This is to certify that the

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N-NITROSAMIDES IN FOOD: FORMATION AND TOXICOLOGY

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N-NITROSAMIDES IN FOOD: FORMATION AND TOXICOLOGY

BY

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ABSTRACT

N-NITROSAMIDES IN FOOD: FORMATION AND TOXICOLOGY

BY

Mohamad Hassan Fooladi

Formation of N-substituted amides was investigated using both a model system and bacon. Fatty acids were shown to react readily with selected -amino acids in model systems at 200°C to give N-substituted amides. This reaction involves decarboxylation of the amino acids and displacement of the alcohol moiety of the fatty esters by the amine that was formed. However, formation of primary amines via the decarboxylation of -amino acids appears to be unlikely at temperatures normally encountered in pan frying of bacon due to insufficient energy for the decarboxylation step. Under these conditions only amines would react readily with fatty acids to yield secondary amides.

N-Substituted amides were shown to be nitrosated readily under acid conditions in a model system. It was demonstrated that the normal pH of foods militates against the formation of N-nitrosamides in foods, even when their precursors are present. It was also demonstrated that

N-nitrosamides are very unstable under conditions commonly encountered in cooking of bacon. The major conclusion from this investigation was that N-nitrosamides are unlikely to be present in heat-processed foods.

In the event that N-substituted amides are formed during processing and cooking of foods, however, they could be nitrosated in vivo. Therefore, the carcinogenicity of these compounds was investigated in a feeding trial using Swiss Mice. None of the N-substituted amides alone or in combination with nitrite or their corresponding N-nitrosamides caused development of tumors in mice within a seven month feeding trial. Only the mice which were fed N-nitrosomethylurea (positive control) had lesions which were localized in the lungs. Since the result of mutagenicity assays showed that N-nitrosopentylpalmitamide and N-nitrosomethylurea are strong mutagens and that N-nitrosomethylpropionamide is a weak mutagen the possibility that these compounds are carcinogenic cannot be ruled out. Therefore, the remaining mice are being held on the same diet and will continue for two years before sacrificing and examining their tissues for tumor development.

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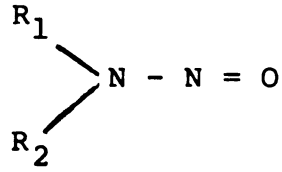
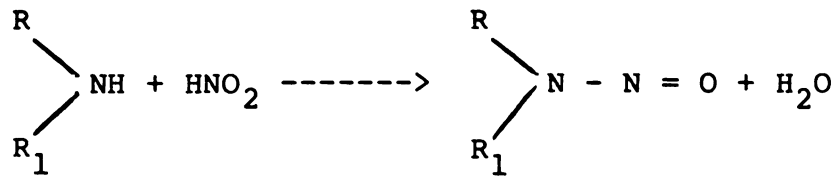
INTRODUCTION

Since the first report of experimental induction of liver cancer in rats by feeding low levels (50 μ/Kg) of N-nitrosodimethylamine (NDMA) (Magee and Barnes, 1956), N-nitroso compounds have been intensively studied as carcinogens. These compounds have been found to produce a variety of tumors at different organ sites, the extent depending on their structure and dosage rate, route of administration and the test species (Preussmann, 1973).

N-Nitroso compounds can be devided into two groups according to their chemical reactivity: (1) N-nitrosamines, and (2) N-nitrosamides. They have the following general formula:



N-Nitrosamines are formed principally from the reaction between secondary amines and nitrous acid. In this reaction, R_1 is an alkyl group while R may be an alkyl, aryl or a wide variety of other functional groups.



N-Nitrosamines can also be formed from tertiary amines, quarternary ammonium compounds and primary polyamines. Many of these N-nitroso compounds have been identified in various food systems (Gray and Randall, 1979). N-Nitrosamines are largely systemic agents and can be converted to reactive metabolites, probably alkylating agents, by enzymatic processes in the mammalian organism (Preussmann, 1973). On the other hand, N-nitrosamides which arise from the reaction of secondary amides with nitrite (Mirvish, 1977), usually have one alkyl residue (R_1) and an acyl residue (R_2). They are chemically reactive compounds and are relatively easily hydrolyzed to alkylating diazoalkanes (Preussmann, 1972). They are considered to exert both local and systemic activity in the carcinogenesis of experimental animals (Preussmann, et al., 1972). Although their powerful carcinogenic responses are well known, there have been only a limited number of studies on the occurrence of non-volatile N-Nitroso compounds in food systems, due in part to their instability under neutral and alkaline conditions (Mirvish, 1971). However, the precursors of N-nitrosamides have been reported in certain foods. Such compounds include uridine and ureas which have been isolated from fish by Mirvish (1975). High concentrations of agmatine, a decarboxylation products of arginine, have been reported in fresh abalone (Kawabata et al., 1978), and citrulline has been reported by Wada (1930) in watermelon.

Recently, Sims and Fioriti (1975) reported that heating fatty acids (or esters) and triglycerides and -amino acids at temperature above 150°C resulted in substantial yields of N-substituted amides. These results were confirmed by Kakuda and Gray (1980a) using a model system containing amino acids or free amines and fatty acids. They reported that presence of a secndary amino group in these compounds makes them susceptible to N-nitrosation. These compounds may represent another source of nitrosatable compounds available for reaction with nitrite.

The present study was undertaken to establish whether N-substituted amides can be formed under conditions encountered in processing and cooking of foods, and thus, be potential precursors of N-nitrosamides. Specific objectives of the present study were: (1) to study the formation of N-substituted amides from reactions between fatty acids and/or triglycerides with -amino acids and amines in both model and bacon systems: (2) to investigate the nitrosation of N-substituted amides by sodium nitrite in both model system and bacon systems: (3) to study the thermal stability of N-nitrosamides during the cooking of food: (4) to determine the mutagenicity of N-substituted amides and their corresponding N-nitrosamides using the Salmonella/microsome mutagenicity test; and (5) to estimate the carcinogenicity of N-substitutes amides and N-nitrosamides in a feeding trial using Swiss mice.

LITERATURE REVIEW

Chemistry of Formation

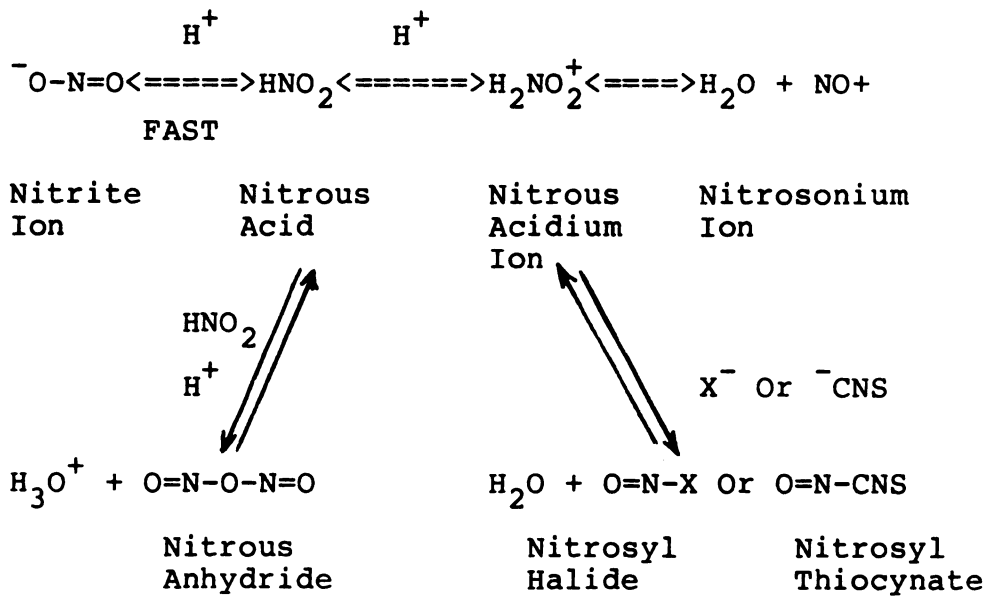
Kinetics of Nitrosation Reactions

N-Nitrosamines are formed principally from the reaction between secondary amines and nitrous acid (Mirvish, 1975). N-Nitrosamines can also be formed from tertiary amines, quarternary ammonium compounds and primary polyamines (Gray and Randall, 1979). In addition, the formation of relatively non-volatile N-nitroso compounds have been suggested by model system studies (Mirvish, 1971; Kakuda and Gray, 1980b).

Mirvish (1975) stated, that for nitrosation to occur, nitrite must first be converted to nitrous acid (HNO_2 - PK_a 3.36), indicating that the reaction is catalyzed by acid. Nitrous acid is then converted to an active nitrosating species. The actual nitrosating species can be one of the following, depending on the reaction conditions: nitrous anhydride (N_2O_3), nitrous acidium ion (H_2NO_2+), free nitrosonium ion (NO^+) and nitrosyl halide (NOX).

The following reactions have been reported by Mirvish (1970) and show the equilibrium equations for the nitrite

ion in aqueous solution:



The kinetics of N-nitrosamines formation from secondary amines and nitrous acid has been studied in detail by Mirvish (1972; 1975). Mirvish (1975) reported that the nitrosation reaction for most secondary amines proceeds via the active nitrosating species, nitrous anhydride. He further proposed that the reaction proceeds according to the overall third order rate equation as shown below:

$$\text{Rate of N-nitrosamine formation} = k\,[\text{total amine}]\,[\text{nitrite}]^2$$

where k is the rate constant.

The equation shows that the reaction rate is proportional to the concentration of the free amine and to the square of the concentration of nitrite. Mirvish (1975) stated that since it is the free amine and not the protonated amine that is nitrosated, both pH and amine basicity may influence N-nitrosamine formation. Sander et al.

(1972) found that there is an inverse relationship between the basicity of amines and the ease of nitrosation. In other words, the lower the basicity of a secondary amine, the easier it is to achieve nitrosation in acid solution, Sander et al. (1972) pointed out that at increasing acid concentrations, a higher proportion of the nitrite is converted to nitrous anhydride, which is the nitrosating agent. At the same time, however, salt formation by the amines is enhanced. Thus, there is an optimal pH value for the nitrosation of secondary amines which is about pH 3.

Sander et al. (1972) reported that nitrosation of alkylamides follows the same principle as that for secondary amines. Because of resonance and inductive effects, however, the ability to nitrosate depends on the structure of the amides. Mirvish (1977) proposed the following equations for nitrosation of alkylamides:

$$HNO_2 + H^+ \Longleftrightarrow H_2NO_2^+ \qquad \text{(I)}$$

$$RNH\ COR^1 + H_2NO_2^+ \Longleftrightarrow RN(NO).COR^1 + H_2O \qquad \text{(II)}$$

$$\text{rate of N-nitrosamide formation} = k_1\ [RNH\ COR^1]\ [HNO_2]\ [H^+] \qquad \text{(III)}$$

$$\text{rate of N-nitrosamide formation} = k_2\ [\text{amide}]\ [\text{nitrite}]\ [H^+] \qquad \text{(IV)}$$

He showed that the nitrosation rate is proportional to the amide and nitrous acidium ion concentrations. Nitrous acidium ion concentration in turn is proportional to H^+ and HNO_2 concentration as shown in equation I. In equation III, k_1 depends on nitrite ionization, and hence on

pH but does not depend on ionization of the amides since amides are usually not ionized above pH2.

Mirvish (1975) suggested that the main nitrosating agent for amides is probably nitrous acidium ion ($H_2NO_2^+$), the protonated nitrous acid. Therefore, nitrosation of secondary amides is pH dependent. In contrast to nitrosation of secondary amines, there is no pH for nitrosation because alkylamide have low basicity. However, the extent of nitrosation increases as the pH of the reaction medium is lowered. Similar results have been reported by Kakuda and Gray (1980b) for the nitrosation of secondary amides. In their model system, they found no apparent pH maximum for the reaction, N-nitrosamide formation increased with increasing hydrogen ion concentration. The rate of nitrosation decreased rapidly as the pH increased, with littly reaction occurring above pH3. They noted that a drop in pH from 2 to 1 increased nitrosation by 5-8 times. The rate constant remained relatively stable over a pH range of 1-3.5, thus supporting the nitrous acidium ion mechanism.

Mirvish (1975) studied the nitrosation of 21 amides and reported that all of them followed the nitrous acidium mechanism. The rate constant values varied some 300,000 times between different amides. They were lowest for simple alkyl- and arylamides and for guanidines, and highest for ethyleneurea. There is no simple rule relating ease of

nitrosation to other properties of the amides as is true for the amines.

Kakuda and Gray (1980b) pointed out that the nitrous acidium ion cannot be considered an important N-nitrosating species in food systems, since low pH is not normally encountered. Thus, the pH of foods would militate against the occurrence of N-nitrosamides, even if their precursors are present. Mirvish (1971), however, showed that *in vivo* N-nitrosation of alkylamides is possible.

In Vivo Formation of N-Nitroso Compounds

in vivo formation of N-nitroso compounds was first indicated by Sander and Burkle (1969). Although they did not perform chemical analyses for N-nitroso compounds in the stomach, they noted esophageal and hepatic tumors in rats fed nitrite together with morpholine and N-methylbenzamine. They also observed tumors characteristic of the corresponding N-nitroso compound in rats upon feeding nitrite plus methylurea, ethylurea and 1,3-dimethylurea. They proposed that nitrosation probably occurs in the stomach and is catalyzed by hydrochloric acid.

Mirvish et al. (1978) measured N-nitrosomethylurea (NMU) formation in the stomach contents of rats fed ^{3}H methylurea and sodium nitrite. The rats were killed 3 hours later and the NMU yield was calculated to be 0.46% of the methylurea. If sodium ascorbate was added to the feed in equimolar amounts, NMU production was completely inhibited.

Between 45-90 minutes after gavage of urea and nitrite to starved rats, Mirvish and Chu (1973) obtained yields of 27% for NMU and 9% for N-nitrosoethylurea (NEU). Gavage of sodium bicarbonate before the methylurea plus nitrite treatment neutralized the stomach content and prevented NMU formation.

Mirvish et al. (1974) showed that starved rats given feed containing sodium nitrite still had 57 and 8% of the nitrite in the stomach after 1 and 5 hours, respectively. The mean nitrite concentration in the acidic glandular part of the stomach was 50% less than that of the non glandular portion. They reported that factors other than emptying of the stomach accounted for about 40% of the nitrite loss, which occurred especially rapidly in the glandular portion of the stomach. They suggested that these factors probably include absorption of nitrite from the stomach, decomposition of nitrite and its reaction with food components. All these processes probably require conversion of nitrite to HNO_2 by the action of gastric HCl. They reported that the transit time in the stomach was 1.2 hours, the pH was 3.6 and the nitrite concentration comprised 11% of that in the food. They concluded that conditions in the stomach would permit significant nitrosation to occur in rodents if the compounds were readily nitrosatable.

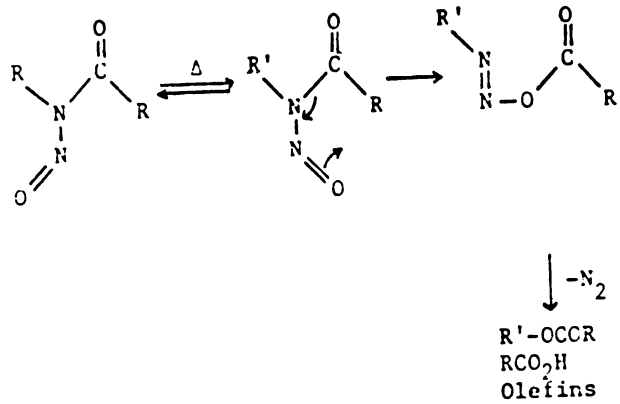
Varghese et al. (1978) also presented evidence supporting *in vivo* formation of N-nitroso compounds. They

detected a bacterial mutagen in ether extracts of freeze dried feces of humans on Western diets. They suggested that the mutagens may be N-nitroso compounds, which were not detected in the diet but were formed in vivo and eliminated in the feces. Sato et al. (1959, 1961) found a correlation between gastric cancer and high intake of salted fish and vegetables in Japan. They suggested that in vivo nitrosation of ureas and methylguanidine, which may be present in fish included in the diet, could be responsible for the high incidence of gastric cancer.

In light of above findings, in vivo nitrosation might present a hazard, especially with the more readily nitrosatable compounds that are present in drugs, agricultural chemicals or as food components (Mirvish, 1977).

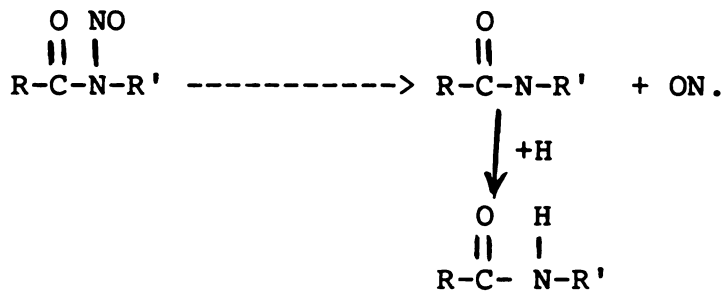
Stability of N-Nitrosamides

Mirvish (1971) reported that decomposition of N-nitrosamides occurs under either neutral or alkaline conditions. Chow (1979) studied the thermal and photolytic decomposition of N-nitrosamides and reported that at temperatures from ambient to 100°C. N-nitrosamides undergo irreversible thermal rearrangements to form diazo esters. He pointed out that diazo esters decompose rapidly to give carboxylic esters or acids and olefins as shown here.



The stability of N-nitrosamides and the final products from diazo esters are strongly dependent on the nature of the R^1 group (primary, secondary, tertiary alkyls, phenyl or benzyl groups, etc), but less so on the R group (Chow, 1979).

Photolytic decomposition of N-nitrosamides in polar and non-polar solvents yield amidyl and nitric oxide radicals, which further undergo typical free radical reacitons as shown by Chow (1979) below:



Chow (1979) reported that the first step in photolysis of N-nitrosamides is the homolytic scission of the N-N bond. The second step is usually abstraction of hydrogen from the alcholic, hydrocarbon or olefinic solvent, which

forms an amide. In the third step, the nitroso radical reacts with the new carbon radical to give ketones from alcoholic solvents or nitrosodimers and nitrite, from hydrocarbons or olefinic solvents (Thomas, 1972).

N-Nitrosamides also decompose readily in neutral and alkaline solutions. In contrast to N-nitrosamines, Mirvish (1971) reported that N-nitrosamide are unstable in neutral and alkaline solutions, and decompose readily to yield alkyldiazo hydroxide as illustrated below:

Druckery (1975) proposed that alkyldiazo hydroxides are proximate alkylating carcinogens. These compounds have been widely used in chemistry as alkylating substances.

There have been only a limited number of reports on the occurrence of N-nitrosamides in food systems. Thus, one may suspect that lack of detection in environmental samples may be due to the instability of the N-nitrosamide linkage.

Precursors of N-Nitroso Compounds

Precursors of N-nitroso compounds constitute nitrite and various amines and amides, many of which are natural constituents of foodstuffs or drugs (Sander and Schweinsberg, 1972). Each of these reactants and then

possible sources in foods will be discussed herein.

Amines in Foods

Amines in foods are formed by both biological and chemical pathways (Maga, 1978). These include: (a) amino acid decarboxylation, which is responsible for the formation of spermidine from methionine (Lakritz, et al., 1975), putrescine from ornithine (Tabor et al., 1958), cadaverine from lysine (Tabor et al., 1958) tyramine from tryosine (Kristoffersen, 1963) and histamine from histidine (Dierick et al., 1974); (b) trimethylamine oxide conversion, such as the enzymatic conversion of trimethylamine oxide to trimethylamine (Tarr, 1940); (c) aldehyde amination as in the amination and transamination of aldehydes, which is the potential pathway for formation of most monoamines associated with foods (Hartmann, 1967; Maier, 1970); (d) phospholipid decomposition, such as the formation of ethanolamine from the splitting of cephalin (Herdlicka and Janicek, 1964); and (e) thermal amino decomposition, which accounts for the appearance of a wide variety of amines, such as ethanolamine, methylamine, propylamine and either iso-or pentylamines, which are formed during heating of cysteine (Mulders, 1973). Velisek and Davidek (1974) also postulated that amines in foods could easily be formed during the nonenzymatic browning process.

