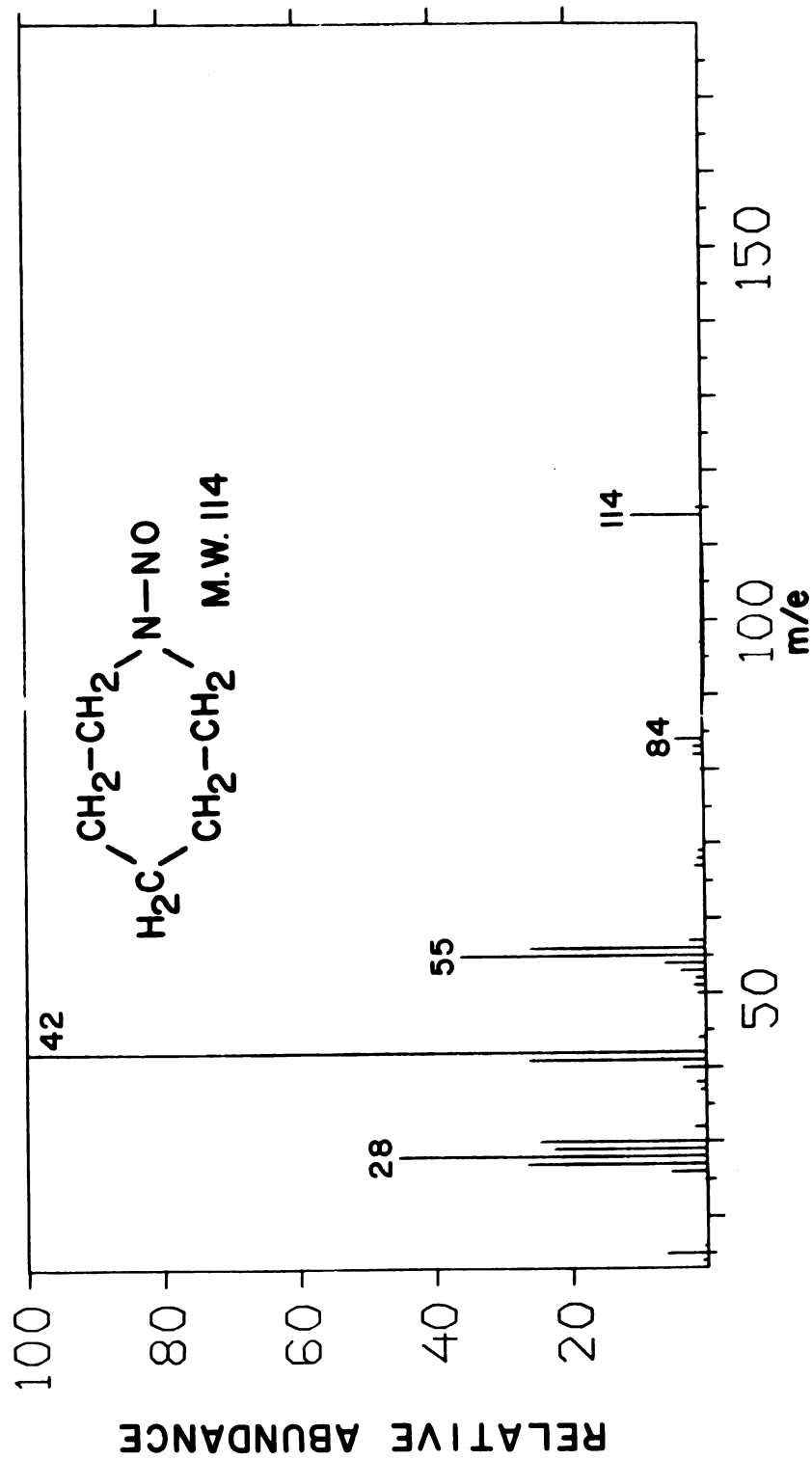

Figure 26.--Mass spectrum of N-nitrosopiperidine purchased from Eastman Kodak Company

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