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Yusef Essanusi El-Mabsout

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CATALYSIS OF N-NITROSAMINE FORMATION BY BACTERIA

By

Yusef Essanusi El-Mabsout

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ABSTRACT

CATALYSIS OF N-NITROSAMINE FORMATION BY BACTERIA

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Yusef Essanusi El-Mabsout

This study was initiated to investigate the role of bacteria in N-nitrosamine formation from immediate precursors (dimethylamine and nitrite), and to investigate the possibility of N-nitrosamine formation from several other compounds (secondary precursors) via the action of selected bacteria. Compounds investigated were trimethylamine, trimethylamine oxide, choline, phosphorylcholine, lecithin, sarcosine, N-nitrososarcosine, proline and N-nitrosoproline.

Each of the following bacterial species were tested for their ability to produce N-nitrosamines from the forementioned precursors: E. coli E-2, E. coli K-12, L. bulgaricus, M. thermosphactum, P. putida, P. putida biotype A, P. fluorescens, two P. schuylkilliensis species and S. aureus.

Growing cultures as well as resting cell suspensions of these bacteria were capable of N-nitrosating dimethylamine when incubated in tryptic soy broth (TSB) or 0.2 M phosphate buffer (pH 8.0), respectively, in the presence of 0.25% dimethylamine and 0.05% sodium nitrite. Bacterial catalysis of the N-nitrosation of dimethylamine was shown to increase with increasing cell concentration. Similarly, N-nitrosamine formation increased with increasing concentrations of nitrite

in the incubation medium.

E. coli E-2, E. coli K-12 and S. aureus were able to form appreciable amounts of N-nitrosodimethylamine (NDMA) when sodium nitrate (0.25%) was used in the incubation medium instead of nitrite. Lower quantities of NDMA were produced from the secondary precursors by various bacterial species used in this study.

None of the bacteria were capable of producing N-nitrosopyrrolidine (NPYR) from proline and nitrite. However, M. thermosphactum produced traces of NPYR as a result of decarboxylation of N-nitrosoproline

Both NDMA and NPYR were detected in two fried Gid-deed samples (salted sundried lamb). The first was treated with refined salt and nitrite, and the second was treated with rock salt. The production of N-nitrosamines in the latter sample indicated that bacteria indigenous to lamb are capable of reducing nitrate impurities in the rock salt to nitrite.

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INTRODUCTION

Historically, the study of the chemistry of aliphatic N-nitrosamines began in 1863 when Geuther obtained N-nitroso-diethylamine (NDEA) by the reaction of diethylamine hydrochloride with sodium nitrite (Crosby and Sawyer, 1976). Twelve years later, Fischer (1875) reported that N-nitroso-dimethylamine (NDMA) was formed in high yield when dimethylamine hydrochloride was acidified with sulfuric acid and treated with sodium nitrite in an aqueous medium.

The present interest in N-nitrosamines started in 1956 when Magee and Barnes demonstrated for the first time the carcinogenicity of NDMA. These investigators reported the induction of liver tumors in rats by feeding NDMA.

Extensive research on N-nitroso compounds (N-nitrosamines and N-nitrosamides) has revealed that the majority of these compounds are carcinogenic (Swann, 1975). Furthermore, some are mutagenic, teratogenic and embryopathic when administered to pregnant animals. In addition, research in the last fifteen years has also revealed significant information on the occurrence of N-nitrosamines and their formation in different foods and beverages. Progress has also been made on their analysis and extraction from food systems.

In 1968, Sander was the first to report N-nitrosamine formation by microorganisms. He demonstrated the ability of four strains of nitrate-reducing enterobacteria to produce N-nitrosamines enzymatically from nitrate and secondary aromatic amines.

This study was initiated to further investigate the role of bacteria in the formation of N-nitrosamines from immediate precursors (dimethylamine and nitrite), and to investigate the possibility of N-nitrosamine formation from several other compounds (secondary precursors) via the action of selected bacteria.

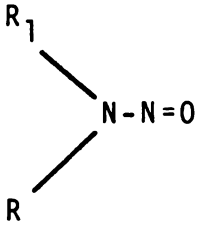
Studies in various laboratories in recent years have shown that a variety of cured meat products contain trace amounts of N-nitrosopyrrolidine (NPYR) and NDMA (Scanlan, 1975; Crosby and Sawyer, 1976; Sen, 1980; Gray, 1981). The preparation of gid-deed (a delicacy food product made from lamb meat in Libya and most Islamic countries in North Africa) involves such steps as dry salting of the meat, sun drying and oil frying. Although nitrates or nitrites are not intentionally added to the product, nitrate may be present as an impurity in the salt and accidentally added in the salting step. Through the action of bacteria, nitrate becomes a source of nitrite which may be available for reaction with the secondary amines present in the meat system. Therefore, one further objective of this study was to investigate the possibility of N-nitrosamine formation

in fried and non-fried gid-deed samples produced under conditions similar to those encountered in the domestic preparation of gid-deed.

LITERATURE REVIEW

N-Nitrosamines are organic compounds that result from the interaction of amines (primary, secondary or tertiary amines) with nitrite. They have been known since the nineteenth century; however, it was not until quite recently that they became the subject of considerable interest and controversy. This renewed interest was generated by the work of Magee and Barnes (1956) who demonstrated the carcinogencity of dimethylnitrosamine (NDMA) when fed to rats. Since then, many workers have investigated the biological effects of N-nitrosamines and the majority of these compounds have been shown to be carcinogenic (Preussmann et al., 1976). Furthermore, some are mutagenic, teratogenic and embryopathic when administered to pregnant animals (Montesano and Bartsch, 1976).

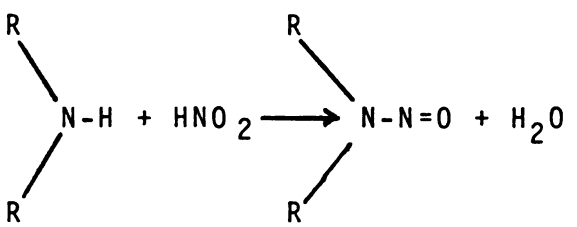
N-Nitrosamines are characteristically yellow, orange-yellow liquids or solids at room temperature (Scanlan, 1975). They have the common chemical formula:



where R is an alkyl group and R^1 is an alkyl, aryl or a wide variety of functional groups (Gray et al., 1979). NDMA, N-nitrosodiethylamine (NDEA) and N-nitrosopyrrolidine (NPYR) are among the classical examples isolated from food systems (Sen, 1972; Fazio et al., 1973; Fong and Chan, 1973; Eisenbrand et al., 1977). NDMA is a symmetrical dialkylnitrosamine while NPYR is a heterocyclic N-nitrosamine.

Precursors of N-Nitrosamines and the Nitrosation Reaction

As stated by Gray et al. (1979), the principal mode of N-nitrosamine formation in foods is the reaction between secondary amines and nitrous acid. When secondary amines are treated with nitrous acid, N-nitrosation proceeds and a



stable N-nitrosamine is formed as shown in the equation above. Quaternary ammonium compounds and tertiary amines can also react with nitrous acid to form N-nitrosamines; however, the yields are much lower compared to secondary amines (Fiddler et al., 1972). When a primary aliphatic amine is reacted with nitrous acid, an unstable N-nitrosamine is formed which tautomerizes to diazohydroxide. Hydrolysis

of this intermediate produces an unstable diazonium ion. The latter loses nitrogen to form a carbonium ion which can undergo additional elimination and rearrangement reactions (Scanlan, 1975). Primary aliphatic amines can also undergo N-nitrosation reactions to produce stable N-nitrosamines (Warthesen et al., 1975). However, the N-nitrosation reaction apparently requires the conversion of the primary amines to secondary amines before the N-nitrosation step.

The N-nitrosation reactions of various secondary amines and amino acids by nitrous acid have been extensively studied by Mirvish (1972, 1975). These studies indicated that the actual N-nitrosating species can be one of the following depending on the N-nitrosation conditions: nitrous anhydride (N_2O_3), nitrous acidium ion (NO^+), nitrosyl halide (NOX) or nitrosyl thiocyanate (NOCNS). The rate of the N-nitrosation reaction is affected by the concentration of nitrite and the secondary amine, the basicity of the amine and the pH of the system (Mirvish, 1970). It is also affected by temperature and the presence or absence of catalysts and inhibitors (Mirvish, 1970; Mirvish, 1972; Foreman and Goodhead, 1975).

In general, the rate of formation of N-nitrosamine from nitrite and secondary amine follows first order kinetics with respect to the amine concentration, and second order kinetics with respect to nitrite concentration, i.e., the rate of reaction doubles with doubling the concentration of the amine and increases four times with doubling the nitrite

concentration (Mirvish, 1970). The rate of formation of N-nitrosamines from secondary amines can be calculated from one of the following equations:

$$\text{Rate} = K \times (\text{unprotonated amine concentration}) (\text{nitrous acid})^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$\text{Rate} = K_1 \times (\text{total amine concentration}) (\text{nitrite})^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

However, it should be pointed out that K in equation (1) is independent of pH, while K_1 in equation (2) is pH dependent. Under standard conditions of temperature and total species concentration, Mirvish (1970) showed that with dimethylamine and nitrite, maximum NDMA formation was obtained at pH 3.4. The percent yield decreased on both sides of this optimum pH. He also reported that over a pH range of 9 to 5, the rate of N-nitrosation increased 10 fold for every unit drop in pH.

The basicity of the amine has also been shown to affect the rate of N-nitrosation reactions. Mirvish (1971) showed that at constant pH around 3, the relative N-nitrosation rate of the total species of amines increased 1.85×10^5 times on preceeding from piperidine (pK_b 2.8) to piperazine (pK_b 8.4) (Table 1). The non-ionized species seemed to have similar reactivities and the rate increased only 4 times for the same amines at pH 3.0. This indicated that the

Table 1. Optimum pH and rate constants for the N-nitrosation of some secondary amines and amino acids.[a]

Amine	pK_a	Optimum pH	Rate Constant
Piperidine	11.2	3.0	0.027
Dimethylamine	10.73	3.4	0.10
Morpholine	8.7	3.0	14.8
Mononitrosopiperazine	6.8	3.0	400.0
Piperazine	5.57	3.0	5000.0
L-Proline	--	2.25	2.9
L-Hydroxyproline	--	2.25	23.0
Sarcosine	--	2.5	13.6

[a]Adapted from Mirvish (1972).

N-nitrosation reaction rate is inversely proportional to the basicity of the amine involved.

The rate of N-nitrosation of amino acids was investigated by Mirvish (1971) who showed that the optimum pH for the reaction is between pH 2.2 and 2.5 (Table 1). Again, only the non-protonated amines in the amino acid molecule were able to undergo N-nitrosation reactions, and sarcosine and hydroxyproline are N-nitrosated much more rapidly than dimethylamine and proline. The complicated ionization of these amino acids has been suggested to be the cause for the observed shift in optimum pH for the N-nitrosation reaction, as well as the reaction rate compared to that of secondary amines (Mirvish, 1972).

The effect of temperature on N-nitrosation reactions has also been studied. As in other chemical reactions, the rate of N-nitrosation is doubled for every 10°C (18°F) rise in temperature (Foreman and Goodhead, 1975).

Prolonged storage conditions have also been shown to influence N-nitrosamine formation even at low temperature. Ender et al. (1967) studied the effect of prolonged storage of N-nitrosamine formation from dimethylamine and nitrite at pH 6.3 and 4°C. The concentration of each reactant was 40 mM. Their data indicated that 1.6 and 133.5 mg/kg of NDMA were formed when the solution were stored for 2 days and 157 days, respectively.

Catalysis and Inhibition of N-Nitrosation Reactions

The presence of weak anions such as bromide, chloride and iodide as well as acetate and thiocyanate in the reaction mixture has been shown to accelerate the N-nitrosation reaction (Boyland et al., 1971; Boyland, 1972; Fan and Tannenbaum, 1973). Keefer and Roller (1973) studied the effect of carbonyl compounds on N-nitrosamine formation. Their results indicated that the N-nitrosation of secondary amines such as dimethyl- and diethylamine and pyrrolidine was actually catalyzed by the presence of formaldehyde and other carbonyl compounds even under alkaline conditions. It was suggested that catalysis occurred as a result of a reaction between the aldehyde and the secondary amine which

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gave rise to an intermediate iminium ion. The latter is directly attacked by the nitrite ion and the resulting complex decomposes to N-nitrosamine and the catalytic aldehyde. However, tertiary and quarternary amines are not affected by such catalysis (Roller and Keefer, 1974).

The role of microorganisms in catalyzing N-nitrosation reactions and N-nitrosamine formation have been studied by many workers (Sander, 1968; Ayanaba and Alexander, 1973; Maduagwu and Bassir, 1979). A detailed discussion of the role of microorganisms of N-nitrosamine formation will be presented elsewhere in this review.

Similarly, the inhibitory effect of certain chemicals when present in the N-nitrosation system has been widely studied. Fiddler et al. (1973) showed that ascorbate and isoascorbate can inhibit NDMA formation in frankfurter cures and in frankfurters. It was also found that the N-nitrosation of morpholine was inhibited by ascorbate (Mirvish et al., 1972) and was completely inhibited when the ratio of ascorbic acid to nitrite was greater than two at pH 4.0 and 25^{o}C (Fan and Tannenbaum, 1973). Other compounds such as α-tocopherol, glutathione, cysteine, propyl gallate and other antioxidants have been shown to inhibit N-nitrosamine formation in foods as well as in vivo (Gray and Dugan, 1975; Mirvish, 1975; Sen et al., 1976). It has been suggested that these N-nitrosation inhibitors perform their function by competing for available nitrite in the reaction

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mixture, thereby reducing the effective nitrite concentration and consequently reducing the reaction rate (Mirvish, 1970; Sen, 1980).

Catalysis of N-Nitrosamine Formation by Microorganisms

Sander (1968) was the first to report N-nitrosamine formation by microorganisms. He demonstrated the ability of four strains of nitrate-reducing enterobacteria to produce N-nitrosamines enzymatically from nitrate and secondary aromatic amines at neutral pH values. Hawksworth and Hill (1971) confirmed the results of Sander (1968) using a number of strains of Escherichia coli isolated from the human intestinal tract. They also showed that some nonnitrate-reducing strains of lactobacilli, group D streptococci, clostridia, bacteroids and bifidobacteria can N-nitrosate secondary amines with nitrite at neutral pH. They have also demonstrated that out of ten strains of E. coli tested, five were able to form N-nitrosamine when incubated aerobically in a nutrient broth medium containing nitrate and the secondary amines, dimethylamine, diethylamine, diphenylamine, piperidine, pyrrolidine and N-methylaniline. They reported that N-nitrosation was actually observed, even when glucose was omitted from the incubation mixture and the pH of the medium did not fall below 6.5. They concluded that the N-nitrosation could not be due to acid catalysis (Hawksworth and Hill, 1971).

Klubes et al. (1972) studied the factors which affect N-nitrosamine formation from secondary amines and nitrates by rat intestinal bacteria. They showed that N-nitrosation of (^{14}C)-dimethylamine by rat intestinal bacteria was suppressed to a certain degree by boiling the bacterial preparation, by omitting glucose, or adding neomycin to the standard incubation system. On the other hand, Klubes et al. (1972) found that N-nitrosation of the secondary amine by bacteria was enhanced by the inclusion of riboflavin in the incubation medium.

Like E. coli, streptococci have been also implicated in N-nitrosamine formation from secondary amines and nitrites. Collins-Thompson et al. (1972) demonstrated that formation of NDMA and NDEA from dimethylamine and diethylamine, respectively, and nitrite by Streptococcus faecalis, S. faecium and S. lactis. However, their results indicated that N-nitrosamine formation by streptococci was non-enzymatic, and the growing and autoclaved cultures or the millipore-filtered culture medium produced equal amounts of N-nitrosamines which were three times greater than the blanks. Their results with E. coli were negative for N-nitrosamine formation. N-Nitrosamine formation by proteus species from dimethylamine and nitrite has also been documented (Thacker and Brooks, 1974). They showed that NDMA was produced by Proteus morganii, P. rettgeri and P. mirabilis but not by three strains of P. vulgaris when grown under the same conditions.

Contrary to the findings of Collins-Thompson et al. (1972) who showed that Staphylococcus aureus was incapable of N-nitrosating dimethylamine, Ishiwata et al. (1976) demonstrated that both S. aureus and S. epidermidis produced appreciable quantities of NDMA when incubated in a culture medium containing brain heart infusion, glucose, dimethylamine-HCl and nitrate. The inclusion of glucose in the medium enhanced the N-nitrosation reaction, but did not change the pH when a short term incubation (2 hr) was used.

Resting cells of E. coli, Cryptococcus terreus, Xanthomonas compestris and Pseudomonas stutzeri have been shown to produce NDMA from nitrite and dimethylamine (Mills and Alexander, 1976). However, only P. stutzeri was capable of forming NDMA during growth.

The acceleratory role of Bacillus species on N-nitrosamine formation was studied by Yang et al. (1977). Their results indicated a 12-49 fold enhancement over the initial rate when B. brevis was incubated with dihexylamine and nitrite at pH 3.5. The increase in the N-nitrosation reaction rate was similar for both boiled and untreated cells.

Molds have also been shown to be active in N-nitrosamine formation. Ayanaba and Alexander (1973) reported that NDMA appeared in cell suspensions of E. coli, Streptococcus epidermidis and in the hyphal mats of Aspergillus oryzae incubated in a solution containing dimethylamine and nitrate.