A wide range of simple aliphatic amines and monoamines, such as tyramine, histamine and tryptamine has been

detected in cheeses (Golovnya and Zhuravleva, 1970; Gray et al., 1979). Spinelli et al. (1974) reported that pyrolysis of proteins on cooking of foods may produce free amino acids and nitrosatable secondary amines. In their analysis of amines in fresh processed pork, they demonstrated the presence of spermidine, spermine, putrescine, cadaverine, tryptamine, tyramine, histamine and ethanolamine. Lijinsky and Epstein (1970) have pointed out that putrescine, which is a decomposition product of arginine, may undergo cyclization to form pyrrolidine during the cooking of fish and meat. Pyrrolidine can undergo nitrosation to form N-nitrosopyrrolidine (NPYR) as reported by Bills et al., (1973).

The aliphatic polyamines, spermidine and spermine are widely distributed in biological material, including viruses, bacteria, plant and animal tissues (Tabor and Tbor, 1964). Lakritz et al. (1975) reported maximum values of 125 mg and 1013 mg of spermidine per 100 g of tissue for fresh pork and putrified pork, respectively. Spermine values were 55.7 mg and 2769 mg per 100 g of fresh and putrified pork, respectively. Formation of NPYR from reactions of spermidine with sodium nitrite upon heating has been reported by Ferguson et al. (1973).

Polyamines and free amino acids can also produce the precursors of N-nitroso compounds. Lien and Nawar (1974a) reported that free amino acids and polyamines are converted to N-nitrosatable secondary amines by thermal degradation. Simmonds et al. (1972) found decarboxylation to be the

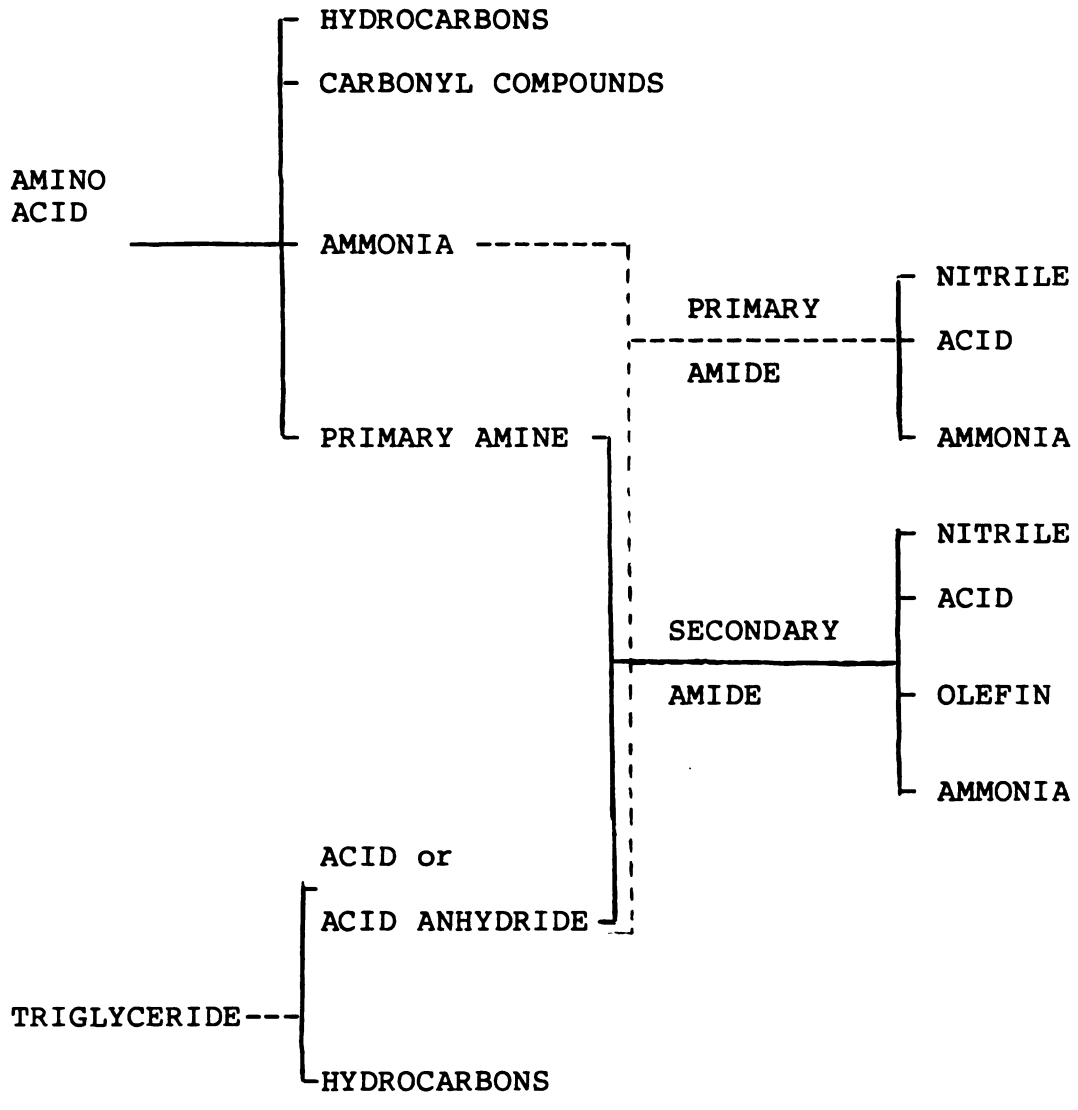
major thermal decomposition pathway for production of free amines from -amino acids.

The free amino acid content of meat increases upon aging due to the actions of the naturally occurring cathepsins (McCain et al., 1968). Bharucha et al. (1979) demonstrated that raw bacon contained 2 to 3 times more free amino acids than was found in fresh pork bellies. Gray and Collins (1977) reported that the free proline concentration in fresh pork bellies increased with storage time. Kakuda and Gray (1980a), however, concluded that formation of free amine via decarboxylation of amino acids under conditions normally encountered in cooking and processing of food seems unlikely, since there is insufficient energy for decarboxylation. They demonstrated that high temperatures (minimum 150^{o}C for 45 min) are required for decarboxylation of norleucine. A high activation energy for decaroxylation of amino acids was previously reported by Sims and Fioriti (1975).

Formation of N-substituted Amides

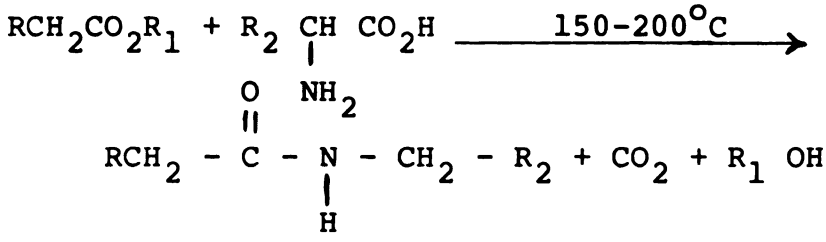
Beckwith (1970) stated that amides can be formed by the acylation of ammonia or amines. This is in agreement with the observations of Lien and Nawar (1974b), who reported formation of amides upon pyrolysis of a mixture of amino acids and triglycerides. Lien and Nawar (1974a) also reported that ammonia and amines are produced by thermal decomposition of amino acids, whereas, acylating agents (i.e.,

carboxylic acid and acid anhydride) can be formed from pyrolysis of triglycerides. They proposed the following reaction pathway for formation of amides:



Sims and Fioriti (1975) have investigated reactions which occur between fatty esters and amino acids under thermal stress. They reported that fatty esters react rapidly with many -amino acids at temperatures as low as 150^{o}C to give N-substituted amides as the major reaction

products. The reaction involves decarboxylation of the amino acid and displacement of the alcohol moiety of the fatty ester by the amine, which is formed. They reported yields of N-substituted amides as high as 50% of theoretical can be obtained. They proposed the following reaction:



Sims and Fioriti (1975) reported that on heating methionine in mineral oil at 200°C for prolonged periods, no CO_2 was evolved, and the methionine could be recovered quantitatively. When fatty acids were present in the mixture however, the corresponding N-substituted amides were formed. They suggested that high temperature reactions of fatty acids and amino acids are consistent with a concerted mechanism. It is possible that two succesive steps are involved, i.e., decarboxylation, which is the rate determining step, and amidation.

Lien and Nawar (1974b) reported formation of caproic amide and isobutylcapric amide upon heating of valine and

tricaproin at 270°C. Breitbart (1977) reported the radiolytic interaction of some amino acids and triglycerides. He identified caproic amides and caproic nitrile from an irradiated lysine and tributyrin mixture.

Kakuda and Gray (1980a) studied the formation of N-substituted amides in a model system containing fatty esters or triglycerides and free amines. They reported that fatty acid ester and free amines reacted readily to form N-substituted amides under conditions involving thermal stress. They showed that free fatty acids are not as reactive as their respective triglycerides, but at high temperature, both reacted readily with amines. They theorized that formation of primary amines via decarboxylation of -amino acids is unlikely under normal cooking conditions, because there is insufficient energy for decarboxylation. They suggested that under conditions encountered in the processing and cooking of foods, only amines would react with fatty acids and/or esters to yield substantial quantities of secondary amides. Furthermore, they reported that the presence of a secondary amino group in the amide compounds would make them susceptible to nitrosation. Thus, they may provide another precursor for N-nitroso compounds.

Nitrate and Nitrite

Nitrate and nitrite have been used in the curing of meat for many years. Nitrite serves several purposes during meat curing, including production of the characteristic

cured meat color (Brooks et al., 1940), contributing to cured meat flavor (Bailey and Swain, 1973), elimination of warmed-over flavor (Bailey and Swain, 1973; Fooladi et al., 1979) and retardation of botulinal toxin formation (Christiansen et al., 1973).

During the past decade however, nitrite has become the center of widespread controversy as a result of its interaction with the naturally occurring amino compounds in meat to produce N-nitroso compounds (Gray, 1976). N-Nitrosamines are formed in cured meat products under certain conditions and have been found sporadically in hams, wieners, bologna, and similar products (Gray, 1976).

The effect of various amounts of nitrite on the formation of NPYR during cooking of bacon has been extensively studied by Sen et al. (1974). They demonstrated a gradual increase in formation of NPYR in fried bacon with increasing nitrite concentrations. The amount of NPYR correlated closely with the initial concentration of nitrite, but not with the residual nitrite level found in raw bacon. Gray and Dugan (1974) showed that the concentration of nitrite and the nitrite to amine ratio are important on formation of nitrosamines. They indicated that minimum or perhaps no N-nitrosamine formation may be expected when nitrite levels are low. They reported maximum formation occurred at or above a nitrite to amine ratio of 2:1.

The rate of N-nitrosation of secondary amines was reported by Mirvish (1970) to be directly proportional to the

square of the nitrite concentration. Thus, the amount of nitrite permitted in curing of meat has received considerable attention. The current levels of nitrate and nitrite used in various meat products as recommended by the USDA Expert Panel (1978) are given in Table 1.

Table 1. Nitrate and nitrite usage recommended by USDA Expert Panel (1978)

Product	Levels of nitrate (mg/kg)	Levels of nitrite mg/kg
Cooked sausages	0	100-156
Fermented sausages	0	60-156
Dry cured cuts	300	100
Pickle cured products	0	110-200
Commercially-sterile products	0	50
Perishable-canned products	0	80-200
Shelf-stable products	0	156
Bacon[a]	0	120

[a] Level established by regulation on May 16, 1978.

The public health aspects of N-nitroso compounds have so far focused mainly on whether or not nitrite should be used to preserve meat products. However, nitrite also occurs in other foods and human saliva. Tannenbaum et al. (1974) reported that nitrite is a normal constituent of human saliva, resulting from the reduction of nitrate by a

variety of microorganisms that inhabit the mouth. They reported that the level of salivary nitrite in healthy individuals is fairly constant and average about 6-10 mg/liter.

According to White (1975) the estimated average daily ingestion of nitrate per capita in the U.S.A. is 80 mg, coming principally from an average daily intake of 306 grams of vegetables. He concluded that the amount varies enormously depending to the type of vegetable and its nitrate content. Nitrate also occurs in water, especially in well water from some rural areas (Comly, 1945; Burden, 1961). The concentration of nitrite in vegetables and water, on the other hand, is usually very low, although fairly high levels have been detected on storage of temperature abused spinach and beets (Heisler et al., 1974).

Tannenbaum et al. (1978) reported that nitrite and nitrate are formed de novo in the human intestine, possibly by heterotrophic nitrification of ammonia or organic nitrogen compounds. They suggested that this reaction takes place in the upper aerobic portion of the intestine. As a result, they concluded that human exposure to nitrite may be much greater than previously recognized.

In light of the above findings on the precursors of N-nitroso compounds the presence of amines, amides, and nitrite in human diets is therefore, unavoidable, even without the consumption of cured meat items.

Toxicology of N-Nitroso Compounds

Carcinogenicity

Lijinsky (1977) reported that N-nitroso compounds can be divided into two classes according to their action as carcinogens: 1) N-nitrosamines 2) N-nitrosamides.

N-Nitrosamines are considered indirect acting carcinogens and require metabolic activation (Lijinsky, 1977). These compounds have been studied extensively as potential environmental carcinogens. Only a few have been found to be non-carcinogenic (Magee and Barnes, 1956). Some of these dialkylnitrosamines have been shown to be carcinogenic to a variety of animal species, including subhuman primates, hamsters, mice, pigs, dogs, parakeets and monkeys (Preussmann et al., 1976; Wishnok, 1979).

N-Nitrosamides on the other hand are direct acting carcinogens and considered not to need metabolic activation (Narisaw et al., 1971). Druckrey (1975) reported that N-nitrosamides, in contrast to dialkyl nitrosamines, are unstable in alkaline solutions and decompose readily to yield alkyldiazo hydroxide which is the proximate alkylating carcinogen. Accordingly they are to be considered as direct acting carcinogens.

Druckrey (1972) stated that N-nitrosamides unlike N-nitrosamines often cause cancer at the site of application in animals. Their biological efficacy corresponds closely to the chemical reactivity of the compound. It is highest

with alkylnitrosocarboxylamides, and decreases in the following order: carbamic esters (urethans), ureas, biurets, and nitroguanidines.

Druckrey (1975) also reported that within individual groups of N-nitrosamides, the smaller the molecule the less carcinogenic it is. The carcinogenicity of N-nitrosamides generally decreases with increasing number of C atoms in both the acyl and alkyl groups. Table 2 lists some of the N-nitrosamides and some of the sites where they induce tumors (Preussmann, 1973).

The data in Table 2 demonstrate that tumors can be produced in a great many tissues. The organ specificity of action depends mainly on the chemical structure of the compound, and to a minor degree on the animal species, the route of application and the dosage rate (Preussmann, 1973).

Mirvish (1977) reported that N-nitrosamides and related compounds like 1-methyl-1-nitroso-3-nitroguanidine, 1-methyl-1-nitroso-3-acetylurea and methylnitrosourea are among the few compounds that induce glandular stomach tumors in rodents. Since N-nitrosamides are rather unstable and decompose readily under the influence of light, temperature and alkaline pH (Chow 1979; Mirvish, 1971), their formation *in vivo* may be more important than their occurrence in foods. Mirvish (1971) stated that if N-nitrosamides are formed *in vivo* in the stomach, they may induce tumors. Sato *et al.* (1959) found a significant

Table 2. Carcinogenicity of N-nitrosamides.

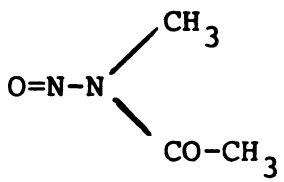
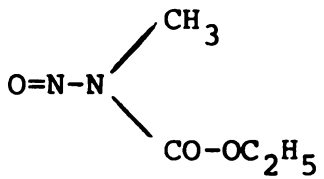
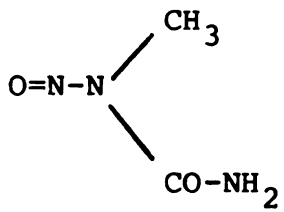
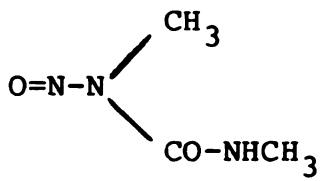
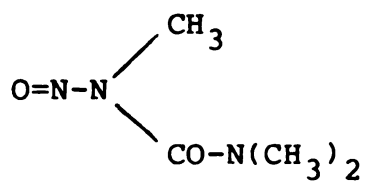
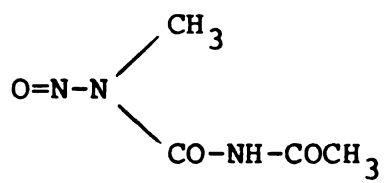
Nitrosamide N-nitroso	Formula	Carcinogenic action	Application	Main target organ
-methylacetamide		++	p.o.	fore-stomach
-N-methylurethane		+++	p.o. iv.	fore-stomach lung
-methylurea		+++	p.o. iv.	fore-stomach brain
-dimethylurea		+++	p.o.	brain, nervous system, spinal cord

Table 2 (cont'd)

Nitrosamide N-nitroso	Formula	Carcinogenic action	Application	Main target organ
-trimethylurea		+++	p.o. iv.	periph. nerves spinal cord
-methyl-N'-acetyl-urea		+++	p.o.	glandular stomach

p.o. = by mouth
iv. = intravenous

positive correlation between the high incidence of gastric cancer and the high intake of salted fish and vegetables in Japan. They suggested that gastric cancer could be due to N-nitrosamides formed *in vivo* from N-nitrosation of ureas and methylguanidine which are present in fish included in the diet (Mirvish, 1971).

Mirvish (1975) failed to induce tumors in rats by feeding nitrite plus the amides, N-methylacetamide, N-methylurethan, N-ethylurethan, phenylurea and 1-methyl-3-acetylurea. However, the corresponding N-nitroso derivatives are known carcinogens. They concluded that of the many amines and amides that may be found in the human environment, in food, and in drugs, only a few are easily nitrosated under the conditions prevailing in the human organism, especially in the stomach.

Mutagenic Action of N-Nitroso Compounds

Magee (1972) pointed out that an identical molecular mechanism may account for both the carcinogenic and mutagenic activity of N-nitroso compounds since much of the evidence favors alkylation as the biochemical mechanism.

N-Nitrosamides, but not N-nitrosamines, are mutagenic in bacterial systems *in vivo*. The N-nitrosamines appear to require metabolic activation by the mammalian enzyme system before they can exert a mutagenic effect. This was demonstrated with NDMA in the host-mediated microbial assay

of Gabridge and Legator (1969) and by the liver microsomal-activated microbial system of Ames et al. (1973).

N-Nitrosoguanidine and other N-nitrosamides have been reported as being among the most powerful mutagens (Magee and Barnes, 1967; Zimmermann, 1971; Mandell and Greenberg, 1960). Although the N-nitrosamides are mutagenically active in bacteria, yeasts, Neurospora, plants and drosophila, N-nitrosamines have generally been reported to be inactive in all the above organisms except drosophila (Magee, 1972). Magee and Barnes (1967) reported that the relatively unstable N-nitrosamines which are believed to require enzymatic decomposition before becoming active carcinogens, are mutagenic only in drosophila and inactive in microorganisms, such as Escherichia coli, Neurospora and Saccharomyces. Magee and Barnes (1967) state that this maybe related to the presence of enzymes capable of -oxidation of the N-nitrosamines in drosophila and their absence in the microorganisms.

Zimmerman et al. (1965) observed that N-methyl-nitrosamides are mutagenic in saccharomyces at pH 2, and that the compounds decomposed to yield nitrous acid. They further demonstrated that deamination of adenine occurred when the base was exposed to some N-nitrosamides at pH 2. These results led them to conclude that N-nitrosamides may exert their mutagenic action via deamination by nitrous acid at low pH and by alkylation at high pH.

