SMOKE-RELATED N-NITROSO COMPOUNDS IN CURED MEAT SYSTEMS

By

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ABSTRACT

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The role of wood smoke in the formation of the heterocyclic N-nitrosamines, (N-nitrosothiazolidine (NTHZ), N-nitrosothiazolidine carboxylic acid (NTCA) and related compounds) in aqueous model systems and in smoked, cured bacon was investigated. The precursors and the mechanisms involved in the formation of these compounds were also studied. A survey of various smoked foods was also conducted in order to determine the distribution of these compounds in foods.

The reaction of smoke condensate with cysteamine/cysteine and nitrite in aqueous model systems resulted in the formation of various 2-substituted N-nitrosothiazolidines and their corresponding N-nitroso derivatives. Formaldehyde present in smoke was responsible for the formation of NTHZ and NTCA, while acetaldehyde, glycolaldehyde and methylglyoxal were involved in the formation of the 2-methyl-, 2-hydroxymethyl- and 2-acetyl- N-nitroso compounds, respectively. NTHZ and 2-hydroxymethyl-N-nitrosothiazolidine (2-HMNTHZ) were the two major N-nitrosamines found in the cysteamine/smoke condensate and nitrite reaction systems.

Various smoked foods were surveyed and were found to contain a wide range of NTCA and 2-hydroxymethyl-N-nitrosothiazolidine carboxylic acid (2-HMNTCA). Smoked, cured poultry products contained the highest amounts of NTCA (1000-1240 µg/kg) and 2-HMNTCA (15-610 µg/kg), while smoked salmon contained the least amounts of NTCA and related compounds.

The investigation into the effects of smoke duration of NTHZ and NTCA formation in bacon showed that the majority of these N-nitrosamines are formed within the first 30 min, followed by a gradual decrease in concentration. Raw bacon contained greater amounts of NTCA than fried bacon. Kinetic studies simulating the pan-frying of bacon, indicated that approximately 2% of NTCA was converted to NTHZ during the frying process. These studies suggested two different mechanisms of NTHZ formation in raw and fried bacon. The first pathway involves NTHZ formation from the interaction of cysteamine/nitrite and formaldehyde in smoked bacon, while the other involves NTCA decarboxylation to NTHZ during frying.

Pork bellies stitch pumped with 0.2% and 0.4% glucose contained only small amounts of NTHZ and NTCA compared to smoked controls. These experiments indicated that NTCA, NTHZ and other related compounds are formed primarily from smoking rather than from breakdown products of glucose.

α-Tocopherol was found to be effective in inhibiting volatile N-nitrosamine formation in fried bacon cooked under standard laboratory conditions and under various home-cooking conditions. However, α-tocopherol had no impact on levels of NTCA. Bacon processed with reduced nitrite levels (80 mg/kg) contained lower concentrations of N-nitrosamines than bacon processed with 120 mg/kg of nitrite.

DEDICATION

Dedicated to my family

Kelly Mandagere and Jr

Dr. Subra and Rukmini Mandagere

Kalyani, Usha, Mike and Jena

for their love, unending support, and sacrifice

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INTRODUCTION

The consistent occurrence of N-nitroso compounds in some foods is a major concern due to their potent carcinogenicity. Laboratories studies have shown that the majority of these compounds elicit carcinogenic responses in a number of animal species (Preussmann et al., 1976; National Academy of Sciences, 1981). The main sources of N-nitrosamines in the human diet are fried bacon, smoked cured fish, various other smoked cured meats, and beer (National Academy of Sciences, 1981). The N-nitrosamines found in these foods are mainly N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR), N-nitrosodiethylamine (NDEA), and N-nitrosopiperidine (NPIP).

Extensive research in the last ten years has resulted in significant reductions in N-nitrosamine levels in cured meats (Havery and Fazio, 1985). These reductions have been brought about by the lowering of ingoing levels of nitrite to 120 mg/kg in bacon, along with the addition of compounds capable of blocking the nitrosation reaction. Examples of blocking agents are α-tocopherol and sodium ascorbate.

Recently, N-nitrosothiazolidine (NTHZ) and N-nitrosothiazolidine carboxylic acid (NTCA) have been identified in bacon and in other cured meats (Gray et al., 1982; Kimoto et al., 1982; Helgason et al., 1983). The formation of NTHZ and 2-methyl-N-nitrosothiazolidine (MNTHZ) in aqueous model systems has been demonstrated by Coughlin (1979) and Sakaguchi and Shibomoto (1979), respectively. The reaction system contained cysteamine / formaldehyde / nitrite and cysteamine / acetaldehyde / nitrite which, when heated, produced NTHZ and MNTHZ, respectively. Gray et al. (1982) hypothesized, on the basis of model system studies, that NTHZ formation in fried bacon could arise from the reaction of glucose fragmentation products with cysteamine and nitrite and by the thermal decarboxylation of NTCA. Subsequent studies by

Pensabene and Fiddler (1983) indicated that wood smoke processing was involved in NTHZ formation in bacon. However, it is not clear which is the primary mechanism of NTHZ formation in bacon and other cured meats.

Initial studies on the mutagenicity of NTHZ using the Ames test by Mihara and Shibomoto (1980) indicated that NTHZ was a strong mutagen when metabolically activated. NTCA has been shown to induce diabetes in experimental animals (Helgason et al., 1983). Onset of diabetes was observed in the male offsprings of mice that had been fed NTCA or smoked cured meats containing high levels of NTCA. Results of epidemiological studies in Iceland indicated a positive correlation between the incidence of juvenile diabetes in male children and the consumption of large quantities of smoked cured mutton by their parents. Further research is required in this area as there appears to be a potential health hazard associated with the consumption of smoked cured meats.

The major objectives of this dissertation were to investigate the following aspects of NTHZ and NTCA formation in bacon:

1. To study the role of wood smoking in the formation of NTHZ and NTCA in bacon, and to elucidate the mechanism(s) of NTHZ and NTCA formation in smoked cured meats.
2. To determine the levels of NTCA in a number of smoked food products.
3. To determine the effects of wood smoking time on NTHZ and NTCA formation in bacon.
4. To investigate the kinetics of the thermal decomposition of NTCA and subsequent formation of NTHZ.
5. To clarify the precursor role of glucose in NTHZ and NTCA formation in raw and fried bacon.
6. To determine the effectiveness of α-tocopherol and sodium ascorbate, along with reduced nitrite levels (80 mg/kg) in inhibiting NTHZ and NTCA formation in bacon.

7. To evaluate the effectiveness of α-tocopherol in reducing N-nitrosamine levels in fried bacon cooked by consumers at home.

REVIEW OF LITERATURE

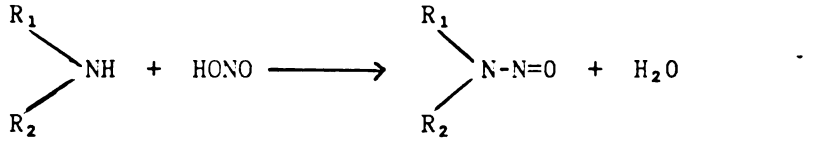
N-Nitroso compounds as a group have been shown to be highly mutagenic and carcinogenic in experimental animals (National Academy of Sciences, 1981). More than 80% of the 300 N-nitroso compounds tested have been found to be carcinogenic in one or more animal species (Magee et al., 1976). N-Nitroso compounds also exhibit mutagenic, teratogenic or embryopathic properties in most species of animals including rats, pigs, rabbits, sheep, mink, mice, guinea pigs and in subhuman primates (Preussmann et al., 1976; Wishnok, 1979; Gray and Randall, 1979). Accidental exposures of humans to NDMA have resulted in extensive liver damage (Preussmann et al., 1979). It is highly probable that N-nitroso compounds, by virtue of their demonstrated carcinogenicity in more than one animal species, are potentially hazardous to man.

Extensive research in the last twenty years has revealed the ubiquitous distribution of these compounds in the environment (Fine, 1977). Human exposure to N-nitroso compounds from sources other than the environment can also occur under physiological conditions. The acidic conditions of the stomach offer an ideal environment for in vivo formation of N-nitrosamines from ingested amines, amino acids and nitrite (Sander, 1967; Sen et al., 1969; Lijinsky et al., 1970; Lane and Bailey, 1973).

The formation and occurrence of N-nitrosamines and their toxicological and human health hazards have been adequately documented (Scanlan, 1975; Crosby and Sawyer, 1976; Gray and Randall, 1979; Sen, 1980; Havery and Fazio, 1985). In this review, the main focus will be on the occurrence and the chemistry of N-nitrosamine formation in cured meat products. However, in an attempt to put this subject into perspective, several related areas will also be discussed.

CHEMISTRY OF FORMATION

Physicochemical properties: N-Nitrosamines are formed primarily from the reaction between secondary amines and nitrous acid, but they can also be formed from primary, tertiary and polyamines (Gray and Randall, 1979).



In this reaction of secondary amines, R_1 is an alkyl group while R_2 may be an alkyl, alcohol, aryl or a wide variety of functional groups. The only common feature of all N-nitroso compounds is the presence of the N-N=O functional group. The physical and chemical properties of the various N-nitroso compounds depend on the substituents on the amine nitrogen. Simple low molecular weight dialkyl N-nitrosamines are water soluble, while the higher molecular weight N-nitrosamines are soluble in lipid and organic solvents. All N-nitrosamines are sensitive to ultraviolet and visible light, and strong acids which cleave the N-nitroso group. They are stable at high temperatures. The chemistry of the reactions of amines has been studied at length and detailed discussions of this subject matter can be found in reviews by Ridd (1961), Mirvish (1972, 1975), Challis (1981).

N-Nitrosamine formation: In acidic environments, nitrite is converted to nitrous acid (HONO) which then can react with primary, secondary and tertiary amines to form N-nitrosamines (Lijinsky et al., 1972). A primary amine can undergo conversion to a secondary amine and ultimately to a N-nitrosamine under cold acidic conditions (Ridd, 1961). The

N-nitrosation reaction occurs via electrophilic attack by the nitrosonium ion $(NO)^+$ on the electron rich amino nitrogen atom (Morrison and Boyd, 1974).

In the case of a primary aliphatic amine treated with nitrous acid, an unstable diazonium ion is formed which loses nitrogen to produce a carbonium ion (Figure 1). The carbonium ion can further undergo addition, elimination or rearrangement to produce a variety of deamination products like nitrogen gas, alkenes and alcohols (Morrison and Boyd, 1974). However, it has been demonstrated that N-nitrosamines can be formed in low yields from primary amines (Warthesen et al., 1976).

When a secondary amine is treated with nitrous acid, nitrosation occurs and a stable N-nitrosamine is formed (Figure 1). The first step for nitrosation of a tertiary amine is similar to the reaction path for primary and secondary amines in that the unshared electron pair on the unprotonated amine reacts with a nitrosating species (nitrous anhydride) to form the nitrosoammonium ion (Figure 1). This ion then undergoes cis-elimination of the nitroxyl ion to form an immonium ion which is hydrolyzed to a carboxyl ion and a secondary amine. The secondary amine is then nitrosated to the corresponding N-nitrosamine (Smith and Loeppky, 1976).

Fiddler et al. (1972) reported that the formation of N-nitrosamines from tertiary amines is less efficient than from secondary amines. When equal molar concentrations of dimethylamine/trimethylamine and nitrite were reacted, ten times more NDMA from the secondary amine was produced than from the tertiary amine (Fiddler et al., 1972).

Kinetics of the nitrosation reaction: The kinetics of nitrosation reactions have been widely investigated (Mirvish, 1972, 1975; Challis, 1981). Aqueous acidic solutions of nitrite salts (or nitrous acid) at pH 2-4 are the best known nitrosating media. Neither nitrous acid nor the nitrite ion react directly with amino compounds. For nitrosation

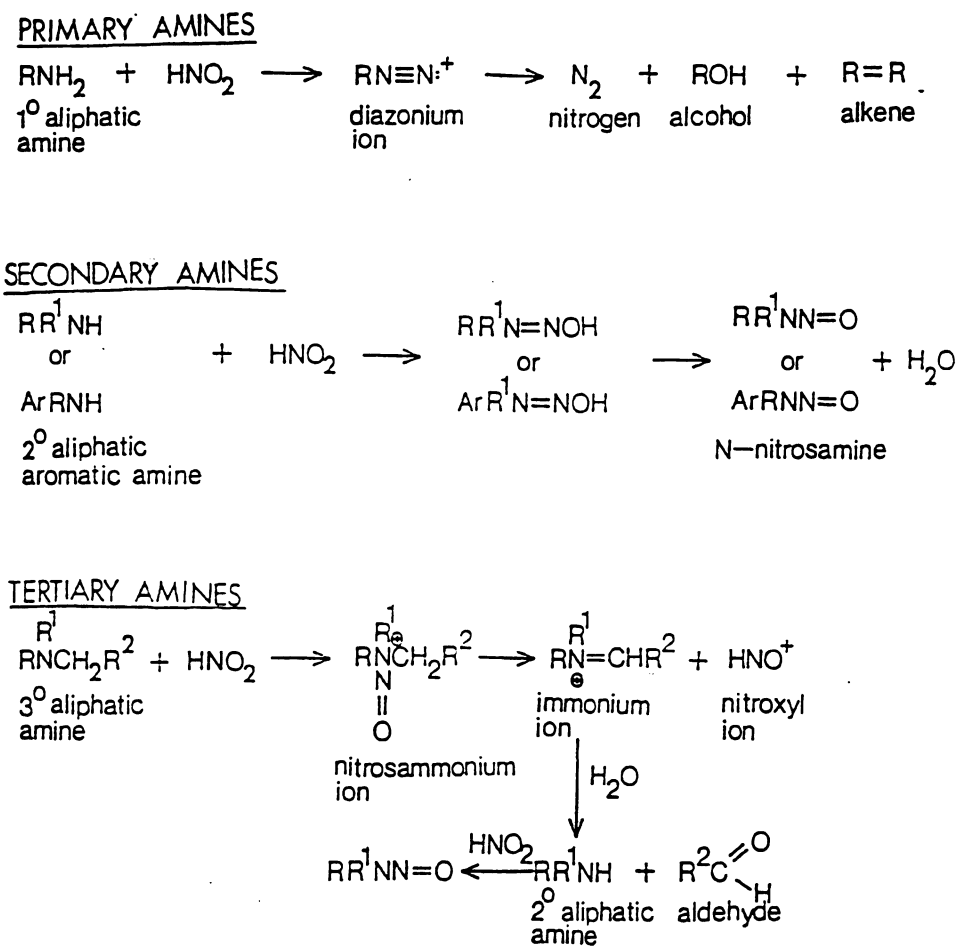
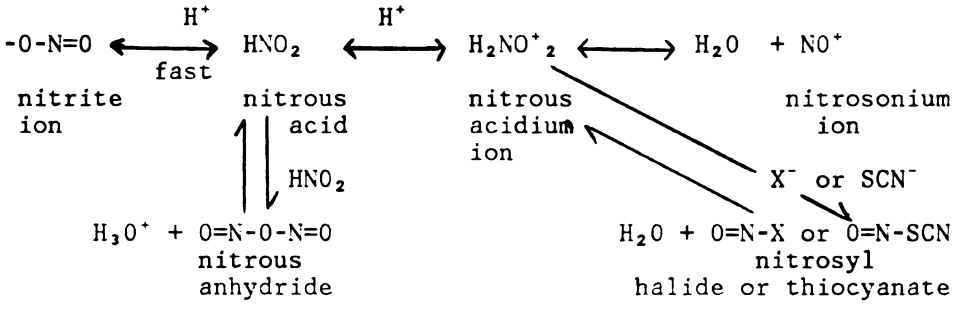


Figure 1: Reactions of primary, secondary and tertiary amines with nitrous acid (Lee, 1981).

reactions to occur, nitrite is first converted to nitrous acid (HNO_2, pKa 3.36) which is then converted to an active nitrosating agent. The actual nitrosating species can be one of the following depending on the reaction conditions: nitrous anhydride (N_2O_3), nitrous acidium ion ($H_2NO_2^+$), free nitrosonium ion (NO^+), nitrosyl halide (NOX) or nitrosyl thiocyanate (NOSCN) as shown below (Challis and Butler, 1968; Mirvish, 1975).



Nitrosation reactions are generally favored by acidic conditions and proceed via a two step mechanism. The first step of the nitrosation reaction involves a fast conversion of nitrite to nitrous anhydride or to the other nitrosating species. In the second step, N-nitrosamine is formed in a slow reaction between the nitrosating species and the unprotonated amine. Mirvish (1975) proposed an overall third order rate equation for the nitrosation of secondary amines.

$$\text{rate of N-nitrosamine formation} = k_1 (RR_1NH) (HNO_2)^2 \quad \text{---------- (a)}$$

$$\text{rate of N-nitrosamine formation} = k_2 (\text{total amine}) (\text{nitrite})^2 \quad \text{---------- (b)}$$

where K_1 and K_2 are the respective rate constants. Nitrosation reactions are first order with respect to amine concentration and second order with respect to nitrite concentrations.

In equation (a), the concentrations expressed are those of the unprotonated amine and undissociated nitrous acid (both of which are pH dependent), and k is independent of pH. In equation (b), the total concentrations are used and k varies with pH and shows a maximum value at pH 3.0 to 3.4 (Mirvish, 1975). In the pH range of 5 to 9, the rate of nitrosation of dimethylamine (DMA) increased ten fold for each unit decrease in pH (Mirvish, 1970).

Rate of nitrosation of a secondary amine is directly related to its basicity (Sander and Schweinburg, 1972). Weakly basic amines are favored over strongly basic amines in the nitrosation reaction since the former produces more unprotonated amine under acidic conditions. The nitrosation of a weakly basic amine follows second order kinetics (equation c)

$$\text{rate} = k\ (HONO_2)^2 \quad \text{---------- (c)}$$

since a decrease in acidity of a weakly basic amine results in an increase in the concentration of the unprotonated amine to the extent that the nitrosation reaction becomes independent of amine concentration (Sander and Schweinburg, 1972; Mirvish, 1975).

Sander and Schweinburg (1972) reported that the yield of N-nitrosamine from the weakly basic diphenylamine (pKa 0.79) was about 1000 times greater than from the strongly basic dipropylamine (pKa 10.9). Mirvish (1970, 1975) studied extensively the relationship between pH, amine basicity and the rate of nitrosation. Through a mathematical procedure it was shown that the pH optimum was 2.4 for nitrosation, assuming nitrosation was via nitrous anhydride. Lane and Bailey (1973) determined the optimum pH for nitrosation of dimethylamine in synthetic gastric juice under physiological conditions to be pH 2.5.

Catalysis: Halides and thiocyanates are very effective nitrosating species in the presence of nitrous acid and change the kinetics of diazotization reactions as shown in equation (d):

$$\text{rate} = k\,(RNH)\,(HONO)\,(H)\,(X) \quad \text{---------- (d)}$$

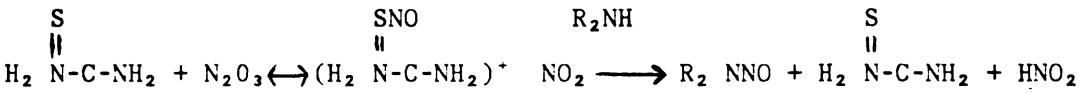
The relative rate of catalysis by these species is I < SCN < Br <<< Cl (Boyland et al., 1971; Fan and Tannenbaum, 1973; Coleman, 1978). As in the case of nitrous anhydride, the nitrosation step is rate limiting except in cases where free amine concentration becomes relatively high. Other anions having an accelerating effect on nitrosation reactions are phthalates, acetates and other anions of weak acids (Telling, 1972). The halide and thiocyanate ions exert their catalytic effects by becoming covalently bonded to nitrosyl derivatives (NOX). NOX are more effective nitrosating agents than nitrous anhydride (Telling, 1972). Since nitrous anhydride is actually the nitrosonium ion $(NO)^+$ covalently bonded to nitrite ion (NO_2^-), it is clear that other anions can form much more effective nitrosating species than nitrous anhydride.

Thiocyanate is present in considerable amounts in normal human saliva (10 - 30 mg/100mL), while saliva of smokers contains three to four times the normal level (Ladd et al., 1983). Therefore, the catalytic effects of thiocyanate in N-nitrosamine formation may be important in in vivo nitrosation reactions (Boyland et al., 1971; Boyland and Walker, 1974; Archer, 1983).

The chloride ion ranks the lowest in terms of catalytic activity. Since sodium chloride is a common food additive and is usually an ingredient in cure mixtures, its effects on the nitrosation of amines in foods have been investigated. Boyland et al. (1971) were able to demonstrate that sodium chloride had a very low catalytic effect around pH 2.0. Hildrum et al. (1975) reported that sodium chloride had a definite accelerating effect at pH 0.5, a slight inhibitory effect at

pH 2.5, and a definite inhibitory effect at pH 4.0 and 5.5. Masui et al. (1974) demonstrated catalysis of dimethylamine nitrosation by thiourea at pH 4.0.

In investigating the mechanism of this effect, Meyer and Williams (1981) showed that catalysis is caused by equilibrium formation of a S-nitroso adduct which acts as a potent transnitrosating agent.



Catalysis occurs in a manner similar to halide ions. However, the nitrosating capacity of the S-nitroso derivative of thiourea is greater than nitrosohalides. The order of efficiency for catalysis of N-nitrosomorpholine (NMOR) formation from morpholine is thiourea >>> SCN > Br in the ratio of 4,200:240:1 (Archer, 1983). Similarly, thiols form thionitrites which act as transnitrosating agents above pH values of 5 (Davies et al., 1978b; Dennis et al., 1979; Kunisaki and Hayashi, 1980). However, at pH 1 to 2 thiols are effective inhibitors of the nitrosation reaction because of the virtual irreversibility of S-nitrosation (Gray and Dugan, 1975; Davies et al., 1978a; Aldred et al., 1982; Williams and Aldred, 1982). Thionitrites present in a polypeptide or protein can also effect transnitrosation (Massey et al., 1978; Davies et al., 1980). This reaction may play an important role in N-nitrosamine formation in nitrite - cured foods, although this is, as yet, unproven.

Formaldehyde can catalyze the nitrosation reaction under neutral or even basic conditions (Keefer and Roller, 1973). The likely mechanism for these reactions, as proposed by Keefer and Roller, (1973), and studied in detail by Casado et al. (1981), involves reaction of the aldehyde and secondary amine to form an iminium ion. Nucleophilic attack by the nitrite ion on the iminium ion results in the formation of a

dialkylamino nitrite ester which then collapses to form the N-nitrosamine.

$$R_2NH + HCHO \longleftrightarrow R_2N = CH \longrightarrow \underset{\underset{O=N-O}{|}}{R_2N-CH} \longrightarrow R_2NNO + HCHO$$

Para-nitrosophenols, which are known to occur in smoked meats, have been reported to catalyze the nitrosation of pyrrolidine and morpholine (Davies and McWeeny, 1977; Davies et al., 1980) and diethylamine (Walker et al., 1979). The catalytic species is thought to be the quinone monoxime tautomer of the nitrosophenol which reacts rapidly with NOX to form the O-nitroso derivative (Figure 2). This is followed by the slower attack of the O-nitroso derivative by the amine, resulting in N-nitrosamine formation and regeneration of the nitrosophenol (Walker et al., 1979; Davies et al., 1980).

Phenols themselves react very rapidly with nitrous acid to produce C-nitrophenols (Challis, 1973) and thus an excess of phenol should inhibit nitrosation reactions. At high nitrite/phenol ratios, however, C-nitrosophenol formation will result in catalysis due to the formation of the O-nitroso derivative. Pignetelli et al., (1980, 1982) showed that 1,3-dihydroxyphenols, but not 1,2- and 1,4-dihydroxyphenols, are potent catalysts of amine nitrosation.

Despite the presence of some of these compounds in foods it is yet unclear as to the extent of their role in N-nitrosamine formation in foods.

Transnitrosation: The formation of N-nitroso compounds through the interaction of secondary and tertiary amines with nitrite and its related nitrosating agents is well documented as discussed earlier. In addition to these nitrosating agents, some C-nitro and C-nitroso, S-nitroso and N-nitroso compounds can act as nitrosating agents through the transfer of their nitroso group to amines, amides, urea and amino acids in acidic

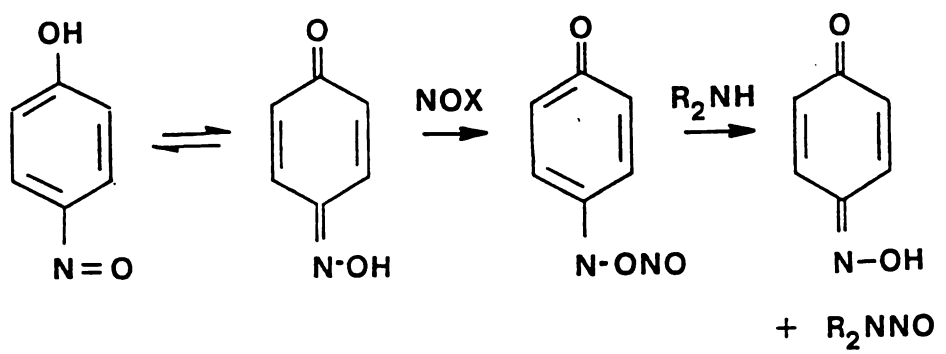


Figure 2: Catalytic role of phenols in N-nitrosamine formation (Ikins, 1982).

conditions, particularly in the presence of thiocyanates (Singer et al., 1977; Davies et al., 1978b; Fan et al., 1978).

Among the most active in these transnitrosating agents are several non-carcinogenic N-nitrosamines, such as N-nitrosoproline, N-nitrosohydroxyproline and nitroso-N-methylpiperazine. The transnitrosating capacity of these N-nitrosoamino acids is dependent on the basicity of the amino nitrogen (Singer et al., 1980). Tetranitromethane is the most powerful transnitrosating agent relative to all other C-nitroso compounds (Archer, 1983). Fan et al. (1978) observed that the presence of an electron withdrawing group like NO or Br bonded to the same carbon atom as the nitro group, makes it a very effective transnitrosating agent due to the weakening of the N-N bond.

Transnitrosating reactions probably occur because N-nitroso compounds release nitrous acid upon treatment with acid. Other organic N-nitro and N-nitroso compounds may liberate nitric oxide or nitrogen dioxide on heating, and these may be converted to active nitrosating species (Archer, 1983).