Yeast, as well, have been shown to catalyze N-nitrosamine formation from immediate precursors. Maduagwa and Bassir (1979) reported that yeast A (a wild yeast isolated from fermenting palm wine) but not Saccharomyces, was able to form N-nitrosamine from dimethylamine and nitrite. The yeast failed to N-nitrosate the secondary amine when nitrite was replaced by nitrate. Similarly, no N-nitrosamine was detected when trimethylamine was used in place of dimethylamine. In another study, Yang et al. (1977) showed that Saccharomyces cerevisia and Saccharomycopsis lipolytica were able to N-nitrosate a group of secondary amines including dimethylamine, diethylamine, dibutylamine, dihexylamine, morpholine and piperidine. The highest rate of N-nitrosation was obtained with dihexylamine when the reaction mixture was adjusted to pH 3.5. They also showed that at a 5 mg/ml cell concentration of S. lipolytica, the rate of N-nitrosation increased 80 fold over the N-nitrosation rate in the blank. Resting cells of C. terreus also N-nitrosated dimethylamine. The growing cell suspension as well as boiled cell suspensions did not enhance N-nitrosamine formation (Mills and Alexander, 1976).

Formation of NDMA from dimethylamine and nitrate by microorganisms used in the baking industry or isolated from raw materials of bakery products was studied by Iubu and Bogovski (1978). They showed that two Saccharomyces cerevisiae strains used in the baking industry, two

Pseudomonas herbicola strains isolated from cereals, one Pseudomonous species isolated from rye, a strain of *Bacillus subtilis* isolated from leaven and another Pseudomonous strain isolated from compressed bakers yeast, all produced NDMA when incubated in a chemically defined medium containing dimethylamine and nitrate.

Role of Microorganisms in N-Nitrosamine Formation

In general, most of the past research involving microbial formation of N-nitrosamines can be classified into three categories:

1. The first deals with the formation of N-nitrosamines from immediate precursors (nitrite and secondary amines).
2. The second relates to organisms capable of either reducing nitrate to nitrite, or metabolizing certain substrates to secondary amines which can react with nitrite to form N-nitrosamines.
3. The last category deals with organisms capable of converting N-nitrosated precursors to N-nitrosamine as their final product.

As far as the first category is concerned, the catalytic role of microorganisms in N-nitrosamine formation has been confirmed beyond any reasonable doubt. However, there are two schools of thought in regard to the catalytic nature. Klubes *et al*. (1972), Ayanaba and Alexander (1973) and

Kunisaki and Hayashi (1979) have concluded that N-nitrosamine formation by microorganisms is enzymatic in nature. They showed that boiled cell suspensions of different organisms were unable to N-nitrosate dimethylamine in the presence of nitrite, while living cells or their extracts did. On the other hand, Collins-Thompson et al. (1972) and Yang et al. (1977) demonstrated that boiled cells were equally active in N-nitrosating secondary amines in the presence of nitrite, and concluded that the N-nitrosation reaction is non-enzymatic. They hypothesized that other cell constituents, but not enzymes, were responsible for N-nitrosation catalysis. Collins-Thompson et al. (1972) suggested that one or more of the metabolic products produced by Streptococci were responsible for catalyzing N-nitrosamine formation, while Yang et al. (1977) proposed that the acceleration of the N-nitrosation reaction in the presence of bacterial cells was caused by the hydrophobic interaction of the precursor amine with cellular constituents, possibly a component of the cell wall, the cytoplasmic membrane or other ultracellular membranous structure. Production of organic acids by microorganisms lowers the pH and favors the chemical N-nitrosation reactions (Sander and Schweinsberg, 1972).

Reduction of nitrates to nitrites by microorganisms is a classical phenomenon in microbiology, and a wide range of microorganisms including certain species of yeasts, molds

and bacteria can convert nitrates to nitrites.

Dimethylamine has been shown to be produced by several bacterial species when incubated with trimethylamine. Ayanaba et al. (1973) reported that microorganisms in sewage converted trimethylamine to dimethylamine with subsequent formation of NDMA when the culture medium was amended with trimethylamine and nitrite. Concurrently, Ayanaba and Alexander (1973) stated that the mold, Mortierella parvispora and an unidentified bacterium converted trimethylamine to dimethylamine, and only the bacterium produced NDMA in the presence of nitrite. Serratia, a soil bacteria, was also shown to be active in the conversion of trimethylamine to dimethylamine, and ultimately used in N-nitrosamine formation in the presence of nitrite (Maduagwu and Bassir, 1979).

Amino acids and N-nitrosamino acids have been shown to be decarboxylated by bacteria producing secondary amines and N-nitrosamines. Kawabata and Miyakoshi (1976) showed that out of 30 organisms tested, 7 were able to decarboxylate L-proline to pyrrolidine. These included two strains of E. coli, Bacillus cereus var. mycoides ATCC 1178, B. megaterium IAM 1166, Bacillus circulans, S. aureus and Flavobacterium xerosis IFO 3752. However, only two Pseudomonas species, Ps. fluorescens AHU 1143 and Ps. schulkilliensis were able to decarboxylate N-nitrosoproline to NPYR giving yields of 0.3 to 0.02% respectively. They also stated that

no close relationship was observed in the distribution pattern of L-proline and N-nitrosoproline decarboxylases among the test organisms, i.e., none of the organisms that decarboxylated L-proline were able to decarboxylate N-nitrosoproline and vice versa.

N-Nitrosamine Degradation by Microorganisms

Very few reports have been published regarding N-nitrosamine degradation by microorganisms. Klubes et al. (1972) stated that preformed (^{14}C) NDMA incubated with cecal bacteria of the rat under standard conditions for 20 hours appears to be stable and was recoverable in 95% yield from incubation mixtures. In a similar study, Rowland and Grasso (1975) reported that a major proportion of bacterial types commonly found in the gastro-intestinal tract of many animals and man were active in degrading N-nitrosodiphenylamine and NDMA, the former being degraded more rapidly than the latter. In this study, approximately 55% of added N-nitrosodiphenylamine, 30% of NPYR, and 4% of NDMA were degraded when the concentrations of the N-nitrosamines were less than 0.05 μmol/ml at pH 7.0 per 20 hour incubation. E. coli, Bacteroides, Bifidobacterium, Lactobacillus and S. faecalis were among the organisms tested, along with several potential pathogens; Klebsiella aerogenes, P. aeruginosa and Proteus species all were found to be active in N-nitrosamine degradation. Recently, Harada (1980) studied the effect of the

conditions of growth and enzymic reaction on the decomposition of N-nitrosamines by Rhizopus oryzae. He showed that a pH of 8.0 and a concentration of 0.03 - 0.12 mmole of N-nitrosamine at 20°C were optimal for NDMA degradation. However, N-nitrosodiphenylamine breakdown was optimal at 30°C and 0.1 mmole concentration. The cell-free enzyme system was also active in degrading N-nitrosamines. The degradation activities were highest at pH 8.0 and 30°C and at N-nitrosamine concentrations greater than 0.1 mmole.

Occurrence of N-Nitrosamines in Foods

As stated by Crosby and Sawyer (1976), the first indication of the presence of N-nitrosamines in the environment came from studies of a disease of mink in Norway which was characterized by extensive liver damage. In 1957, the injurious effects were first observed and the toxic factor was later isolated and identified as NDMA (Ender et al., 1964). The carrier was herring meal which has been processed with high levels of nitrite and fed to the animals. Since then, several N-nitrosamines have been shown to occur in a wide variety of food products and alcoholic beverages. However, NDMA and NPYR are, by far, the most frequently detected N-nitrosamines in food products.

N-Nitrosamines in Cured Meats. As far as the occurrence of N-nitrosamines in foods is concerned, cured meat products are the products of major significance (Gray, 1981). Cured meats such as bacon (cooked or fried) and to a much lesser

extent, ham and certain cured sausage products including dry sausages, uncooked salami and frankfurters, are the major contributors of N-nitrosamines in the American diet (Sen, 1972; Fazio et al., 1973; Gray et al., 1977; Sen, 1980). A summary of reports on the occurrence of N-nitrosamines in cured meat products is presented in Table 2.

Among all cured meat products tested, cooked bacon has consistently been shown to contain relatively high levels of N-nitrosamines, mainly NPYR and traces of NDMA (Sen, 1980). Since NPYR has not been detected in raw bacon, NPYR is believed to be formed during the high cooking temperatures utilized in the frying process (Sen, 1980; Gray, 1981).

Although several compounds have been suggested as possible precursors for NPYR in cooked bacon (putrescine, collagen, spermidine, pyrrolidine), free proline which is present in concentrations of approximately 20 mg/kg in pork belly, is believed to be the major precursor (Gray, 1976; Lakritz et al., 1976; Gray and Collins, 1977; Bharucha et al., 1979). Model system studies have indicated that N-nitrosation of proline followed by decarboxylation is the major pathway by which NPYR is formed in cooked bacon as opposed to decarboxylation of proline to pyrrolidine and subsequent N-nitrosation to NPYR (Bharucha et al., 1979; Lee, 1981).

The level of NPYR in the cooked product depends upon well documented factors (Gray, 1981). These include nitrite

Table 2. N-Nitrosamines detected in various cured meat products

Meat product	N-nitrosamine	(μg/kg)	Investigator
Fried bacon Cooked-out fat	NPYR	trace-40	Crosby et al. (1972)
Fried bacon Cooked-out fat	NPYR	4-25	Sen et al. (1972)
Fried bacon Cooked-out fat	NDMA, NPYR	trace 44	Sen et al. (1973)
Fried bacon Cooked-out fat	NPYR	4-100	Havery et al. (1973)
Fried bacon Cooked-out fat	NPYR	trace-41	Gray et al. (1977)
Fried bacon Cooked-out fat	NPYR, NDMA	2-34	Sen et al. (1979)
Fried bacon Cooked-out fat	NPYR	2-34	Pensabene et al. (1980)
Frankfurters	NDMA	11-84	Wasserman et al. (1972)
Dry sausage uncooked salami	NDMA	20-80	Sen (1972)
Smoked horse meat	NDMA, NDEA	trace-25	Groenen et al. (1976)

concentration, frying temperature and time, and the presence or absence of N-nitrosamine inhibitors such as ascorbates and lipophilic inhibitors.

N-Nitrosamines in Fish and Fish Products. Fish, fish meal and other seafoods have also been shown to contain appreciable amounts of NDMA, and to a lesser extent NPYR and NDEA (Table 3). As an example, Fong and Chan (1973)

Table 3. N-Nitrosamines reported in fish and other seafoods.

Fish Product	N-nitrosamine (μg/kg)	Investigator
Herring meal (nitrite treated)	NDMA (15,000-1000,000)	Ender et al. (1964) Sakshaug et al. (1965)
Smoked fish	NDMA, trace-40	Ender and Ceh (1967)
Smoked fish, nitrite or nitrate treated	NDMA, 4-26	Fazio et al. (1971)
Salted and dried fish	NDMA, 10-1000	Fong and Chan (1973)
Various Chinese seafoods	NDMA, NPYR, trace-37	Fong and Chan (1977)

have detected fairly high levels (10-1000 μg/kg) of NDMA in salted and dried marine fish. The presence of nitrate (as an impurity) in crude salt appears to be instrumental in N-nitrosamine formation. When crude salt was replaced by refined salt, and benzoic acid was added to control

microbial growth, apparently only small amounts of N-nitrosamine were detected (Fong and Chan, 1976). Herring meal, treated with nitrite has been found to contain as high as 100 mg/kg of NDMA (Sakshaug et al., 1965). Significantly lower levels (450 µg/kg) were detected in untreated fish meal (Sen et al., 1972).

The presence of NDMA in salted and/or cured fish products is not unexpected since dimethylamine, trimethylamine and trimethylamine oxide have been reported to be present at fairly high concentrations in fish and fish products (Shewan, 1951; Kawamura et al., 1971; Singer and Lijinsky, 1974).

N-Nitrosamines in Dairy Products. Trace amounts to several parts per billion of NDMA, NDEA, N-nitrosopiperidine (NPIP) and NPYR have been detected in a variety of cheeses including Cheddar, Gouda, Edam, Camembert and Havarti cheese (Crosby et al., 1972; Eisenbrand et al., 1977; Sen et al., 1977). Table 4 lists some of the dairy products from which N-nitrosamines have been isolated.

N-Nitrosamines in Other Food Products. NDMA has been shown to be present in a variety of alcoholic beverages including beer (Spiegelhalder et al., 1979; Havery et al., 1981), palm wine (Bassir and Maduagwu, 1978) and Scotch whiskey (Goff and Fine, 1979). NDMA also has been detected in edible oils and margarine. Levels of 0.22-1.01 µg/kg were reported in several commercial oils including corn,

Table 4. N-Nitrosamines (μg/kg) detected in selected dairy products

Dairy Product	N-Nitrosamine and level present	Investigator
Cheese		
Havarti and Gouda	NDPA[a], NDIPA[b], 5-10	Kroeller (1967)
Cheshire	NDEA, NPYR, 1	Alliston et al. (1972)
Gouda, Havarti, Camembert, Cheddar	NDMA, NDEA, NPIP, trace-19	Sen et al. (1977)
Wine cheese	NDMA, NDEA, 7-68 NPIP	Sen et al. (1977)
Yogurt	Not detected	Gough et al. (1977)
Dried buttermilk	NDMA 0.9-1.8	Libbey et al. (1980)
Nonfat dried milk	NDMA trace-4.5	Libbey et al. (1980)

[a]NDPA, N-nitrosodipropylamine

[b]NDIPA, Nitrosodiisopropylamine

olive, sunflower and soybean oils and margarine (Fiddler et al., 1981). However, higher levels (up to 23 and 28 µg/kg for NDMA and NDEA, respectively) had been reported earlier by Hedler et al. (1979). Commercial curing premixes containing nitrates, nitrites, and spices such as black pepper, paprika and salt have also been shown to contain excessively high levels of N-nitrosamines (850-25,000 µg/kg). Sen et al. (1973) found that when nitrite was mixed with paprika, NPYR was formed, whereas nitrite plus black pepper resulted in NPIP formation. Similar results were reported later by Gough and Goodhead (1975).

Precursors and N-Nitrosamine Formation in Foods

In foods, N-nitrosamines are formed principally by the same chemical or biochemical reactions discussed earlier, where naturally occurring amines react with nitrites added to the food system or produced by bacterial reduction of naturally occurring nitrates (Gray, 1981). The N-nitrosation reactions are also affected by the same factors: pH, concentration of nitrite and secondary amine, basicity of the amine being N-nitrosated and the presence or absence of inhibitors and catalysts.

Amines in Foods.

a) Fish and fish products. Historically, amines have been associated with fish products (Maga, 1978). Table 5 summarizes some of the amines reported to be present

Table 5. Some of the amine compounds reported to be present in fish and fish products.

Fish product	Amine	Level (mg/kg)	Investigators
Raw sardine	Dimethylamine	5.9	Kawamura et al. (1971)
Roasted sardine	"	48.6	"
Oiled sardine	"	179.6	"
Raw pollack roes	"	116.6	"
Roasted pollack roes	"	205.7	"
Flaked tuna	"	12.6	"
Oiled tuna	"	13.1	"
Canned tuna	"	23.2	Singer and Lijinsky(1976)
Perch	"	180	"
Bass	"	110	"
Cod	"	738	"
Trout	"	7	"
Raw crab	"	0.3	Kawamura et al. (1971)
Raw shrimp	"	4.0	"
Raw oyster	"	0.1	"
Fish protein concentrate	"	151.0	Miller et al. (1973)
Fish	Trimethylamine	1400	"
Fish protein	"	5.1	"
Trout	Dipropylamine	0.4	Singer and Lijinsky (1976)

in some fish products.

Trimethylamine, trimethylamine oxide and dimethylamine are found at fairly high concentrations in various fish, particularly those of marine origin. Concentrations as high as 100-185 mg/100 g of fish have been reported (Shewan, 1951). Several other amines such as methylamine, propylamine, butylamine and pentylamine along with tyramine, putrescine and cadaverine have been reported to be present in some fish products (Basco and Barrera, 1972; Cantoni et al., 1974; Golovnya et al., 1967; Minakowski and Mathias, 1967).

