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Title: IDENTIFICATION AND QUANTITATION OF 3-HYDROXY-N-NITROSOPYRROLIDINE IN FRIED BACON AND FRIED-OUT FAT.

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The formation of 3-hydroxy-N-nitrosopyrrolidine from decarboxylation of 4-hydroxy-N-nitrosoproline was demonstrated in model heating system designed to simulate the conditions of frying bacon. The identification of the nitrosamine was achieved by comparison of its TLC $R_f$ value, gas chromatographic retention time, and mass spectral fragmentation pattern with those of the authentic compound.

3-Hydroxy-N-nitrosopyrrolidine was isolated from commercial fried bacon and fried-out fat. The identification of the compound was achieved by combined gas chromatography-mass spectrometry after trimethylsilyl derivatization of the nitrosamine. Additional verification of identity was obtained from coincident retention time data on a combined gas chromatography-thermal energy analyzer.

Quantitative determination of 3-hydroxy-N-nitrosopyrrolidine was accomplished without derivatization and was greatly facilitated by the use of a thermal energy analyzer. Quantitative analysis by combined gas chromatography-thermal energy analyzer did not require as extensive cleanup as was required for identification by combined gas chromatography-mass spectrometry.

The levels of 3-hydroxy-N-nitrosopyrrolidine occurring in five samples of fried bacon were 0.5 to 3.9 ppb while the levels in fried-out fat ranged from 0.3 to 2.2 ppb. These levels are lower than the concentrations generally reported for nitrosopyrrolidine in fried bacon.

This study, although the number of samples analyzed was limited, suggests that low ppb levels of 3-hydroxy-N-nitrosopyrrolidine appear fairly consistently in fried bacon.

# Identification and Quantitation of 3-Hydroxy-N-nitrosopyrrolidine in Fried Bacon and Fried-out Fat

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# IDENTIFICATION AND QUANTITATION OF 3-HYDROXY-N-NITROSOPYRROLIDINE IN FRIED BACON AND FRIED-OUT FAT

## I. INTRODUCTION

Many N-nitrosamines have been shown to be carcinogenic when fed to test animals and concern has arisen regarding the occurrence of these compounds in food. N-nitrosamines, which are generally produced by the reaction of nitrite with secondary amines, have been reported in several foods. Dimethylnitrosamine has been sporadically detected in several cured meat products while nitrosopyrrolidine (NPYR) has been reported to occur consistently in cooked bacon.

Methods for the detection of volatile nitrosamines have been dramatically improved and many investigators have succeeded in disclosing the presence of these compounds in the human environment. Nonvolatile N-nitrosamines, however, have scarcely been studied until recently, because of the lack of analytical methodology.

It has been speculated that 3-hydroxy-N-nitrosopyrrolidine (HNPYR) might be formed in fried bacon via reactions analogous to the formation of NPYR. NPYR has been shown to be carcinogenic to animals and due to the similarity in chemical structure between

the two compounds, it has been speculated that HNPYR would also be a potent carcinogen.

The purpose of this study was to isolate, identify and quantify HNPYR from commercial fried bacon.

# II. REVIEW OF LITERATURE

The detection of nitrosamines in foods has been an active area of investigation. Scanlan (1975) and Crosby and Sawyer (1976) have recently reviewed in detail the subject of nitrosamines in foods.

Nitrosamines have occurred in foods sporadically at the low ppb level. Generally, it can be concluded that nirtosamines occur sporadically in cured meats except that NPYR occurs consistently in cooked bacon (Scanlan, 1975).

Nitrosamines are formed by a reaction between nitrite and amine containing compounds. Nitrite, which is a permitted food additive, can also be derived from nitrate by the action of bacteria. Even though primary, tertiary and quaternary amines can form nitrosamines (Lijinsky, 1974; Warthesen et al., 1975), the principal amines for nitrosamine formation are secondary amines (Ridd, 1961; Ingold, 1969; Challis and Butler, 1968). Polyamines (Hildrum et al., 1976) and certain secondary amino acids (Lijinsky et al., 1970a; Warthesen et al., 1975).

Some of the early reports of nitrosamines in foods were based on detection methods lacking specificity and the results may be erroneous (Thewlis, 1968; Pensabene et al., 1972; Wolff and Wasserman, 1972). The identification of nitrosamines in foods today must

be carefully confirmed, usually by combined gas chromatography-mass spectrometry (GC/MS).

Analytical methodology for volatile nitrosamines has been very well established and many investigators have successfully determined nitrosamines in foods and in the environment. Non-volatile nitrosamines, however, pose difficult problems in isolation and cleanup procedures; thereby, studies of nonvolatile nitrosamines have been very rare until recently.

The biological effects of nitrosamines have been reviewed by Druckrey, et al. (1967), Magee and Barnes (1967), Magee (1971), Shank (1975), and Swann (1975). A great deal of work has been undertaken which demonstrates the carcinogenicity of nitrosamines in test animals (Swann, 1975). There is indirect evidence that man would also be adversely affected by nitrosamines even though no cases of cancer in man have been attributed to nitrosamines (Magee, 1973; Weisburger and Rainen, 1975). Recently Cairns (1975) stated that almost all human cancers appear to be caused by exposure to factors in the environment and the most promising approach for controlling the disease is to identify the causative factors and eliminate them.

## N-Nitrosoproline

Nitrosoproline can be formed by the interaction of free proline,

either present as the free amino acid or produced by hydrolysis of protein, and nitrite. Nitrosation of N-terminal proline which is followed by hydrolysis would also yield nitrosoproline (Mirvish et al., 1973).

Lijinsky and Epstein (1970) pointed out that conditions of the mammalian stomach are favorable for nitrosation of amino acids. In addition, nitrosation can take place in cured meat products and nitrosoproline has been isolated from raw bacon (Ivey, 1974; Kushnir et al., 1975; Dhont and Ingen, 1976). The concentration of nitrosoproline in raw bacon was generally much higher than the concentration of NPYR in fried bacon.

The high level of proline in connective tissue (Ramachandran, 1967) might be related to nitrosoproline formation. Lakritz et al. (1976) reported a dramatic increase in free amino acids in pork bellies during storage. Nitrosoproline can be formed from nitrosation of ornithine followed by rearrangements (Warthesen et al., 1975). The significance of nitrosoproline is that it may be an immediate precursor of NPYR in cured meat products. Nitrosoproline has failed to demonstrate carcinogenic activity in rats (Nixon et al., 1976). There have been a number of studies on nitrosoproline as a precursor of NPYR (Nakamura et al., 1976; Hwang and Rosen, 1976).

Nitrosoproline, being a nonvolatile and polar compound, cannot be analyzed by the well established analytical methods for volatile nitrosamines which involve cleanup by distillation, followed by gas chromatographic determination. Investigators attempted to solve the problem mainly by the use of two different approaches.

One approach was to make use of volatile derivatives of the nitrosoproline, either the methyl ester (Kushnir et al., 1975; Dhont and Ingen, 1976) or the trimethylsilyl derivative (Eisenbrand et al., 1975). Kushnir et al. (1975) and Dhont and Ingen (1976) determined nitrosoproline in cured meat products including raw bacon. Kushnir et al. used diazomethane, and Dhont and Ingen used methanol containing 3% HCl in forming the methyl ester of nitrosoproline. The trimethylsilyl derivative has been shown to be a promising alternative (Eisenbrand et al., 1975).

The other approach that has been explored was the use of differential pulse polarography. Chang and Harrington (1975) reported that nitrosoproline could be determined quantitatively as low as 10 ppb in dichloromethane by differential pulse polarography. Hasebe and Osteryoung (1975) isolated nitrosoproline from raw bacon by the use of gel permeation chromatography and determined the nitrosamine by differential pulse polarography.

## N-nitrosopyrrolidine

### Occurrence

A recent review by Gray (1976) dealt with NPYR in terms of precursors, pathways of the formation, and factors influencing formation. Contrary to sporadic reports of nitrosamines in other foods, NPYR has been found in approximately 80% of all fried bacon samples analyzed (Crosby et al., 1972; Fazio et al., 1973; Sen et al., 1973). Recent research has indicated that NPYR occurs in fried bacon but not in other cured meat products. Fazio et al. (1973) found no NPYR in fried ham or Canadian bacon but they were able to isolate the nitrosamine from fried bacon and fried-out fat.

Two explanations based on the difference in fat to lean ratio have been suggested for the occurrence of NPYR solely in fried bacon. Fazio et al. (1973) theorized that since NPYR was fat soluble, it was protected from volatilization from bacon fat during frying while NPYR which is formed in leaner cuts of Canadian bacon and ham escaped by volatilization.

The other explanation suggested by Scanlan (1975) was that different internal temperatures are reached in lean and adipose when bacon is fried. The different temperatures result from the differences in moisture content and specific heats of the two tissues.

Higher internal temperatures might be reached in the adipose tissue because of its lower moisture content and lower specific heat.

NPYR has been found in cooked bacon but not in raw bacon (Fazio et al., 1973; Sen et al., 1973; Pensabene et al., 1974). The amount formed appears to be dependent upon many factors. Although concentrations of NPYR as high as 108 μg/kg (Fazio et al., 1973) have been noted, the quantities usually found are in the range of 10-20 μg/kg.

Recently Gough et al. (1976) reported the distribution of NPYR in cooked bacon, cooked-out fat, and in the vapor produced during cooking. Up to 25 and 35% of the NPYR was found in bacon and in cooked-out fat respectively. However, 23 out of 36 samples lost more than 75% of the NPYR formed during frying by volatilization. Sen et al. (1976) also reported that 50% of the total NPYR produced during cooking volatilized in the fumes.

Mysliwy et al. (1974) reported in vivo formation of NPYR in a dog's stomach by concurrent feeding of sodium nitrite and pyrrolidine.

## Precursors

Investigators have suggested a number of possible amine precursors for the heat induced formation of NPYR (Gray et al., 1976). Collagen has been shown to be capable of producing NPYR

under conditions of high temperature and high nitrite concentration (Huxel et al., 1974; Gray and Dugan, 1975). Patterson et al. (1976), however, concluded that collagen is unlikely to be a significant contributor to NPYR in bacon because they found NPYR almost exclusively in the fatty tissue, not in lean and rind which have higher collagen contents than fatty tissue.

Putrescine, which is a decomposition product of arginine, may undergo cyclization to pyrrolidine (Lijnsky and Epstein, 1970; Scanlan, 1975) and react with sodium nitrite to yield NPYR in an oil-water system (Bills et al., 1973). Putrescine has been reported in amounts of 1.7 to 189.3 mg/100g of fresh pork and 1.1 to 50.4 mg/100g of butt portions of commercially cured and smoked hams (Lakritz et al., 1975).

The aliphatic polyamines, spermidine and spermine, are widely distributed in biological materials (Tabor and Tabor, 1964; Moruzzi and Caldavera, 1964; Wang, 1972). NPYR, along with at least four other nitrosamines (Hildrum et al., 1976) was produced by heating spermidine with sodium nitrite (Bills et al., 1973; Ferguson et al., 1974).

Other compounds that have been investigated as NPYR precursors include L-propylglycine, glycyl-L-proline and hydroxyproline (Huxel et al., 1974; Gray and Dugan, 1975); the first two compounds produced NPYR.

The most probable precursor of NPYR in bacon, however, appears to be proline either via pyrrolidine or via nitrosoproline. Many workers reported the formation of NPYR from proline and nitrite (Bills et al., 1973; Gray and Dugan, 1975), pyrrolidine and nitrite (Huxel et al., 1974; Nakamura et al., 1976) or nitrosoproline (Nakamura et al., 1976; Hwang and Rosen, 1976). Proline is a natural component of many foods and is especially abundant in connective tissue. Swine collagen is reported to contain about 12% proline and 10% 4-hydroxyproline (Ramachandran, 1967). Gray (1977) also reported that the free proline content of bacon samples ranged from 18.3 to 31.6 μM per 100 g.

Hwang and Rosen (1976) recently reported work which concluded that proline is more likely to be the precursor of NPYR in fried bacon than either spermidine or putrescine. Labeled NPYR was not detected in fried bacon when raw bacon was spiked with $^{14}C$ labeled spermidine or putrescine. Raw bacon containing $^{14}C$ labeled proline and nitrosoproline produced $^{14}C$ labeled NPYR upon frying.