In an acidic environment, the transnitrosation reaction is similar in many respects to nitrosation by nitrous acid. The acidic environment of the stomach along with the presence of a catalyst such as thiocyanate from saliva can facilitate in vivo formation of strong carcinogenic N-nitroso compounds from weak or non-carcinogenic N-nitroso compounds via transnitrosation reactions (Cardy et al., 1979).

Heating aromatic N-nitroamines will facilitate the transnitrosation reaction (Buglass et al., 1975). Heating cleaves the N-N bond and liberates nitric oxide which can undergo oxidation in the presence of oxygen to nitrogen dioxide (Outram, 1979). Similarly, nitrite esters can undergo thermal and photolytic decomposition to produce an alkoxy radical (RO) and nitric oxide (Forrest et al., 1978; Coombes, 1979). Transnitrosation by nitrite esters may be important due to their

solubility in lipid media. Like C-nitroso compounds, electron withdrawing groups (X) at the beta position will lead to N-nitrosamine formation by the direct nucleophilic attack of the amine on the neutral nitrite ester (Challis and Shuker 1979, 1980; Challis et al., 1980).

As discussed earlier, the transnitrosating capacities of C-nitro and S-nitroso compounds are dependent on relative nitrite/phenol concentrations and pH, respectively (Davies et al., 1978; Archer, 1983). Thionitrites present in a polypeptide or protein can also effect transnitrosation (Massey et al., 1978; Davies et al., 1980). This reaction may play an important role in in vivo N-nitrosamine formation from ingesting nitrite - cured foods, although this has not been proven.

Inhibitors of N-nitrosamine formation: Since nitrite is a reactive chemical participating in redox reactions, the potential inhibitors for nitrosation reactions are numerous. Basically, any chemical which reacts with nitrite can compete with amines for the available nitrite. These include sulfhydryl compounds, certain aromatic compounds, ascorbates, tocopherols and phenols. Of these, ascorbic acid is perhaps the most extensively studied. This reductant is particularly valuable since it is acceptable for human consumption and has been shown to inhibit in vivo nitrosation reactions (Ohshima and Bartsch, 1981; Mirvish, 1981). Both ascorbic acid and ascorbate anion (pKa 4.3) rapidly reduce the nitrosating agent, NOX, to nitric oxide (Figure 3). Oxidation of ascorbic acid proceeds by the initial attack on the 3-hydroxy group by the nitrosating species, forming the nitrite ester, which decomposes to the semiquinone.

Further reaction of the semiquinone with an additional mole of nitrosating agent yields dehydroascorbate (Archer, 1983). Mirvish et al. (1972) first showed that ascorbic acid effectively blocked N-nitrosamine formation from pH 1 to pH 4. The ascorbate anion was more effective in inhibiting N-nitrosamine formation than ascorbic acid due to its greater

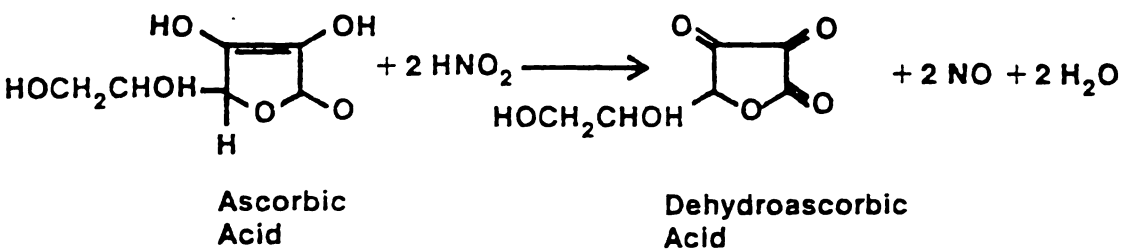


Figure 3: Reaction of ascorbic acid with nitrite (Mirvish, 1981).

nucleophilic activity (Kawabata et al., 1974; Mirvish and Schubik, 1974; Mottram et al., 1975; 1979). At pH 3 to 5, where the proportion of anion is very high, reaction with nitrite is so rapid that the formation of NO is rate limiting (Mirvish et al., 1972; Mirvish and Schubik, 1974).

Since ascorbic acid is water soluble, it is most effective as a nitrite scavenger in aqueous environments. Various ascorbyl and erythorbyl fatty acid esters and α-tocopherol are more effective inhibitors in lipophilic environments such as the fat phase of foods (Sen et al., 1976a; Pensabene et al., 1978; Fiddler et al., 1978; Mergens et al., 1978; Mergens and Newmark 1979, 1980; Gray et al., 1982).

α-Tocopherol is oxidized to a quinonoid product by the NOX with the subsequent formation of NO (Figure 4). In a mechanism analogous to the nitrite -ascorbic acid reaction, α-tocopherol reduces the nitrosating agent to a non-nitrosating species. Unlike many phenolic compounds, α-tocopherol cannot undergo C-nitrosation due to its fully substituted ring and thus cannot form a catalytic species (Walker et al., 1979). Pensabene et al. (1978) mixed α-tocopherol with the emulsifier Polysorbate 20 in order to facilitate its water solubility. When used in a two-phase (aqueous buffer/corn oil) model system at a concentration of 500 mg/kg, α-tocopherol inhibited N-nitrosopyrrolidine (NPYR) formation in the oil phase by 80% and 67% in the aqueous phase. Gray and Dugan (1975) reported over 90% inhibition of the nitrosation of dimethylamine (DMA) by α-tocopherol in an aqueous model system.

Coleman (1978) studied the effects of antioxidants on nitrosation reactions in model systems and reported inhibition by butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and ethoxyquin. Ethoxyquin was more inhibitory than BHT or BHA. Overall, the inhibitory effects of these antioxidants were relatively low compared to that of ascorbic acid (Coleman, 1978).

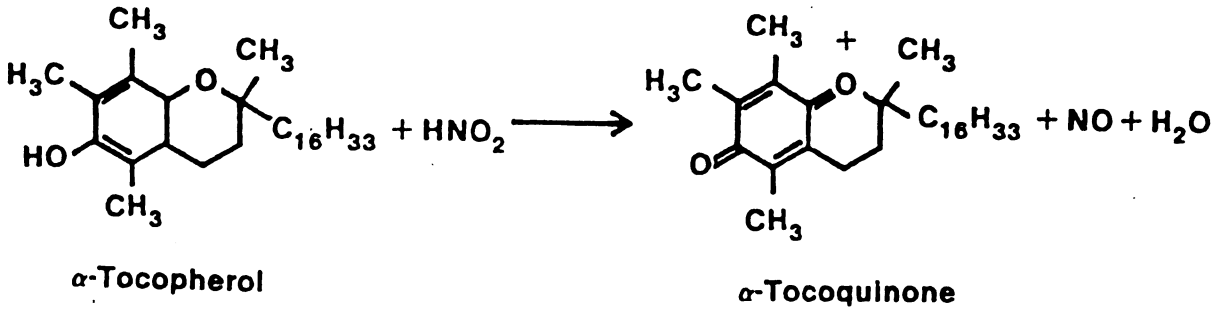


Figure 4: Reaction of α-tocopherol with nitrite (Mergans and Newmark, 1980).

PRECURSORS OF N-NITROSAMINES IN FOOD SYSTEMS

Nitrates, nitrites and oxides of nitrogen: Nitrates and nitrites are widely distributed in nature, particularly in plants and forage. Large concentrations of nitrate (1000 to 3000 mg/kg) are sometimes formed in green vegetables (Ashton, 1970; White, 1975; Lin and Lue, 1979; Lin and Yen, 1980). Nitrates also occur in water from natural sources or by contamination of ground water by agricultural practices (Comley, 1945; Burden, 1961). Other sources of nitrate include fruits and fruit juices, baked goods, cereals, milk and dairy products (White, 1975, 1976).

Nitrites, on the other hand, occur in only small concentrations in water and in vegetables, although high levels have been detected in storage-abused spinach and beets (Heisler et al., 1974). In addition to the sources listed above, nitrate and nitrite can originate in foods as intentional food additives. These chemicals are used in many countries for the preservation of fish, meat, cheese and other food products. They are mainly used for their role in inhibiting the outgrowth of Clostridium botulinum spores (Christiansen et al., 1973; Tompkin et al., 1978; Lucke and Leistner, 1979). Reduction of nitrate to nitrite brings about the antimicrobial activity. It has been shown by Rowe et al. (1979) and Yarbrough et al. (1980) that nitrite inhibited the active transport, oxygen uptake and oxidative phosphorylation in bacteria. Apart from their preserving action, these chemicals are believed to play an important role in (1) producing the characteristic cured meat color, nitrosyl hemochrome, (Brooks et al., 1940; Dryden and Birdsall, 1980); and (2) contributing the characteristic cured meat flavor (Bailey and Swain, 1973; Pearson et al., 1977; MacDonald et al., 1980).

Varying concentrations of nitrogen oxides are found in air (outdoors and indoors), in the work place, tobacco smoke, and during the smoking of foods (World Health Organization, 1978; Ehreneberg et al., 1980;

Eslandsson, 1981; Newmark and Mergens, 1981). Nitrogen oxides are generated by the chemical and microbial reduction of nitrite and nitrate salts, and are common environmental pollutants produced during combustion of fossil fuels. Approximately half the oxides of nitrogen in urban air arises from automobiles and electric power utilities. Four of these compounds have been implicated in the formation of N-nitroso compounds: nitrogen dioxide (NO_2), dinitrogen tetroxide (N_2O_4), dinitrogen trioxide (N_2O_3) and nitric oxide (NO) (National Academy of Sciences, 1981). The first three compounds react unaided with amines, but nitric oxide requires either oxidation to nitrogen dioxide or the presence of certain metal salts, iodine or hydrogen iodide. The formation of N-nitroso compounds from oxides of nitrogen is usually faster and more extensive than from aqueous nitrous acid (Challis and Kyrtopoulos, 1979). The average concentration of nitrogen oxides in the atmosphere is approximately 90 $\mu g/m^3$, although their concentration in air of smog laden cities may reach 1.9 mg/m^3. Newmark and Mergens (1981) observed that the intake of nitrogen oxides can be as high as 1 µMol in cities during smog formation. Cigarette smoke is also an important source of nitrogen oxides. Bokhoven and Neissen (1961) reported that almost all of the nitrogen oxides inhaled in cigarette smoke are retained in the body. Oda et al. (1981) reported that inhalation of nitrogen dioxide leads to the appearance of large concentrations of nitrate and nitrite in the blood of rats. Pryor and Lightsey (1981) reported that nitrogen dioxide can react with unsaturated lipids to produce nitrite and that this could occur in vivo. Estimates of human exposure to nitrate and nitrite originating from nitrogen oxides is conjectural since data on the actual conversion of nitrogen oxides to nitrite are incomplete. A National Academy of Sciences (1981) report listed the average concentrations of nitrate, nitrite and oxides of nitrogen used to estimate human exposures (Table 1).

Table 1 - Average concentrations of nitrate, nitrite and nitrogen oxides used to estimate exposure of humans[1,2]

Sources	Nitrate	Nitrite	Nitrogen Oxides
Cured meats	40 mg/kg	10 mg/kg	
Fresh meats	10 mg/kg	1 mg/kg	
Vegetables	86 mg/kg	0.2 mg/kg	
Fruits	20 mg/kg	negligible	
Baked goods and cereals	12 mg/kg	2.4 mg/kg	
Milk and dairy products	0.5 mg/liter	negligible	
Water	1.3 mg/kg	negligible	
Ambient atmosphere			1 $\mu g/m^3$
Tobacco smoke			513 µg/cigarette
Saliva[2]	30 mg/liter	8.62 mg/liter	

1 Adapted from the National Academy of Sciences (1981)

2 Adapted from White (1976)

White (1975, 1976), Food and Drug Administration (1979), Birdsall (1981), and Hartman (1982) have estimated the average daily ingestion of nitrate and nitrite for U.S. residents and evaluated the relative significance of various dietary sources. The data from these studies show that vegetables are the major source (86 - 97%) of nitrate in the average American diet. The remaining nitrate originates from salivary excretion and cured meats (0 - 9%). The predominant portion of the ingested nitrite, however, comes from the bacterial reduction of nitrate in the saliva (76 - 80%) and a significant amount from cured meats (20 - 60%) National Academy of Sciences, 1981).

Various studies have shown that the nitrite level in saliva can increase markedly after consumption of nitrate-rich foods such as vegetables (Spiegelhalder et al., 1976; Tannenbaum et al., 1976). These results imply that the ingested nitrate is converted in vivo to nitrite and then secreted in the saliva. Despite the high volume of daily excretion of saliva (up to 1000 ml), the average nitrite content of the stomach is very small since it is delivered in small doses. However, high concentrations of nitrite in saliva (as observed after a nitrite-rich diet) can be important in the formation of N-nitroamines in the human stomach (Wanger and Tannebaum, 1985).

Amines in foods: Like nitrates and nitrites, amines and amino compounds are widely distributed in the environment (National Academy of Sciences, 1981). Their reactivity with nitrosating agents varies considerably and may be influenced by pH, temperature, and the presence of catalysts or inhibitors.

Formation of amines in foods is governed by type of amino acid, proteins, metabolic enzymes, bacteria, preprocessing, storage and cooking conditions (National Academy of Sciences, 1981). Amino acid decarboxylation is the most common mode of formation of many of the amines in animals, plants and bacteria. The formation of spermidine from

methionine (Lakritz et al., 1975), putrescine from ornithine, cadaverine from lysine (Tabor et al., 1958; Johnson, 1976), tyramine from tyrosine (Kristoffersen, 1963), and histamine from histidine (Dierick et al., 1974) can occur through decarboxylation reactions. The enzymatic conversion of trimethylamine oxide to trimethylamine was observed mainly in salt water fish by Tarr (1940). Aldehyde amination and transamination is another common pathway for amine formation in plants (Hartmann, 1967; Maier, 1970). Thermal degradation of amino acids accounts for the appearance of a wide variety of amines, such as ethanolamine, methylamine, propylamine, and iso- or pentylamine (Mulders, 1973). The diamine, putrescine, and the polyamines, spermidine, and spermine probably occur universally in animals, plants and in most bacteria. In all organisms, spermine and spermidine are formed from putrescine by successive donation of one or more aminopropyl groups from decarboxylated S-adenosylmethioninie (Janne et al., 1978). Conversion of polyamines like putrescine and cadaverine to the nitrosatable secondary amines pyrrolidine and piperidine, respectively, was observed in some foods by Singer and Lijinsky (1976a). Tobacco and cigarette smoke was found to contain higher levels of dimethylamine (100 mg/kg) and pyrrolidine (240 mg/kg) than in most foods (Singer and Lijinsky, 1976b). It is possible for smokers to be exposed to greater levels of DMA and pyrrolidine from tobacco than from foods (Lijinsky and Singer, 1976b).

Bacterial action has been reported to be responsible for the presence of high levels of dimethylamine, trimethylamine and trimethylamine oxide in various marine fish (Shewan, 1951; Castell et al., 1971; Golovnya, 1976), and in mature Gouda cheese (Ruiter, 1973). In addition, a wide range of simple aliphatic amines and monoamines such as tyramine, histamine and tryptamine have been detected in cheese (Voight and Eitenmiller, 1974; Gray et al., 1979). A survey of secondary amines in commercial foods by Kawamura et al. (1971) revealed that modified

powdered milk contains five times more dimethylamine than milk, while only trace amounts were found in butter and cheese.

In a later survey conducted by Singer and Lijinsky (1976b), ham and frankfurters were found to contain 2 to 5 mg/kg of DMA and 0 to 0.2 mg/kg of piperidine. These investigators also reported high levels of DMA (730 - 745 mg/kg) in cod and at much lower levels (20 - 25 mg/kg) in canned tuna. In a more extensive survey, Neurath et al. (1977) reported the distribution of various primary and secondary amine levels below 10 mg/kg in fresh vegetables, bread, cheese, fish and fish products. Higher concentrations of DMA (20 - 120 mg/kg) were found in herring, some cheese and in radishes. Besides DMA and diethylamine (DEA), the most prevalent secondary amines found were pyrrolidine, piperidine, N-methylbenzylamine, N-methylalanine and N-methylphenylamine. The highest content of secondary amine was found in red radishes (38 mg/kg pyrrolidine and 20 mg/kg piperidine) (Neurath et al., 1977).

Common spices like paprika, cayenne pepper and black pepper contain high levels of the cyclic amines, pyrrolidine and piperidine (Marion, 1950; Gough and Goodhead, 1975; Singer and Lijinsky, 1976a). Since these spices are widely used in the preparation of various foods, they may contribute significantly to the total intake of amines in our diet.

Low levels of simple amines have also been reported occasionally in various meat products (Landmann and Batzer, 1966; Cantoni et al., 1969; Patterson and Mottram, 1974). The monoamines (histamine, tryptamine and ethanolamine) and polyamines (spermidine, spermine, putrescine and cadaverine) have been identified in fresh pork bellies (Spinelli et al., 1974) at concentrations ranging from 0.3 mg/kg for cadaverine to 81 mg/kg for spermine. Processing into bacon did not significantly affect the amine content. Similar amines were identified in fresh ham with concentrations ranging from 5 mg/kg for tyramine to 1890 mg/kg for

putrescine in fresh tissue (Lakritz et al., 1975). It was also demonstrated that cooking resulted in a substantial decrease in amine concentration which may be due to volatilization. Significant increases in spermine, spermidine, putrescine and cadaverine concentrations occurred during putrefaction. The volatile amines (methylamine, dimethylamine, trimethylamine, ethylamine, n-propylamine and isopropylamine) were detected in pork carcass meat used for processing into Wiltshire bacon (Patterson and Mottram, 1974). The highest concentration detected was 1.9 mg/kg of methylamine in fresh meat which decreased during the curing process. Rice et al. (1976) have reported the presence of histamine, putrescine, tyramine and 2-phenylethylamine in dry and semi-dry sausages. A survey conducted by Nakamura et al. (1976) for the presence of polyamines in Japanese fresh and processed pork tissue showed similar results as obtained by Spinelli et al. (1974).

Proline and hydroxyproline will undergo nitrosation to form N-nitrosoamino acids (NAA); however, they have been found to be non-carcinogenic (National Academy of Sciences, 1981). It has been proposed that during cooking they can decarboxlate to N-nitrosamines or transnitrosate secondary amines. Bharucha et al. (1979) reported the presence of 18 different amino acids in raw pork bellies and in raw bacon at concentrations ranging from 10 to 220 mg/kg. The concentration of proline was found to be between 10 - 26 mg/kg in pork bellies, while raw bacon contained 30 - 80 mg/kg. Hydroxyproline was found at very low concentrations (2 - 4 mg/kg). Generally, raw bacon contained 2 to 3 times the amount of free amino acids as those found in pork bellies.

Gray and Collins (1977a,b) reported that free proline concentrations in pork bellies increased with storage time. Whole pork bellies stored at 2°C for 7 days contained 45% more free proline, while lean and adipose tissue concentrations increased by 33% and 86%, respectively. They also reported that free proline concentrations ranged from 18 - 30 µMol/100 g

of tissue in commercial bacon. Collagen contains relatively large amounts of proline and hydroxyproline and has been shown to produce NPYR under conditions of high temperature and nitrite (Huxel et al., 1974; Gray and Dugan, 1975).

The extensive distribution of nitrite, nitrate and the amines in foods clearly precludes the possibility of eliminating them from the human diet. While much data have been gathered on preformed N-nitrosamines in foods, there is a growing interest in the area of in vivo N-nitrosamine formation and their potential health hazard.

ENVIRONMENTAL DISTRIBUTION OF N-NITROSAMINES

N-Nitrosamines have been found to be ubiquitous in the environment. Human exposure to N-nitrosamines from the environment has been classified by the following categories (National Academy of Sciences, 1981):

1. Occupational - working in rubber and leather tanning industries, pharmaceutical and chemical factories, and machine shops.
2. Life style - smoking and chewing of tobacco, and use of cosmetics and drugs.
3. Atmospheric - working or living next to chemical plants, smog.
4. Food - cured meats and fish, beer and liquor.

In this review, the main focus will be on the occurrence of N-nitrosamines in cured meat products. More extensive information can be found in the following publications - Eisenbrand et al. (1976); Preussmann et al. (1979); and National Academy of Sciences, (1981).

N-Nitrosamines in foods: Extensive compilations on the occurrence of N-nitrosamines in a wide range of foods have been published by Scanlan (1975), Gough et al. (1977), Havery et al. (1978), Kawabata et al. (1979), Preussmann et al. (1979), National Academy of Sciences (1981),

Gray (1981), and the International Agency for Research on Cancer (1978, 1980).

The majority of foods with the exception of bacon, some cured meats and beer, contain less than 1 µg/kg of N-nitrosamines. Of the large number of samples of fresh meat, vegetables, edible oils, fruits, cheese and non-fat dry milk analyzed, only a small percentage of them were found to contain measurable amounts (0.1 - 2 µg/kg) of N-nitrosamines (Sen et al., 1977; Gough et al., 1977; Kawabata et al., 1979; Webb and Gough, 1980; Fiddler et al., 1981).

The main sources of N-nitrosamines in the human diet are fried bacon, smoked cured fish cooked in gas ovens, and beer. The N-nitrosamines found in these foods are mainly NDMA, NPYR, N-nitrosodiethylamine (NDEA) and N-nitrosopiperidine (NPIP).

Beer, malt and fish: A high percentage of beer samples analyzed by Spiegelhalder et al. (1979) in Germany contained 2 to 60 µg/kg of NDMA. Similar studies in the U.S. by Goff and Fine (1979) revealed the presence of NDMA in beer at similar concentrations. Subsequent research revealed that malt was the main source of NDMA. When malt was dried in a direct fired kiln, the amines present in malt were nitrosated by oxides of nitrogen from the kiln (Spieglhalder et al., 1980; Mangino and Scanlan, 1982). Consequently, changes in malting procedures brought about a 15 - 30 fold reduction in NDMA formation in beer and in malt. N-Nitrosamine formation in malt was also greatly reduced by adding sulfur to the open flame kiln or switching to an indirect malt drying process (Havery and Fazio, 1985).

Fish samples were originally suspected to contain high levels of N-nitrosamines due to the presence of large concentrations of dimethylamine and diethylamine. However, studies by Gough et al. (1978) and Webb and Gough (1980) on raw and cooked fish revealed NDMA levels of less than 1 µg/kg. Kawabata et al. (1979), however, reported that gas

oven-cooked fish contained higher levels of NDMA and NPYR than fish cooked in an electric oven. Oxides of nitrogen from gas combustion were suspected as the source of nitrosating agent.

Cured meats: Generally, cured meat products such as corned beef, luncheon meat, frankfurters, sausage, salami, pepperoni, smoked beef and ham contain relatively low levels (1 - 5 µg/kg) of N-nitrosamines. However, high levels (1000 - 25,000 µg/kg) of NPYR and NPIP have been reported and these have been attributed to the practice of pre-mixing cure spices, sodium nitrite and nitrate (Sen et al., 1973a; Sen and McKinley 1974). Black pepper and paprika contain high levels of piperidine and pyrrolidine, which could react with nitrite to produce NPIP and NPYR, respectively. Gough and Goodhead (1975) and Havery et al. (1976) confirmed the findings of Sen and co-workers. Consequently, changes in premix packings resulted in a lowering of the N-nitroamine levels (less than 1 µg/kg) in cured meat products (Sen and McKinley 1974; Gough et al., 1977).

Bacon: NPYR is the major N-nitrosamine in fried bacon, although lower levels of NDMA, NDEA and NPIP are also present (Table 2). NPYR is produced during the cooking process and the levels depend on a number of factors including cooking method, frying temperature, length of cooking time, lean to adipose tissue ratio, preprocessing procedures, nitrite concentration, and presence of inhibitors of the nitrosation reaction (Skrypec et al., 1985). The majority of N-nitrosamines produced during the frying process are volatalized into the vapor phase. This phenomenon has been investigated by several researchers, who reported a wide range of values for the percentage of N-nitrosamines found in the vapors (Table 3). Factors such as cooking temperature, moisture content and ratio of lean to adipose tissue in bacon samples influence the amount of N-nitrosamines volatalized. In the early 1970's, NPYR and NDMA levels in excess of 100 µg/kg, were reported in bacon and were believed to be due

Table 2 - N-Nitrosamine concentrations (µg/kg) in fried bacon and cook-out fat (drippings)

Investigators	Number of Samples Analyzed	N-Nitrososopyrrolidine		N-Nitrodimethylamine	
		Bacon	Cook-Out Fat	Bacon	Cook-Out Fat
Sen et al. (1973b)	8	13 (ND-25)[1]	NR[2]	6	NR
Fazio et al. (1973)	8	63 (10-108)	100 (45-207)	NR	NR
Pensabene et al. (1974)	6	20 (11-38)	25 (16-39)	NR	NR
Pensabene et al. (1979)	8	20 (2-45)	30 (7-49)	5 (2-9)	15 (7-28)
Sen et al. (1979)	12	9 (2-22)	22 (15-34)	3 (ND-17)	6 (3-12)
Gray et al. (1982)	5	20 (14-27)	18 (13-28)	5 (4-6)	5 (3-6)
Skrypec et al. (1985)	5	6 (4-9)	13 (NR)	3 (1-4)	7 (NR)
Havery et al. (1986)	18	21 (1-65)	27 (1-75)	7 (1-44)	4 (ND-11)
Vecchio et al. (1986)	39	17 (tr-130)	32 (tr-170)	4 (tr-23)	5 (tr-33)

[1] Not detected

[2] Not reported in study

Table 3 - Percentages of N-nitrosamine in the fumes produced during the frying of bacon[1]

Investigators	N-Nitrosamine (%) NPYR	NDMA	Sample
Gough et al. (1976)	60-95	75-100	bacon
Hwang and Rosen (1976)	14-37	--	bacon
Warthesen et al. (1976)	20-40	--	pork belly[2]
Sen et al. (1976)	28-82	28-92	bacon
Gray and Collins (1977a)	27-49	--	pork belly[2]
Mottram et al. (1977)	57-75	73-80	bacon
Gray et al. (1978)	--	56-80	pork belly[2]
Bharucha et al. (1979)	Up to 32	Up to 62	bacon

1 Adapted from Gray (1981).

2 Contained added nitrite.

to the addition of 156 µg/kg of nitrite and 2000 mg/kg of nitrate (Fazio et al., 1973). Consequently, ingoing levels of nitrite were reduced to 120 mg/kg, the use of nitrate was eliminated and 550 mg/kg of ascorbate were added to the cure (Mulhern, 1975). This, in association with improved control of processing procedures, has resulted in significant reductions in N-nitrosamine levels in fried bacon (Sen et al., 1977; Havery et al., 1978). In spite of these improvements, no further reduction has occurred in N-nitrosamine levels in bacon since then (Havery and Fazio, 1985).