As stated by Koprowski (1968), Dyer (1952) cited three sources of trimethylamine and/or trimethylamine oxide in fish. First, it may be present in the food supply. It has been reported that marine algae and plants as well as zooplankton contain more of these amines than do the comparable fresh water species (Tarr, 1941; Groninger, 1959). Secondly, trimethylamine and trimethylamine oxide can be formed by bacterial degradation of choline and choline-containing substances such as phosphorylcholine and lecithin (Dyer and Wood, 1947; Bilinski, 1962). Aerobacter aerogenes, P. rettgeri, P. ichthyosmins, P. mirabilis and Schigella alkalescens have been shown to be capable of decomposing choline to trimethylamine (Dyer and Wood, 1947). The third mechanism which has been suggested was de novo synthesis of trimethylamine from ammonia through a series of methylations on the ammonia molecule to form methylamine, dimethylamine

and then trimethylamine (Dyer, 1952). Trimethylamine can be oxidized in fish liver to trimethylamine oxide as proposed by the same investigator. Trimethylamine oxide is present at higher concentrations in marine fish than in fresh-water fishes because it is necessary for osmotic pressure regulation in marine fish, while it is not necessary for that purpose in fresh-water fish and is simply excreted in the urine (Dyer, 1952). Trimethylamine levels have been used to judge the freshness of fish, as influenced by storage. It has been reported that good quality sable fish contains 147 mg trimethylamine/kg of fish whereas the level in spoiled sable fish was in excess of 1100 mg/kg (Gruger, 1972).

b) Meats and meat products. As has been mentioned previously amines are naturally present in meats and meat products. Dimethylamine, diethylamine and dipropylamine, as well as spermine, spermidine and tyramine, have been identified in a variety of meat products (Spinelli et al., 1974; Lakritz et al., 1975; Singer and Lijinsky, 1976). Gray and Collins (1977) determined the free proline concentration, a possible precursor of NPYR (Bills et al., 1973; Gray and Dugan, 1975) in five green pork bellies stored at $2^{o}C$ for 1, 8, 15 and 28 days. Their results indicated an average of 11.8 µM free proline/100 g of whole tissue sample (lean and adipose). Storage of samples at $2^{o}C$ for 28 days resulted in an approximate three-fold increase in proline content in both whole belly and lean tissue and a four-fold increase

in the adipose tissue. Similarly, 18.3 to 31.6 μmoles of free proline were found to be present in 100 g tissue for five commercial bacon samples (Gray et al., 1977). The effect of heating and putrefaction on pork amine levels have been studied by Lakritz et al. (1975). They reported that putrefaction dramatically increased the levels of spermine, spermidine and putrescine in pork, while cooking reduced them slightly. Similar trends were observed with the amines, cadaverine, histamine, tryptamine and tyramine. Lecithin, phosphorylcholine and choline (amine containing compounds), which have been proposed as possible precursors for NDMA (Pensabene et al., 1975; Gray et al., 1978), occur naturally in meats. The lecithin content of fresh hog belly was quantitated by Kuchmak and Dugan (1963) who reported levels of 0.34 g per 100 g of fresh hog belly. Sarcosine, which can be N-nitrosated much more rapidly than dimethylamine, occurs widely in nature as a result of protein degradation. Degradation of proteins under anaerobic conditions by microorganisms may significantly increase the free amine content of histamine, tyramine, piperidine, putrescine and cadaverine (Frazier, 1967).

c) Dairy products. Several secondary amines, dimethylamine, diethylamine, dipropylamine, dibutylamine, pyrrolidine and methylbutylamine have been isolated and identified in a number of dairy products including evaporated whole milk, low fat yogurt and a wide variety of cheeses

Golovnya and Mironov, 1968; Ney and Wirotama, 1971; Singer and Lijinsky, 1976). Trimethylamine, triethylamine and tripropylamine have been reported to be present in Russian cheese (Golovnya and Mironov, 1968; Golovnya and Zhuravleva, 1970). De Vuayst et al. (1976) analyzed several cheeses for histamine, tyramine, putrescine and cadaverine and reported that blue cheese had the highest total amine content, and the highest histamine level (4.093 mg/g cheese). However, cheddar contained more of the other amines than all the other cheeses analyzed.

d) Amines in other foods and alcoholic and non-alcoholic beverages. As reviewed by Maga (1978), primarily volatile amines are present in beer. Dimethylamine, pyrrolidine and dimethylbutylamine have been reported to be present at relatively low concentrations (0.65 mg/kg, trace, 0.16 mg/kg, respectively), with ethylamine being present in the highest concentration among the volatiles (1.04 mg/kg (Palmand et al., 1971; Slaughter et al., 1971; Singer and Lijinsky, 1976).

Dimethylamine and trimethylamine were also reported to be present in cocoa and tea leaves, and in coffee along with other polyamines (Serekov and Proiser, 1960; Amorim et al., 1977; Marion et al., 1967). Various spices, such as paprika and black pepper, contain high levels of amines including pyrrolidine and piperidine (Gough and Goodhead, 1975).

e) Biologically active amines in foods. Biologically active amines are normal constituents of many foods, but they usually do not represent any hazard to individuals unless large amounts are ingested, or the natural mechanism for one or more of the amines is inhibited or genetically deficient (Rice et al., 1976). They are defined as aliphatic, alicyclic or heterocyclic organic bases of low molecular weight which are metabolically produced in plants, animals and microorganisms (Franzen and Eyesell, 1969). These compounds include phenethylamine, dopamine, norepinephrine and tyramine (vasoactive pressor amines which increase blood pressure when ingested) and serotonin and histamine, strongly vasoactive amines. Histamine is a strong capillary dilator and can produce hypotensive effects i.e., reduce blood pressure (Goodman and Gilman, 1965).

Biologically active amines have been detected in many foods including cheese, fish products, meat extract, sauerkraut, wines and yeast extract (Blackwell and Mabbitt, 1969; Sen, 1969; Ienisten, 1973; Mayer and Pause, 1972). As an example, tuna fish has been reported to contain as high as 5000 µg histamine/g while the histamine content of cheese varies from 0 to 2600 µg/g (Ienisten, 1973; Voigt et al., 1974), depending on the type of cheese and on how long it is aged.

Generally speaking, fresh foods contain small amounts of tyramine and histamine; however, fermentation and/or

spoilage of these products via microbial degradation of amino acids increase their amine content (Rice et al., 1976). It has been reported that several strains of S. faecalis along with C. sporogenes, C. aerofoetidum, E. coli, P. mirabilis and Ps. reptilivora possess tyrosine decarboxylase activity and can product tyramine from tyrosine (Gale, 1940; Mossell, 1968). Histidine decarboxylase activity have also been detected in four Lactobacillus species including Lactobacillus 30a (Rodwell, 1953).

Nitrates and Nitrites in Foods

Nitrates are natural components of the environment and constitute the primary source of fixed nitrogen for green plants (Gray and Randall, 1979). They occur in relatively high concentrations in vegetables such as lettuce, cabbage, cauliflower, spinach, celery, carrots, beets and radishes. Sometimes values as high as 1000-3000 mg/kg have been reported (White, 1975). Nitrates also occur in drinking water, and when their concentration exceeds 20 mg/kg, water becomes the major source of nitrate dietary intake (Hawksworth and Hill, 1971). Nitrates also enter our foods as impurities in crude salt and as intentional food additives in some meat curing premixes. Nitrates were added along with nitrites in cure preparations for ham, bacon, frankfurters, cured fish and corned beef. They were added to act as a reserve for nitrites. Nitrates are also added to cheese milk in some European countries to inhibit the growth of Clostridium

tyrobutyricum spores which may survive the normal pasteurization process (Goodhead et al., 1976). It was estimated that out of the 99.3 mg average daily consumption of nitrates in the U.S., only 9.4% comes from cured meat, and a large proportion, 86.3%, comes from vegetables (White, 1976).

Nitrites are present at lower concentrations in our diet than are nitrates. However, the latter can readily be reduced to nitrite by a variety of microorganisms, such as E. coli, various salmonella and staphylcocci (Bolotou et al., 1972) or in vivo in the human body (Tannenbaum et al., 1976). Nitrites are added to meats as curing ingredients to inhibit the outgrowth of Clostridium botulinum spores. These organisms produce a very powerful neurotoxin, which may cause death if ingested even at minute quantities (Frazier, 1967; Sofos et al., 1979). Nitrites are also added in the curing of meat products to inhibit oxidative rancidity and warmed over flavor (WOF), to fix the desirable pink color, to modify the flavor and improve the texture of cured meat products (Lechowich et al., 1978; Sofos et al., 1979). It was originally thought that cured meats accounted for approximately 21.2% of the estimated average daily ingestion for U.S. residents (White, 1976). Other sources included fruits, vegetables, bread and saliva. The latter accounted for 76.8% of the average daily ingestion for U.S. residents. More recent estimates, however, indicate that only 2% of the human exposure to nitrite in the United States is a consequence of

consumption of meats cured with nitrite (CAST, 1978). The remaining 98% of the exposure is due to other sources, which appear to be almost exclusively dietary nitrogenous substances other than nitrite that undergo transformation in the digestive tract with the production of some nitrite (Tannenbaum et al., 1974; Tannenbaum et al., 1978).

In Vivo N-Nitrosamine Formation

In view of the conditions under which N-nitrosamines are formed, and the fact that N-nitrosation reactions proceed at a faster rate at relatively low pH values close to those of the human stomach, researchers have become interested in examining the possibility that trace quantities of N-nitroso compounds may be formed under the acidic conditions of the human stomach. Studies on concurrent administration of nitrite and various secondary amines to experimental animals have shown that N-nitrosamine formation can indeed occur in the gastro-intestinal tract. Hashimoto et al. (1976) detected NDMA in the contents of the stomach, intestine and cecum of rats fed a diet supplemented with 0.1% dimethylamine and 0.4% potassium nitrate using 5 strains of NDMA forming bacteria. NDMA was not detected in the control group (their diet did not contain bacteria). As reviewed by Sen (1980), esophageal and liver tumors developed in experimental rats when their diets included nitrites and secondary amines (Sander and Burkle, 1969). NPYR was also detected

in the feces of two out of three rats tested when they were fed diets containing N-nitrosoproline (Kawabata and Miyakoshi, 1976). Formation of N-nitrosamines from secondary amines and nitrite *in vitro* has also been studied by several workers. Sen *et al*. (1969) demonstrated the formation of NDEA when diethylamine and nitrite were incubated with gastric juices from rats, rabbits, cats, dogs and man. They also showed that human and rabbit gastric juices (pH 1-2) produced more NDEA than did rat gastric juice (pH 4-5). *In vivo* N-nitrosation of diethylamine was demonstrated in cats and rabbits. Lane and Bailey (1973) studied the effect of pH on NDMA formation *in vitro* in human gastric juice. Their results indicated that over a pH range of 1.7-4.5, N-nitrosation of dimethylamine appeared to be optimal at pH 2.5 although NDMA was formed over the entire pH range tested. N-Nitrosamines were also detected in non-normal human gastric contents. Lakritz *et al*. (1978) analyzed the stomach contents (gastric) of fasting patients, hospitalized for a variety of conditions and undergoing routine clinical gastric examinations. Their preliminary data indicated the presence of 5-30 μg NDEA per kilogram in 4 samples, 2 μg NDMA in two samples tested. Dimethylamine, trimethylamine, histamine, cadaverine, putrescine, ethanolamine and tryptamine were detected (qualitatively) in a pooled sample (Lakritz et al., 1978).

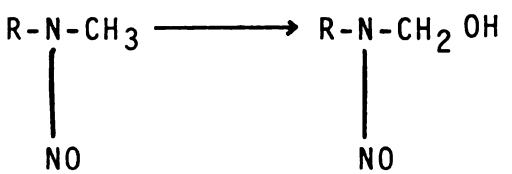
Carcinogenicity of N-Nitrosamines

Among carcinogens, N-nitrosamines are the most broadly acting and among the most potent. Over 100 N-nitroso compounds have been tested and the vast majority have been found to be carcinogenic (Preussman et al., 1976; Lijinsky, 1977; Lijinsky, 1979). Thus far, there is no direct evidence that N-nitrosamines are carcinogenic to man (Gray and Randall, 1979). However, several animal species including sub human primates have been tested and none of these species were able to resist the carcinogenic effect of N-nitrosamines (Sen, 1980). Therefore, it is generally assumed that man is not the exception and these compounds are considered potential hazards to humans unless proven otherwise.

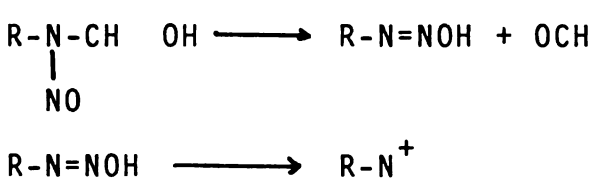
According to their mode of action as carcinogens, N-nitroso compounds can be divided into two classes; N-nitrosamides, which act directly, and N-nitrosamines which require enzymatic activation before they exert their carcinogenic effect (Magee, 1971; Lyle et al., 1979; Michejda et al., 1979). N-Nitrosamines are considered to be among the most versatile carcinogens known to man. They can induce tumors in practically all important organs including liver, kidney, lungs, brain and nervous system, esophagus, stomach, urinary bladder and blood generating organs (Griciute, 1978). Target specificity is governed by the chemical structure of the carcinogenic compound,

dosage, mode of administration and duration of exposure. It also depends on animal species and strain (Crosby and Sawyer, 1976; Griciute, 1978). The specificity of certain N-nitrosamines in their ability to attack a specific tissue is explained by the assumption that certain tissues are more capable in metabolizing a specific N-nitrosamine than others. Therefore, liver cancer is more frequent in the animal species tested, because liver is the primary site in which xenobiotics are metabolized (detoxicated or activated).

The series of events which take place after N-nitrosamines are ingested and which lead to cancer development are unknown. However, many of the mechanisms which have been proposed suggest that methyl alkyl nitrosamines are enzymatically oxidized and/or hydroxylated on the methyl group to give α-hydroxymethyl N-nitrosamine (Baldwin et al., 1976; Mechejda et al., 1979). This α-hydroxynitrosamine can lose an aldehyde and form the primary alkyl N-nitrosamine



which rapidly rearranges and forms an alkyl diazonium ion



which has the ability to alkylate nucleic acids. β-Hydroxylation reactions have also been postulated as a possible mechanism for N-nitrosamines with side chains larger than ethyl. Degradation of these compounds via β-hydroxylation, as suggested, will produce active methylating agents (Kruger, 1973). Whatever the case might be, it has been suggested that the methylating agent alkylates the third position of cytosine or the seventh position of guanine in the DNA and/or the RNA molecule (nucleic acids) in the target tissue and interferes with their normal functions (Shank, 1975). This also explains both the mutagenic and teratogenic activity of these N-nitroso compounds when tested in bacteria and pregnant animals, respectively (Tomatis et al., 1975; Baldwin et al., 1976; Montesano and Bartsch, 1976).

The quantitative aspects of exposure in N-nitrosamine carcinogenesis have been studied by several investigators. Magee and Barnes (1956) demonstrated a high incidence of malignant liver tumors in rats fed a diet containing 50 mg/kg NDMA. Kidney tumors were observed when higher doses were used. Preussmann et al. (1977) studied the dose response relationship of NPYR in rats and they reported that a statisticaly significant increase in liver cancer occurred when rats were fed daily (in water) 10, 3, 1 mg/kg of body weight. A daily dose of 0.3 mg/kg did not increase significantly the rate of tumor development compared to untreated controls.

A similar study with NDMA has shown that all doses tested (2, 5, 10, 20, 50 mg/kg) in the diet of rats caused a significant number of liver tumors, except the lowest dose, which resulted in liver tumors only in one rat. However, no safe dose was established since the untreated controls did not develop liver tumors (Terracine et al., 1967).

Gid-deed Preparation

Gid-deed, a delicacy food product, is prepared traditionally almost in every household in Libya, especially on Aid Al-akbar (a religious day which coincides with the day Ibrahim, the prophet, was asked to sacrifice his son). Animals, preferably yearlings are slaughtered, skinned and eviscerated. Part of the meat is used on the same or the next day and the remaining meat is deboned, heavily salted by rubbing dry salt on meat slices. The salted meat is sun-dried for 2-3 days, depending on the season. The sun-dried product is either stored as is in glazed clay containers at room temperature, or fried in olive oil and stored for use as a delicacy, and used when fresh meat is not available. Originally, the method may have been developed to preserve the meat for later consumption. Today, refrigerators are available for the majority of households but gid-deed is still used in the same manner.

Nitrates or nitrites are not intentionally added to the product; however, nitrate may be present as an impurity in the salt and accidentally added in the salting step. This is supported by personal observations of some gid-deed samples which have a bright red color when cooked or after oil frying. No data are available regarding the nitrate content of local salt or N-nitrosamine formation in the final product.

EXPERIMENTAL

Materials

Sarcosine, NDMA and NPYR were obtained from Eastman Organic Chemical Division, Eastman Kodak Co. (Rochester, NY), trimethylamine hydrochloride (TMA-HCl) and trimethylamine-N-oxide dihydrate (TMA-N-O. $2H_2O$) from Aldrich Chemical Company, Inc. (Milwaukee, Wis.), dimethylamine hydrochloride and phosphatidylcholine from Fisher Scientific Company Chemical Manufacturing Division (Fair Lawn, NJ), and choline chloride and phosphorylcholine chloride from Sigma Chemical Company (St. Louis, MO). The microbiological media, including tryptic soy broth (TSB) and brain heart infusion (BHI), were purchased in a dehydrated form from Difco Laboratories (Detroit, Michigan). Other chemicals used were reagent grade and were obtained from commercial suppliers.

N-Nitrosoproline and N-nitrososarcosine were synthesized according to the method of Hansen et al. (1974). L-proline (hydroxyproline-free) or sarcosine, obtained from Nitritional Biochemicals Company (Cleveland, Ohio), was dissolved in deionized distilled water. A ten fold excess of sodium nitrite was added and the pH was adjusted to 3.0 with

hydrochloric acid. The N-nitrosation reaction was allowed to proceed in the dark, with stirring, for 18 hours at room temperature. The pH was readjusted to 1.0 and the reaction mixture was freeze dired. N-Nitrosoproline was extracted with methylene chloride. The extract was dried over anhydrous sodium sulfate, filtered, and the methylene chloride evaporated in a rotary evaporator at 40°C. Both N-nitrosoproline and N-nitrososarcosine were stored in dark bottles at -20°C until required.

Methods

Bacteriology

A total of 10 strains of various bacteria consisting of E. coli E-2, E. coli K-12, S. aureus, Microbacterium thermosphactum, Lactobacillus bulgaricus, Pseudomonas fluorescens ATCC 13430, Pseudomonas putida biotype A ATCC 15070, Pseudomonas putida ATCC 27212 and two strains of Pseudomonas schuylkilliensis ATCC 15916 and ATCC 15917 were examined for their ability to N-nitrosate dimethylamine, to decarboxylate N-nitrosoproline (to NPYR) and to produce NDMA from various precursors in the presence of nitrite. All the organisms were grown in a nutrient broth medium (NB) at the optimum temperature for each organism (see later) in screw capped test tubes for 24 hours and then stored under refrigeration at 4°C. Fresh transfers were made in the same medium every 4 weeks.