## Pathway of Formation

The pathway for the formation of NPYR in fried bacon is not yet firmly established. Several pathways have been proposed (Fiddler et al., 1974). Starting from proline, the generally accepted

major precursor, there are two possible pathways: via pyrrolidine and via nitrosoproline (Figure 1). Many studies have been carried out and contradicting results have been reported. Since NPYR does not occur in raw bacon the involvement of heat induced reactions for NPYR formation is generally accepted.

The pathway via nitrosoproline was supported by the following investigators. Kushnir et al. (1975) isolated up to 1.18 mg/kg nitrosoproline from raw bacon which indicated that nitrosoproline was an available precursor for NPYR. Other workers (Bills et al., 1973; Pensabene et al., 1974; Hwang and Rosen, 1976) proved that nitrosoproline could be decarboxylated under conditions approximating frying bacon. Finally, Hwang and Rosen (1976) unequivocally demonstrated that nitrosoproline was decarboxylated to yield NPYR in bacon by the frying process. The authors further reported that the NPYR concentrations found in fried bacon corresponded with nitrosoproline concentration found in raw bacon considering a decarboxylation yield of 1.4%.

Further support for the idea that nitrosoproline is formed prior to frying comes from the work of Sen et al. (1974) who reported that the amount of NPYR formed in bacon correlated with initial nitrite concentrations but not with nitrite concentrations at the time of frying. Since the frying process is of relatively short duration, it is diffucult to imagine that the concentration of nitrite at the time

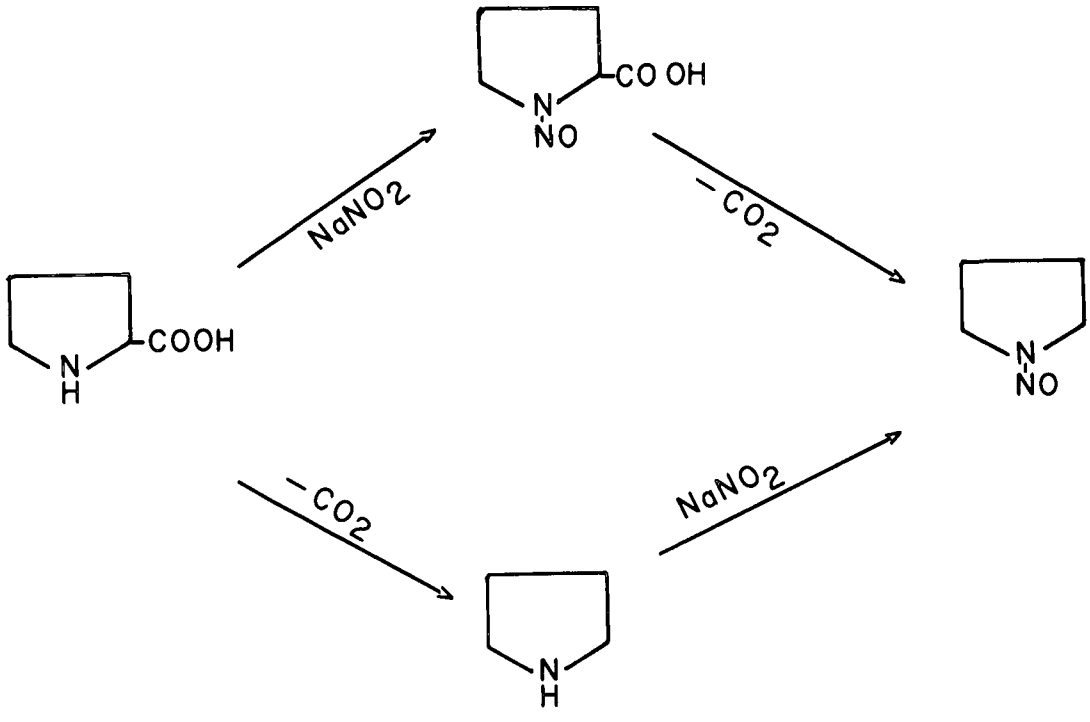


Figure 1. Suggested pathways for nitrosopyrrolidine formation from proline.

of frying has no effect on NPYR yield if NPYR is formed by nitrosation of pyrrolidine in the frying process.

The other pathway, formation of NPYR via pyrrolidine, was suggested by Ender and Ceh (1971) who studied the formation of NPYR from proline and nitrite in a dry starch matrix. More recently, this pathway was supported by Sen et al. (1976a) who showed that treatment, just prior to frying, of normal nitrite-cured bacon with 1000 ppm of propyl gallate, or other nitrosation inhibitors, markedly reduced the formation of NPYR during cooking. Addition of these additives would not inhibit the formation of NPYR from nitrosoproline. The authors, therefore, concluded that the additives inhibit NPYR formation in bacon by interfering with reactions other than the decarboxylation of nitrosoproline.

At present, it might be concluded that both pathways may contribute to the formation of NPYR (Figure 1).

## Factors Influencing Formation

Even though it is still not fully understood, many investigators have contributed information concerning the influence of certain parameters on the formation of NPYR during cooking of bacon. Among the factors which can influence formation of NPYR are methods of cooking, cooking temperature, initial nitrite concentration, salt concentration, presence of inhibitors or catalysts,

water content, and the amount of adipose tissue.

Standard pan frying produced the highest amounts of NPYR while microwave cooking produced lower levels (Herring, 1973; Pensabene et al., 1974). Frying temperature, rather than cooking time, was shown to be a major factor (Fiddler, 1973; Pensabene et al., 1974). Pensabene et al. (1974) reported 17 ppb of NPYR from bacon fried at 204° C for four min while no NPYR was produced when bacon was fried for 105 min at 99° C to same "doneness".

The rate of nitrosation of secondary amines is directly proportional to the square of the nitrite concentration (Kalatzis and Ridd, 1966; Mirvish, 1970). Sen et al. (1974) demonstrated the effect of increased nitrite concentration on the formation of NPYR and showed that the amount of NPYR correlated well with the initial concentration of nitrite but not with the nitrite concentration at the time of cooking.

Sodium chloride, an important ingredient in cure mixtures, affects nitrosation of proline differently at various acidities due to the nature of the nitrosating species at different pH levels (Hildrum et al., 1975a). Since the pH of most foods to which nitrite and chloride are added is above 4.0, chloride ions would be expected to produce an inhibitory rather than an accelerating effect on N-nitrosamine formation (Scanlan, 1975).

The effect of increasing amounts of ascorbic acid on inhibition of NPYR formation in bacon which contains typical sodium nitrite cures has been clearly demonstrated by Herring (1973) and Sen et al. (1976a). Greenberg (1973) was concerned about the possibility of botulinal toxin formation in meat products when they were treated with excess ascorbic acid. Bowen et al. (1974), however, reported that the presence of sodium ascorbate in vacuum-packed wieners did not adversely alter the effectiveness of nitrite in controlling botulinal toxin formation.

Higher water content, in a model system, increased the amount of NPYR produced from collagen and nitrite, even though it lowered the maximum temperature reached during heating (Gray and Dugan, 1975). The authors speculated that the presence of water in the system increased hydrolysis of collagen and consequently the amount of available proline.

Fiddler et al. (1974) and Patterson et al. (1976) studied the roles of lean and adipose tissue in the formation of NPYR in fried bacon and reported that NPYR was derived from the adipose and not the lean portion. They concluded that the precursors of NPYR exist in the adipose tissue of raw bacon. However, the free proline content of lean was almost four times that of adipose tissue (Lakritz et al., 1976) and the higher NPYR content in adipose tissue may be

better explained by the higher temperature attained during cooking (Scanlan, 1975).

## 4-hydroxy-N-nitrosoproline

Although 4-hydroxy-N-nitrosoproline has not been detected in cured meat products, the possibility of formation of this compound was recognized after nitrosoproline was isolated from raw bacon (Scanlan, 1975). The kinetics of N-nitrosation of 4-hydroxyproline, along with its abundance in connective tissues, further supports this possibility. At pH 2 and 25$^{o}$ C the pH-dependent rate constant for nitrosation of hydroxyproline was almost 10 times that of proline (Mirvish et al., 1973). According to the authors (Mirvish et al., 1973), N-nitrosation of peptides with N-terminal 4-hydroxyproline followed by hydrolysis would also yield 4-hydroxy-N-nitrosoproline and the nitrosation rate constants would be greater than that for the free amino acid.

Dhont and Ingen (1976) attempted to isolate 4-hydroxy-N-nitrosoproline from cured meat products. The method involved extraction with ethyl acetate, subsequent purification by ion exchange, and conversion of the compound to the methyl ester. The authors could not detect the methyl ester of 4-hydroxy-N-nitrosoproline by GC/MS while methyl esters of other nitrosamino acids could be identified. The difficulty was attributed to the free hydroxyl group

of 4-hydroxy-N-nitrosoproline. The authors (Dhont and Ingen, 1976) claimed, however, that the methyl ester of synthetic 4-hydroxy-N-nitrosoproline could be quantitatively determined by photolysis.

Eisenbrand et al. (1975) reported that trimethylsilyl derivatives of nitrosamino acids could be easily gas chromatographed. The trimethylsilyl derivative of 4-hydroxy-N-nitrosoproline eluted after the trimethylsilyl derivatives of nitrososarcosine and nitrosoproline under the gas chromatographic conditions employed. The authors (Eisenbrand et al., 1975), citing their unpublished results, stated that the methyl ester of 4-hydroxy-N-nitrosoproline gave a strongly tailing peak and low recovery due to nonspecific adsorption of the free hydroxyl group. The trimethylsilylation process silylates both the carboxyl and hydroxyl groups simultaneously, and consequently leads to a derivative with satisfactory gas chromatographic properties.

Derivation of 4-hydroxy-N-nitrosoproline by use of methyl iodide and sodium hydride might be an alternative. This process would esterify the carboxyl group while converting the hydroxyl group into a methyl ether. The resulting derivative, having no free hydroxyl group, may be quite suitable for gas chromatographic analysis.

Several studies have shown a null carcinogenic effect of nitrosamino acids. Greenblatt and Lijinsky (1972, 1973) failed to

produce tumors in experimental animals after concurrent feeding of sodium nitrite and amino acids. Garcia and Lijinsky (1973) fed low levels of nitrosoproline and 4-hydroxy-N-nitrosoproline to rats for 75 weeks and found no carcinogenic activity. Nixon et al. (1976) found no tumorigenic effect in rats given large doses of nitrosoproline or 4-hydroxy-N-nitrosoproline.

## 3-hydroxy-N-nitrosopyrrolidine

Work on the detection of HNPYR in foods is very rare. Scanlan (1975), however, suggested the possibility of formation of HNPYR by analogous reactions to those for formation of NPYR. Furthermore, decarboxylation of nitrosamino acids, including 4-hydroxy-N-nitrosoproline, to form corresponding nitrosamines was reported by Lijinsky et al. (1970). To date, however, the only report on detection of HNPYR in a biological system is the work of Krüger and Bertram (1975) who isolated the compound from rat urine after application of NPYR.

Without seriously exploring methodology for the isolation of HNPYR from food matrix, several investigators examined the possibility of HNPYR production in model systems. Huxel et al. (1974) did not detect HNPYR or NPYR in the model system in which 4-hydroxyproline was heated with sodium nitrite either in dry state or in pH 6.2 buffer. Gray and Dugan (1975) investigated the

formation of HNPYR from 4-hydroxyproline and nitrite in a low moisture carboxymethylcellulose (CMC) system. The authors failed to detect HNPYR while 6.5% theoretical yield of NPYR was produced from proline and nitrite in the same system at 180° C.

Gray et al. (1977) employed a polar solvent for isolating the HNPYR from a freeze-dried matrix of aqueous solution and achieved 20% recovery starting from an aqueous solution of HNPYR. The same authors, however, employed steam distillation in an attempt to isolate HNPYR from fried bacon. The efficiency of recovery of HNPYR by steam distillation was not reported. The authors could not detect HNPYR in fried bacon while NPYR, at levels up to 23 ppb, was found in the same samples. It is speculated that Gray et al. (1977) did not find HNPYR because of the low recovery of the relatively nonvolatile nitrosamine by steam distillation.