Microorganisms, i.e., certain species of bacteria, are most commonly used for testing for mutagenic effects. The chemical in question is introduced into the grwoth medium of these organisms. The changes if any, both in metabolism and in the genetic material of the cell, are examined for mutagens. Magee and Barnes (1967) stated that the validity of such tests is questioned if the results are compared to humans, because most chemicals ingested and absorbed through the samall intestine in human pass through the liver first. The liver has many mechanisms for detoxifying chemicals, rendering them harmeless to the body. They are then excreated in the urine. Bacteria do not have this property.

The mutagenic test receiving the widest attention at the present time is the reverse mutation test developed by Ames et al. (1975). This test uses a mutant strain of Salmonella typhimurium that lacks coding for the enzyme phosphoribosyl ATP synthetase, which is required for histidine synthesis, thus, this strain is unable to grow in a histidine-deficient medium unless a reverse mutation has occrred. Since many chemicals are not mutagenic or carcinogenic unless they are biotransformed to a toxic product by the endoplasmic reticulum (microsomes), rat liver microsomes are usually added to the medium containing the mutant strain and the reverse mutation is then quantitated by the growth of the strain on a histidine-deficient medium. The principle of the test is based on the use of mutants with

an unique type of DNA damage. Ames (1975) introduced a base substitution strain TA 1535 and two different kinds of frameshift mutations, TA 1537, TA 1538, for detecting mutations by the sensitive and convenient back mutation test. The sensitivity of the test strains has been increased by adding to them an additional deletion mutation, designated as UVrB. This effectively eliminates any DNA repair system that normally would protect salmonella from mutation by ultraviolet light. The effect of choosing strains carring the UVrB defect has been shown to increase the sensitivity of the strains to most chemical mutagens. Still another mutation, rfa, was also incorporated into the test which changed the nature of the lipopolysaccharide bacteria cell wall. It became more permeable to chemicals like the polycyclic and heterocyclic hydrocarbons and thus more sensitive to the mutagenic activity of such compounds. Two new test strains (TA 100 and TA 98) have recently been introduced by McCann *et al*. (1975b). They were developed by transferring a resistance transfer factor, PKM 101, to both standard TA 1535 and TA 1538. This made new test strains much more sensitive to reversion with a variety of potent carcinogens, such as aflatoxin B_1.

Ames *et al*. (1975) stated that the sensitivity of the bacterial mammalian-microsomal system makes it useful as a tool for rapidly obtaining information on the mutagenic and potential carcinogenic activity of uncharacterized compounds in complex mixtures. This test commonly known

as the Ames test, can be used as an assay to identify the mutagenic components of complex mixtures. The test is highly selective for the detection of carcinogens. The Ames salmonella tester strains have been used by McCann et al. (1976) to screen large numbers of carcinogens for mutagenic activity. The results showed a positive correlation of 90% between mutagenicity and carcinogenicity. Ames and McCann (1976) reported that out of 106 non carcinogens tested about 85% were non-mutagenic. Sugimura et al. (1976) determined the correlation between mutagenicity and carcinogenicity of various substances. Using carcinogenicity data from the published literature and data on mutagenicity obtained in their own laboratory, they concluded that many mutagenic compounds are also carcinogenic while many non-mutagenic compounds are not carcinogenic. They found that number of mutagenic compounds that are noncarcinogenic, and conversely, the number of nonmutagenic compounds that are carcinogenic are very small. Their work demonstrated that most carcinogenic compounds give positive tests by the Ames procedure, whereas, very few noncarcinogens give positive results. This indicates the validity of the Ames mutagenicity test as a rapid screening procedure for determining environmental carcinogens.

MATERIALS AND METHODS

Reagents

All chemicals and solvents employed were of analytical grade and used without further purification. All fatty acids and their methyl esters were purchased from Fisher Scientific Co. (Fair Lawn, N.J.). Pentylamine, norleucine, valine, methionine and N-methylpropionamide were purchased from Eastman Kodak Co. (Rochester, N.Y.). Column packing materials were obtained from Supelco, Inc., Bellefonte, PA. Lard was purchased from a local retail market. Pork bellies were purchased from a local supplier soon after slaughter and stored in a cooler at 2°C until used.

Preparation of Amides in Model Systems

(i) Amino Acids plus Saturated Fatty Acids

Palmitic acid (1 mole) and norleucine (1.5 moles) were heated together for 1 hour at 200°C in a 150-ml round bottom flask fitted with a water-cooled condenser. After cooling, the reaction mixture was slurried in warm (30°C) diethyl ether, and any unreacted amino acid was removed by filtration. The solvent was evaporated in a Buchii rotary

evaporator. The residue was redissolved in a minimum of warm (40°C) petroleum ether and then left at room temperature to crystallize. The crude amide mixture was dissolved in 100 ml of methanol and then made alkaline with 1 N methanolic KOH. The solution was stirred for 1 hour before evaporating to dryness under vacuum. The dried residue was extracted with diethyl ether and filtered under suction. The ether extract was washed twice with aqueous 0.1 N KOH, followed by two washings with water. A series of amides was prepared in a similar manner by reacting norleucine with lauric, myristic and stearic acids, and by reacting lauric acid with valine and methionine.

(ii) Amino Acids plus unsaturated Fatty Acids

A 4 g aliquot of oleic (or linoleic) acid was heated with 1.6 g of norleucine at 200°C for 1 hour as described previously. Removal of the unreacted amino acid and evaporation of the diethyl ether left an oily liquid. This crude product was purified by column chromatography using Supelcosil (ATF 061) as described by Sims and Fioriti (1975). A 3 g sample of the crude product was dissolved in a 4 ml of petroleum ether and applied on the column. The material on the column was eluted with 200 ml of petroleum ether, which was discarded. A 250 ml aliquot of petroleum ether/diethyl ether (90:10, v/v) was then used to elute a second fraction which was collected and evaporated down until only an oily liquid comprising the amide remained.

(iii) Amino Acids plus Lard or Pork Bellies Adipose Tissue

Lard or pork belly adipose tissue (4 g) and 1.6 of norleucine were heated for 1 hour at 200°C. The reaction mixture was slurried in warm diethyl ether and filtered. The filtrate was evaporated to a paste and purified by thin-layer chromatography (Sims and Fioriti, 1975). The TLC plates were developed in a solvent system of petroleum ether/diethyl ether/acetic acid (40:60:1, v/v) dried, and sprayed with water to visualize the bands. The amide-containing bands were scraped off the plates, dried and the amides eluted from the absorbent with diethyl ether.

Nitrosation of N-Substituted Amides

Preparation of N-Nitroso-N-pentylpalmitamide.

The nitrosation procedure was based on the method described by White (1955) with a few modifications as outlined by Kakuda and Gray (1980b). A 3.25 g aliquot of recrystallized N-pentylpalmitamide (prepared as previously described) was dissolved in a solvent mixture containing glacial acetic acid (50 ml), acetic anhydride (50 ml) and chloroform (95 ml). The mixture was cooled in an ice bath and 15 g of sodium nitrite were slowly added with stirring over a 4-5 hour period. After reacting overnight at 4°C, the mixture was carefully poured into ice water. The chloroform phase was collected and the water phase was extracted

with another 100 ml aliquot of chloroform. The pooled chloroform extracts were washed with water, 5% K_2CO_3 solution and again with water before evaporating to dryness under vaccum. The crude N-nitrosamide preparation remaining was partially purified by precipitating the unreacted amide in cold petroleum ether (4°C) followed by vacuum filtration of the cold mixture. The clear yellow filtrate was placed on a Supelcosil AFT 061 column and eluted with 60 ml of petroleum ether. All the N-substituted amides, which were prepared previously from the reactions of norleucine, valine and methionine with capric, lauric, myristic, stearic, oleic and linoleic acids were nitrosated in the same manner.

Preparation of N-Nitroso-N-methylpropionamide (NOMP)

A 25-g aliquot of N-methylpropionamide (Eastman Kodak Co., Rochester, N.Y.) was dissolved in a mixture containing 120 ml of glacial acetic acid and 138 mL of acetic anhydride and cooled to 0°C. Sodium nitrite (61 g) was added slowly to the mixture over a 4 hour period. After allowing the mixture to react overnight at 4°C, the N-nitrosamide was extracted with chloroform. The chloroform extract was washed successively with water, 5% K_2CO_3, and again with water. The solvent was removed by vacuum evaporation. The remaining N-nitroso-N-methylpropionamide was purified by vacuum distillation, which was repeated a second time. The distillations were conducted at 40°C (15.0 mm Hg) and

distillates were collected in a receiving falsk packed in ice. The first and final 5-10 mL portions of the distillate were discarded during each distillation.

Analysis and Identification of Amides and N-Nitrosamides

The purity of the N-substituted amides and N-nitrosamides was determined by gas liquid chromatography (GLC) while their identities were confirmed by gas liquid chromatography-mass spectometry (GLC-MS).

A Hewlett Packard gas chromatograph (Model 5830A) equipped with a flame ionization detector (FID) and a Hewlett Packard 18850A GC therminal was used for analysis of the amides and N-nitrosamides. A glass column (2m x 2mm, i.d.) was packed with 3% OV-101 on 80/100 Supelcoport (Supelco Inc., Bellfonte, PA). The chromatograph was operated under the following conditions:

Initial temperature (T_1)	:	240°C (30°C for N-methylpropionamide and NOMP)
Time at T_1 (t_1)	:	1 min
Final temperature (T_2)	:	260°C
Time at T_2	:	10 min
Injection port temperature	:	250°C
Flame ionization detector temperature	:	350°C
Chart speed	:	1 cm per min
Attenuation	:	variable

Slope sensitivity	:	0.5
Carrier gas	:	Nitrogen
Carrier gas flow rate	:	30 ml per min
Hydrogen flow rate	:	30 ml per min
Air flow rate	:	200 ml per min

The samples were injected using a 10 µl Hamilton Syringe (Hamilton Co., Reno, Nevada). The volumes of sample injected varied from 0.5 to 1.5 µl.

The GC/MS system was a Hewlett Pakard 5985A gas chromatograph/Hewlett Packard mass spectrometer (Hewlett Packard Corp., Arondale, PA). The column was the same as that used for GC analysis. Helium was the carrier gas with a flow rate of 25 ml/min. The analysis were carried out using a temperature program from 240 to 260^{o}C at 5^{o}C/min, with a one minutes hold time at 240^{o}C.

The ion source and analyzer temperature of the mass spectrometer were maintained at 200^{o}C. The electron multiplier voltage was 2000 V and the ionization potential was 70 eV.

Formation of N-substituted Amides in Pork Belly Slices

Processing of Pork Bellies

A fresh pork belly weighing approximately 8 lbs was sliced to 1/8 inch in thickness. The slices were divided

into two groups. One of the groups was sprayed on the surface wtih pentylamine, while the other group of slices was freeze dried and then rehydrated with water containing pentylamine, followed by equilibration for 48 hours.

Two pork bellies approximately the same size were selected and stitch pumped with water containing pentylamine and/or norleucine. All treated bellies were smoked for 4 hours at 58°C (dry bulb) and 3 hours at 52°C (dry bulb) at ambient relative humidity in a laboratory smoke house (Drying System Inc., Chicago, IL). Smoke was applied throughout cooking with a midget size Mepaco Smoke generator (Meat Packers Equipment Co., Oakland, CA) utilizing mixed hard wood sawdust. The smoked bellies were transferred to a tempering cooler (-2°C) where they were held overnight prior to slicing.

Frying of the Bacon Slices

The treated slices were fried in a Sunbeam (Sears and Roebuck, Chicago, IL) electric fry pan. Each group of slices was fried such that half of them were held at 180°C for 4 minutes on each side and the other half for an additional 4 minutes on each side. Cook-out fat was taken for analysis after each cooking interval.

Analysis of N-substituted Amides in Fried Pork Belly Slices and Cook-out Fat.

Twenty grams of fried pork belly slices were homogenized with 10 ml distilled water in a Waring Blender and extracted three times with 50 ml of methylene chloride. The extract and tissue residue were then transferred to a medium grade sintered glass funnel and filtered under vacuum. The homogenizer and the residue in the funnel were washed with an additional volume of methylene chloride and filtered.

The extract was quantitatively transferred to a 500 ml separating funnel and distilled water (10% by volume) was added and throughly mixed. The mixture was allowed to separate into two phases until the interface was clear. The upper phase was transferred to a 500 ml volumetric flask and evaporated to dryness in a vacuum Rotavapor-R (Buchi, Switzerland) at 30-40^{o}C. The crude product was purified by column chromatography (Supelcosil-ATF 061). The crude dried extract was dissolved in 5 ml of petroleum ether and applied on the column. The sample was eluted with 200 ml of petroleum ether, followed by 250 ml of petroleum ether/diethyl ether (90:10, v/v). The first fraction was discarded and the second fraction was collected and evaporated to dryness. The purified amide was dissolved in 5 ml diethyl ether for GLC analysis.

Formation of N-Nitrosamides in Pork Belly Slices

A solution of N-pentylpalmitamide in diethyl ether (100 mg in 3 ml) and sodium nitrite in water (0.5 g in 3 ml) was injected into 50 g of pork belly slices with a Hamilton syringe and stored 24 hours at 4°C. The slices were fried as previously described. The fried pork belly slices and cooked-out fat were extracted with chloroform. The pooled chloroform extracts were washed with water and evaporated to dryness. The crude product was partially purified by precipitating the unreacted amide in cold petroleum ether (4°C) and vacuum filtering. The filtrate was placed on a Supelcosil ATF-061 column eluted with 100 ml of petroleum ether. The petroleum ether was evaporated and the purified compound was dissolved in 5 ml of diethyl ether for GLC analysis.

Thermal Stability of N-Nitrosamides under Frying Conditions of Bacon.

100 mg of N-nitrosopentylpalmitamide were dissolved in 3 ml of diethyl ether and injected into 100 g of pork belly slices with a Hamilton microsyringe. 50 g of pork belly slices were fried as before. The fried pork belly slices and cooked-out fat were extracted and analyzed for N-nitrosopentylpalmitamide. The remaining 50 g of raw pork belly were extracted and analyzed in a similar manner and used as the control.

Determination of Fatty Acid Composition of Lard and Pork Belly Adipose Tissue.

The fatty acid composition of lard and pork belly adipose tissue was determined by gas chromatographic analysis of their fatty acid methyl esters.

Preparation of Methyl Esters for GLC Analysis

Esterification of the fatty acides was carried out according to the procedure of Morrison and Smith (1964). An aliquot of the lipid was placed in a test tube fitted with a teflon-lined screw cap. Boron trifluoride-methanol reagent was added under nitrogen in the proportions of 1 ml of reagent per 4-16 mg of lipid and the tube was closed with the screw cap. The tube was then heated in a boiling water bath for 30 minutes, cooled and opened. The esters were extracted by adding 2 volumes of pentane, then 1 volume of water, shaking briefly and centrifuging until both layers were clear. The top organic layer was removed using a micropipette and placed in a clean vial. The solution was concentrated by slowly removing part of the solvent in stream of nitrogen.

A Hewlett Packard gas chromatograph (Model 5830A) equipped with a flame ionization detector (FID) and Hewlett Packard 18850A GC therminal was used for the analysis of the fatty acid methyl esteres. The glass column (2m x 2mm. i.d.) was packed with 15% diethylene glycol succinate (DEGS) on Chromosorb W 60/80 mesh. The instrument was

operated under the following conditions:

Initial temperature (T_1)	:	190°C
Time at T_1 (t_1)	:	20 min
Final temperature (T_2)	:	260°C
Time at T_2	:	10 min
Ingection port temperature	:	210°C
FID temperature	:	350°C
Chart speed	:	1 cm per min
Attenuation	:	8
Nitrogen carrier gas	:	30 ml per min
Hydrogen flow rate	:	30 ml per min
Air flow rate	:	200 ml per min

Standard fatty acid methyl esters were prepared under identical conditions and used for identification and quantitating the fatty acids in the samples.

Effects of N-substituted Amides and N-Nitrosamides on Tumor Development in Mice

160 female mice were allotted into 9 groups of 20 mice each, except for groups 8 and 9 had only 10 mice. The mice were idenfified by numbering from 1 though 10 by clipping their toes. The animals were watered and fed purified diet (Appendix 11) containing one of the following additives as indicated in Table 3.

Table 3. Additives that has been added to purified diet for different groups of mice in feeding trial.

Group	Compound
Group 1:	Control
Group 2:	(300 mg/kg) N-Methylpropionamide
Group 3:	(300 mg/kg) N-Methylpropionamide + (300 mg/kg $NaNO_2$)
Group 4:	(300 mg/kg) N-Pentylpalmitamide
Group 5:	(300 mg/kg) N-Pentylpalmitamide + 300 mg/kg $NaNO_2$
Group 6:	(300 mg/kg) Nitrosomethylpropionamide
Group 7:	(300 mg/kg) Nitrosopentylpalmitamide
Group 8:	(300 mg/kg) Nitrosomethylurea (positive control)
Group 9:	(300 mg/kg) Nitrosopentyldecanamide

Food and water were given ad libitum and the intake per group of 10 mice was recorded. After 7 months on the diet, 10 mice from each group were sacrificed by etherizing (except groups 8 and 9 in which 4 mice were sacrificed). Dr. Stuart D. Sleight of the department of Pathology grossly examined the internal organs and glands for tumors and other abnormalities. Samples of liver, lungs, spleen, heart and stomach were then placed in 10% neutral formalin. Fixed tissues were processed routinely, embedded in paraffin and cut into 6-μm sections-tissue. Sections were stained with hematoxylin and eosin and examined by light microscopy, and the histologically observed lesions were characterized as benign or malignant.

The remaining mice were kept on the same diet which will continue for a 2 year period.

Ames Test

The Ames test was performed according to the procedure described by Ames et al. (1975). The compounds were tested for mutagenecity using the Salmonella strain G46 (mutation in TA 1535) with and without S-9 mix. Instead of the standard plate test, a liquid suspension test was used. This involves pre-incubatian of chemicals with S-9 mix and bacteria in liquid suspension for approximately 20 minutes at 37^{o}C before plating.

The S-9 mix was prepared following the procedure of Ames et al. (1975). Male rats (300 g) were injected

interaperitoneal (ip) with Aroclor 1254 (500 mg/kg). Five days before exsanguination, the 9000x g supernatant was collected and frozen until needed.

RESULTS AND DISCUSSION

Preparation of N-Substituted Amides

Long chain and short chain N-substituted amides were prepared in gram quantities and purified by crystallization in petroleum ether, or by column chromatography. The formation of these N-substituted amides was confirmed by GLC-MS analysis.

Figure 1 shows a gas chromatogram of N-pentylstearamide (NPS), prepared by reaction of norleucine and stearic acid at 200°C for two hours. The reaction involves decarboxylation of norleucine and replacement of the alcohol moiety of the fatty acid with the amine group which is formed (Sims and Fioriti, 1975). The mass spectrum of NPS is shown in Figure 2. and exhibits major ion peaks at m/e 129, 297, 353 (M^+), 267, 57, and 30.