Recently a new N-nitrosamine, N-nitrosothiazolidine (NTHZ) was identified in fried bacon (Gray et al., 1982; Pensabene and Fiddler, 1983a,b). These investigators speculated that the presence of NTHZ in bacon was associated with wood smoke itself. Nitrosation of thiazolidines has been demonstrated by Coughlin (1979) and Sakaguchi and Shibamoto (1979) in aqueous model systems. Reaction systems containing cysteamine/formaldehyde/nitrite and cysteamine/acetaldehyde/nitrite when heated to 90°C for 5 hours yielded NTHZ and N-nitroso-2-methyl-thiazolidine (MNTHZ). Mutagenic studies on NTHZ and its 2-alkyl derivatives using the Ames test indicated that NTHZ was strongly mutagenic, while the 2-alkyl derivatives of NTHZ were relatively weak mutagens (Sekizawa and Shibomoto, 1980). Fiddler et al. (1983) reported that NTHZ did not show any mutagenic activity when synthesized from thiazolidine. However, a mutagen was formed when NTHZ was synthesized from cysteamine, formaldehyde and nitrite reaction mixture.

N-Nitrosopyrrolidine: The presence of NPYR in fried bacon has led to an intensive search for the precursors and the mechanism(s) involved in its formation. Model system studies have implicated a number of compounds including proline (PRO), pyrrolidine (PYR), collagen, putrescine, and spermidine as possible precursors of NPYR (Gray, 1976). The most probable precursor in bacon appears to be PRO. Investigations by Fiddler

et al. (1974), Lakritz et al. (1976), Nakamura et al. (1976), Gray and Collins (1977b), and Bharucha et al. (1979) showed that pork bellies contained 20 - 90 mg/kg of free PRO.

Two pathways have been proposed for the formation of NPYR from PRO, but the exact mechanism involved has not been fully resolved (Gray, 1976; Bharucha et al., 1979). The proposed pathways involved are: 1) PRO is nitrosated to NPRO which then undergoes thermal decarboxylation to yield NPYR, 2) PRO is thermally decarboxlyated to PYR which is subsequently nitrosated to form NPYR (Figure 5). Of the two pathways, the one involving NPRO formation seems to be the more likely route for NPYR formation, since the decarboxylation of NPRO occurs at a much lower temperature than the decarboxylation of PRO to PYR (Bharucha et al., 1979; Lee et al., 1983). However, experimental evidence generated by Sen et al. (1976a), Hansen et al. (1977), and Bharucha et al. (1979) indicates the decarboxylation of preformed NPRO may not be the primary route of NPYR formation, as shown by the fact that ascorbyl palmitate, when added to bacon, inhibits NPYR formation (Sen et al., 1976). However, Bharucha et al. (1979) reported that NPRO is formed at the high temperatures attained during the frying of bacon.

Bharucha et al. (1979) postulated a free radical mechanism rather than an ionic mechanism for NPYR formation during the frying of bacon. Their hypothesis is supported by the fact that NPYR is produced substantially towards the end of the frying process when most of the water has been removed. Essentially, only the lipid phase remains during the final frying process which tends to act as an excellent heat transfer medium. The high temperature, the catalytic effects of lipid hydroperoxides, and the absence of the inhibitory effects of water and antioxidants support the involvement of a free radical mechanism in NPYR formation (Coleman, 1978).

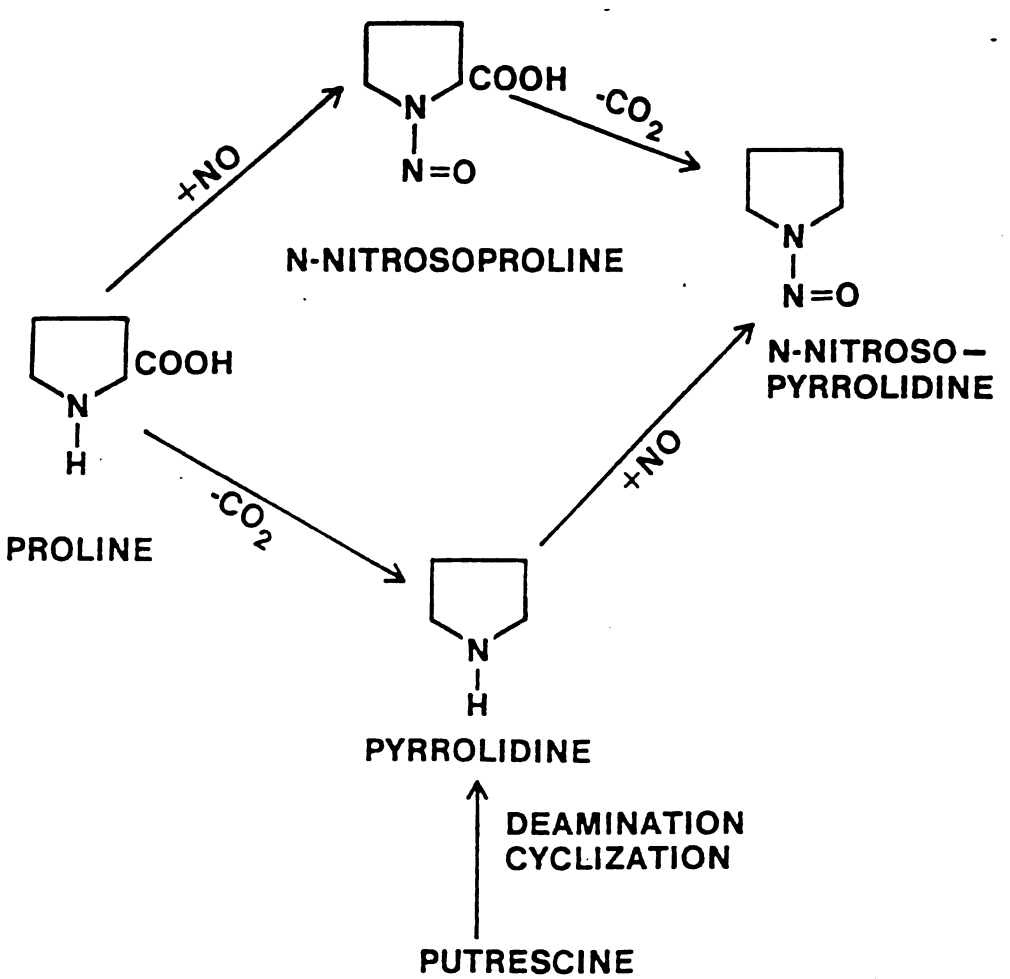


Figure 5: Possible pathways of N-nitrosopyrrolidine formation in bacon (Lee, 1981).

Bharucha et al. (1979) proposed a free radical pathway in the formation of NPYR during the frying of bacon (Figure 6). As the water is vaporized during the frying process, nitrous acid is converted to N_2O_3 which, in turn, undergoes dissociation at high temperatures(>> 100°C) to NO and NO_2 radicals. It is possible that the NO_2 radical can abstract a proton from the nitrogen atom of PRO to form a proline radical which, in turn, reacts with NO radical to form NPRO.

N-Nitrosodimethylamine: NDMA has been consistently found in bacon and is more carcinogenic than NPYR (Magee and Barnes, 1967; Druckrey et al. 1969). However, there has been little research into the mechanism of NDMA formation in bacon. Several compounds including dimethylamine, trimethylamine, sarcosine, lecithin, and quarternary ammonium compounds have been shown to form NDMA in model system studies (Ender and Ceh, 1971; Fiddler et al., 1972; Mohler and Hallmayer, 1973; Scanlan et al., 1974; Pensabene et al., 1975; and Eisenbrand et al., 1976).

Gray et al. (1978), after investigating several precursors of NDMA found in bacon, concluded that sarcosine and choline - containing compounds were the most probable amine precursors of NDMA in bacon. Patterson and Mottram (1974) reported that dimethylamine concentrations in pork increased with storage and after processing from 200 µg to 520 µg/kg. However, studies on levels on sarcosine, choline type compounds and other precursors of NDMA in pork bellies are lacking.

FACTORS INFLUENCING N-NITROSAMINE FORMATION IN BACON

As previously stated there are several factors that influence N-nitrosamine formation in bacon. These factors include the cooking method, frying temperature and time, lean to adipose tissue ratio, presence of inhibitors, nitrite concentration, storage of pork bellies prior to processing, and smoking (Gray, 1976; Gray and Randall, 1979; Sen, 1980; Skrypec et al., 1985).

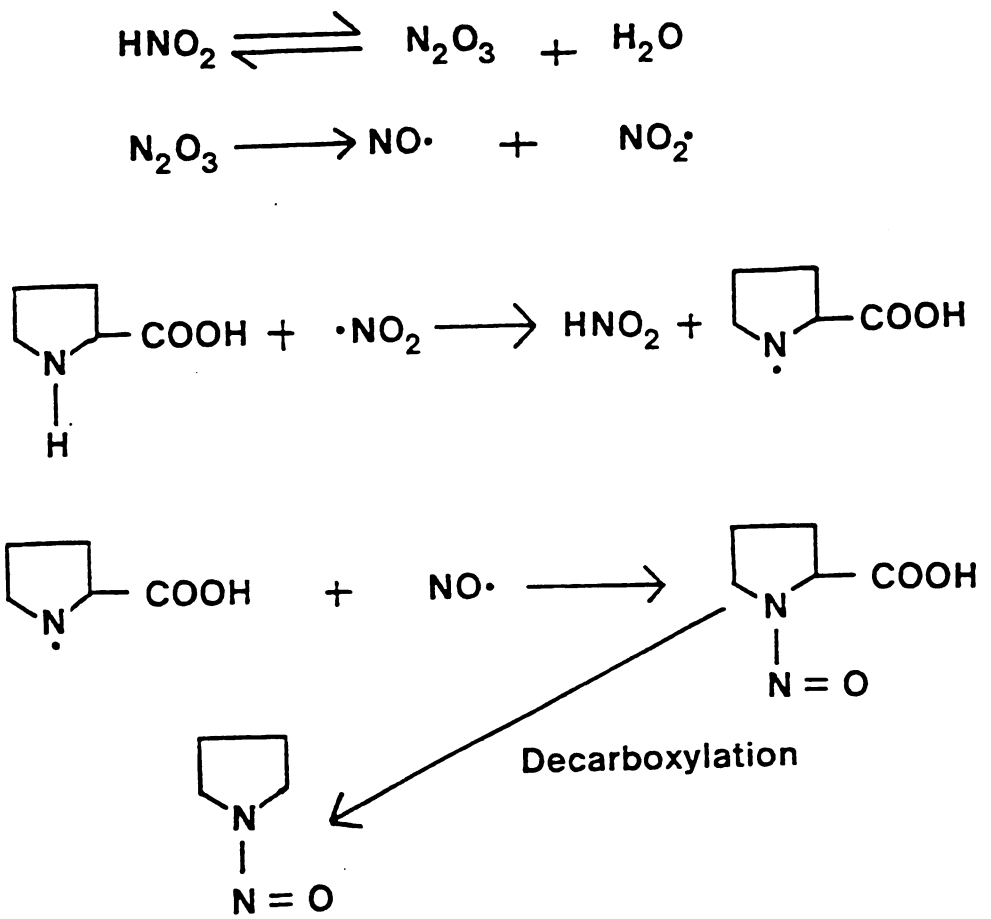


Figure 6: Free radical mechanism of N-nitrosopyrrolidine formation in bacon (Bharucha et al., 1979).

Cooking method: Pan frying has been shown to produce the greatest amount of N-nitrosamines in bacon (Wasserman et al., 1978), while microwave cooking (Herring, 1973; Pensabene et al., 1974) and grilling (Bharucha et al., 1979) produce smaller amounts. These differences can be explained by the low internal temperatures reached during microwave cooking (Wasserman et al., 1978). Similarly, the draining of cook-out fat (drippings) during grilling results in the product attaining lower internal temperatures (Bharucha et al., 1979). Temperature and length of cooking time does play an important role in N-nitrosamine formation. The maximum concentrations of N-nitrosamines are produced when bacon was fried at 360°F for 12 minutes after starting from a cold pan (Bharucha et al., 1979). Similarly, Pensabene et al. (1974) showed that bacon samples from the same package produced no N-nitrosamines when fried at 100°C for 105 minutes while samples fried to the same "doneness" at 204°C for 4 minutes produced 17 μg/kg of N-nitrosamines. The N-nitrosamine content of cooked bacon begins to increase after 4 minutes and reaches a maximum at about 12 minutes, and then declines. Pensabene et al. (1974) determined that the optimum temperature for N-nitrosamine formation was 185°C, near the normal pan frying temperature of bacon. They found only 10% NPYR formation at 100°C compared to NPYR produced at 180°C. Coleman (1978) reported that during frying of bacon strips, the internal temperature of lean reached a maximum temperature of 110°C. However, when the majority of the water had been volatilized, the temperature of the rashers reached 180°C. It was proposed that the majority of N-nitrosamines are produced after most of the water has been volatilized (Pensabene et al., 1974; Coleman, 1978; Bharucha et al., 1979).

Nitrite concentration: Nitrite concentration is one of the primary factors involved in N-nitrosamine formation. The rate of the nitrosation reaction is directly proportional to the square of nitrite concentration (Mirvish, 1975). Sen et al. (1974) fried bacon samples prepared with 0,

50, 100, 150 and 200 mg/kg levels of nitrite. The NDMA and NPYR levels in the fried samples correlated well with initial concentrations of nitrite, but not with the residual nitrite levels found in raw bacon prior to frying. In a similar study, Bailey (1980) added nitrite at concentrations of 120, 200, 400 and 625 mg/kg. The formation of NPYR and NDMA in fried bacon was directly proportional to the ingoing levels of nitrite. While a strong correlation exists between initial nitrite concentrations and N-nitrosamines formed in fried bacon, no clear pattern could be established for residual nitrite levels (Sen et al., 1974). However, later studies by Dudley (1979) and Sebranek (1979) indicated that the lowest residual nitrite gives the least probability of N-nitrosamine being formed.

Consequently, USDA passed a ruling that the ingoing nitrite levels for bacon be reduced from 156 to 120 mg/kg, along with the inclusion of 550 mg/kg of sodium ascorbate or erythorbate to inhibit N-nitrosamine formation (Federal Register, 1975).

Fatty acid composition: It has been clearly established by Pensabene et al. (1974), Coleman (1978), and Bharucha et al. (1979) that the majority of N-nitrosamines are formed in the adipose tissue during bacon frying. Many studies have been performed to elucidate the mechanism involved in the formation of N-nitrosamine in adipose tissue. The involvement of unsaturated fatty acids as transnitrosating agents has been investigated by Walter et al. (1979). They indicated a possible interaction between nitrite (nitric oxide) and the unsaturated carbon-carbon bonds of fatty acids. They demonstrated that the α-nitroso-nitrite esters of unsaturated triglycerides transnitrosate secondary amines and suggested that similar derivatives of unsaturated lipids may be involved in N-nitrosamine formation in adipose tissue. Mirvish and Sam (1983) showed that a nitrosating agent can be produced from the reaction of methyl linoleate with nitrogen dioxide and that the

reaction occurs at a slower rate with methyl stearate and methyl oleate. These investigators speculated that active nitrite esters might arise by simple addition of NO to an ethylene group to produce a nitrosate, or by a more complex series of free radical reactions beginning with an electron abstraction by nitrogen dioxide (Pryor and Lightsey, 1981). This reaction not only initiates the autoxidation of alkenes in the presence of oxygen or air, but also leads to the production of nitrous acid rather than a product containing a nitro group attached to a carbon atom. The nitrous acid can react with a secondary amine to produce N-nitrosamines.

Skrypec et al. (1985) investigated the influence of pig diet and subsequent fatty acid composition of bacon adipose tissue on N-nitrosamine formation. Their data clearly indicated that higher levels of fatty acid unsaturation in bacon adipose tissue results in higher NPYR and NDMA levels in fried bacon. Mottram et al. (1977) found that cooking bacon in highly unsaturated corn oil resulted in marked enhancement of NPYR formation. These studies support the hypothesis that NPYR formation during frying of bacon could proceed through the intermediate formation of a nitroso-nitrite ester derivative of unsaturated lipids. Recent studies by Hotchkiss and Vecchio (1985) showed extensive N-nitrosamine formation in ham, sausage and other cured meats fried in corn oil. They also showed that when fish, liver, eggs, bacon, and chicken were cooked in cook-out bacon fat (drippings), large amounts of NDMA (5.9 - 198 µg/kg), NPYR (2.5 - 38.2 µg/kg) and NTHZ (0.2 - 2 µg/kg) were formed in the vapors. However, only trace amounts were actually present in the edible portions. These studies clearly show the transnitrosating capacity of unsaturated lipids in a cooking medium.

Preprocessing: Pensabene et al. (1980) reported that aging of bellies prior to processing resulted in higher levels of NPYR formation in fried bacon compared to bacon made from fresh bellies. The increase in NPYR

levels might be due to the increase in both amines and amino acids that occur during extended storage (Pensabene et al., 1980; Amundson et al., 1982). Lakritz et al. (1976) and Gray and Collins (1977b) have shown that the free proline content in whole and lean tissue of pork bellies increased approximately 50% after storage at 2°C for one week. The free proline content in the adipose tissue increased approximately 90% over the same period of time.

Smoking: Bacon and other foods are smoked in order to enhance preservation and to impart characteristic smoked flavor. Smoke is a complex matrix composed of gaseous, particulate and condensable (volatile organic compounds) phases (Wistreich, 1979). The volatile constituents of the condensable phase of smoke are responsible for the desirable characteristics like color and flavor development. The undesirable qualities of smoking are deposition of carcinogens on the surface of the food and reduction in nutritional value, especially in respect to lysine (Ruiter, 1979). The main constituents of the condensable phase are the acids, carbonyls, phenols, furans, lactones, alcohols, esters and the polycyclic aromatic hydrocarbons (Hamm, 1977).

The acids (aliphatic) have been reported to be responsible for the bacteriostatic effects of smoke (Clifford et al., 1980) and contribute to the smoke color and flavor development (Toth and Potthast, 1984).

A number of carbonyls have been identified in wood smoke (Toth and Potthast, 1984), and include formaldehyde, acetaldehyde, glycolaldehyde, and methyl glyoxal. These compounds have been shown to be responsible for the typical smoked food color formation via Maillard type browning reaction.

The phenolics compounds present in wood smoke that play a major role in flavor and aroma development are guaiacol and syringol and their derivatives (Wasserman, 1966; Daun, 1979). High molecular weight phenols

contribute to the smoke color formation by cross-linking collagen via hydrogen bonding (Caurie et al., 1974).

Some of the components of wood smoke such as formaldehyde and phenols (nitroso phenols) could possibly be involved in the N-nitrosation reactions. Due to the complex nature of the interaction between meat and smoke components, it is difficult to assess the overall effects of smoking on N-nitrosamine formation in bacon. Bharucha et al. (1980) reported that smoked bacon samples generally contained lower levels of NPYR and NDMA than unsmoked bacon samples, presumably due to a lowering of pH by the acidic smoke components. The effects of smoke appear to be a combination of pH lowering and direct C-nitrosation of phenolic compounds to reduce the nitrite concentration in the product (Knowles, 1974).

Inhibitors of N-nitrosamine formation in bacon: As hypothesized by Gray and Dugan (1975), any compound that can successfully compete with a meat matrix for reaction with nitrite is likely to lower the potential for N-nitrosamine formation. Consequently, several compounds have been investigated as potential blocking agents in bacon (Table 4). The first compound to be recognized as a N-nitrosamine inhibitor was ascorbic acid or its isomer, erythorbic acid (Mirvish et al., 1972b). This information has been utilized in regulatory form to ensure that all bacon is processed with maximum levels (550 mg/kg) of ascorbate or erythorbate (Sebranek, 1979). Although these compounds are quite effective, they are not completely successful as N-nitrosamine inhibitors because of their limited solubility in adipose tissue.

Consequently, ascorbyl palmitate has been found to be more effective than sodium ascorbate in reducing N-nitrosamine formation (Sen et al., 1976a). Similar conclusions were reached by Bharucha et al. (1980), who showed that ascorbyl palmitate reduced N-nitrosamine formation in bacon by 70 - 90% when used at the 500-1000 mg/kg level. However, its activity

Table 4 - Effects of Various Blocking Agents on N-Nitrosamine Formation in Fried Bacon.

Compound	Level (mg/kg)	Percent Inhibition Fried Bacon	Cooked-out fat	Investigators
Ascorbic acid	1000	100	--	Greenberg (1973)
Ascorbyl palmitate	1000	59-87	--	Sen et al. (1976a)
	500-1000	--	70-90	Bharucha et al. (1980)
Ascorbic acid acetal	1000	62-88	90-98	Bharucha et al. (1980)
Piperazine	1000	90-91	--	Sen et al. (1976a)
Propyl gallate	1000	50-97	--	Sen et al (1976a)
	500	65-94	--	Mandagere (1979)
TBHQ	500-1000	55-64	--	Anon. (1977)
	500	up to 80	--	Fiddler et al. (1978)
α-Tocopherol	500	up to 85	--	Mergens and Newmark (1979)
	125-750	50 to 96	--	Gray et al. (1982)
Glucose	20,000	70-82	--	Mandagere (1979)
	20,000-60,000	60-90	--	Bailey (1980)
Sodium ascorbate and α-tocopherol	550	60-80	--	Gray et al. (1982)

Analysis conducted on combined bacon and cook-out fat extracts.

tends to decrease with storage time. The long chain acetals (C12, C14, C16, C18, C18:1) of ascorbic acid have also been reported to be effective in bringing about a 93-98% reduction of N-nitrosamines in the cook-out fat when applied to the surface of bacon slices at a 1000 mg/kg level (Bharucha et al., 1980). The C12 ascorbyl acetal, and to a much lesser extent, the C14 homologue left a soapy after-taste. However, the bacon samples treated with ascorbyl C16, C18 and C18:1 acetals were indistinguishable from commercial samples. The major drawback to this group of compounds as N-nitrosamine blocking agents in bacon is that they are not approved for use in food systems.

The inhibition of NPYR formation in fried bacon by the use of cure-solubilized α-tocopherol has been demonstrated by Fiddler et al. (1978). This compound was dispersed with polysorbate emulsifiers to obtain adequate distribution in the product and produced a significant reduction of N-nitrosamines when used at a level of 250 - 500 mg/kg. Walters et al. (1979) also reported reduced levels of N-nitrosamines in the vapors during frying of bacon in fat containing α-tocopherol. Mergens and Newmark (1979) reported that α-tocopherol dispersed quite effectively during frying of bacon slices; therefore, application to bacon may be made by spray or dip to overcome the problems of water insolubility. Recently, Gray et al. (1982) processed dry cure and brine-cured bacon with α-tocopherol coated salt at various concentrations (250, 500, and 750 mg/kg) along with 550 mg/kg of sodium ascorbate. The fried bacon samples contained consistently lower levels of NPYR and NDMA than the control samples. The optimum inhibition of α-tocopherol was observed in bacon samples treated with 500 mg/kg of α-tocopherol and 550 mg/kg of ascorbate. Similar studies by Pensabene and co-workers (1978) have shown that a combination of α-tocopherol and ascorbate was more effective in inhibiting N-nitrosamine formation than either compound acting alone. This is due to the fact that ascorbate inhibits

N-nitrosamine formation in the aqueous phase, while α-tocopherol acts in the lipid phase, thus providing maximum inhibition of N-nitrosamine formation in fried bacon.

Several other potential N-nitrosamine blocking agents have been studied. Sen et al. (1976a) reported relatively successful use of propyl gallate when applied to the bacon slices immediately before frying. Tertiary butylhyroxyquinone (TBHQ) has also been reported to function as a blocking agent (Anonymous, 1977). Dextrose has been shown to be an effective inhibitor of N-nitrosamine formation by competing with nitrite for the available amine (Bailey and Mandagere, 1980). In a similar study, Bailey (1980) prepared dry cured bacon with 120, 200, 400 and 625 mg/kg of nitrite. The resulting fried bacon was found to contain unacceptable levels of NPYR. However, these higher levels were reduced to below 16 µg/kg for all samples except the 625 mg/kg nitrite sample by including 1 - 2% dextrose. However, bacon cured with high levels of dextrose underwent extensive Maillard type browning reactions when fried, resulting in an unacceptable product.

TOXICOLOGY OF N-NITROSO COMPOUNDS

Many N-nitroso compounds are potent carcinogens. Thus far, about 300 N-nitroso compounds have been tested and approximately 80% of them have been shown to be carcinogenic (Preussmann et al., 1976; National Academy of Sciences, 1981). NDMA has been shown to be carcinogenic in 6 species and NDEA in about 20 animal species including subhuman primates (Magee et al., 1976; Sen et al., 1980). No animal species tested thus far is resistant to the carcinogenic effects of NDEA. N-Nitrosamines are organ specific. Thus a given N-nitrosamine will produce a liver or esophageal tumor regardless of the route of administration. NDMA and NPYR, the most widely occurring N-nitrosamines in foods, cause mostly liver tumors and occasionally kidney tumors in experimental animals (Druckey et al., 1967,

1969; Hecker et al., 1979; Preussmann et al., 1979; Cottrell et al., 1979).