Recently Sen et al. (1976b) reported a method by which low levels of HNPYR could be determined by GC/MS. The authors made use of a stable volatile derivative of the nitrosamine, namely 3-methoxy-1-nitrosopyrrolidine. The outline of the procedure is given in Figure 2. The methylation technique used was very similar to a procedure used by Williams et al. (1960) and Lawrence and Iverson (1975). The reaction proceeded smoothly at room temperature and gave a yield of 90%. The procedure as reported,

however, does not provide for the isolation of HNPYR from the food matrix.

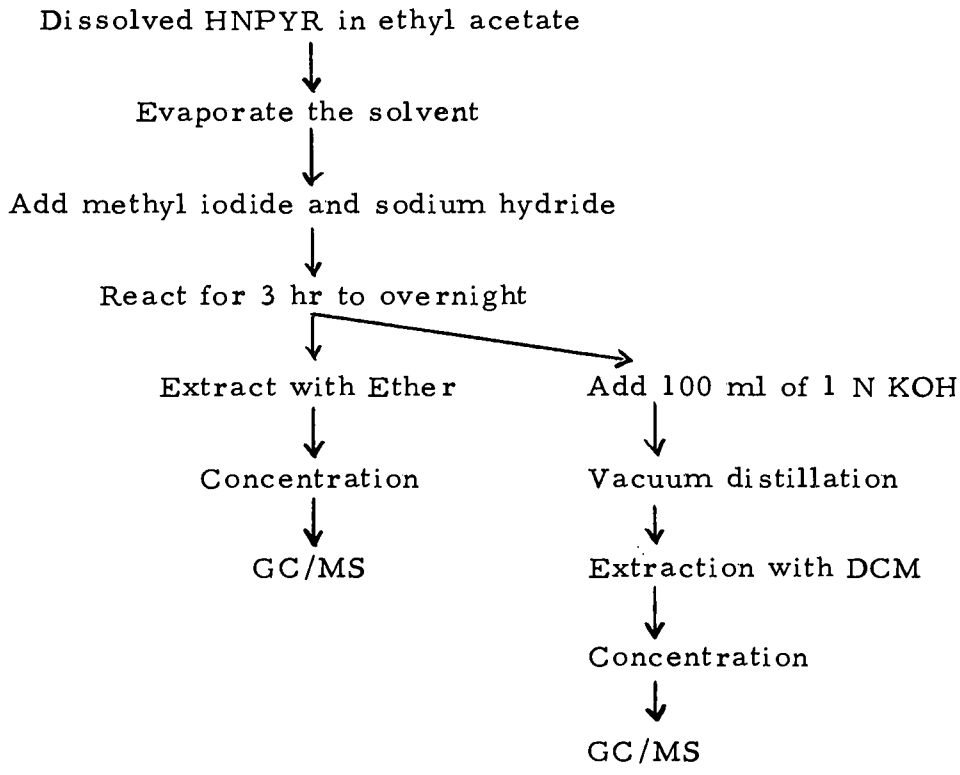


Figure 2. Formation and determination of 3-methoxy-1-nitrosopyrrolidine (From Sen et al. 1976 Journal of Chromatography 128:169-173).

Although there has been no study on the carcinogenicity of HNPYR, Stoltz and Sen (1977) recently reported the potent mutagenicity of the nitrosamine by use of Salmonella typhimurium. The authors predicted that HNPYR would be carcinogenic, based on the correlation between carcinogenicity and mutagenic activity for the S. typhimurium strains developed by Ames (1975).

## Analysis of Nitrosamines

Analysis of nitrosamines has been a difficult task because of the trace levels at which these compounds have been found in foods and the need for absolute confirmation. Several reviews deal with the various techniques employed (Eisenbrand, 1973; Foreman and Goodhead, 1975; Fiddler, 1975; Scanlan, 1975; Crosby and Sawyer, 1976). The majority of the work to date has been directed toward the more volatile nitrosamines.

Analysis of nitrosamines in foods usually consists of five steps: isolation from the food, cleanup, separation, detection, and confirmation of identity. Often the problems encountered in isolation, cleanup, and separation of nonvolatile nitrosamines are more difficult than those encountered with volatile nitrosamines.

### Volatile Nitrosamines

Methodology for the analysis of volatile nitrosamines has been developed and a number of procedures are available. Distillation, either under atmospheric pressure (Crosby et al., 1972) or vacuum (Telling et al., 1971), has been most commonly employed for isolation and preliminary cleanup of volatile nitrosamines. Extraction also has been used as a preliminary isolation method (Fiddler et al., 1971; Fazio et al., 1972; White et al., 1974),

dichloromethane being the most widely used solvent. Further purification has generally been accomplished by column chromatography (Fazio et al., 1972; Eisenbrand, 1974), even though thin-layer chromatography (TLC) has been also used (Eisenbrand et al., 1970).

TLC can be used for separations of nitrosamines (Sen and Dalpe, 1972). Gas chromatography (GC), however, has been the choice of most investigators for separation and detection of nitrosamines. In addition to the normal attributes of GC, it has the added advantage of being capable of interfacing with a mass spectrometer (MS) (Gough and Sawyer, 1974). It has been recommended that N-nitrosamines in foods be confirmed by mass spectral identification (Telling et al., 1971; Fazio et al., 1971; Scanlan, 1975; Gough and Sugden, 1975).

## Nonvolatile Nitrosamines and Nitrosamino Acids

Many of the nitrosatable amines, including the secondary amine containing amino acids, would lead to nonvolatile rather than volatile nitrosamines. The analysis of nonvolatile nitrosamines has been especially difficult because the use of distillation, a valuable isolation and cleanup method, is limited. Gas chromatographic applications are also limited due to nonvolatile nature of the compounds.

Solvent extraction is an approach which has been used for the removal of nonvolatile nitrosamines from foods. This is usually followed by cleanup methods involving liquid-liquid extraction, freeze drying, adsorption-desorption and/or gel permeation chromatography.

Dhont and Ingen (1976) used methanol:water (3:1) for extraction of nitrosamino acids from meat products. The aqueous extract, after evaporation of methanol was extracted with ethyl acetate.

As an alternative to liquid-liquid extraction, Kushnir et al. (1975) freeze dried an aqueous extract of bacon and the nitrosamino acids were extracted with methanol from the freeze-dried matrix. The extract was filtered, concentrated, centrifuged and then applied to silicic acid under nitrogen. The nitrosamino acids were removed from the silicic acid by continuous extraction with ether.

Another method of recovering nonvolatile nitrosamines from the aqueous solution was the use of activated carbon. The nitrosamines adsorbed to activated carbon were recovered by refluxing with methanol. The efficiency of desorption was not complete for all nitrosamines and the presence of lipid reduced significantly the ability of the carbon to adsorb (Walters et al., 1974). This, however, could be a method of choice for preliminary cleanup of nonvolatile nitrosamines which could not be efficiently recovered by distillation (Walters et al., 1970). Hasebe and Osteryoung (1976)

reported efficient separation of nitrosoproline from lipid materials by gel permeation chromatography using Sephadex LH-20.

In situ conversion of the nitroso group into a volatile inorganic derivative was another approach to isolate nonvolatile nitrosamines from the food matrix. Denitrosation of nitroso compounds by thionyl chloride (Lunt et al., 1973; Walters et al., 1974), however, produced variable recovery. Downes et al. (1976) claimed that the most suitable denitrosating agent was hydrogen bromide which produces nitrosylbromide. Nitrosylbromide was dehalogenated and nitrosamines were determined as nitric oxide by using a chemiluminescence analyzer. The detection limit was claimed to be below 6 ng. In situ denitrosation, however, would provide no information regarding the identity or the amounts of individual nitrosamines.

Derivatization was used in order to facilitate separation of nonvolatile nitrosamines by GC. Methyl esters of nitrosamino acids can be formed either by treating nitrosamino acids with diazomethane (Kushnir et al., 1975) or methanol plus HCl (Dhont and Ingen, 1976). The trimethylsilyl derivatives of nitrosamino acids were also satisfactory for gas chromatographic analysis. This derivative was particularly useful for 4-hydroxy-N-nitrosoproline (Eisenbrand et al., 1975). Methyl iodide and sodium hydride has been used by Sen et al. (1976) to form 3-methoxy-1-nitrosopyrrolidine from HNPYR.

High performance liquid chromatography (HPLC), being able to handle nonvolatile compounds without derivatization, is a promising alternative for the separation of nonvolatile nitrosamines. Fine et al. (1976) analyzed nonvolatile nitroso compounds at ug/kg level by using a combined high performance liquid chromatography-thermal energy analyzer (HPLC/TEA). The chromatographic column was interfaced directly to a specially designed TEA. Fine et al. (1976) further applied HPLC-TEA to fried bacon and reported 80 ± 9% recovery of spiked N-nitrosobenzylphenylamine.

## Detection of Nitrosamines

Methods used for the detection and quantitation of nitrosamines include the use of spray reagents in conjunction with thin-layer chromatography (Ender and Ceh, 1971; Sen and Dalpé, 1972), polarography, chemiluminescence, U.V. photoelectron spectroscopy, spectrophotometry, TEA, and various gas chromatographic detections. Several nitrogen sensitive or specific detectors for gas chromatography such as the Coulson electrolytic conductivity detector (CECD), alkali flame ionization detector, and the electron capture detector have been utilized (Foreman and Goodhead, 1975).

Eisenbrand and Preussmann (1970) introduced spectrophotometric detection of nitroso compounds as inorganic nitrite following denitrosation. Jhonson and Walters (1971) demonstrated the

specificity of this approach. Differential pulse polarography, as a means of nitrosamine detection, has been explored (Walters et al., 1970; Chang and Harrington, 1975; Hasebe and Osteryoung, 1975; Hasebe and Osteryoung, 1976). Pulse polarography has the advantage of providing sensitive rapid analysis with simple inexpensive equipment. In principle, it requires less demanding sample cleanup than the GC/MS technique (Hasebe and Osteryoung, 1975).

Betteridge and Hasanuddin (1976) suggested the possibility of using UV photoelectron spectroscopy as a nitrosamine detector in gas chromatography.

A fluorescence technique combined with HPLC, developed by Wolfram et al. (1977), could detect nitrosoproline at the level of 0.5 ng while the detection limit of GC/MS for methyl ester of nitrosoproline was 7 ng. The fluorescence technique, however, involve denitrosation of nitrosoproline and derivatization of the proline into a fluorescent compound prior to detection. Free proline contained in food products has to be removed prior to analysis and generation of proline prior to derivatization has to be prevented.

The TEA, a recently developed nitroso compounds detector, is based on thermoluminescence. The principle of operation (Fine et al., 1975) and design parameters (Fine et al., 1975; Fine and Rufeh, 1975) have been described in detail by Fine et al. The N-NO

bond in nitroso compounds is ruptured in a pyrolyzer with release of the nitrosyl radical (· NO). The nitrosyl radical is oxidized with ozone in an evacuated reaction chamber to give electronically excited nitrogen dioxide ($NO_2$*). The excited nitrogen dioxide decays back to ground state with the emission of characteristic radiation which is detected by means of a sensitive photomultiplier tube.

The outstanding advantage of TEA, in addition to high sensitivity and specificity, is that it does not require as extensive cleanup as the other gas chromatographic detectors. The TEA has been successfully used as a volatile nitrosamine detector coupled with gas chromatography (GC/TEA, Fine and Rounbehl, 1975). Although no work has been reported, GC/TEA could be readily applied to the analysis of nonvolatile nitrosamines, provided the compounds are converted into volatile derivatives prior to GC/TEA analysis.

As an alternative method for nonvolatile nitroso compounds, Oettinger et al. (1975) developed a HPLC/TEA interface in which the effluent from the HPLC was introduced directly into the TEA catalytic furnace by means of a specially designed liquid-gas interface nozzle. Although the HPLC/TEA chromatograms are well defined, the technique was not as sensitive as the GC/TEA. Work is underway to improve sensitivity and to provide the HPLC with a solvent programming capability (Fine et al., 1976). Fine et al.

(1976) demonstrated the practical application of HPLC/TEA by isolating and detecting nonvolatile nitrosamines from spiked meat products. Analysis of nitrosamines in air has been achieved by GC/TEA and HPLC/TEA (Fine et al., 1976b; Fine et al., 1976c).

Fine et al. (1976d) compared the TEA, as a nitrosamine detector, with other gas chromatographic detectors in terms of specificity and sensitivity. The authors concluded that the specificity and sensitivity claims for the TEA were justified. They based their conclusion on the fact that results obtained by GC/TEA on dilute and dirty extracts were comparable to results obtained on clean concentrates by GC/CECD and GC/MS.