The most intense peak in the mass spectrum of NPS is a rearrangement ion at m/e 129, which is correlated with cleavage of the C-C bond beta to the carbonyl group, and is accompanied by rearrangement of a hydrogen atom (Gilpin, 1959). The breakdown of NPS to form the m/e 129 peak is

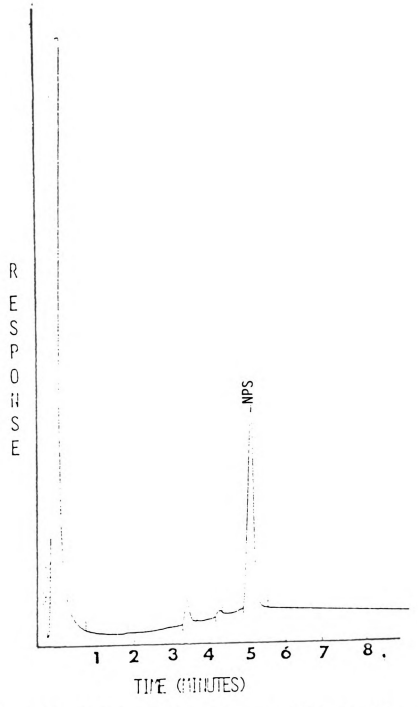
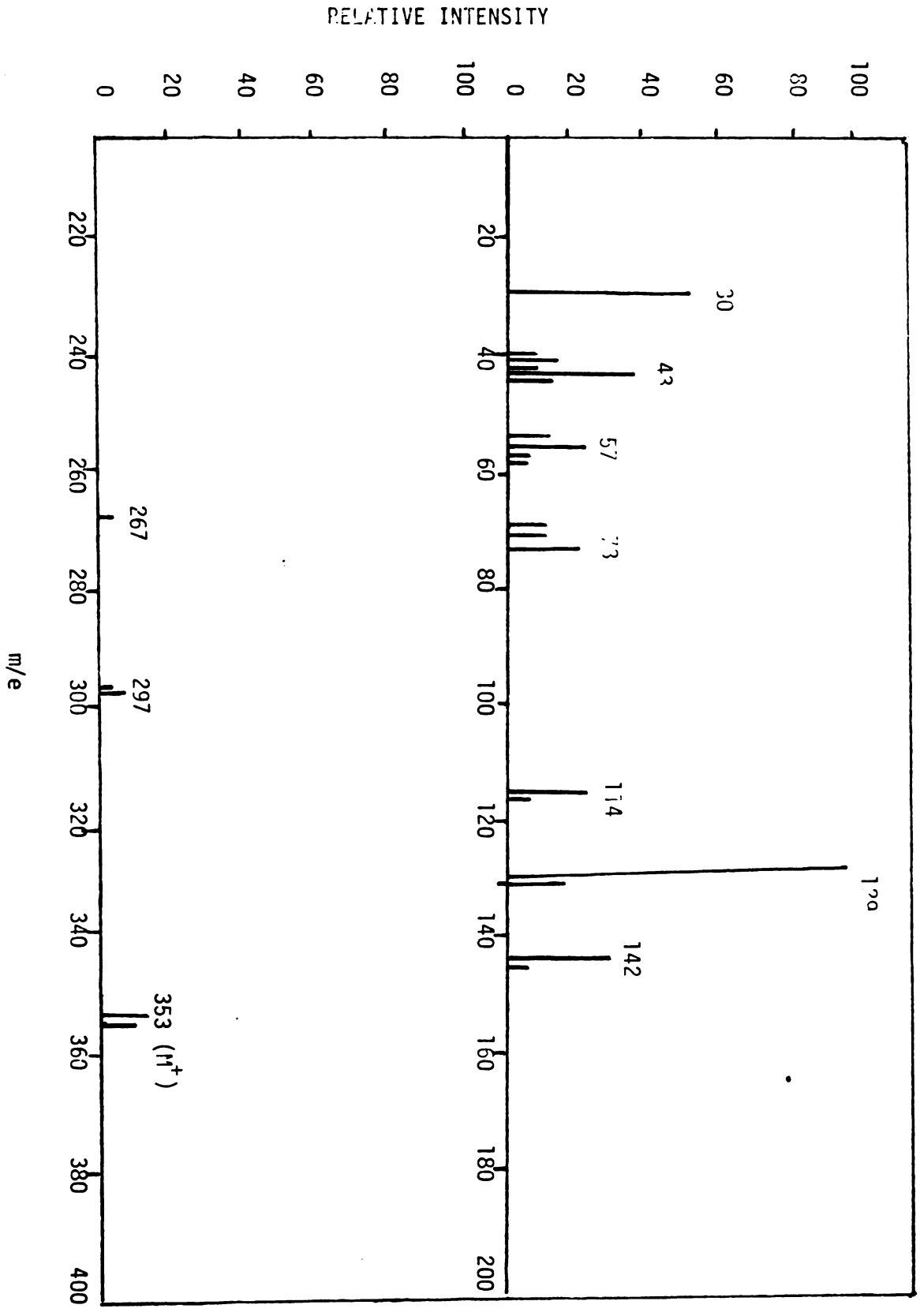
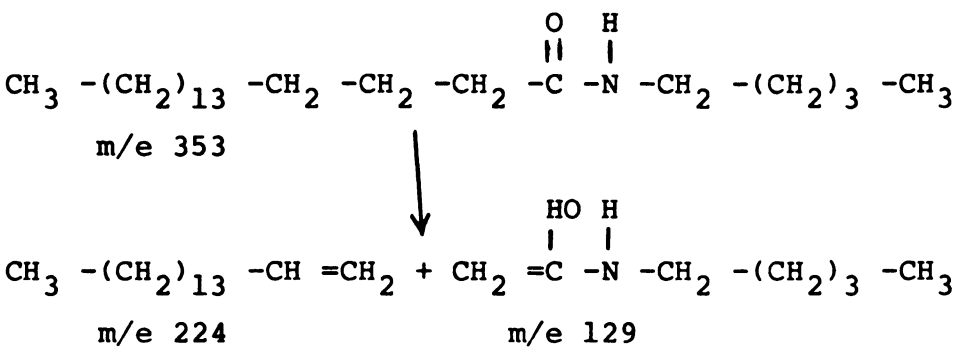


Figure 1. Gas chromatogram of N-pentylstearamide.

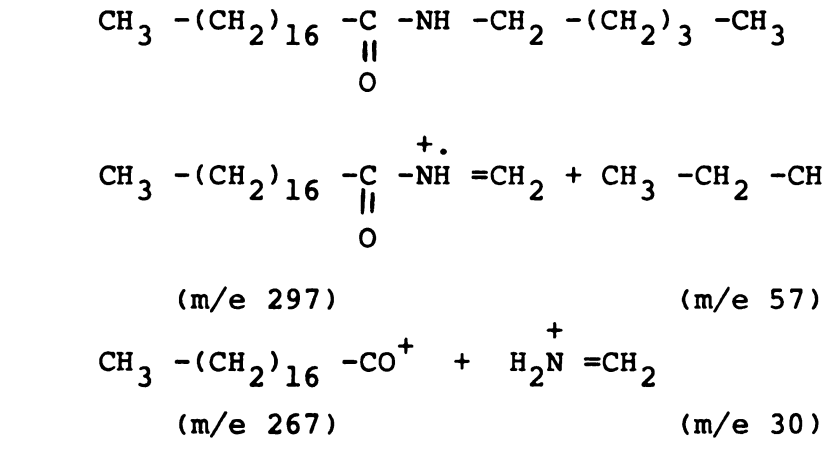
Figure 2. Mass spectrum of N-pentylstearamide prepared from the interaction between norleucine and stearic acid.


RELATIVE INTENSITY
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
30
43
57
73
114
120
142
267
297
353 (M+)
m/e

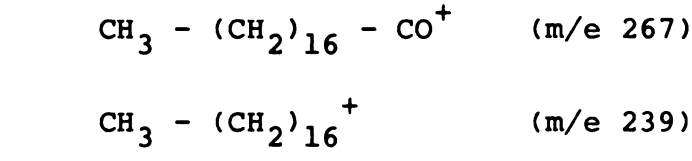
shown below:



The second most intense peak (m/e 30) resulted from cleavage of the C-C bond beta to the nitrogen atom. It was accompanied by a hydrogen rearrangement on the nitrogen-containing fragment (Budzikiewicz et al., 1967) as illustrated below:



Other important peaks in the spectrum and their structures are shown below:



The mass spectrum of N-pentylpalmitamide exhibited major ions at m/e 129, 296, 325 (M), 239, 268, and 30. A similar fragmentation pattern has been reported by Kakuda and Gray (1980a) for NPP. The mass spectrum of the other amides are presented in Appendices (1-6) and confirmed their formation from amino acids (valine and norleucine) and fatty acids (lauric, myristic, oleic and linoleic).

A mixture of purified amides was prepared and used as standards for identificaiton on the basis of retention times of the amides formed in pork belly slices. Figure 3 shows the GLC chromatogram of the standard mixture of N-pentylmyristamide, N-pentylpalmitamide, N-pentylstearamide, N-pentyloleamide and N-pentyllinoleamide. Figure 4 shows the GLC chromatogram of N-isobutymyristamide, N-isobutyl-palmitamide and N-isobutylstearamide and were derived from reaction of valine with the corresponding fatty acids.

Fatty Acid Composition of Lard and Pork Belly Adipose Tissue.

The fatty acid composition of lard and pork belly adipose tissue was determined by gas chromatographic analysis of their fatty acid methyl esters. Figure 5 shows that pork belly adipose tissue contains myristic, palmitic, stearic, oleic, and linoleic acids. The analysis revealed that lard contained 1.0, 19.5, 17.0, 51.0 and 10.6% of myristic, palmitic, stearic, oleic and linoleic acids, respectively.

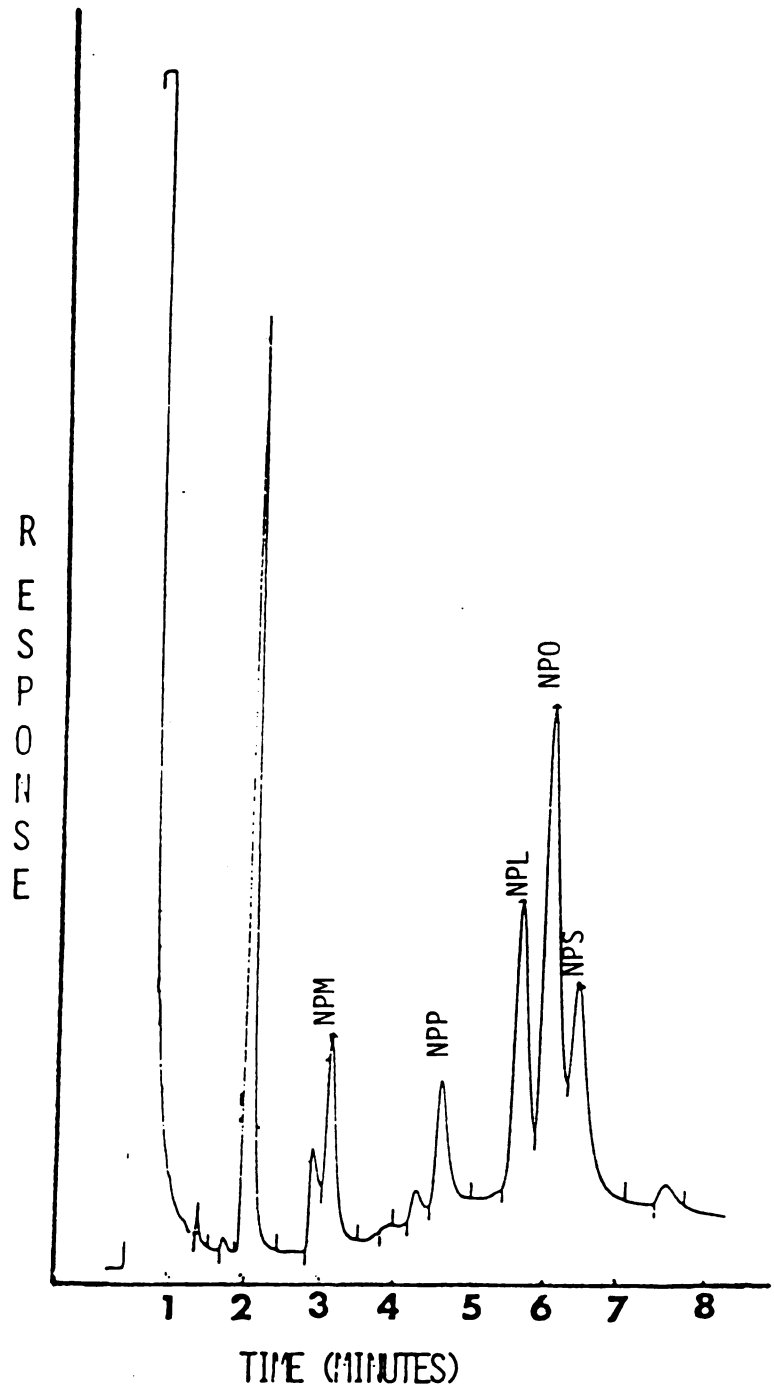


Figure 3. Gas chromatogram of standard N-pentylmyristamide (NPM), N-pentylpalmitamide (NPP), N-pentyllinoleamide (NPL), N-pentyloleamide (NOP) and N-pentylstearamide (NPS) formed from reaction of pentylamine and fatty acids.

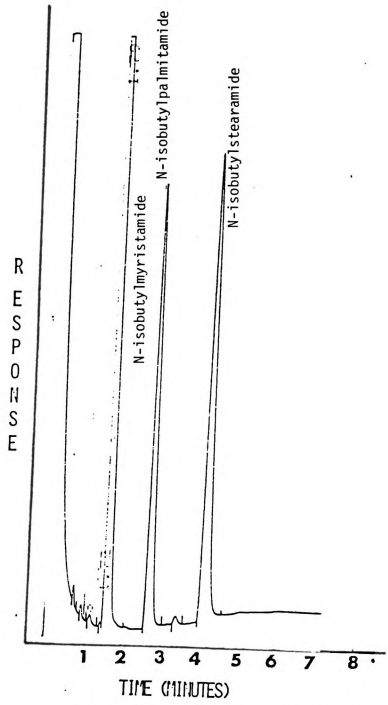


Figure 4. Gas chromatogram of standard mixture containing N-isobutylmyristamide, N-isobutylpalmitamide, N-isobutylstearamide prepared from reactions of valine and corresponding fatty acids.

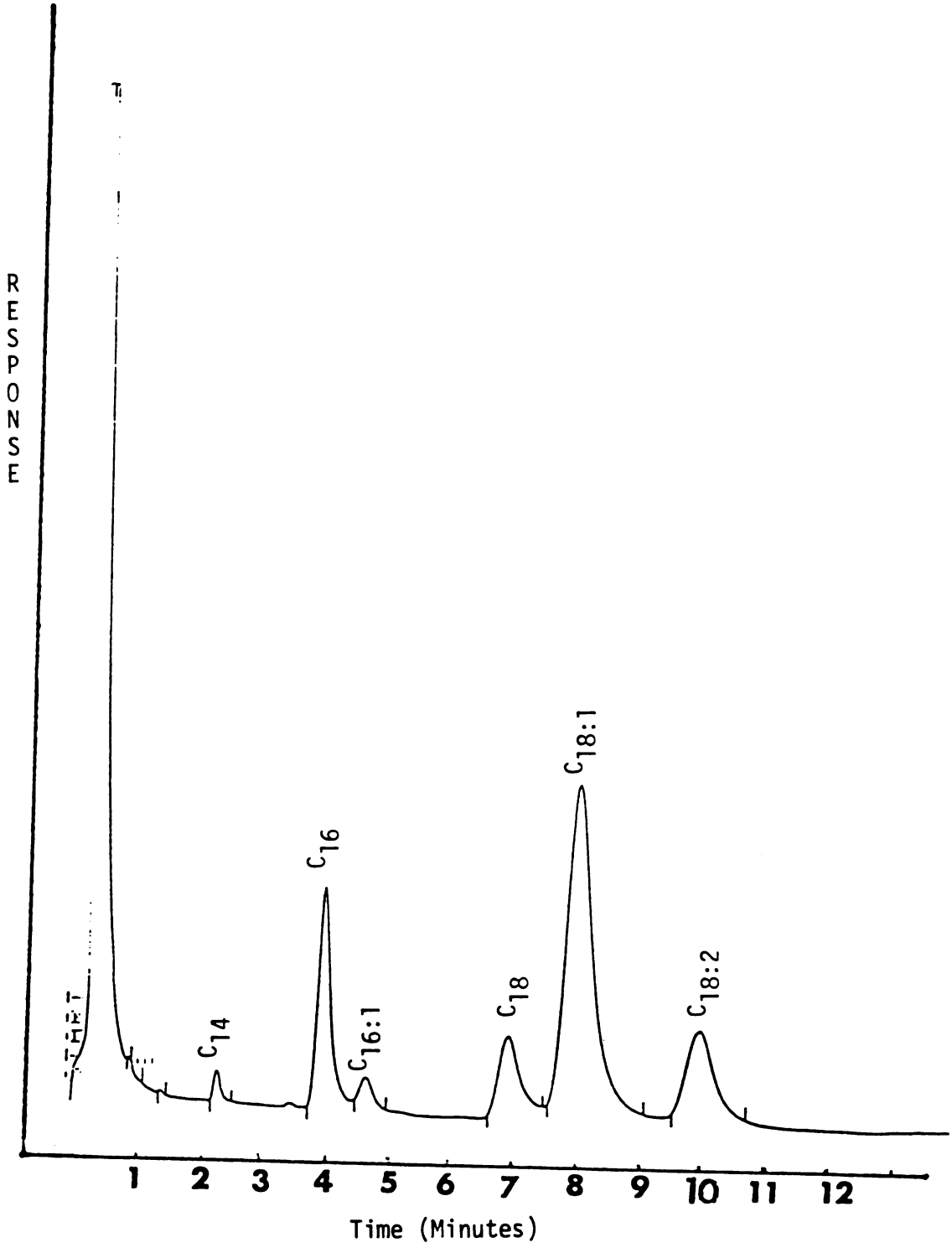


Figure 5. Gas chromatogram showing fatty acid composition of pork belly adipose tissue.

Formation of Amides in Pork Belly Slices Treated with Norleucine and/or Pentylamine

Freeze dried pork belly slices were rehydrated in water containing 1000 mg/kg of pentylamine. After equilibration for 24 hours, the treated slices were fried 4 minutes on each side at 175°C and analyzed for amide formation. The analysis revealed that there was neither amide formation in the cook-out fat nor in the fried bacon after heating. After heating of the fried bacon and cook-out fat for an additional 8 minutes, five N-substituted amides were identified in the cook-out fat and cooked residue (Figure 6), indicating that the time of frying is the limiting factor in their formation. It was noted that the relative percentages of the various amides formed closely approximated the fatty acid composition of pork belly adipose tissue as indicated in Table 4.

Similar results were obtained when pentylamine was sprayed on the surface of pork belly slices, followed by frying. N-Substituted amides were formed from the reaction of added pentylamine and the fatty acids naturally present in the pork belly slices. The results are in agreement with the findings of Kakuda and Gray (1980a), who reported that free amines react readily upon heating with fatty acids and fatty esters. They repoted that a temperature of 100°C for 15 minutes was sufficient for amide formation from the reaction of pentylamine with tripalmitin or palmitic acid.