N-Nitrosamines are considered indirect acting carcinogens and require metabolic activation (Druckrey et al., 1973, Lijinsky, 1977). Low molecular weight dialkyl nitrosamines (e.g. NDMA and NDEA) are potent carcinogens; whereas high molecular weight, highly branched and polar N-nitrosamines (N-nitrososarcosine, N-nitrosodiphenylamine and N-nitrosodiethanolamine) are generally weak carcinogens. The alpha position of N-nitrosamines has been associated with the carcinogenic action of these compounds (Wishnok, 1979). The metabolic activation of NDMA involves enzymatic α-hydroxylation on the methyl group to give α-hydroxydimethylnitrosamine (Druckrey et al., 1973; Koepke et al., 1983). The very unstable hydroxy N-nitrosamine then loses formaldehyde to form the primary alkylnitrosamine, which rapidly rearranges to the alkyldiazonium ion. The latter compound, being a powerful electrophile, alkylates various nucleophiles including DNA, RNA and other macromolecules (Michejda et al., 1979).

Long chain dialkyl nitrosamines have been postulated to undergo beta hydroxylation, which is converted to a sulfate by sulfonetransferase (Kruger, 1973; Michejda et al., 1979; Okada, 1983). Since the sulfate groups are excellent leaving groups in nucleophilic displacement reactions, it acts as a direct alkylating agent (Michejda et al., 1979; Umbenhauer and Pegg, 1981).

The role of nucleic acid methylation by N-nitrosamines in acute toxicity and carcinogenesis has been extensively studied. Alkylation of the 7 position of guanine appears to be closely associated with acute toxic injury. Methylation of messenger RNA resulting from NDMA poisoning effectively inhibits translation in protein synthesis (Shank, 1975). These workers have also shown that alkylation of cytosine at the 3 position may be more closely related to carcinogenesis than alkylation

of the 7 position of guanine. At this time, the mechanism for carcinogenesis via alkylation of nucleic acids and macromolecules is not known.

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CHAPTER I

Role of Wood Smoke in N-Nitrosothiazolidine Formation in Bacon

ABSTRACT

The role of wood smoke in N-nitrosothiazolidine (NTHZ) formation in bacon was investigated in model systems in which wood smoke condensate was reacted with combinations of nitrite and cysteamine, cysteine, or cystine. NTHZ formation was most pronounced in the system containing nitrite and cysteamine. When the smoke condensate was treated initially with sodium bisulfite before reaction with nitrite and cysteamine, only trace amounts of the N-nitrosamine were obtained. Pork bellies at various stages of processing and cooking were also analyzed for NTHZ. Higher levels of NTHZ were found in raw pork belly which had been cured and smoked than in the fried counterpart.

INTRODUCTION

Recently, a new N-nitrosamine, N-nitrosothiazolidine (NTHZ), has been identified in raw and fried bacon (Gray et al., 1982; Kimoto et al., 1982; Pensabene and Fiddler, 1983). This compound and its 2-alkyl derivatives, when prepared from the Maillard browning reaction (glucose and cysteamine) and nitrite, have been shown to be direct acting frameshift mutagens by the Ames mutagenicity assay procedure (Sekizawa and Shibamoto, 1980). However, Fiddler et al. (1983) have reported recently that NTHZ, when synthesized by the direct nitrosation of thiazolidine, is not mutagenic to Salmonella typhimurium TA 100 over a 2-log dose range. These investigators concluded that it was likely that other workers were not detecting the mutagenicity of NTHZ, but of some other compound.

At present, the exact mechanism of NTHZ formation in bacon has not been established. Thiazolidine, the parent amine, has not been reported in foods (Sekizawa and Shibamoto, 1980). Model system studies have shown that heterocyclic compounds including thiazolidine, 2-methylthiazolidine and 2-ethylthiazolidine are formed on heating a cysteamine/glucose model browning system (Sakaguchi and Shibamoto, 1978). Similarly, Russell (1983) reviewed the formation of NTHZ from the reaction of cysteamine/formaldehyde/nitrite. It has been proposed, based on model system studies, that NTHZ formation in bacon may be due to the reaction of glucose fragmentation products with cysteamine (Reddy et al., 1982a). While this mechanism cannot be completely discounted and may be operative towards the end of the frying process, subsequent studies indicate that the primary mode of NTHZ formation in bacon is related to the smoking process. Pensabene and Fiddler (1983) reported NTHZ data for bacon and other cured meat products which strongly suggest NTHZ is formed as a result of wood smoking. However, it was not resolved whether NTHZ was

formed during smoking and deposited on the surface of bacon during processing, or one or more of the smoke components reacted with meat constituents to form the N-nitrosamine. The objectives of the present study were to determine the mechanism of NTHZ formation during smoking, and to identify the compounds involved in its formation.

EXPERIMENTAL

Wood smoke and its role in NTHZ formation: Hickory wood smoke condensate was obtained during a typical bacon smoking process as described by Gray et al. (1982). The condensate was collected in a stainless steel tray in which was placed a 4-liter Erlenmeyer flask filled with ice. Aliquots of the condensate were reacted with nitrite, cysteamine and nitrite, cystine and nitrite, and cysteine and nitrite. Another aliquot was treated overnight with sodium bisulfite to remove the aldehydes from the condensate, vacuum distilled and the distillate reacted with cysteamine and nitrite. The pH of the reaction mixture was adjusted to pH 5.0 and the reactions carried out for 1 hr at 30°C. The reaction systems were extracted with dichloromethane and analyzed for NTHZ.

NTHZ levels were determined using a Varian 3700 gas chromatograph containing a 3 m x 2 mm i.d. glass column packed with Carbowax 20M on 80/100 mesh Chromosorb W (Supelco Inc., Bellefonte, PA) and interfaced with a thermal energy analyzer (Model 502 LC, Thermo Electron Corp., Waltham, MA). Gas chromatographic conditions included: temperature programming, 100-160°C at 20°C/min; carrier gas (nitrogen) flow rate, 30 ml/min; TEA pyrolyzer temperature 475°C; and oxygen flow rate 10 ml/min.

NTHZ formation in pork bellies: To further investigate the possible role of smoking in NTHZ formation in bacon, pork bellies were analyzed for NTHZ at various stages during processing and cooking. NTHZ levels were determined using a modified mineral oil distillation procedures. Ammonium sulfamate (2 g) was added to a 25 g portion of the pork sample (ground raw or fried) and mixed thoroughly. Mineral oil (25 ml) was added and distillation under vacuum was carried out as described previously by Reddy et al. (1982b).

RESULTS AND DISCUSSION

Wood smoke condensate was reacted with various compounds to determine the possible role of smoking in NTHZ formation in cured meats (Table 1). No NTHZ was detected in wood smoke condensate, even after the addition of sodium nitrite. However, when cysteamine was added to the condensate, NTHZ was isolated and its identity confirmed by mass spectrometry. The presence of small amounts of NTHZ in the model system containing only wood smoke condensate and cysteamine suggests the probable presence of nitrogen oxides generated during the combustion process (NAS, 1981). These results indicate that wood smoke does not contain NTHZ or thiazolidine per se, but that some component(s) of wood smoke is/are capable of reacting with cysteamine to form thiazolidine. To further test this hypothesis, the aldehydes in the wood smoke condensate were removed by forming the bisulfite addition complexes (Morrison and Boyd, 1976). After filtering and adjusting the pH to 10, the condensate was vacuum distilled, and the distillate reacted with cysteamine and nitrite as before.

Only traces of NTHZ were detected, thus suggesting that a carbonyl compound, most probably formaldehyde, is involved in the formation of this N-nitrosamine. Formaldehyde is present in wood smoke at concentrations of approximately 80 mg/100 g sawdust (Gorbatov et al.,

Table 1 - N-Nitrosothiazolidine formation in a wood smoke condensate model system

Model System[1]	NTHZ[2] (µMol)	MNTHZ (µMol)	NMOR (µMol)
Wood smoke condensate	ND[3]	ND	ND
Wood smoke condensate + nitrite	ND	ND	tr[4]
Wood smoke condensate + cysteamine	tr	ND	tr
Wood smoke condensate + cysteamine + nitrite	13.1	0.1	tr
Wood smoke condensate + cystine + nitrite	ND	ND	tr
Wood smoke condensate + cysteine + nitrite	ND	ND	tr
Wood smoke condensate treated with $NaHSO_3$ overnight, vacuum distilled, + cysteamine + nitrite	tr	ND	ND

[1] Consisted of 100 ml of wood smoke condensate, to which were added cysteamine (25 mg), cystine (25 mg), L-cysteine (25 mg), or nitrite (25 mg) where appropriate. The pH of the systems was adjusted to 5.0; reaction time 1 hr at 30°C.

[2] NTHZ, N-nitrosothiazolidine; MNTHZ, 2-methyl-N-nitrosothiazolidine, NMOR, N-nitrosomorpholine.

[3] ND, not detectable.

[4] tr, Trace

1971). Similarly, Ruiter (1979) reported a formaldehyde concentration of 710 mg/kg in a smoke solution obtained by exposing water-filled Petri dishes to smoke. Another N-nitrosamine, 2-methyl-N-nitrosothiazolidine (MNTHZ) was detected in the model system containing wood smoke condensate, cysteamine, and nitrite (Table 1). Acetaldehyde, which has been implicated in the formation of this N-nitrosamine (Sakaguchi and Shibamoto, 1978) has been identified in the low-boiling components of hickory smoke (Doerr et al., 1966).

Pork bellies at various stages of processing and cooking were analyzed for NTHZ (Table 2). Ammonium sulfamate was added to the distillation flask immediately before distillation to prevent possible artifactual N-nitrosamine formation during the extraction. Higher levels of NTHZ were found in raw pork belly which had been cured and smoked than in the fried counterpart. Although this study is somewhat limited in terms of the number of pork bellies analyzed, these preliminary data are in general agreement with those of Pensabene and Fiddler (1983). These investigators reported that the NTHZ level in raw bacon was higher than that found in fried bacon and the cook-out fat combined.

While it is apparent that the primary mode of NTHZ formation in bacon is related to the smoking process, other possible sources of this N-nitrosamine cannot yet be overlooked (Figure 1). Further studies are necessary to determine whether glucose in bacon can undergo fragmentation during frying to produce formaldehyde.

Furthermore, although the concentrations of various amines in fresh and processed pork have been determined (Lakritz et al., 1975), there is a paucity of data regarding the cysteamine content of meat products. It is possible that formaldehyde can react with cysteine to form thiazolidine carboxylic acid (thioproline), which then could be converted to NTHZ by either of the two pathways shown in Figure 1. An indication of the relative ease of decarboxylation of thioproline and its

Table 2 - N-Nitrosothiazolidine levels in pork bellies at various stages of processing and cooking[1]

Sample	NTHZ (µg/kg)[1]
Pork belly	ND
Pork belly, smoked	tr
Pork belly, cured	ND
Pork belly, cured and smoked	2.5
Pork belly, fried	ND
Pork belly, smoked and fried	ND
Pork belly, cured and fried	ND
Pork belly, cured, smoked and fried	1.4

[1] Average value of two bellies per treatment, triplicate analyses per belly.

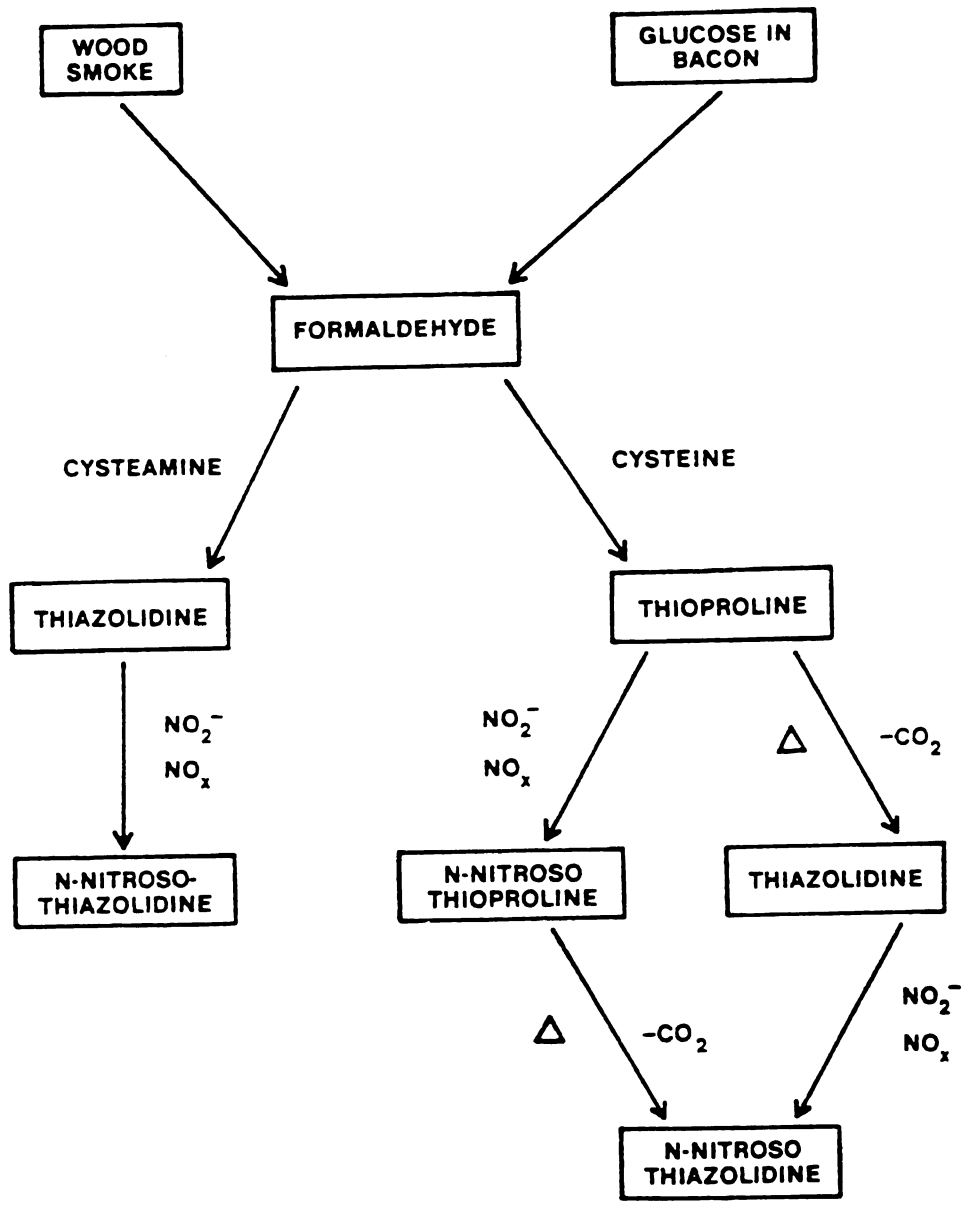

Figure 1. Possible mechanism for the presence of N-nitrosothiazolidine and N-nitrosothiazolidine carboxylic acid in smoked cured meats.

N-nitrosated derivatives was obtained from differential scanning calorimetric studies (Mandagere, unpublished results). While no thermal change was observed with thiazolidine carboxylic acid in the 80-175°C range, N-nitrosothiazolidine carboxylic acid (NTCA) underwent an endothermic change at 108°C. Thus, decarboxylation of NTCA to NTHZ occurs more readily than the transformation of thiazolidine carboxylic acid to thiazolidine. This mechanism, however, cannot account for the presence of NTHZ in raw smoked bacon as the normal smoking temperature is approximately 58°C (135°F) and is not high enough to effect the decarboxylation reaction. Further studies are required to determine cysteamine and NTCA levels in cured meats and also to ascertain whether NTHZ is formed during the frying process.

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CHAPTER II

N-Nitrosothiazolidine Carboxylic Acid and Related Compounds in Smoked Foods

ABSTRACT

Various smoked foods including raw and fried bacon, poultry products and beef were analyzed for their N-nitrosothiazolidine carboxylic acid (NTCA) contents. The highest concentrations of NTCA were observed in smoked duckling (up to 1240 µg/kg) and pheasant (up to 1009 µg/kg), while smaller concentrations (<600 µg/kg) were recorded for hams, bacon, and beef. It was also established that NTCA levels were consistently higher on the exterior of the smoked meat products than in the less exposed interior portions. Another N-nitroso compound, 2-hydroxymethyl -3-N-nitrosothiazolidine- 4-carboxylic acid (2-HMNTCA) was identified in the smoked products at concentrations ranging from 15 to 610 µg/kg. This compound, believed to arise from the reaction of glycolaldehyde in the smoke with cysteine and nitrite in the meat product, can undergo decarboxylation during the frying of bacon to produce the corresponding 2-hydroxymethyl- N-nitrosothiazolidine (2-HMNTHZ). The latter compound was not detected in raw bacon. Three smoked cheese samples contained >1000 µg/kg of 2-HMNTCA, while NTCA or related compounds were not identified in smoked salmon.

INTRODUCTION

Recently, N-nitrosothiazolidine (NTHZ) and/or N-nitrosothiazolidine carboxylic acid (NTCA) have been reported in smoked cured meats (Pensabene and Fiddler, 1983a,b; Helgason et al., 1984; Mandagere et al., 1984; Skrypec et al., 1985; Sen et al., 1985, 1986; Ikins et al., 1986). It has been proposed that the formation of these N-nitroso compounds in smoked meats is due to the reaction of formaldehyde in the smoke with cysteamine and cysteine, respectively, followed by nitrosation (Mandagere et al., 1984; Sen et al., 1985, 1986). Sen et al. (1986) surveyed various smoked foods for NTHZ and NTCA and reported that most meat and bacon samples and a few smoked fish contained traces of NTHZ and considerably high levels of NTCA (up to 13,700 μg/kg). Their data indicated a positive correlation between NTCA levels in raw bacon and the NTHZ content of fried bacon, but not with that of raw bacon. Sen et al. (1986) also reported preliminary data which indicated that raw bacon processed by the traditional wood smoking methods contained higher levels of NTCA than bacon processed with liquid smokes. Similar results were obtained by Ikins et al. (1986) who demonstrated that the incorporation of liquid smokes into curing brines resulted in raw bacon with lower concentrations of NTCA than bacon processed by the traditional wood smoke process.

It is now widely accepted that formaldehyde in the wood smoke is intimately involved in the formation of NTHZ and NTCA in smoked cured meats (Skrypec et al., 1985; Pensabene and Fiddler, 1985a,b; Sen et al., 1985, 1986). It is also clearly established that wood smoke contains other carbonyl compounds including glycolaldehyde, methylglyoxal (pyruvaldehyde), glyoxal, acetaldehyde, and propionaldehyde (Ruiter, 1970, 1979; Gilbert and Knowles, 1975). These compounds can react with cysteamine in aqueous solution to form heterocyclic compounds (Sakaguchi

and Shibamoto, 1978), all of which are capable of undergoing nitrosation. However, there is very little information pertaining to their presence in smoked cured meat products. Mandagere et al. (1984) reported small amounts of 2-methyl-N-nitrosothiazolidine (2-MeNTHZ) in a model system containing wood smoke condensate, cysteamine, and nitrite. Ikins et al. (1986) also detected 2-MeNTHZ when five liquid smoke condensates were reacted with cysteamine and nitrite. This would indicate the presence of acetaldehyde in the smoke preparations and would support the hypothesis that formaldehyde in smoke is the precursor of NTHZ. Toth and Potthast (1984) reported that formaldehyde and acetaldehyde were present in wood smoke at concentrations of 200 mg and 1150 mg per 100 g of wood, respectively.

The initial objective of this study was to determine NTCA levels in a number of smoked food products. However, during the course of the survey, a number of unidentified peaks were observed in the gas chromatograms of the sample extracts. The identification of several of these peaks is also reported herein.

EXPERIMENTAL

Safety: N-Nitrosamines are potent carcinogens and must be handled with appropriate safety precautions.

Reagents: NTCA and N-nitrosopipecolic acid (NPIC) were synthesized by nitrosating thiazolidine carboxylic acid and pipecolic acid (Sigma Chemical Co., St Louis, MO) as described by Lijinsky et al. (1970). Formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, glyoxal, pyruvaldehyde (methylglyoxal), glycolaldehyde, hexanal, cysteamine, cysteine, and sodium nitrite were purchased from Sigma Chemical Co. All other chemicals were of reagent grade and were used without further purification.

Synthesis and purification of 2-substituted N-nitrosothiazolidines and thiazolidine carboxylic acids: N-Nitrosothiazolidines and N-nitrosothiazolidine carboxylic acids were synthesized by reacting the respective aldehydes with cysteamine or cysteine at pH 11.0 according to the method described by Ratner and Clark (1937), followed by nitrosation (Lijinsky et al., 1970). NTCA and the 2-substituted N-nitrosothiazolidine carboxylic acids were converted to their methyl esters by reacting with diazomethane (prepared from Aldrich N-methyl-N-nitroso-P-toluene-sulfonamide as directed).

NTHZ and NTCA methyl ester and their 2-substituted analogues were purified by preparative thin layer chromatography (Gray and Dugan, 1975). Dichloromethane solutions of the N-nitroso compounds were streaked on a 20 x 20 cm silica gel G (Woelm) TLC plate and developed in a solvent system containing hexane, dichloromethane and ethyl acetate (4:3:3). The band containing the N-nitroso compound was monitored by covering the TLC plate with another glass plate in such a way that half-inch wide strips of the TLC plate were exposed on the two vertical sides. The exposed edges were sprayed with Griess reagent and exposed to UV light. The band between the two Griess-positive spots was removed from the plate and the N-nitroso compound eluted from the silica gel by shaking with dichloromethane. The dichloromethane extract was concentrated to 0.5 ml for gas chromatography-mass spectrometry analysis.

Gas chromatography-thermal energy analyzer (GC-TEA) analyses: The N-nitroso compounds were analyzed using a GC-TEA system comprised of a Varian 3700 gas chromatograph coupled to a TEA model 502 LC (Thermo Electron Corp., Waltham, MA). The column used was a 3 mm x 2 mm i.d. glass column packed with 2% OV-17 and 1% OV-210 on 80-100 mesh Chromosorb W (Supelco Inc., Bellefonte, PA). Gas chromatographic conditions included: oven temperature programming, 100-180°C at 5°C/min;

carrier gas (nitrogen) flow rate, 30 ml/min; TEA pyrolyzer temperature, 475°C; TEA vacuum, 1.0 mmHg; liquid nitrogen cold trap.

Gas chromatography-mass spectrometry (GC-MS): A Hewlett Packard Model 5880 GC-MS system was used under the following conditions: Ionization voltage, 70 eV; electronic multiplier voltage 2400 V; scanning range 40-300 mass units; source and transfer line temperature, 200°C. Gas chromatographic column and oven conditions were as described for the GC-TEA analyses except that helium was used as the carrier gas.

Analysis of smoked cured food product samples: Various smoked cured food products were purchased from local supermarkets and from a family processing operation specializing in smoked meats. All samples with the exception of the bacon products were analyzed without cooking. Bacon was fried in a preheated teflon-coated electric frying plan for 6 min at 171°C (340°F) as described by Ikins et al. (1986). The bacon was fried on each side for 3 min, removed from the frying pan and dried on paper towels, and then stored overnight at -20°C until analysis.

Quantitation of NTCA and other class compounds: The meat samples were ground and thoroughly mixed with a Hobart grinder (Model 84181D). N-Nitrosopipecolic acid (1 ml of 5 µg/ml NPIC in methanol), equivalent to 200 µg/kg, was added to the meat sample (25 g) which was then blended in 200 ml distilled water. Following blending (3 min at high speed in a Waring blender), the contents were quantitatively transferred to a 250 ml glass centrifuge bottle and centrifuged at 5000 rpm for 10 min in a Model K centrifuge (International Centrifuge, Needham Hts, MA). The supernatant was filtered through glass wool. The residue was reextracted with another 100 ml aliquot of distilled water and centrifuged and filtered as before. The pooled filtrates were treated with 5 ml of 20% ammonium sulfamate in 1.5N H_2SO_4 and 40 g NaCl. After sitting at room temperature for 10 min, the mixture was centrifuged for 15 min at 5000 rpm and the supernatant filtered through Whatman No. 1 filter paper.

The filtrate was transferred to a 500 ml separatory funnel and extracted with three 100 ml aliquots of ethyl acetate. The pooled ethyl acetate fractions were dried over anhydrous sodium sulfate and then concentrated to 1 ml on a rotary evaporator. The concentrated sample was quantitatively transferred to a test tube and treated with diazomethane. Following methylation for 30 min at 30°C, the sample was concentrated to 1 ml under a steady stream of nitrogen. NTCA and other class compounds were quantitated using the GC-TEA procedure previously described.

Average recoveries of 78.6 ± 3.2% were observed for unsmoked samples spiked with NTCA at 50, 100, and 150 µg/kg, while average recoveries of 83.5 ± 4.6% were obtained for the internal standard, NPIC. Mass spectral confirmation was carried out on those samples containing 100 µg/kg or more NTCA or other class compounds. Prior to mass spectral analysis, the methylated sample extracts were subjected to TLC clean-up using the chromatographic conditions described earlier. The band (Rf 0.4-0.48) containing the N-nitrosothiazolidine carboxylic acid methyl esters was scraped off the plate and eluted with dichloromethane.

Quantitation of NTHZ and other class compounds in raw and fried bacon was achieved using the same extraction procedure as that described for NTCA. A known aliquot of the ethyl acetate extract was streaked on silica gel G TLC plates and developed in the solvent system, hexane/dichloromethane/ethyl acetate (4:3:3). A large Griess reagent-positive band with an Rf value of 0.71-0.75 was found to contain the volatile N-nitrosamines including NTHZ and N-nitrosopyrrolidine (NPYR). The N-nitrosamines were eluted from the silica gel with dichloromethane and quantitated using the GC-TEA system. N-Nitrosothiomorpholine (200 ng) was routinely added to each sample at the beginning of the analysis to monitor the efficiency of the analytical process. The results were not corrected for percent recoveries (79 ± 5%) of the internal standard. Similarly, addition of 2,6-dimethylmorpholine

(100 µg/kg) to the extraction system indicated that N-nitroso-2,6-dimethylmorpholine was not artifactually formed during the extraction, clean-up, and concentration steps.

Model system study: Hickory wood smoke condensate was obtained during a typical bacon process as described by Mandagere et al. (1984). Aliquots of the condensate (10 ml) were reacted with nitrite (5 mMol) and cysteine/cysteamine (1 mMol). The pH of the reaction mixtures was adjusted to 5.5 with concentrated NaOH and the reactions carried out for 1 hr at 55°C. After the addition of 5 ml of 20% ammonium sulfamate in 1N H_2SO_4, the reaction systems were extracted with dichloromethane when cysteamine was used as a reactant, and with ethyl acetate when cysteine was used in the model system. Analyses for the N-nitroso compounds were performed as previously described.