## Confirmation of Identity

At present MS is the only generally accepted method for confirmation of identity of nitrosamines. The most convenient way to introduce small amounts of nitrosamines into the mass spectrometer is via a gas chromatographic column. For volatile nitrosamines, GC/MS can be easily employed with clean, concentrate samples (Telling et al., 1971; Stephany et al., 1976). Nonvolatile nitroso compounds, however, have to be derivatized into more volatile compounds to be gas chromatographed for GC/MS analysis. Nitrosamino acids can be converted either into methyl esters (Ishibasi et al., 1975; Kushnir et al., 1975; Dhont and

Ingen, 1976) or trimethylsilyl derivatives (Eisenbrand et al., 1975). HNPYR has been converted into a methyl ether (Sen et al., 1976) for GC/MS analysis.

# III. EXPERIMENTAL PROCEDURE

## Gas Chromatographic Conditions

Three different gas chromatographic columns were employed in this study.

1. Gas chromatographic conditions used for separation and identification of HNPYR from a model heating system. A Varian GC, Series 1200, equipped with a flame ionization detector and a stainless steel column (0.3 cm o.d. x 1.5 m) packed with 1% Carbowax 20 M on Chromosorb G was employed. Temperatures of injector, column, and detector were 210° C, 200° C, and 265° C, respectively. The flow rate of the carrier gas (He) was 25 ml/min at 200° C.

2. Gas chromatographic conditions used for determining partition coefficients of HNPYR and for quantitative determinations of HNPYR in commercial bacon and fried-out fat. A Varian GC, series 1400, equipped with a stainless steel column (0.3 cm o.d. x 0.75 m) packed with 10% SP 2340 on 100/120 Supelcoport (Supelco Inc.) was employed. The effluent from the column was monitored by a TEA as described by Fine and Rounbehler (1975). The TEA operating conditions were: furnace 400° C; cold trap, -150° C; reaction chamber, 4 mm Hg. Temperatures of GC injector and

column were 210° C and 200° C, respectively. The flow rate of the carrier gas (He) was 50 ml/min at room temperature.

3. Gas chromatographic conditions for the identification of the trimethylsilyl derivative of 3-hydroxy-N-nitrosopyrrolidine (TMS-HNPYR) from fried bacon and fried-out fat. A Varian GC, Series 1400, equipped with a stainless steel capillary column (0.76 mm i.d. x 152 m) coated with SF-96 was coupled to a MS (Hildrum, et al. 1975b). Temperatures of the injector and column were 200° C and 140° C, respectively. The flow rate of the carrier gas (He) was 15 ml/min at 150° C.

## Authentic Compound

### Synthesis

The method of Lijinsky et al. (1970) was used to synthesize 4-hydroxy-N-nitrosoproline. Synthesis of HNPYR was accomplished as follows. Into a 50 ml round-bottom flask was introduced 2 g of 3-hydroxypyrrolidine (Pfaltz and Bauer), 3 ml of 10 N HCl, 4 ml of water, and a magnetic stirring bar. The flask and its contents were cooled in an ice bath, and with constant stirring, 2 g of sodium nitrite dissolved in 4 ml of water was added dropwise. The reaction mixture was allowed to stand for one hour in the ice bath. The aqueous phase was saturated with anhydrous sodium

sulfate and extracted with four 20 ml portions of anhydrous ethyl ether. The combined extract was dried over anhydrous sodium sulfate and concentrated to a volume of 4 ml in a Kuderna-Danish apparatus equipped with a Snyder tube.

## Thin-layer Chromatography

In order to purify the synthesized compound, the concentrated extract was subjected to TLC. Precoated 20 x 20 cm x 2.0 mm thick silica gel G plates (Macherey-Negel and Company) were used. The plates were developed in ether:methanol (3:1, V/V) as the mobile phase. Following development, one end of the plates was sprayed with modified Griess reagent (Fan and Tannenbaum, 1971) and exposed to UV light to reveal the location of the compound. The remaining portions of the plate at the proper $R_f$ were scraped and the HNPYR was taken up with methanol.

The authentic HNPYR appeared as a single peak at a retention time of 18.2 min when analyzed by GC/MS (Gas chromatographic conditions 1).

## Partition Coefficients

Two ml of each solvent were placed into a small vial with a screw-cap. After spiking with 3 μg HNPYR the vial was shaken for two hours to equilibrate the compound between the two solvents.

After equilibration, aliquots of the two solvents were analyzed by GC/TEA (Gas chromatographic conditions 2). The areas of the peaks were measured by the cut and weigh method and the partition coefficients were calculated.

For the partition coefficients between sodium sulfate saturated aqueous phases and the organic solvents, 100 ml of saturated solutions were prepared by stirring distilled water or the water-methanol mixture for two hours with excess sodium sulfate. For the determination of partition coefficients, two ml of saturated solution were placed into the vial along with several crystals of sodium sulfate to ensure saturation.

## Model Heating System

### Heating

The system consisted of a 200 ml balloon flask containing 50 ml of steam-stripped vegetable oil, 0.2 g of 4-hydroxy-N-nitrosoproline dissolved in 2.0 ml of $H_2O$, and a boiling chip. The temperature of the contents of the flask was brought to 170° C in about 10 min and held at 170° C for an additional 10 min under reflux (Bills et al., 1973).

In some of the heating trials, the aqueous phase was neutralized with NaOH prior to heating in order to bring the pH of the system to

that of bacon. Without neutralization, the aqueous phase had a pH of approximately 2 since 4-hydroxy-N-nitrosoproline is a strong organic acid.

## Extraction

Following the heating process, the contents of the balloon flask were transferred to a 500 ml separatory funnel and extracted with 50 ml of methanol. The methanol extract was centrifuged to separate and remove residual lipid material. The clear methanol extract was then dried over anhydrous sodium sulfate and concentrated to a volume of 3 ml in a Kuderna-Danish apparatus equipped with a Snyder column. The extract was further concentrated under a stream of nitrogen to a volume of 1 ml in a Chromaflex sample tube.

## Thin-layer Chromatography

The concentrated extract was subjected to TLC. Silica gel G plates of 250 microns thickness were used with ether: methanol (3:1). Following development, the plates were sprayed with modified Griess reagent (Fan and Tannenbaum, 1971) and then exposed to UV light to produce a pink spot for N-nitrosamines.

## Identification of 3-Hydroxy-N-nitrosopyrrolidine

Identification of HNPYR was based on coincidence of GC retention times (Gas chromatographic conditions 1), coincidence of TLC $R_f$ values, and comparison of mass spectral fragmentation patterns with those obtained from the authentic compound. The MS was a Finnigan model 1015C operated under the following conditions; filament current, 400 μA; electron voltage, 70 eV; multiplier voltage 2.4 KV. Data acquisition and processing were accomplished with a System Industries (System 250) data system. The effluent of the gas chromatographic column was introduced into the MS ion source through a Gholke glass jet helium separator.

## Inactivation of Nitrite

In the determination of HNPYR in fried bacon and fried-out fat it was desirable to inactivate residual nitrite in order to prevent nitrosation between residual nitrite and amines during the analytical procedure. Two hundred ml of extracting solvent mixture (3 parts distilled water plus 2 parts methanol), were spiked with 7.5 mg sodium nitrite (0.37 mM). This amount of nitrite corresponds to a level of 50 ppm in 100 g of fried bacon. Actually, residual nitrite in fried bacon rarely exceeds 20 ppm (Davidson, 1977). The

mixture was adjusted to pH 2 with sulfuric acid and made 0.011 M with ammonium sulfamate (250 mg). Absorbance at 220 nm was used as a measure of nitrite concentration and was measured on a Beckman DB spectrophotometer before adding ammonium sulfamate and at 3 min intervals after adding ammonium sulfamate. Standard nitrite solutions at several concentrations were prepared to relate absorbancy to nitrite concentration.

The same kind of solvent mixture, 3 parts distilled water plus 2 parts methanol, was used in all initial extractions of fried bacon or fried-out fat throughout this study.

## Isolation and Identification of 3-Hydroxy-N-nitrosopyrrolidine in Commercial Fried Bacon and Fried-out Fat

For the identification of HNPYR, 6.80 kg of raw bacon were used.

### Frying and Initial Extraction

Commercial bacon was fried to a well-done crispness (7-9 min) on an electric frying pan at 177° C. Seven strips of bacon were usually fried at one time, and fried-out fat was drained after each group was fried. Temperature of the fried-out fat during frying was 170° C. Approximately 100 g of fried bacon and 250 g of fried-out fat were obtained from a 450 g package of raw bacon.

Fried bacon was broken into smaller pieces and ground in a Waring Blendor for four min with 250 ml of extracting solvent. The mixture was centrifuged in a cold room at 13,000 g for 30 min (from power on to power off) and the supernatant was placed into a separatory funnel to remove unsolidified fat and oil. Usually 200 ml of extract were collected.

Fried-out fat was placed into a separatory funnel along with 500 ml of extracting solvent and was intermittently shaken for 20 min. The solvent was drained after it separated from fat. Approximately 450 ml of extract was obtained.

The extracts, from bacon and fried-out fat, were separately carried through exactly the same analytical procedure until they were pooled together after they were cleaned up with alumina column chromatography.

Inactivation of Nitrite

The extract was adjusted to pH 2 with sulfuric acid and made 0.011 M with ammonium sulfamate (250 mg). The extract was then stirred for 30 min with a magnetic stirrer and neutralized to pH 8 with a saturated sodium hydroxide solution.

Continuous Liquid-liquid Extraction

The neutralized extract was stirred for 30 min with sufficient

anhydrous sodium sulfate to bring the mixture to saturation. The saturated extracting solvent was then continuously extracted with 50 ml of dichloromethane for 12 hours. The dichloromethane extract was dried by passing through an anhydrous sodium sulfate bed and then concentrated to a volume of 3 ml in a Kuderna-Danish apparatus equipped with a Snyder column. The dichloromethane extract was further concentrated to 1 ml under a stream of nitrogen in a Chromaflex sample tube.

Clean-up with Alumina Column

Alumina, activated at 800° C for 4.5 hours and kept overnight at 200° C, was packed in 14 mm i.d. Chromaflex columns fitted with a 250 ml reservoir to the length of about 15 cm. The concentrated dichloromethane extract (1 ml) was diluted with 25 ml of diethyl ether and applied to the column. This was then eluted by a series of solvents in the following order; 50 ml of diethyl ether containing 2.5 ml of ethanol, 50 ml of diethyl ether containing 5 ml of ethanol, and 50 ml of diethyl ether containing 10 ml of ethanol.

After initial diethyl ether was discarded, the eluates were collected in 10 ml fractions numbering 1 to 15. Each fraction was blown down to 100 µl under a stream of nitrogen and analyzed by GC/TEA (Gas chromatographic conditions 2) to check for the presence of HNPYR. Those fractions containing a significant amount of HNPYR

(usually fraction 4-8) were pooled together in an Activial with mininert values (Pierce Chemical Company) for derivatization.

## Derivatization

The pooled eluates were blown to dryness under a stream of nitrogen and kept overnight in a desiccator over $P_2O_5$. The vial was then subjected to vacuum for 10 min and 100 µl of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was added to the vial.

## Identification of Trimethylsilyl derivative of 3-hydroxy-N-nitrosopyrrolidine

Identification of TMS-HNPYR was based on coincidence of GC retention times, selective sensitivity of TEA detection, and comparison of mass spectral fragmentation patterns with those obtained with authentic TMS-HNPYR. GC/TEA was followed by GC/MS (Gas chromatographic conditions 3). Peak enhancement was used to verify the retention time of TMS-HNPYR in the GC/TEA analysis.

Mass spectra were obtained using the SF-96 capillary column with a Finnigan 1015C GC/MS; this system incorporated an all-glass jet helium separater and a Varian 1400 GC. The conditions were: high vacuum, $5 \times 10^{-5}$ Torr; ionizing current, 400 µA; electron voltage 70 eV; multiplier voltage 2.80 KV and mass range m/e 10-200, which was scanned every 10 seconds.