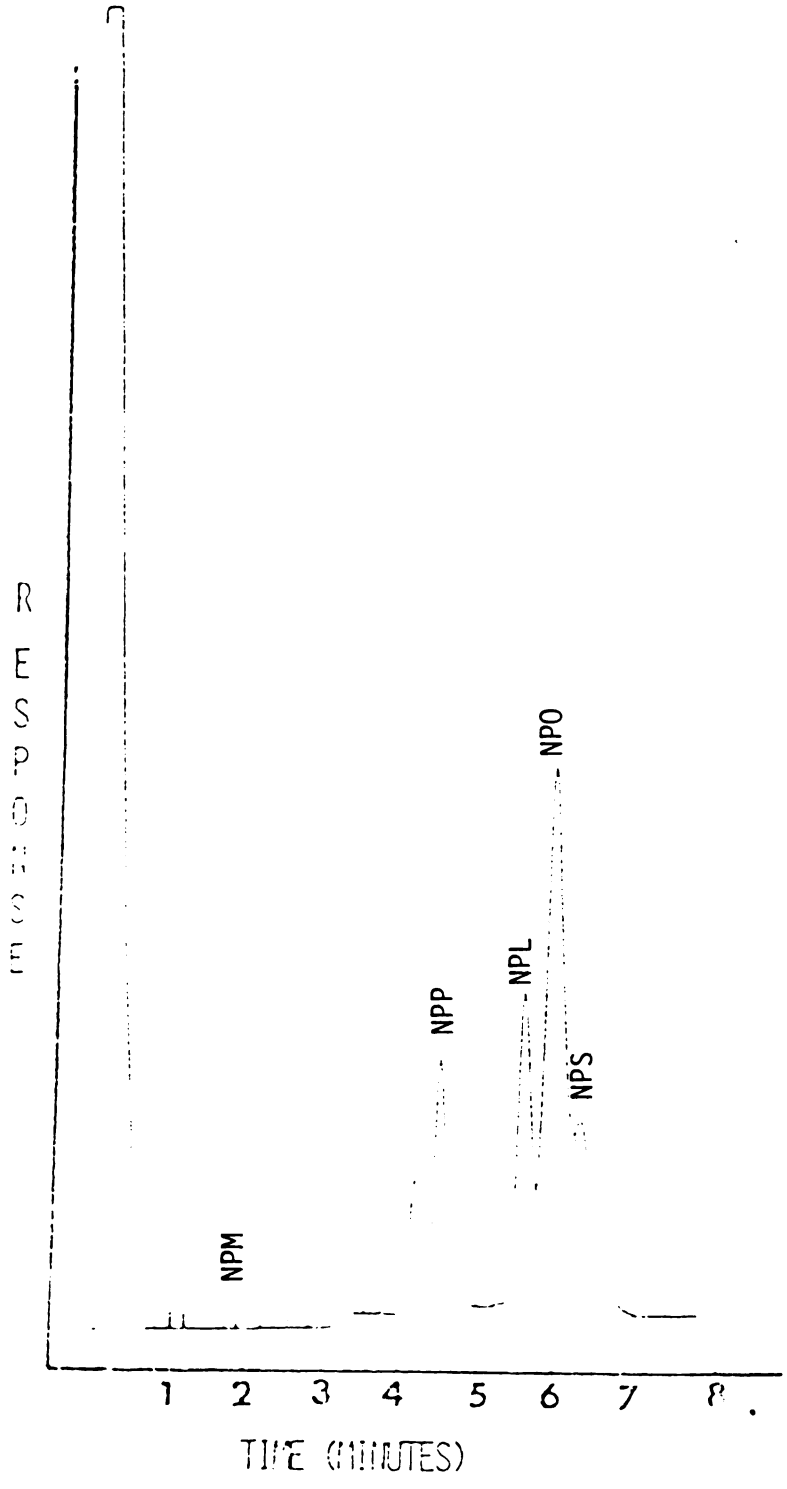


Figure 6. Gas chromatogram of N-substituted amides (N-pentylmyristamide, N-pentylpalmitamide, N-pentyllinoleamide, N-pentyloleamide and N-pentylstearamide) present in cook-out fat from rehydrated freeze dried pork belly slices containing pentylamine, heated for 16 minutes at 175°C.

Table 4. Fatty acid composition of pork bellies and N-substituted amides which formed upon heating pork bellies containing pentylamine

Fatty Acid	Percent	% Amide	N-Substituted Amide
C_{14}	1.3	0.4	Pentylmyristamide
C_{16}	16.5	16.3	Pentylapalmitamide
C_{18}	11.1	14.	Pentylstearamide
C18:1	49.8	43.	Pentyloleamide
C18:2	15.4	15.5	Pentyllioleamide

When the study was repeated with norleucine, amide formation did not take place in the cook-out fat or fried bacon, even after an additional heating period of 8 minutes at 175°C. However, on heating norleucine and pork belly adipose tissue or lard for one hour at 200°C, five N-substituted amides were identified (Figure 7). These results indicate that time and temperature of frying was not sufficient for decarboxylation of norleucine. Sims and Fioriti (1975) reported that decarboxylation of an amino acid in the presence of a fatty acid ester is zero order and is much slower than the aminolysis reaction.

When pork bellies were stitch pumped with pentylamine or norleucine and trilaurine, followed by smoking, slicing and frying, amide formation was not evident in either the smoked belly, the smoked and fried belly, or in the cook-out fat. This was true even though previous experiments had

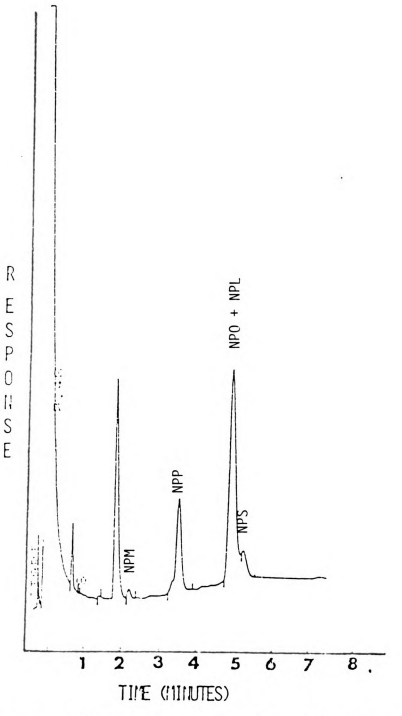


Figure 7. Gas chromatogram of N-pentylmyristamide (MPM), N-pentylpalmitamide (NPP), N-pentyloleamide (NPO), N-pentyllinoleamide (NPL) and N-pentylstearamide (NPS) present in pork belly adipose tissue with added norleucine heated 1 hour at 200°C.

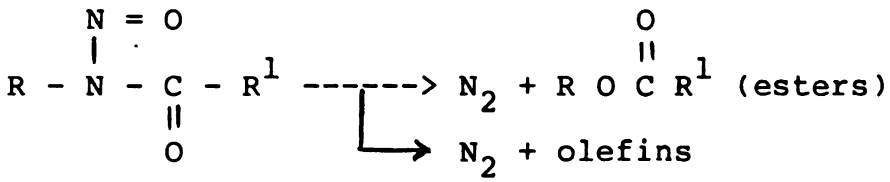
confirmed the formation of amides in pork belly slices treated with pentylamine under identical frying conditions. Patterson and Mottram (1974) reported that the concentration of volatile amines in pork carcass meat decreased during the curing and processing. Therefore, these results suggest that low temperature and long periods of smoking resulted in volatilization and loss of pentylamine. The smoking conditions and frying temperature were not sufficient to decarboxylate the norleucine, since, smoked pork belly slices which contained norleucine, when heated 1 hour at 200^{o}C formed five N-substitutes amides corresponding to the fatty acid content of pork belly adipose tissue.

These results indicate that formation of primary amines via the decarboxylation of amino acids appear to be unlikely under norml cooking conditions. There does not appear to be sufficient energy for the decarboxylation reaction. Kakuda and Gray (1980a) reported high temperatures (minimum 150^{o}C for 45 minutes) were required for the decarboxylation of norleucine. However, the amount of free amines in foods is not limited to those formed by thermal decarboxylation. Many enzymatic and bacterial decarboxylation reactions are known to occur in many foodstuffs (Maga, 1978), and these reactions may serve as sources of free amines. The presence of amines, fatty acids, and high temperatures during cooking and processing may lead to formation of secondary amides in foods.

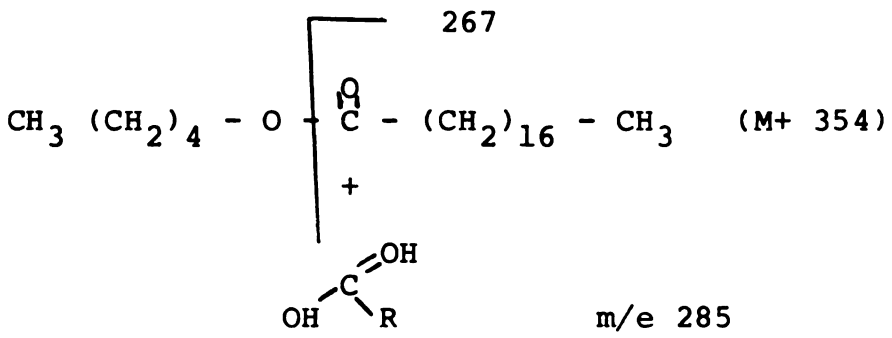
Nitrosation of N-Substituted Amides

Gram quantities of different N-nitrosamides were prepared and purified by column chromatography or vacuum distillation. The purity of the N-nitrosamides was determined by GLC and their identity was confirmed by GLC-MS analysis. All compounds had purities in excess of 95%.

Figure 8 shows the chromatogram of N-nitrosopentylstearamide (NOPS) which was prepared by nitrosation of recrystallized N-pentylstearamide. N-nitrosopentylstearamide contained small amounts of N-pentylstearamide (NPS). Figure 9 shows the mass spectrum of NOPS and represents only the corresponding ester produced by thermal elimination of nitrogen. White (1955b) repoted that formation of esters and olefins occur according to the following scheme:



The compound detected by GLC-MS analysis of NOPS corresponded to pentylstearate as shown below:



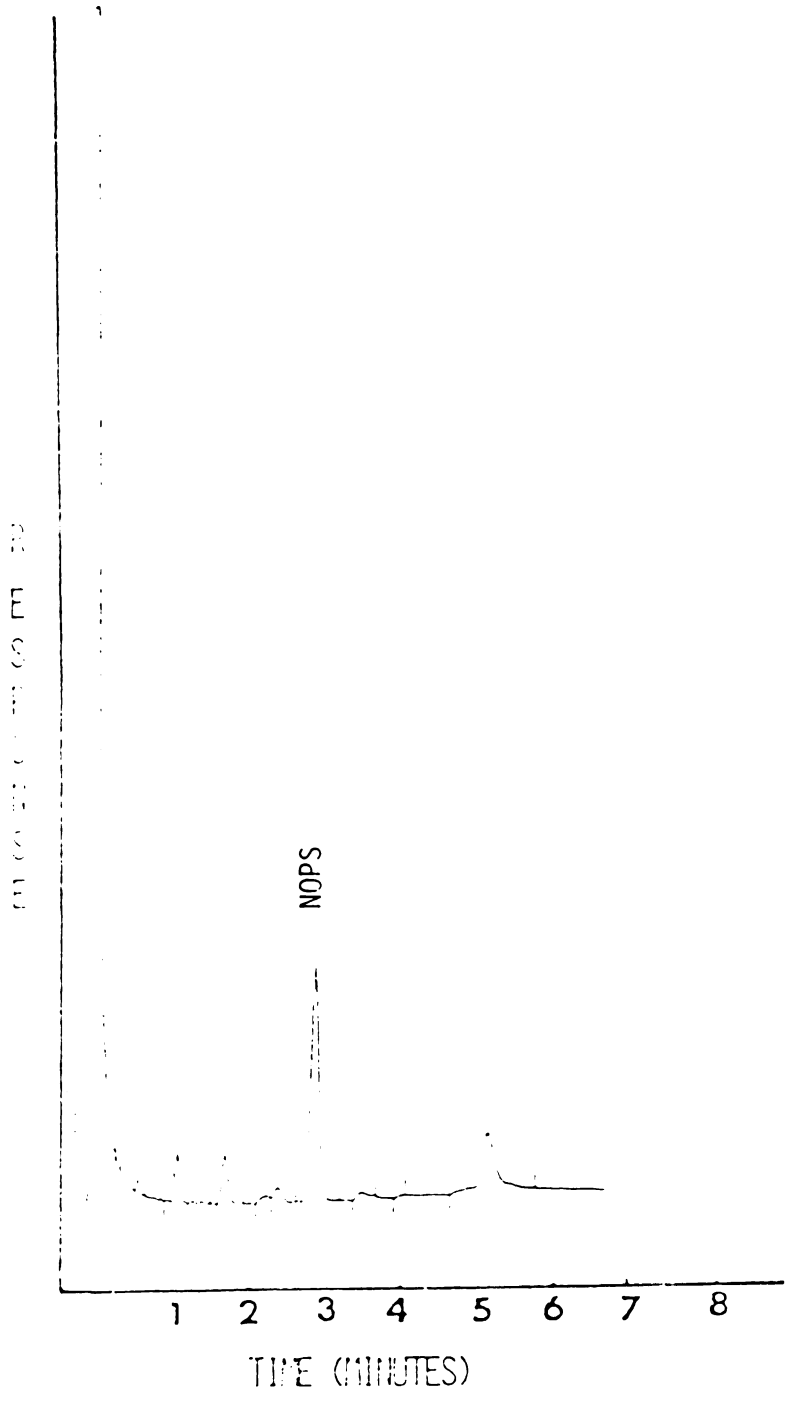
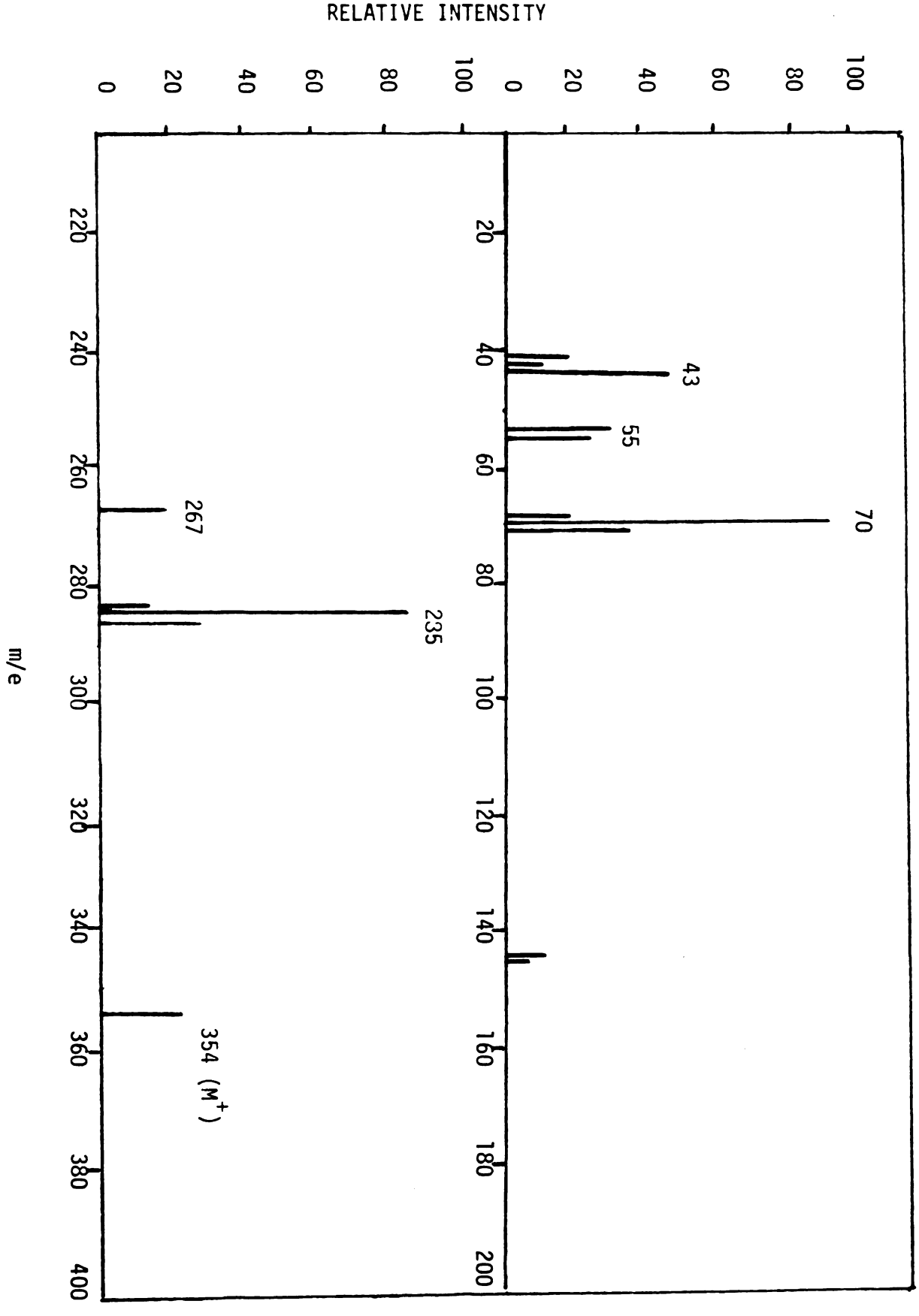


Figure 8. Gas chromatogram of N-nitrosopentylstearamide.

Figure 9. Mass spectrum of pentyl stearate, a breakdown product of N-nitrosopentylstearamide.


RELATIVE INTENSITY
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
m/e
43
55
70
267
235
354 (M+)

The major ions present were m/e 70, 43, 267, 354 (M^+) and 285. The mass spectrum of N-nitrosopentylpalmitamide (NOPP) exhibited major inos at m/e 70, 43, 326 (M^+) and 239. A similar fragmentation pattern was reported by Kakuda and Gray (1980b) for NOPP.

The mass spectra of the other N-nitrosamide confirmed their formation by nitrosating their corresponding N-substituted amides. A standard mixture of purified N-nitrosopentylmyristamide, N-nitrosopentylpalmitamide, N-nitrosopentylstearamide, N-nitrosopentyloleamide and N-nitrosopentyllinoleamide was prepared (Figure 10) and used as standards for the identification of the N-nitrosamides in bacon.

Formation of N-Nitrosamides in Bacon

Nitrosation of secondary amides under acid conditions in the model system was confirmed by GLC-MS analysis. In order to determine whether nitrosation of amides can occur in bacon, a solution of N-pentylpalmitamide and sodium nitrite were injected separately into the pork belly slices and stored 24 hours at $4^{o}C$. The bacon was then analyzed for the presence of N-nitrosopentypalmitamide before and after frying. Results showed that N-nitrosamide formation did not occur in the raw or cooked bacon or in the cook-out fat. This is not surprising since the main nitrosating agent for amides is the nitrous acidium ion (Mirvish, 1975a). Thus, nitrosation of secondary amides requires low pH. The extent of nitrosation of secondary amides decreases

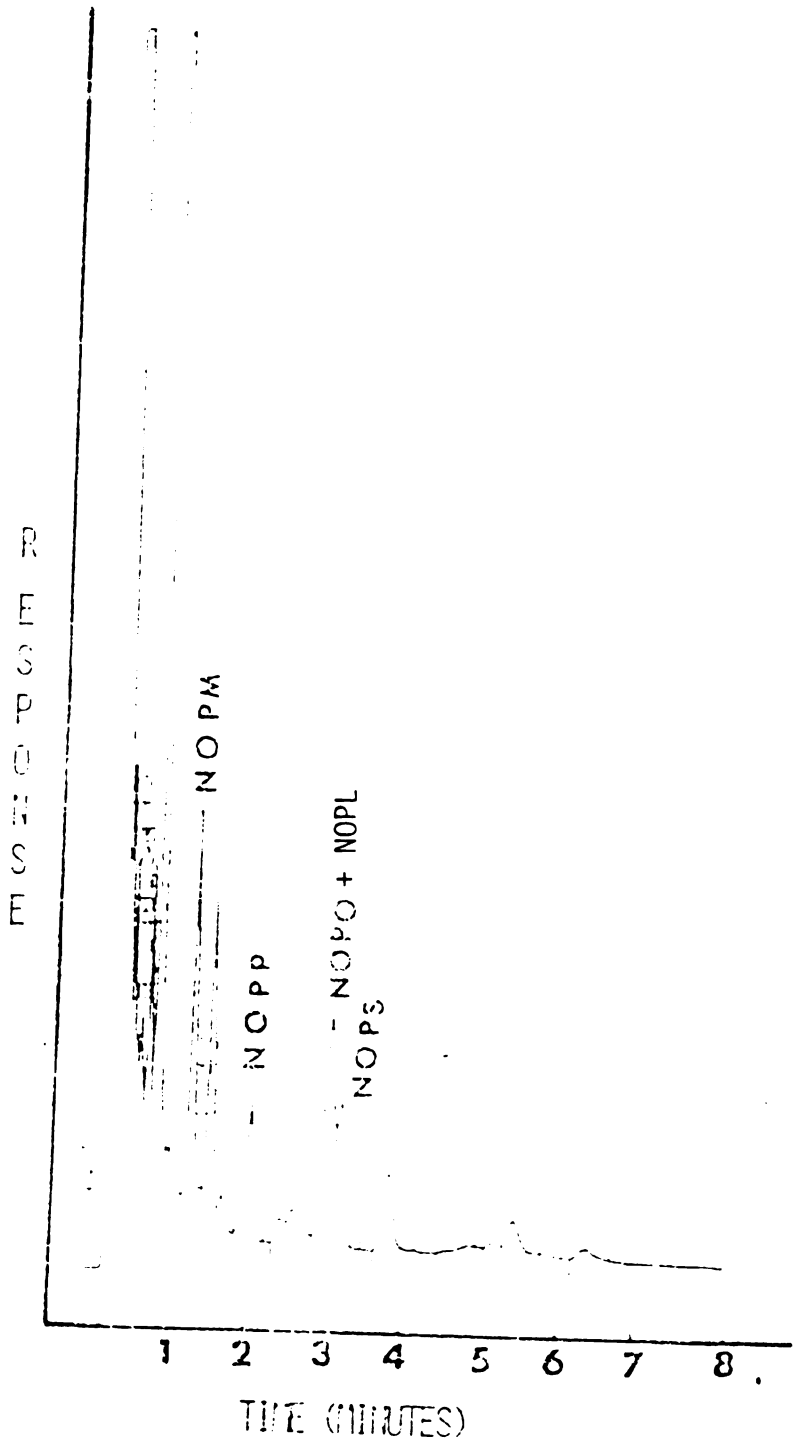


Figure 10. Gas chromatogram of standard mixture containing N-nitrosopentylmyristamide (NOPM), N-nitrosopentylpalmitamide (NOPP), N-nitrosopentyloleamide (NOPO), N-nitrosopentyllinoleamide (NOPL), and N-nitrosopentylstearamide (NOPS) derived from nitrosation of their corresponding N-substituted amides.

as the pH of the environment increases and little reaction occurs above pH 3 (Kakuda and Gray 1980b). Thus, the pH of foods would militate aginst the occurrence of nitrosamides, even if their precursors are present. However, in vivo nitrosation of alkylamides has been reported by Mirvish (1971).