N-Nitrosation of Dimethylamine with Nitrite. Fresh cultures (1 ml), as prepared above, were inoculated into 200 ml of TSB medium containing 0.05% (w/v) $NaNO_2$ and 0.25% (w/v) DMA-HCl (0.1 g and 0.5 g, respectively) in 500 ml Erlenmeyer flasks. Flasks were incubated for 48 hours at the optimum temperatures (37°C for E. coli, L. bulgaricus and S. aureus, and 25°C for the remaining cultures) in a gyratory New Brunswick controlled-environment incubator (Model G-25) set at a speed of 200 rpm.

N-Nitrosation of Dimethylamine with Nitrate. Fresh cultures were inoculated into 200 ml of TSB medium containing 0.25% (w/v) DMA-HCl and 0.25% (w/v) $NaNO_3$ in 500 ml Erlenmeyer flasks. The samples were incubated as above.

Decarboxylation of N-nitrosoproline and N-nitrososarcosine. The N-nitrosoproline and N-nitrososarcosine decarboxylase reactions of test organisms were carried out according to Kawabata and Miyakoshi (1976) with minor modifications. Test organisms were inoculated in 100 ml of a medium containing 0.2% (w/v) N-nitrosoproline or N-nitrososarcosine and incubated at their optimum growth temperature with shaking and aeration in a gyratory NBS controlled-environment incubator (Model G-25) at 200 rpm for 48 hours. The resulting NPYR and NDMA (from the decarboxylation of N-nitrosoproline and N-nitrososarcosine respectively) were extracted with methylene chloride and analyzed with a combined gas chromatograph-thermal energy

analyzer (GC-TEA) system.

NDMA Formation from Other Precursors. The possibility of N-nitrosamine formation via microbial degradation of secondary precursors of NDMA was investigated using the following precursors: trimethylamine hydrochloride (TMA-HCl), trimethylamine oxide-dihydrate (TMA-O: $2H_2O$), choline chloride, phosphorylcholine chloride, phosphatidylcholine (PC) and sarcosine.

For each precursor, ten 500 ml Erlenmeyer flasks containing 200 ml of TSB medium and 0.25% (w/v) of the test precursor were prepared. Each flask was inoculated with one of the test cultures (48 hour culture, 1 ml/200 ml medium).

The flasks were incubated at the appropriate temperature with shaking on a NBS controlled environment incubator for 48 hours, extracted and prepared for N-nitrosamine analysis using the GC-TEA system.

Effect of Incubation Time and Temperature on NDMA Formation by Growing Bacterial Cultures. Nine 500 ml Erlenmeyer flasks containing 200 ml of TSB media and 0.25% (w/v) dimethylamine-HCl and 0.05% (w/v) nitrite were inoculated each with 1 ml of P. schuylkilliensis. The flasks were incubated in the dark at different temperatures as specified below:

Group 1	(3 flasks)	at $5^{o}C$
Group 2	(3 flasks)	at $25^{o}C$
Group 3	(3 flasks)	at $32^{o}C$

One flask from each group was extracted with methylene chloride (as previously described) every 4 days. The extract was concentrated and injected into the GC-TEA system for NDMA analysis.

Resting Cells Studies

a) Preparation of resting cells. Cultures were inoculated in 500 ml of the TSB medium in 100 ml Erlenmeyer flasks. The flasks were incubated for 48 hours at the appropriate temperature with shaking. The cells were harvested with centrifugation in a Sorval II refrigerated centrifuge at 6000 rpm for 20 minutes at 2°C. The cell pellet was resuspended in sterile deionized distilled water and recentrifuged. The clean cells were suspended in phosphate buffer (0.2 M, pH 8.0) and stored under refrigeration (4°C) until required.

b) Effect of cell concentration on NDMA formation. Four 50 ml suspensions of E. coli in phosphate buffer (0.2 M, pH 8.0) were prepared in screw capped milk dilution bottles. The cell concentration was determined as mg protein per ml and was adjusted to 7, 5.6, 4.2 and 2.8 mg protein/ml of suspension. Nitrite and dimethylamine were added at the levels used in the previous experiment. The bottles were incubated in the dark at 37°C for 5 hours. After incubation, the samples were immediately extracted with methylene chloride and checked for NDMA using GC-TEA. Similar suspensions were boiled and treated the same way to serve as

blanks.

i) Protein determination. Two ml of the bacterial suspension in phosphate buffer (0.2 M, pH 8.0) were digested and analyzed for nitrogen according to standard micro-Kjeldahl procedure described by AOAC (1970). The nitrogen content was multiplied by a factor of 6.25 to determine the protein content.

c) Effect of nitrite concentration on NDMA formation. Four 50 ml S. aureus suspensions (5 mg protein/ml) in phosphate buffer (0.2 M, pH 8.0) containing 0.25% (w/v) dimethylamine were prepared in screw-capped milk dilution bottles. The nitrite content was adjusted to 0.01, 0.05, 0.1, and 0.2 g per 100 ml of suspension. The bottles were incubated in the dark at 37°C for 5 hours. After incubation, the cultures were immediately extracted with methylene chloride. The extract was concentrated to 1.0 ml and injected into the GC-TEA system for NDMA identification and quantitation.

Extraction of N-Nitrosamines from Bacterial Cultures

Direct extraction of liquid bacterial cultures with methylene chloride was used throughout this study. Forty-eight hour cultures were centrifuged in a Sorval II super refrigerated centrifuge for 10 minutes at 2°C and 6000 rpm. The supernatant was collected, the cells were washed two times with sterile, distilled and deionized water and

recentrifuged each time. The combined supernatants were saturated with NaCl, extracted with three 50 ml portions of redistilled methylene chloride. The methylene chloride extracts were combined, and the aqueous phase discarded. The combined extracts were dried over anhydrous sodium sulfate and filtered through Whatman No. 42 filter paper. The filtrate was collected in a 1000 ml Kuderna Danish concentration flask, to which was added 2-3 "Boileezers". Snyder columns were fitted to the flasks and the methylene chloride extracts were concentrated to 1 ml by heating the flasks in a steam bath. The concentrated samples were quantitatively recovered into 2 dram screw capped glass vials. The volume was adjusted to 1.0 ml using nitrogen gas to evaporate excess solvent. The samples were stored at -20^{o}C until GC-TEA and GC-MS analyses.

GC-TEA Analysis

Samples, standard NDMA and NPYR were analyzed using the GC-TEA system. The gas chromatograph was a Varian Model 3700 gas chromatograph equipped with a stainless steel column (2 m x 3.0 mm i.d.) packed with 10% Carbowax 20 M + 5% KOH on Chromsorb W (80/100 mesh) obtained from Supelco Inc. (Bellefonte, Pennsylvania). The TEA system was a Thermo Electron thermal energy analyzer model 502 (Thermo Electron Corporation, Analytical Instrument Division, Waltham, MA).

GC Operating Conditions

The following conditions were found to be satisfactory and were employed:

Carrier gas (nitrogen): 35 ml/min.

Injection port temperature: 180°C

Column temperature: 180°C, isothermal

TEA Conditions

Prolyzer furnace temperature: 475°C

Trap temperature: -196°C liquid nitrogen

Oxygen flow rate: 10 ml/minute

Pressure: 1.5 atmospheres

Heated transfer line (between GC and TEA): 190°C

Appropriate volumes of standard solutions of NDMA and NPYR and sample extracts were injected into the GC-TEA system. The TEA detector response was recorded on a recorder chart. Peak heights and retention times of standard N-nitrosamines were recorded. The peak heights of all compounds in the samples corresponding to the retention times of the standard N-nitrosamine were measured. The N-nitrosamine contents of the samples were calculated according to the following equation:

$$S = \frac{Sph \times St_V \times St_{con}}{Stph \times Sv} \times Vsc \times 1000$$

where S = ng N-nitrosamine/total volume of culture

Sph = sample peak height (arbitrary units)

St_v = standard volume injected (μl)

St_{con} = standard concentration (ng/μl)

St_{ph} = standard peak height (arbitrary units)

Sv = sample volume injected (μl)

Vsc = total volume of the sample concentrate (μl)

Confirmation of the Identity of N-Nitrosamines

GC-MS Analysis. The identity of NDMA in some of the positive N-nitrosamine samples from GC-TEA analysis were confirmed by mass spectral analysis according to the following procedure:

The samples were further concentrated to 0.3 ml in a stream of nitrogen. A 3 μl aliquot was injected into the GC-MS system. The mass spectrometer was a Hewlett Packard Model 5985 low resolution quadrupole mass analyzer (Hewlett Packard Corp., Avondale, PA). The gas chromatograph was a Hewlett Packard Model 5840 A equipped with a glass column (2 m x 2 mm i.d.) packed with 10% Carbowax 20 M + 5.0% KOH on Chromosorb W (80/100 mesh).

GC Conditions

Carrier gas: helium (35 ml/min)

Injection port temperature: 180°C

Column temperature: 140-180°C, programmed at 10°C/min with an initial hold of 2 minutes

Mass Spectrometer Conditions

Ion source temperature: 200°C

Electron impact: 70 eV

Electron multiplier: Volt: 2000

Start-stop masses: 40-200

UV Irradiation. Irradiation of samples was carried out according to Doerr and Fiddler (1977) with some modifications. Positive N-nitrosamine samples from GC-TEA analysis were exposed to ultraviolet light for 24 hours in a carton paper box laminated with aluminum foil. The light source was a long wave (366 nm) Black-Ray UV lamp Model UVL-21 (Ultra Violet Products Inc., San Gabriel, CA). The extent of photolysis was measured by injecting the samples into the GC-TEA system and analyzing for N-nitrosamines before and after UV irradiation.

N-Nitrosation of Sarcosine by Bacteria and Detection of N-Nitrososarcosine by High Performance Liquid Chromatography (HPLC)

Five bacterial cultures were used in this study. These were E. coli, S. aureus, L. bulgaricus, M. thermosphactum and Ps. fluorescens. A 1 ml culture was inoculated into 200 ml of TSB medium containing 0.25% (w/v) sarcosine and 0.05% (w/v) $NaNO_2$. The cultures were incubated at the appropriate temperatures for 48 hours with shaking and aeration (except for L. bulgaricus). The cultures were

filtered through a Millipore filter (0.45 μ) and the supernatants collected and refiltered. The pH of the supernatants was adjusted to 3.1 using H_3PO_4. A 50 μl aliquot of the supernatants was injected into the HPLC system for identification and quantitation of N-nitrosarcosine.

The chromatographic system consisted of a Waters Associates liquid chromatograph, equipped with a chromatography pump Model 6000 A, and a μBondapak C_{18} reverse phase column (32 cm x 6 mm i.d.), a Model 440 absorbance detector and strip chart recorder (Linear Instruments).

Appropriate volumes of a standard N-nitrososarcosine solution (2 mg/100 ml) and samples were injected into the HPLC and the samples eluted with a 0.1 M sodium phosphate buffer (pH 3.0) at a flow rate of 1.0 ml/min, and detected by ultraviolet absorption at 254 nm. The UV detector response was recorded. Peak heights and retention times of a standard N-nitrososarcosine solution were recorded. The peak heights of compounds in the samples corresponding to the retention time of standard N-nitrososarcosine were measured.

The N-nitrososarcosine content of samples were calculated from a standard curve of peak height vs standard N-nitrososarcosine concentration.

Gid-deed Processing

A one half lamb carcass weighing approximately 18 lbs was obtained from a commercial meat distributor in the East Lansing area 10 hours after slaughter. The carcass was cut into three sections, front leg, middle section and hind leg, and deboned. Each section was divided longitudinally into four equal pieces. Four representative samples (A, B, C and D) were pooled from the three sections (1350 g each). The meat in each sample was further sliced to about 2 cm in thickness. The first sample (A) was heavily salted by rubbing an unknown amount of dry salt (refined salt flakes) on the meat slices until a satisfactory product was obtained. The amount of salt used (126 g) was calculated backwards by weighing the remainder of a pre-weighed stock. Samples B, C and D were treated as follows:

Treatment	Salt
B	126 g refined salt + 120 mg/kg of nitrite
C	126 g refined + 500 mg/kg of nitrate
D	126 g crude salt

The samples were hung on a rope in the open air in the sun for 3 days. Representative samples from each treatment were fried in olive oil to a medium "doneness" on a slow flame. The temperature of the frying oil was allowed to increase from room temperature to 190^{o}C in approximately 7 minutes, after which the frying was discontinued. The fried gid-deed was removed from the oil and placed on a

filter paper to remove excess oil. Both the salted-sundried and the fried samples were analyzed for the presence of N-nitrosamines using vacuum distillation and GC-TEA as described later.

Free Amino Acid Analysis of the Meat Samples

Samples from fresh and salted-sundried lamb meat were analyzed for their free sarcosine and proline contents according to the procedure of Clark et al. (1966), as modified by Gray and Collins (1977). The meat samples were homogenized in a Waring blender with 1000 ml of 3% 5-sulfosalicylic acid and were immediately centrifuged for 10 min in Sorval II super refrigerated centrifuge at 4000 rpm. The supernatant was removed and the residue was again extracted with 500 ml of the sulfosalicylic acid solution and centrifuged as before. The combined supernatants were freeze-dried and the residue dissolved in 100 ml of citric acid buffer (0.2 M) at a pH of 2.1. The sample solution was extracted with 100 ml of n-hexane to remove residual lipids, filtered through a Millipore filter (0.2 μ) and stored at -20^{o}C until analysis. A Dionex amino acid analyzer fitted with a DC-4 resin (Durrum) cation exchange column (column bed 26 cm x 3 mm) was used for amino acid determination. Amino acids in the samples and reference amino acids (including proline) were eluted using a lithium citrate buffer system and reacted with ninhydrin to produce the

colored chromogen for quantitation.

Vacuum Distillation and Extraction of N-Nitrosamines in the Gid-Deed Samples

Twenty-five grams of the ground meat samples (fried and unfried) were distilled under reduced pressure according to the procedure described by Robach et al. (1980) using paraffin oil as the distillation medium and ammonium sulfamate (0.5 g/25 g sample) to prevent N-nitrosamine formation during the distillation step. Sample distillates were recovered in vacuum traps immersed in liquid nitrogen. The distillation continued until an internal temperature of 115^{o}C was reached in the distillation flasks. The distillates were allowed to thaw in the dark and were extracted using methylene chloride as described by Robach et al. (1980). Preptubes^{TM} (Thermo Electron Corp., Waltham, Mass.) were used to remove H_2O and reduce impurities in the methylene chloride extract. The extracts were concentrated as before and injected into the GC-TEA system for N-nitrosamine analysis.

Nitrite Analysis

Nitrite analyses were carried out according to the standard Association of Official Analytical Chemists (AOAC) procedure (1970), with the following modification: N-1-naphthyl-ethylene-diamine dihydrochloride was used to produce the

colored chromogen instead of α-naphthylamine since the latter is a recognized carcinogen.

RESULTS AND DISCUSSION

Separation and Identification of N-Nitrosamines by GC-TEA

The chromatographic separation of volatile N-nitrosamines by the GC-TEA system using TEA as a detector provides a very powerful tool for the separation and identification of these compounds. The method is relatively specific, and requires a minimum of purification steps (Fine et al., 1975a). The TEA detector is extremely sensitive to N-nitrosamines, and is almost non-responsive to other impurities that might be present in the sample (Fine et al., 1975b).

A standard mixture of NDMA and NPYR in methylene chloride was injected into the GC-TEA system whenever sample extracts were analyzed. Usually 2.5 µl of the standard mixture containing 10 ng of each of NDMA and NPYR (4 ng of each/1 µl) were used. Figures 1 and 2 illustrate typical chromatograms obtained when standard N-nitrosamines and sample extracts (in methylene chloride) were injected, under the employed conditions, into the GC-TEA system.

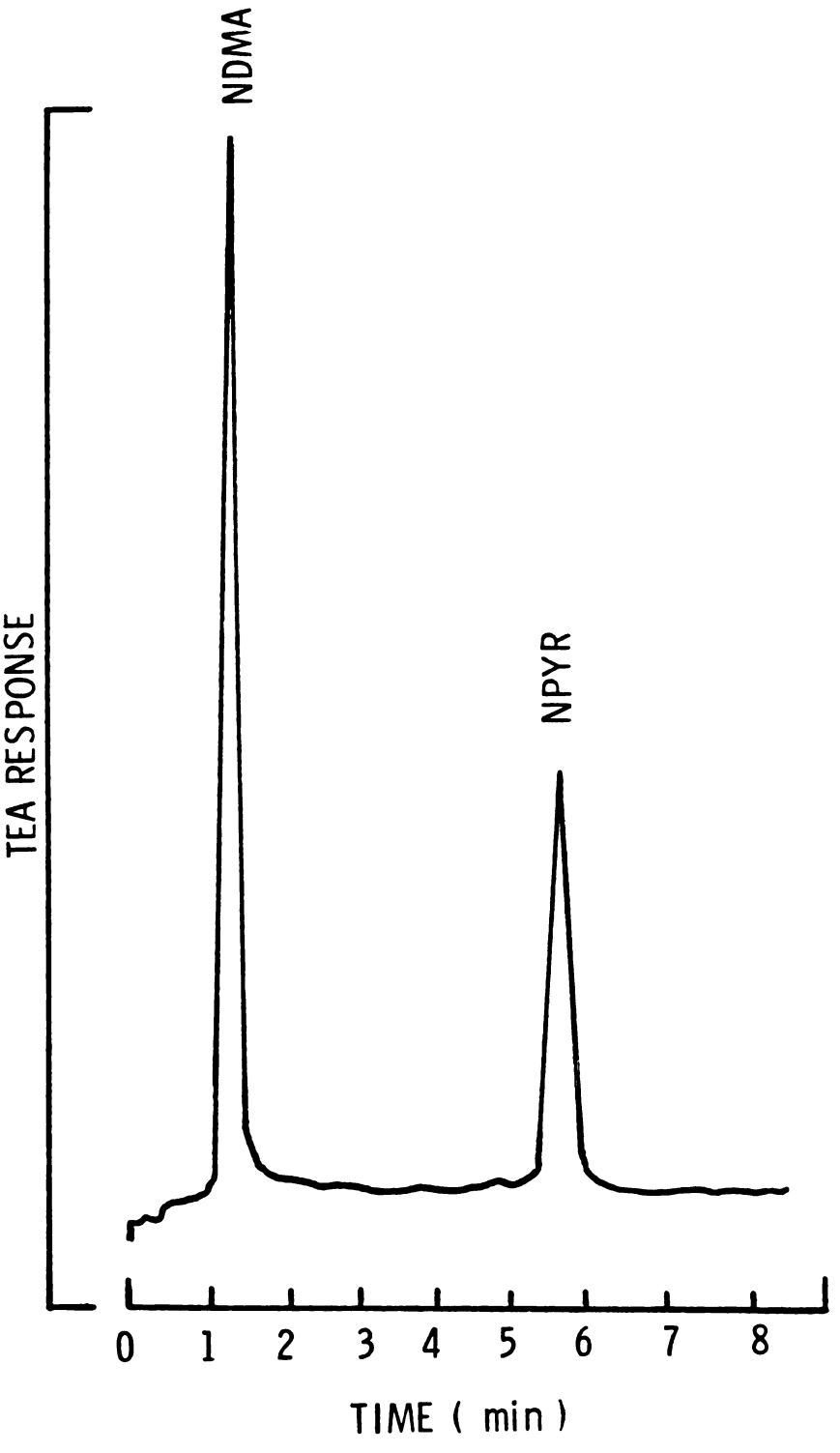


Figure 1. GC-TEA chromatogram of standard N-nitroso-dimethylamine and N-nitrosopyrrolidine.