Mass spectral data was acquired and processed by a System Industries System 250 data system. The MS was controlled by the data system using the IFSS (integration time as a function of signal strength) mode; this resulted in a nearly constant signal-to-noise ratio. The data system incorporated a Tektronix 4010 display terminal, which greatly facilitated data output. The compound of interest, TMS-HNPYR, was located by requesting a limited mass search for m/e 42 which is the base peak of this compound. Once located, background subtracted hard copy plots of the compound were drawn and compared with the standard spectrum of TMS-HNPYR.

## Examination of Artifactual Nitrosamine Formation

It was important to check for the possibility of HNPYR being generated during the analytical procedure, either by 1) nitrosation of 3-hydroxypyrrolidine, or 2) decarboxylation of 4-hydroxy-N-nitrosoproline. The following experiments were carried out for this purpose.

1. Two hundred ml of extracting solvent was spiked with 7.5 mg sodium nitrite and 2.5 mg 3-hydroxypyrrolidine. This corresponds to 50 ppm nitrite and 25 ppm 3-hydroxypyrrolidine in 100 g fried bacon.

2. Two hundred ml of extracting solvent spiked with 200 μg of 4-hydroxy-N-nitrosoproline.

3. Two hundred ml of extracting solvent was spiked with 2 μg of HNPYR. This corresponds to 20 ppb HNPYR in 100 g fried bacon. This sample served as check for recovery of HNPYR.

All of the extracting solvents were carried through the analytical procedure and analyzed for HNPYR by GC/TEA (Gas chromatographic conditions 2).

## Quantitative Analysis of Commercial Bacon and Fried-out Fat

### Preparation of Nitrite Free Bacon

Nitrite free bacon was prepared as follows: Raw pork bellies were soaked in a nitrite free pickle (4 kg sodium chloride, 1.35 kg sucrose in 15 kg of water) for 10 days at $3^{o}$ C. The bellies were rinsed with fresh water and heated to an internal temperature of $63^{o}$ C in a smoke house. The smoking process took approximately 16 hours. The bellies were cut into 900 g pieces, wrapped and frozen until used.

### Estimation of Percent Recovery

The nitrite free bacon was analyzed for HNPYR. The fried

bacon and fried-out fat contained no, or less than 0.2 ppb (lower detection limit) HNPYR.

In determining the percent recovery of HNPYR from fried bacon, 50 g of fried nitrite free bacon were spiked with 500 ng of HNPYR. The fried bacon and nitrosamine were ground in a Waring Blendor for 4 min with 150 ml of extracting solvent, and centrifuged at 13,000 g for 30 min in a cold room. The supernatant was carefully poured into a beaker (Extract A). One hundred ml of extracting solvent was added into centrifuge tubes and the residue was mixed well by use of a spatula and vigorous hand shaking for 1 min. This was centrifuged and the supernatant was collected in the same way as above (Extract B). Meanwhile, extract A was cleaned from the remaining fat and oil with a separatory funnel, cooled in ice water for 20 min, and then filtered through a Buchner funnel to remove any remaining bacon residue and fat. Extract B was treated in the same way as extract A, and then both were pooled.

The pooled extract was made 0.011 M with ammonium sulfamate, adjusted to pH 2.0 with sulfuric acid and stirred for 30 min. The extract was neutralized to pH 8 with saturated sodium hydroxide solution, saturated with sodium sulfate and centrifuged at 13,000 g for 10 min. The supernatant was continuously liquid-liquid extracted with dichloromethane for 12 hours. The dichloromethane extract was then concentrated by a Kuderna Danish apparatus and

evaporated under a stream of nitrogen to 500 μl. The concentrated extract was analyzed by GC/TEA (Gas chromatographic conditions 2) and the areas of the peaks were quantitated by the cut and weigh method.

In determining percent recovery of HNPYR from fried-out fat 100 g of fried-out fat from nitrite free bacon were spiked with 1 μg HNPYR. This was extracted with 230 ml of extracting solvent using a separatory funnel by intermittently shaking for 20 min. The extract was cooled in ice water for 20 min, filtered through a Buchner funnel, made 0.004 M with ammonium sulfamate and brought to pH 2 with sulfuric acid. After stirring for 30 min, the extracting solvent was carried through the remainder of the procedure and quantitatively analyzed by GC/TEA in the same way as previously described for recovery of HNPYR from nitrite free bacon.

Quantitation

The same analytical procedure for determining the percent recovery of HNPYR from nitrite free fried bacon was employed for quantitative analysis of commercial bacon except that the amount of bacon was increased to 200 g. Correspondingly the amounts of extracting solvent and ammonium sulfamate were increased by four times as well.

For analyzing commercial fried-out fat, the analytical procedure for determining percent recovery of HNPYR from nitrite free fried-out fat was employed except that the amount of fat was increased to 300 g. The amounts of extracting solvent and ammonium sulfamate were also increased by three times. The HNPYR peaks were verified by the peak enhancement technique on the GC/TEA and the peak areas were quantitated by the cut and weigh method.

## Safety Precautions

Many nitrosamines have been determined to be potent carcinogens in test animals and it is likely that these compounds are also hazardous to humans. As a matter of good laboratory practice, all N-nitroso compounds, whether previously demonstrated to be carcinogenic or not, should be handled with the respect that cancer-causing agents deserve.

In this investigation, measures were taken to avoid skin contact, inhalation, and accidental spills. Experiments with nitrosamines were conducted in a fume hood. The gas chromatograph was operated with the detector vented into a fume hood. All glass wares and solutions were discontaminated as outlined by Eisenbrand and Preussmann (1970). Absorbent covers were used in all work areas.

# IV. RESULTS

## Partition Coefficients

Partition coefficients of HNPYR in several solvent systems are shown in Table 1. The values demonstrate the polar nature of HNPYR which contributes to the difficulty encountered in isolating the compound from food. The information in Table 1 also indicates that dichloromethane would be the best solvent for extracting HNPYR from the saturated extracting solvent and continuous extraction would be required for an efficient recovery.

Table 1. Partition coefficients of 3-hydroxy-N-nitrosopyrrolidine.

| Solvent System | Partition Coefficients[1] | |
|---|---|---|
| | Aqueous Phase Unsaturated | Aqueous Phase Saturated with Sodium Sulfate |
| DCM/Water | 0.073 | 0.201 |
| DCM/Water/Methanol (3:2) | 0.175 | 0.221 |
| Ether/Water | - | 0.086 |

[1] Partition coefficient = $\frac{\text{concentration in solvent}}{\text{concentration in aqueous}}$

## Formation of 3-Hydroxy-N-nitrosopyrrolidine from Model Heating System

When 4-hydroxy-N-nitrosoproline was heated in seven trials under the conditions described, a Griess-positive product was observed each time as a pink spot on the TLC plates on which the concentrated extract was spotted and developed. Under the conditions employed, the TLC $R_f$ value for the new compound was 0.45 whereas the original compound (4-hydroxy-N-nitrosoproline) was found to elongate only slightly from the point of application. At this point the compound from the heated system was tentatively identified as HNPYR since authentic HNPYR had an $R_f$ value of 0.45 under the same TLC conditions.

The Griess-positive product at $R_f$ 0.45 was observed when the pH of the aqueous phase of the model system was adjusted to 7.0 or when it was left unadjusted prior to heating. However, 4-hydroxy-N-nitrosoproline was completely absent following heating of the systems in which the pH was not adjusted, but was still present to some extent in the heated systems in which the pH was adjusted to 7.0. Also, the yield of the Griess-positive product at $R_f$ 0.45 was lower in the pH adjusted treatment.

When the Griess-positive product was scraped from a TLC plate (which had been developed but not sprayed with Griess reagent), taken up in solvent, and analyzed by GC/MS, it was identified as

HNPYR by comparison of its GC retention time and mass spectral fragmentation patterns with those of the authentic compound (Figure 3).

## Isolation and Identification of 3-Hydroxy-N-nitrosopyrrolidine from Fried Bacon and Fried-out Fat

HNPYR was isolated from commercial fried bacon and fried-out fat and identified as the TMS-HNPYR. Three independent trials produced same results. Identification of HNPYR was based on: GC/TEA response at the correct retention time and comparison of mass spectral fragmentation patterns with those obtained with authentic TMS-HNPYR. The relative retention time for the isolated TMS-HNPYR compared to NPYR was 2.28 whereas that of authentic TMS-HNPYR compared to NPYR was 2.32 under gas chromatographic conditions 3. Figure 4 shows the mass spectrum of the authentic TMS-HNPYR (A) and that of TMS-HNPYR isolated from commercial fried bacon and fried-out fat (B). An examination of these spectra shows that they are practically identical.

## Inactivation of Nitrite

Preliminary experiments indicated ammonium sulfamate effectively inactivated residual nitrite (Table 2). The values in Table 2 indicate that ammonium sulfamate quickly reduced the

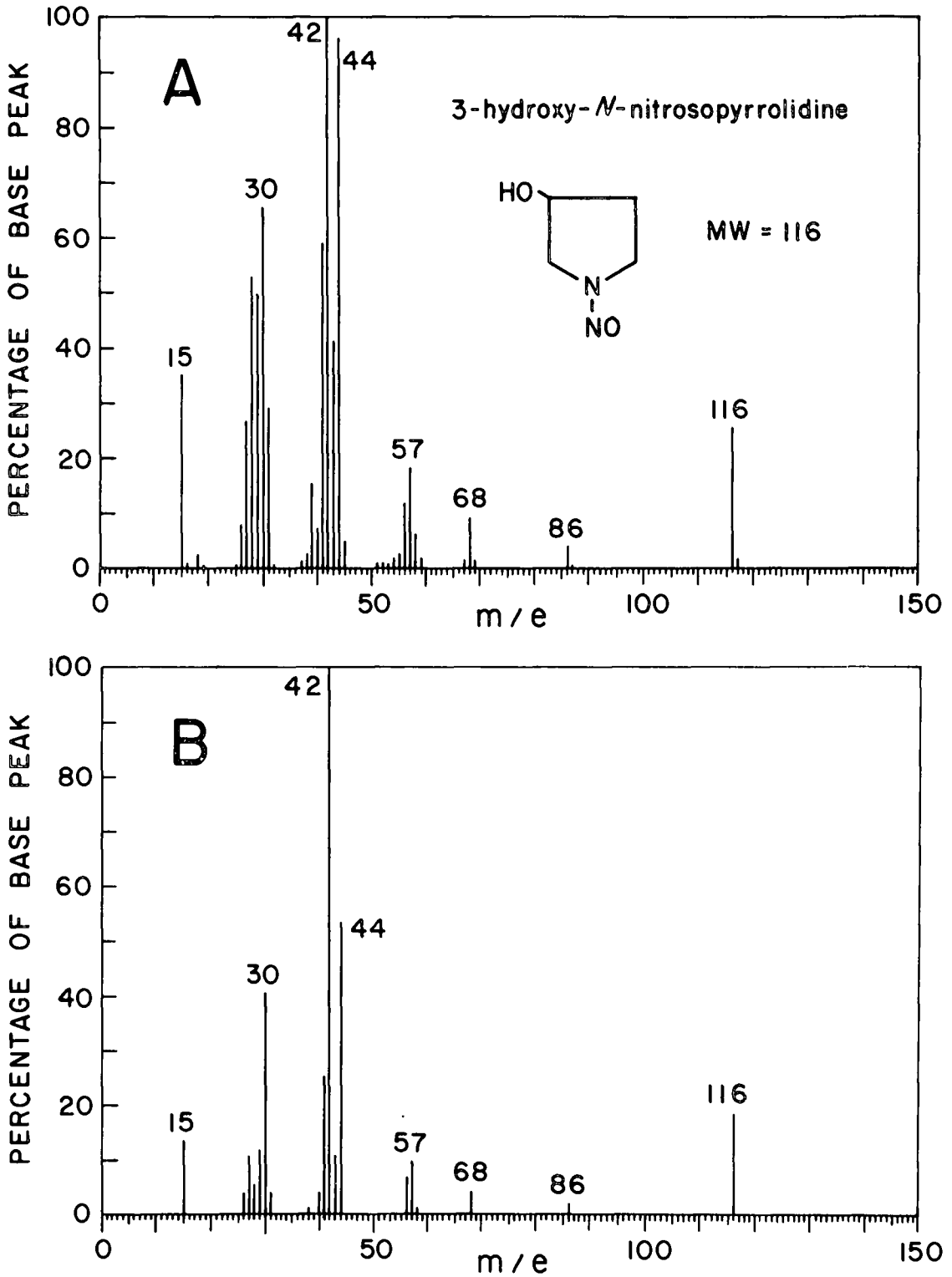


Figure 3. Mass spectra of 3-hydroxy-N-nitrosopyrrolidine: (A) Authentic compound, (B) Isolated from heated 4-hydroxy-N-nitrosoproline.