RESULTS AND DISCUSSION

Model system study: Preliminary analysis of several selected smoked cured meat products indicated the presence of NTCA at concentrations ranging from 10 to 1200 µg/kg. Smaller quantities of 2-MeNTCA (35-95 µg/kg) were observed in many of the samples. During the course of these analyses, however, several peaks, one of which was quite large, were observed on the GC-TEA chromatograms that did not correspond to any of the traditionally studied N-nitroso compounds. When portions of the sample extracts were exposed overnight to ultraviolet light (365 nm), a substantial reduction in size of the large unidentified peak and that corresponding to NTCA was observed. This strongly suggested the presence of other N-nitroso compounds (Doerr and Fiddler, 1977), which could conceivably arise from the reaction of smoke carbonyls with selected meat components and nitrite.

To evaluate this hypothesis, several aldehydes known to be present in woodsmoke were reacted with cysteamine/cysteine and nitrite to produce

the corresponding N-nitrosothiazolidine-class compounds. The identities of these compounds were confirmed by mass spectral analyses. Model system studies involving the reaction of wood smoke condensate, cysteine/cysteamine and nitrite, were then initiated to evaluate further the role of smoke carbonyls in N-nitrosamine formation in smoked cured meats. Previous studies with a similar model system revealed only the formation of NTHZ when cysteamine was used as a reactant (Mandagere et al., 1984). Ikins et al. (1986) reported not only NTHZ but also 2-MeNTHZ formation when wood smoke condensate and four commercial liquid smoke preparations were reacted with cysteamine and nitrite. Analytical conditions included the use of the polar Carbowax 20M as the stationary phase in the gas chromatographic column.

In the present study, Carbowax 20M was replaced by a mixed stationary phase of 2% OV-17/1% OV-210 and this produced chromatograms similar to those illustrated in Figure 1 for solvent extracts of the reaction products. Chromatographic retention times and mass spectral analysis confirmed the presence of large amounts of NTHZ and 2-hydroxymethyl-N-nitrosothiazolidine (2-HMNTHZ) in model systems when cysteamine was a reactant, and NTCA and 2-hydroxymethyl-N-nitrosothiazolidine carboxylic acid (2-HMNTCA) when cysteine was utilized. Due to the polar nature of 2-HMNTHZ and 2-HMNTCA, these compounds could not be resolved on the Carbowax 20M column as used in the earlier study by Mandagere et al. (1984). Retention times of 4.1 min for NTHZ, 10.3 min for 2-HMNTHZ, 9.3 min for NTCA methyl ester, and 14.3 and 15.5 min for 2-HMNTCA methyl ester were recorded under the GC-TEA and GC-MS conditions used. Massey et al. (1985) have recently identified 2-HMNTCA in smoked bacon and implicated glycolaldehyde in wood smoke as a precursor. These investigators reported that capillary GC-TEA analysis of the trimethlsilyl derivative of 2-HMNTCA indicated the

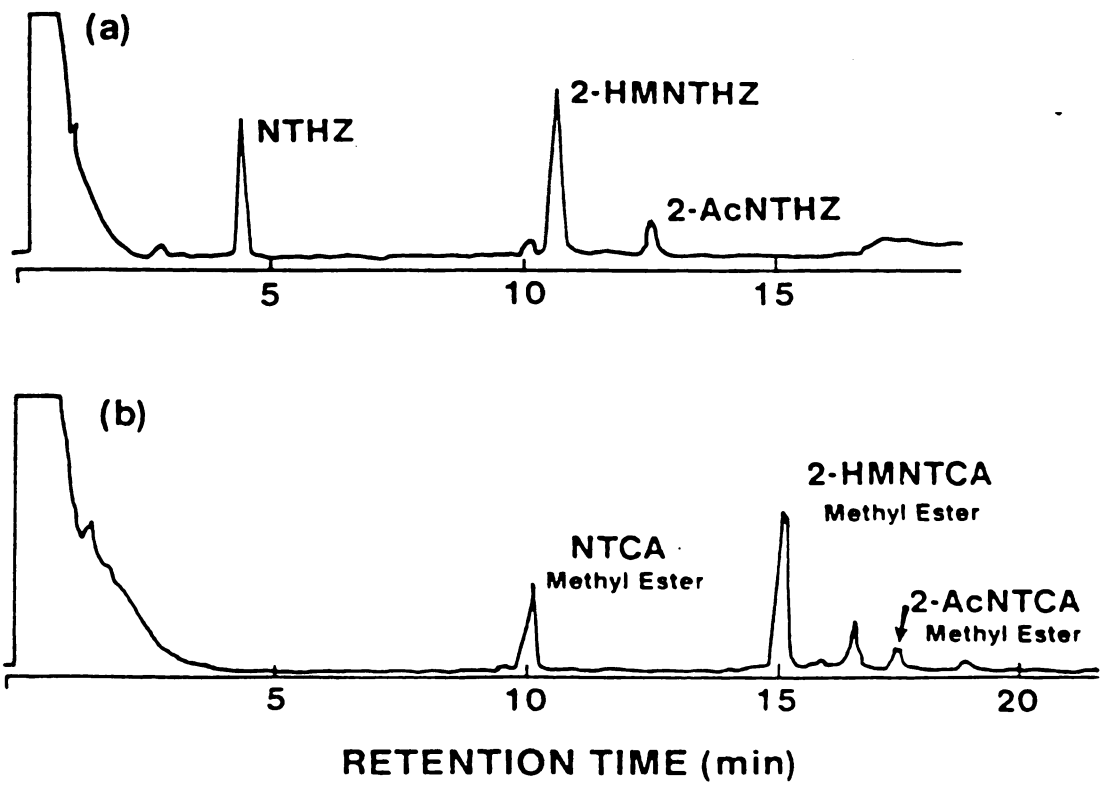

Figure 1. GC-TEA chromatograms of solvent extracts of model systems containing (a) wood smoke condensate, cysteamine, and nitrite, and (b) wood smoke condensate, cysteine, and nitrite. Extracts analyzed on the mixed phase (2% OV-17 and 1% OV-210) column. NTHZ, N-nitrosothiazolidine; 2-HMNTHZ, 2-hydroxymethyl- N-nitrosothiazolidine; 2-AcNTHZ, 2-acetyl-N-nitrosothiazolidine; NTCA, N-nitrosothiazolidine carboxylic acid; 2-HMNTCA, 2-hydroxymethyl-N-nitrosothiazolidine carboxylic acid; 2-AcNTCA, 2-acetyl-N-nitrosothiazolidine carboxylic acid.

presence of two peaks, corresponding to the two isomeric forms of the compound.

Mass spectral analysis of the isolated 2-HMNTCA methyl ester showed that the spectrum was identical (95% match) to that of its synthesized counterpart. Its mass spectrum is shown in Figure 2. The major

fragments common to both compounds are as follows: m/z (% relative abundance) 86 (100), 59 (65.5), 146 (28.8), 87.2 (16.7), 60 (11.2), 175 (8.8), and 176 (3.9). The major ion (86) arises from the loss of NO, CH_2OH, and $-C-OCH_3$, while m/z 59 corresponds to $CH_3-O-C=O^+$. The ion m/z 60 is due to cleavage of the thiazolidine ring (Coughlin, 1979), while m/z 175 and 176 arise from loss of CH_2OH and NO, respectively. The molecular ion (M=206) was not observed in any of the two spectra.

The mass spectrum of 2-HMNTHZ, isolated from the model system containing cysteamine, smoke condensate, and nitrite, is shown in Figure 2 and exhibits the following major ions: m/z 60 (100), 88 (55.2), 87 (43.7), 59 (46.9), 118 (29.9), and 117 (20.6). The numbers in parentheses are the relative abundances. The major ion (m/z 60) arises from the cleavage of the thiazolidine ring, while m/z 118 arises from the loss of NO. The molecular ion was m/z 148.

Two other small peaks with retention times of 12.1 and 17.6 min were also observed during the analysis of extracts of the wood smoke model systems (Figure 1). The mass spectra (Figure 3) of the two peaks matched those obtained for standard 2-acetyl-N-nitrosothiazolidine (2-AcNTHZ) and 2-acetyl-N-nitrosothiazolidine carboxylic acid methyl ester (2-AcNTCA), respectively. These latter compounds were synthesized by reacting methylglyoxal with cysteamine/cysteine and nitrite. The retention times of the two standard compounds were identical to those of the two compounds present in the model system reaction mixtures. Mass spectra data for these compounds are summarized as follows: 2-AcNTHZ, m/z (% relative abundance): 60 (100), 88 (93.3), 87 (64.2), 160 (42.8),

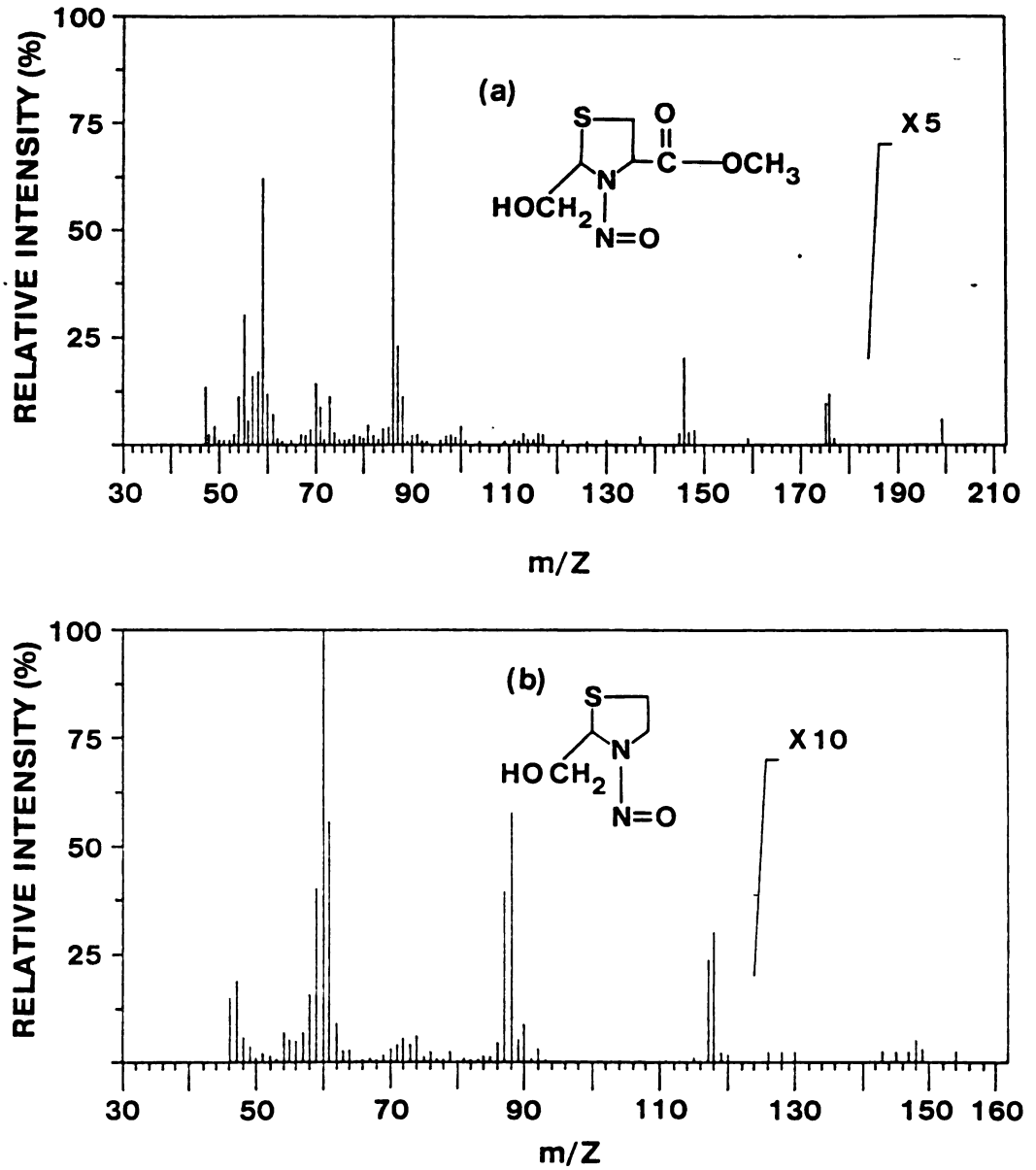

Figure 2. Mass spectra of (a) 2-hydroxymethyl-N-nitrosothiazolidine carboxylic acids (isolated as the methyl ester) and (b) 2-hydroxymethyl-N-nitrosothiazolidine formed in the wood smoke condensate model systems.

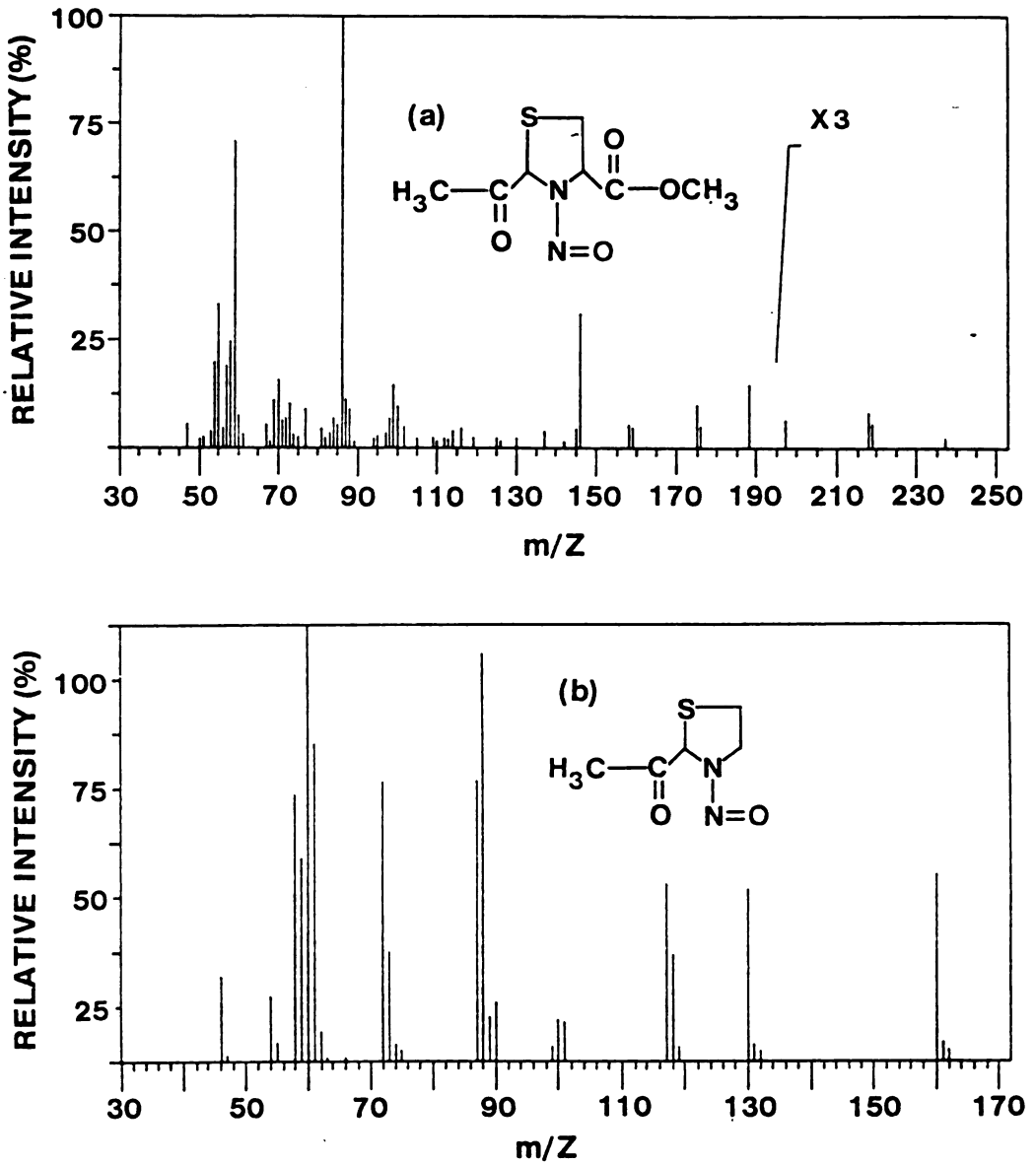

Figure 3. Mass spectra of (a) 2-acetyl-N-nitrosothiazolidine carboxylic acid (isolated as the methyl ester) and
(b) 2-acetyl-N-nitrosthiazolidine formed in the wood smoke condensate model systems.

117 (40.7), 130 (39.3), 118 (24.2), and 161 (3.4). The major ion m/z 60 arises from thiazolidine ring cleavage, m/z 88 from loss of NO and $-CH_3C=O^+$, m/z 117 from loss of the acetyl group, and m/z 130 from loss of NO. The molecular ion is m/z 160. 2-AcNTCA methyl ester, m/z (% relative abundance): 86 (100), 59 (70.7), 146 (30.9), 188 (14.6), 175 (10.0), 159 (4.9), and 218 (2.7). The major ion, m/z 86 arises from loss of NO, acetyl and methyl ester groups, while the next major ion 59 is due to the methyl ester group. The molecular ion (M^+) is m/z 218.

Relative concentrations of N-nitroso compounds formed in the cysteamine/cysteine, smoke condensate and nitrite reaction model systems are presented in Table 1. 2-HMNTCA and 2-HMNTHZ were the major components present in the cysteine and cysteamine systems, respectively. The levels of 2-HMNTCA and 2-HMNTHZ were approximately 1.7 times greater than those obtained for NTCA and NTHZ. This ratio is consistent with the relative levels of glycolaldehyde (1500 mg/kg) and formaldehyde (710 mg/kg) in a smoke solution as reported by Ruiter (1979). While the relative concentrations of methylglyoxal (830 mg/kg) and formaldehyde in the smoke solution were approximately equal (Ruiter, 1979), the 2-AcNTCA/2-AcNTHZ levels in the model system reaction mixtures were less than 10% of the NTCA/NTHZ levels, respectively. This large difference in concentrations indicates that formaldehyde is much more reactive than methylglyoxal with respect to cysteine/cysteamine.

NTCA and other class compounds in smoked foods: The levels of NTCA, 2-MeNTCA and 2-HMNTCA in various smoked foods are presented in Table 2. All samples assayed with the exception of smoked salmon were found to contain NTCA. The highest concentrations of NTCA were observed in smoked duckling (830-1204 µg/kg) and pheasant (901-1009 µg/kg). These products were obtained from a local processor specializing in the production of smoked meat products and they could be visually described as being heavily smoked. Commercially processed hams, bacon, and beef had smaller

Table 1. N-Nitrosamine formation in the wood smoke condensate model system

System[1]	NTHZ	2-HMNTHZ	2-AcNTHZ	NTCA	2-HMNTCA	2-AcNTCA
	Concentration[2] in µMol					
Cysteine, nitrite, smoke condensate	--	--	--	3.6 (2.8-4.3)	7.8 (6.3-9.2)	0.28 (0.23-0.40)
Cysteamine nitrite, smoke condensate	57.6 (51.7-63.6)	75.0 (64.9-84.5)	1.6 (0.4-1.9)	--	--	--

[1]Model system consisted of smoke condensate (10 ml), nitrite (5mMol), and cysteamine/L-cysteine (1 mMol), reaction period 1 hr, temperature 55°C; pH 5.5, total volume, 100 ml.

[2]Average of two determinations, duplicate experiments.

Table 2. N-Nitrosothiazolidine carboxylic acid and other class compounds in various smoked food products

Sample[1] type	Number of Samples[2] Analyzed	NTCA Mean (Range)	2-MeNTCA Mean (Range)	2-HMNTCA Mean (Range)
		µg/kg		
Bacon, raw	40	465 (18-501)	21 (ND[3]-26)	442 (22-618)
Bacon, fried	25	82 (5-136)	ND (ND)	54 (14-72)
Ham	3	361 (219-490)	14 (ND-21)	285 (196-475)
Summer sausage	5	44 (6-68)	(ND)	ND
Pepperoni	3	37 (28-56)	(ND)	11 (8-14)
Pork chops	3	68 (46-98)	1 (ND)	ND
Beef	3	492 (328-570)	13 (ND-28)	168 (129-255)
Turkey ham	3	177 (145-225)	10 (5-13)	124 (112-140)
Duckling	3	1067 (829-1240)	76 (35-97)	439 (409-462)
Turkey	5	205 (119-237)	20 (12-27)	216 (198-245)
Capon	3	467 (391-493)	61 (27-81)	357 (310-390)
Pheasant	4	969 (901-1008)	92 (76-98)	59 (24-75)
Salmon	3	ND	ND	
Cheese	3	15 (5-24)	ND	1182 (1062-1328)

[1]All samples were smoked and cured with the exception of cheese and pork chops. These samples were smoked only.

[2]Duplicate analysis per sample, N-nitrosamine data uncorrected for recovery.

[3]ND = none detected (detection limit for reliable measurement, 5 µg/kg).

NTCA levels, generally in the range 218-570 µg/kg. The presence of high concentration of NTCA in some samples is consistent with previous literature reports. Helgason et al. (1984) reported NTCA levels of 1000-4000 µg/kg in smoked mutton and bacon, while Pensabene and Fiddler (1985b) obtained values up to 1400 µg/kg for cure-pumped bacon.

Sen et al. (1985, 1986) surveyed various smoked meats and fish and reported NTCA levels as high as 9000 µg/kg in raw bacon samples. These investigators also reported that raw bacon samples processed by old-fashioned direct smoking methods contained the highest level of NTCA (>1000 µg/kg), whereas those processed with liquid smoke contained either undetectable or extremely low levels of NTCA. Ikins et al. (1986) also reported that the incorporation of liquid smokes into curing brines resulted in raw bacon with lower NTCA levels than bacon processed by the traditional wood smoke process.

The ham, duckling, capon, and pheasant samples were further evaluated for NTCA distribution. NTCA content was found to be consistently higher on the exterior than in the less exposed interior portion of the meat products. Similar findings were reported by Pensabene and Fiddler (1983a) for NTHZ distribution in raw bacon. These observations indicate that formaldehyde is primarily deposited on the surface of the product and gradually migrates into the interior. The amount of formaldehyde absorbed by the product depends on the formaldehyde concentration in the smoke, the duration of exposure to smoke, and the temperature and relative humidity in the smokehouse (Ruiter, 1979). Toth and Potthast (1984) reported that formaldehyde was present in wood smoke at a concentration of 200 mg per 100 g of wood. As formaldehyde is deposited and absorbed into the food products, it reacts with cysteine and/or cysteamine to form thiazolidine carboxylic acid and thiazolidine, respectively, which in turn reacts with nitrite in the product to form the N-nitroso compound (Fiddler et al., 1986).

Three salmon samples were negative for NTCA, the minimum level of reliable measurement being 5 µg/kg (Table 2). Helgason et al. (1984) also reported negligible quantities of NTCA in smoked salmon. However, Sen et al. (1986) analyzed 20 fish and seafood products and reported a mean NTCA content of 67 µg/kg. Eight of the samples analyzed contained low levels of NTCA, while one smoked herring sample contained an extremely high (1,600 µg/kg) level of NTCA. These investigators speculated that oxides of nitrogen present in smoke, or nitrite, produced by microbial reduction of nitrates in water or present in salt (as are impurity) are possible sources of the nitrosating species.

2-MeNTCA was observed in the majority of the products tested. However, the levels of 2-MeNTCA were only 5-10% of those for NTCA. These findings are generally consistent with the results of model system studies conducted by Mandagere et al. (1984) and Ikins et al. (1986). When cysteamine and nitrite were reacted with wood smoke condensate, the levels of 2-MeNTHZ observed were less than 10% of the NTHZ levels. This would indicate that formaldehyde reacts much more rapidly with cysteamine and cysteine than does acetaldehyde. Consequently, 2-MeNTHZ was not detected in any of the fried bacon samples analyzed in the survey (limit of detection 3 µg/kg).

Smoked cured foods that were found to contain 2-HMNTA were ham, bacon (raw and fried), turkey, duckling, capon, pheasant, and beef at concentrations ranging from 16-610 µg/kg (Figure 4, Table 2). The levels of 2-HMNTCA detected were generally less than or equal to those recorded for NTCA. This trend was opposite to that previously observed in the model system studies where 2-HMNTCA was the major N-nitroso compound identified (Table 1). However, meat is a much more complex system and contain other amino compounds, e.g., lysine, that can compete with cysteine for the smoke carbonyls. Glycolaldehyde is an active browner with amino groups (Ruiter, 1979) and it is likely that a large portion of

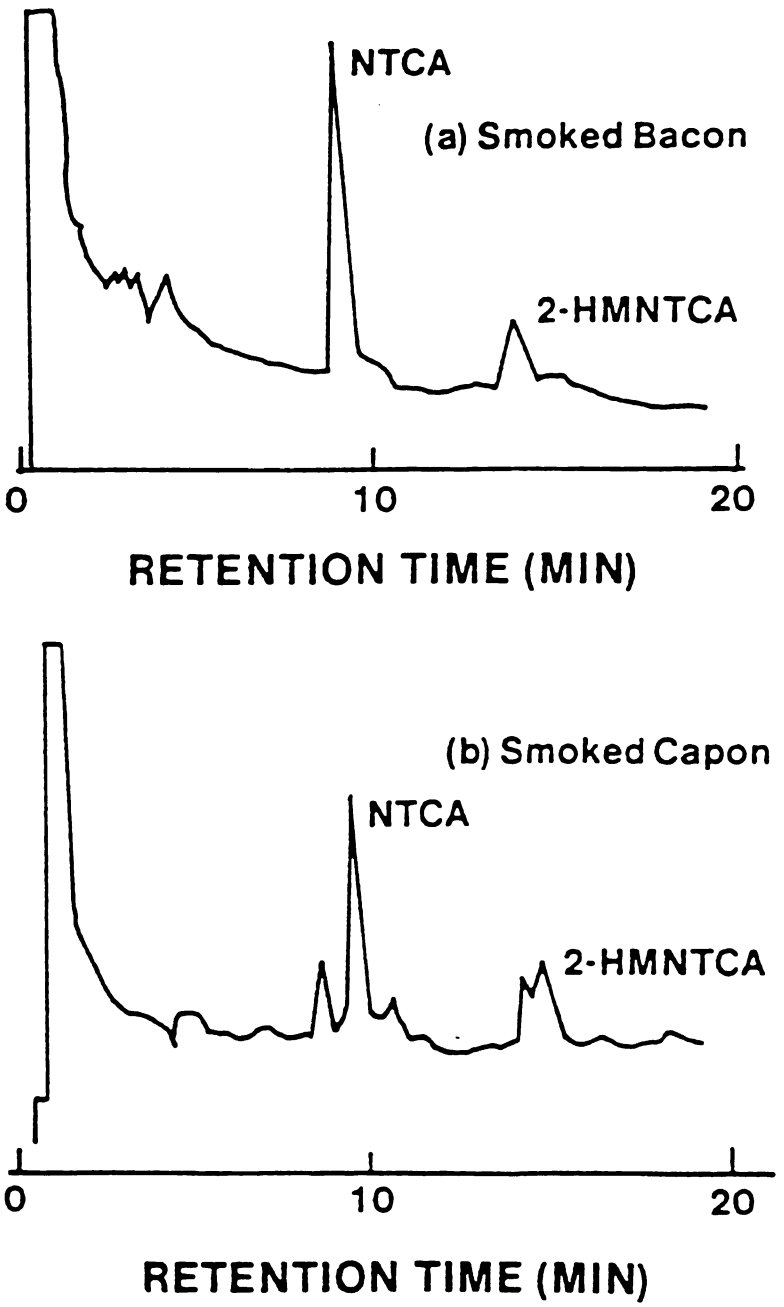

Figure 4. GC-TEA chromatograms of ethyl acetate extracts of (1) raw bacon and (b) capon showing the presence of 2-hydroxymethyl-N-nitrosothiazolidine carboxylic acids and N-nitrosothiazolidine carboxylic acid (isolated as the methyl esters). The internal standard (N-nitrosopipecolic acid) was not added to those samples.

this aldehyde reacts with amines other than cysteine during smoking of meats.