The nitrosation of amides require acid conditions, which occur in gastric juices plus sufficient quantities of nitrite. The eating of vegetables and vegetable juices results in increases in salivary nitrite, 3 levels of hundreds of parts per million being reported by Tannenbaum, et al., 1976. Also, de novo synthesis of nitrate and nitrite in the upper aerobic portion of the intestine has been reported by Tannenbaum et al., (1978). Thus, in vivo nitrosation of N-substituted amides in the digestive tract is possible.

Thermal Decomposition of N-Nitrosamides

The stability of NOPP under frying conditions similar to those used in the cooking of bacon was also studied. An aliquot (1000 mg/kg) of NOPP solution in corn oil was injected into pork belly slices and stored overnight at 4°C, and then fried. A 95% recovery of the NOPP was obtained from the raw pork belly slices (Figure 11). Analysis revealed that only 8 and 5% of the original NOPP was found in cook-out fat (Figure 12) and fried bacon (Figure 13), respectively. This indicates that 87% of the

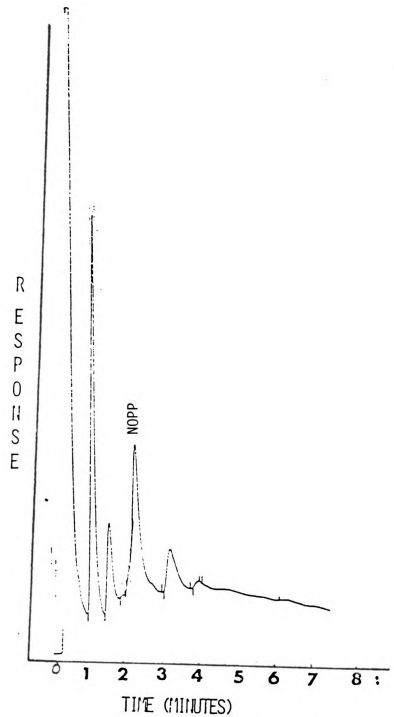


Figure 11. Gas chromatogram of recovery of N-nitroso-pentylpalmitamide from raw bacon.

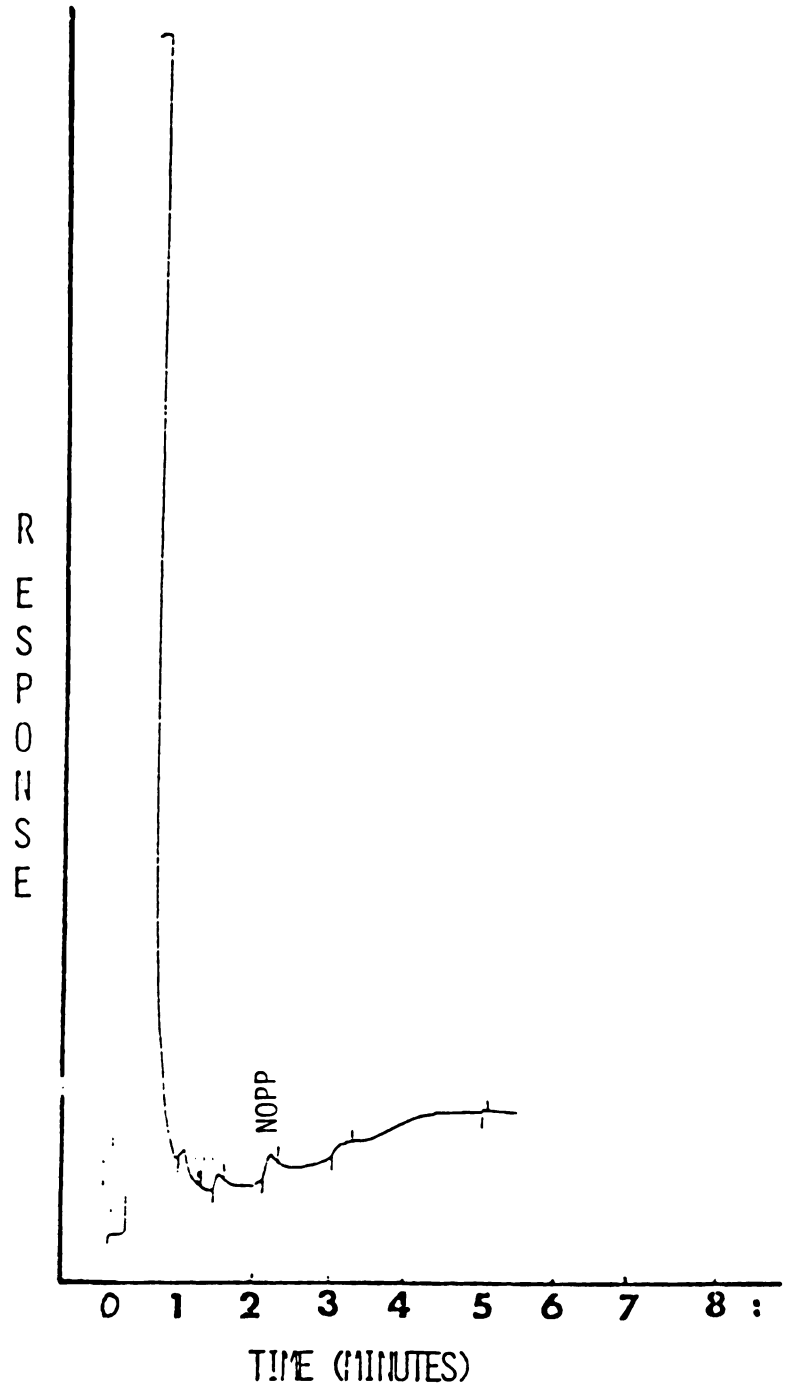


Figure 12. Gas chromatogram of N-nitrosopentylpalmitamide residue in cook-out fat.

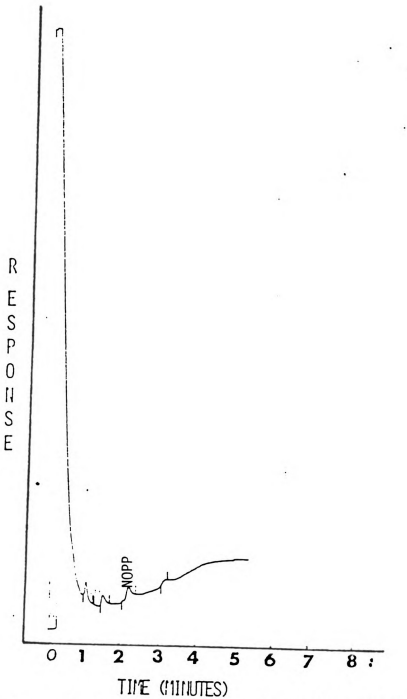


Figure 13. Gas chromatogram of N-nitrosopentylpalmitamide residue in fried bacon.

original amount of NOPP was lost during heating. These results support the finding of Kakuda and Gray (1980b) who reported N-nitrosamides are much less stable than volatile N-nitrosamines. In their thermal decomposition studies on N-nitrosamides utilizing heating conditions commonly encountered in the pan frying of bacon or in the oven roasting of pork, Kakuda and Gray (1980b) found that NOPP was degraded to the extent of 74-97% compared to 3-14% for NPYR and NDMA. Chow (1979) reported that at temperatures ranging from ambient to 100°C, N-nitrosamides undergo irreversible thermal rearrangements to form diazo esters. Diazo esters, in turn, decompose rapidly to give carboxylic esters or acids and olefins. The instability of N-nitrosamides under alkaline and neutral pH and their rapid thermal decomposition leads to the conclusion that the occurrence of N-nitrosamides in food systems is unlikely. The major contribution of N-substituted amides, if present in foods, may be as precursors of N-nitroso compounds formed by *in vivo* nitrosation reactions.

Mutagenicity of N-Substituted Amides and Their Corresponding N-Nitrosamides

The mutagenicity of some short chain and long chain N-substituted amides and their corresponding N-nitrosamides was tested by using Salmonella strain G 46 (mutation in TA 1535) with and without S-9 mix. The results of the test are

presented in Table 5. The numbers shown in Table 5 represent the frequency of histidine-positive revertants per plate after substraction of the spontaneous mutation rate (background).

The starred values (*) deserve consideration as possible mutagenic compounds. None of the N-substituted amides which were tested showed mutagenic potential. N-Nitrosopentylpalmitamide was the only N-nitrosamide which gave a strong mutagenic response without S-9 activation. N-Nitrosomethylpropionamide showed weak mutagenic activity at the concentration tested upon addition of S-9 mix. N-Nitrosomethylurea was not mutagenic in the Ames test but microsomes from induced rat liver (S-9 mix) can convert it to derivatives, which are mutagenic. These results are in contrast to the findings of Magee (1972), who reported that N-nitrosamides are mutagenic without metabolic activation. McCann et al. (1975a) reported that N-nitrosomethylurea is mutagnic in Salmonella strain TA 100 and TA 1535 without the addition of S-9 mix. Brundrett et al. (1979) studied the mutagenecity of seven N-nitrosoureas and nitrosamides and reported that mutagenecity of none of the compounds was enhanced by the addition of S-9 extract to the standard Ames test. In fact, mutagenic activity was often decreased slightly by S-9 addition. Furthermore, these authors showed a decrease in mutagenecity of N-nitrosoacetamide and nitrosoureas with an increase in N-alkyl chain length.

Table 5. Mutagenicity potential of N-substituted amides and their corresponding N-nitrosamides.

Concn.	Compound	His+ Revertants/plate (+) S-9	(-) S-9
1 mM	N-pentylpalmitamide	ND	ND
1 mM	N-nitrosopentylpalmitamide	ND	2350*
1 mM	N-methylpropionamide	ND	ND
1 mM	N-nitrosomethylpropionamide	280*	ND
1 mM	N-nitrosomethylurea	1086*	43
1 mM	N-(3-methylbutyl) myristamide	ND	ND
1 mM	N-(3-methylbutyl) stearamide	ND	ND
1 mM	N-nitroso-(3-methylbutyl) stearamide	ND	ND

ND=non detectable

The present observations on mutagenecity of N-nitrosamides which can be considered only as preliminary tests are inconsistent with the literature. The contradictory nature of the results could be due to the instability of the N-nitrosamides and rapid decomposition via photolysis, alkaline pH or temperature (ambient to 100^{o}C). Therefore, The observed mutagenicity reponse could be due to the breakdown products. Further studies on mutagenicity of these compounds with different strains of Salmonella are required before any positive conclusions can be drawn.

Carcinogenicity of N-Nitrosamides

The effect of N-substituted amides and N-nitrosamides on tumor development was investigated in feeding trials using Swiss mice. Table 6 presents the data on feed and water consumption per mouse per day in each group.

The data on Table 6 indicate that the feed and water consumption of the mice in group 6 was greatly reduced, being only half as much as for the other groups. within two weeks on the diet, two mice from group 6 died and after 3 weeks, the remaining mice began sneezing, chattering and showing signs of respiratory distress. They refused to consume their feed and water and started to lose the hair from their backs. Two mice from this group were sent to the Animal Health Diagnostic Laboratory at Michigan State University. The laboratory examination report indicated that a light brownish red discoloration was observed in the

Table 6. Effect of additives on feed and water consumption of mice

Group		Additives[a]	g feed/ mouse/day	mL H_2O/ mouse/day	mg additives/ mouse/7 months
Control	1	none	4.2	7.98	0
	2	NMP	4.2	7.61	264.6
	3	NMP+$NaNO_2$	4.00	7.93	252
	4	NPP	3.82	7.52	239.4
	5	NPP+$NaNO_2$	4.90	8.10	245.7
	6	NOMP	2.1*	4.23*	163.8
	7	NOPP	3.85	7.87	242.5
	8	NMU	3.93	8.02	241.9
	9	NOPD	3.84	7.89	247.6

a. NMP = N-methylpropionamide
NPP = N-pentylpalmitamide
NOMP = N-nitrosomethylpropionamide
NOPP = N-nitrosopentylpalmitamide
NMU = N-nitrosomethylurea
NOPD = N-nitrosopentyldecanamide

carnial lung lobes of these mice. The problem was suspected to be a viral disease triggered by the stress induced by NOMP. However, the laboratory examination was unable to identify a specific disease in these mice. 18 mice out of 20 were dead within 7 months while mice in other groups appeared quite normal.

After seven months on the experiment, 10 mice from each group were sacrificied except for groups 8 and 9 with 4 mice each and group 6 with 2 mice. Table 7 presents the results of the gross examination of the mice for tumors and other abnormalities.

Microscopic examination of liver, lungs, spleen, heart and stomach tissues showed that the only mice that had any consistent number of lesions were in group 8, which was fed the purified diet plus N-nitrosomethylurea. Two of four mice in this group had adenocarcinomas in the lungs and the other two had adenomas. These lesions were seen grossly in three of four mice.

The carcinogenicity of N-nitrosomethylurea has been demonstrated in several species for a variety of tissues depending on animal species, the route of application and the dosage rate. Preussmann (1973) reported that NMU developed tumors of the forestomach in mice when the compound was administered by mouth, and tumors of the lungs by intravenous (i.v.) route of application. Denlinger et al. (1978) reported that metastasis occurred to liver, spleen, lymph nodes, lungs and adrenal of purebred Boxer

Table 7. Data on gross examination of the mice for tumors and other abnormalities upon autopsy.

Group	Abnormalities
1	Normal
2	Mouse #1 had enlarged uterus, filled with olive green watery fluid
3	All mice normal
4	Mouse #2 had enlarged spleen - 1 1/2 times normal size
5	Mice #3, 8 and 9 had enlarged uterus filled with olive green liquid
6	Only 2 mice survived. The hair was sparse on both
7	All normal
8	Small modules in lungs found in four mice that were examined
9	All normal

dogs when given weekly i.v. injections (5 mg/kg) of NMU for 36 weeks and observed for approximately 3 years.

Although the hair was sparse, skin sections from the surviving 2 mice in group 6 had no histological lesions. Sections of spleen, kidney, stomach and intestine were normal. N-Nitrosopentylpalmitamide and N-nitrosopentyldecanamide also did not develop any tumors in the above tissues. These compounds are high molecular weight with long chain alkyl and acyl groups, therefore, the possibility that these compounds are not carcinogenic exists. Druckrey (1975) reported that the carcinogenic potential of N-nitrosamides corresponds closely to the chemical reactive of the compound and generally decreases with increasing number of carbon atoms in the acyl and alkyl groups.

The only conclusion from these results would be that N-substituted amides with and without the addition of nitrite, and their corresponding N-nitrosamides did not cause tumor development in mice given the dosages and time periods used in this study. However, some of these compounds (NOPP and NOMP) were found to be mutagenic by the Ames test. Since there was no evidence of tumors at this time, the remaining mice are being kept on the same diet and will continue to be held for two years at which time will sacrifice and examined for tumor.

SUMMARY AND CONCLUSION

A study was conducted to establish the formation of N-substituted amides from the reaction of amino acid or free amines and fatty acids. GLC-MS analysis confirmed their formation at 200°C. Formation of N-substituted amides under frying condition of bacon was investigated by rehydrating freeze dried pork belly slices with water contianing pentylamine. After equilibrating for 24 hours at 4°C, the slices were fried for 4 minutes on each side at 175°C in a preheated pan. Analysis revealed no amide formation in either the fried slices or cook-out fat. However, upon heating for an additional 6 minutes, N-substituted amides were formed in proportion to the fatty acid composition of pork belly adipose tissue. When the study was repeated with norleucine, no amide formation took place in the cook-out fat or in fried slices, even after the additional heating period. However, heating pork belly adipose tissue and norleucine together at 200°C for 1 hour resulted in formation of N-substituted amides proportional to the fatty acid composition of the pork belly adipose tissue. Furthermore, when pork bellies were stitch-pumped with pentylamine or norleucine and trilaurin, smoked, sliced and fried, amide

formation was not evident in either the bacon or in cook-out fat. It is concluded that under the conditions encountered in the frying of bacon, only amines would react with fatty acids to yield secondary amides. It was further concluded that the temperature of frying is not sufficient for decarboxylation of the amino acids and formation of N-substituted amides.

Nitrosation of N-substituted amides was also carried out under acid conditions. Formation of N-nitrosamides was confirmed by GLC-MS. However, the normal pH of bacon would militate against the formation of N-nitrosamides, even if their precursors were present.

The stability of N-nitrosopentylpalmitamide under frying conditions used for bacon was also studied. The overall decomposition was approximately 87%. The major conclusion from this investigation was that N-nitrosamides are unlikely to be present in heat processed foods.

In the event that N-substituted amides are formed during processing and cooking of foods, they could be nitrosated in vivo. Therefore, the carcinogenicity of these compounds was investigated in a feeding trial using Swiss mice. None of the N-substitutes amides alone or in combination with nitrite or their corresponding N-nitrosamides developed tumors in mice within 7 month. Only the mice which were fed N-nitrosomethylurea (the positive control) had lesions in the lungs. Since the results of mutagenicity

assays showed that N-nitrosopentylpalmitamide and N-nitrosomethylurea are strong mutagens and that N-nitrosomethylpropionamide is a week mutagen, the possibility exists that some of these compounds are carcinogenic. Therefore, the remaining mice have been kept on the same diet and will continue for a two year period before sacrificing and determining the number of tumors.