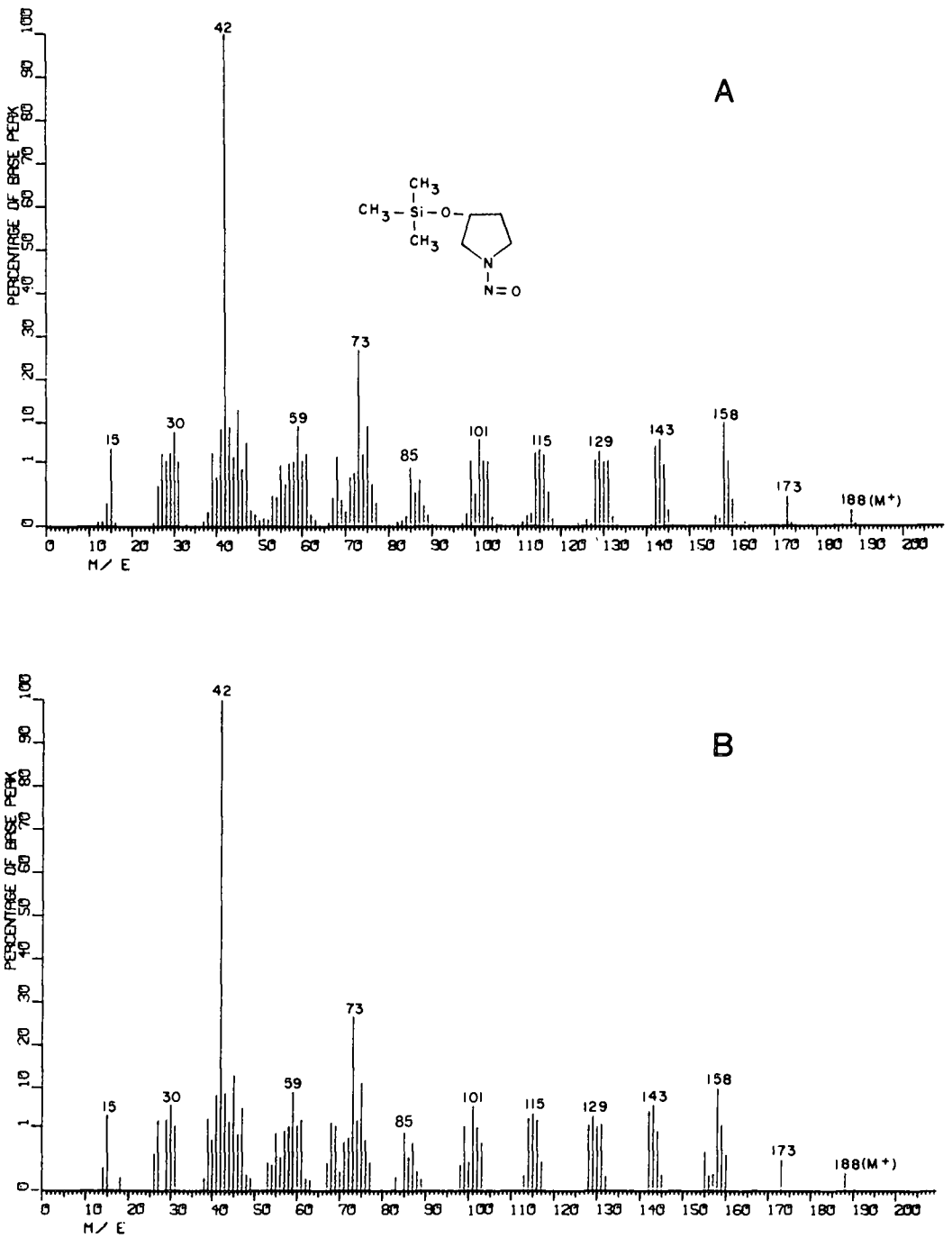


Figure 4. Mass spectra of trimethylsilyl derivative of 3-hydroxy-N-nitrosopyrrolidine: (A) Authentic compound (B) Isolated from commercial fried bacon and fried-out fat.

nitrite concentration and completely eliminated the nitrite after 24 min.

Table 2. Inactivation of nitrite by ammonium sulfamate.

| Time (min) | Residual Nitrite (ppm) | |
|---|---|---|
| | 25 ppm nitrite in Water:Methanol (3:2) at pH 2 | 25 ppm nitrite in Water:Methanol (3:2) at pH 2, 0.011 M in ammonium sulfamate |
| 0.5 | 12 | 5.7 |
| 3 | 5.7 | 3.2 |
| 6 | 4.8 | 0.8 |
| 9 | 4.2 | 0.4 |
| 12 | 3.6 | 0.2 |
| 15 | 2.6 | 0.2 |
| 18 | 2.4 | 0.2 |
| 21 | 2.0 | 0.1 |
| 24 | 1.6 | 0 |
| 27 | 1.4 | 0 |
| 30 | 1.0 | |

## Examination of Artifactual Nitrosamine Formation

The possibility of artifactual HNPYR formation during the analytical procedure both by nitrosation and decarboxylation was examined and the results of these experiments are summarized in Table 3. The experiments conducted to determine whether any nitrosation occurred proved that no, or less than 7 ng HNPYR formed via nitrosation of precursors from 100 g of fried bacon. Seven ng HNPYR was the lower detection limit for this experiment.

If 7 ng HNPYR were formed, it would amount to 0.07 ppb in 100 g of fried bacon. In the decarboxylation experiment, it was shown that no, or less than 7 ng, HNPYR was produced from 200 μg of 4-hydroxy-N-nitrosoproline. This means that 100 g of fried bacon containing 200 μg of 4-hydroxy-N-nitrosoproline (2 ppm) would produce, at most, 0.1 ppb HNPYR.

Table 3. Examination of Artifactual Nitrosamine Formation.

| Object of Test | 200 ml. extracting solvent spiked with: | Amount of HNPYR |
|---|---|---|
| Artifactual nitrosation | 7.5 mg sodium nitrite plus 2.5 mg 3-hydroxy-pyrroline | ND[1] |
| Artifactual decarboxylation | 200 μg 4-hydroxy-N-nitrosoproline | ND[1] |
| Recovery | 2.0 μg HNPYR | 1.4 μg |

1 ND = none detected. Lower detection limit 7 ng.

## Percent Recovery of 3-Hydroxy-N-nitrosopyrrolidine

The results of six replicate analyses for determining percent recovery are shown in Table 4. The recoveries of the individual trials were 58.5, 59.0, 65.4, 54.5, 69.7, and 62.5 percent for fried bacon and 62.5, 64.0, 59.4, 69.5, 60.7, and 60.0 for fried-out fat.

Table 4. Percent recovery of 3-hydroxy-N-nitrosopyrrolidine.

| | N | $\overline{X}$ | S | 95% Confidence Limit |
|---|---|---|---|---|
| Fried bacon | 6 | 61.6 | 5.4 | 67.26-55.94 |
| Fried-out fat | 6 | 62.7 | 3.75 | 66.67-58.77 |

## Quantitative Analysis

The results of the quantitative analysis in commercial fried bacon and fried-out fat are shown in Table 5. The HNPYR concentration in fried bacon and fried-out fat appears to be lower than values generally reported for NPYR (Fazio et al., 1973; Sen et al., 1973; Pensabene et al., 1974). This may be due to the fact that free 4-hydroxyproline occurs at lower levels than free proline in raw bacon (Gray, et al., 1977). Distribution of HNPYR between fried bacon and fried-out fat is different from that reported for NPYR (Fiddler et al., 1974; Patterson et al., 1976; Gough et al., 1976). HNPYR, being the more polar compound, occurs in slightly higher concentrations in fried bacon than in fried-out fat while the reverse seems to be true for NPYR.

Table 5. Quantitative analysis of commercial fried bacon and fried-out fat for 3-Hydroxy-N-nitrosopyrrolidine.

| Commercial Bacons[1] | Cure Ingredients | Cooking Yield (%) | | HNPYR (ppb) |
|---|---|---|---|---|
| 1 | sugar, sodium phosphate sodium ascorbate | Bacon<br>Fat | 25.5<br>42.4 | 0.4<br>0.3 |
| 2 | sodium phosphate, flavoring, sodium erythorbate | Bacon<br>Fat | 21.9<br>46.0 | 2.2<br>2.2 |
| 3 | sugar, sodium phosphates sodium erythorbate | Bacon<br>Fat | 20.4<br>40.9 | 2.0<br>1.2 |
| 4 | Sugar, sodium phosphates, maple syrup, vanillin, imitation maple flavoring | Bacon<br>Fat | 19.3<br>49.2 | 2.6<br>2.3 |
| 5 | sodium phosphates, sodium erythorbate, flavoring | Bacon<br>Fat | 19.9<br>51.9 | 3.9<br>1.9 |

[1] All brands contain water, salt, and $NaNO_2$.

# V. DISCUSSION

HNPYR was formed by decarboxylation of 4-hydroxy-N-nitrosoproline on heating in the model system consisting of vegetable oil and water. The results suggest that 4-hydroxy-N-nitrosoproline, if present in raw bacon, could be decarboxylated to yield HNPYR by frying. The mass spectrum of HNPYR isolated from the model heating system is practically identical to that of the authentic HNPYR (Figure 3).

The ion at m/e 86 indicates the loss of NO, which is characteristic of most nitrosamines. The ion at m/e 68 indicates further loss of $H_2O$ from m/e 86, which is known to occur in certain organic compounds. The base peak at m/e 42 is a characteristic ion in most nitrosamines and the suggested structure is $CH_2{=}N{=}CH_2$ (Budzikiewicz et. al., 1962). The ion at m/e 30 is due to NO. The ion at m/e 28 is often interpreted to come from an air leak in the system. This interpretation, however, may not be valid, particularly when a negligible quantity of ion at m/e 32 is observed. The ion at m/e 32, due to oxygen, should also occur when there is a leak in the system. Since the spectrum of HNPYR shows an ion at m/e 28 but practically none at m/e 32, the m/e 28 is not likely due to an air leak. The ion at m/e 28, moreover, is commonly present in mass spectra of nitrosamines. The formula for the ion is speculated to be $C_2H_4$.

Other prominent ions are at m/e 57, 44, and 15. The suggested mass spectral fragmentation scheme is given in Figure 5.

Although model heating systems are useful tools for preliminary studies, the results of model systems should be supported by information obtained from actual meat products. Even the best devised model system is far from duplicating the conditions of meat tissues. Bacon, for example, contains a mixture of food additives, in addition to the complicated natural components of meat. Any of these components or additives may have an effect on the chemical reactions which occur when bacon is fried. For example, investigations on the precursors of NPYR in bacon by using various model systems produced contradicting results. Spermidine and putrescine produced NPYR in an oil-water system (Bills et al., 1973; Hildrum et al., 1976) but failed to do so when bacons containing these amines were fried (Hwang and Rosen, 1976).