Raw bacon samples contained higher levels of 2-HMNTCA and NTCA than the corresponding fried bacons. It is possible that 2-HMNTCA can undergo thermal decarboxylation during frying of bacon to produce the corresponding N-nitrosamine. Results of Sen et al. (1985, 1986) and Ikins et al. (1986) have implicated the heat-induced decarboxylation of NTCA during the frying of bacon. Furthermore, Sen et al. (1986) have provided data which indicate a positive correlation between NTCA levels in raw bacon and the NTHZ content of fried bacon. Consequently, we analyzed a number (10) of fried bacon samples and found 2-HMNTHZ to be present at the 3-16 µg/kg level (average 8.5 µg/kg). A typical GC-TEA chromatogram of a TLC extract of fried bacon is shown in Figure 5. The identity of 2-HMNTHZ was confirmed by mass spectral analysis. NTHZ concentrations in the range of 3-18 µg/kg (average 9.8 µg/kg) were also recorded. However, the analytical conditions used in this study did not provide as good a resolution of the NTHZ peak as that obtained using a Carbowax column (Ikins et al., 1986). The NTHZ values were somewhat higher than those previously reported by our laboratory for bacon processed under controlled conditions at the Meat Laboratory at Michigan State University (Mandagere et al., 1984; Ikins et al., 1986), but were in general agreement with those reported by Vecchio et al. (1986) for consumer-cooked commercial bacon. Two other peaks at 6.3 and 8.3 min could not be identified by mass spectral analysis due to low concentrations and high background interference.

When the mineral oil distillation procedure (Ikins et al., 1986) was applied to the fried bacon samples, 2-HMNTHZ was not detected, possibly due to the low volatility of the N-nitrosamine. The dual column chromatographic method developed by Pensabene and Fiddler (1982) for NTHZ in fried bacon was not used in this study, although it seems likely that

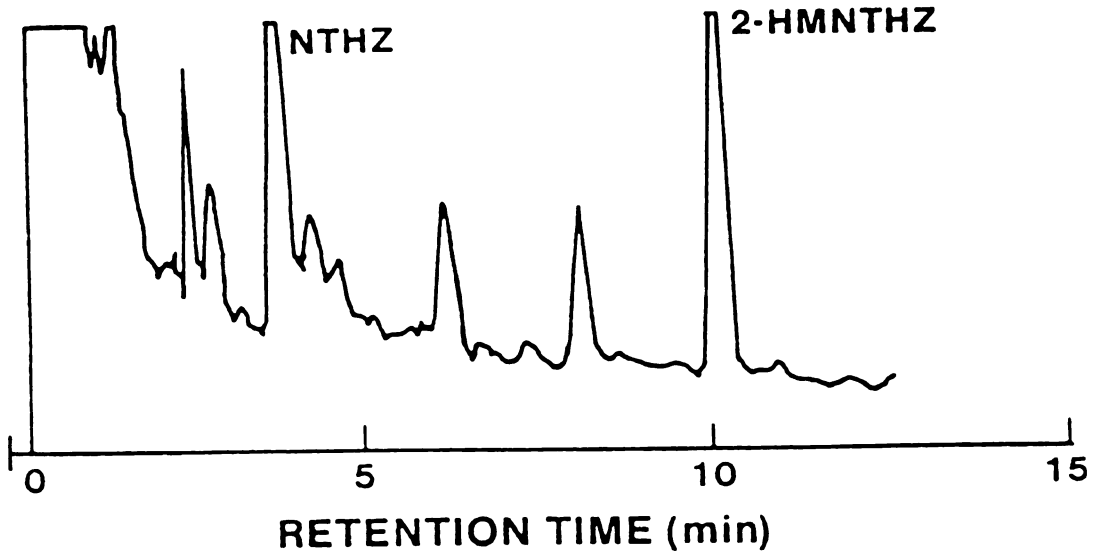

Figure 5. GC-TEA chromatogram of a TLC fraction of a fried bacon extract showing the presence of 2-hydroxymethyl-N-nitrosothiazolidine and N-nitrosothiazolidine, when analyzed on a 2% OV-17 and 1% OV-210 column.

this method would provide a more rapid and more efficient isolation of 2-HMNTHZ from fried bacon than the TLC procedure used in this study.

None of the meat samples assayed was found to contain 2-AcNTCA. However, this was not unexpected, based on the low amounts of 2-HMNTCA and 2-AcNTCA formed in the model system containing cysteine, smoke condensate and nitrite.

One of the most surprising observations of our survey was the high levels of 2-HMNTCA (1062-1328 µg/kg) in the three smoked cheese samples analyzed. Mass spectral analysis confirmed the presence of this compound in the cheeses. These levels appear to be very high for a product that contains relatively low concentrations of nitrite (NAS, 1981). Sen et al. (1986) also reported an extremely high level (1,600 µg/kg) of NTCA in one sample of smoked herring that was not nitrite-cured. It is possible that the small amounts of nitrate in cheese (10.0 mg/kg, NAS 1981) might undergo bacterial reduction to nitrite. Nitrogen oxides gases in smoke are also likely to be involved in the nitrosation process. Clearly, a much more comprehensive study of smoked cheese products is warranted in order to establish the concentrations of N-nitrosothiazolidine compounds and to determine the source(s) of the nitrosating species.

Results of this study indicate that a number of N-nitroso derivatives of heterocyclic carboxylic acids are present in smoked cured meats. In addition to the widely studied NTCA (Pensabene and Fiddler, 1985 a,b; Sen et al., 1985, 1986), many of the smoked cured foods surveyed contained 2-HMNTCA. This compound has also been recently identified in smoked cured bacon by Massey et al. (1985). A possible mechanism of formation of this compound is presented in Figure 6.

Glycolaldehyde present in wood smoke can react with cysteine in the meat product to form the thiazolidine carboxylic acid which is then readily nitrosated. 2-HMNTHZ was also detected in fried bacon and a

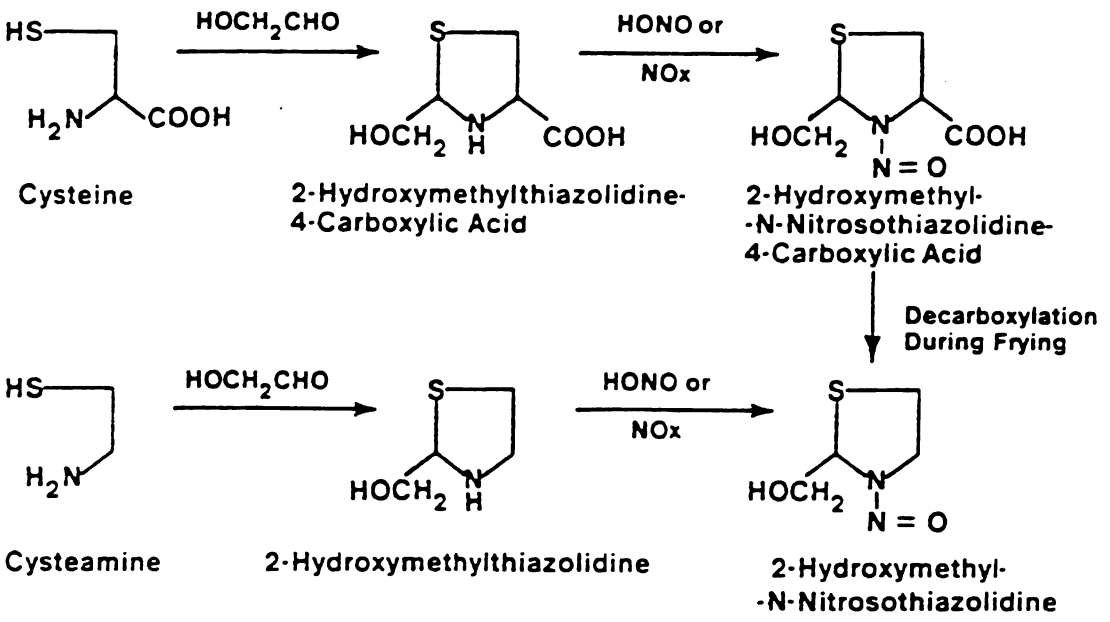

Figure 6. Possible pathways for the formation of 2-hydroxymethyl-N-nitrosothiazolidine carboxylic acid and 2-hydroxymethyl-N-nitrosothiazolidine in bacon.

likely pathway appears to be the decarboxylation of 2-HMNTCA. However, more definitive studies are necessary to confirm its mode of formation. Sen et al. (1986) have reported a positive correlation between NTCA levels in raw bacon and the NTHZ content of fried bacon, while Pensabene and Fiddler (1985b) have shown in a bacon model system that NTCA must be present in large amounts to contribute to NTHZ formation when bacon is fried. These latter investigators also have presented evidence from both model system and frying experiments with NTCA and its precursors which suggests that NTCA decarboxylation to NTHZ is not the principal pathway to NTHZ formation in uncooked bacon. While 2-HMNTHZ was not detected in raw bacon in our survey (limit of detection 3 ug/kg), the prevalence of glycolaldehyde in smoke and the levels of 2-HMNTCA in raw bacon and other smoked cured meats, would suggest that the mechanisms currently being cited for NTHZ formation in raw and cooked bacon (Pensabene and Fiddler, 1985b; Sen et al., 1986) would also apply to 2-HMNTHZ formation in bacon.

CONCLUSIONS

This study demonstrates a much wider and a more complex involvement of smoke in N-nitrosamine formation in smoked foods than was previously anticipated. Further studies are needed to assess the toxicological significance of these compounds in food systems. Although very little is known about the toxicity of NTCA or NTHZ, Helgason et al. (1984) implicated NTCA as the causative agent of the high incidence of diabetics in juveniles whose mothers consumed heavily smoked meats during pregnancy. It seems likely that other N-nitroso heterocyclic carboxylic acids will elicit similar responses.

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CHAPTER III

The Effects of Smoking Time and Frying on N-Nitrosothiazolidine Formation in Bacon

ABSTRACT

Formation of N-nitrosothiazolidine carboxylic acid (NTCA) and N-nitrosothiazolidine (NTHZ) in raw bacon as a function of smoking time was studied. Maximum formation of these compounds occurred after 30 minutes of smoking. There was a significant decrease in NTCA levels in bacon smoked for 60, 120, 240, and 480 min as compared to the 30 min samples. Kinetic studies with a pork belly model system indicated that, under conditions simulating the pan-frying of bacon, approximately 2% of the NTCA present in bacon is converted to NTHZ. The amount of NTHZ formed, however, does not account for the large differences in the NTCA contents of raw and fried bacon. The NTHZ levels in raw and fried bacon strongly suggest that the majority of NTCA decomposed during frying is converted to products other than NTHZ. The NTCA and NTHZ data also suggest the intermediacy of NTCA in NTHZ formation in fried bacon.

INTRODUCTION

Considerable amounts of processed meat products in the United States are smoked by the traditional wood smoking process. Recently, smoking has been implicated in the formation of N-nitrosothiazolidine (NTHZ) in cured meats (Pensabene and Fiddler, 1983; Mandagere et al., 1984). Initial studies on the mutagenicity of NTHZ by Sekizawa and Shibamoto (1980) indicated that NTHZ was an indirect mutagen. However, later studies by Fiddler et al. (1984) with NTHZ prepared by the direct nitrosation of thiazolidine indicated that it was not mutagenic.

While the experimental evidence for mutagenicity of NTHZ is inconclusive, N-nitrosothiazolidine carboxylic acid (NTCA) has been shown to induce diabetes in experimental animals (Helgason et al., 1984). Onset of diabetes was observed in the male offspring of mice that had been fed NTCA or smoked cured meats containing high levels of NTCA. Epidemiological studies in Iceland by these investigators indicated a positive correlation between the incidence of juvenile diabetes in male children and the consumption of large quantities of smoked, cured mutton by their parents. Although further research in required in this area, there appears to be a potential health hazard associated with the consumption of smoked cured meats.

To date, the mode of formation of these two N-nitroso compound in smoked meats has not been clearly established, although it appears that formaldehyde in wood smoke is directly involved in their formation (Mandagere et al., 1984; Pensabene and Fiddler, 1985a,b; Sen et al., 1986).

Surveys of smoked cured meats reveal that NTCA levels are consistently much higher than NTHZ levels (Helgason et al., 1984; Pensabene and Fiddler, 1985b; Sen et al., 1985, 1986; Ikins et al., 1986). Recent studies by Sen et al. (1986) and Ikins

et al. (1986) indicate that NTCA levels in raw bacon correlated with those for NTHZ in the corresponding fried bacon samples. Pensabene and Fiddler (1983), however, have postulated that NTHZ in fried bacon originates from that present in the raw bacon and that none is formed during frying. In a later study, Pensabene and Fiddler (1985b) concluded that NTCA must be present in large amounts in raw bacon to contribute to NTHZ formation when bacon is fried. Their results also indicated that while NTCA was present in raw bacon at concentrations considerably higher than normally obtained for NTHZ, the concentrations were sufficiently low as not to significantly contribute to NTHZ formation during normal frying. The major purpose of this study was to further delineate the mechanism of NTHZ and NTCA formation in bacon. Specific objectives of the study were to determine the effect of smoking time on NTHZ and NTCA formation in bacon, and to investigate the kinetics of the thermal decomposition of NTCA and subsequent formation of NTHZ.

EXPERIMENTAL

Safety: N-Nitrosamines are potent carcinogens and must be handled with appropriate safety precautions.

Materials: NTHZ, NTCA, N-nitrosothiomorpholine and N-nitrosopipecolic acid (NPIC) were synthesized from thiazolidine, thioproline (thiazolidine carboxylic acid), thiomorpholine, and pipecolic acid (Sigma Chemical Co., St. Louis, MO) according to the method described by Lijinsky et al. (1970).

Effect of smoking time on N-nitrosamine formation in bacon: Eighteen bellies (approximately 4-5 kg) were obtained from a commercial supplier soon after slaughter and stored for no more than 2 days in a coooler at 2°C. All bellies were stitch pumped to 110% of their green weight with a brine containing 15% sodium chloride, 5% sucrose, 3.5% sodium tripolyphosphate, 1,200 mg/kg sodium nitrite, and 5,500 mg/kg sodium

ascorbate, and smoked as described by Reddy et al. (1982). Three bellies were selected at random and removed from the smoke house during the smoking cycle at time intervals of 0, 30, 60, 120, 240, and 480 min. The smoked bellies were transferred to a holding cooler (2°C) where they were held overnight before slicing and vacuum packaging. The sliced and packaged bacon samples were held for one week at 2°C. Two packages randomly selected from each belly were analyzed for NTHZ and NTCA. Similar samples were selected and fried in a preheated electric frying pan (340°F) for 6 min (3 min per side). The fried samples were ground and analyzed for NTHZ, NTCA and other N-nitrosamines. The study was repeated three times and all samples were analyzed in duplicate.

N-Nitrosamine analyses: NTHZ and other volatile N-nitrosamine concentrations in raw and fried bacon were determined by a modified mineral oil vacuum distillation procedure as described by Reddy et al. (1982). A 25 g aliquot of ground bacon was mixed with 1 ml of 20% ammonium sulfamate in 1N H_2SO_4, along with 1 ml of 0.2 µg/ml of N-nitrosothiomorpholine as internal standard. Average recoveries of 83 ± 5% were achieved for the internal standard. Ammonium sulfamate in 1N H_2SO_4 was added in order to prevent artifactual formation of N-nitrosamines during the analysis. Addition of 2,6-dimethylmorpholine (100 µg/kg) to the distillation system indicated that N-nitroso-2,6-dimethylmorpholine was not an artifact. N-Nitrosamine levels were quantitated using a CG-TEA system comprised of a Varian 3700 Model gas chromatograph interfaced to a TEA Model 502 LC (Thermo Electron Corp., Waltham, MA). The N-nitrosamines were separated on a 3 m x 2 mm i.d. glass column packed with 10% Carbowax 20M TPA on 80/100 mesh Chromosorb W (Anspec Comp., Inc., Ann Arbor, MI.). GC-TEA conditions included: oven temperature program, 140-180°C at 15°C/min; carrier gas (nitrogen) flow rate 30 ml/min; TEA pyrolyzer temperature, 475°C; TEA vacuum, 1.0 mm; oxygen flow rate, 10 ml/min.

Quantitation of NTCA in bacon: The NTCA contents of the bacon samples (raw and fried) were determined using the ethyl acetate extraction procedure described previously (Mandagere et al., 1986). NPIC was used as the internal standard, the average recovery being 79 ± 4%. NTCA was analyzed as its methyl ester which was prepared by methylating the extracted NTCA with diazomethane, prepared from N-methyl-1-N-nitroso-p-toluene-sulfonamide (Aldrich Chemical Co., Milwaukee, WI) as directed by the manufacturer.

Model system study: Hickory wood smoke condensate was collected at various time intervals (30, 60, 120, 240, and 480 min) during the smoking of bellies in order to assess the potential of the various smoke fractions for NTHZ and NTCA formation (Mandagere et al., 1984). Ten ml aliquots of the smoke condensate were reacted with 5 mMol of nitrate and 1 mMol of cysteamine (or cysteine) in 100 ml of water at pH 5.5 for 1 hr at 55°C. The reaction was stopped by the addition of 5 ml of 20% ammonium sulfamate in 1N sulfuric acid. The reaction systems were extracted with three 30 ml aliquots of dichloromethane when cysteamine was used as a reactant, and with three 30 ml of aliquots of ethyl actate when cysteine was included in the model systems. The pooled solvent extracts were dried over anhydrous sodium sulfate and the final volumes adjusted to 100 ml.

NTHZ and NTCA (as its methyl ester) were quantitate using the GC-TEA conditions previously described for the bacon analyses.

Statistical treatment of N-nitrosamine data: Statistical analyses of N-nitrosamine data for the model system study and the different bacon treatments were performed using Bonferroni t statistics (Gill, 1978).

Kinetics of NTCA thermal decomposition in green pork belly: Fresh green pork bellies obtained from a commercial supplier within 24 hr of slaughter, were ground twice in a Oster food grinder (Model 945-08-H, Oster Corp., Milwaukee, WI). Aliquots (0.5 g) of the ground pork belly

were mixed with 2.5 µMol of NTCA in 10 ul of methanol, transferred to a 10 ml glass vial (Wheaton Scientific Co., Millville, NJ) and flame sealed. The vials were heated at preset temperatures (75-175°C at 25°C intervals) for 3, 6, 9, and 12 min in Reacti-Therm™ heating modules (Pierce Chemical Co., Rockport, IL) filled with silicone oil. A thermometer was placed in an oil well to monitor the temperature during the heating process.

After heating, the vials were cooled rapidly in a dry ice/actone bath and the contents were ground thoroughly with 5 g anhydrous sodium sulfate in a mortar. The free-flowing samples were spiked with 5 µg NPIC and 1 µg N-nitrosomorpholine (NMOR) in 100 µl methanol and mixed for an additional 30 seconds. The samples were quantitatively transferred to a chromatographic column (300 x 15 mm) and an additional 5 g of sodium sulfate added to the top of the sample in the column. The mortar and pestle were rinsed with two 10 ml aliquots of 10% dichloromethane in n-pentane which were then poured into the column. Another 30 ml portion of the pentane solution was added to the column. The eluate containing NTHZ and NMOR was collected at 1-2 ml per min. After approximately 40 ml of eluate were collected, the receiving flasks were changed and the residual NTCA and NPIC were eluted from the column with 50 ml of 25% methanol in ethyl acetate. The pentane fractions were concentrated to 0.5 ml in a Kuderna-Danish apparatus, while the methanol/ethyl acetate fractions were concentrated to 0.5 ml in a rotary evaporator and then methylated with diazomethane. NTHZ, NMOR and the methylated NTCA and NPIC were quantitated using the GC-TEA conditions described earlier in this paper. Average recoveries of 81 ± 3% for NPIC and 88 ± 5% for NMOR were recorded for the internal standards.

Control samples consisting of 0.5 g of ground pork belly spiked with varying amounts (0 to 2.5 µMol) of NTCA and NTHZ were analyzed to determine the percent recovery of the added N-nitroso compounds. Average

recoveries of 78 ± 5% for NTHZ and 82 ± 4% for NTCA were observed in these unheated samples.

The rate constants for NTCA decomposition and NTHZ formation were calculated by the first order rate equation (Fan and Tannenbaum, 1972):

$$\text{rate} = k\ (\text{reactant})$$

The activation energies for the above reactions were estimated from the slope of the plot of the log k (rate constant) against the reciprocal of absolute temperature.

RESULTS AND DISCUSSION

Model system studies: Wood smoke condensate fractions collected at various time intervals during the smoking of bacon were reacted with cysteamine/cysteine and nitrite to promote N-nitrosamine formation. The amounts of NTHZ and NTCA formed in these systems were used as a measure of the distribution of aldehydes in the smoke fractions. N-Nitrosamine data indicate that the levels of NTHZ, NTCA and 2-methyl-N-nitrosothiazolidine (2-MeNTHZ) formed were relatively constant over the entire smoking period (correlation coefficient 6.3%, 5.8%, and 5.3%, respectively, Table 1). These results show that the N-nitrosamine-forming capacity of wood smoke was relatively constant throughout the smoking process.

These results imply that formaldehyde, the carbonyl believed to be responsible for NTHZ and NTCA formation in smoked meats is produced evenly throughout the smoking cycle. Toth and Potthast (1984) reported that formaldehyde was present in wood smoke at a concentration of 200 mg per 100g of wood. Glyoxal, also present in wood smoke, has also been shown to produce thiazolidine when reacted with cysteamine (Sakaguchi and Shibamoto, 1978). However, its Maillard reaction potential in minimal compared to formaldehyde and other carbonyls (Ruiter, 1970) and is thus unlikely to contribute to NTHZ formation in cured meats.

Table 1 N-nitrosamine formation in woodsmoke condensate model systems[1]

Smoke Cycle (min)	Duration of Collection (min)	N-Nitrosamines (μMol)[2] 2-MeNTHZ[3]	NTHZ[4]	NTCA[5]
0-30	30	0.85 ± 0.23	62.3 ± 3.0	4.6 ± 0.25
30-60	30	0.87 ± 0.15	54.9 ± 2.8	3.9 ± 0.12
60-120	60	0.92 ± 0.15	52.8 ± 1.0	4.4 ± 0.25
120-240	120	0.91 ± 0.08	54.3 ± 1.7	4.0 ± 0.31
240-480	240	0.93 ± 0.16	60.9 ± 1.5	4.2 ± 0.25
0-480	480	1.0 ± 0.15	59.6 ± 1.7	4.4 ± 0.12

[1] Model System consisted of smoke condensate (10 ml), sodium nitrite (5 mMol), cysteamine/cysteine (1 mMol), reaction period 1 hour, pH 5.5, temperature 55°C, total volume 100 ml

[2] Average of two determinations, triplicate experiments

[3] Correlation coefficient 5.3%

[4] Correlation coefficient 6.3%

[5] Correlation coefficient 5.8%

The formation of 2-MeNTHZ in the model system would indicate the presence of acetaldehyde in the smoke and would support the hypothesis that formaldehyde in smoke is the precursor of NTHZ. The concentrations of 2-MeNTHZ produced in the model systems were much lower than the NTHZ concentrations, even though the acetaldehyde concentrations in smoke has been reported to be as high as 1150mg/100g wood (Toth and Potthast, 1984). This indicates that formaldehyde reacts much more rapidly with cysteamine and cysteine than does acetaldehyde, an observation also made recently by Ikins et al. (1986). This would explain why only small amounts of 2-methyl-N-nitrosothiazolidine carboxylic acid (2-MeNTCA) have been reported in cured meats relative to NTCA concentrations (Mandagere et al., 1986).

The major reason for this phase of the study was to establish that formaldehyde concentration would not be a limiting factor in subsequent studies on the effect of smoking time on NTCA and NTHZ formation in bacon. Results of this model system study clearly establishes that formaldehyde is uniformly produced throughout the entire smoking cycle.