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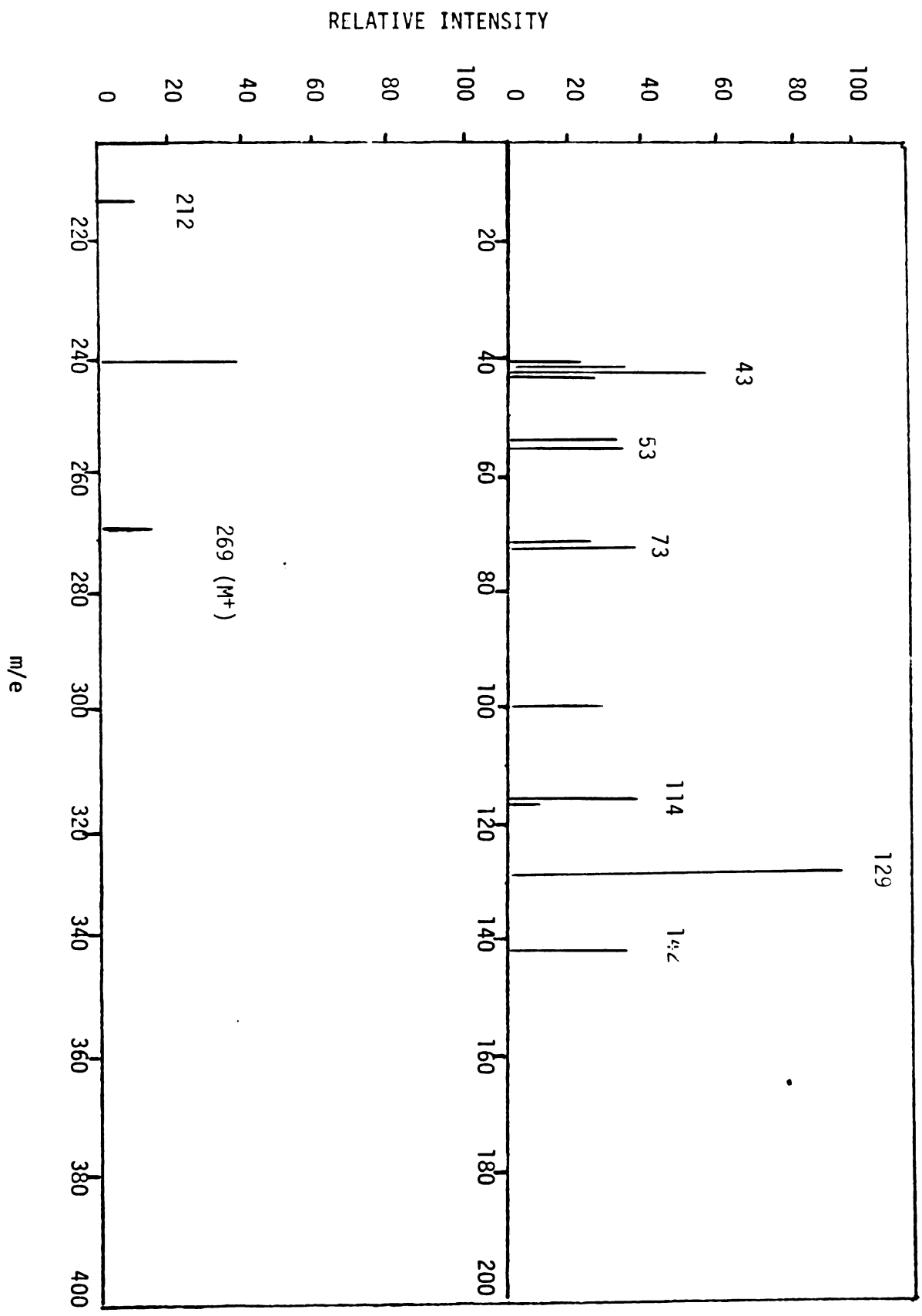
APPENDICES

Appendix 1: Mass spectrum of N-pentyllauramide prepared from reaction of norleucine and lauric acid at 200°C.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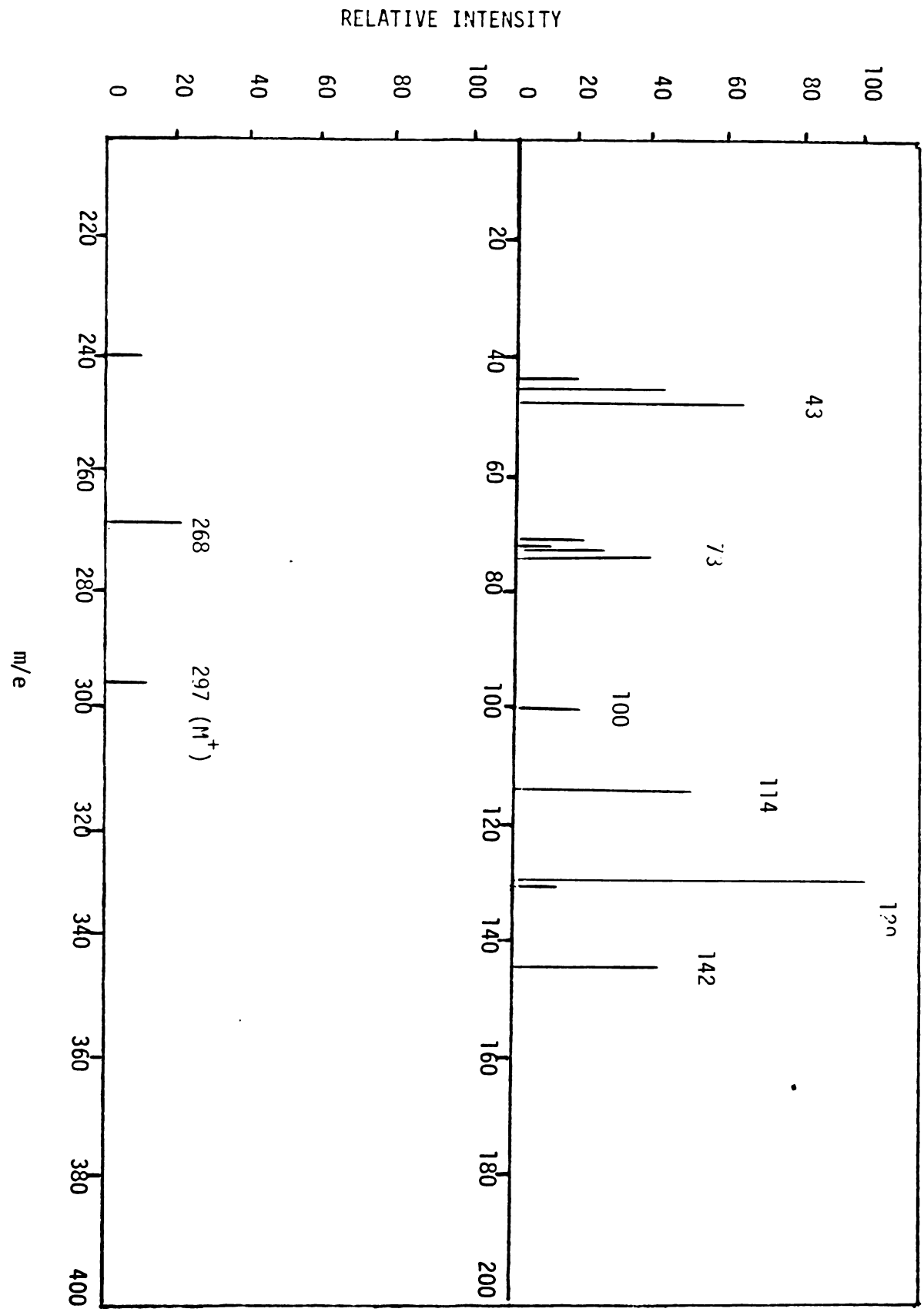
m/e
43
53
73
114
129
142
212
269 (M+)

Appendix 2: Mass spectrum of N-pentylmyristamide prepared from reaction of norleucine and myristic acid at 200^{o}C.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m/e
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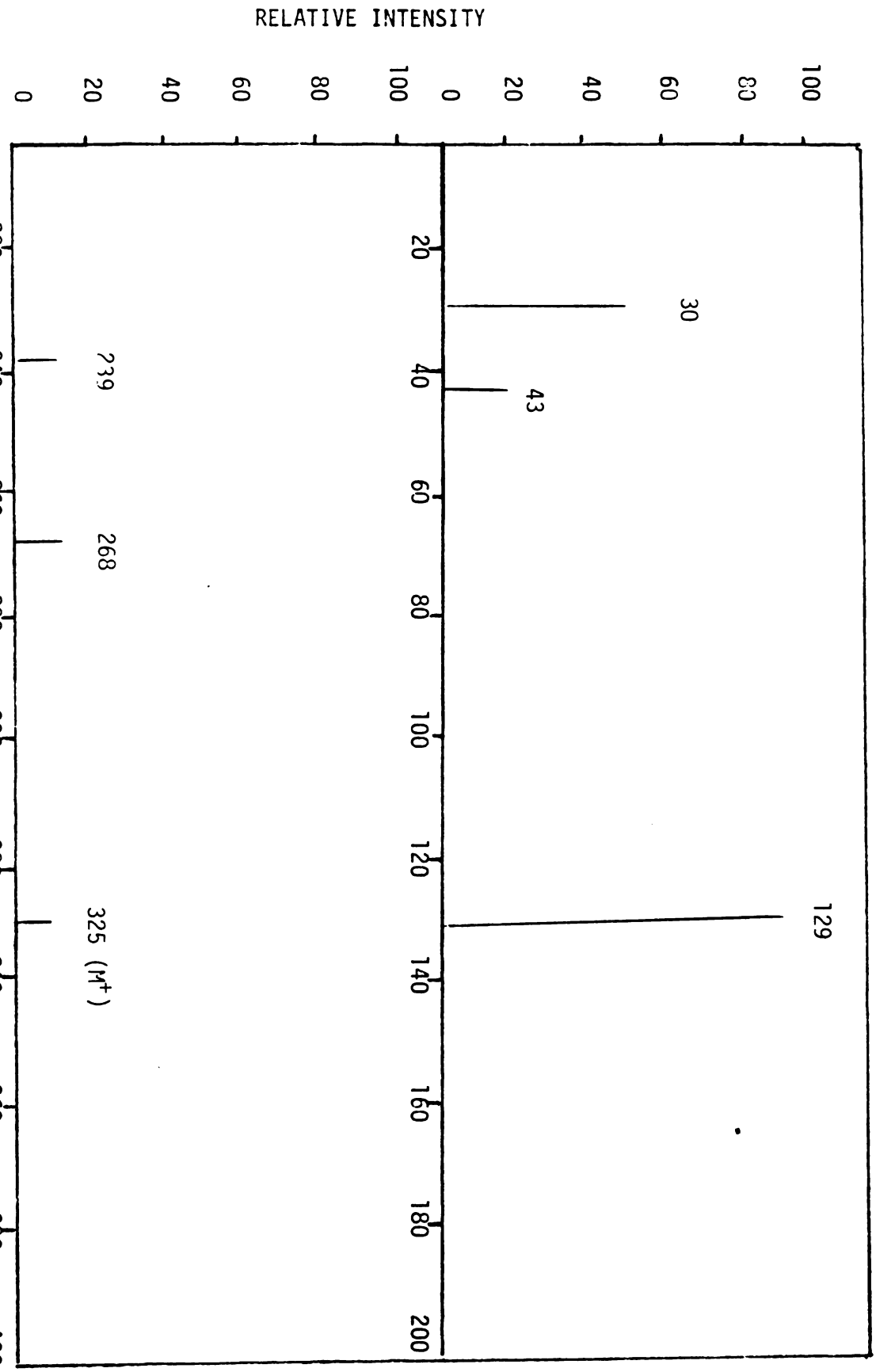
268
297 (M+)

Appendix 3: Mass spectrum of N-pentylpalmitamide prepared from reaction of norleucine and palmitic acid at 200°C.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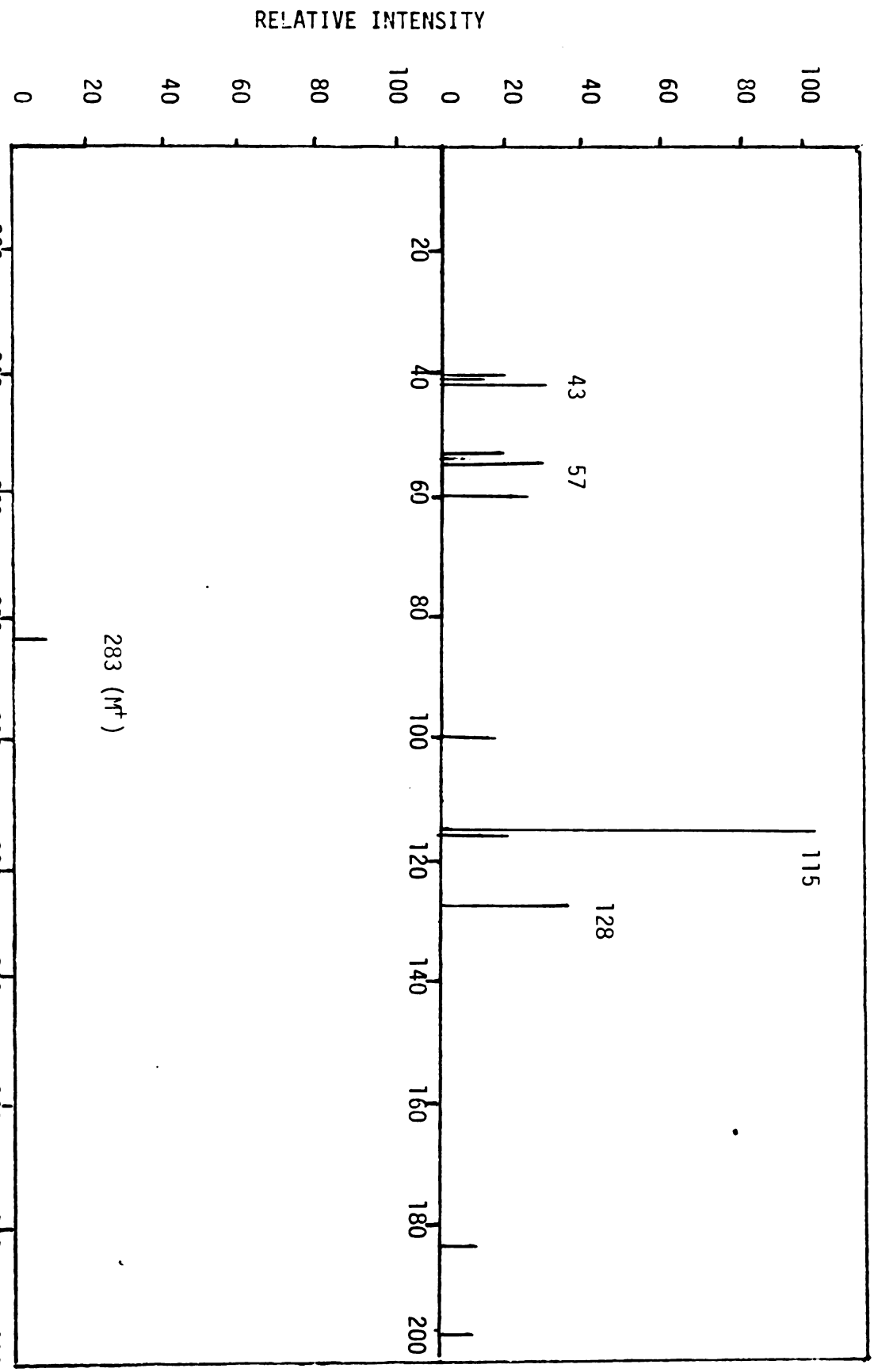
Appendix 4: Mass spectrum of N-isobutylmyristamide prepared from reaction of valine and myristic acid.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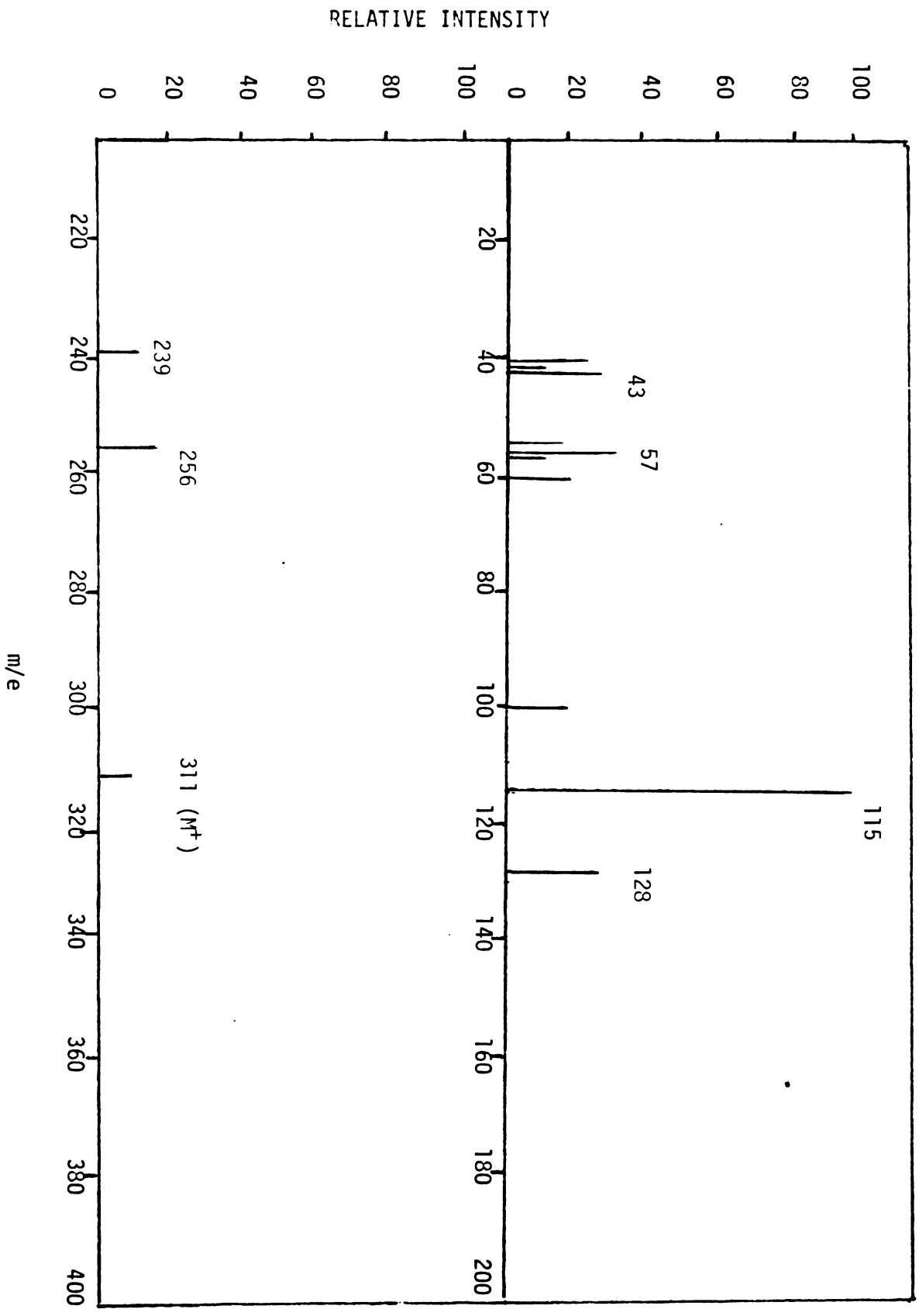
128
283 (M+)