Possible explanations for the failure of earlier studies to detect HNPYR as a product of heating 4-hydroxy-N-nitrosoproline or 3-hydroxypyrrolidine plus nitrite can be based upon the polar nature of HNPYR. Bills et al. (1973), Huxel et al. (1974), and Gray and Dugan (1975) used dichloromethane to extract HNPYR in their model system experiments. Later it was shown that neither dichloromethane nor diethyl ether are effective in extracting HNPYR from aqueous solution (Gray et al., 1977), particularly when extraction was

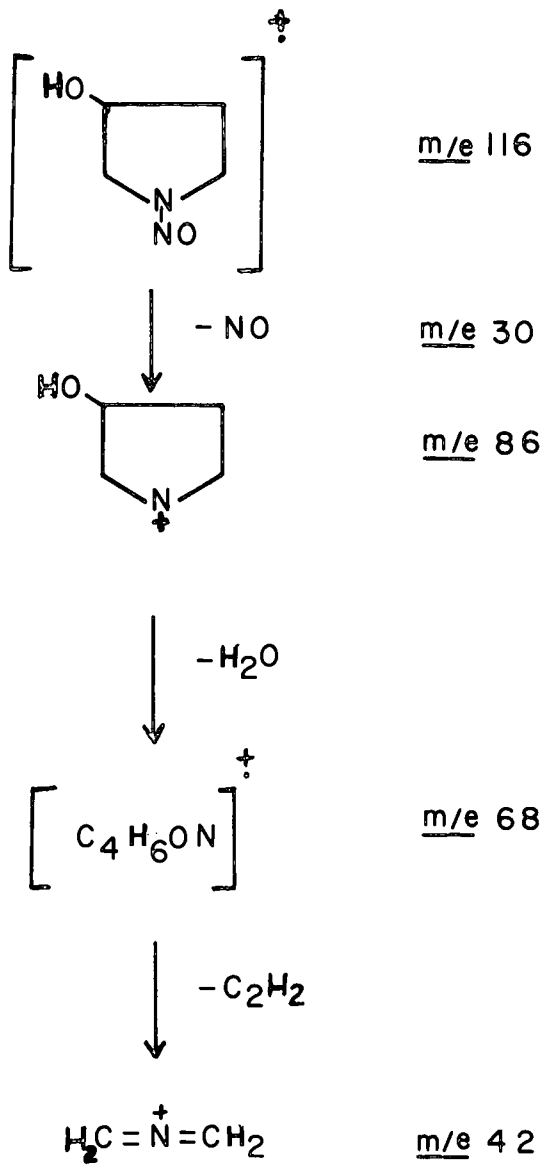


Figure 5. Suggested mass spectral fragmentation scheme of 3-hydroxy-N-nitrosopyrrolidine.

carried out in a separatory funnel. Partition coefficients of authentic HNPYR, the data in Table 1, clearly demonstrates the problem and indicates the need for continuous liquid-liquid extraction for efficient recovery. Based on this data, we chose polar water: methanol (3:2) for the initial extraction from bacon followed by continuous liquid-liquid extraction with dichloromethane as a cleanup step.

Cleanup procedures such as those devised by Fazio et al. (1970) might be expected to remove the compound prior to analysis, since it would partition favorably into the aqueous washes.

Gas chromatographic columns and conditions typically employed for the analysis of volatile nitrosamines would be unsuited for HNPYR as the compound would not be eluted with a reasonable retention time. Huxel et al. (1974) used a stainless steel column (0.32 cm o.d. x 1.5 m) packed with 5% carbowax 20 M at an oven temperature of 120° C isothermal. Gray and Dugan (1975) employed an oven temperature of 110° C isothermal to provide good separation of the more volatile nitrosamines.

Non-polar stationary phases or lower loadings of polar stationary phases, higher oven temperatures and/or shorter columns are required in order to elute HNPYR from a gas chromatographic column within a reasonable period of time. In this study, a stainless steel column (0.32 cm o.d. x 1.8 m) packed with 1% Carbowax 20 M

was operated at an oven temperature of 200° C isothermal.

HNPYR tended to tail strongly on most gas chromatographic columns, presumably because of the nonspecific adsorption due to its free hydroxyl group (Eisenbrand et al., 1975). A short stainless column (0.45 m) packed with 8.5% Silar 10C, however, gave the most satisfactory results among those columns that were tried. Precoated SP 2340, which is of the same chemical nature as Silar 10C with refined quality, was later obtained from Supelco Inc. and used satisfactorily in this study.

The frying temperature employed, 177° C, was the temperature recommended by the pan manufacturer. Since each package of bacon required a different length of time to reach the same "doneness", the frying time could not be standardized (7-9 min). The "doneness" of the fried bacon was standardized, which is probably closer to the practice which is followed when bacon is fried in the home.

Initial attempts were made to isolate and identify HNPYR in commercial bacon samples by the same procedure that was employed for isolation and identification of the nitrosamine in the model heating system. The commercial bacon, however, was far more complicated than the oil-water model system. The polar lipids, for example, were extracted into methanol in the initial extraction of the nitrosamine from fried bacon. Attempts to use

TLC to separate the HNPYR from these interferring substances were not successful.

As a result of these problems the extracting solvent was changed to water:methanol (3:2). Intensive cleanup procedure employing liquid-liquid extraction, column chromatography, and thin-layer chromatography has been developed. Although this procedure provided the cleanup requirements for detection by GC/TEA, the ultimate confirmation of HNPYR by GC/MS under gas chromatographic conditions 1 was not achieved due to impurities that cochromatogramed with the compound.

In view of this difficulty it was decided to explore the use of a high efficiency gas chromatographic capillary column for identification of HNPYR by GC/MS. Since HNPYR is nonvolatile and did not elute from the column even at the oven temperature of 180° C, the temperature limit for the SF-96 capillary column, derivatization into a more volatile compound was necessary. The trimethylsilyl derivative of authentic HNPYR was prepared and it was found that the derivative possessed satisfactory gas chromatographic properties. It eluted from the capillary column even at a lower temperature (140° C) and also appeared as a sharp peak even at a long retention time. The TMS-HNPYR should be kept in a refrigerator in a sealed container filled with Drierite. The derivative was not stable over long periods of time.

For the identification of TMS-HNPYR by GC/MS, the compound was located by requesting a limited mass search for m/e 42 which is a base peak of the compound. Although the real time gas chromatogram did not show a peak for the compound, a limited mass search for characteristic major ions give the exact location (Figure 6). Once located, background subtracted hard copy plots were drawn and these were compared with the standard spectrum of authentic TMS-HNPYR.

The mass spectrum of TMS-HNPYR is shown in Figure 4. The parent ion at m/e 188 is very small and represents about 0.3% of base peak. This is presumably because the TMS-HNPYR is not stable and rapidly undergoes fragmentation. The ion at m/e 173 is loss of $CH_3$ which may further loses NO to give an ion at m/e 143. The loss of NO from the parent ion gives m/e 158 which may further lose $CH_3$ to give m/e 143. The ion at m/e 115 is loss of $Si(CH_3)_3$. The ions at m/e 42 and 30 are characteristic of most nitrosamines. A suggested mass spectral fragmentation scheme of TMS-HNPYR was shown in Figure 7.

Recently, Gray et al. (1977) independently reported the isolation of HNPYR from heated 4-hydroxy-N-nitrosoproline in a model system. A polar solvent was employed to extract the compound and higher oven temperature (200° C) was used in gas chromatographic determination. The authors, however, could not

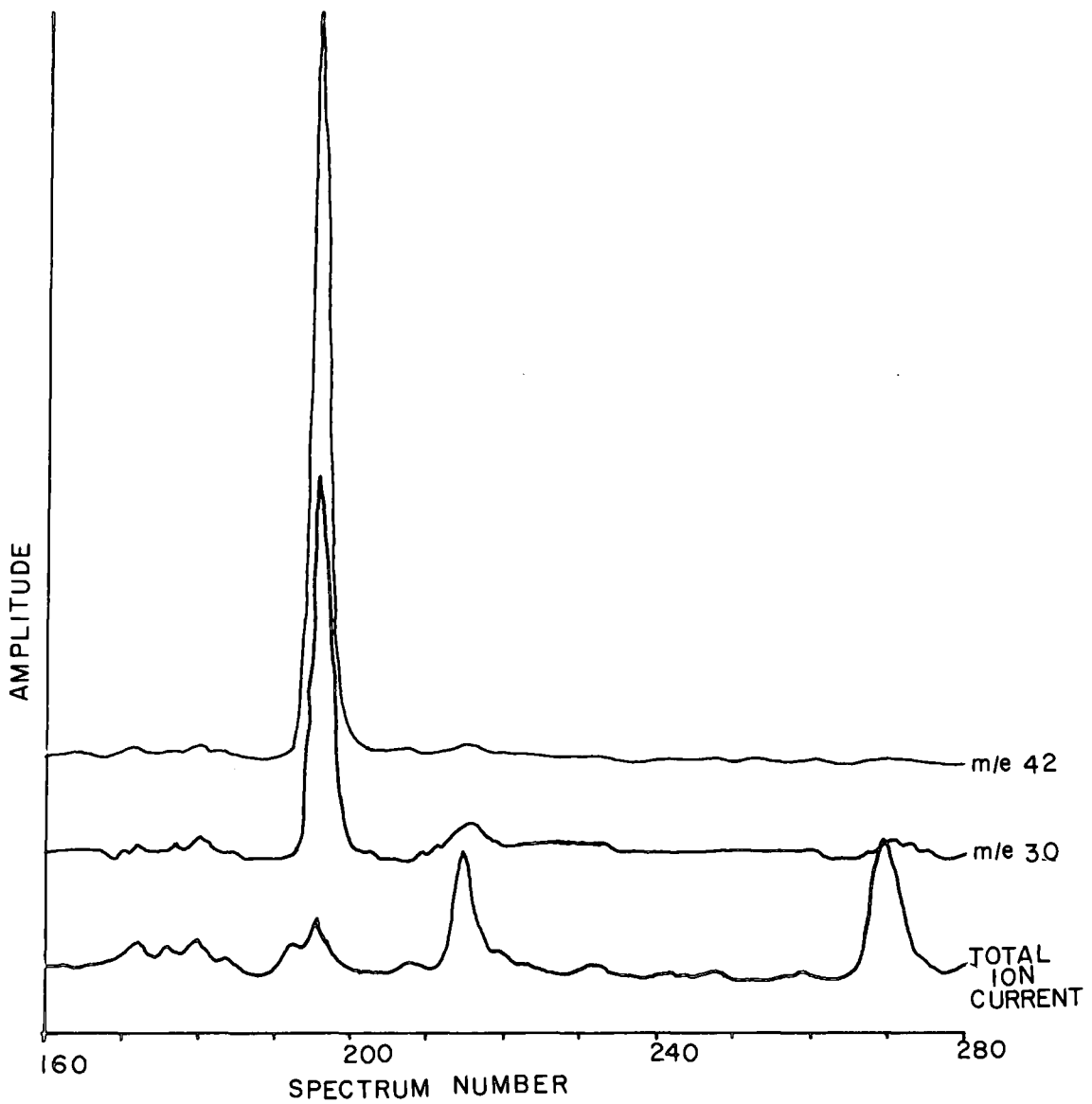


Figure 6. Total ion current plot and limited mass searches for m/e 30, 42.