Kinetics of NTCA decomposition in ground pork belly: Results of the heat-induced decomposition of NTCA (2.5 µMol) to NTHZ in the pork belly model systems are presented in Table 2. The decomposition of NTCA as a function of time and temperature indicated that no major changes occurred at 75°C and 100°C. From 125° to 175°C, NTCA levels were reduced at a rapid rate. These results are consistent with the differential scanning calorimetric data of Mandagere (cited by Mandagere et al., 1984) that show an endothermic change at 110°C. At the higher temperatures (125, 150, and 175°C), the decomposition of NTCA appears to have two different rates. Initially (0-3 min), there was a rapid reduction in NTCA levels followed by a more gradual decrease from 3 to 12 min. The rate of NTCA decomposition appeared to be mainly temperature-dependent and much less time-dependent at 150°C and 175°C. Lee et al. (1983a) reported that the

Table 2 Effects of temperature and time on the formation of N-nitrosothiazolidine from the decomposition of N-nitrosothiazolidine carboxylic acid (2.5 µMol) in pork belly

Heating Temp (°C)	Heating Times (min) 3	6	9	12
75	N.D[1] 4.4[2,4]	N.D. 3.2	N.D. 2.6	N.D. 3.6
100	0.01[3,5] 7.6	0.02 14.4	004 17.8	0.5 22.6
125	0.4 37.6	0.52 50.4	0.64 52.0	0.8 62.8
150	0.8 67.4	1.2 68.8	1.52 74.8	1.6 76.4
175	1.8 92.6	2.08 94.9	2.3 95.6	2.4 96.7

[1] None detected, detection limit 0.5 ng

[2] % decomposition of NTCA, corrected for recovery and blank

[3] % Yield of NTHZ, corrected for recovery and blank

[4] Standard deviation ±3.5 to 7.5% (n=3)

[5] Standard deviation ±0.2 to 0.45% (n=3)

average internal temperature reached in bacon slices during frying at 175°C in a preheated skillet was 164°C. However, the moisture and lipid contents of the bacon had a significant influence on the internal temperatures attained. High moisture and low lipid contents decreased the internal temperatures to 154°C. Based on these temperatures and on data in Table 1, it can be calculated that the NTCA content of bacon could be reduced by 70 to 80% when fried at 175°C for 6 min.

While NTCA appeared to undergo a large percent decomposition between 150 and 175°C, there was only a small percent yield of NTHZ (1.2 - 2.1%, 6 min) at these temperatures (Table 2). The low yields of NTHZ indicate the possible involvement of intermediate(s) in NTHZ formation from NTCA, thus introducing rate limiting processes for NTHZ formation. These results support the preliminary findings of Sen et al. (1985) who reported a 1-3% conversion of NTCA to NTHZ during the frying of bacon. It is possible that NTCA undergoes rapid initial denitrosation and decarboxylation instead of a simple decarboxylation to NTHZ. Sakaguchi and Shibamoto (1978) also indicated that thiazolidines might undergo dehydrogenation during heating to form thiazoles and thiazolines.

The rate constants, $t_{1/2}$, and the rates of decomposition of NTCA are presented in Table 3. While $t_{1/2}$ values of NTCA showed an overall decrease with increase in temperature, NTHZ showed no recognizable trend, again indicating the possible involvement of intermediates in NTHZ formation. Similarly, the rate constant (k) for NTCA decomposition showed an overall increase with increase in temperature, while the rate of NTHZ formation demonstrated no recognizable trends in terms of temperature effect. The overall activation energy for NTCA decomposition was calculated from the Arrhenuis plot (log k versus $1/^{0}K$) and found to be 7.13 Kcal/mole. This value compares favorably with the Ea of 5.99 Kcal/mole calculated from differential scanning calorimetry data

Table 3 Kinetic data for the decomposition of N-nitrosothiazolidine carboxylic acid in green pork belly at various temperatures

Temp °C	NTCA decomposition $t_{1/2}$ (min)	k min-1	rate of decomposition µMol Min-1	NTCA---> NTHZ $t_{1/2}$ (min)	k min-1	rate of formation µ Mol/min-1
75	333.8	2.0×10^{-3}	5.0×10^{-3}	70.45a	1.4×10^{-1}a	3.5×10^{-1}a
100	32.75	2.1×10^{-2}	5.25×10^{-2}	47.63	1.91×10^{-1}	4.78×10^{-1}
125	7.53	7.46×10^{-2}	1.87×10^{-1}	66.16	7.63×10^{-2}	1.91×10^{-1}
150	2.85	1.05×10^{-1}	2.62×10^{-1}	55.43	7.72×10^{-2}	1.93×10^{-1}
175	1.67	2.45×10^{-1}	6.12×10^{-1}	106.69	3.18×10^{-2}	7.96×10^{-2}
Ea(Kcal/mol)	7.13			Ea(Kcal/mol)	4.66	

a Calculated value

(Mandagere et al., 1984). However, there are no other published values available for comparison.

From these kinetic data, it is clear that the amounts of NTHZ formed in fried bacon via NTCA decomposition depend upon the initial levels of NTCA in raw bacon and on the internal temperatures reached in the bacon during frying. Sen et al. (1986) have recently determined that NTCA level in the raw bacon and the cooking conditions appear to be the most important factors involved in the formation of NTHZ in the fried bacon. Pensabene and Fiddler (1985b) have also concluded that NTCA must be present in large amounts to contribute to NTHZ formation when bacon is fried. Similar conclusions can be drawn from the results of this study of the effects of smoking time on N-nitrosamine formation in bacon.

Relationship between smoking time and N-nitrosamine formation in bacon

Raw Bacon: Results of the N-nitrosamine analyses of raw bacon removed from the smoke house at various times during the smoke cycle are summarized in Table 4. Bacon samples smoked for 30 min were found to contain the greatest amounts of NTCA (average 766 µg/kg). There was a significant decrease ($p<0.01$) in NTCA levels in bacon smoked for periods of 60, 120, 240, and 480 min as compared to the 30 min samples. A similar trend was observed for NTHZ levels, with the 30 min samples containing significantly greater ($p<0.01$) levels than those smoked for longer periods. However, the observed NDMA levels did not show any apparent trends. These results are consistent with those of previous studies which indicate that NDMA levels in raw bacon are low and quite variable (Gray et al., 1982). As expected, little or no N-nitrosopyrrolidine (NPYR) was detected in the raw bacon as this compound is mainly produced during the frying process (Bharucha et al., 1979; Lee et al., 1983b).

Table 4 N-Nitrosamine levels in raw bacon prepared by smoking cured pork bellies for various times

Smoking Time (min)	N-Nitrosamine (μg/kg)[1,2] NDMA	NPYR	NTHZ	NTCA
0	2.5 ± 0.4	Tr[4]	Tr	41 ± 10[3]
30	2.6 ± 0.7	Tr	3.0 ± 1.4	766 ± 68
60	0.8 ± 0.3[3]	Tr	1.3 ± 0.3[3]	467 ± 45[3]
120	3.0 ± 0.5	Tr	1.8 ± 0.3[3]	380 ± 37[3]
240	1.0 ± 0.4[3]	Tr	2.1 ± 0.5[3]	469 ± 28[3]
480	1.5 ± 0.4[3]	Tr	1.7 ± 0.8[3]	356 ± 32[3]

[1] NDMA, N-nitrosodimethylamine; NPYR, N-nitrosopyrrolidine; NTHZ, N-nitrosothiazolidine; NTCA, N-nitrosothiazolidine carboxylic acid

[2] Average of duplicate analyses, three replicates per smoke time, three bellies per replicate

[3] Significantly different from the 30 min smoke time ($p \leq 0.01$)

[4] Tr, trace, <1μg/kg

Maximum formation of NTCA and NTHZ occurred within the first 30 min of the smoking operation and the subsequent decrease could be due to a number of factors. NTCA formation occurs primarily at the surface of the bacon (Pensabene and Fiddler, 1983; Mandagere et al., 1986). The loss of NTCA during the smoking cycle could be due to thermal decomposition and/or chemical decomposition brought about by various acidic components of smoke, or lost along with the drip during smoking. Thermal decomposition of NTCA appears to be highly unlikely at smoke processing temperatures (58°C). Differential scanning calorimetric (Mandagere et al., 1984) and kinetic studies indicated no decomposition below 100°C. When NTCA (1 µMol) was incubated for 8 hr in 10 ml of smoke condensate at pH 5.5 and 58°C, no major loss of NTCA was observed.

Drip from the smoked bellies was collected during the entire smoking operation and was found to contain substantial amounts of NTCA (10-20% of the levels found in the 30 min smoked samples). However, this still leaves large amounts of NTCA unaccounted for. Further research is required in this area in order to elucidate the exact mechanism of NTCA loss during smoking.

Similarly, the NTHZ levels in raw bacon showed a marked decrease when the cured bellies were smoked for longer than 30 min. This decrease could be due to volatilization. Presence of hot gases and high humidity in the smoke house could facilitate the volatilization of NTHZ from the surface of the pork bellies.

The levels of NTHZ present in raw bacon smoked for various time intervals are consistent with those values cited previously by Mandagere et al. (1984), Pensabene and Fiddler (1985b) and Sen et al. (1986) for raw bacon. It appears that cysteamine might be the primary precursor of NTHZ in raw smoked bacon, (Fiddler et al., 1986), although at present there have been no published data on either cysteamine or thiazolidine content in meat products.

Fried bacon: The results of the analyses of volatile and nonvolatile N-nitroso compounds in fried bacon are presented in Table 5. As with the raw bacon, the concentrations of NTCA in the bellies smoked for 30 min were significantly greater ($p<0.01$) than in the pork bellies smoked for longer periods of time. NTHZ levels also showed a similar trend, the 30 min smoked samples containing the highest levels of NTHZ (average 18.3 µg/kg). The levels of NTHZ in fried bacon for all smoking periods were greater than those of the raw bacon counterparts. These findings agree with the results reported by Sen et al. (1985, 1986) for NTHZ levels in raw and fried bacon. It appears, therefore, that the observed correlation between NTCA levels in raw bacon and NTHZ levels in the fried product is consistent with the theory of formation of NTHZ by heat-induced decarboxylation of NTCA. This intermediacy of NTCA in NTHZ formation in fried bacon was previously proposed by Mandagere et al. (1984), Sen et al. (1985, 1986) , and Ikins et al. (1986).

A comparison of NTCA and NTHZ levels in raw and fried bacon is presented in Table 6. The N-nitrosamine levels in fried bacon were adjusted for fat and moisture loss during frying to facilitate a comparison with raw bacon data. This adjustment was performed by frying several packages of randomized sliced bacon per treatment and calculating the loss in weight of the bacon as a consequence of frying. The adjusted NTCA levels in fried bacon indicated that 63-81% of the NTCA in the raw bacon was decomposed during frying. These values are consistent with kinetic study data which show approximately 60-80% decomposition of NTCA at 150-165°C after 6 min of heating. The average internal temperature reached by bacon sliced during hot skillet frying at 175°C for 6 min was reported to be 164°C by Lee et al. (1983b). These investigators also reported that moisture and fat contents affect the internal temperatures reached in bacon. It is expected that bacon samples smoked for 0, 30, and 60 min had higher moisture levels than the bacon samples smoked for

Table 5 N-Nitrosamine levels in fried bacon prepared by smoking cured pork bellies for various times

Smoking Time (min)	N-Nitrosamines (μg/kg)[1,2]			
	NDMA	NPYR	NTHZ	NTCA
0	3.1 ± 0.5	8.6 ± 0.5[3]	1.3 ± 0.2[3]	33 ± 6[3]
30	4.9 ± 0.4	16.0 ± 1.7	18.3 ± 2.6	549 ± 39
60	3.7 ± 0.6	8.1 ± 1.0[3]	7.6 ± 1.5[3]	433 ± 57[3]
120	6.6 ± 0.5	7.8 ± 1.5[3]	4.6 ± 1.1[3]	288 ± 38[3]
240	5.4 ± 0.8	9.7 ± 2.1[3]	6.6 ± 1.0[3]	340 ± 38[3]
480	2.1 ± 0.7	6.1 ± 1.6[3]	4.2 ± 0.8[3]	242 ± 38[3]

[1] NDMA, N-nitrosodimethylamine; NPYR, N-nitrosopyrrolidine; NTHZ, N-nitrosothiazolidine; NTCA, N-nitrosothiazolidine carboxylic acid

[2] Average of duplicate analyses, three replicates per smoke time, three bellies per replicate

[3] Significantly different from 30 min smoke time ($p<0.01$)

Table 6 N-Nitrosothiazolidine and N-nitrosothiazolidine carboxylic acid levels in raw and fried bacon prepared by smoking cured pork bellies for various times

Smoking Time (min)	NTCA[1] (µg/kg)				NTHZ[1] (µg/kg)		
	Raw Bacon	Fried Bacon Adjusted	% Decomposition	Theoretical[3] Yield of NTHZ (µg/kg)	Raw Bacon	Fried Bacon	% Change (Adjusted)
0	41 ± 10	15 ± 3[4]	63	0.8	Tr[6]	Tr	---
30	766 ± 68	209 ± 15[4]	73	15.2	3.7	6.9 ± 1.0[4]	+87.6
60	467 ± 45	139 ± 18[4]	70	9.3	1.3	2.4 ± 0.9[5]	+86.9
120	380 ± 37	95 ± 12[4]	80	7.6	1.8	1.5 ± 0.4[5]	-18.3
240	469 ± 28	109 ± 12[4]	77	9.3	2.1	2.2 ± 0.3[5]	+3.8
480	356 ± 32	68 ± 11[4]	81	7.1	1.7	1.2 ± 0.3[5]	-30.6

[1] NTCA, N-nitrosothiazolidine carboxylic acid; NTHZ, N-nitrosothiazolidine

[2] Fried bacon NTCA and NTHZ values were adjusted for fat and moisture loss for comparison with raw bacon data

[3] Theoretical yields of NTHZ is based on a 2% NTCA conversion to NTHZ in kinetic studies

[4] Significantly different from raw bacon smoke time counterpart ($p \leq 0.01$)

[5] Not signficantly different from raw bacon smoke time counterpart ($p \leq 0.05$)

[6] Trace, <1 µg/kg

120, 240, and 480 min. It is possible that the higher moisture contents resulted in lower internal temperatures in the 0, 30, and 60 min smoked bacon which in turn resulted in lower levels of NTCA decomposition.

The NTHZ levels in raw and fried bacon along with the kinetic data on NTCA decarboxylation to NTHZ strongly suggest that the majority of NTCA decomposed is converted to products other than NTHZ. Kinetic studies show that approximately 2% of NTCA is converted to NTHZ under frying conditions (150-175°C, 6 min). Sen et al. (1985) spiked raw bacon with known amounts of NTCA and ascertained that 1 to 3% of the added NTCA was converted to NTHZ under their frying conditions. Data in Table 6 indicate that when the bacon samples smoked for 30 min were fried, there was a net increase of approximately 3 µg/kg NTHZ. However, assuming a 2% conversion of NTCA to NTHZ, the 30 min bacon samples should contain approximately 15 µg/kg NTHZ, based on the decomposition of 556 µg/kg NTCA (difference between NTCA levels in raw and fried bacon). It is likely that the remainder of the NTHZ produced during frying is steam-volatilized since bacon drippings (cook-out fat) retains only trace amounts of NTHZ (Sen et al., 1985; Vecchio et al., 1986).

The N-nitrosamine data in Table 6 also support the observations of Sen et al. (1985, 1986) and Ikins et al. (1986) that the NTHZ levels in the fried bacon seem to correlate the the NTCA levels in the corresponding raw bacon. However, the 120, 240, and 480 min -fried bacon samples showed a net loss or no change in NTHZ levels compared to the raw bacon. These results are consistent with the hypothesis that a large portion of the NTHZ produced from the decarboxylation of NTCA is steam-volatilized during frying of bacon. This may also explain the observation of Pensabene and Fiddler (1985b) who showed with a bacon model system the NTCA must be present in large amounts to contribute to NTHZ formation when bacon is fried. Sen et al. (1986) also alluded to the possible low concentrations of NTCA in raw bacon as an explanation

for results of an earlier study by Pensabene and Fiddler (1983) which showed that raw bacon contained higher levels of NTHZ than either fried, baked, or broiled bacon.

The NTHZ levels in both raw and fried bacon reflect the involvement of two separate mechanisms for NTHZ formation in bacon. In raw bacon, NTHZ appears to form via the cysteamine-thiazolidine pathway (Pensabene and Fiddler, 1985b; Fiddler et al., 1986), while NTCA seems to be the primary source of NTHZ in fried bacon. However, the mechanism of NTHZ formation in bacon requires further investigation. The quantitation of cysteamine and cysteine levels in pork bellies would assist in evaluating the pathways involved in NTHZ formation in bacon.

Of the other N-nitrosamines present in fried bacon, NDMA levels did not exhibit any trends in concentration as a result of the duration of the smoking process (Table 5). NPYR, on the other hand, showed similar trends to that of NTHZ in fried bacon, in that maximum NPYR formation occurred in bacon receiving only a 30 min smoke. It is not clear as to the mechanism behind the reduction in NPYR levels in bacon smoked for longer periods. It is possible that longer smoking resulted in lower residual nitrite levels in the bacon. Sleeth et al. (1982) reported that the acid nature of liquid smokes promotes the reduction of nitrite to volatile nitric oxides, thus lowering the residual nitrite concentration. Theiler et al. (1984) explained the significant reduction in NPYR levels in a fried pork belly system as being due to depletion of the residual nitrite by the unneutralized organic acids in liquid smoke. Results of a recent study by Ikins et al. (1986) on N-nitrosamine formation in fried bacon processed with liquid smoke preparations support the observation that the lower the residual nitrite level, the lower the level of NPYR in the fried product. NTHZ levels did not appear to be influenced by residual nitrite. Sen et al. (1986) also failed to observe a correlation between residual nitrite and NTHZ levels in raw bacon and other smoked

meats. No nitrite data are available in the present study to substantiate this explanation for the higher NPYR levels in the bacon samples smoked for 30 min.

CONCLUSIONS

In conclusion, we have shown that under conditions simulating the pan-frying of bacon, approximately 2% of the NTCA present in bacon will be converted to NTHZ. The amount of NTHZ formed, however, does not account for large differences in the NTCA contents of raw and fried bacon. It is likely that the thiazolidine compounds will undergo further heat-induced decomposition to form a mixture of products including thiazoles and thiazolines. There appears to be two mechanisms operative for the formation of NTHZ in bacon - one involving cysteamine for NTHZ formation in raw bacon and the other involving the intermediacy of NTCA in NTHZ formation in fried bacon.

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CHAPTER IV

An Investigation Into Glucose As A Potential Precursor Of N-Nitrosothiazolidine In Bacon

ABSTRACT

The possible involvement of glucose in the formation of N-nitrosothiazolidine (NTHZ) in bacon was investigated by pumping pork bellies with brines containing added glucose (2 and 4%). Raw bacon samples processed with glucose had significantly lower levels of N-nitrosothiazolidine carboxylic acid than the smoked control bacons. When the bacon samples with glucose were fried for 6 and 12 min, no NTHZ was detected in the fried products. N-Nitrosopyrrolidine (NPYR) levels in these samples were much lower than those in the fried control samples. Results of this study demonstrate that the contribution of glucose to NTHZ formation in bacon is minimal relative to that of formaldehyde in wood smoke.

INTRODUCTION

In recent years, considerable attention has focused on the presence of N-nitrosothiazolidine (NTHZ) and N-nitrosothiazolidine carboxylic acid (NTCA) in smoked cured meats (Pensabene and Fiddler, 1983a,b; Mandagere et al., 1984; Ikins et al., 1986). Sen et al. (1986) reported trace amounts of NTHZ in various smoked meat samples and a few smoked fish and considerably higher (up to 13,700 µg/kg) levels of NTCA. These investigators also provided evidence that NTCA will undergo decarboxylation during frying of bacon, and that the formation of NTHZ was dependent on frying temperature and frying time.

It is widely accepted that NTHZ and NTCA formation in smoked cured meats results from the interaction of formaldehyde in the smoke with cysteamine and cysteine, respectively, followed by nitrosation (Pensabene and Fiddler, 1985; Sen et al., 1986). Mandagere et al. (1984) speculated that formaldehyde may also be formed from the fragmentation of glucose during frying of bacon. This speculation was based on results of model system studies which showed that heterocyclic compounds including thiazolidine, 2-methylthiazolidine and 2-ethylthiazolidine are formed on heating a cysteamine/glucose model browning system (Sakaguchi and Shibamoto, 1978). The objective of this study was to clarify the precursor role, if any, of glucose in NTHZ and NTCA formation in raw and fried bacon.

EXPERIMENTAL

Bacon processing: Fifteen skinned pork bellies were obtained from a local slaughterhouse within 24 hr postmortem and stored for no more than 2 days in a cooler at 2°C. The bellies were randomized into three groups with five in each. Bellies in group 1 were stitched pumped to 110% of their green weight with a brine containing 15% sodium chloride,

5% sucrose, 3.5% sodium tripolyphosphate, 1,200 mg/kg sodium nitrite, and 5,500 mg/kg sodium ascorbate, and smoked for 8 hr by a standard smoking process (Reddy et al., 1982). Bellies in groups 2 and 3 were stitch-pumped with brines containing 2% and 4% glucose instead of 5% sucrose, respectively. All other ingredients of the brine were similar to those in the control (group 1). The bellies were pumped to 110% of their weight and held at 2°C for 2 days. The glucose-treated samples were cooked in the smoke house at 58°C for 8 hr in the absence of woodsmoke. The bacon samples were sliced and vacuum packaged and held at 2°C for 1 week. Two randomly selected packages from each belly were fried in a preheated electric frying pan at a thermostat setting of 340°F. The control samples (group 1) were fried for 6 min (3 min per side) while the glucose samples were fried for 6 and 12 min (3 and 6 min per side, respectively). The fried samples were ground and analyzed for NTCA, NTHZ, and other volatile N-nitrosamines.

N-Nitrosamine analysis: NTHZ and other volatile N-nitrosamines in raw and fried bacon were determined by the mineral oil vacuum distillation procedure described in detail by Ikins et al. (1986). All samples were analyzed in duplicate and precautions were taken to avoid artifactual N-nitrosamine formation during sample preparation (Ikins et al., 1986). Percent recovery of the added internal standard, N-nitrosothiomorpholine, was 86 ± 5%. N-Nitrosamine values were not corrected for recovery of the internal standard.

The NTCA contents of the bacon samples (raw and fried) were determined using the ethyl acetate extraction procedure described by Mandagere et al. (1986a). The average recovery of the internal standard (N-nitrosopipecolic acid) was 79 ± 4%. The gas chromatographic - thermal energy analyzer conditions were as described by Mandagere et al. (1986a).

Statistical analysis: Statistical analyses of N-nitrosamine data were carried out according to the methods described by Gill, (1978).

Safety note: N-Nitrosamines are potent carcinogens and should be handled with appropriate safety precautions.

RESULTS AND DISCUSSION

N-Nitrosamine data for the raw and fried bacon samples are summarized in Table 1. The raw bacon samples processed with glucose (groups 2 and 3) contained significantly lower ($P<0.01$) levels of NTCA compared to the smoked control samples. This clearly demonstrates that NTCA formation in bacon is primarily due to the smoking process. The very low levels of NTCA in the glucose-bacon sample likely arise from the residual smoke in the smokehouse. Similar levels of NTCA were found in a limited number (3) of control bacon samples (i.e. containing sucrose) which were cooked simultaneously with the glucose samples in the smokehouse. N-Nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR) levels in all treatments were somewhat similar, the small differences most likely being due to compositional differences in the bellies (Pensabene et al., 1979).

NTCA levels in fried bacon decreased during the frying process. Mandagere et al. (1986b) have shown that approximately 2% of the NTCA present in raw bacon is converted to NTHZ during frying. NTCA was not detected in the glucose samples, the limit for reliable detection being 5 µg/kg. Added glucose (0.2 and 0.4%) in bacon did not appear to influence NTHZ formation during frying. Similarly, increasing frying time from 6 min to 12 min did not induce NTHZ formation which indicates that glucose fragmentation to formaldehyde is of minor importance. Sakaguchi and Shibamoto (1978) showed that model systems containing glucose and cysteamine produced large amounts of thiazolidine when refluxed for 2 hr at 100°C. If this reaction had occurred during the frying of bacon containing the added glucose, large amounts of thiazolidine would have been produced. Thiazolidine, because of its weak

Table 1. N-Nitrosamine levels in bacon processed with two levels of glucose

Treatment Group	N-nitrosamine concentration (μg/kg)[1] NDMA	NPYR	NTHZ	NTCA
RAW BACON				
(1) Control (0.5% sucrose)	2.5 ± 0.8	1.0 ± 0.2	1.9 ± 0.5	365 ± 16
(2) 0.2% glucose	3.1 ± 0.9	1.0 ± 0.3	ND[5]	8 ± 3[2]
(3) 0.4% glucose	3.8 ± 0.7	1.5 ± 0.7	ND	12 ± 3[2]
FRIED BACON				
(1) Control (6 min)	2.1 ± 0.7	5.0 ± 1.7	4.5 ± 0.8	189 ± 8
(2) 0.2% glucose (6 min)	3.9 ± 0.9	2.5 ± 0.3[3]	ND	ND
(2) 0.2% glucose (12 min)	2.8 ± 0.6	1.0 ± 0.3[3,4]	ND	ND
(3) 0.4% glucose (6 min)	2.5 ± 0.8	2.6 ± 0.6[3]	ND	ND
(3) 0.4% glucose (12 min)	1.5 ± 0.5	ND[3,4]	ND	ND

[1]N-Nitrosamine levels represent average of two determinations, five bellies per treatment

[2]Significantly different from control ($p<0.01$)

[3]Significantly different from control ($p<0.05$)

[4]Significantly different 6 min sample of the sample glucose concentration ($p<0.01$)

[5]ND, not detected; limit of detection (1 μg/kg for NTHZ; 5 μg/kg for NTCA)

basicity, would have undergone rapid nitrosation to NTHZ (Coughlin, 1979). Since no NTHZ was detected in the glucose-treated bacon (limit of detection 1μg/kg), the major pathway for NTHZ formation in bacon must involve the formaldehyde in the wood smoke (Figure 1).

While the contribution of glucose to NTHZ and NTCA formation appears to be minimal, it has been established that glucose can undergo Maillard-type reactions with cysteamine (Bonner and Meyer Zu Reckendorff, 1961) and with cysteine (Schubert, 1939; Weitzel et al., 1959) to form 2-[D-glucopentahydroxypentyl(1)]-thiazolidine and the carboxylic acid derivative, respectively. Coughlin, (1979) reported that the D-glucose/L-cysteine adduct, being weakly basic, should be nitrosated quite rapidly in nitrite-containing foods and in vivo in the gastro-intestinal tract following ingestion of the adduct. The nitrosated compound is also a β-oxidized N-nitrosamine and many of these compounds are proximate carcinogens and/or mutagens (Coughlin, 1979).