Appendix 5: Mass spectrum of N-isobutylpalmitamide prepared from reaction of valine and palmitic acid at 200°C.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m/e
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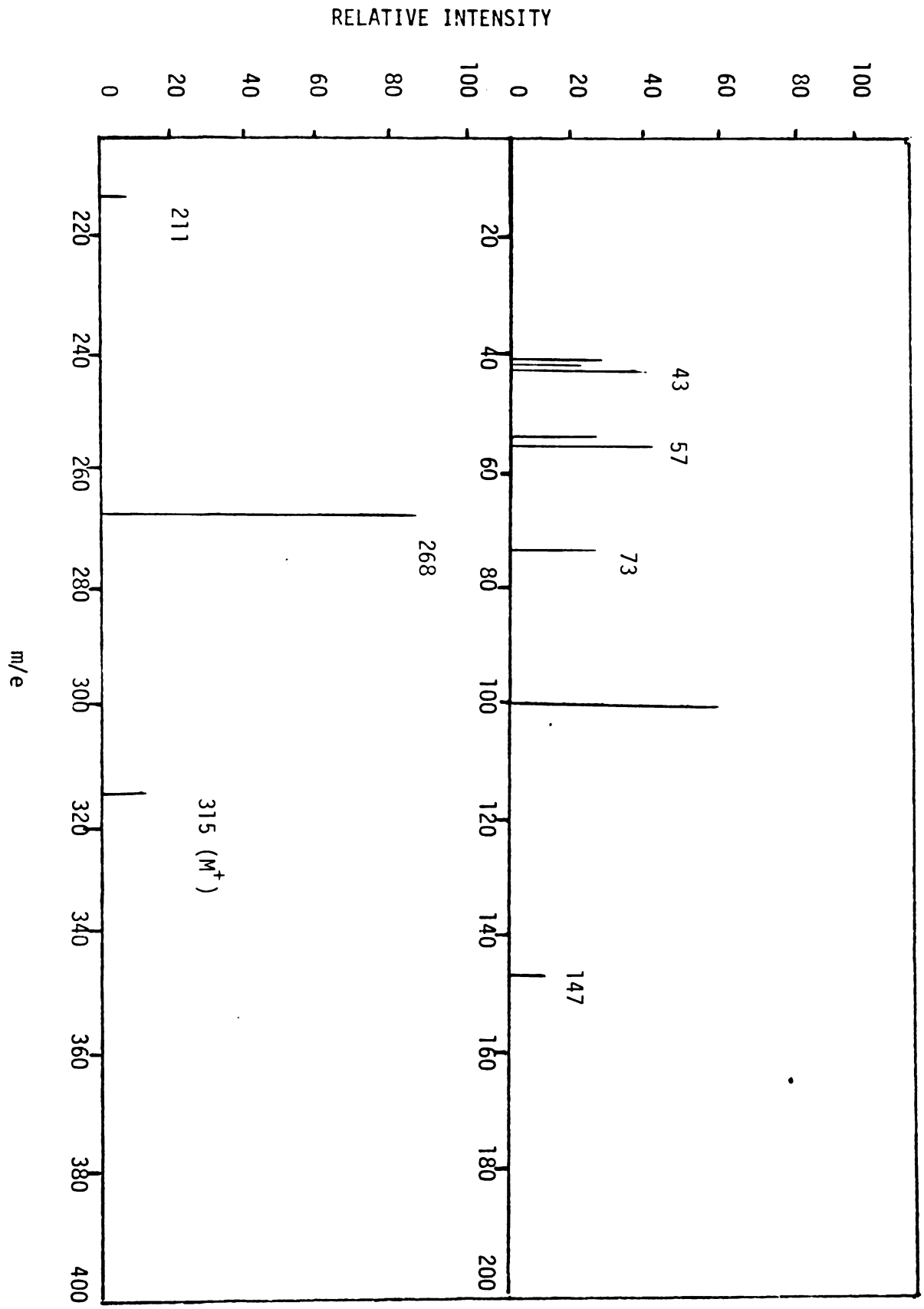
115
128
239
256
311 (M+)

Appendix 6: Mass spectrum of N-3-methylthiopropylmyristamide prepared from reaction of methionine and myristic acid at 200°C.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340
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380
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m/e
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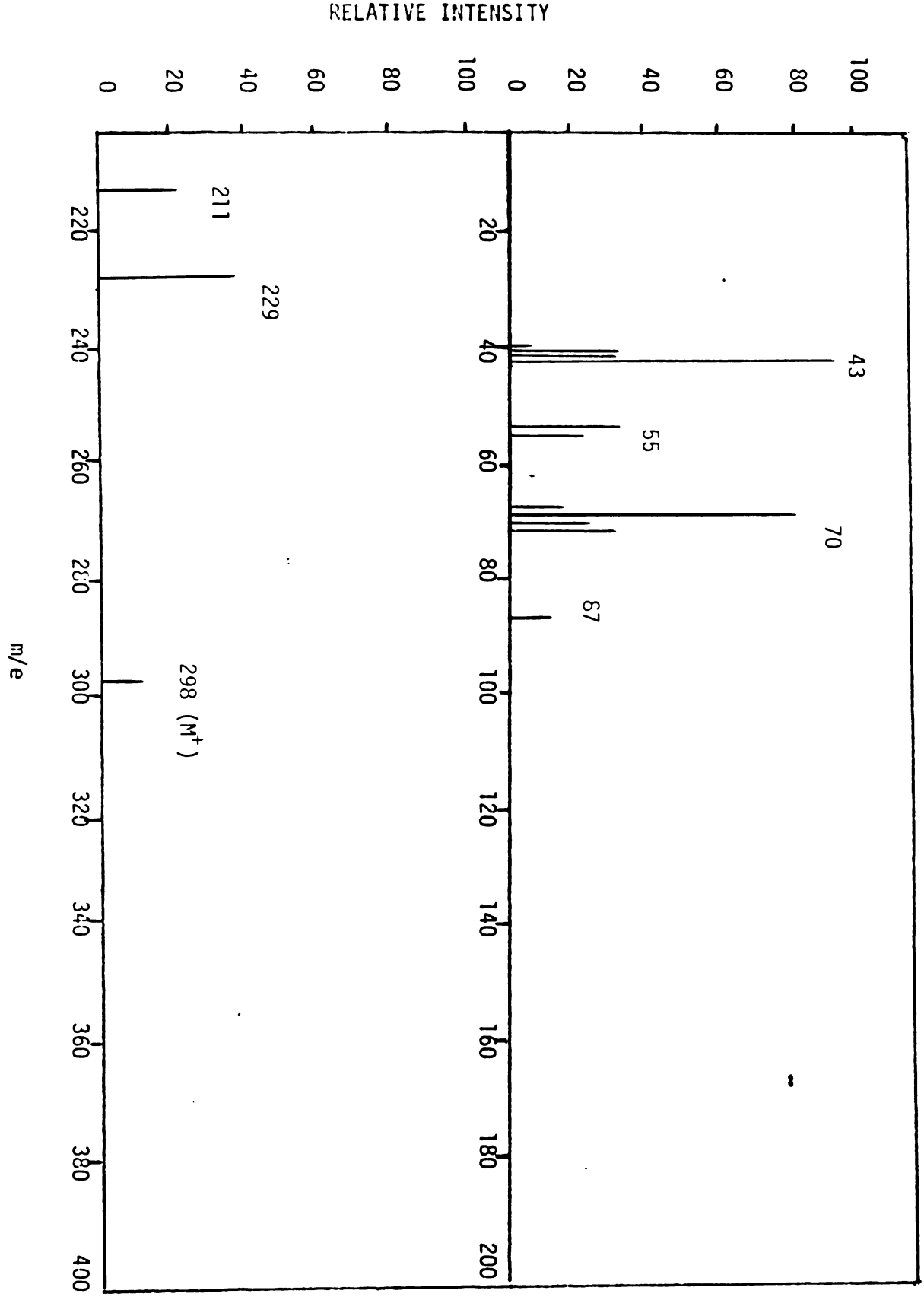
73
147
211
268
315 (M+)

Appendix 7: Mass spectrum of pentyl myristate a breakdwon product of N-nitrosopentylmyristamide.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380
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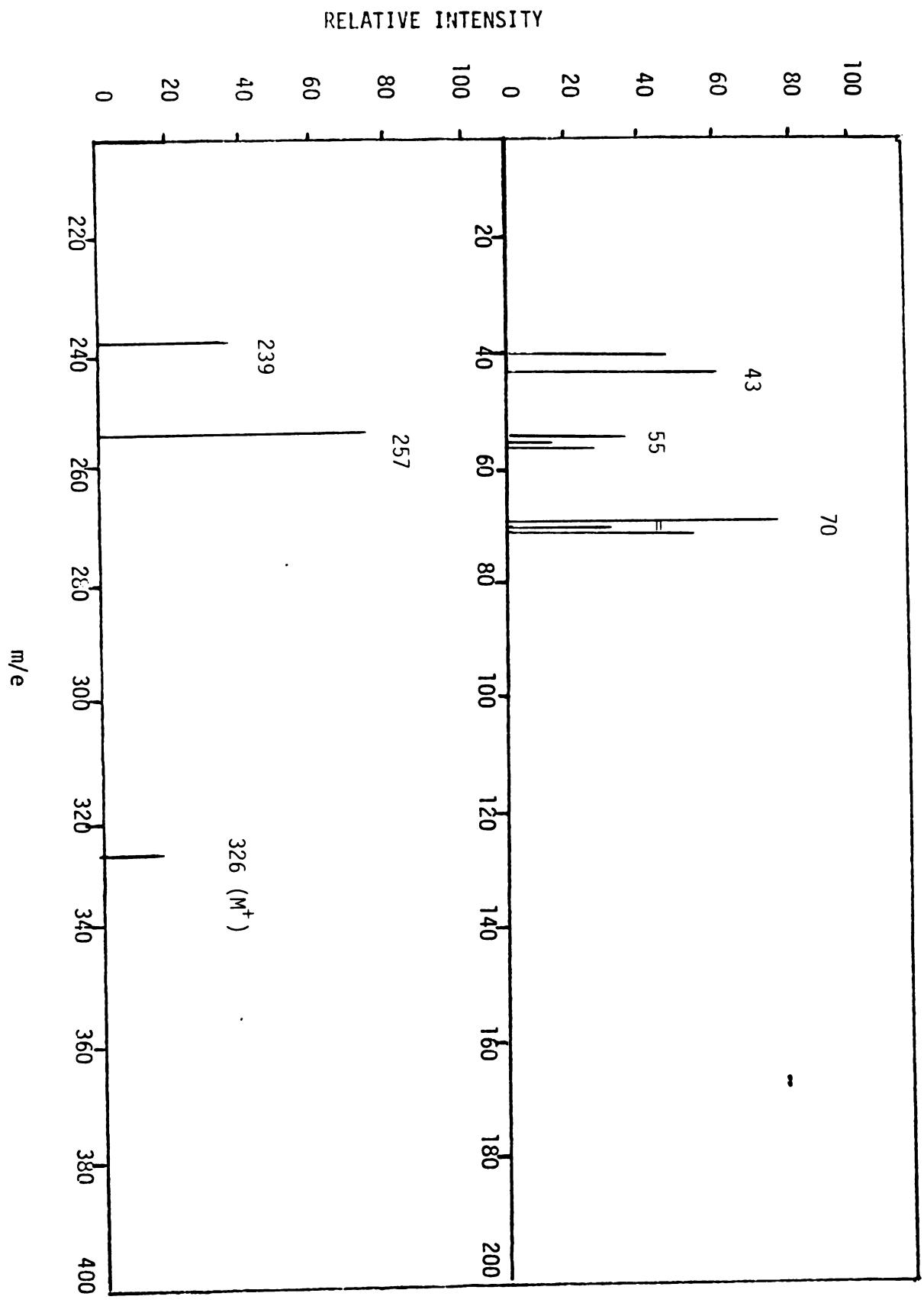
211
229
298 (M+)
m/e

Appendix 8: Mass spectrum of pentyl palmitate a breakdwon product of N-nitrosopentylpalmitamide.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380
400
m/e
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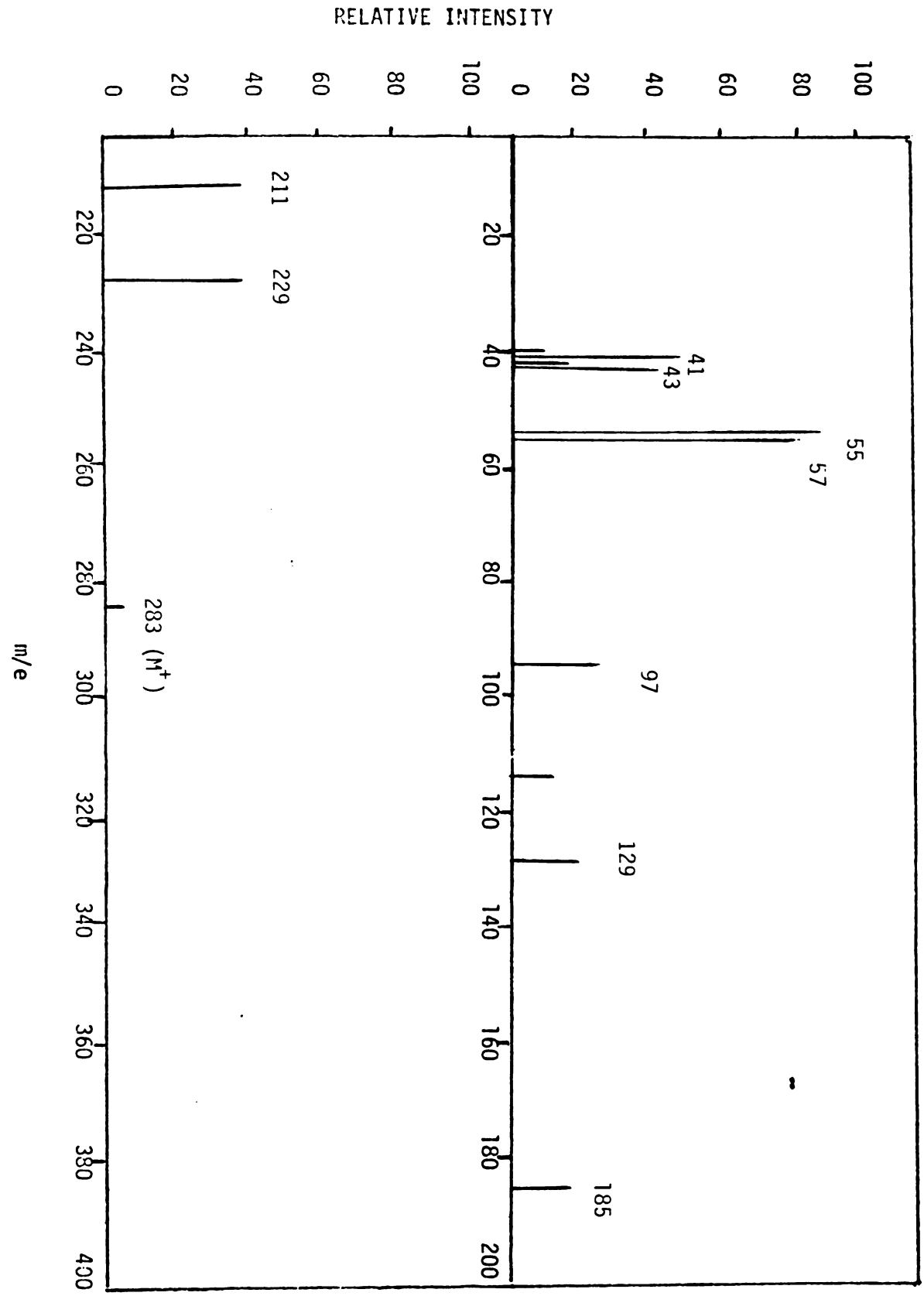
70
239
257
326 (M+)

Appendix 9: Mass spectrum of isobutyl myristate a breakdwon product of N-nitrosoisobutylmyristamide.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340
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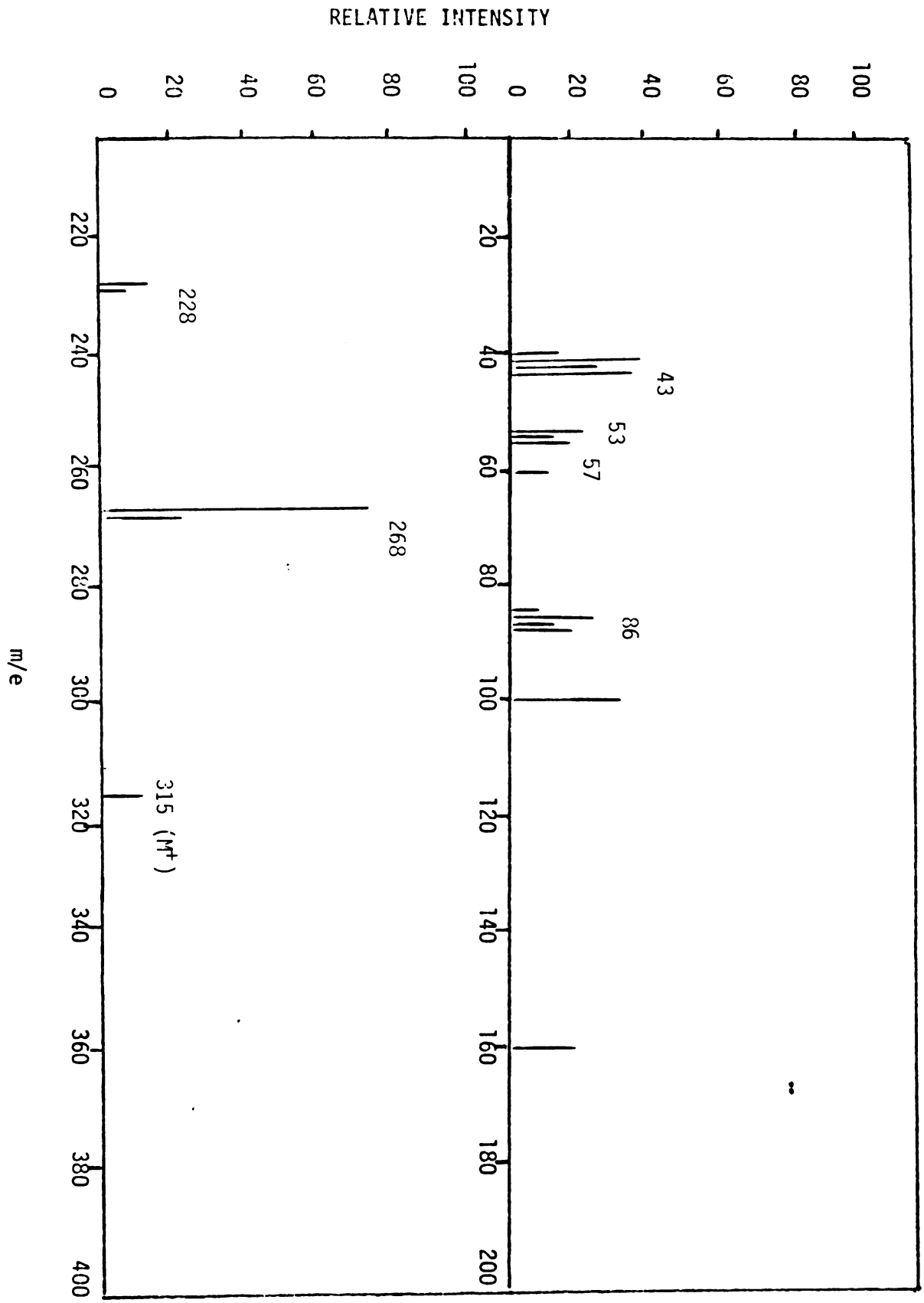
43
55
57
97
129
185
211
229
283 (M+)
m/e

Appendix 10: Mass spectrum of 3-methylthiopropyl myristate a breakdwon product of N-nitroso-3-methylthiopropylmyristamide.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380
400
m/e
43
53
57
86
228
268
315 (M+)

Appendix 11: Composition of purified diet

Ingredient	Amount (g)
D-Dextrose	649
Vitamine Free Casein	200
Corn oil	100
Salt mix	
$CaCO_3$	6.54
$CaHPO_4.2H_2O$	14.7
NaCl	4.3
K_2HPO_4	3.09
$K_3C_6H_5O_7.H_2O$	9.46
$MgCO_3$	1.64
$FeC_6H_5O_7.3H_2O$	0.64
$MnSO_4.H_2O$	0.055
$ZnCO_3$	0.018
$CuSO_4.5H_2O$	0.007
KI	0.0018
Vitamin mix	
Vitamin A as retinyl palmitate	25,000 I.U.
Vitamin D as ergocalciferol	2,000 I.U.
Menadione (Vitamin K)	1 mg
biotin	0.1 mg
Vitamin B_{12}	0.1 mg
Calcium pantothenate	10 mg
folic acid	1 mg
niacin	25 mg
pyridoxine HCl	5.0 mg
riboflavin	10 mg
choline chloride	5 g
selenium	.04 mg