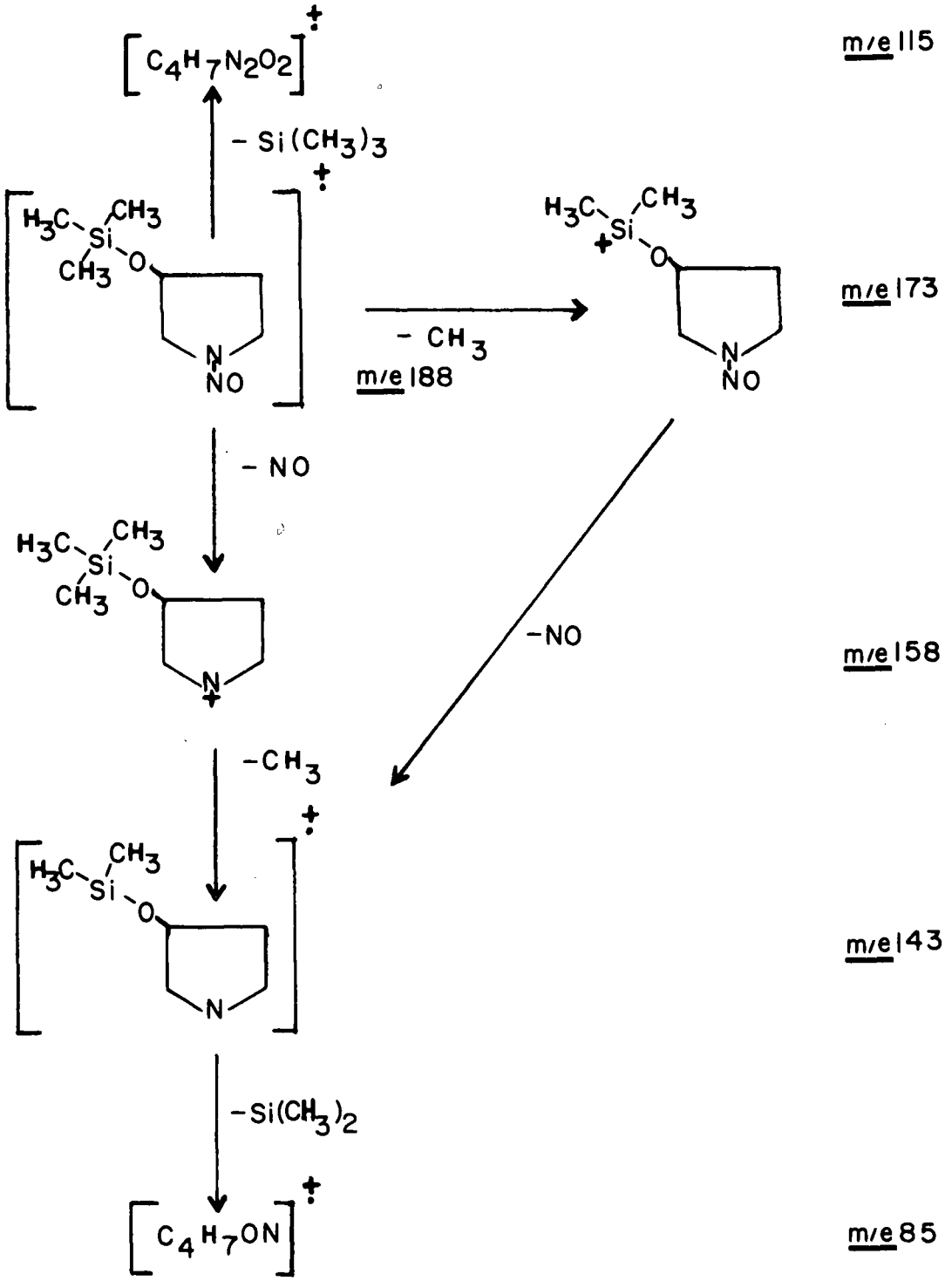


Figure 7. Suggested mass spectral fragmentation scheme of trimethylsilyl derivative of 3-hydroxy-N-nitrosopyrrolidine.

identify HNPYR in commercial fried bacon and attributed the negative result to either low recovery (20% recovery of HNPYR from aqueous solution) or the low level of free hydroxyproline in raw bacon. Considering the trace level of HNPYR (0.5 ppb to 3 ppb) determined in this study, it can be concluded that the analytical procedure employed by Gray et al. (1977) was not sensitive enough. The lower detection limit for spiked HNPYR was reportedly 50 ppb. The low recovery of HNPYR (Gray et al., 1977) was probably due to the poor efficiency of steam distillation which was used for the isolation of HNPYR from bacon. Steam distillation had been explored initially in this study and it has been shown that the recovery of HNPYR from an aqueous slurry of bacon was lower than 10%.

Although TMS-HNPYR has been successfully used in this laboratory, the methyl ether of HNPYR has been employed by Sen et al. (1976). The authors applied the technique to an organic solution of HNPYR and reported that the derivative could be identified by GC/MS. Since the methyl ether derivative would probably be more stable than TMS-HNPYR, the parent ion from the ether might be larger than the parent ion from TMS-HNPYR. Unfortunately the authors (Sen et al., 1976) did not report the mass spectrum.

Occasionally, high resolution mass spectrometry has been suggested as the only reliable technique for confirmation of identity of nitrosamines (Sen et al., 1976; Stephany et al., 1976). This,

however, may be true only when adequate resolution (10,000 or above) is used and a unique choice can be made among possible structures written for an empirical formula. Furthermore, high resolution mass spectrometry may not be adequate for the analysis of the extremely low levels of nitrosamines, especially when extensive cleanup work is required. The identification of TMS-HNPYR by GC/MS in this laboratory was supported by an additional specific detector for nitrosamines, namely the TEA.

Quantitation of HNPYR in commercial fried bacon and fried-out fat was greatly facilitated by the use of TEA. The TEA demonstrated its sensitivity and specificity in quantitative analysis of HNPYR from commercial bacon, where the cleanup procedure was minimized in order to obtain higher recovery. The concentrated dichloromethane extract obtained from continuous liquid-liquid extraction of the water:methanol extract of bacon was clean enough to be analyzed by GC/TEA. It was desirable, however, to remove most of the fats and oil from the water:methanol extract of fried bacon prior to continuous liquid-liquid extraction with dichloromethane. In addition to its specificity and sensitivity, another advantage of the GC/TEA was its capacity for accepting relatively large injections. Large quantity injections were necessary to avoid loss of the compound by excessive concentration. Large

injections of dichloromethane or diethyl ether were also required to obtain measurable peaks in the determination of partition coefficients.

Although the number of samples analyzed in this study was limited, our data suggests that low ppb levels of HNPYR appear fairly consistently in fried bacon and in fried-out fat. The levels of HNPYR in fried bacon are lower than the amounts reported for NPYR. One explanation for this was suggested by Gray et al. (1977) who attributed the low level of HNPYR to extremely low levels of free 4-hydroxyproline in raw bacon.

It was necessary to demonstrate that the analytical procedure was free from artifactual HNPYR formation. Artifactual HNPYR could be formed by (1) nitrosation of 3-hydroxypyrrolidine or (2) decarboxylation of 4-hydroxy-N-nitrosoproline during the analytical procedure. Preliminary experiments demonstrated the efficiency of ammonium sulfamate for inactivating nitrite. The level of nitrite employed in the experiment (50 ppm of 100 g fried bacon) was a reasonable level because the nitrite concentration of fried bacon is less than 20 ppm (Davidson, 1977). The nitrite concentration of fried-out fat is much lower.

The actual proof that the analytical procedure was free from artifactual HNPYR was achieved by two experiments conducted to examine the two possibilities for formation, the concentration of 3-hydroxypyrrolidine (25 ppm) in the experiment to check artifactual

nitrosation was probably quite high. While there is no data on 3-hydroxypyrrolidine concentration in raw or cooked bacon, the 3-hydroxypyrrolidine level in baked ham was lower than 0.1 ppm (Singer et al., 1976) and the free hydroxyproline content of bacon was reported to be almost negligible (Gray et al., 1977).

The level of 4-hydroxy-N-nitrosoproline (2 ppm in 100 g fried bacon) in the experiment to check the possibility of HNPYR generation by decarboxylation of 4-hydroxy-N-nitrosoproline seems reasonable. While no information on 4-hydroxy-N-nitrosoproline in bacon is available, the nitrosoproline content in raw bacon has been reported to be 0.38-1.18 ppm (Kushnir et al., 1975). Considering that nitrosoproline and 4-hydroxy-N-nitrosoproline may undergo decarboxylation and/or decomposition in the frying process, the residual content of these nitrosamino acids in fried bacon would probably be lower.

The results of the two experiments indicated that neither nitrosation nor decarboxylation contributed to HNPYR formation during the analytical procedure.

Although no work has been done regarding the pathway for the formation of HNPYR in fried bacon, it has been suggested that HNPYR is formed by analogous reactions to the formation of NPYR in bacon (Scanlan, 1975). At present, it is assumed that NPYR may be formed either by nitrosation of pyrrolidine or decarboxylation of nitrosoproline, or a combination of both routes (Figure 1).

The formation of HNPYR likewise may be suggested as a dual pathway (Figure 8). Decarboxylation of 4-hydroxy-N-nitrosoproline was demonstrated in the oil-water model system designed to simulate bacon frying conditions. The occurrence of 4-hydroxy-N-nitrosoproline in bacon has not yet been reported. This may be attributed to the lack of analytical methodology for nonvolatile nitrosamines, and active explorations in the area are underway.

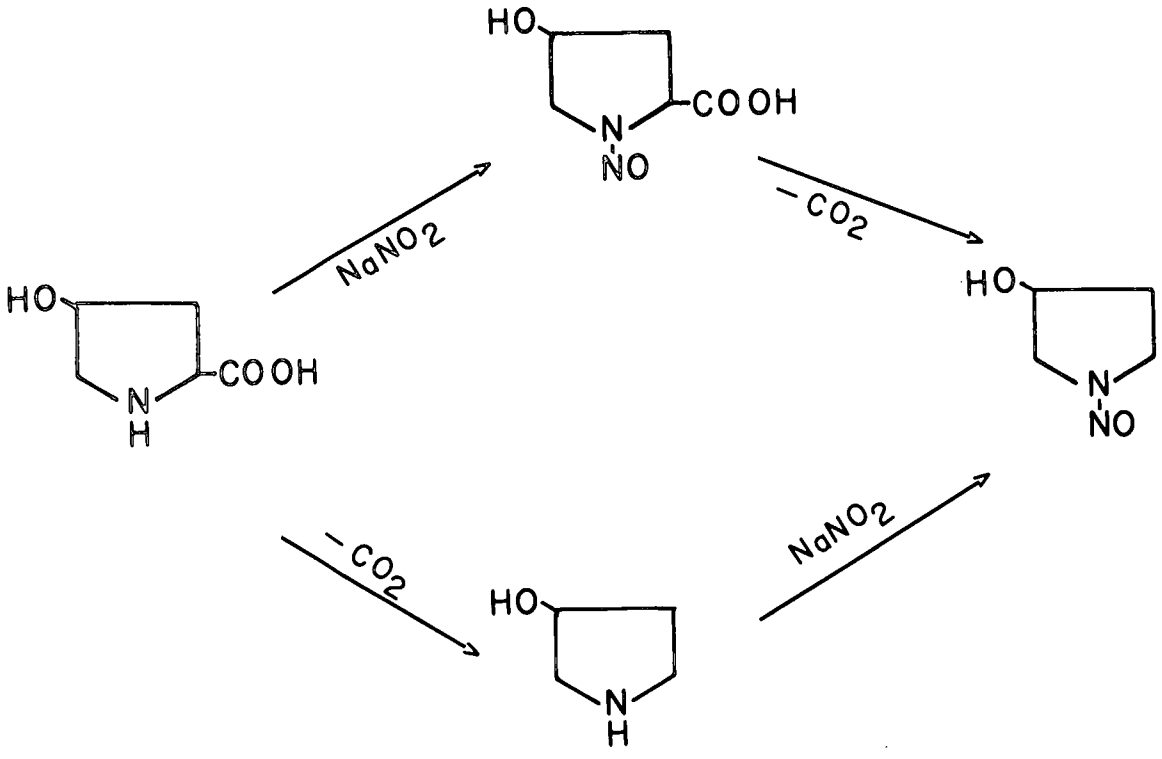


Figure 8. Suggested pathway for the formation of 3-hydroxy-N-nitrosopyrrolidine.

To date, no study on the carcinogenicity of HNPYR has been conducted. This makes an assessment of the public health significance of HNPYR in fried bacon very difficult. Stoltz and Sen (1977), however, recently reported the active mutagenicity of HNPYR with Salmonella typhimurium. The authors predicted, based on the correlation between mutagenicity and carcingenicity developed by Ames et al. (1975), that HNPYR would also be carcinogenic.

# VI. SUMMARY AND CONCLUSIONS

The formation of 3-hydroxy-N-nitrosopyrrolidine from decarboxylation of 4-hydroxy-N-nitrosoproline was demonstrated in model heating system designed to simulate the conditions of frying bacon. The identification of the nitrosamine was achieved by comparison of its TLC $R_f$ value, gas chromatographic retention time, and mass spectral fragmentation pattern with those of the authentic compound.

3-Hydroxy-N-nitrosopyrrolidine was isolated from commercial fried bacon and fried-out fat. The identification of the compound was achieved by combined gas chromatography-mass spectrometry after trimethylsilyl derivatization of the nitrosamine. Additional verification of identity was obtained from coincident retention time data on a combined gas chromatography-thermal energy analyzer.

Quantitative determination of 3-hydroxy-N-nitrosopyrrolidine was accomplished without derivatization and was greatly facilitated by the use of a thermal energy analyzer. Quantitative analysis by combined gas chromatography-thermal energy analyzer did not require as extensive cleanup as was required for identification by combined gas chromatography-mass spectrometry.

The levels of 3-hydroxy-N-nitrosopyrrolidine occurring in five samples of fried bacon were 0.5 to 3.9 ppb while the levels in fried-out fat ranged from 0.3 to 2.2 ppb. These levels are lower

than the concentrations generally reported for nitrosopyrrolidine in fried bacon.

This study, although the number of samples analyzed was limited, suggests that low ppb levels of 3-hydroxy-N-nitrosopyrrolidine appear fairly consistently in fried bacon.

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