To date, no nitrosated Amadori compounds have been isolated from food systems. Scanlan and Reyes (1985) reported that these compounds can be isolated and identified by a reverse phase high performance liquid chromatographic-thermal energy analyzer system. However, due to the basic incompatibility between the two systems, little progress has been made in studying the formation of nonvolatile nitrosated Amadori compounds in foods.

The addition of glucose to bacon also had a marked influence on NPYR levels in the fried product (Table 1). Significant reductions ($p<0.05$) in NPYR levels were observed when the bacon samples were fried for 6 min. These results are in agreement with the findings of Bailey and Mandagere (1980) and Thieler et al. (1984), who reported major reductions in NPYR formation in fried bacon cured with glucose and other reducing sugars. Theiler et al. (1984) initially hypothesized that the

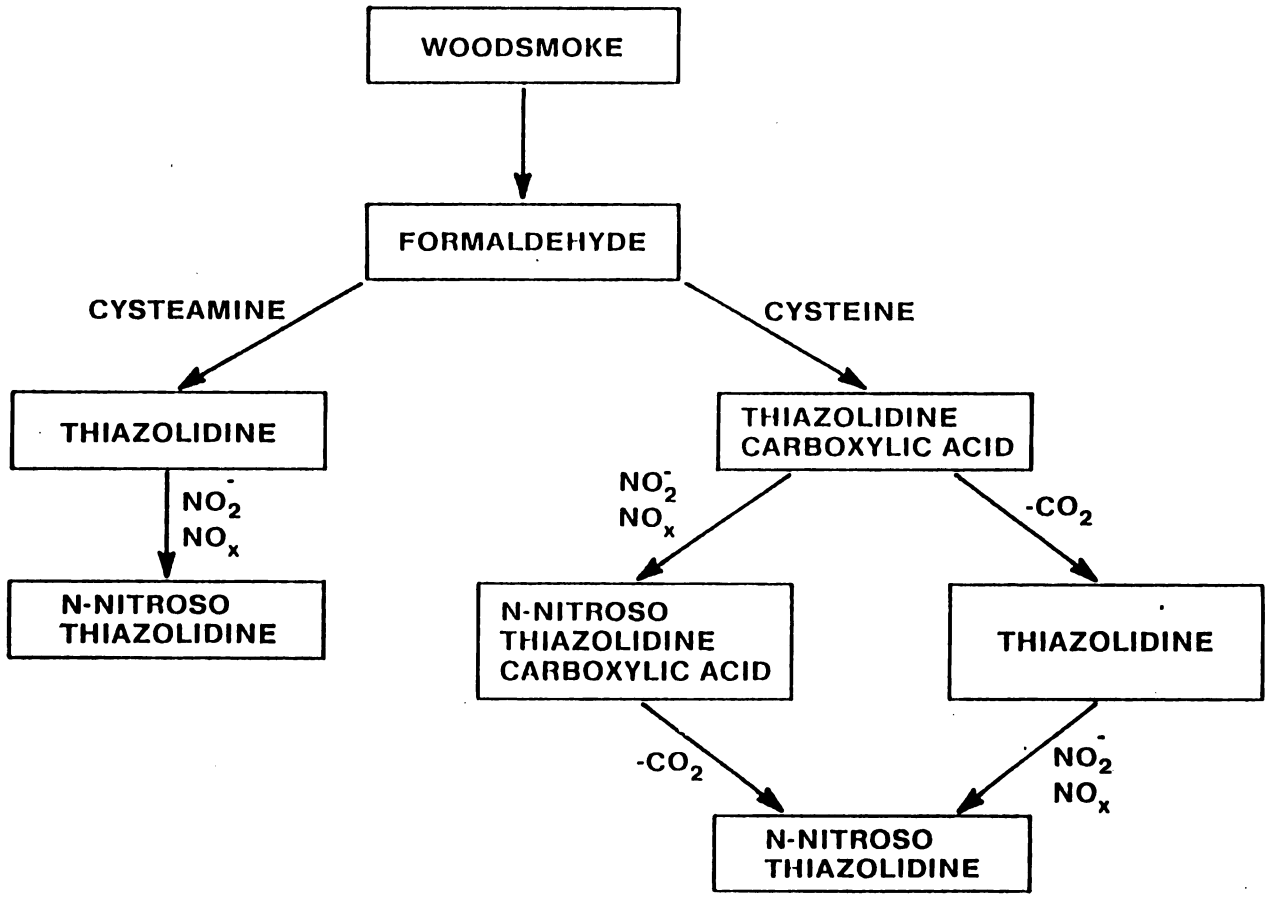

Figure 1. Possible pathway of N-Nitrosolthiazolidine formation in bacon

mechanism of reducing sugar inhibition involved the amine-sugar browning reaction (Maillard reaction) and that the products formed (Amadori compounds) were capable of competing with NPYR precursors such as proline in the nitrosation reaction. Results of further studies with the nonreducing sugars, α- and β-methylglucose, did not support this hypothesis as NPYR levels were reduced to the same extent as with glucose. These investigators concluded that the classic Maillard reaction occurring between added reducing sugars and primary amines did not adequately explain the mechanism of inhibition.

N-Nitrosamine data in Table 1 also indicate that the NPYR levels in the glucose-bacon samples fried for 12 min were significantly lower ($p<0.01$) than those fried for 6 min. This difference could be due to volatilization of the NPYR formed and/or creation of additional secondary amino compounds as a result of the extended frying period. NDMA levels in the fried bacon were generally not affected by the presence of glucose in the bacon. Statistical treatment of the NDMA data is not reported due to the lower levels of NDMA present, levels that can be affected by duration of frying, and compositional differences between pork bellies (Pensabene et al., 1979).

CONCLUSIONS

In conclusion, it has been demonstrated that the contribution of glucose to NTHZ and NTCA formation in bacon is minimal relative to that of formaldehyde in the smoke. Addition of glucose to bacon, although effective in reducing NPYR formation during frying, has practical limitations because of its tendency to produce excessive browning in the fried product. Furthermore, formation of N-nitroso Amadori compounds is also undesirable because of their potential mutagenicity/carcinogenicity.

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CHAPTER V

Reduction of N-Nitrosamine Formation in Smoked Bacon

ABSTRACT

The effectiveness of α-tocopherol (300 mg/kg) and reduced nitrite concentrations (80 mg/kg) in reducing N-nitrosamine formation in pump-cured bacon was investigated. N-nitrosopyrrolidine (NPYR) levels in fried bacon were reduced by 60 percent when α-tocopherol was added to the curing brine. A similar reduction was achieved in reducing the ingoing nitrite level to 80 mg/kg. N-Nitrosothiazolidine (NTHZ) levels in the fried bacon were similarly reduced by these treatments. N-Nitrosothiazolidine carboxylic acid (NTCA) levels in raw bacon were lower in the α-tocopherol-treated and lower nitrite samples compared to the control samples (120 mg/kg). α-Tocopherol was also shown to be effective as a N-nitrosamine inhibitor in bacon cooked under home conditions.

INTRODUCTION

The consistent occurrence of N-nitrosamines in bacon has led to an intensive search for both the precursors and mechanisms that could account for its formation. Similarly, much effort has gone into developing ways of reducing the formation of N-nitrosamines in bacon.

It has been well established that the N-nitrosamine levels of fried bacon are dependent upon the ingoing levels of nitrite. Significant reductions in N-nitrosamine levels can be achieved by reducing ingoing levels of nitrite. However, nitrite is a very effective antibotulinal agent and cannot be eliminated without incorporating an antibotulinal alternative. Many such systems have been evaluated, including sorbate (Sofos et al., 1979; Paquette et al., 1980), hypophosphite (Pierson et al., 1981), fumarates (Huhtanen et al., 1981) and irradation (Rowley et al., 1983). These compounds (including irradiation) have been used in conjunction with low levels of nitrite necessary for color and flavor development (Widdus and Busta, 1982). However, none have been approved for use in bacon processing.

The other approach to reducing N-nitrosamine levels in fried bacon involves the addition of an inhibitor of the nitrosation reaction to bacon during processing. Alpha (α)-tocopherol and sodium ascorbate, when used in combination, effectively reduce N-nitrosamine levels in bacon (Fiddler et al., 1978; Mergens and Newmark, 1979; Gray et al., 1982; Pensabene and Fiddler, 1985; Skrypec et al., 1985). α-Tocopherol functions as an inhibitor of the nitrosation reaction through its ability to reduce nitrite to a non-reactive species. It, in turn, is oxidized to a quinone (Mergens and Newmark, 1979). N-Nitrosamine formation in fried bacon can be effectively reduced by the inclusion of α-tocopherol as a curing adjunct. Fiddler et al. (1978) demonstrated that this compound, when dispersed with polysorbate emulsifiers in the curing brine to obtain

adequate distribution in the cured pork bellies, significantly reduced NPYR and NDMA formation in bacon when used at a concentration of 500 mg/kg. α-Tocopherol can also be applied to bacon by spray or dip to overcome the problem of water insolubility (Mergens and Newmark, 1979). These authors stressed the importance of obtaining good distribution of the blocking agent in the adipose tissue. Pensabene and Fiddler (1985) have further demonstrated that the combination of sodium ascorbate (550 mg/kg) and α-tocopherol (500 mg/kg) effectively reduced NTHZ formation in bacon.

The effect of α-tocopherol on the antibotulinal properties of bacon formulated with sodium nitrite was investigated by Tanaka (1982). Bacon, made with 0 or 0.9% sucrose and 3 levels of α-tocopherol (0, 500, and 1000 mg/kg), was inoculated with heat shocked spores of C. botulinum types A and B and incubated at 27°, 25° or 23°C in vacuum packages. The results of this study formation without diminishing the antibotulinal effectiveness of nitrite.

Recently, d-α and dl-α-tocopherols were affirmed as generally recognized as safe (GRAS) for use as inhibitors of N-nitrosamine formation in pump-cured bacon (Federal Register, 1984). In July 1985, the Food Safety and Inspection Service (FSIS) amended the federal meat inspection regulations to permit the use of these blocking agents as curing adjuncts (Federal Register, 1985).

Gray et al. (1982) introduced α-tocopherol into the brine on the surface of salt of high surface area. These coated salts effectively inhibited N-nitrosamine formation in pumped cured (Gray et al., 1982) and dry-cured bacon systems (Bernthal et al., 1986).

In order to effectively disperse α-tocopherol in the curing brine, lecithin is included as part of the α-tocopherol-salt system. In addition, silicone dioxide is added to provide free-flowing properties to the salt systems. The application of α-tocopherol at ingoing levels of

500 mg/kg results in the introduction of silicone dioxide into the bacon at levels which are not currently acceptable to the regulatory agencies (Wilkens, personal communication). Lower ingoing levels of α-tocopherol would also reduce the amount of silicone dioxide to an acceptable level in bacon. Thus, there is a need to evaluate the anti-N-nitrosamine efficacy of reduced levels of α-tocopherol (300 mg/kg) in pump-cured bacon.

N-Nitrosamine levels can also be reduced by curing bacon with lower ingoing levels of nitrite (less than 120 mg/kg). Recently, Tanaka et al. (1985 a, b) processed bacon with 40 and 30 mg/kg nitrite and reported much lower N-nitrosamine levels than those in bacon processed with 120 mg/kg of sodium nitrite.

Another important variable that influences N-nitrosamine levels in bacon is cooking temperature. Consistently higher levels of N-nitrosamines have been reported in bacon cooked at high temperatures (171°C - 190°C) (Pensabene et al., 1974; Lee et al., 1983; Hotchkiss and Vecchio, 1985). The accepted method of frying bacon by the majority of researches and regulatory agencies has been 340°F (171°C) for three min per side. While this standardized method of frying is essential for comparison of research and monitoring program data, it may not accurately reflect the levels of N-nitrosamines in consumer-cooked bacon due to considerable variability in temperature, duration, and types of utensils used (Vecchio et al., 1986). While Wasserman et al. (1978) and Vecchio et al. (1986) have analyzed consumer-cooked bacon, there has been no evaluation of the effectiveness of α-tocopherol in blocking N-nitrosamine formation in bacon cooked by the consumer.

The objectives of this study were:

1. To determine the effectiveness of ingoing α-tocopherol levels of 300 mg/kg in inhibiting N-nitrosamine formation in pump-cured bacon using α-tocopherol-coated salts.
2. To determine the effectiveness of lower ingoing levels of sodium nitrite (80 mg/kg) along with α-tocopherol (300 mg/kg) in reducing N-nitrosamine formation in pump-cured bacon.
3. To evaluate the effectiveness of α-tocopherol (300 mg/kg) in reducing N-nitrosamine levels in fried bacon cooked by consumers at home.

EXPERIMENTAL

Preparation of brine-cured bacon: Sixty four pork bellies (approximately 4.5 to 5 kg) were obtained from a local supplier soon after slaughter. The bellies were divided into four groups and processed into bacon by stitch pumping to 110% of their green weight using brines having the composition shown in Table 1. Four replicate experiments (four bellies per treatment) were carried out over a two-week period in order to minimize belly to belly variation. The bellies were smoke-cooked at a temperature of 58°C (dry bulb) for 4 hr, followed by three further hours of cooking at 52°C (dry bulb) at ambient relative humidity in a smoke house. The smoked bellies were transferred to a tempering cooler (-2°C) where they were held overnight before slicing and packaging as described by Robach et al. (1980).

Bacon frying: One week after packaging, representative portions of the bacon samples were fried in a preheated electric skillet set at 171°C (340°F). The bacon was fried on each side for three min, removed, and drained on paper towels. Both the edible portion and the cook-out fat were retained for N-nitrosamine analysis.

Analysis of bacon: Raw and fried bacon was analyzed for N-nitrosamines by the modified method of Robach et al. (1980). A 2 ml aliquot of 20% ammonium sulfamate in H_2SO_4 was added to the distillation flask before the distillation step of the mineral oil distillation procedure. The N-nitrosamines were quantitated by GC-TEA method described by Mandagere et al. (1984). N-Nitrosothiazolidine carboxylic acid levels in raw bacon were determined as described by Mendagere et al. (1986). Nitrite levels in the uncooked bacon samples were determined at the time of frying using the AOAC (1984) procedure. Analysis of the N-nitrosamine data was accomplished by ANOVA analysis (Gill, 1978).

Table 1 Brine formulations used in the mg/kg preparation of bacon samples[1]

Ingredient	Percent of total brine[2]			
	Standard Control Brine	α-Tocopherol Brine[3]	Reduced Nitrite Control Brine	Reduced Nitrite α-Tocopherol Brine[3]
α-Tocopherol-salt	0	15.75	0	15.75
Dry salt	15.00	0	15.00	0
Sodium nitrite	0.12	0.12	0.08	0.08
Sodium ascorbate	0.55	0.55	0.55	0.55
Sugar	5.00	5.00	5.00	5.00
Phosphate	3.50	3.50	3.50	3.50
Water	75.83	75.08	75.87	75.12

[1]Bellies were pumped at a 10% target pump level for brine formulation at 100% yield.

[2]Total salt in both formulations = 15.00%.

[3]Target α-tocopherol level in finished bacon was 300 mg/kg.

Preparation of bacon for consumer frying study: In the second experiment, two sets of paired bellies were obtained from pigs raised on the swine farm at Michigan State University. The bellies of each set were pumped with the brines listed in Table 1, (treatments 1 and 2). The bellies were smoked, tempered, sliced, packaged, and stored at 4°C for six days. One package of bacon from each belly was then given to ten volunteers from the Department of Food Science and Human Nutrition, Michigan State University. These participants were instructed to cook the four samples of bacon as they would routinely do with commercial bacon. All samples in this study were cooked seven days after packaging and the participants were asked to record their cooking conditions and the general "doneness" of the samples. The cooked bacon slices were put in plastic bags and stored overnight in a refrigerator. All samples were returned to the investigators the following morning for analysis. The bacon samples were analyzed for N-nitrosamines as described previously. N-nitrosamine data were statistically analyzed using the procedure of ANOVA analysis (Gill, 1978).

RESULTS AND DISCUSSION

In the first study, the α-tocopherol treatment produced no changes in N-nitrosamine levels in raw bacon (Table 2). No significant differences ($p<0.01$) in NDMA and NPYR levels were observed between the four treatment groups. NTHZ levels were too low in all treatments for any evaluation. These results are consistent with previous reports on N-nitrosamine levels of raw bacon (Gray et al., 1982). However, NTCA levels showed marked reduction in bacon processed with 80 mg/kg nitrite. α-Tocopherol, however, did not appear to have any inhibitory effect on NTCA formation.

Table 2: N-Nitrosamine levels and percent inhibition of N-nitrosamine formation in raw brine-cured bacon.

Treatment[1]	(N-Nitrosamine concentration µg/kg) NDMA	NPYR	NTHZ	NTCA
Control (120 mg/kg nitrite)	1.8 ± 0.8 (0.9 - 3.9)	0.62 ± 1.0 (ND - 3.1)	0.5 ± 0.9 (ND - 3.0)	114.5 ± 24 (77 - 145)
α-Tocopherol (300 mg/kg) nitrite (120 mg/kg)	1.5 ± 0.3[2] (1.0 - 2.1)	0.44 ± 0.7[2] (ND - 2.0)	ND[4]	107 ± 14[2] (87 - 140)
Reduced Nitrite (80 mg/kg) Control	1.4 ± 0.8[2] (0.7 - 4.2)	0.5 ± 0.5[2] (ND - 2.5)	ND[4] (ND - 0.9)	74 ± 19[3] (44 - 105)
α-Tocopherol (300 mg/kg) reduced nitrite (80 mg/kg)	1.1 ± 0.5[2] (ND - 1.8)	0.43 ± 0.7 (ND - 2.3)	ND[4]	66 ± 16[3] (43 - 99)

[1]Sixteen bellies per treatment.

[2]Not significantly different from control (120 mg/kg NO_2)($P \leq 0.01$).

[3]Significantly different from control (120 mg/kg NO_2)($P \leq 0.05$).

[4]Not detected, (detection limit 0.5 µg/kg)

In fried bacon, α-tocopherol had a much more significant impact on all N-nitrosamine levels (Table 3). α-Tocopherol treatment resulted in approximately 60% reduction in NPYR levels compared to the standard cure formulation (120 mg/kg NO_2). NDMA concentrations in bacon samples were also reduced although the amount of inhibition was smaller than that achieved for NPYR. Similarly, NTHZ formation was inhibited by the inclusion of α-tocopherol as a curing adjunct, although the levels obtained for this N-nitrosamine were generally below 2 μg/kg.

Bacon samples prepared with the lower level of nitrite (80 mg/kg) contained significantly ($P<0.01$) lower levels of NPYR and NTHZ compared to control (120 mg/kg nitrite) bacon. The levels of all N-nitrosamines tested were less than 2 μg/kg. However, addition of α-tocopherol to the 80 mg/kg nitrite treated bacon did not significantly ($P<0.01$) alter the percent inhibition of N-nitrosamine levels.

In order to further evaluate the anti-N-nitrosamine efficacy of α-tocopherol (300 mg/kg), two matched pairs of pork bellies were processed into bacon. One belly in each pair was pumped with a control brine, while the other belly was pumped with the brine containing α-tocopherol. Bacon from each of the four bellies were given to ten participants who fried the samples as they would routinely do in the preparation of a meal at home. N-Nitrosamine data for these samples are summarized in Table 4. Inhibition of 62% and 69% for NPYR and NTHZ were observed, respectively. These results are similar to those reported earlier for bacon processed with 300 mg/kg α-tocopherol and fried under the FSIS frying protocol.

In this study, half of the samples were fried in an electric skillet, while the remaining samples were cooked in frying pans on the top of a stove. No correlation between N-nitrosamine content and method of cooking was evident. However, the combination of higher cooking temperatures for shorter times appeared to result in greater NPYR

Table 3: N-Nitrosamine levels and percent inhibition of N-nitrosamine formation in fried pump-cured bacon processed with α-tocopherol-coated salt

Treatment[1]	Nitrite (mg/kg)	NPYR[3] (µg/kg)	Inhibition (%)	NDMA[3] (µg/kg)	Inhibition (%)	NTHZ (µg/kg)	Inhibition (%)
Control (120 mg/kg NO_2)	37 ± 9.9 (28 - 64)[4]	4.2 ± 0.9 (2.7 - 5.4)	--	2.1 ± 1.2 (ND - 4.1)[4]	--	1.6 ± 1.1	--
α-Tocopherol (300 mg/kg) (120 mg/kg NO_2)	37 ± 13.3 (19 - 66)	1.7 ± 0.4[2] (0.9 - 2.4)	60	1.2 ± 1.1 (ND - 3.2)	33	0.5 ± 0.8[2] (ND - 2.4)	69
Control (80 mg/kg)	30 ± 11.9 (18 - 49)	1.55 ± 0.9 (ND - 3.8)	63	1.38 ± 0.7 (ND - 2.8)	34.3	0.4 ± 0.6[2] (ND - 2.6)	75
α-Tocopherol (300 mg/kg) +NO_2(80 mg/kg)	34.5 ± 12.9 (28 - 47)	1.6 ± 0.7[2,3] (0.8 - 3.4)	62	1.3 ± 0.9 (ND - 2.7)	38.1	0.38 ± 0.5[2,3] (ND - 1.2)	76

[1]Sixteen bellies per treatment.

[2]Significantly different from 120 mg/kg NO_2 control ($P \leq 0.01$).

[3]Not significantly different from 80 mg/kg NO_2 control ($P \leq 0.0$).

[4]ND not detected, detection limit 0.5 µg/kg.

Table 4: Effect of α-tocopherol (300 mg/kg ingoing) on N-nitrosamine formation (µg/kg) in brine-cured bacon fried under home conditions[1]

	Belly Pair 1			Belly Pair 2		
	Control	α-Tocopherol	% Inhibition	Control	α-Tocopherol	% Inhibition
NDMA	7.4 (2.0 - 17.5)	3.4[2] (ND - 6.5)	55	7.4 (4.0 - 12.4)	3.2[2] (ND - 8.6)	54
NPYR	5.8 (2.9 - 13.6)	2.2[2] (0.5 - 4.4)	65	5.3 (2.8 - 9.6)	2.1[2] (0.7 - 4.3)	61
NTHZ	1.8 (ND - 4.5)	0.6[2] (ND - 2.1)	69	1.7 (ND - 3.5)	0.5[2] (ND - 3.2)	69

[1]Bacon samples were fried by 10 participants.

[2]Significantly different from control ($P<0.005$).

[3]ND, not detected (detection limit 0.5 µg/kg).

formation. Similar observations were made by Vecchio et al. (1986), who reported that bacon samples above the median in NPYR levels generally were fried with higher heat settings. This value was higher than the mean NPYR concentrations observed in the study.

N-Nitrosamine data indicate that α-tocopherol, when used at the 300 mg/kg level, will effectively reduce NPYR formation in bacon fried under normal household conditions. This is an important observation as it is well established that frying conditions strongly affect N-nitrosamine formation in bacon (Pensabene et al., 1974; Lee et al., 1983; Vecchio et al., 1986). N-Nitrosamine levels determined under controlled laboratory conditions may not accurately reflect the levels in bacon being consumed.

Similar trends for NDMA and NTHZ were observed in the home-cooked bacon samples. NDMA levels in the control bacon samples were generally below 5 μg/kg, and were reduced by 55% with the inclusion of α-tocopherol in the bacon. The mean NTHZ level in the control bacon samples was 1.8 μg/kg, while that in the α-tocopherol-treated samples was less than 1 μg/kg.

CONCLUSIONS

In conclusion, results of these studies clearly demonstrate that N-nitrosamine levels of fried bacon can be effectively reduced by incorporating α-tocopherol in conjunction with sodium ascorbate into the cure. Further, similar reductions can also be accomplished by reducing the ingoing level of nitrite from 120 mg/kg to 80 mg/kg. However, the reduction of nitrite levels to 80 mg/kg may present possible problem with outgrowth of Clostridium botulinum if no antibotulinal alternative are incorporated (Tanaka et al., 1985 a, b). Recent Food and Drug Administration regulations allow 100 or less (40-80) mg/kg of nitrite (ingoing) in bacon provided 550 mg/kg of sodium ascorbate along with

lactic acid producing bacteria such as pediococcus acidilactici or other bacteria proven effective are added (Federal Register, 1986). On the average, the consumer home cooking of α-tocopherol treated bacon shows similar levels of reductions in N-nitrosamines as FSIS bacon frying method. However, large variations can occur in N-nitrosamines levels depending on cooking temperature and duration of the cooking process.

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SUMMARY AND CONCLUSIONS

The role of wood smoke in NTHZ and NTCA formation in bacon was investigated. Further, the precursors and the mechanism(s) behind NTHZ formation were also explored. As a result of these studies, several conclusions pertaining to the formation of NTHZ and NTCA in bacon can be made. These are as follows:

1. The primary mode of NTHZ and NTCA formation in smoked cured bacon is due to the smoking process. Formaldehyde appears to be the main carbonyl compound involved in the formation of these compounds.
2. Glucose does not appear to play a significant role in NTHZ formation in fried bacon. However, glucose was very effective in inhibiting NPYR formation in fried bacon.
3. Small amounts of 2-MeNTCA were detected in many of the smoked foods surveyed. Its formation is most likely due to acetaldehyde present in wood smoke.
4. Further, a number of N-nitroso derivatives of heterocyclic carboxylic acids were present in smoked cured foods in addition to the widely studied NTCA. Many smoked cured foods surveyed contained 2-HMNTCA. Glycolaldehyde present in wood smoke appears to be the carbonyl precursor responsible for 2-HMNTCA formation.
5. 2-HMNTHZ was also detected in fried bacon and a likely pathway appears to be the decarboxylation of 2-HMNTCA during frying.
6. Small amounts of 2-AcNTHZ and 2-AcNTCA were detected in model system containing smoke condensate, cysteamine/cysteine, and nitrite. However, these compounds were not observed in any of the smoked food surveyed. Methylglyoxal in smoke condensate appears to be the carbonyl responsible for these N-nitrosamines.