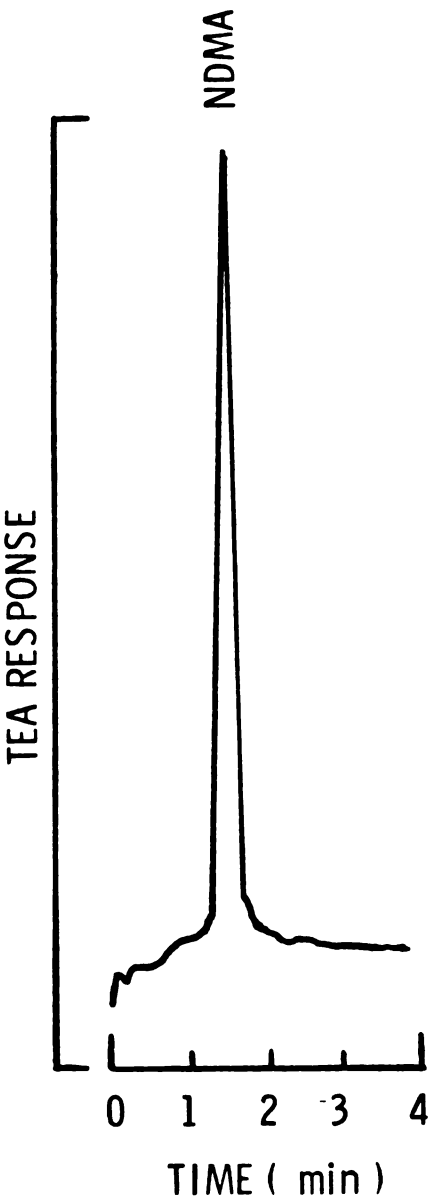


Figure 2. GC-TEA chromatogram of a typical methylene chloride extract of a bacterial culture containing precursors of N-nitrosodimethylamine.

Confirmation of the Identity of N-Nitrosamines by GC-MS Analysis and UV Irradiation

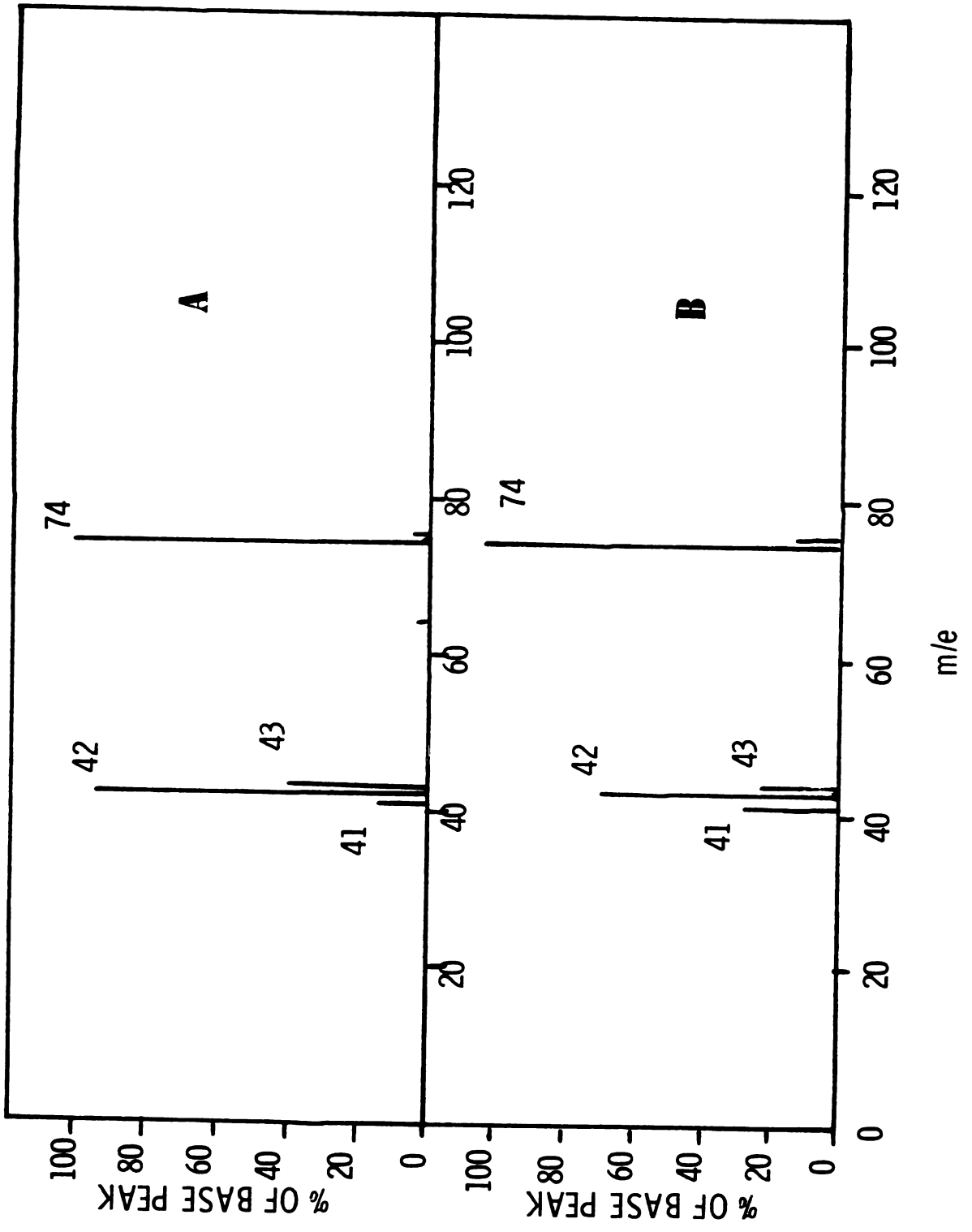
The identity of NDMA in some of the positive N-nitrosamine samples was confirmed by mass spectral analysis and UV irradiation. The fragmentation patterns of NDMA in the samples were similar to those of authentic NDMA and to the fragmentation patterns of the N-nitrosamine reported in literature (Kawabata et al., 1977). Typical spectra of NDMA are shown in Figure 3.

Photolysis of the NDMA and N-nitrosodibutylamine (NDBA) in the samples was evident when the methylene chloride extracts were subjected to UV irradiation. Doerr and Fiddler (1977) have stated that, for low levels of N-nitrosamines that can not be confirmed by mass spectral analysis, photolysis at 366 nm of a small portion of the sample concentrate offers a simple, rapid and sensitive method for the presumptive evidence of N-nitrosamines. Therefore, the formation of N-nitrosamines in the bacterial cultures can be more or less positively identified by the combination of TEA analysis and UV photolysis.

NDMA Formation by Growing Bacterial Cultures from Dimethylamine and Nitrite

The bacterial cultures were grown in 200 ml of TSB medium containing 0.25% (w/v) dimethylamine and 0.05% (w/v) sodium nitrite. The samples were extracted after 48 hours

Figure 3. Mass spectra of: (A) N-nitrosodimethylamine standard, and (B) N-nitrosodimethylamine isolated from bacterial cultures containing dimethylamine and nitrite/or nitrate.


A
B
74
42
43
41
% OF BASE PEAK
m/e
0
20
40
60
80
100
120

of incubation and the amount of NDMA produced in the incubation medium was determined by the GC-TEA system (Table 6). The

Table 6. NDMA formation by growing bacterial cultures in 200 ml of tryptic soy broth containing dimethylamine (0.5 g) and nitrite (0.1 g).

Organism	NDMA µg/200 ml Culture[a]	% Conversion (DMA → NDMA)
E. coli E-2	384.0	0.056
E. coli K-12	342.9	0.055
L. bulgaricus	1481.5	0.236
M. thermosphactum	173.2	0.028
P. fluorescens	11.8	0.002
P. schuylkilliensis	42.1	0.007
P. schuylkilliensis	23.1	0.004
P. putida biotype A	12.9	0.002
P. putida	14.2	0.002
S. aureus	297.1	0.004
Control (pH 7.2)	6.1	0.001

[a]Corrected for control determination

highest yield of NDMA was produced by *L. bulgaricus* being 1481.4 µg, followed by the two *E. coli* species and *S. aureus* which produced 384.0, 342.9 and 297.1 µg, respectively. The lowest N-nitrosamine yield was obtained by *P. putida* biotype A, (12.92 µg). These results correlate very well with published literature regarding N-nitrosamine formation by bacteria. Hawksworth and Hill (1970) showed that out of

ten strains of E. coli tested, five were able to form N-nitrosamines when incubated aerobically in a nutrient broth medium containing nitrate or nitrite and the secondary amines, dimethylamine, diethylamine, diphenylamine, piperidine, pyrrolidine and N-methylaniline. Similarly, NDMA has been shown to be produced from dimethylamine and nitrite by S. aureus, S. epidirmidis and Psuedomonas species (Mills and Alexander, 1976; Ishiwata et al., 1976; Uibu and Bogovski, 1978).

The starting pH of the culture medium was 7.2 ± 0.1 and the final pH varied with bacterial species tested. For E. coli species, the final pH was 5.6. Similarly, the final pH of L. bulgaricus and M. thermosphactum was 5.4 and 5.7, respectively. All other species had final pH values above 7.2. These pH values indicate that chemical N-nitrosation catalyzed by acid production in the culture media may have been partly responsible for N-nitrosamine formation. Mirvish (1970) showed that for dimethylamine and nitrite, under standard conditions of temperature and total species concentration, a maximum NDMA formation was obtained at pH 3.4. The percent yield decreased on both sides of this optimum pH. He also reported that over a pH range of 9 to 5, the rate of N-nitrosation increased 10 fold for every unit drop in pH. Accordingly, L. bulgaricus and E. coli are expected to produce more NDMA in the incubation medium than the other species used. This was borne out by the

experimental data obtained.

NDMA Formation by Resting Cells at pH 8.0

To investigate the catalytic nature of N-nitrosamine formation from dimethylamine and nitrite by bacterial species and to ascertain whether the bacterial catalysis of N-nitrosamine formation is merely the result of a change in pH or some other phenomenon, resting cells of the test organisms are suspended in 100 ml of 0.2 M phosphate buffer at pH 8.0. Dimethylamine and nitrite were added at the same level as in the growing cultures (0.05% (w/v) nitrite and 0.25% (w/v) dimethylamine). The reaction mixtures were incubated for 5 hours at the optimum temperatures for the bacterial suspensions. The total NDMA formed in each suspension was determined, after correcting for the blank determinations (Table 7). These data indicate that NDMA formation from dimethylamine and nitrite was catalyzed by the presence of bacteria, even at pH 8.0. This is a pH value, at which chemical N-nitrosamine formation is minimal (Mirvish, 1970). Therefore, it could be assumed that another catalytic mechanism is involved in N-nitrosamine formation by these bacterial species. Klubes (1972), Ayanaba and Alexander (1973), and Kunisaki and Hayashi (1979) reported that boiled bacterial cells including those of E. coli B were unable to N-nitrosate dimethylamine in the presence of nitrite. However, NDMA was produced in the

Table 7. NDMA formation by resting bacterial cells in 100 ml phosphate buffer (0.2 M, pH 8.0) containing 0.25 g dimethylamine and 0.05 g sodium nitrite.

Organism	NDMA µg/100 ml Buffer	% Conversion[a] (DMA→NDMA)
E. coli E-2	0.089	2.8
E. coli K-12	0.035	1.2
L. bulgaricus	0.120	3.8
M. thermosphactum	0.510	16.2
P. fluorescens	0.533	17.0
P. schuylkilliensis	0.056	1.8
P. schuylkilliensis	0.083	2.6
P. putida biotype A	0.143	4.6
P. putida	0.238	7.6
S. aureus	0.125	4.0
Control (no culture)	0.007	0.24

[a] $\times 10^5$

presence of living cells. They concluded that N-nitrosamine catalysis by bacteria was enzymatic in nature. On the other hand, Collins-Thompson *et al*. (1972) and Yang *et al*. (1977) showed that boiled cells were equally active in catalyzing N-nitrosamine formation from secondary amines and nitrites and concluded that the N-nitrosation reaction is non-enzymatic. Data presented in Tables 6 and 7 also indicate that

under growing conditions, L. bulgaricus, E. coli species and S. aureus, respectively, produced the greatest amount of NDMA after incubating for 48 hours in a TSB medium. At pH 8.0 in a 0.2 M phosphate buffer solution and after 5 hours of incubation, a major shift in N-nitrosamine formation was observed. Under these conditions, P. fluorescens and M. thermosphactum were shown to be the most active in catalyzing NDMA formation from dimethylamine and nitrite. This phenomenon indicated that a pH-dependent mechanism is primarily involved in the N-nitrosation reaction when growing bacterial cultures are involved.

Effect of Nitrite Concentration on NDMA Formation

Resting cells of S. aureus species were incubated for 5 hours in 50 ml of 0.2 M phosphate buffer (pH 8.0) containing a fixed concentration of dimethylamine (0.25% w/v) and increasing nitrite concentrations (0.01, 0.05, 0.1 and 0.2% w/v). N-Nitrosamine analysis of the incubated suspensions indicated that NDMA formation increased with increasing nitrite levels (Table 8). Furthermore, NDMA formation was doubled as the nitrite concentration was increased from 0.01% to 0.05% and increased four times when the nitrite concentration was increased from 0.05% to 0.1%. However, NDMA formation was increased only by a factor of 0.5 as the nitrite concentration was increased from 0.1% to 0.2%. The N-nitrosation reaction is second order with respect to

Table 8. Effect of nitrite concentration on NDMA formation by S. aureus using a fixed dimethylamine concentration at 37°C and pH 8.0 for 5 hours.

Concentration of nitrite (%)	NDMA ng/50 ml buffer	% Conversion[a] (DMA→NDMA)
0.01	103.23	6.6
0.05	225.22	14.6
0.1	1013.51	64.9
0.2	14358.11	91.9

[a] $\times 10^5$

nitrite concentration, i.e., the rate of the reaction is increased 4 times with doubling of the nitrite concentration (Mirvish, 1970). In this study, this criterion was only evident when the concentration of nitrite was increased from 0.05% to 0.1%. This is probably due to the fact that N-nitrosation of secondary amines in a bacterial system operates under kinetic rules different from those followed in a chemical model system. Kunisaki and Hayashi (1979) reported that the formation of N-nitrosamines by the resting cells of E. coli B was proportional to the incubation time and cell concentration. They also indicated that the reaction followed Michaelis-Menten kinetics and was inhibited by high concentration of the substrates, dimethylamine and nitrite.

Effect of Cell Concentration on NDMA Formation

Resting cells of E. coli K-12 were suspended in a 50 ml of phosphate buffer (0.2 M, pH 8.0) containing 0.25% (w/v) dimethylamine and 0.05% (w/v) nitrite. The cell concentration was adjusted to 7.0, 5.6, 4.2, and 2.8 mg protein/ml. Data in Table 9 show the total amount of NDMA

Table 9. Effect of cell concentration of E. coli K-12 on NDMA formation at constant pH (8.0) using fixed concentrations of nitrite and dimethylamine at 37°C for 5 hours.

Cell concentration (mg protein/ml)	NDMA ng/50 ml	% Conversion[a] (DMA→NDMA)
7.0	899	5.75
5.6	577.8	3.70
4.2	486.1	3.11
2.8	355.8	2.28
Blank (Boiled cells) 7.0	166.7	1.07

[a] $\times 10^4$

accumulated in each suspension. These results indicated that NDMA formation increased with increasing cell concentration. NDMA was also detected in the boiled cell suspension (blank or control), the amount being approximately 18.5% of that produced by the living cells. These results indicated that a major proportion of NDMA formed by E. coli K-12 was catalyzed by a heat sensitive mechanism, possibly

enzymes.

NDMA Formation by Growing Bacterial Cultures from Dimethylamine and Nitrate

Data presented in Table 10 indicate that out of the 10 bacterial species tested, only 3 were capable of producing

Table 10. NDMA formation by growing bacterial cultures from sodium nitrate (0.5 g) and dimethylamine (0.5 g) in 200 ml TSB.

Organism	NDMA µg/200 ml culture	% Conversion[a] (DMA→NDMA)
E. coli E-2	26.57	4.24
E. coli K-12	32.85	5.25
L. bulgaricus	--	--
M. thermosphactum	traces	--
P. fluorescens	0.21	0.03
P. schuylkilliensis	0.14	0.02
P. schuylkilliensis	0.12	0.02
P. putida biotype A	0.22	0.04
P. putida	0.05	0.01
S. aureus	320.0	51.11

[a] $\times 10^3$

appreciable amounts of NDMA when the growing cultures were incubated in TSB media in the presence of nitrate and dimethylamine. These species were: E. coli E-2, E. coli K-12 and S. aureus. Other cultures produced relatively

smaller amounts similar to those obtained in the control (blank). The production of NDMA by these bacterial species from dimethylamine and nitrate indicated that these organisms are capable of reducing nitrate to nitrite which will subsequently N-nitrosate dimethylamine. Similar results have been reported by Hawksworth and Hill (1970) and Ishiwata *et al*. (1976). Hawksworth and Hill (1970) showed that out of ten strains of *E. coli* tested, five were able to form N-nitrosamine when incubated aerobically in a nutrient broth medium containing nitrate and secondary amines. *S. aureus* has also been shown to produce NDMA when incubated in brain heart infusion (BHI) containing dimethylamine and nitrate (Ishiwata *et al*., 1976). Nitrate reduction by bacteria through nitrate reductase activity results in the accumulation of nitrites; however, certain bacterial species have been shown to reduce nitrate to gaseous nitrogen (Atlas and Bartha, 1981). In this case nitrite does not accumulate, and NDMA is not expected to be formed in appreciable quantities,

$$NO_3^- \longrightarrow NO_2^-$$
$$NO_3^- \longrightarrow N_2$$

even if dimethylamine is present at a high concentration in the incubation medium. Similarly, NDMA will not be formed if the organisms are not endowed with nitrate reductase activity. Previous studies have indicated that the actual

N-nitrosating species can be one of the following depending on N-nitrosation conditions: nitrous anhydride (N_2O_3), nitrous acidium ion (NO^+), nitrosyl halide (NOX) or nitrosyl thiocyanate (NOCNS) (Challis and Butler, 1968; Mirvish, 1972; Mirvish, 1975).

Effect of Incubation Time and Temperature on NDMA Formation by P. schuylkilliensis Growing Cultures

Three sets of growing cultures of P. schuylkilliensis were prepared. Each set contained three 500 ml Erlenmeyer flasks. Each flask contained 200 ml of TSB medium to which were added 0.25% (w/v) dimethylamine and 0.05% (w/v) sodium nitrite along with 1.0 ml of a P. schuylkilliensis active culture. The first set was incubated at 5°C. The second and the third sets were incubated at 25°C and 32°C, respectively. A flask from each set was removed every four days, and the contents extracted and analyzed for NDMA. Data in Table 11 show the total NDMA formed in each flask. These data indicated that N-nitrosamine formation by P. schuylkilliensis was, as expected, dramatically increased by increasing the incubation temperature. The NDMA formed after 4 days of incubation increased approximately six times as the incubation temperature was increased from 5 to 25°C, and 67 times as the incubation temperature was increased to 32°C. However, these differences were less pronounced after 8 to 12 days of incubation. These results also indicated

Table 11. Effect of incubation time and temperature on NDMA formation by P. schuylkilliensis growing in 200 ml of TSB medium containing 0.1 g nitrite and 0.5 g dimethylamine.

Days of incubation	µg NDMA formed/200 ml culture[a]		
	5°C	25°C	32°C
4	0.5	3.0	33.6
8	0.32	0.63	7.51
12	0.32	0.74	1.77

[a]Values are the average of two replicates.

that NDMA was reduced in the 25 and 32°C cultures as the incubation time was extended beyond 4 days. However, the quantity of NDMA in the 5°C cultures remained almost unchanged as the incubation time was extended to 8 and 12 days. As for other chemical reactions, N-nitrosation reaction rates have been shown to be doubled for every 10°C rise in temperature (Foreman and Goodhead, 1975). The results of this study indicated a similar trend.

Prolonged storage conditions have been reported to increase N-nitrosamine formation even at low temperature (Ender et al., 1967). In the present study, longer incubation (8 and 12 days) resulted in lower levels of NDMA, especially at the 25°C and 32°C incubation temperatures. This loss of NDMA could be due to degradation of the preformed N-nitrosamine by P. schuylkilliensis or by volatilization of the NDMA to the atmosphere. In both cases, the

loss is expected to be much higher at 25°C and 32°C incubation temperatures.

Degradation of preformed NDMA by various bacteria have been reported by Klubes et al. (1972) and Rowland and Grasso (1975). Both reported a loss of approximately 5% of the N-nitrosamine when incubated with bacteria under standard conditions and pH 7.0 for 20 hours. Similar results were reported earlier by Hawksworth and Hill (1971) who demonstrated that NDMA and NDEA were degraded to the parent secondary amine and nitrite by washed-cell suspensions of five E. coli strains (out of ten strains tested), 3/10 of the clostridia and 3/10 of non-spore forming anaerobes tested. They also indicated that the enzyme responsible for the degradation was of low activity (maximum level of breakdown observed after overnight incubation was only 0.025%), was located in the cytoplasmic material of the cell and had a pH optimum range of 7 to 8.

NDMA Formation by Growing Bacterial Cultures from Trimethylamine and Trimethylamine-N-Oxide

Bacterial cultures were incubated in TSB media containing 0.25% (w/v) trimethylamine or trimethylamine oxide and 0.05% (w/v) sodium nitrite. Results of NDMA analysis indicated that most of the bacterial cultures had a higher N-nitrosamine content when incubated with trimethylamine-oxide than with trimethylamine after 48 hours of incubation

(Table 12). The highest conversion was observed in the S. aureus culture containing trimethylamine oxide. Both trimethylamine and trimethylamine oxide have been implicated in NDMA formation when heated in chemical model systems in the presence of nitrite (Scanlan et al., 1974; Ohshima and Kawabata, 1978). The latter group reported that NDMA was produced in yields as high as 53% from trimethylamine and 55% from trimethylamine oxide when the amines were reacted with nitrites at pH 3.0 and 100°C for 2 hours. However NDMA yields were much lower (2-3% from trimethylamine and 0.02% from trimethylamine oxide) at pH 6-7 under the same heating conditions.

Similarly, trimethylamine has also been shown to be converted to dimethylamine which is subsequently N-nitrosated to NDMA in the presence of bacteria and nitrates or nitrites (Ayanaba et al., 1973; Ayanaba and Alexander, 1973; Maduagwu and Bassir, 1979).

Similar to nitrate, trimethylamine oxide has been shown to serve as a terminal electron acceptor in anaerobic respiration of bacteria in which a variety of hydrogen donors are oxidized at the expense of trimethylamine oxide which is reduced to trimethylamine (Strom, 1979). Therefore, trimethylamine oxide is ultimately reduced to trimethylamine, then possibly demethylated to dimethylamine, and N-nitrosated to NDMA via bacterial activity. This process takes place as bacteria attain higher counts in the incubation media.

Table 12. NDMA formation by growing bacterial cultures from triamines (0.5 g) and nitrite (0.1 g) in 200 ml of tryptic soy broth.

	µg NDMA/200 ml culture			
	TMA	% Conversion[a] (TMA→NDMA)	TMA-O	% Conversion[a] (TMA → NDMA)
E. coli E-2	Traces	--	0.14	0.31
E. coli K-12	Traces	--	0.28	0.63
L. bulgaricus	1.39	2.6	2.43	5.43
M. thermosphactum	0.33	0.62	0.53	1.18
P. fluorescens	Trace	--	0.20	0.45
P. schuylkilliensis	0.26	0.49	0.12	0.27
P. schuylkilliensis	0.14	0.26	0.20	0.45
P. putida biotype A	Traces	--	0.17	0.38
P. putida	0.13	0.24	0.11	0.25
S. aureus	0.13	0.24	107.39	240.05

TMA = Trimethylamine-hydrochloride

TMA-O = Trimethylamine-N-oxide: dihydrate

[a] $\times 10^4$

Compared to trimethylamine oxide, trimethylamine is extremely volatile (bp. 3.7°C). This may explain the higher yields of NDMA from trimethylamine oxide as opposed to those obtained from trimethylamine.

Mechanism of NDMA Formation from Tertiary Amines

Two possible mechanisms have been suggested for the formation of NDMA from tertiary amines and nitrite (Loeppky and Smith, 1967; Lijinsky et al., 1972; Sander et al., 1975). The first involves the oxidative cleavage of one N-R bond before N-nitrosamine formation. The second mechanism involves nonoxidative cleavage of one N-R bond when the R group is in an oxidation state appropriate for its removal by hydrolysis (Lijinsky et al., 1972). In both mechanisms, the tertiary amines are converted to secondary amines before the final N-nitrosation step. This conversion is suggested to take place as follows: the tertiary amine is directly attacked by the nitrous acidium ion to form an adduct which decomposes spontaneously to form an iminium ion. In the presence of water, the iminium ion is hydrolyzed to the secondary amine and an aldehyde. The secondary amine is N-nitrosated by a nitrite ion to form a N-nitrosamine. An alternative mechanism was proposed later by Ohshima and Kawabata (1978) in which NDMA may be formed directly from trimethylamine or trimethylamine oxide by a pathway not involving secondary amines. In this mechanism, the iminium

ion may undergo nucleophilic attack by the nitrite ions to form an unstable complex which directly decomposes to NDMA and formaldehyde.

NDMA Formation by Growing Bacterial Cultures from Choline and Choline-Containing Compounds

Data presented in Table 13 indicate that NDMA, in the range of trace amounts to 1.23 µg, were produced when the bacterial cultures were incubated for 48 hours in 200 ml of TSB medium containing 0.05% (w/v) nitrite and 0.25% (w/v) of each of these compounds, separately. Again, the total NDMA accumulated varied with the bacterial species and the compound used. However, higher N-nitrosamine yields were obtained when lecithin was utilized as a precursor. Choline, phosphorylcholine and lecithin have been cited as possible precursors for NDMA in foods, and were shown to result in NDMA formation when heated with nitrite in a chemical model system (Gray et al., 1978). On the other hand, N-nitrosamine formation from these compounds via microbial activity has not been reported so far. However microbial degradation of choline and choline compounds have been reported to result in dimethylamine formation (Asatoor and Simenhoff, 1965; Hawksworth, 1970; Hawksworth and Hill, 1971).

As far as NDMA formation is concerned, lecithin, when used as a precursor, gave higher NDMA yields with most bacteria than did choline or phosphorylcholine. This could

Table 13. NDMA formation by growing bacterial cultures in 200 ml of TSB containing 0.05% (w/v) nitrite and 0.25% (w/v) of each of the choline-containing compounds.

Organism	Choline-Chloride		Phosphorylcholine-Cl		Lecithin	
	µg NDMA	% Conversion[a] (choline →NDMA)	µg NDMA	% Conversion[b] (Ph. choline →NDMA)	µg NDMA	% Conversion[c] (Lecithin →NDMA)
E. coli E-2	0.054	0.15	0.053	2.7	0.471	0.72
E. coli K-12	0.13	0.36	0.079	4.0	0.446	0.67
L. bulgaricus	0.49	1.34	Trace	--	0.357	0.55
M. thermosphactum	0.01	0.03	Trace	--	0.535	0.82
P. fluorescens	0.08	0.22	0.21	10.6	1.232	1.89
P. schuylkilliensis	0.051	0.14	0.077	3.9	0.49	0.75
P. schuylkilliensis	0.06	0.16	0.12	6.1	0.55	0.84
P. putida biotype A	0.09	0.25	0.08	4.0	0.61	0.93
P. putida	0.11	0.30	0.035	1.8	0.80	1.22
S. aureus	0.96	2.63	0.065	3.3	0.422	0.65
Control	Trace	--	Trace	--	0.027	
		$^{a}\times 10^{4}$		$^{b}\times 10^{5}$		$^{c}\times 10^{3}$

possibly be due to one or more of the following factors:

1) The presence of trace amounts of dimethylamine in the commercial lecithin preparation as reported by Pensabene et al. (1975).

2) Lecithin (phosphatidylcholine) is a chemical compound that has both hydrophobic and hydrophilic properties and can form spherical molecular aggregates (micelles) in aqueous media. The presence of these micelles in the N-nitrosation reaction mixture has been reported to significantly increase the rate of N-nitrosation of secondary amines (Okun and Archer, 1977).

3) Nitrite to precursor ratio. At the levels used, choline, phosphorylcholine and phosphatidylcholine had different nitrite to precursor molar ratios. The highest ratio was observed in lecithin (2.27) followed by phosphorylcholine and choline for which the ratios were 0.75 and 0.41, respectively. The higher NDMA yield for lecithin under these conditions could be explained based on the nitrite/lecithin ratio.

The rate of formation of N-nitrosamines from nitrite and secondary amines follows second order kinetics with respect to the nitrite concentration (Mirvish, 1970). Therefore, increasing the nitrite to precursor ratio could increase the rate of formation of N-nitrosamines as evidenced by the results of this study.

Mechanism of NDMA Formation from Choline and Choline-Containing Compounds

As has been mentioned earlier, several model chemical studies have implicated choline and choline-containing compounds as possible precursors of NDMA. However, the mechanisms as to how these compounds are converted to NDMA are yet to be discovered. The following is a suggested pathway for NDMA formation from choline-containing compounds via bacterial activity (Figures 4 and 5).

Lecithin can be converted through a series of biochemical reactions into several intermediates. Betaine and betaine aldehyde can be N-nitrosated to produce NDMA in a manner similar to that suggested for NDMA formation from trimethylamine and trimethylamine oxide (Figure 5). However, N-nitrosamine formation from these compounds would require an additional N-nitrosation cleavage of an N-R group to form a tertiary amine. The tertiary amine is subsequently converted to NDMA via the iminium ion as suggested by Smith and Loeppky (1967) and Lijinsky *et al*. (1972).

NPYR Formation from L-Proline and N-Nitrosoproline

In model chemical systems, proline has been demonstrated to be a major precursor of NPYR in fried bacon (Gray, 1981). In this study, none of the bacterial cultures used were able to produce NPYR when incubated for 48 hours in TSB or BHI media containing 0.25% (w/v) L-proline and 0.05% (w/v)

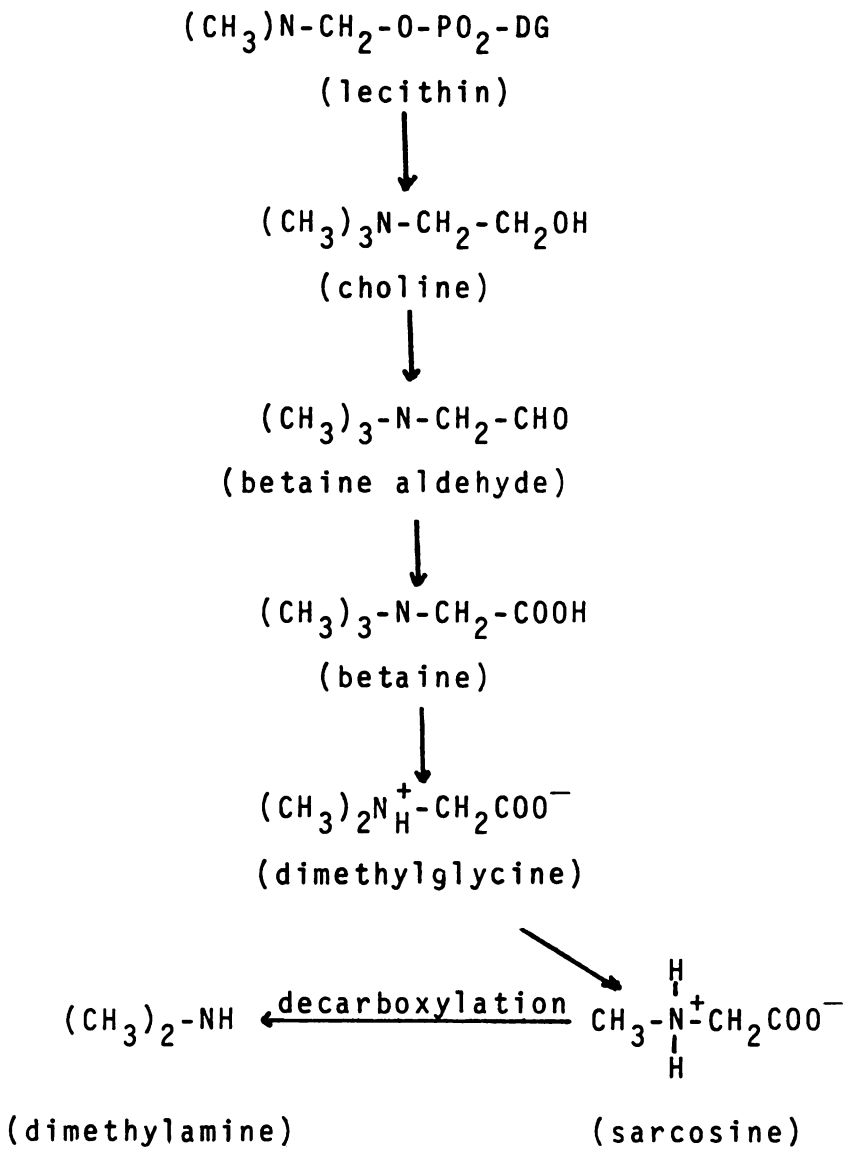


Figure 4. Biological degradation of lecithin (adopted from Anonymous, Metabolic Pathways, 1968)

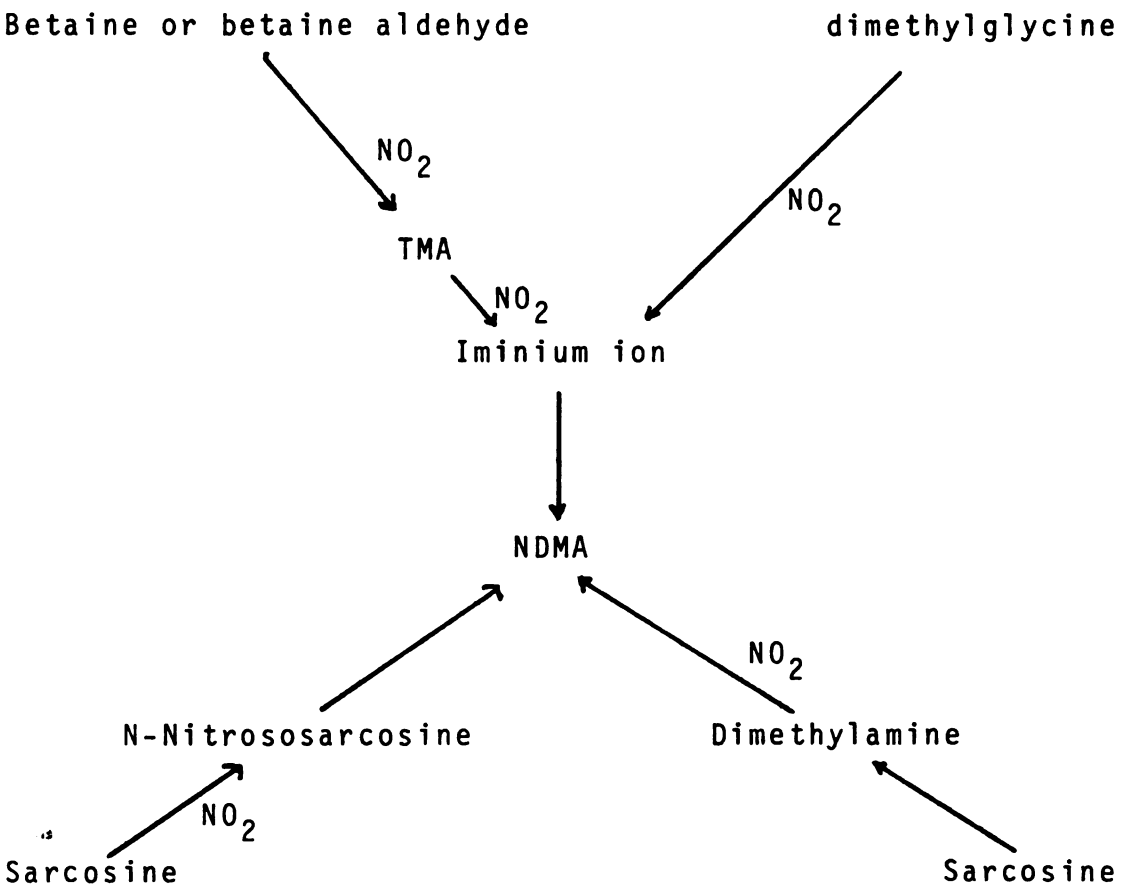


Figure 5. Suggested pathway for N-nitrosamine formation from nitrite and choline degradation intermediates.

sodium nitrite. These results conformed with those of a Moller decarboxylase test which showed that none of the bacterial cultures tested were able to decarboxylate L-proline to pyrrolidine.

The fact that NPYR was not detected in the final methylene chloride extract does not eliminate the possibility of N-nitrosoproline formation in the incubation medium. The latter may have been discarded in the water phase in the extraction process (Lee, 1981). In a similar study, Kawabata and Miyakoshi (1976) reported that no close relationship was observed in the distribution pattern of L-proline and N-nitrosoproline decarboxylases among the test organisms i.e., none of the cultures that decarboxylated L-proline were capable of decarboxylating N-nitrosoproline. Decarboxylase activity was observed only in M. thermosphactum culture when incubated in TSB or BHI containing 0.2% (w/v) N-nitrosoproline (Table 14). However, even in the case of M. thermosphactum, N-nitrosoproline decarboxylase activity was quite low since only trace amounts of NPYR were detected (Table 14). These results give evidence to the suggestion that the distribution of bacteria capable of decarboxylating N-nitrosoproline is narrow as stated by Kawabata and Miyakoshi (1976).

Table 14. The effect of growing bacterial cultures in NPYR formation from N-Nitrosoproline (0.2%, w/v) in 200 ml of TSB medium.

Organism	µg NPYR formed/200 ml of culture
E. coli E-2	ND
E. coli K-12	ND
L. bulgaricus	ND
M. thermosphactum	Traces-0.5
P. fluorescens	ND
P. schuylkilliensis	ND
P. schuylkilliensis	ND
P. putida biotype A	ND
P. putida	ND
S. aureus	ND

ND = Not detected, limit of detection 1.0 ng

NDMA Formation by Growing Bacterial Cultures from Sarcosine and Nitrite

Sarcosine (N-methylglycine) is one of the most commonly occurring N-nitrosatable secondary amines which is involved in transmethylation reactions (Friedman, 1972). It has been quantitated in the cat by Tallan et al. (1954), who reported that sarcosine was present at the levels of 1.3 mg/100 g of bladder and 4.3 mg/100 g of liver. Relatively large amounts of free sarcosine have been found in the muscles of

cartilaginous and ganoid fish (Vul'fson, 1961; Schaefer, 1962).

In low moisture model system studies involving the heating of samples under conditions similar to those encountered in the pan-frying of bacon, sarcosine was suggested to be a major precursor of NDMA (Gray et al., 1978). The present study indicated that only trace amounts of NDMA (0 to 0.57 µg) were detected when the test organisms were incubated for 48 hours in TSB medium containing 0.25% (w/v) sarcosine and 0.05% (w/v) sodium nitrite (Table 15). The

Table 15. NDMA formation by growing bacterial cultures from sarcosine (0.5 g) and sodium nitrite (0.1 g) in 200 ml of TSB medium.

Organism	µg NDMA formed/ 200 ml culture	% Conversion[a] Sarcosine ⟶ NDMA
E. coli E-2	0.05	0.09
E. coli K-12	0.08	0.14
L. bulgaricus	0.11	0.19
M. thermosphactum	0.21	0.37
P. fluorescens	0.07	0.12
P. schuylkilliensis	0.07	0.12
P. schuylkilliensis	0.05	0.09
P. putida biotype A	0.09	0.16
P. putida	Trace	--
S. aureus	0.57	1.00

[a] x 10^4

highest NDMA yield was observed with S. aureus and M. thermosphactum cultures. These results indicated one of three possibilities:

1) Sarcosine was N-nitrosated to N-nitrososarcosine, but bacteria were not capable of decarboxylating the resulting N-nitroso amino acid to NDMA.

2) Sarcosine was not N-nitrosated.

3) Sarcosine was decarboxylated by bacteria to dimethylamine but the decarboxylation process was not sufficiently fast to accumulate sufficient quantities of dimethylamine for the N-nitrosation reaction.

The last possibility did not prove to be true since all the test organisms failed to produce typical Moller decarboxylase reactions even after extended periods of incubation.

Detection of N-Nitrososarcosine by High Performance Liquid Chromatography

To ascertain whether sarcosine was N-nitrosated during the course of the incubation period, five 48 hour cultures were analyzed with HPLC for their N-nitrososarcosine content. Results of this analysis (Table 16) indicated that M. thermosphactum, P. fluorescens and S. aureus are very active in N-nitrosating sarcosine to N-nitrososarcosine. The amounts of N-nitrososarcosine produced in each culture were 540, 640 and 2000 µg/200 ml, respectively

Table 16. Determination of N-nitrososarcosine in bacterial cultures by HPLC.

Organism	N-nitrososarcosine µg/200 ml	% Conversion Sarcosine → N-nitrososarcosine
E. coli E-2	ND	--
L. bulgaricus	ND	--
M. thermosphactum	540	0.08
S. aureus	2000	0.30
P. fluorescens	640	0.10

ND = Not detected; limit of detection 0.5 ng

These results were compared to those obtained in Table 15 in which NDMA formation by growing bacterial cultures from sarcosine and nitrite was evaluated. The comparison showed that only a minor fraction of N-nitrososarcosine formed was decarboxylated to NDMA. Again, these results confirmed the data previously obtained by the Moller decarboxylase test.

Chemical N-nitrosation of sarcosine has been reported to proceed at a faster rate than the N-nitrosation of dimethylamine (Mirvish, 1971). The present study indicated similar results. Mirvish et al. (1973) suggested that the faster N-nitrosation rate of sarcosine (at optimum pH) compared to that of the secondary amine, dimethylamine, is due to the complex ionization of the amino acid which has two pK values, one for the amine group and one for the carboxylic group.

Formation of NDMA from N-Nitrososarcosine by Growing Bacterial Cultures

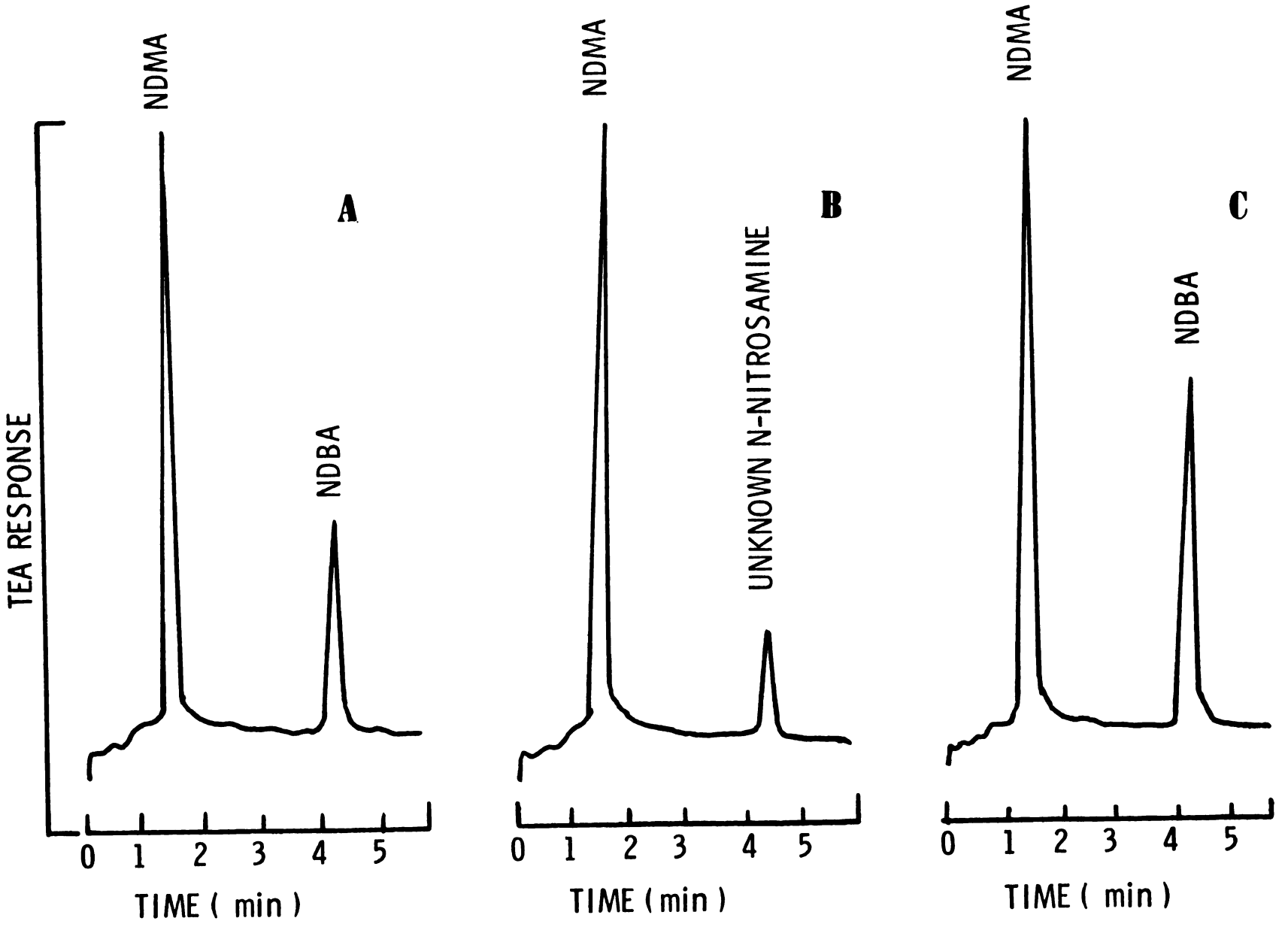
None of the bacterial cultures used, except L. bulgaricus, were able to grow when incubated for 48 hours in a TSB medium containing 0.1% (w/v) N-nitrososarcosine. Although L. bulgaricus demonstrated visible growth, N-nitrosamine analysis of the methylene chloride extract of the culture was negative for NDMA. Failure of these bacteria to grow in the presence of 0.1% (w/v) N-nitrososarcosine may be due to a fatal mutagenic activity associated with this compound. Baldwin et al. (1976) reported that some N-nitroso compounds were shown to be mutagenic in bacterial tests. Stanier et al. (1976) reported that the most powerful class of mutagens are the alkylating agents, some examples of which are mustard gas, ethylmethane sulfonate and N-methyl-N-nitro-N-nitrosoguanidine (N-Nitrosoguanidine). The latter is the strongest chemical mutagen known. It induces one or more mutations within each surviving cell when used to treat E. coli under optimal conditions (Stanier et al., 1976). However, the bacteria will not grow if the mutation involves an essential growth mechanism or induces a nutritional requirement (which is not provided in the growth medium). The growth of L. bulgaricus under these conditions, i.e., in the presence of N-nitrososarcosine, could be due to inherent impermeability of these bacterial cells to N-nitrososarcosine (Momose, Personal Communication, 1981). Ames et al.

(1973) reported that the lipopolysaccharide that normally coats the bacteria Salmonella typhimurium, acts as a barrier to penetration of mutagens to the cell membrane, and the removal of this barrier by mutation, improves the sensitivity of these test strains (S. typhimurium) to mutagenic compounds.

Identification of the Unknown N-Nitrosamine

An unidentified compound was detected (unknown peak) when the methylene chloride extract of Pseudomonas cultures were injected into the GC-TEA system for NDMA analysis. This unknown peak was observed only when sarcosine or trimethylamine oxide were used as the precursors of NDMA. To identify this peak, several authentic N-nitrosamines in methylene chloride were injected into the GC-TEA system. By comparison, it was found that this peak had a retention time similar to that of N-nitrosodibutylamine (NDBA). Multiple injection of the unknown N-nitrosamine (in the sample) and standard NDBA gave a single peak which coincided exactly with the retention time of the standard NDBA (details are illustrated in Figure 6). Therefore, the N-nitrosamine was tentatively identified as NDBA. Photolysis of the unknown N-nitrosamine was also evident when the methylene chloride extracts were subjected to UV irradiation at 366 nm. This confirmed the identity of N-nitrosamine of the sample as suggested by Doerr and Fiddler (1977).

Figure 6. GC-TEA chromatogram of a standard N-nitrosodibutylamine and N-nitrosodimethylamine (A), Pseudomonas culture methylene chloride extract (B), and Pseudomonas culture extract plus standard N-nitrosodibutylamine (C).


TEA RESPONSE
NDMA
NDBA
A
0 1 2 3 4 5
TIME (min)
NDMA
UNKNOWN N-NITROSAMINE
B
0 1 2 3 4 5
TIME (min)
NDMA
NDBA
C
0 1 2 3 4 5
TIME (min)

Precursors of NDBA

Several experiments were designed in an attempt to identify the possible precursors of NDBA. These included incubation of P. fluorescens in a TSB medium containing nitrite and one of the following compounds: dibutylamine, n-butylamine, and butanal. N-Nitrosamine analysis of these cultures (after standard incubation and extraction) showed that only the dibutylamine-containing culture was positive for NDBA. These results indicated that neither butylamine, nor butanal was a precursor for NDBA under these experimental conditions. This also meant that dibutylamine had to be either originally present in the incubation media (TSB) or synthesized by the bacterial cultures (Pseudomonas species) as a result of their metabolic activity.

Preparation and Analysis of Gid-deed

Residual Nitrite and N-Nitrosamines in Gid-deed

Historically, meat preservation with salt preceeded the intentional use of nitrate and nitrite by many centuries. Binkerd and Kolari (1975), who reviewed the history and use of nitrate and nitrite in the curing of meat, indicated that meat preservation with salt was first practiced in the saline desert of Hither Asia and in coastal areas. During the late Roman times, it was noticed that red patches were formed on the surface of meat preserved with salt (Sofos

et al., 1979). Desert salts (rock salt) often contain nitrate and borax as impurities (Rubin, 1977). The thought that the reddening effect was due to nitrate impurities of the salt led to the deliberate addition of saltpeter or nitre ($Ca(NO_3)_2$) which was formed by nitrifying bacteria and found on the walls of caves and stables (Jensen, 1954; Crosby and Sawyer, 1976).

The traditional method of Gid-deed preparation and processing is somewhat similar to dry curing of ham and bacon. In both cases, dry salt (NaCl) is rubbed on the meat surface, and the final Gid-deed product is cooked or fried in a similar manner to bacon.

N-Nitrosamine analysis of the salted-sun dried gid-deed samples and the fried samples (Table 17) indicated that NPYR

Table 17. N-Nitrosamine levels (µg/kg) in raw (salted-sun-dried) and oil fried gid-deed.

Treatment	Salted-Sundried		Oil Fried	
	NDMA	NPYR	NDMA	NPYR
(A) refined salt	ND	ND	ND	ND
(B) refined salt + NO_2^-	ND	ND	0.43	0.55
(C) refined salt + NO_3	ND	ND	ND	ND
(D) rock salt	ND	ND	0.30	0.33

ND = not detected; limit of detection 1.0 ng.

and NDMA were not present in the salted-sundried samples. However, oil frying of samples B and D (in which 120 mg/kg of nitrite and refined salt or rock salt were used, respectively) resulted in detectable levels of both NPYR and NDMA. These preliminary results indicated that N-nitrosamine formation can be avoided by using refined salt in gid-deed preparation. Similar observations were made by Fong and Chen (1976) who reported that, when crude salt was replaced by refined salt and benzoic acid was added to control microbial growth, lower N-nitrosamines were detected in salted and dried marine fish.

The detection of N-nitrosamines in the rock salt-treated samples and their absence in the samples treated with nitrate could be due to a number of reasons including: ingoing nitrate level was higher in the rock salt-treated sample; the bacterial load of the rock salt was higher or more active in reducing nitrate to nitrite, and finally rock salt may contain other impurities that catalyze N-nitrosamine formation.

Nitrite analysis of the salted-sundried samples indicated that N-nitrosamine formation in the fried samples is correlated to their residual nitrite content. Both the nitrite- and the crude salt-treated samples (B and D) had residual nitrite contents of approximately 25 mg/kg (Table 18). The nitrate-treated sample (sample C) contained slightly less (18 mg/kg) residual nitrite after sun drying than both

Table 18. Residual nitrite (mg/kg) in raw (salted-sundried) gid-deed.

Treatment	Nitrite Level (mg/kg)
(A) refined salt	ND
(B) refined salt + NO_2^-	25
(C) refined salt + NO_3^-	18
(D) rock salt	25

ND = not detected

samples B and D. Neither N-nitrosamines nor residual nitrites were detected in sample (A) in which refined salt was used. Recent studies have indicated that it is the residual and not the initial nitrite level that influences N-nitrosamine formation in bacon (Dudley, 1979; Sebranek, 1979). It is also noted that the higher the residual nitrite in bacon, the higher the possibility of N-nitrosamine formation (Sebranek, 1979). The present study indicated similar trends, i.e., when residual nitrite was detected, N-nitrosamines were produced, although the presence of 18 mg/kg of residual nitrite in the nitrate-treated sample did not seem to result in any detectable N-nitrosamines after frying.

The fact that N-nitrosamines were not detected in the unfried sundried samples indicated that their formation is associated with the high temperature of frying. In this respect, N-nitrosamine formation in gid-deed is quite

similar to their formation in bacon, i.e. they are frequently detected in fried bacon but not in the raw product. However, the quantities of N-nitrosamines formed in gid-deed during oil frying are much less than those encountered in fried bacon. In bacon, the fat is located in close proximity with the precursors of N-nitrosamines. Being an excellent heat transfer medium, fat in the adipose tissue causes the internal temperature of bacon to increase rapidly towards the end of the frying period and accelerate N-nitrosamine formation. On the other hand, lamb portions which are usually used in gid-deed preparation are much leaner than pork bellies, therefore a frying medium (usually olive oil) is used. In this respect, gid-deed frying is similar to the frying of other cured meats such as ham. In these products, the internal temperature increases at a slower rate during the course of frying. This is also reflected in the levels of N-nitrosamine which are formed. Based on a survey of N-nitrosamines in Canadian cured meats, Holland et al. (1981) stated that processed meats other than bacon do not contain significant amounts of NPYR, NPIP, NDBA, NDEA and NDMA. Similarly, the Nitrite Safety Council (1980) concluded that cured meat products such as cooked sausages, semi-dry and dry sausages, as well as fried slices of most dry-cured hams and shoulders, were found to be free of N-nitrosamines. Also, the application of several typical home or institutional cooking procedures did not cause N-nitrosamine

formation in the various sausages (Nitrite Safety Council, 1980). The detection of NPYR and NDMA in the gid-deed sample to which nitrite was not added (crude salt-treated sample) indicated that nitrates may be present in the crude salt, which may have been reduced to nitrite by the meat-reducing conditions (e.g. NADPH, $NADH+H^+$) or via bacterial nitrate reductase activity. The resulting nitrites ultimately participated in N-nitrosamine formation.

From a quantitative standpoint, NPYR and NDMA were detected in gid-deed samples B and D in relatively low concentrations compared to the levels which have been encountered in bacon. This is probably due to differences in fat distribution in lamb meat and pork bellies especially if the suggestion of Bharucha et al. (1979) is true: that N-nitrosamine formation during frying of bacon occurs essentially if not entirely in the fat phase, after the bulk of the water is removed. The presence of increased concentrations of salt in the gid-deed samples (1.5%) may have reduced the chance for N-nitrosamine formation. Fan and Tannenbaum (1973) reported that increasing the chloride ion would result in the formation of NOCl which decreases the efficiency of the N-nitrosation reaction.

Precursors of NPYR and NDMA in the Fried Gid-deed

As stated by Gray (1981), free proline appears to be the most probable precursor of NPYR in bacon. However, the

exact mechanism of how proline is converted to NPYR is not fully understood. Recent evidence suggests that N-nitrosation of proline to N-nitrosoproline followed by decarboxylation to NPYR is the most probable mechanism for NPYR formation in bacon (Gray, 1976; Lee, 1981). The total amino acid composition of muscle protein is quite constant regardless of the species or the muscle from which it is obtained (Schweigert et al., 1945; Blum et al., 1966). Schweigert and Payne (1956) assayed fresh beef, lamb and pork for their amino acid composition. As a percentage of protein, proline made up 5.4% of beef muscle proteins, and 4.6% and 4.8% of pork and lamb muscle proteins, respectively. Free proline has been reported to be present in lamb in fairly high concentrations (0.28 mg/g) (Baldwin et al., 1976). Therefore, it is very probable that free proline is the major precursor of NPYR in fried gid-deed. Also, the mechanisms of NPYR formation in bacon are expected to be operative in NPYR formation in fried gid-deed. Based on this assumption, the free proline content of gid-deed and fresh lamb meat was determined.

Although model system studies have implicated a number of compounds including dimethylamine, trimethylamine, quarternary ammonium compounds, sarcosine and lecithin as possible precursors of NDMA, the actual precursor of NDMA in fried bacon as well as the exact mechanism of its formation remain to be determined (Fiddler et al., 1972;

Pensabene et al., 1975; Eisenbrand et al., 1976). Gray et al. (1978) concluded that both sarcosine and phosphatidylcholine can contribute to NDMA formation during the frying of bacon. Since most of these compounds are naturally present in meat, regardless of the species differences, NDMA formation in fried gid-deed is not unexpected. Furthermore, the same compounds are suggested as possible precursors of NDMA in fried gid-deed.

Free Proline and Free Sarcosine Content of Fresh Lamb and Gid-deed

Results of the free amino acid analysis of the fresh lamb samples (Table 19) indicated that free proline was

Table 19. Free proline and free sarcosine in fresh lamb and raw (salted-sundried) gid-deed.

Sample	free proline (μmoles/100 g)	free sarcosine
fresh lamb	92.97	X
raw gid-deed	265.62	4X

present at a level of 92.97 μmoles/100 g sample. Baldwin et al. (1976) reported that for fifteen raw, deboned lamb leg samples, the mean free proline content on dry, fat-free basis was 0.28 mg/g (243.5 μmoles/100 g). They also showed that free proline was not detected in both raw beef and pork longissimus muscles. Contrary to these findings, measurable

quantities of free proline have been reported in pork bellies, ham and bovine muscles (McCain et al., 1968; Field and Chang, 1969; Field et al., 1971; Lakritz et al., 1976; Gray and Collins, 1977). As an example, Field et al. (1971) reported that 10 longissimus and 10 biceps femoris beef muscles had an average of 0.46 μmoles of free proline per gram of fresh muscle. In a similar study, Lakritz et al. (1976) reported that 6 green pork bellies had an average free proline content of 14.9 μM per 100 g of wet intact tissue. Lean and adipose tissues had free proline contents of 23.9 and 5.7 μM per 100 tissues, respectively.

Aging of ham and refrigerated storage of pork bellies and beef muscles have been reported to increase the free proline content in these products (McCain et al., 1968; Bowers, 1969; Field et al., 1971; Lakritz et al., 1976; Gray and Collins, 1977). The magnitude of the free proline increase varied with the length and temperature of storage. The results of this study (Table 19) showed that three-fold increases in the free proline content were observed after salting and sun drying for three days. Part of the increase in the free amino acid content is attributed to loss of moisture caused by plasmolysis during the addition of salt and sun drying (approximately 40% of the fresh weight). Based on the original moisture content of fresh lamb, the free proline content was calculated and found to be 159.37 μmoles/100 g of gid-deed. The other part of the increase is

probably due to protein hydrolysis caused by the proteolytic enzymes of the muscle or microbial degradation of proteins (Iodice et al., 1966; Parrish et al., 1966; Ayres et al., 1980).

Sarcosine is one of the most commonly occurring N-nitrosatable secondary amines (Friedman, 1972). Involved in transmethylation reactions, sarcosine has been quantitated in the cat ranging from 1.3 mg/100 g bladder to 4.3 mg/100 g liver (Tallan et al., 1954). Relatively large amounts of free sarcosine have also been found in the muscles of cartilaginous and ganoid fish (Vul'fson, 1961; Schaefer, 1962). As far as the results of this study are concerned, no specific values as to the presence of free sarcosine in the fresh lamb or gid-deed can be reported. This is due to the fact that in the ion exchange chromatographic analysis of free amino acids, no quantitative data were generated for sarcosine. However, relative peak areas of free sarcosine in fresh lamb apparently increased three folds during salting and sun drying. Part of the increase of the free sarcosine content in the samples could probably be due to the degradation of the phospholipid, lecithin (Anonymous, 1968). The other part could be attributed to protein degradation and transmethylation. In both cases, microbial enzymes are expected to be major catalytic factors along with natural meat enzymes.

SUMMARY AND CONCLUSIONS

The catalysis of N-nitrosamine formation by selected bacteria from dimethylamine and nitrite was studied. NDMA was detected in the incubation media of all the bacterial cultures used. However, growing cultures of L. bulgaricus, M. thermosphactum, S. aureus and the two E. coli strains E-2 and K-12, produced higher amounts of NDMA than the remaining species when incubated in 200 ml of TSB containing dimethylamine (0.5 g) and sodium nitrite (0.1 g).

Resting cell suspensions of these bacteria also were shown to be capable of N-nitrosating dimethylamine when incubated in 0.2 M phosphate buffer at pH 8.0. Under these conditions, P. fluorescens and P. putida were found to be the most active in N-nitrosating dimethylamine to NDMA in the presence of sodium nitrite.

Bacterial catalysis of the N-nitrosation of dimethylamine has been shown to increase with increasing cell concentration. Similarly, N-nitrosamine formation increased with increasing concentrations of nitrite in the incubation medium. NDMA formation (by bacteria) also increased as the incubation temperature was increased, and decreased when the incubation time was extended beyond four days.

E. coli E-2, *E. coli* K-12 and *S. aureus* were able to form appreciable amounts of NDMA when sodium nitrate (0.25%) was used in the incubation medium instead of nitrite.

Lower quantities of NDMA were detected when the secondary precursors were incubated with bacteria in 200 ml of TSB medium containing 0.5 g of each of the precursors (separately) and sodium nitrite (0.1 g).

None of the bacterial species investigated were capable of producing NPYR from proline and nitrite. However, *M. thermosphactum* produced traces of NPYR from N-nitrosoproline.

Trace amounts (0.30 to 0.55 μg/kg) of NPYR and NDMA were detected in some fried gid-deed samples. The N-nitrosamine-positive gid-deed samples were those to which nitrite or crude salt have been added.

The residual nitrite content of gid-deed was determined and was found to be highly correlated to N-nitrosamine formation in the fried samples, except in the case of the nitrate-treated sample.

The free proline and sarcosine contents of fresh lamb were also determined and were found to increase during salting and sun drying; however, part of the increase was due to loss of moisture during the drying of the samples.

The conclusions reached as a result of this study are summarized below:

1. Bacteria can possibly contribute to N-nitrosamine formation in vivo or in food systems, if enough nitrate or

nitrite is present.

2. Bacterial catalysis of N-nitrosamine formation from immediate precursors (dimethylamine and nitrite) is probably enzymatic in nature. However, other catalytic factors may have contributed to the total NDMA formation.

3. Higher bacterial counts are necessary before any detectable levels of N-nitrosamine can be formed. In this respect, foods will show signs of spoilage which will provide a built-in safety factor and thus reduce the possibility of ingesting food products that contain preformed N-nitrosamines.

4. The bacterial species which are capable of N-nitrosating dimethylamine in the presence of nitrite are widely distributed in nature. However, those which are capable of producing NDMA from nitrate and dimethylamine are not as numerous.

5. The distribution of bacteria which are capable of decarboxylating L-proline, and N-nitrosoproline is narrow i.e. very few bacteria are capable of decarboxylating these compounds.

6. Very few bacterial species are capable of decarboxylating sarcosine or have the ability to grow in a medium containing N-nitrososarcosine.

7. In the presence of bacteria, N-nitrosamines can be synthesized even in the absence of the traditionally recognized precursors.

8. Bacteria can also degrade preformed N-nitrosamines; however, the distribution of these bacteria in nature is yet to be determined.

PROPOSALS FOR FUTURE RESEARCH

This study has raised several important questions. One is related to the possibility of N-nitrosamine formation in food products in which bacteria, molds or yeasts are added and attain very high concentrations. Such products include yogurt, buttermilk, roquefort cheese, sauerkraut, some oriental fermented foods and pickles. It would be interesting to investigate the possibility of N-nitrosamine formation in these food products under normal production procedures.

All the NDMA precursors utilized in this study were used at a constant concentration of 0.5 g precursor/200 ml of TSB medium (0.25% w/v). Similarly, the nitrite concentration was adjusted to 0.05% w/v (0.1 g sodium nitrite/200 ml of TSB medium), therefore resulting in a different nitrite/precursor ratio for each compound. Since NDMA yield is affected by the aforementioned ratio, it would be very desirable to design another experiment in which equimolar concentrations of all precursors are used, and compare NDMA yields and relate that to NDMA formation from dimethylamine and nitrite under similar conditions.

The ratio of nitrite/precursor was less than 1.0 for all the compounds used in this study; therefore, it is justified

to examine NDMA formation under the same conditions using excess of nitrite, i.e. using nitrite/precursor ratios of 1, 2, 3 and 4.

This study has also shown that NDMA formation by bacteria from nitrite and dimethylamine was primarily catalyzed by a heat sensitive, pH dependent mechanism, possibly enzymes. These data supported the results of Sander (1968) and Kunisaki and Hayashi (1979). However, no conclusive evidence has been presented so far on whether N-nitrosamine formation by bacteria is enzymatic or otherwise. Therefore, further studies should be pursued utilizing nitrite, secondary amines and cell free systems to disclose the nature of N-nitrosamine formation by bacteria. Furthermore, attempts should be made to isolate the enzyme(s) (if any) responsible for the catalysis and elucidate their kinetics.

Finally, betaine aldehyde, betaine and dimethylglycine (choline biodegradation intermediates) were suggested in this study as possible precursors of NDMA. It would be interesting to add these chemical compounds (if they are available) to a microbiological system (one at a time) along with nitrite and monitor NDMA formation.

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تلخيص

تحفيز تكون النيتروزامينات بواسطة البكتيريا

بقلم

يوسف السباعي البسوط

أجريت هذه الدراسة لبحث دور البكتيريا في تكون النيتروزامينات "١" من المصادر الأولية المباشرة (ثنائي أمين المتابل وأملاح النترات)، وبحث الكائنات تكون نيتروزامينات عند من المركبات الكيمادية (مصادر ثانوية) عبر طريقه النشاط البكتيري، وقد استعملت في هذه الدراسة عشرة اصناف من البكتيريا، خمس منها من جنس الزيدوموناس والخمس الأخرى موزعة كما يلي: لاكتوباسلس بوجاريكس ستافيلوكوكس اوريانس، ميكروباكتيريم ثرموسفاكتم وثلاثة من الانتيريشيا كولي، وقد أظهرت الدراسة عينة النتائج التالية:

١- تمكنت جميع أنواع البكتيريا المستعملة في هذه الدراسة من "تصنيع مادة" ثنائي ميثايل النيتروزامين من المصادر الأولية المباشرة. حدث ذلك في حالة نمو البكتيريا في بيئة نهرضوم فول الصويا "ترتيك صوي بروث" وفي حالة التوقف عن النمو في محلول الفسفات المعادل الأس الهيدروجيني ٨.

٢- تناسب تكون النيتروزامينات تناسبا طرديا مع زيادة عدد الخلايا البكتيرية وتركيز مادة النترات ومع ارتفاع درجة حرارة التحضين.

٣- تمكنت بعض أنواع البكتيريا المستعملة من انتاج ثنائي ميثايل النيتروزامين من بعض المصادر الثانوية مثل الكولين وفوسفاتيدات الكولين والليسيثين وثنائي ميثايل الأمين.

٤- تبين من خلال هذه الدراسة أيضا أن القدر المستعمل في ليبيا يمكن أن يكون محتويا على هذه المركبات بكميات قليلة جدا، إذا تم تخليته في الزيت على درجة حرارة مرتفعة، شريطة أن يكون الملح المستعمل من نوع غير مكرر ومحتوى على كمية كافية من أملاح المغنيزيات، أما القدر الغير مقلى فقد كان خاليا من هذه المركبات.

"١" مواد ثبت من خلال التجارب السابقة أنها تسبب أنواعا عديدة من السرطان مثل سرطان الكبد والرئة والمرئ والكلية في حيوانات التجارب ويشتبه ان تكون كذلك في الانسان